The Effect of Processing Conditions on the Bioactive Compounds, Aroma Profile and Sensory Attributes of Wine from *Hibiscus sabdariffa*

Idolo Ifie

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School of Food Science and Nutrition

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The candidate confirms that this work submitted is his/her own, except part of the work that was jointly published and has been included in this thesis which is mentioned below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Abstract

Hibiscus sabdariffa (*H. sabdariffa)* is a rich source of bioactive phytochemicals with potential health benefits. Based on its reported use in folk medicine for the treatment of hypertension and recently type 2 diabetes, the plant has gained considerable commercial importance as a functional food. Three varieties of *H. sabdariffa* were analyzed for their phytochemical content and inhibitory potential on carbohydrate-digesting enzymes as a basis for selecting a variety for wine production. The dark red variety was chosen as it was highest in phenolic content and partially inhibited α–glucosidase (maltase), with delphinidin 3-*O*-sambubioside, cyanidin 3-*O*-sambubioside and 3-*O*-caffeoylquinic acid accounting for 65 % of this activity. None of the varieties significantly inhibited α–amylase. Regarding *H. sabdarifa* wine, the effect of fermentation (20 and 30 ˚C) and ageing (6, 15 and 30 °C) temperatures on the phytochemical composition and bioactivity of Roselle wine was investigated for a period of 40 days and 12 months respectively. The main change in phytochemical composition observed during fermentation was the hydrolysis of 3-*O*-caffeolquinic acid and the concomitant increase of caffeic acid irrespective of fermentation temperature. Wine fermented at 20 ˚C was slightly more active for α–glucosidase inhibition with more fruity aromas, but there were more flowery notes at 30 ˚C. Although ageing temperature did not produce an obvious trend in concentrations of most of the volatiles analysed, its impact was clearly demonstrated in the final concentrations of diethyl succinate which increased with higher ageing temperature. The data on the overall quality rating of roselle wine by a sensory panel showed that neither fermentation nor ageing temperatures produced any significant difference ($p \geq 0.05$) in the wine samples. This study demonstrates that processing of *H. sabdariffa*

into wine represents a promising alternative to expanding the functional properties of this crop.

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Abbreviations

BIBD	Balanced incomplete block design
CD	Colour density
CS	Cyanidin 3-*O*-sambubioside
DNS	3,5-Dinitrosalicylic acid
DS	Delphinidin 3-*O*-sambubioside
EGCG	(-)-Epigallocatechin gallate
ELSD	Evaporative light scattering detector
GC-MS	Gas chromatography mass spectrometry
GLUT2	Glucose transporter type 2
Hibiscus sabdariffa	*H. sabdariffa*
HPLC	High performance liquid chromatography
HS-SPME	Head space solid-phase micro extraction
HT	Hue tint
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
LSD	Least square difference
MLF	Malolactic fermentation
M3A	Myricetin 3-arabinogalactoside
PA	Polymeric anthocyanin
PBS	Phosphate buffer saline
RSD	Relative standard deviation
SD	Standard deviation

SGLT1	Sodium dependent glucose transporter type 1
SPE	Solid phase extraction
Q3S	Quercetin 3-sambubioside
TA	Titratable acidity
TPC	Total phenolic content
3-CQA	3-*O*-caffeolquinic acid
4-CQA	4-*O*-caffeolquinic acid
5-CQA	5-*O*-caffeolquinic acid

Chapter 1 Introduction

1.1 *Hibiscus sabdariffa*: An underutilised powerhouse of plant bioactives

1.1.1 Origin and cultivation

Hibiscus sabdariffa L. (*H. sabdariffa*) is a short-day annual shrub of the Malvaceae family grown in many tropical and subtropical countries (Aurelio *et al.*, 2008). The origin of *H. sabdariffa* is quite uncertain although there is some evidence that it was first domesticated by the black populations of western Sudan and later transported to other regions of the world. In addition, its use for food and medicinal purposes dates back to 5000 BC, spanning from Egypt, West Indies, China, India and South America (Mohamed *et al.*, 2012). Today, the leading producers of *H. sabdariffa* include Jamaica, Thailand and China, with the quality of Sudan rated the best in the world. *H. sabdariffa* is known by different names across the world as shown in Table 1-1.

Table 1-1 Vernacular names of *H. sabdariffa* in different countries.

Country	Vernacular name
England	Roselle
U.S	Roselle
France	O seille
Jamaica	Flor de Jamaica
Switzerland, Sudan and Arabia	Karkade
Nigeria	Zobo
Senegal	Byssap

Source (Ismail *et al.*, 2008)

1.1.2 Botanical description and morphology

The shrub *H. sabdariffa* grows up to 2.4 m, and the leaves, which are 7.5 -12.5 cm, are green, having reddish veins and long or short petioles. The flowers, borne singly in the leaf axils, are up to 12.5 cm wide, yellow or buff with a rose (maroon eye), which turn pink upon withering at the end of the day. Thereafter, the typically red calyx (3.2 - 5.7 cm long), which consists of 5 large sepals with a collar (epicalyx) of 8 to 12 slim, pointed bracts (or bracteoles) around the base, begins to enlarge, becomes fleshy, crisp and juicy, which fully encompasses the velvety capsule that turns brown and splits open when mature and dry (Morton, 1987). The seeds which are covered with minute, stout and stellate hairs, are light brown, 3 mm long and kidney-shaped (Morton, 1987). There is also a lesser known variety of *H. sabdariffa* referred to as white roselle with its distinct green/whitish calyces and commonly consumed as a vegetable.

1.1.3 Cultivation and harvesting of *H. sabdariffa*

The plant thrives in tropical and subtropical regions and is very susceptible to damage from fog and frost. *H. sabdariffa* requires an average rainfall of about 182 cm (72 inches) and propagation is by seeds, although it can be cultivated by shoot cuttings. The seedlings may be raised in the nursery and later transplanted to the field when they are 7.5 -10 cm (3 to 4 inches) high. It is necessary to carry out weeding during the first month, while the application of fertilizer varies amongst regions, although *H. sabdariffa* responds favourably to nitrogen application. The fruit consisting of the large reddish calyces is harvested 2-3 weeks after the onset of flowering, while the rest of the crop is left in the field until the seeds are ready for threshing.

1.1.4 Uses of *H. sabdariffa*

Food uses

The calyces, fresh or sometimes dried, are used in the production of teas, ice cream, jams, marmalade, sherbets and sauces. In addition, wines, jellies, beverages and other desserts can also be produced from the calyces (Tsai *et al.*, 2002; Herrera-Arellano *et al.*, 2004). In Nigeria, a refreshing drink known as "Soborodo" is prepared by boiling the calyces along with sugar and spices, while in Mexico a drink processed in similar manner is referred to as "Agua de Jamaica". The seeds can be taken as a coffee substitute, roasted or ground in meals. It is also fermented to produce a condiment known as "Mungza ntusa" in the northern parts of Nigeria (Omobuwajo *et al.*, 2000), while in China and certain parts of western Africa, the seeds are used for oil production (Atta and Imaizumi, 2002). Furthermore, the seeds are employed in the production of feed for both poultry and sheep. The leaves are also edible and are eaten raw, dried or cooked in Sudan and Malaysia (Ismail *et al.*, 2008). They also serve as a good source for animal fodder and fibre.

Traditional and folk medicine

The calyces of *H. sabdariffa* have been used in folk medicine for the treatment of various health conditions such as hypertension, diabetes, nerve disorders and heart ailments. Furthermore, several ethnomedicinal studies have reported the use of infusions of the calyces for treatment of hyperlipidaemia, obesity, and urinary tract infections (Hopkins *et al.*, 2013; Patel, 2014). In Guinea, the leaves are deemed to be diuretic, refrigerant and sedative, while the seeds are considered antiscorbutic and laxative. In addition, the leaves are employed in the treatment of cough and abscesses in parts of Central Africa and Angola (Duke, 1983).

1.1.5 Phytochemistry

H. sabdariffa is a rich source of bioactive phytochemicals (Tables 1-2 & 1-3) which may be responsible for the reported health benefits. The phytochemical profile of *H. sabdariffa* calyces varies between studies, probably due to factors such as geographical location, varieties, genetics, ecology and harvesting conditions. The main bioactive constituents in the calyces related to its pharmacological activities are the anthocyanins, organic acids, flavonoids and polysaccharides (Borrás-Linares *et al.*, 2015). Moreover, the seeds have also been reported to be rich in polyunsaturated fatty acids and lipid soluble antioxidants (Mahadevan *et al.*, 2009).

1.1.5.1 Anthocyanins

These are a group of flavonoid derivatives responsible for the red, blue and purple pigments found in *H. sabdariffa* and they account for about 1.7 to 2.5 % dry weight of the calyces. The first anthocyanin to be isolated from the calyces of *H. sabdariffa* was "hibiscin" which was later renamed delphinidin 3-*O*-sambubioside (DS) (Yamamoto and Osima, 1932). Thereafter, three different anthocyanins were also identified in the calyces namely cyanidin 3-*O*-sambubioside (CS), delphinidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside (Du and Francis, 1973). In most published studies, delphinidin 3-*O*-sambubioside and cyanidin 3-*O*-sambubioside have been reported as being the main anthocyanins present within the calyces accounting for over 75 % of the total anthocyanins (Ramirez-Rodrigues *et al.*, 2011; Sindi *et al.*, 2014).

Table 1-2 Phytochemicals present in *H. sabdariffa.*

Class	Compound	Reference(s)
Anthocyanins	Delphinidin 3-*O*-sambubioside (DS)	(Du and Francis, 1973; Ramirez-Rodrigues *et al.*, 2011)
	Cyanidin 3-*O*-sambubioside (CS)	(Wong *et al.*, 2002; Borrás-Linares *et al.*, 2015)
	Delphinidin 3-*O*-glucoside	(Sindi *et al.*, 2014)
	Cyanidin 3-*O*-glucoside	(Rodriguez-Medina *et al.*, 2009)
	Cyanidin-3,5-*O*-diglucoside	(Segura-Carretero *et al.*, 2008)
Organic acids	Hibiscus acid	(Rodriguez-Medina *et al.*, 2009; Ramirez-Rodrigues *et al.*, 2012)
	Hibiscus acid glucoside	(Ramirez-Rodrigues *et al.*, 2011; Ramirez-Rodrigues *et al.*, 2012)
	Hibiscus acid 6-methyl ester	(Ramirez-Rodrigues *et al.*, 2011; Borrás-Linares *et al.*, 2015)
	Hydroxycitric acid	(Mahadevan *et al.*, 2009; Borrás-Linares *et al.*, 2015)
	Malic acid	(Wong *et al.*, 2002; Jung *et al.*, 2013)
	Oxalic acid	(Wong *et al.*, 2002; Ali *et al.*, 2005)
	Citric acid	(Wong *et al.*, 2002; Ali *et al.*, 2005)
	Succinic acid	(Wong *et al.*, 2002)
	Tartaric acid	(Mahadevan *et al.*, 2009)
Phenolic acids	3-*O*-caffeoylquinic acid	(Rodriguez-Medina *et al.*, 2009; Borrás-Linares *et al.*, 2015)
	4-*O*-caffeoylquinic acid	(Ramirez-Rodrigues *et al.*, 2011)
	Chlorogenic acid	(Jiménez-Ferrer *et al.*, 2012; Borrás-Linares *et al.*, 2015)
	5-*O*-caffeoylshikimic acid	(Rodriguez-Medina *et al.*, 2009; Borrás-Linares *et al.*, 2015)
	N-Feruloyltyramine	(Rodriguez-Medina *et al.*, 2009; Borrás-Linares *et al.*, 2015)
	Caffeic acid	(Ramirez-Rodrigues *et al.*, 2011; Ramirez-Rodrigues *et al.*, 2012)
	Coumaroylquinic acid	(Borrás-Linares *et al.*, 2015)
	Gallic acid	(Ramirez-Rodrigues *et al.*, 2012)
	Protocatechuic acid	(Mahadevan *et al.*, 2009; Ramirez-Rodrigues *et al.*, 2011)
Flavonols	Quercetin	(Fernandez-Arroyo *et al.*, 2011; Fernandez-Arroyo *et al.*, 2012)
	Quercetin 3-*O*-glucoside	(Ramirez-Rodrigues *et al.*, 2011; Borrás-Linares *et al.*, 2015)
	Quercetin 3-sambubioside (Q3A)	(Rodriguez-Medina *et al.*, 2009)
	Myricetin	(Borrás-Linares *et al.*, 2015)
	Myricetin-3-arabinogalactoside (M3A)	(Rodriguez-Medina *et al.*, 2009)
	Rutin	(Rodriguez-Medina *et al.*, 2009)
	Kaempferol-3-*O*-glucoside	(Fernandez-Arroyo *et al.*, 2011)
Sterols	Ergosterol	(Ali *et al.*, 2005; Mahadevan *et al.*, 2009)
	Stigmasterol	(Ali *et al.*, 2005)
	β-Sitosterol	(Mahadevan *et al.*, 2009)

Table 1-3 Volatiles present in *H. sabdariffa.*

Class	Compound	Reference(s)
Volatiles	1-octen-3-ol	(Ramírez-Rodrigues *et al.*, 2011)
	1-Hexanol	(Ramírez-Rodrigues *et al.*, 2011; Ramirez-Rodrigues *et al.*, 2012)
	Hexanal	(Ramírez-Rodrigues *et al.*, 2011)
	Heptanal	(Ramírez-Rodrigues *et al.*, 2011)
	Eugenol	(Ramírez-Rodrigues *et al.*, 2011)
	Linalool	(Ramírez-Rodrigues *et al.*, 2011)
	2-Hexenol	(Chen *et al.*, 1998)
	Limonene	(Chen *et al.*, 1998)
	4-Methyl-2,6-di-*tert*-butylphenol	(Jung *et al.*, 2013)
	2-Pentylfuran	(Jung *et al.*, 2013)

However, a study also reported the absence of delphinidin-3-*O*-glucoside in the extracts of some strains (Da-Costa-Rocha *et al.*, 2014). The chemical structure of the main anthocyanins and some phytochemicals found in *H. sabdariffa* are presented in Figure 1-1.

1.1.5.2 Organic acids

The calyces of *H. sabdariffa* contain a myriad of organic acids which include malic acid, citric acid ascorbic acid, hibiscus acid and hydroxycitric acid (Wong *et al.*, 2002). In addition, tartaric acid, oxalic acid, succinic acid and malic acids have also been identified in the calyces (Ali *et al.*, 2005). Citric acid and malic acid were the first acids to be isolated in the calyces and their contents varies widely between studies (Buogo and Picchinenna, 1937). The reported concentration of ascorbic and malic acids in *H. sabdariffa* varied between 80 - 120 mg/100 g, while succinic, oxalic and tartaric acids content ranged from 170 – 510 mg/100 g (Wong *et al.*, 2002; Mohamed *et al.*, 2012). Hydroxycitric acid and its lactone form (+) -allo-hydroxycitric acid (hibiscus acid) are the main organic acids found in the calyces of *H. sabdariffa*. Hydroxycitric acid - (2S,

3R) is different from the (2S, 3S) - hydroxycitric acid isolated from the leaves of *Garcinia spp.* (Da-Costa-Rocha *et al.*, 2014).

1.1.5.3 Phenolic acids

The main phenolic acid present in *H. sabdariffa* is chlorogenic acid which belongs to the family of esters formed between certain trans-cinnamic acids (caffeic acid, ferulic acid and p-coumaric acid) and quinic acid (Clifford *et al.*, 2003; Borrás-Linares *et al.*, 2015). Chlorogenic acid and its derivatives have been identified in many studies involving extracts of *H. sabdariffa* (Salah *et al.*, 2002; Rodriguez-Medina *et al.*, 2009; Ramirez-Rodrigues *et al.*, 2011). Another phenolic acid isolated from the dried flowers of *H. sabdariffa* and assigned the structure of 3, 4-dihydrobenzoic acid is protocatechuic acid, while gallic and ellagic acids have also been reported in *H. sabdariffa* (Peng *et al.*, 2011; Lin *et al.*, 2011; Lin *et al.*, 2012).

1.1.5.4 Flavonoids

H. sabdariffa calyces contain different classes of the flavonoids namely flavonols, flavones and flavan-3-ols. These include quercetin and its glucosides, alongside myricetin, rutin, kaempferol, luteolin and its glucoside all being present in *H. sabdariffa* (Salah *et al.*, 2002; Rodriguez-Medina *et al.*, 2009; Fernandez-Arroyo *et al.*, 2012). The flavan 3-ols found in *H. sabdariffa* include catechin and gallocatechins gallates (Yang *et al.*, 2009).

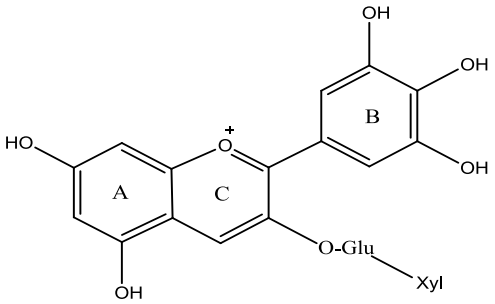
1.1.5.5 Polysaccharides

The petals of *H. sabdariffa* yield 65 % (dry weight) of mucilage, which on hydrolysis produces galacturonic acid, glucose and rhamnose (El-Hamidi *et al.*, 1967). Furthermore, three water soluble polysaccharides have also been isolated from the flower buds, with the neutral fractions composed of arabinans and arabinogalactans. The major fraction was

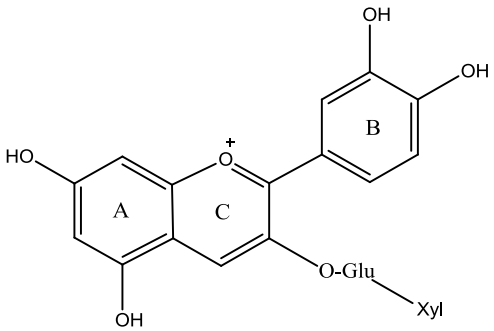
shown to be a pectin-like molecule (Mr = 105 Da) linked together by α-1,4-galacturonic acid and 1,2-rhamnose bonds. Furthermore, side chains of galactose and arabinose are connected to the main chain through C- 4 bond of every third rhamnose (Ali *et al.*, 2005).

1.1.5.6 Volatile composition

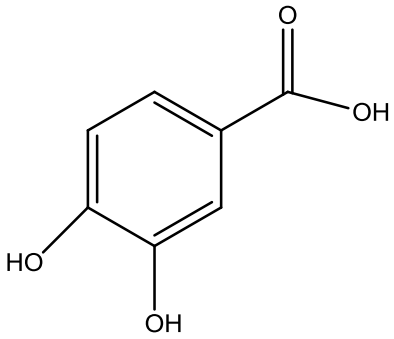
The volatile composition in *H. sabdariffa* calyces was analysed by (Gonzalez-Palomares *et al.*, 2009). Twenty volatile compounds were identified in the ethanol extract, which includes terpenoids, esters, hydrocarbons and aldehydes. However after drying, only fourteen volatile compounds were identified indicating that some of the volatiles were lost during processing. In another study, a total of 28, 25, 17, and 16 volatiles were identified in the dried hot extract (DHE), dried cold extract (DCE), fresh hot extract (FHE), and fresh cold extract (FCE) samples, respectively (Ramírez-Rodrigues *et al.*, 2011). The most potent aroma compounds common to all four extracts were 1-octen-3-ol and nonanal. Furthermore, linalool and 2-ethyl-1-hexanol were detected only in beverages from fresh hibiscus, while furfural and 5-methyl furfural derived from the drying process were found only in dried hibiscus extract. The same observation regarding furfural and 5-methyl furfural production after drying of *H. sabdariffa* calyces was also reported by (Chen *et al.*, 1998). Furthermore, the analysis of four differently treated *H. sabdariffa* samples: (untreated, frozen, hot-air-dried at 50 °C, and hot-air-dried at 75 °C), revealed more than 37 volatile compounds characterized by GC-MS. These were the fatty acid derivatives, sugar derivatives, phenolic derivatives, and terpenes.



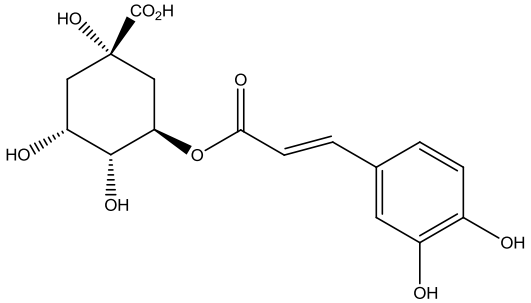
Delphinidin 3-*O*-sambubioside



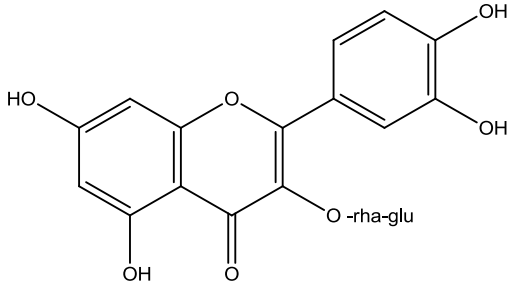
Cyanidin 3-*O*-sambubioside



Protocatechuic acid



Chlorogenic acid



Quercetin 3-*O* rutinoside

Figure 1-1 Chemical structures of some phytochemicals found in *H. sabdariffa.*

The results also showed higher amounts of the aliphatic C6 lipid derivative (responsible for green aroma notes) in the fresh calyces, while only trace amounts were found in the frozen and air-dried samples.

1.1.6 Pharmacological and biological activities of *H. sabdariffa*

H. sabdariffa has been shown to exhibit some pharmacological properties, making it a suitable candidate for therapeutic application as highlighted in Figure 1-2. However, the scope of this study is limited to its antidiabetic properties through the inhibition of carbohydrate digesting enzymes (α-amylase and α-glucosidase). The justification being that so far the antihypertensive properties of *H. sabdariffa* have been the most researched and reported health benefit found in the literature, while the antidiabetic activities of *H. sabdariffa* have been scarcely documented and this warrants further investigation (Wahabi *et al.*, 2010).

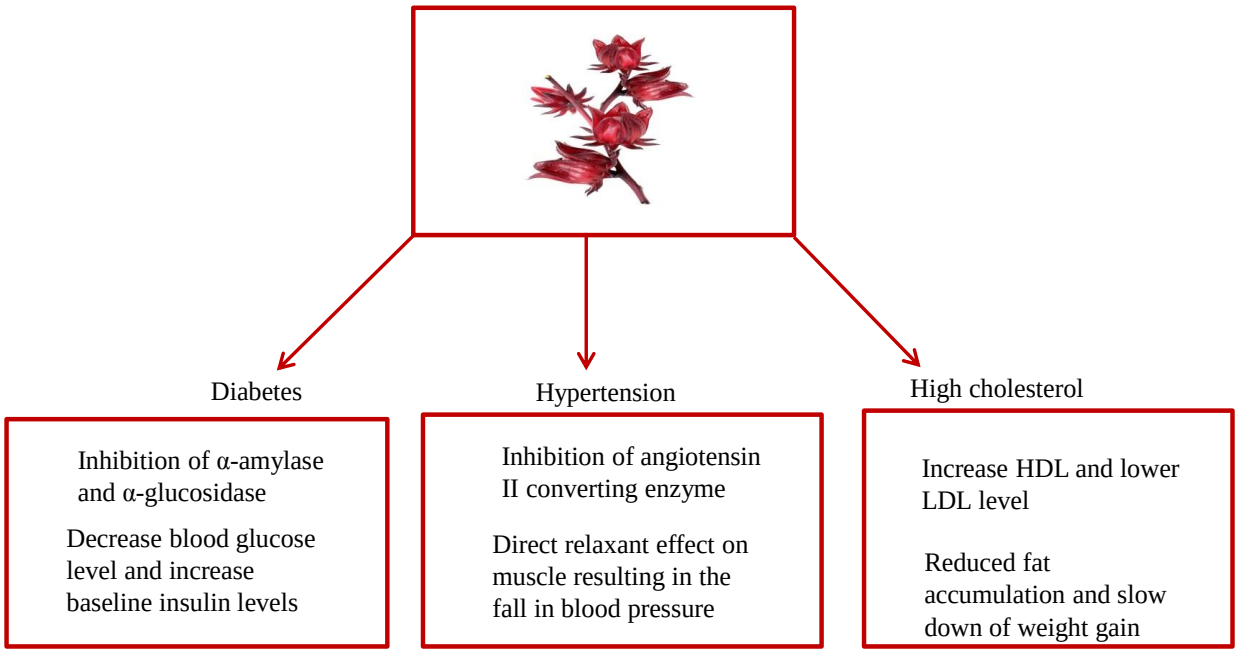


Figure 1-2 Pharmacological activities of *Hibiscus sabdariffa*. The scheme has been modified from (Patel, 2014).

1.1.6.1 Inhibition of carbohydrate digesting enzymes

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both (American Diabetes Association 2010). One of the therapeutic approaches adopted thus far to ameliorate postprandial hyperglycemia involves the retardation of glucose absorption via the inhibition of carbohydrate-hydrolyzing enzymes including α-amylase and α-glucosidase in the digestive organs (Williamson, 2013). The α–amylase (α-1,4-glucan-4-glucanohydrolases; E.C. 3.2.1.1) found in microorganisms, plants and higher organisms is one of the major secretory products of the pancreas (about 5–6 %) and salivary glands, playing a role in digestion of starch and glycogen. It constitutes a family of endo-amylases that catalyse the initial hydrolysis of starch into shorter oligosaccharides through the cleavage of α-D-(1-4) glycosidic bonds. Neither terminal glucose residues nor *α*-1,6-linkages can be cleaved by α-amylase and the end products of α-amylase action are oligosaccharides with varying length with an α-configuration and α-limit dextrins. A second enzyme, α-glucosidase (EC 3.2.1.20) located in the brush border surface of intestinal cells catalyzes the end step of digestion of starch releasing glucose from the non-reducing end side of substrate, which is then transported into the blood by the transporters sodium-dependent glucose transporter type 1 (SGLT1; SLC5A1) and glucose transporter type 2 (GLUT2; SLC2A2) (Nyambe-Silavwe *et al.*, 2015).

The powerful synthetic α-glucosidase and α-amylase inhibitors, such as acarbose (Figure 1-3), miglitol, and voglibose, function by reducing the sharp increases in glucose levels that occur immediately after food consumption (Lee *et al.*, 2010). However, the potency of acarbose is not without side effects, such as diarrhoea, flatulence and nausea. An alternative strategy as postulated by Williamson *et al.* (2013) is the use of small molecules like polyphenols which might have acarbose-like effects. The interaction between

flavonoids and human α-amylase was investigated and the mode of inhibition was in agreement with the mechanism of action proposed for acarbose (Lo Piparo *et al.*, 2008). The study also showed that the potency of inhibition is correlated with the number of hydroxyl groups on the B ring of the flavonoid skeleton (Figure 1-4). The interaction occurs with the formation of hydrogen bonds between the hydroxyl groups in position R6 or R7 of the ring A and position R4' or R5' of the ring B of the polyphenol ligands and the catalytic residues of the binding site and formation of a conjugated-system that stabilizes the interaction with the active site. This finding has given rise to a number of studies conducted on bioactivity of polyphenols in the search for natural α-amylase inhibitors that induce no deleterious side effects (Nyambe-Silavwe *et al.*, 2015).

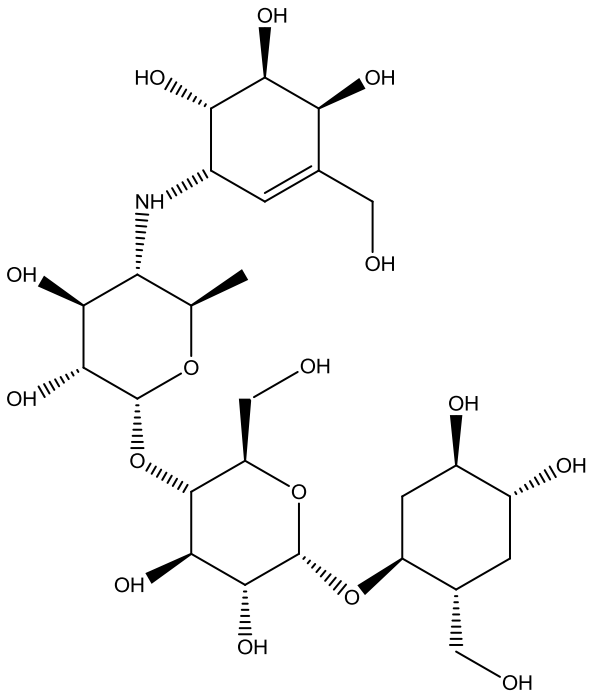


Figure 1-3 Structure of acarbose.

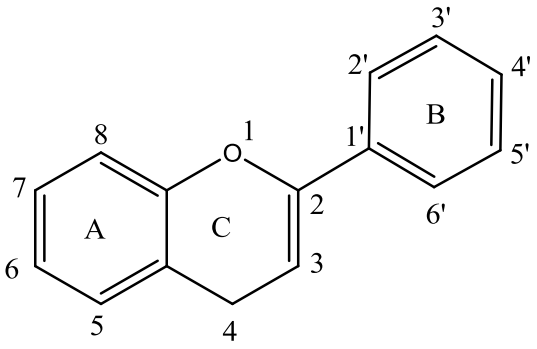


Figure 1-4 Flavonoid carbon skeleton structure.

1.1.6.2 Inhibition of α-amylase and α-glucosidase by *H. sabdariffa*

There are a few research studies reporting the inhibition of α-amylase and α-glucosidase by *H. sabdariffa*. The inhibition of α-amylase inhibition has been carried out using enzymes from either porcine pancreas or bacteria and under different assay conditions, making comparison between results difficult. Hansawasdi *et al.* (2000) investigated the inhibitory activities of 43 polyphenol rich extracts (100 mg/mL) including *H. sabdariffa* on porcine α-amylase were evaluated and *H. sabdariffa* exhibited the highest inhibitory activity. Consequently, it was chosen for isolation of the active principles and their subsequent inhibitory potentials. The active compounds were identified as hibiscus acid and hibiscus acid ester with their IC_{50} values being 3.22 mM and 1.10 mM respectively. Similarly a dose-dependent inhibition of both porcine α-amylase and α-glucosidase from rat intestinal powder by *H. sabdariffa* extract was reported (Adisakwattana *et al.*, 2012). The extract showed to be a more potent inhibitor of α-amylase with an IC_{50} of 3.52 mg/mL, while that of α-glucosidase was above 5 mg/mL. In contrast, Ademiluyi and Oboh (2013) found that *H. sabdariffa* extracts from two varieties (red and white) were better inhibitors of α-glucosidase than α-amylase. The IC_{50} also showed that the red

variety (25.2 μg/mL) exhibited higher α-glucosidase inhibitory activity than the white variety (47.4 μg/mL), while the white variety (90.5 μg/mL) exhibited higher α-amylase inhibitory activity than the red variety (187.9 μg/mL). A look at the IC_{50} values reported shows a wide variation in the results and warrants further investigations. The proposed direction for future studies should include: (1) characterisation of the phenolic compounds in the extract used for the experiment, (2) optimisation of assay conditions to ease comparison of results and (3) identification of the main principles responsible for the effect and thereafter elucidate the possible mechanism for the inhibitory activities.

1.1.7 *H. sabdariffa* and translational research

To ascertain the level of translational research on *H. sabdariffa* products, a patent search at the Espacenet patent database of the European patent office was performed in 2013 (Da-Costa-Rocha *et al.*, 2014). The results showed a range of patents for the manufacture of diverse functional foods and nutraceuticals from *H. sabdariffa*, which shows a growing interest in novel technological applications of potential products from the plant. A brief highlight of some of the patented products alongside possible future application in the food and nutraceutical industries is discussed in section 1.1.7.1.

1.1.7.1 Beverage industry

The beverage industry comprises of four main sectors as presented in Figure 1-5. The soft drink industry can be sub-divided into five main sectors (bottled water; carbonated soft drinks; dilutables, also known as squash and including powders, cordials and syrups; 100 % fruit juice, and nectars with 25–99 % juice content; still drinks, including ready-to-drink (RTD) teas, sports drinks and other non-carbonated products with less than 25 % fruit juice). The alcoholic beverage industry is also divided into subcategories namely,

wine, beer spirits, cider, sake and flavoured alcoholic beverages – sometimes referred to as pre-mixed spirits (Roethenbaugh, 2005).

H. sabdariffa has found a place in the beverage industry (hot drinks and soft drinks sectors) owing to its appealing red colour, refreshing properties and the awareness of its potential health benefit. However, the highlighted portion in Figure 1.5 shows the sectors yet to be fully exploited. Some of the products of *H. sabdariffa* in the beverage industry currently in the market are described below.

Hibiscus Lemon Bissap

The product is manufactured by the company Adina for Life founded in 2004 and based in California, U.S.A. The company uses certified organic hibiscus blossoms from Senegal under fair trade agreements as ingredients in its drink. This has served to improve the livelihood of the peasant farmers and offered them decent incomes to raise their standard of living. The drink marketed as a New Age Beverage, with all natural ingredients and good for you appeal serves retailers throughout the United States including Whole Foods and Wegmans (Ramirez *et al.*, 2010; Rodrigues, 2010).

Squish Hibiscus Pressé

Produced and marketed in New Zealand, Hibiscus Presse has as ingredients, *H. sabdariffa* extract from water, fructose and ascorbic acid. The label on the 375 mL plastic bottle appeals to consumers, enlightening them about hibiscus's use in folklore and folk medicine in the Caribbean, China, Egypt, Sudan, and Asia. It also informs consumers that "Hibiscus is stimulating scientific inquiry into possible health benefits and its antioxidant properties" (Rodrigues, 2010).

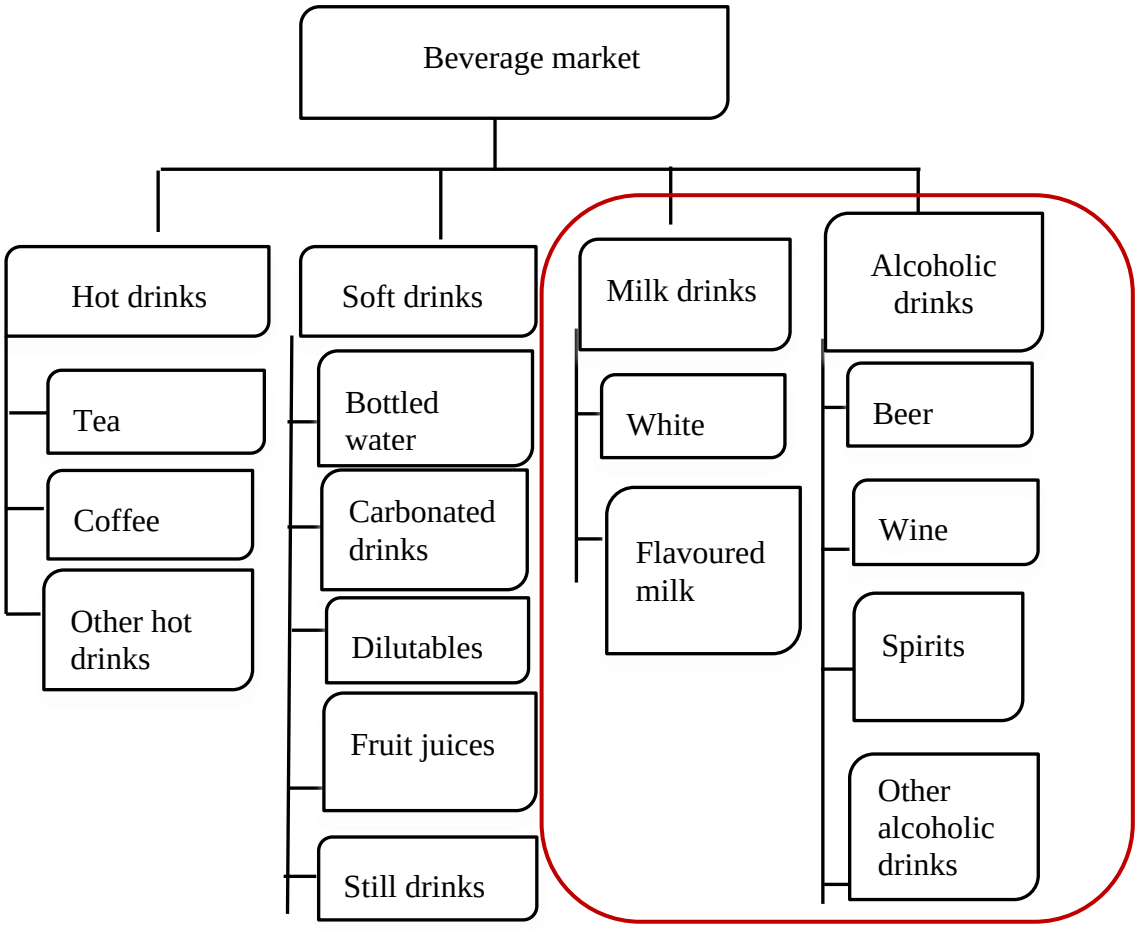


Figure 1-5 Schematic representation the beverage sector and segments. (Source Rodrigues, 2010). The highlighted portion shows sectors of the market yet to be fully exploited in the production of hibiscus based drinks.

Simply Hibi

The beverage available in 500 mL glass bottles is produced by Hibi Organics based in the UK and the raw material sourced from very special variety of hibiscus grown by farmers in Uganda. The product also known as Hibiscus Elixir is prepared from infusions of the calyx blended together with sugar to make a concentrate which needs to be diluted before consuming it. In addition, the company in conjunction with Britania Allied Industries in Kampala, one of the biggest names in the East African Juice Market, produces Simply Hibi, a single strength juice as part of the 'Splash' range of juices

through the extensive network of Splash distributors across East Africa. The label informs consumers that the product contains the same high quality, goodness and great taste as the Simply Hibi enjoyed in the UK (Rodrigues, 2010).

Yomm Hibiscus drink

Produced by Yomm Beverage in Canada, the refreshing hibiscus drink comes in four different flavours original, strawberry, lemon and sugar-free. The ingredients include *H. sabdariffa* extract, water, organic agave syrup, organic cane sugar and natural flavour and the 335 mL bottle contains 0.270 g and 0.095 g of polyphenols and anthocyanins as stated on the product label.

Hibiscus Tea

Being known as a caffeine-free herbal drink, hibiscus tea tends to be widely available and produced by different tea companies in tea bags or as loose flower petals. It is known by different brand names and while some contain only hibiscus, some contain it as a major component plus other ingredients to give different flavours.

1.1.7.2 Potential applications of *H. sabdariffa*

Although the use of *H. sabdariffa* has gained foray into the food beverage industry, there are still other potential uses of *H. sabdariffa* in the beverage industry as highlighted in Figure 1-5. Its high content of anthocyanins can be exploited and used as a natural colourant for beverages like milk and yogurt -based drinks as well as in the production of hibiscus wine. The judicious use of *H. sabdariffa* in food processing can promote the manufacture of novel functional foods that may ultimately provide health benefit at low cost (Patel, 2014). However, the amount of bioactive components in these finished products which influences the overall quality is dependent on the processing method applied. In this context, section 1-2 highlights the effect of food processing on the phenolic groups of bioactive compounds.

1.2 Food processing

Food processing can be defined as the methods and techniques employed to convert raw ingredients into food or to transform food into other forms fit for consumption by humans or animals either in the home or by the food industry.

The broad objectives of food processing are:

1. To extend the shelf life or the duration of time in which the food is deemed to be wholesome, free of biochemical and microbiological spoilage and nutritious to consumers.
2. To make more bioaccessible nutrients required for health
3. To spice up the diet by introducing variety to the organoleptic properties (colour, texture, aroma and flavour) of foods.
4. To restore and/or raise nutrient levels in food.
5. To make a profit for food companies and provide employment.

Most food processing techniques involve a sequence of operations to bring about the desired change in the raw material. Furthermore, each unit operation has its own particular effect on the food constituents (nutrients, phytochemicals and organoleptic properties) and the final product quality is determined by the combination of the effects from each operation (Fellows, 2009). In addition, for bioactive compounds to exert their positive health effect, they first have to withstand the food processing conditions, be released from the food matrix and be bioaccessible in the gastrointestinal tract, undergo metabolism and reach the target tissue of interest. Consequently, the role that food processing has on plant bioactives is important, as it represents the first step in the challenging journey of bioactive compounds reaching the target tissues. In addition, because of their possible biological effects in humans, it is necessary that the changes in phenolic compounds during processing be evaluated to better assess the dietary value of the processed products

(Skrede *et al.*, 2000). In the light of the above mentioned reasons, sections 1.2.1 examines existing data on the impact of different processing techniques on phenolic compounds, and for emphasis food sources rich in these compounds are mentioned.

1.2.1 Effect of processing on phenolic compounds

1.2.1.1 Anthocyanins

Anthocyanins are bioactive compounds that are present in fruits and vegetables. They differ with respect to their anthocyanidin skeleton, type of sugars and potential aliphatic and aromatic acyl moieties, and their substitution positions (Andersen and Jordheim, 2010). Although there are several anthocyanidins occurring in nature (Table 1-4), the six most prevalent occurring in fruits and vegetables are cyanidin 50 %, pelargodinin 12 %, peonidin 12 %, delphinidin 12 %, petunidin 12 %, and malvidin 7 %. Anthocyanindin stability is affected by the ring B substituents (Figure 1-4) and additional methoxyl or hydroxyl groups attached to the B ring decreases its stability in neutral media; this makes pelargodinin the most stable anthocyanindin. Glucose, galactose, rhamnose and arabinose are the sugars mostly found, usually as 3-*O*-glycosides or 3,5-*O*-diglycosides. In addition, the presence of rutinosides (6-*O*-α-L-rhamnosyl-D-glucose), sambubiosides (β-D-xylosyl-(1→2)-β-D-glucose), 3-7-diglycosides and 3-triosides do appear. Many anthocyanins are seen to be acylated by aliphatic or aromatic acids, with the most frequent acyl groups being coumaric, caffeic, ferulic, benzoic, synapic, malonic, acetic, succinic, oxalic, and malic acids (Clifford, 2000). The most frequent acyl moiety occurring in about 25 % of anthocyanins is malonic acid. The tertiary structure adopted by anthocyanins in intact plant tissues offers them protection from nucleophilic attack by water and produces the hyperchromic and bathochromic effects. However, once detached from their primary

environment and protection offered by co-pigmentation they become unstable and breakdown easily.

The factors that influence its stability include the pH, oxygen, temperature, light, metal ions enzymes and sugars (Clifford, 2000). With respect to pH, anthocyanins exist in four different forms (Figure 1-6) depending on the pH. At pH 1, the flavylium cation (red colour) predominates and is responsible for the purple red colours. When the pH is between 2 and 4, the quinoidal blue species dominates. At pH 5 and 6, the colourless species which are the carbinolpseudobase and a chalcone exist together and when the pH is above 7, the anthocyanins degrade depending on their substituent groups (Castañeda-Ovando *et al.*, 2009).

During processing operations like juice extraction, cutting and dicing, disruption of the cell arrangement occurs allowing for substrate and the enzyme to mix together. In the process, enzyme hydrolysis occurs and the sugar at position C3 is cleaved off, thereby exposing the resulting chalcone, which being unstable breaks down to 2,4,6-trihydroxyphenylacetaldehyde and a benzoic acid. To slow down the rate of the breakdown of the pigment, the enzyme can be inactivated by applying a mild heating procedure known as blanching; this process has been shown to have a positive effect on anthocyanin retention and stability (Skrede *et al.*, 2000).

Table 1-4 Structural identification of anthocyanidins (aglycones).

			Substitution pattern						
Name	Abbreviations	Colour	3	5	6	7	3′	4′	5′
Apigeninidin	Ap	Orange	H	OH	H	OH	H	OH	H
Aurantinidin	Au	Orange	OH	OH	OH	OH	H	OH	H
Capensinidin	Cp	Bluish-red	OH	OMe	H	OH	OMe	OH	OMe
Cyanidin	Cy	Orange-red	OH	OH	H	OH	OH	OH	H
Delphinidin	Dp	Bluish-red	OH	OH	H	OH	OH	OH	OH
Europinidin	Eu	Bluish-red	OH	OMe	H	OH	OMe	OH	OH
Hirsutidin	Hs	Bluish-red	OH	OH	H	OMe	OMe	OH	OMe
6-Hydroxycyanidin	6OHCy	Red	OH	OH	OH	OH	OH	OH	H
Luteolinidin	Lt	Orange	H	OH	H	OH	OH	OH	H
Malvidin	Mv	Bluish-red	OH	OH	H	OH	OMe	OH	OMe
5-Methylcyanidin	5-MCy	Orange-red	OH	OMe	H	OH	OH	OH	H
Pelargonidin	Pg	Orange	OH	OH	H	OH	H	OH	H
Peonidin	Pn	Orange-red	OH	OH	H	OH	OMe	OH	H
Petunidin	Pt	Bluish-red	OH	OH	H	OH	OMe	OH	OH
Pulchellidin	Pl	Bluish-red	OH	OMe	H	OH	OH	OH	OH
Rosinidin	Rs	Red	OH	OH	H	OMe	OMe	OH	H
Tricetinidin	Tr	Red	H	OH	H	OH	OH	OH	OH

Source: (Castañeda-Ovando *et al.*, 2009).

Another factor that plays a major role in anthocyanin degradation either through direct oxidative mechanism or through the action of polyphenol oxidases (PPO) is oxygen. Although the anthocyanins are not substrates for PPO, those possessing a dihydroxyl B-group react with the o-quinones derived from the oxidation of phenolic compounds by PPO to form brown condensation products. The anthocyanins not having the dihydroxyl B-group form adducts with such quinones (Clifford, 2000). The degradation products formed from these reactions are generally unstable and degrade further to form colourless compounds. To overcome this, the addition of sulphur dioxide as an antioxidant to fruits and vegetable is frequently employed as it has been reported to slow down anthocyanin degradation (Bakker and Bridle, 1992).

Thermal degradation of anthocyanins during processing results in the production of different degradation products which vary according to the heating temperature (Figure 1-7). The opening of the pyrylium ring and chalcone formation has been suggested as the initial step for anthocyanin breakdown. In addition, the hydrolysis of the sugar moiety and the aglycone probably due to the formation of cyclic–adducts has also been proposed as a possible initial mechanism for its degradation (Patras *et al.*, 2010). The terminal degradation products from the chalcone were identified to be phenolic acids and phloroglucinaldehyde. The stability of anthocyanins can be enhanced by co-pigmentation. Co-pigmentation is a phenomenon in which pigments and other non-coloured organic components form molecular associations or complexes resulting in a brighter and more stable colour (Boulton, 2001).

This occurrence is critical because colour remains a major quality criteria influencing consumer acceptability of a product. Co-pigmentation and polymerisation reactions have been reported to be responsible for the stability of wine colour (He *et al.*, 2012b).

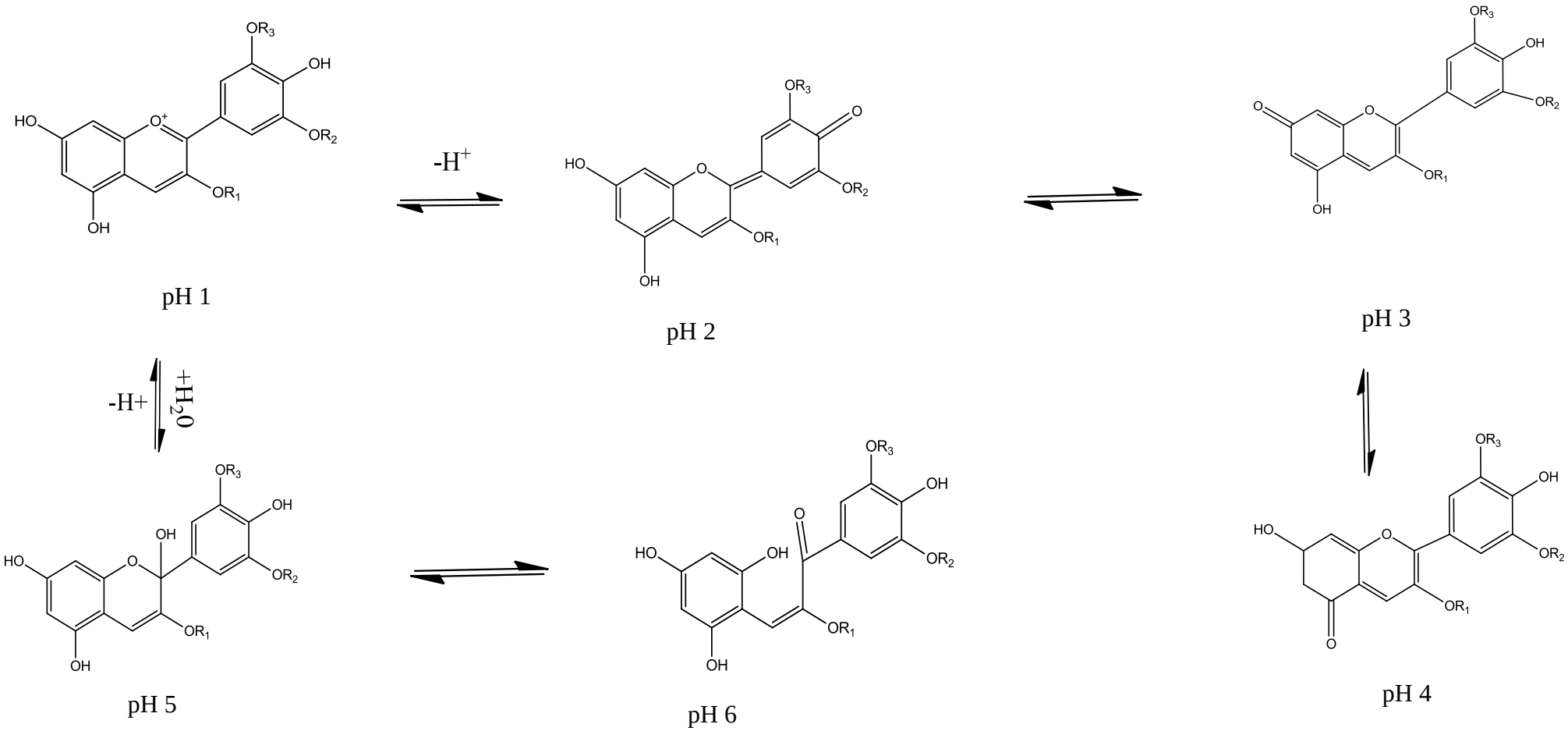


Figure 1-6 Anthocyanins chemical form depending on pH and degradation reaction for anthocyanins. Where R1 = H or a saccharide, R2 and R3 = H or Methyl (Castañeda-Ovando *et al.*, 2009).

1.2.1.2 Phenolic acids

Hydroxybenzoic and hydroxycinnamic acid derivatives are major phenolic acids present in bound form in plant cells where they play a vital role in plant defence, in the maturation processes and development of fruit flavour quality (Fallico *et al.*, 1996). The hydroxybenzoic acid derivatives are present as sugar derivatives and organic acids in plant foods as well as present in lignins and hydrolysable tannins, while the trans-4-hydroxycinnamic acids (ρ-coumaric, caffeic, ferulic, and sinapic) occur in the form of esters and glycosides. Ellagic acid, ρ-hydroxybenzoic and gallic acids have been reported to be the main phenolic acids present in the berry (strawberry, raspberry, and cloudberry) family Rosaceae (Häkkinen *et al.*, 2000; Veberic *et al.*, 2008). The main cinnamic compound found in apples, pears, and potatoes is chlorogenic acid (3-*O*-caffeoylquinic acid). In grapes, caftaric acid (caffeoyltartaric) is most abundant, while ferulic acid esters are typical of citrus fruit. For cereals, the most abundant hydroxycinnamic acid is ferulic acid and its oxidative products, diferulic acids, while other hydroxycinnamic acids (sinapic acid, ρ-coumaric acid, caffeic acid) and benzoic acid derivatives also occur in small quantities in various grains and derived products (Dao and Friedman, 1992; Fallico *et al.*, 1996; Gallardo *et al.*, 2006; Nayak *et al.*, 2015). The changes that occur in free and bound forms of phenolic acids during processing depends on the type of fruit, vegetable, plant food, and the processing technique employed (Nayak *et al.*, 2015). Furthermore, temperature, oxygen and enzymes are also major factors affecting the stability of phenolic acids (Dewanto *et al.*, 2002). For instance, an increase in ellagic acid content in strawberry fruit kept under modified atmosphere (5 °C for 10 days) has been reported. The same effect was also seen when strawberries were processed into puree juice and stored at 4 °C (Häkkinen *et al.*, 2000; Oszmiański and Wojdyło, 2009). The increase in ellagic acid can be attributed to the hydrolysis of ellagitannins during processing and

ageing. On the other hand, the processing of blue berries into jams produced a 20 % reduction in ellagic acid levels, possibly due to the antioxidant activities of ellagic acid during the manufacturing process (Häkkinen *et al.*, 2000). Similarly, food processing involving heat has been shown to decrease the chlorogenic acid content of potatoes. The decrease varied according to the type of the heat applied, with oven-baked potatoes having the greatest reduction and microwaved treated retaining the highest amount (Dao and Friedman, 1992)**.** The study also demonstrated that the nature of heating influences final levels of chlorogenic acid and controlling processing temperatures is critical in processing potato products. For ferulic acid esters, thermal processing and storage of orange juice results in the hydrolysis of the esters and release of the free acids which may later undergo decarboxylation, leading to the formation of 4-vinyl guaiacol which imparts an unpleasant odour to the final product. Subsequently, 4-vinyl guaiacol may undergo thermal degradation to produce other phenolic compounds like vanillin, 4-ethyl or 4-methyl guaiacol (Lee and Nagy, 1990). Therefore monitoring the levels of these compounds is important from the nutritional as well as organoleptic point of view.

In cereals, most of the phenolic acids occur in the bound form. However, during extrusion processing of wheat, barley oat and rye (temperatures between 120 – 200 °C), an increase of 200 to 300 % in free forms of vanillic, syringic and ferulic acids was observed indicating that hydrothermal processing of cereal grains may release phenolic acids and their derivatives from the wall cells (Zielinski *et al.*, 2001).

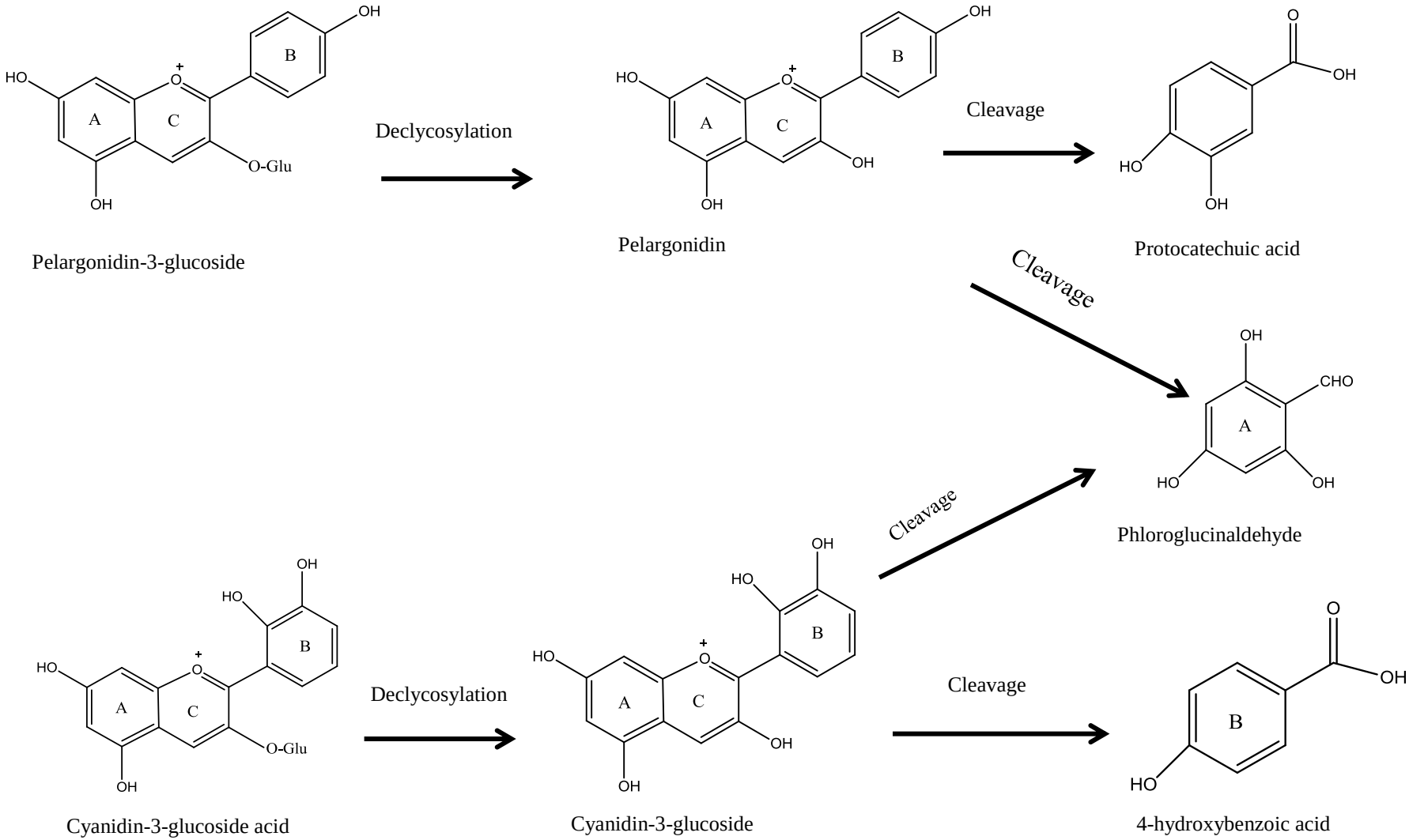


Figure 1-7 Proposed mechanism for thermal degradation of anthocyanins (modified from Sadilova *et al.*, 2007). The figure shows the hydrolysis of sugar moiety and aglycone formation as initial degradation step possibly due to the formation of cyclic-adducts which later decomposes upon heating into a chalcone structure, the latter being further transformed into a coumarin glucoside derivative with a loss of the B-ring.

Similarly during the fermentation of rye and wheat, increased levels of ferulic acid and other phenolic acids were reported probably due to the activity of hydrolytic enzymes (Katina *et al.*, 2007; Bhanja *et al.*, 2009). Overall, processing tends to have both positive as well as detrimental effects on phenolic acids in foods depending on the plant matrix and the processing method employed. Therefore, it is critical to determine not only optimal processing conditions that extend the shelf life of products, but in addition reduce the degradation of bioactive compounds present in them.

1.2.1.3 Flavonoids (flavan-3-ols, flavonols and isoflavones)

There is growing evidence to support the cardioprotective effects of flavan-3-ols (present in tea and cocoa), flavonols (quercetin found in onion and most leafy vegetables) and isoflavones, present in soy (Perez-Vizcaino and Duarte, 2010; Curtis *et al.*, 2012). However during processing, these phenolic compounds undergo significant changes in their structure that may impact on their biological activities. This is observed during black tea processing where catechins are acted upon by the oxidative enzymes, polyphenol oxidase and peroxidase, to form theaflavins and thearubigins during fermentation (Muthumani and Kumar, 2007). Furthermore, these oxidation products along with catechin are responsible for the taste and astringent character of black tea. Similarly, during the fermentation of cocoa beans, polyphenols undergo oxidation to condensed high molecular weight insoluble tannins which results in decreases in catechin and epicatechin contents (Wollgast and Anklam, 2000; Nazaruddin *et al.*, 2006). The reverse occurs during the roasting of cocoa bean where (-)-catechin levels increase when bean temperature exceeds 70 °C probably due to the epimerisation of (-)-epicatechin (Payne *et al.*, 2010).

Flavonols constitute an integral part of the human diet with onions and asparagus being rich sources of quercetin 3,4'-*O*-diglucoside and quercetin 4'-*O*-glucoside. The impact of

domestic processing (blanching, chopping, maceration and boiling) on flavonol content in onions and asparagus was assessed: chopping caused a significant decrease in rutin content of asparagus but did not affect the levels of quercetin 3,4'-*O*-diglucoside and quercetin 4'-*O*-glucoside in onions (Makris and Rossiter, 2001). Boiling for 60 min produced the highest reduction in total flavonols, indicating that the impact of thermal heating on flavonols in these plant sources cannot be ignored. On the contrary, processing of tomatoes into juice and puree increased the levels of free quercetin in the tomato-based products; this may be attributed to the hydrolysis of rutin and other quercetin conjugates (Stewart *et al.*, 2000). For soy products, e.g. defatted soy (minimal heat during processing), the major isoflavone present is the malonylglucoside conjugates. When the processing temperature is increased to 100 °C (manufacture of soy milk, tofu, and soy molasses), the isoflavone β-glucosides are mostly present. In the production of fermented soy foods (tempeh), the reduction of the malonylglucosides and the concomitant increase of the unconjugated aglycones (genistein and daizein), is observed as fermentation time increases (Barnes *et al.*, 1994; Kwon *et al.*, 2010). In conclusion, while heat processing produces diverse effects on these phenolic compounds, fermentation does seem to have a positive effect in altering their structural composition, which in turn may have an impact on the bioavailability and bioefficacy of these compounds.

1.2.2 Effect of processing on the bioavailability of phenolic compounds

The biological effects or bioefficacy of phenolic compounds is dependent on their bioavailability once consumed (Scalbert and Williamson, 2000; Rein *et al.*, 2013). The factors that play a major role in the bioavailability of phenolic compounds include the concentration within the cell wall, variations in cell wall structure, location of glycosides in cells, molecular structure and the binding of compounds within the food matrix (Parada and Aguilera, 2007). Most polyphenols present in food substances occur as glycosides,

esters or polymers which cannot be easily absorbed. Before glycosylated polyphenols can be absorbed, it is necessary for the sugar moiety to be removed by enzymes present in the gastrointestinal mucosa, or in the colonic microflora, or through food processing (Scalbert and Williamson, 2000). The role food processing plays in altering the bioavailability of glycosylated polyphenols is demonstrated in the manufacture of tomato puree, where increased levels of free flavonols was reported due to hydrolysis and extraction from the food matrix (Stewart *et al.*, 2000). Similarly, during fermentation of soy foods, the breakdown of glycosylated isoflavones by microorganisms into the aglycones may be beneficial, as it has been demonstrated that the isoflavone aglycones of soymilk absorb more efficiently and in greater amounts than their glucosides in humans (Kano *et al.*, 2006). Furthermore, in cases where polyphenols occur as esters bound to hemicellulose or as large molecular weight compounds (proanthocyanidins or hydrolysable tannins), their bioavailability is reduced as these ester-linked substitutions or polymerisation chain reactions have a marked effect on the biological properties of the polyphenols (Scalbert and Williamson, 2000). Thermal processing has proven to be effective in such cases as observed in the release of ferulic acid during extrusion processing of cereal grains (Zielinski *et al.*, 2001), and in the breakdown of hydrolysable ellagitannins into ellagic acid during processing of jam from strawberry (Häkkinen *et al.*, 2000).

1.2.3 Future direction of food processing

Existing data on the effect of processing on phenolic compounds shows that food processing plays a significant role when it comes to bioaccessibility and bioavailability of polyphenols. High temperature processing which ensures food safety has both positive and detrimental effects on phenolic compounds. The reviewed literature also suggests that if food processing is judiciously applied, it can serve as a means of improving

bioavailability of polyphenols through structural modification or breakdown of the parent compound.

Therefore, food processing research should be tailored towards optimisation of both thermal and non-thermal methods that have the potential of retaining, releasing (bioaccessible) or at best transforming these compounds into more bioavailable forms (Nayak *et al.*, 2015). Fermentation as a non-thermal processing technique, seems to impact positively on the bioavailability of phenolic compounds. Since one of the main objectives of this work is to make wine from *H. sabdariffa*, section 1.3 reviews the process of wine-making along with the changes that occur in the phytochemical profile and its relation to the bioactivity and quality attributes of the product.

1.3 Wine: A complex mixture of bioactive and aroma compounds

Wine in the strictest term is the alcoholic fermentation of grape juice. However in recent times, wine has been produced from other fruit and flowers and is designated by the name of the raw material (e.g. apple wine, pomegranate wines, elderberry wine etc.). The four leading producers of wine are France, Italy, Spain and the U.S.A, with 60 % of the world's wine produced in these countries (Cholette *et al.*, 2005). The process of turning grapes into wine is called vinification which involves the conversion of sugars in grapes (glucose and fructose) by enzymes from yeast into alcohol and carbon dioxide in approximately equal proportions with the liberation of heat.

1.3.1 Chemical composition of grapes and wines

Grape juice when freshly squeezed contains about 70 to 80 % water and other dissolved solutes. These dissolved substances from the winemaking perspective include; sugars, polysaccharides, ethanol, acids, phenolic compounds, minerals and aroma compounds (Margalit, 2012).

1.3.1.1 Sugars

A large portion of soluble solids in grapes is sugar with the sugar content of ripe grape varying between 150 to 250 g/L. The main sugars in grapes are glucose and fructose with glucose predominating during the unripe stage and in equal ratio when mature, however when the grapes are overripe, fructose levels become higher (Soleas *et al.*, 1997). Sucrose is rarely found in *Vitis vinifera* grapes, while other sugars are found in insignificant proportions. The standard wine yeast, *Saccharomyces cerevisiae* (*S. cerevisiae*) uses glucose and fructose for its metabolic processes but has limited ability to ferment other substances. The residual sugars in wine generally below 1.5 g/L, consists of unfermentable pentoses like arabinose, xylose and rhamnose (Lonvaud-Funel, 1999).

1.3.1.2 Polysaccharides

Polysaccharides found in wine are derived both from cell walls of grapes or microorganisms (Vidal *et al.*, 2003). The grape-derived polysaccharides are the type II arabinogalactan-proteins (AGP), and rhamnogalacturonans (RG-II), while those from yeast cell walls are mainly mannoproteins (Vidal *et al.*, 2004). Polysaccharides in wine play different roles in the quality of the finished product. They contribute to the mellowness of wines and impact on the organoleptic properties of wines owing to their interaction with polyphenols (Vidal *et al.*, 2004). In addition, they contribute to protein and tartaric stability and act as protective colloids (Boulet *et al.*, 2007). Their levels in finished wines range from 0.2–2 g/L.

1.3.1.3 Ethanol

Ethanol is the most abundant alcohol in wine and it is important to the stability, ageing and sensory properties of wine (Soleas *et al.*, 1997). The production of ethanol as fermentation progresses limits the growth of other microorganisms allowing yeast to

dominate during the fermentation process. It is also an important solvent for the extraction of grape phenolics and in particular tannins which play a role in wine colour and taste. The stability of wine during storage is due to the inhibitory activities of ethanol, the wine acidity and added potassium metabisuphite. Ethanol concentration in table wine ranges from 10 - 14 %, v/v (Fischer and Noble, 1994).

1.3.1.4 Acids

The main acids in grape are tartaric, malic and citric acids. They are the main determinant of the pH of wine which affects the colour, microbial and chemical stability of wines. They also influence the tartness (sourness) of wine. Apart from the acids present in grape, the combined metabolism of yeasts and bacteria during fermentation generates other by products (volatile acids) which contribute to the pool of wine acids. Succinic acid is the major acid produced by yeast during fermentation, while lactic is mostly from the breakdown of malic acid by lactic acid bacteria (Volschenk *et al.*, 2006). Another group of acids found in wine are the volatile acids of which acetic acid is the most significant, being responsible for the sour vinegary taste in wines. The other volatile acids present in lower concentrations include formic, butyric and propionic acids. The titratable acidity in wine is generally within 5.5 to 8.5 g/L (Rankine *et al.*, 1971).

1.3.1.5 Phenolic compounds

Phenolic compounds are important constituents of grapes and wines as they play a vital role in influencing wine colour and flavour. Phenolic compounds take part in browning reactions in grapes and wines and are also involved in the ageing and maturation of wines (Fernandez-Zurbano *et al.*, 1995; Kallithraka *et al.*, 2015). Wine phenolics which are discussed in section 1.3.3 include the non-flavonoids: hydroxycinnamic acids, hydroxybenzoates and the stilbenes. The flavonoids consist of the flavan-3-ols, flavonols

and the anthocyanins. The anthocyanins and flavonols are predominantly located in the skins, whereas the flavan-3-ols (catechins) and leukoanthocyanins are located mainly in the seeds and stems. White wines are usually fermented with little skin or no contact and the phenolic content ranges from 96 - 260 mg/L. For red wine, they are produced in contact with skins and seeds and the phenolic content varies between 938 - 3659 mg/L (Stratil *et al.*, 2008)

1.3.2 Grape processing and vinification

The process of turning grapes into red wine involves a series of processing stages which is summarised in Figure 1-8. This section briefly elaborates on the unit operations involved during the wine-making process.

1.3.2.1 De-stemming

The most common practice after harvesting of grapes is to separate the berries from the leaves, stem and grape stalks. This is to prevent the excessive uptake of phenols and lipids from vine components. Stem phenols generally produce more astringent and bitter tastes than the phenols released from the skins and the seeds (Del Llaudy *et al.*, 2008). The grapes are crushed immediately after de-stemming and the crushed fruit consisting of pulp, skin and seed, called must is transferred to a container and SO_2 is added to prevent the growth of unwanted microbes.

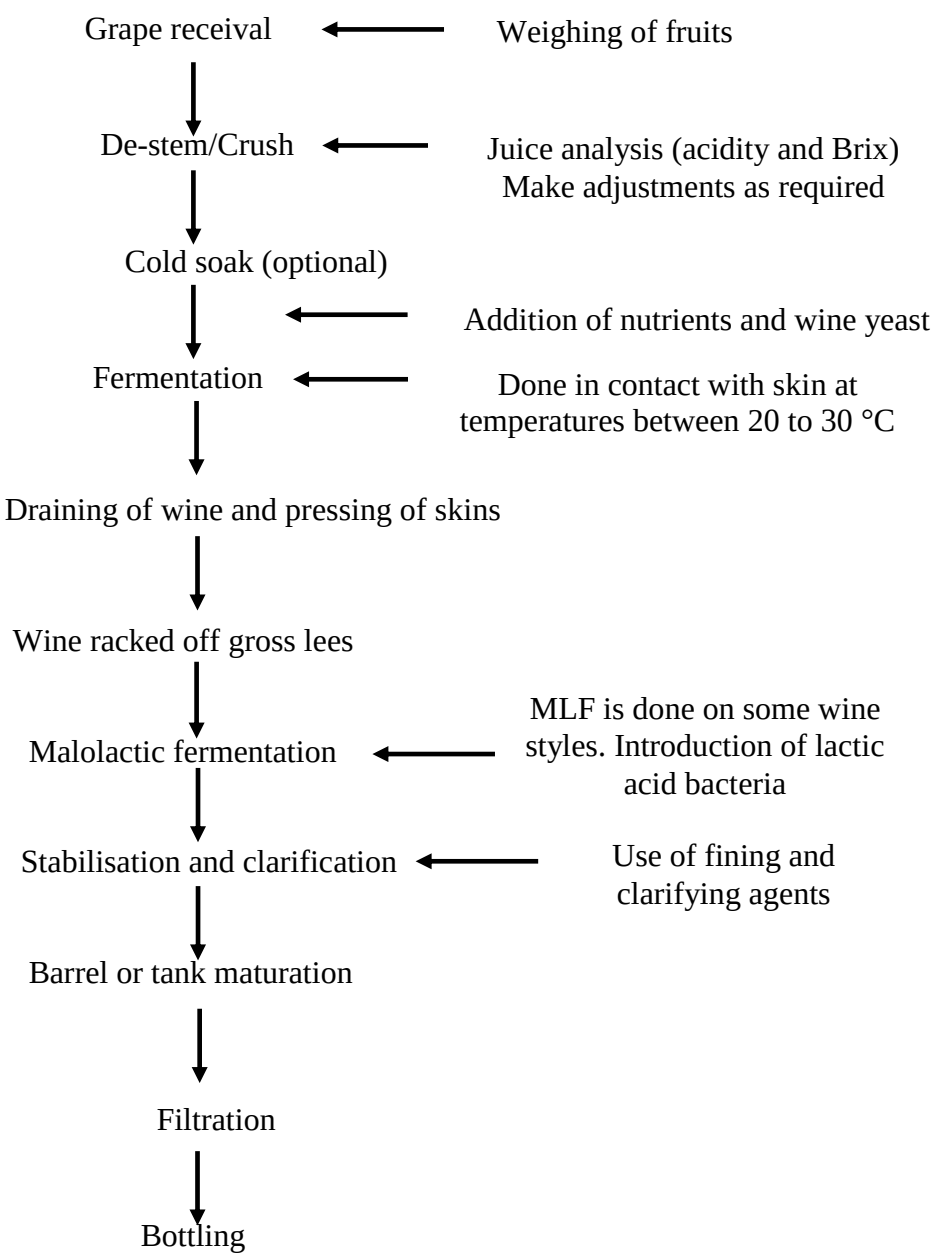


Figure 1-8 Flowchart for red wine production.

1.3.2.2 Must adjustment and treatment

Grapes are generally harvested at 22 to 24 °Brix (Brix indicates the percent of sugar by weight in grams per 100 mL of unfermented juice) for red wine production. However, some grape varieties may not have enough sugar and sugar may be added to the must. In addition, if the grape is low in acidity (less than 5 g/L) then the acidity should be raised by the addition of tartaric acid (Christaki and Tzia, 2002). The process of adjusting the sugar and acid ratio is termed juice amelioration. In order to facilitate the release of pigments, tannins and polysaccharides, commercial preparations of pectolytic enzymes may be added to the must (Romero-Cascales *et al.*, 2012). Thereafter, the addition of diammonium phosphate and yeast nutrients containing essential vitamins is recommended to prevent the formation of H_2S and ensure a clean fermentation (Marks *et al.*, 2003).

1.3.2.3 Fermentation

During the primary fermentation of wine, grape sugars, primarily glucose and fructose are converted to alcohol (ethanol) by the fermenting yeast. The by-products of primary fermentation are aromas and flavours, carbon dioxide, and heat (Sablayrolles, 2009). The fermentation is an exothermic process which implies that the temperature of the fermentation vessel will rise, and will require action to cool it down in order to prevent the development of unwanted flavours. Red wine are generally fermented between 21 - 30 °C, while for white wines, the fermentation is usually conducted in the range of 8 - 14 °C (Butzke and Singleton, 2007; Margalit, 2012).

1.3.2.4 Pressing

The must is fermented until it is reaches dryness, i.e. all the fermentable sugar is used up. Thereafter, the must is pressed according to the desired wine style when an optimum

amount of colour, tannins flavour and other constituents have been extracted. After pressing, the young wine is transferred to a secondary container and the fermentation is allowed to finish (if unfermented sugar remains).

1.3.2.5 Malolactic fermentation

Malolactic fermentation (MLF) is a biological process that involves the conversion of dicarboxylic L-malic acid (malate) to the monocarboxylic L-lactic acid (lactate) and carbon dioxide. The benefits of MLF include the reduction in wine acidity, addition of flavour and bacterial stability of the product. MLF also referred to as secondary fermentation usually occurs at the end of alcoholic fermentation by yeasts, although it sometimes occurs earlier. This process is normally carried out by lactic acid bacteria (LAB) isolated from wine, with *Oenococcus oeni* being the preferred strain (Knoll, 2011). In some cases, MLF is allowed to occur spontaneously, however it may expose the wine to other spoilage LAB species. The limiting factors on MLF include total and free sulphur dioxide (SO_2) concentration, alcohol content, with the most crucial being the pH, and temperature (Malherbe *et al.*, 2007). Generally MLF requires a must pH of ≥ 3.2 and a temperature range of 20 to 25 °C.

1.3.2.6 Wine clarification and racking

Young wine is generally cloudy and turbid due to the presence of particles in suspension. The particulate matter includes grape fragments, crystalline compounds, colloidal compounds and yeast lees (Butzke and Singleton, 2007). The first clarification is usually carried out by sedimentation where the wine is left for the solids to settle out, after which the wine is racked off the solid lees by pumping or gravity. In addition, during storage, many of the particles settle to the bottom leaving the wine clearer. However in order to

achieve greater clarity, wines are sometimes subjected to fining using different clarification agents.

1.3.2.7 Wine fining

The purpose of fining is to assist in creating a product, which is almost perfect in terms of colour, clarity, taste and bouquet (Castillo-Sánchez *et al.*, 2008). Red wines are rich in pigments and phenolic compounds such as tannins that confer on wine a harsh and astringent taste. The concentration of these wine constituents can be reduced by the addition of reactive and/or adsorptive substances. Generally, fining agents can be classified under the following groups (1) proteins, e.g. casein, gelatin, isinglass and albumin, (2) clays, e.g. bentonite, kaolin, (3) synthetic polymers, e.g. polyvinylpolypyrolidine (PVPP), (4) polysaccharide, e.g. agar, gum arabic and (5) carbons, e.g. activated charcoal (Soleas *et al.*, 1997).

1.3.2.8 Wine ageing and maturation

Wine ageing refers to a group of reactions that tend to improve the taste and flavour of a wine over time. The term wine "maturation" refers to changes in wine after fermentation and before bottling (Garde-Cerdán and Ancín-Azpilicueta, 2006). The intricate interaction between the acids, sugars, alcohols, esters and phenolic compounds is what brings about the modification of the aromas during the ageing process. The containers used for wine storage in cellars include wooden barrels, stainless steel tanks and in some cases bottles. For wines aged in bottles, a very humid environment produces mould growth on the cork, which may contaminate the wine and when the humidity is too low, the cork will dry out and crack exposing the wine to oxidation. Ideally, the best environmental condition for ageing of wine is a humidity of around 70 % and a temperature range of 15 to 20 ºC (de Simón *et al.*, 2010; Scrimgeour *et al.*, 2015).

1.3.2.9 Wine filtration and bottling

Filtration of wines is done using membrane filters made from cellulose acetate, cellular nitrate and polysulfone. They have controlled pore sizes and can be set up so that the flow is either perpendicular to and through the membrane or parallel to the surface also referred to as cross flow filtration (El Rayess *et al.*, 2011). These membranes rely on their pore size to exclude larger particles like microorganisms, proteins, gums and tannins (Umiker *et al.*, 2013).

1.3.3 Wine phenolics and health benefits

Wine phenolics can be grouped into two categories, the flavonoids and non-flavonoids. The flavonoids include the anthocyanins (e.g, malvidin 3-*O*-glucoside), flavan-3-ols (e.g., catechin), and flavonols (e.g., quercetin). The non-flavonoids present in wine are hydroxycinnamic acids (e.g., caffeic acid), hydroxybenzoic acids (e.g., gallic acid), stilbenes (e.g., resveratrol) and hydrolysable tannins, which are ester linked oligomers of gallic or ellagic acids.

The French paradox first mentioned in 1992 by Prof. Serge Renaud in the Lancet could be defined as the protection from cardiovascular diseases by moderate consumption of alcohol and in particular red wine (Renaud and de Lorgeril, 1992; Biagi and Bertelli, 2015). Since then the debate as to whether the protective effect of red wine on health is due to the non-alcoholic fraction, mainly polyphenols and/or ethanol is still far from concluded (Artero *et al.*, 2015). Notwithstanding, a number of studies have supported the notion that phenolic compounds present in wine have a major role in the health-promoting effects of moderate wine consumption (Soleas *et al.*, 1997; Chiva-Blanch *et al.*, 2013).

1.3.3.1 Inhibition of α-amylase and α-glucosidase by wine phenolics

As mention earlier (section 1.1.6.1), α-amylase and α-glucosidase are the key enzymes involved in the breakdown of starch and intestinal glucose absorption respectively. Therefore, the inhibition of these enzymes can help control postprandial rise in glucose levels after consuming carbohydrate rich foods. Red wine phenolics (RWP) when tested for their inhibitory effect on carbohydrases did not inhibit porcine α-amylase but strongly inhibited α-glucosidase (Kwon *et al.*, 2008). The results also revealed that wine samples with higher levels of gallic acid, caffeic acid and quercetin were more potent in the inhibitory activities. However, McDougal *et al*. (2005) reported the inhibition of salivary α–amylase by RWP extract, attributing the activity to the tannins in the wines samples. The discrepancies in the results can be due to assay conditions under which the experiments were done and also to the phenolic profile of the wine samples. As observed in a study (El Rayess *et al.*, 2011), most of the phenolic compounds present in wine have been reported to be in *H. sabdariffa* extract. Therefore, it is expected that the health benefits attributed to wine can be somewhat interrelated with *H. sabdariffa* wine.

1.3.4 Wine quality parameters

Wines are known by their colour, brightness, aroma and taste and a good wine is judged by its strength, beauty, fragrance, coolness and briskness (Margalit, 2012). In other words, consumers rate wine from the standpoint of its appeal to human sight, olfactory and taste system.

1.3.4.1 Wine colour

Colour is one of the attributes of a red wine which is relatively easy to assess and which is generally associated with quality. In mature wines, the colour is stabilised by co-pigmentation and polymerisation reactions. Co-pigmentation reactions can take place

through intramolecular interactions, in which case, an organic acid, an aromatic acyl group, or a flavonoid (or sometimes in combination) is covalently linked to an anthocyanin chromophore, or through loose intermolecular interactions, in which colourless flavonoids or other phenolic compounds react with weak hydrophobic forces with anthocyanins (Eiro and Heinonen, 2002). In the latter (phenolic compound) the behaviour results from the chemical phenomena known as charge-transfer complex formation or π-π interactions which occur when opposite charge compounds interact. Therefore, in the rings linked by the weak bond, the electronic density is transferred from the rich to the poor ring; since the flavylium ion in the anthocyanin is positively charged, it becomes a suitable compound for the formation of complexes by charge transfer with rich electron substrates. The other pathways in which co-pigmentation occurs are: (1) self-association if the co-pigment is an anthocyanin, (2) a complexation when the interaction is with a metal and (3) which is the most complex, when the co-pigmentation involves an aglycone, sugar and protons all at the same time (Castañeda-Ovando *et al.*, 2009). Co-pigmentation reactions determine the characteristic intense red-purple colour and has been suggested as the first step leading to the formation of polymeric anthocyanins which are the pigments present in aged wines (Gutiérrez *et al.*, 2005). During vinification and maturation, these conglomerates tend to dissociate freeing these anthocyanin molecules into the acidic wine environment resulting in the loss of their bluish colour; although a few co-pigmentation complexes may survive and form during fermentation (He *et al.*, 2012a). Furthermore during maturation, the individual anthocyanin molecule tends to lose their sugar and acyl constituents making them more susceptible to oxidation leading to browning. To limit these occurrences, it is vital that wines have significant quantities of flavonoids and condensed tannins to form stable polymers. The concurrent extraction of flavonoids with anthocyanins during fermentation

is crucial to long-term stability in red wine as these compounds begin to polymerise with the anthocyanins almost immediately (Jackson, 2009). Therefore, the red colour of wines is a reflection not only of the anthocyanins (amount, nature and state), but also the types and amounts of flavonoids extracted and retained during and after vinification.

1.3.4.2 Wine aroma and flavour

Amongst the various factors that contribute to the enjoyment of wine, its aroma and flavour are possibly the most important (Rapp and Mandery, 1986). The term aroma is normally associated with the smell of a young fresh wine, while the wine bouquet is used to describe older wines which are less fresh and more complex in their aroma notes (Swiegers *et al.*, 2005). Wine flavour is related to the sweetness, acidity, bitterness, saltiness and overall mouth-feel. These structural elements should be in balance and harmony as they are not assessed in isolation, but in relation to one another. The aroma of wine is derived from: (1) The aroma which originates from the grapes (or parent material) and modification during grape processing, (2) aromas produced by the activities of yeast and bacteria during fermentation, (3) the bouquet (from oak wood extraction and chemical reactions during storage) which results during the ageing of wines (Rapp and Mandery, 1986). The majority of volatile compounds derived from grapes responsible for the varietal aroma of wines are reported as constituents of the volatile fractions of many other fruits. These include the aliphatic alcohols, aldehydes, C13-norisoprenoids, ketones terpenes and benzene derivatives with their final concentrations dependent on factors such as the grape variety, culture practices, soil type, and climate (Slegers *et al.*, 2015). For instance, terpenes, such as linalool, geraniol and nerol, have been identified as compounds responsible for the flowery aromas of the Muscat grape variety (Karagiannis *et al.*, 2000). However, the substantial portion of wine aroma and flavour is derived from yeast fermentation which includes esters, higher alcohols, carbonyl compounds, sulphur

compounds, volatile phenols, amongst others (Figure 1-9). Furthermore, the grapery odour of the juice is superimposed by these fermentation aromas which then form the vinous associated with wine wines (Rapp and Mandery, 1986). During wine ageing (depending on the storage conditions), several chemical reactions occur influencing the composition of volatile constituents in wine and transforming the aroma into bouquet. For wines aged in oak barrels, aromatic elements of wood are extracted into the wine without dominating the wine character afterwards. In addition, decreases in acetates responsible for fruit like aromas occur gradually, until they approach equilibrium with their corresponding acids and alcohols. Although over 800 volatiles have been identified in wines, only a relatively small number of specific key aroma active compounds have been identified by sensory analysis (Aznar *et al.*, 2001). This is because the particular importance of each compound to the final aroma is related to its odour perception threshold, which is defined as the lowest concentration that can be detected by smelling (Table 1-5). Therefore, the concentration/threshold ratio, known as the "odour activity value" (OAV), measures the contribution of a specific compound to the aroma of a wine and also distinguishes between one wine style from another (Rocha *et al.*, 2004; Gómez-Míguez *et al.*, 2007). For example, when the aroma compounds of six premiums red wines in Spain were analysed, 40 odorants had odour activity (OAVs $\geq$ 1), while in another study on the volatiles of Chardonnay white wine in China, only thirteen compounds had OAVs $\geq$ 1 (Tao and Zhang, 2010). It is important to state that the calculated threshold values found in the literature of wine aroma compounds vary as they are influenced by the solvent involved, type of wine, presence of other aromatic compounds and the acuity of the taster during sensory evaluation of wine aroma.

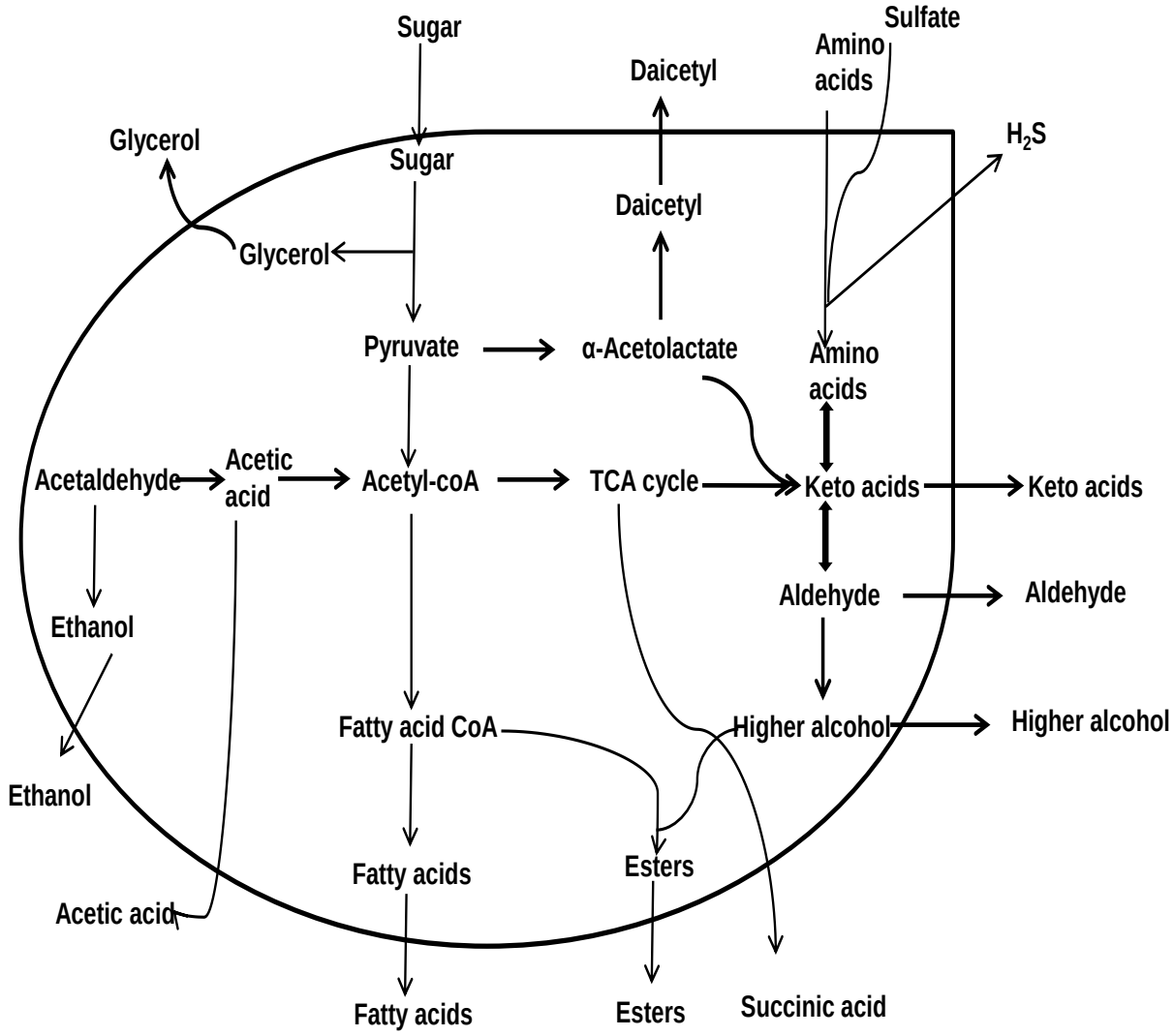


Figure 1-9 Schematic representation of the derivation and synthesis of flavour-active compounds from sugar, amino acids and sulphur metabolism adapted from Swiegers *et al.* (2005)

Table 1-5 Concentrations, aroma thresholds and descriptors of some odour–active compounds found in wines.

Compound	Concentration in wine (mg/L)	Aroma threshold (mg/L)	Aroma descriptors
Acids			
Acetic acid	100 - 1150	200	Sour, pungent
Octanoic acid	0.5 - 4.97	0.5	Sweat, cheese
Hexanoic acid	0.8 - 5.8	0.42	Sweat,
Decanoic acid	0.06 – 2	1	Rancid, fat
Acetates			
Ethyl acetate	22.5 - 63.5	7.5	Vinegar, nail, polish
Isoamyl acetate	0.1 - 3.4	0.03	Banana
Phenyl ethyl acetate	0 - 18.4	0.25	Rose, honey tobacco
Ethyl esters			
Ethyl hexanoate	0.03 - 3.4	0.05	Green, apple
Ethyl octanoate	0.05 - 3.8	0.02	Sweet, soap
Ethyl decaonote	0 - 2.1	0.2	Floral, soap
Ethyl butanoate	0.01 - 1.8	0.02	Floral, fruity
Alcohols			
Isoamyl alcohol	6.0 – 490	30	Whiskey, malt, burnt
2-phenyl ethanol	40 – 19.7	14	honey , spice rose
1-Hexanol	0.3 - 12.0	8	Green, cut grass
Butanol	0.5 - 8.5	150	Fusel, spirituous
Phenols			
Eugenol	0.001 - 0.015	0.006	Clove, honey
4-Ethylguaiacol	0.001 - 0.44	0.11	Spice, clove
4-Vinylguaicol	0.0014 - 0.71	0.01	Clove, curry
4-Ethyl phenol	0.012 - 6.5	0.44	Phenol, spice
Monoterpenes			
Linalool	0.0017 - 0.010	0.025	Rose
Geranoil	0.001 - 0.044	0.03	Rose-like
Citronellol	0.015 - 0.042	100	Citronella
Miscellaneous			
Diacetyl	≤ 5	2.8	
Acetoin	0.6 – 254	150	Butter, cream
Acetaldehyde	10 – 75	100	Sherry, nutty
2-3 Butanedoine	200 – 3390	0.1	Butter

The compilation is from the combination of published data from (Francis and Newton, 2005; Swiegers *et al.*, 2005; Jackson, 2009), The threshold values reported in the table are derived from either 10 % ethanol solution, red wine or synthetic wine.

1.3.5 Wine sensory evaluation

Sensory evaluation is a scientific discipline used to evoke measure, analyse and interpret reactions to the characteristic of foods and materials as they are perceived by the senses of sight, smell, taste, touch, and hearing (Dijksterhuis, 2008). In a competitive environment, it is important to base decisions about wine, new products and wine improvements on the best information possible. Furthermore, the effects of changes in processing conditions on sensory attributes can be measured and the impact of the processing decision weighed. The results of sensory evaluation can be interpreted statistically, providing a basis on which decisions can be made. Sensory analysis of wine involves a series of techniques to study wine attributes, how they are perceived, and how they relate to features such as chemical, varietal, regional, or stylistic origin. These include both quantitative and qualitative analysis.

Quantitative wine assessment usually entails two components either evaluation or analysis. Wine evaluation usually involves the differentiation and/or ranking of wines. It can vary from consumer tastings, intended to assess purchaser preference, to trained panels designed to assess differences generated by experimental treatments or to choose the formula to blend (Jackson, 2009). In contrast, wine analysis involves detailed investigation of a wine's sensory attributes. Sensory evaluation may or may not use trained panels, whereas sensory analysis essentially uses panels specifically trained for the task (Jackson, 2009). Quantitative wine analysis is primarily a research/development tool, therefore it is essential that the assessment be conducted in a quiet, neutral environment, devoid of taster interaction or prior knowledge of the sample origin. For qualitative wine analysis, the emphasis is on the expression of the wine's varietal, stylistic, regional or artistic attributes. The method of fermentation or processing usually stamps the wine with a distinct set of aroma attributes. This analysis can be quite daunting

as most wines do not readily express varietal or regional character. Despite these difficulties, and possibly because of them, wine provides much pleasure and intrigue and provokes an endless source of discussion (Jackson, 2009).

1.4 Justification of the study

Increased interest in human health, nutrition and disease prevention has enlarged consumers' demand for functional foods including fruits and their products such as wine (Rupasinghe and Clegg, 2007). The literature reviewed (section 1.1.6) revealed that *H. sabdariffa* contains a myriad of beneficial phytochemicals that may well reduce risk factors associated with type 2 diabetes and cardiovascular diseases. Despite these perceived benefits, the processing of *H. sabdariffa* into a range of functional foods is yet to be fully exploited. Furthermore, under-utilisation of this botanical elixir has led to increased postharvest losses (particularly in developing countries) justifying the need for alternative processing techniques like wine production. During winemaking, both fermentation and subsequent ageing entail the transformation of native phenolic substances into secondary metabolites able to have an impact on the quality of the final product (Ginjom *et al.*, 2011). Furthermore, phenolic compounds also contribute greatly to the organoleptic qualities of wine by modifying colour and flavour, and consequently influencing consumer preferences. Indeed, processing and storage conditions play a vital role in wine colour, aroma and taste and these conditions should be monitored for product quality, particularly if hibiscus wine is to be marketed as a novel functional fruit wine. In addition, wine quality sensory evaluation aimed at understanding the effect of processing conditions on the final product quality is needed. In the light of the above, research into the phytochemical composition and aroma profile throughout the winemaking stages will provide information that will help improve the wine processing, establish quality

parameters, relate it to sensorial and organoleptic properties of the wine and finally promote health beneficial research (Mena *et al.*, 2012).

1.5 Hypothesis and research questions

Statement of the problem: This research work seeks to investigate the effect of processing conditions on bioactive compounds, aroma and sensory profile of wine from *Hibiscus sabdariffa*.

General research questions:

- *Can fermentation alter the bioavailability of polyphenolic compounds in wines processed from H. sabdariffa?*
- *What is the resultant effect of fermentation and ageing on the bioactivity of polyphenols in H. sabdariffa extract?*
- *What is the impact of wine ageing temperature on volatile compounds?*
- *Will the wine processing condition have an impact on the sensory rating of the wine samples?*

Hypothesis:

1. *The fermentation of hibiscus juice into wine will transform the phenolic compounds into more bioavailable forms that may influence the biological activity.*
2. *Wine aged under lower temperature will retain higher levels of phytochemicals that will have a positive impact on sensorial qualities of the wine*.

Chapter 2 General analytical procedures

This chapter describes the general analytical procedures that apply to more than one chapter.

2.1 Chemicals

Commercial standards of delphinidin 3-*O*-sambubioside, cyanidin 3-*O*-sambubioside, delphinidin 3-*O*-glucoside, rutin, quercetin, protocatechuic acid, 3-*O*-caffeoylquinic acid (Extrasynthase 49915 and a gift from Mike Clifford), catechin and quercetin 3-*O*-glucoside (all HPLC grade) were purchased from Extrasynthese (France), while 5-*O*-caffeolquinic acid (5-CQA) (Sigma C3878), trifluoroacetic acid, gallic acid, caffeic acid, caftaric acid, Folin-Ciocalteu's reagent and ethanol were from Sigma Aldrich (USA). Sucrose, citric acid, malic acid, maleic acid, oxalic acid, tartaric acid and succinic acid were from Sigma-Aldrich (UK); while glucose, fructose and sodium carbonate were from Fisher Scientific (UK). All other standards used in the study were either analytical grade, HPLC or GC grade.

2.2 Planting and harvesting

Although Nigeria lies wholly within the tropical zone, there are wide climatic variations in different areas of the country. In southern Nigeria, there are two distinct seasons: the raining season that starts in March and lasts till the end of July, with a peak period in June. The other season referred to as the dry season starts around October and lasts till around mid-March with a short dry season generally experienced in August which lasts for about 3 - 4 weeks. In this study, the planting of *H. sabdariffa* seeds was carried out during both seasons.

Three varieties of *H. sabdariffa* seeds (dark red, light red and white variety) were planted in mid-July, 2013, in the nursery and transplanted as seedlings to the field in August,

2013, at the National Horticultural Research Institute (NIHORT), Ibadan, South-Western Nigeria. Harvesting was done in mid-December, 2013, after which the calyces were allowed to dry in a storage chamber with temperatures between 28 - 30 °C. Samples were then vacuum-packed, transported to the UK and stored in a freezer at -20 °C. The time of planting described above falls into the dry season. A second planting of only the seeds of the dark red variety was done in early March, 2014 which signifies the commencement of the raining season under the same conditions. The samples were harvested at the beginning of August, 2014, and processed under identical conditions as samples for the dry season.

2.3 Sample preparation of *H. sabdariffa* varieties

H. sabdariffa extracts for phytochemical analysis and inhibition of starch digestion were prepared by weighing out 2 g of each variety and grinding them into a powder with the use of a pestle and mortar. Extraction was done using 100 mL of distilled water. The sample was immediately transferred to a water bath (GLS Aqua 12 plus) at 50 °C and extraction was carried out for 30 min with stirring intermittently. The sample was then centrifuged (2500 g; 10 min), filtered through a Whatman no.1 filter paper and used for the analysis. The extraction was repeated in triplicate.

2.4 Colour analysis

2.4.1 Principles of the analysis

Spectrophotometers play an important role and are an essential tool for any wine analysis. Using light absorption measurements, spectral data can be used to predict the different stages of wine fermentation and the subsequent ageing. This is done by placing wine samples in a cuvette and ultraviolet or visible light at a certain wave length transmitted through the sample (Giusti and Wrolstad, 2001). In this study, three different spectral

measurements were determined which were the colour density, hue tint and the polymeric anthocyanins. The colour density is a measure of the addition of the absorbance both at 420 nm and 520 nm and in some cases measurement at 700 nm is taken to account for errors arising from the turbidity of wine samples. The hue tint (wine colour hue) measures the ratio of the absorbance at 420 nm and 520 nm and is used to interpret colour changes that occur during ageing of wine (shift from purple red to brown tones). The polymeric colour measurement is done after the addition of excess bisulfite to wine samples and the principle of the reaction is that polymeric anthocyanins, responsible for aged wine colour are resistant to the bleaching effects of SO2 (Birse, 2007). The limitation of the colour measurement's derived from spectrophotometric readings is that the results cannot be translated to human colour measurement. This limitation is addressed with the use of the colorimeter which describes all colours visible to the human eye. In the CIELAB (Figure 2-1) 1976 colour space (Hernández *et al.*, 2011), L* is the approximate correlate of the perceived attribute of lightness. Coordinate a* records positive values for reddish colours and negative values for greenish colours. The coordinate b* records positive values for yellowish colours and negative values for bluish colours. L*, a* and b* form a rectangular coordinate system in a three-dimensional, approximately uniform colour space and any point in this colour space can be also defined by the cylindrical coordinates L*, c* and hab. The c* and hab coordinates are the approximate correlates with the perceived attributes of chroma and hue respectively and they are the polar coordinates in the a*and b* plane defined by:

$$c* = \sqrt{(a*)^2 + (b*)^2} \qquad (1)$$

$$\text{hab} = \arctan\frac{(b*)}{(a*)}. \qquad (2)$$

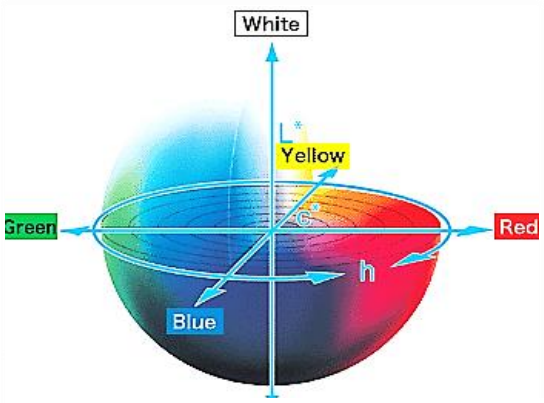


Figure 2-1 The L-*c * h colour. space.

In this study, the colour measurement L* (lightness), a* (redness), b* (blue to yellow), c* (chroma) and h* (hue angle) was performed using a colorimeter (Lovibond RT 100, Tintometer Series II, UK) calibrated with the instrument's standard white tile with XYZ colour scale values as (X = 81.5, Y = 85.8 and Z = 88.0). Samples (5 mL) were put in a Petri dish placed on a white tile and the parameters recorded in total transmittance mode, illuminant D65, and 10° observer angle. In addition to the rapid colour measurement, the following spectrophotometric wine colour parameters including: colour density (CD), hue tint (HT), polymeric anthocyanin (PA) were evaluated according to the method of (Giusti and Wrolstad, 2001) using a spectrophotometer (CECIL CE 3021 Series). An aliquot of sample (*H. sabdariffa* extracts or wine) (200 µL) was placed in a 1 mm pathlength cuvette and the colour indices were determined as follows:

$$CD = [(A420\ nm - A700\ nm) + (A520\ nm - A700\ nm)] \quad (3)$$

$$HT = [(A420\ nm - A700\ nm) +/ (A520\ nm - A700\ nm)] \quad (4)$$

$$\%\ PA = [(A420\ nm - A700\ nm) + (A520\ nm - A700\ nm)]\ SO2/CD \times 100 \quad (5)$$

2.5 Physicochemical analysis

The pH and titratable acidity (TA) was measured with the use of a pH meter (HANNA HI2211) calibrated with pH 4.0 and 7.0 buffer. A sample volume (extract or wine) of 10 mL was used, and the TA was determined by titration with 0.1 N NaOH until pH 8.1 and expressed as percentage of malic acid (g/100 mL) (Ramirez-Rodrigues *et al.*, 2011).

2.6 High performance liquid chromatography (HPLC) analysis

2.6.1 Principle of HPLC

HPLC has been a method of choice for the separation and quantification of polyphenolics in fruits and plant extracts. The separation of compounds by HPLC depends on the interaction of analyte molecules with the stationary phase and the mobile phase The chromatographic conditions commonly used for the separation of polyphenols by HPLC methods include the use of, almost exclusively, a reversed-phase (RP) C18 column; UV-Vis diode array detector, and a binary solvent system containing acidified water (solvent A) and a polar organic solvent (solvent B) (Ignat *et al.*, 2011). Solvent A usually includes aqueous acids or additives such as phosphates, while acidified methanol or acetonitrile are normally used as solvent B. The separation of compounds by RP-HPLC, is governed by the mixture of hydrophobic, polar and ionic interactions (Jandera, 2011). Consequently, method development aims at modulating stationary phase and mobile phase conditions to optimize these interactions and achieve a specific separation. Based on this principle, a RP-HPLC method was developed in the laboratory and optimised for the analysis of phenolic compounds in *H. sabdariffa*. The identification and quantification of phenolics in *H. sabdariffa* extracts was carried out using a UFLCXR system (Shimadzu) consisting of a binary pump, a photodiode array with multiple wavelength (SPD-20A), a solvent delivery module (LC-20AD) coupled with an online unit degasser

(DGU-20A3/A5) and a thermostat autosampler/injector unit (SIL-20A). The photodiode array detector was set at wavelengths of 265, 280, 320, 360 and 520 nm for the simultaneous detection of hydroxybenzoic acids, flavan-3-ols, hydroxycinnamic acids, flavonols, and anthocyanins respectively. A two phase gradient system consisting of 0.1 % (v/v) trifluoroacetic acid mobile phase (A) and trifluoroacetic acid/acetonitrile/water (50:49.9:0.1) mobile phase (B) was employed for the analysis. The gradient conditions were as follows: the initial condition started with 92 % A and was increased to 18 % solvent B at 3.50 min, 32 % B at 18 min, 60 % B at 28 min, reaching 100 % B at 32 min, held at 100 % B for 4 min, and returning to the initial conditions for 3.5 min for the next analysis. The chromatographic separation was performed on a Phenomenex Gemini C18 column (5 µm, 250 mm x 4.6 mm) at a flow rate of 1 mL/min. The temperature of the column was maintained at 35 °C and the injection volume was 10 µL. Stock solutions of individual phenolic compounds were made by dissolving known amounts in 70 % ethanol solution from which at least six different working concentrations within the range commonly reported in published data were prepared.

The identification of phenolic compounds in *H. sabdariffa* extracts was done based on comparison with standard phenolic compounds run under similar conditions in terms of the retention time, UV-visible spectrum, spiking of the sample with the corresponding standard phenolic compound and LC-MS data (section 2.7). With the use of the system software, the similarity index (SI) was used to match each phenolic compound.

2.6.2 Validation of the HPLC method for quantification of phenolic compounds

Validation of the HPLC method was carried out in terms of repeatability (inter-day and intra-day values), specificity, linearity and accuracy, in terms of recovery. Quantification of phenolic compounds was achieved using a minimum of six point regression graphs of the UV-visible data collected at the absorption maxima of the analytes investigated. In

the case of compounds for which standards were not available, they were quantified as equivalents of the most related member of its phenolic group. The limit of detection (LOD) was determined from the amount of analyte required to give a signal to noise ratio of 3 and the limit of quantification (LOQ) was defined as the amount needed to give a signal to noise ratio of 10 (Currie, 1995). Peak identification was based on comparison of retention times and spiking with authentic standards, while peak quantification was based on the external standard method. The concentration of compounds for which there was no pure reference available as well as unidentified compounds was approximated by using the same calibration graphs as one of the compounds with the most similar and relevant chemical structure. Gallic acid, 5-*O*-caffeolquinic acid (5-CQA) (Sigma C3878), caffeic acid, quercetin and catechin were used for quantitation of hydroxybenzoic acids, chlorogenic acid isomers, hydroxycinammic acids, flavanols and flavan-3-ols respectively.

2.7 Liquid chromatography mass spectrometry (LC-MS) analysis

2.7.1 Principle of LC-MS

One of the limitations of using HPLC-DAD or PDA detector method for analysis of compounds (polyphenols) is that there are cases where the UV-Vis spectra of two analytes may be very similar making the identification of compounds difficult. In addition, when the analysis involves complex matrices like plant extracts, the identification of compounds becomes nearly impossible using HPLC-DAD alone. The combination of the data from the DAD and a mass spectrophotometer are powerful tools for the identification of compounds in complex matrices (Cuyckens and Claeys, 2004). When an LC is connected to a mass spectrophotometer, the effluent from the LC enters into the interface where the mobile phase is evaporated and the compounds get ionised in an ionisation

chamber and are then directed to the mass analyser which sorts and separates the ions according to their mass to charge ratio (m/z value) for identification (Ignat *et al.*, 2011). In this study, the identity of polyphenols in *H. sabdariffa* was confirmed using a LC-MS method developed in the laboratory. A HPLC (LC-2010 HT) coupled with a 2020 quadrupole mass spectrometer (Shimadzu) fitted with an electro spray ionisation source (ESI-MS) operated in single ion monitoring (SIM) was used in positive mode for anthocyanins and negative mode for other polyphenols. The other operating parameters were detector -1.80 kV, DL temperature 250 °C and nebulizing gas flow and drying gas flow set as 1.50 and 15 L/min respectively. The mobile phase A was 0.5 % formic acid in water and mobile phase B was a mixture of acetonitrile/ water/formic acid (50:49.5:0.5). Formic acid was used as an acidifier instead of trifluoroacetic acid because of problems associated with ionisation of the latter. The flow rate was 0.5 mL/min and the total time of analysis was increased to 60 min to make adjustments for the change in flow rate. All other conditions were identical to the HPLC analysis described in section 2.6.1.

2.8 Total phenolic content analysed by Folin–Ciocalteau's method

2.8.1 Principle of Folin's assay

The Folin's assay measures the total reducing capacity of the sample and the principle behind the assay is that at alkaline conditions, phenolate anions reduce the yellow molybdenum in the Folin-Ciocalteu reagent to blue colour (Everette *et al.*, 2010). Polyphenols in plant extracts react with the Folin-Ciocalteu's reagent to form the blue complex that can be quantified by visible-light spectrophotometry (Berker *et al.*, 2013). The assay modified from (Singleton and Rossi, 1965) contained 1 mL of sample diluted with 80 % methanol solution (1:10), 5 mL of diluted Folin-Ciocalteu's phenol reagent

(1:10) and 4 mL of 75 g/L sodium carbonate solution. The mixture was then kept in a water bath (GLS Aqua 12 plus) at 26 °C and the absorbance reading measured at 765 nm with a spectrophotometer (Cecil 3000 series) after 2 h. The quantification of phenolic content was performed using gallic acid as standard.

2.9 HPLC-evaporative light scattering detector (ELSD) analysis of sugars

2.9.1 Principle of the ELSD

The ELSD works by nebulising the effluent from the LC and forming droplets in a gas stream. The mobile phase is then evaporated from the droplets and when the analyte is less volatile than the mobile phase, it remains in the gas stream as a dry solute particle which is exposed to the detector. The particles scatter the light beam and the amount of scattered light is a measure of the concentration of the analyte (Kroll *et al.*, 2000).

The UFLCXR system (Shimadzu) in section 2.6.1 for *H. sabdariffa* polyphenol quantification was attached to an ELSD and used for sugar identification and quantification. The analysis was performed under isocratic conditions with an analysis run time of 30 min using a Grace Davison Prevail Carbohydrate Es column (5 µm, 250 mm x 4.6 mm). The mobile phase was 75 % acetonitrile (v/v) delivered at a flow rate of 0.5 mL/min and the sample volume was 10 µL. The operating conditions of the ELSD were as follows: signal 0.00, gain 4, pressure 350 kPa, with the drift tube temperature set at 40 °C. Peak identification was based on comparison of retention times and spiking with authentic standards, while peak quantification was based on the external standard method.

2.10 HPLC determination of organic acids

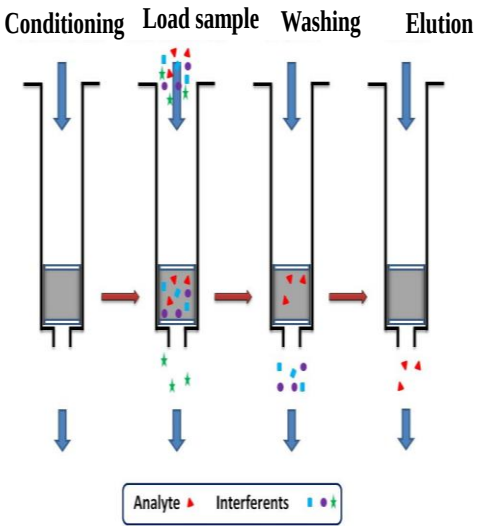
Organic acid determination in must and wines is important not only because it influences the balance of the flavour, but also the chemical stability particularly during the fermentation and ageing process. The complexity of *H. sabdariffa* extracts and wine

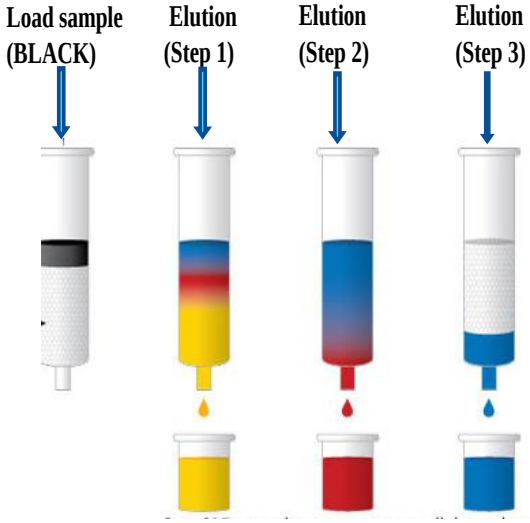
necessitates the use of some form of sample preparation (sample clean up) prior to the HPLC analysis to avoid interferences from sugars or colourants (anthocyanins), which could co-elute with organic acids (Mato *et al.*, 2005). This was achieved using solid phase extraction (SPE).

2.10.1.1 Principle of SPE

Solid phase extraction (SPE) is chromatography. It involves the selective partitioning of one or more components between two phases, one of which is a solid sorbent as depicted in Figure2-2. The organic acids in *H. sabdariffa* extracts and wine were analysed as follows: An aliquot of the sample (1 mL) diluted 1:5 with phosphoric acid (0.005 M) was passed through the cartridge (Oasis Max 60 mg, Waters Corporation Ltd, USA) previously conditioned with methanol (1 mL) and 0.005 M phosphoric acid (1 mL). The cartridge was washed with 1 mL of phosphoric acid (0.005 M) and the eluted organic acid fractions pooled together. The HPLC analysis was performed with the same equipment used for polyphenol quantification (section 2.6.1) using a Thermoscientific Acclaim TM Organic acid column (5 µm, 250 x 4.6 mm), with column oven set at 20 °C.

The chromatographic separations of the acids were achieved in 10 min under an isocratic procedure. The operating conditions were as follows: flow rate 0.5 mL/min, mobile phase 10 mM KH2PO4 (pH 2.6), injection volume 5 µL and the detection wavelength set at 210 nm.





SPE can be performed by:

(1) adsorbing matrix interferences while components of interest pass through the cartridge

(2) adsorbing components of interest while matrix interferences pass through the cartridge

SPE Manifold

Note: Different strength solvents can be used to separate the dyes on a single cartridge

Figure 2-2 Principle and operation of SPE.

2.11 Analysis of volatile compounds by head space-solid phase micro extraction-gas chromatography mass spectrometry (HS-SPME–GC-MS).

The aroma of wine is determined traditionally by liquid–liquid, solid–liquid extraction, and dynamic headspace (Torrens *et al.*, 2004). However, in recent years, solid-phase micro extraction (SPME) has been introduced to study wine flavour composition (Arthur and Pawliszyn, 1990).

2.11.1 Principle of SPME

Solid-phase micro extraction was developed to address the need to facilitate rapid sample preparation. The theory (Figure 2-3) consists of exposing a thin polymeric coating to the sample matrix (headspace) for a predetermined amount of time. SPME extraction is completed when the analyte concentration has reached distribution equilibrium between the sample matrix and the fibre coating. The advantages of using this technique are that it is relatively cheap, fast with higher sensitivity for the wine volatile compounds and the lower interferences because of the more polar substances. The selectivity and sensitivity of this technique depends majorly on the fibre composition. A wide range of commercial fibres can be found, these include polydimethylsiloxane (PDMS) which is most widely used. In addition, other fibres used in wine analysis include polyacrilate (PA) useful for the more polar compounds (aldehydes and acids), and carbowax (CAR)–divinylbenzene (DVB) which is employed to detect esters, acids, and volatile phenols

2.11.2 Optimisation of the HS-SPME conditions

The method was optimised by evaluating different extraction times, sample dilution and agitation, and by comparing the efficiency of the different fibres (PDMS/DVB and CAR/DVB/PDMS). The optimised conditions used for the analysis are in section 2.11.3.

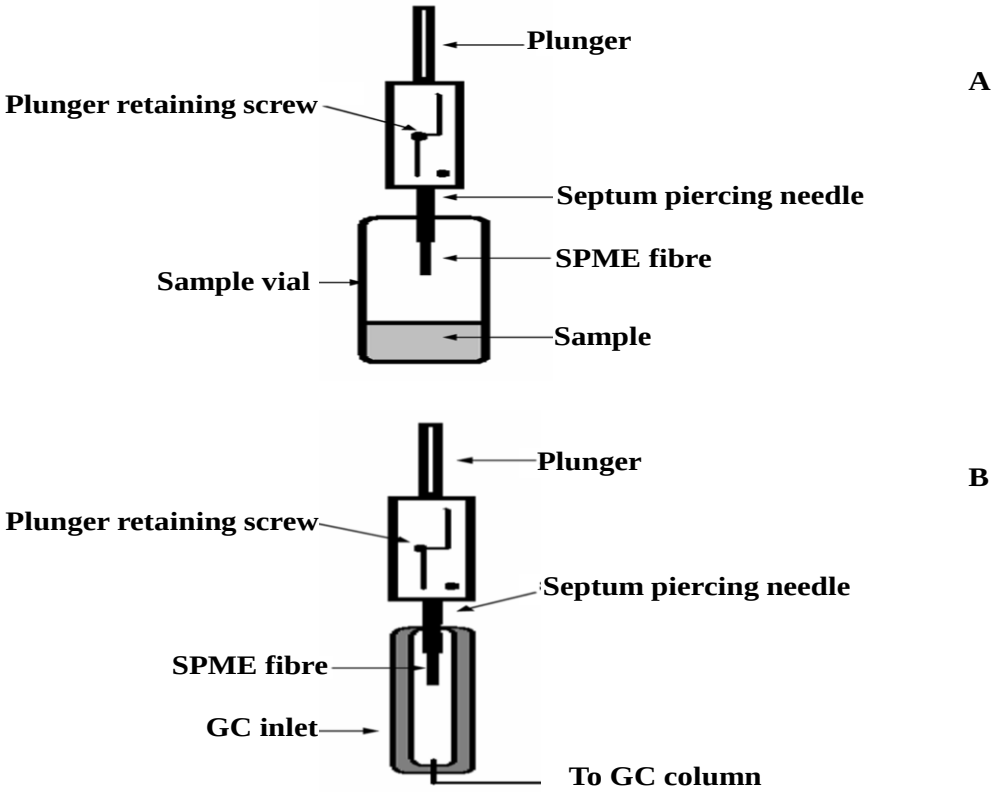


Figure 2-3 Principle and operation of SPME. (A) Fibre exposed to the head space of sample matrix and (B) Injecting volatiles extracted by fibre into the GC column).

Previously, it had been reported that the PDMS/DVB and CAR/DVB/PDMS were suitable for the extraction of a range of wine volatiles; consequently these two fibres were selected for the purpose of optimisation of the method of analysis (Rodríguez-Bencomo *et al.*, 2002; Torrens *et al.*, 2004; Carrillo *et al.*, 2006)

2.11.3 Extraction of headspace volatiles

Extraction of the headspace volatiles was performed with an automated SPME combipal equipped with a 65 µm PDMS/DVB fibre (Supelco, Bellefonte, PA., USA). An aliquot of sample (10 mL) and 2-octanol as internal standard (10 µL) were added to a 20 mL magnetic crimp sample vial. To facilitate the release of the volatile compounds, 1.5 g of NaCl was added to the sample vial which was then equilibrated for 30 min at 40 °C with

agitation on and off at 30 s interval. The SPME fibre was then exposed to the head space for 20 min and inserted into the GC injector to desorb the analytes.

2.11.4 Principle of GC-MS analysis

The GC works on the principle that a mixture will separate into individual substances when heated as they are carried through a column (stationary phase) with an inert gas. As the separated substances emerge from the column opening, they flow into the MS where they are bombarded by a stream of electrons causing them to fragment (electron ionisation). The identification of compounds is by the mass of the analyte molecule as each compound has a unique fingerprint and in addition, software is readily available to provide a library of spectra for unknown compounds.

2.11.5 GC-MS analysis

Volatile compounds were analysed in a GC (Varian 3800) equipped with an MS detector (Saturn 2200) and a CTC CombiPAL autosampler (CTC analytics, Zwingen, Switzerland). The separation was performed on a Phenomenex ZB-WAX column (30 m × 0.32 mm × 0.50 µm film thickness). The injector temperature was 250 °C and helium was the carrier gas set at a flow rate of 1.0 mL/min. The oven programme started at 40 °C (held for 10 min), increased to 100 °C at 15 °C/min (held for 5 min) and finally to 250 °C at 15 °C/ min with a final hold of 5 min. The MS was operated in electron ionisation mode (70 eV) and scanning was programmed for a m/z range of 29-300. Identification of volatile compounds was achieved by comparison with a reference standard, match spectrum from the NIST 2.0 library and fragmentation patterns for compounds reported in the literature.

2.12 Results and discussion

2.12.1 Validation of the HPLC method for analysis of polyphenols in *H. sabdariffa*

The precision of the method was evaluated by measuring the inter-day and intra-day repeatability of both retention time and peak area of a selected concentration of phenolic standards. Inter-day repeatability was assessed by analysis of the results of six determinations conducted within the same day with (n = 6), while intra-day analysis was carried out weekly over a month (n = 4). The results presented in Table 2-1 show that the % RSD for inter-day and intra-day variations in retention time was ≤ 0.25 % and ≤ 2.83 % respectively, while inter-day and intra-day variations for peak areas were lower than 0.96 and 3.66 respectively. The accuracy of the method was determined by spiking a representative class of phenolic compounds identified in *H. sabdariffa* extracts with three concentrations of protocatechuic acid as a representative of the hydroxybenzoic acids, caffeic acid for hydroxycinnamic acids and delphinidin 3-*O*-glucoside for anthocyanins. The % recovery calculated on the basis of the difference between total concentration in spiked and non-spiked samples ranged from 92.1 to 112.6 % (Table 2-2). Furthermore, the LOD and LOQ are also presented in Table 2.1.

Table 2-1 Validation of HPLC method for quantification of polyphenols in *H. sabdariffa.*

Analyte	Retention time (min) average	Intra-day variation RSD (%)	Inter-day variation RSD (%)	ʎ (nm)	LOD (mg/L)	LOQ (mg/L)	Linearity range (mg/L)	Calibration curve equation	Correlation coefficient
Gallic acid	6.18	0.06	0.53	265	0.325	0.98	0.5 - 100	y = 28935x - 24958	0.9992
Protocatechuic acid	9.01	0.05	0.91	265	0.083	0.25	0.5 - 100	y = 33614x + 2129	0.9999
Caftaric acid	12.17	0.25	0.89	320	0.2	0.6	0.5 - 100	y = 28370x + 405.3	0.9993
Caffeic acid	15.84	0.06	1.22	320	0.098	0.3	0.5 - 100	y = 54095x - 22632	0.9998
Delphinidin 3- *O*-sambubioside	14.02	0.13	1.71	520	1.08	3.28	10 - 500	y = 27350x - 98573	0.9997
Cyanidin 3-*O*-sambubioside	16.57	0.05	1.66	520	1.43	4.35	10 - 500	y = 28728x - 41143	0.9996
Delphinidin 3-*O*-glucoside	14.79	0.06	2.83	520	0.06	0.2	10 - 50	y = 31357x - 47122	0.997
Quercetin	32.8	0.007	0.71	360	0.39	1.2	5 - 100	y = 43119x - 53557	0.999

Table 2-2 Recovery analysis of after spiking samples of dark red *H. sabdariffa* with standard concentration of analytes.

Analyte	Concentration in sample (mg/L)	Amount added (mg/L)	Theoretical value after spiking (mg/L)	Amount recovered (mg/L)	% Recovery
Protocatechuic acid	3.47	10	13.47	15.17	112.62
		20	23.47	24.96	106.34
		30	33.47	36.08	107.79
Caffeic acid	4.9	10	14.90	14.06	94.36
		20	24.90	23.81	95.58
		30	34.90	32.71	93.69
Delphinidin 3-*O*-glucoside	5.96	20	25.96	26.56	102.31
		30	35.96	33.12	92.10
		50	55.96	61.00	109.00

Values are the means of three determinations (n = 3).

2.12.2 Linearity of HPLC-ELSD method for sugar analysis in *H. sabdariffa*

Sugars available in juices are fermented by yeast during the process of wine-making. Most fruits and some grape varieties do not have enough sugar in them, hence the need to adjust the sugar levels. A mixed standard solution of fructose, glucose and sucrose ranging from 100 mg/L to 2500 mg/L was used to generate the calibration curves for estimating the concentrations of these sugars in *H. sabdariffa* extracts and wine (Figure 2-4, 2.5 & 2.6). Although the method efficiently separated the analysed sugars, a slight disadvantage observed with the ELSD is the sensitivity and accuracy of the equipment particularly when the concentration of the analyte is low.

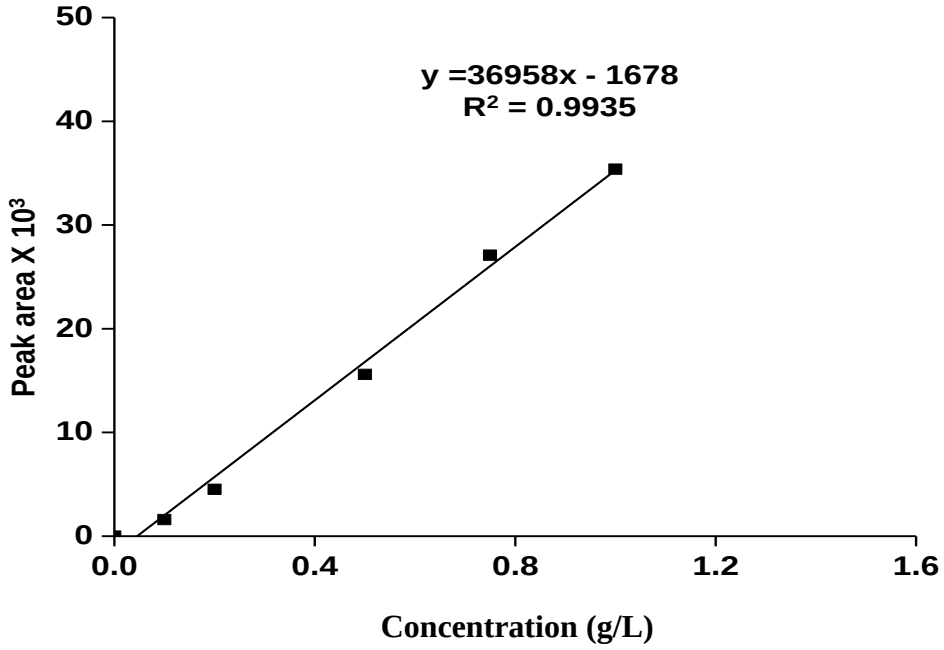


Figure 2-4 Calibration curve of fructose.

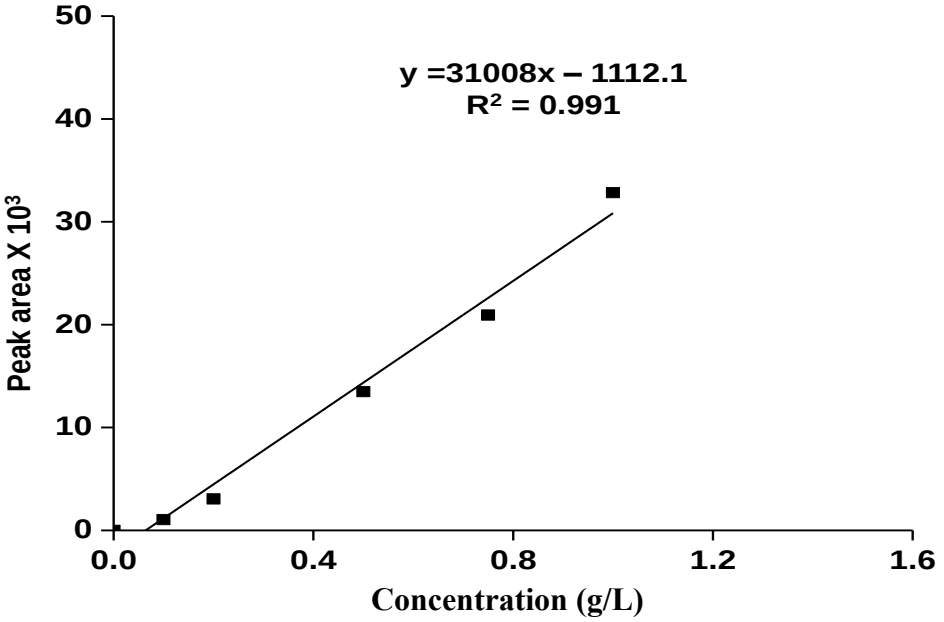


Figure 2-5 Calibration curve of glucose.

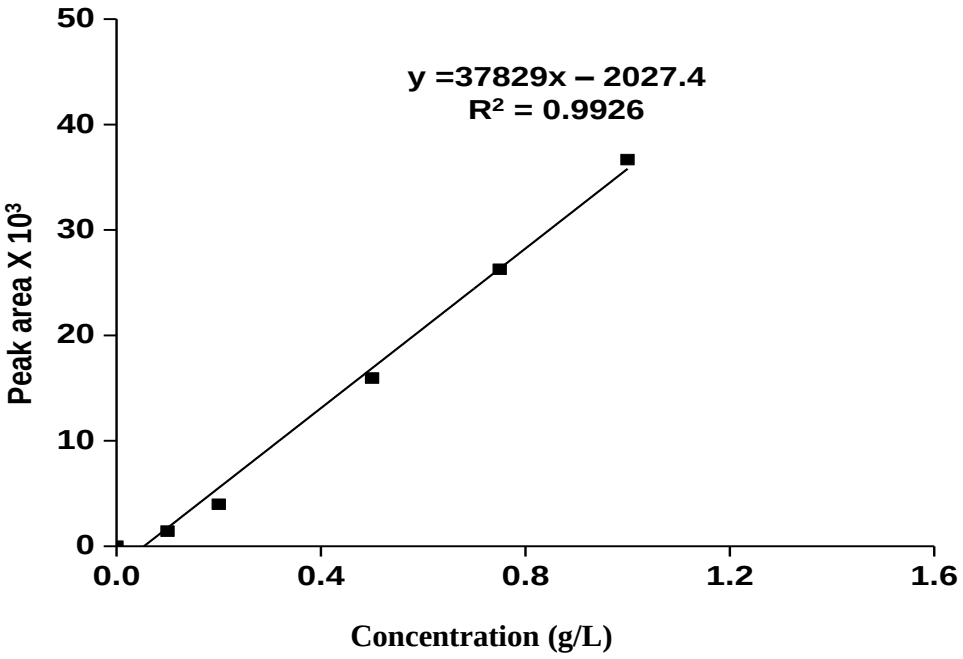


Figure 2-6 Calibration curve of sucrose.

2.12.3 Linearity of HPLC method for quantification of organic acids in *H. sabdariffa*

Firstly, a stock solution of mixed standards of the organic acids (tartaric, oxalic, malic, citric and succinic) was used to prepare different concentrations of the acids ranging from 100 to 1000 mg/L. Thereafter, to investigate the organic acids resulting from fermentation and ageing of roselle wine, a stock solution of acetic and lactic acid was also prepared in the same concentration range as the other organic acids. The correlation coefficient of the calibration curves used for estimating the quantity of these acids ranged from 0.9993 to 0.9999 (Table 2-3). An example of the chromatogram of the mixed standards of organic acids is presented in Figure 2-7. In order to resolve the peaks of oxalic and tartaric acid for accurate estimation of their peak areas, manual integration using the equipment

software was performed on the chromatograms and the resulting peak areas obtained from the manual integration were used for generating the standard curve calibration equation of these organic acids. To be consistent, the same manual integration parameter was also applied for estimating the concentrations of these organic acids in *H. sabdariffa* extracts and wine.

Table 2-3 Linearity range, calibration curve and correlation coefficient used for identification and quantification of organic acids in *H. sabdariffa* extracts and wine.

Analyte	Retention time (min)	Linearity range (mg/L)	Calibration curve equation	Correlation coefficient
Oxalic acid	2.8	0.5 - 100	y = 5455.5x + 11180	0.9999
Tartaric acid	2.9	0.5 - 100	y = 28935x – 24958	0.9995
Malic acid	3.3	0.5 - 100	y = 501.49x + 19291	0.9939
Lactic acid	3.6	0.5 - 100	y =322.19x +13188	0.9929
Acetic acid	3.8	0.5 - 100	y = 54095x – 22632	0.9938
Citric acid	5.4	0.5 - 100	y = 27350x – 98573	0.9998
Succinic acid	5.7	0.5 - 100	y = 28728x – 41143	0.9995

2.12.4 Results of optimisation of the HS-SPME/GC-MS method

The effect of the following analytical conditions (extraction time of 5, 10, 20 and 30 min, sample agitation/without agitation, and wine sample dilution (1:10)/without dilution) on the extraction of the main volatiles in roselle wines was determined by comparing the peak areas of volatile compounds under the different extraction conditions. The conditions which resulted in higher response of total area of wine volatiles were then chosen for the HS-SPME analytical conditions. Thereafter, the suitability of two different

fibres (PDMS/DVB and CAR/DVB/PDMS) for the targeted volatiles in the wine samples was evaluated by comparing their peak areas under the optimised conditions. The results presented in Table 2-4 showed that the PDMS fibre was more suitable for the targeted volatiles as the peak areas of the compounds were higher than the DVB fibre. Furthermore, the OAV known as the "odour activity value" (OAV), for roselle wine volatiles was calculated from the ratio of the concentration of the specific aroma compound in the wine samples to its threshold value reported in the literature.

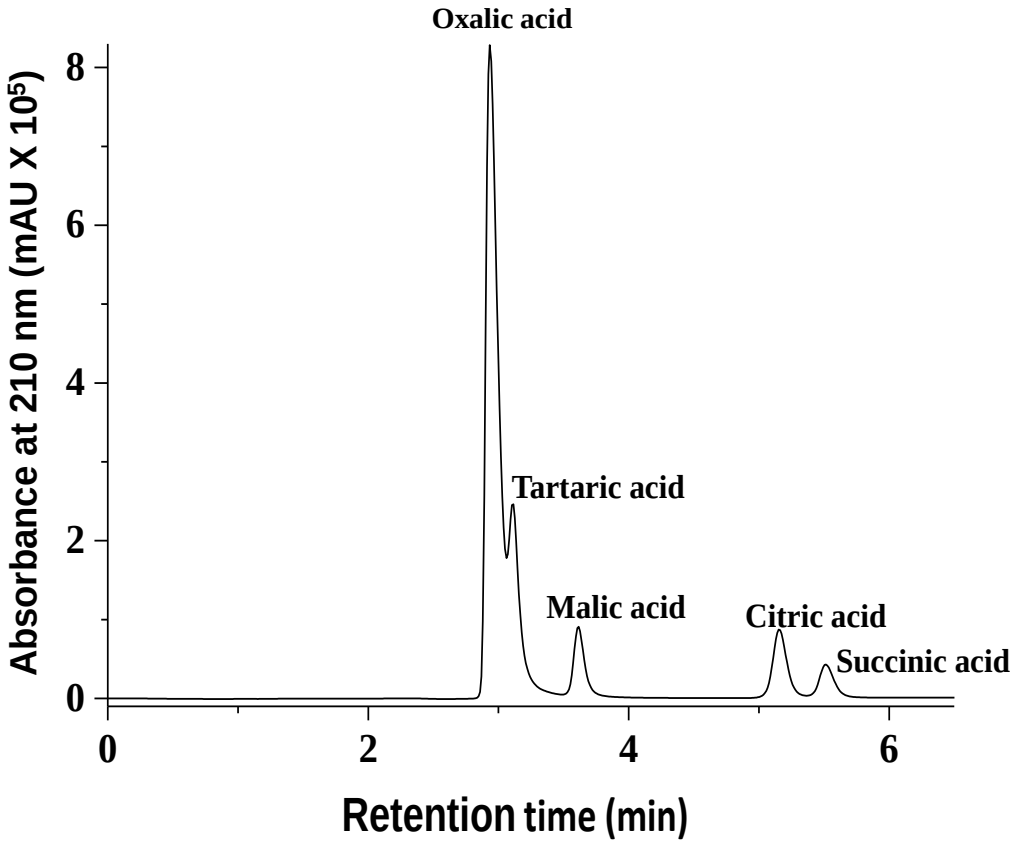


Figure 2-7 An example of HPLC chromatogram of mixed standards of organic acids.

Table 2-4 Efficiency of extraction of extraction of main volatiles in roselle wine using two commercial SPME fibres.

	Average peak area	
Analyte	PDMS	DVB
isoamyl alcohol	7784000	3967667
2- phenyl ethanol	863091	203878
ethyl hexanoate	918609	754820
ethyl octanoate	1117941	944284
ethyl decanoate	714628	206870

Values are the means of six determinations (n = 6).

2.13 Conclusion

The methods described in chapter 2 were all optimised and used for the experiments reported in chapters 3-5. They all gave good reproducibility as well as precision and were therefore suitable for the analysis carried out on *H. sabdariffa* extracts and wine.

Chapter 3 Phytochemical and physicochemical profile of three varieties of *H. sabdariffa* (Linn)

3.1 Abstract

H. sabdariffa is a potent source of various phytochemicals, but different methods can be applied to obtain the extracts. The variety and extraction conditions used contribute to the phenolic and physicochemical attributes of the extracts making comparisons between compositional data in the literature very difficult. The objective of this research was to compare the phenolic and physicochemical properties of three varieties of *H. sabdariffa* (dark red, light red and white/green) processed under identical conditions with the aim of selecting the ideal variety for manufacture of *H. sabdariffa* wine. Seeds of three *H. sabdariffa* varieties were planted and harvested in Nigeria. They were dried, vacuum packed and transported to the UK for further analysis. Hibiscus extracts were made from the three varieties under identical conditions using water as the solvent. Physicochemical properties were measured using a pH meter, colorimeter, and spectrophotometer, while polyphenols were identified and quantified with the use of the LC-MS and HPLC-PDA. Results of the phenolic composition of hibiscus varieties revealed that dark red *H. sabdariffa* contained the highest levels of anthocyanins, and flavonols, while the white variety alone contained flavan-3-ols. Furthermore, glucose was the main reducing sugar, while malic acid was the major organic acid in the three varieties. Regarding the physicochemical properties, the pH values of the extracts were ≈ 3.1, while colour density was highest for the dark red. The results from this study showed that the dark red variety contains the highest level of phytochemicals and therefore makes it the ideal variety for processing into wine.

3.2 Introduction

It has been suggested that if flavonoid intake is important to long-term human health, then the varieties of fruits and vegetables consumed is a critical factor to be considered (Crozier *et al.*, 1997). Furthermore, within a fruit and vegetable family, the quality and quantity of the phenolic pool may change with the cultivar, growth stage, season and environmental conditions (drought, salinity, heat/cold etc.) (Pérez-Gregorio *et al.*, 2010). The influence of cultivar on phenolic composition (mainly flavonols) have been studied previously e.g. in onions, tomatoes, pear juices, red raspberry juices, apricot juices and jams, and red wines (Häkkinen and Törrönen, 2000). For instance, in onions, the red pigmented variety, in addition to having anthocyanins, has higher levels of quercetin and its glycosides than the white variety, while in tomatoes wide variation in quercetin was found in different varieties (Patil *et al.*, 1995; Pérez-Gregorio *et al.*, 2010). Similarly, in another study, "Round" lettuce, which is cheap and used widely in the U.K, contained about eight times less quercetin than "Lollo Bionda" and more than 50 times less quercetin than the red-leaved "Lollo Rosso" (Crozier *et al.*, 1997). Therefore, it is only expedient that functional foods should be developed using varieties with higher quality and quantity of phenolics.

Regarding *H. sabdariffa*, although compositional data on the phytochemicals, and mainly polyphenols, present in *H. sabdariffa* have been documented, a common limitation of most of the research is that the geographical origin as well as variety is not specified, thus making comparison between the phytochemical and biological properties difficult. This warrants more investigation in order to clarify some of the discrepancies that exist in the literature concerning the compositional data on phytochemicals present in *H. sabdariffa* (Borrás-Linares *et al.*, 2015).

Regarding wine, grape (*Vitis vinefera*) stands out as the ideal raw material with the right balance of sugar and acidity needed for alcohol production and keeping quality. Notwithstanding, there are few varieties like muscadine that do not have enough sugar in them to meet the standard for alcohol content of wines. Consequently, in preparing the must for fermentation, sugar is generally added to the juice to increase the soluble solids content. Furthermore, most fruits that have been used for making wine are generally low in acidity and acids, in the form of tartaric or citric is added to adjust the pH and acidity of the must. It is in this context, that assessing the physicochemical properties of *H. sabdariffa* extracts like the acidity, sugar and colour attributes is needed to provide information on the required adjustment needed for roselle must. Based on the above reasons, the aim of this study was to evaluate the physicochemical properties and phytochemical profile of three varieties of *H. sabdariffa* with the intention of selecting the ideal variety for production of roselle wine.

3.3 Materials and methods

3.3.1 Physicochemical analysis, identification and quantification of polyphenols and total phenols in *H. sabdariffa* extracts

These analyses were determined according to the methods described in chapter 2 sections 2.4, 2.5, 2.6, 2.7 and 2.8 respectively. In addition, simple sugars (fructose, glucose and sucrose) and organic acids (oxalic, tartaric, malic, citric and succinic) were analysed under the conditions reported in chapter 2 sections 2.9 and 2.10 respectively.

3.3.2 Seasonal variation on phytochemicals and simple sugars in dark red *H. sabdariffa*

To investigate this (effect of seasonal variations on phenolic compounds in dark red *H. sabdariffa*), planting of the seeds was carried out on the same planting site and under the

same planting and harvesting conditions within two distinctive seasons (wet and dry) established in Nigeria as mentioned in section 2.2.

3.4 Results and discussion

3.4.1 Physicochemical analysis

The results of the physicochemical properties analysed (Table 3-1) showed that the extracts from the three varieties prepared in water exhibited a pH of ≈ 3.12, which falls within the range of values (2.5 - 3.2) reported in the literature (Fasoyiro *et al.*, 2005; Jung *et al.*, 2013). With respect to the colour parameters evaluated, the extract of white variety gave the highest L value, which is a measure of the degree of lightness of the extracts. The highest a* value (measure of redness) and colour density (CD) recorded for the dark red variety can easily be related with the results from the quantification of anthocyanins in the three varieties. Furthermore, the b* value (measure of yellow tones) which measures the absorbance at 420 nm is indicative of the quantity of other phenolic compounds (flavonols) aside anthocyanins present in *H. sabdariffa*. It is therefore expected that the dark red variety would have the highest levels of both anthocyanins and other phenolics since it recorded much higher a* and b* readings than the other varieties.

3.4.2 Analysis of sugars present in *H. sabdariffa*

The result of the sugar analysis by HPLC identified fructose, glucose and sucrose as the main sugars prevalent in *H. sabdariffa* extracts (Figure 3-1). Glucose recorded the highest amount in all three varieties (Table 3-1) and this agrees with the results obtained in a previous study, an unspecified red variety (Jung *et al.*, 2013). The concentration of sugar present in *H. sabdariffa* is low when compared to grape (180 mg/g); therefore to produce wine from this plant, the addition of sugar generally referred to as juice amelioration is needed to boost alcohol production.

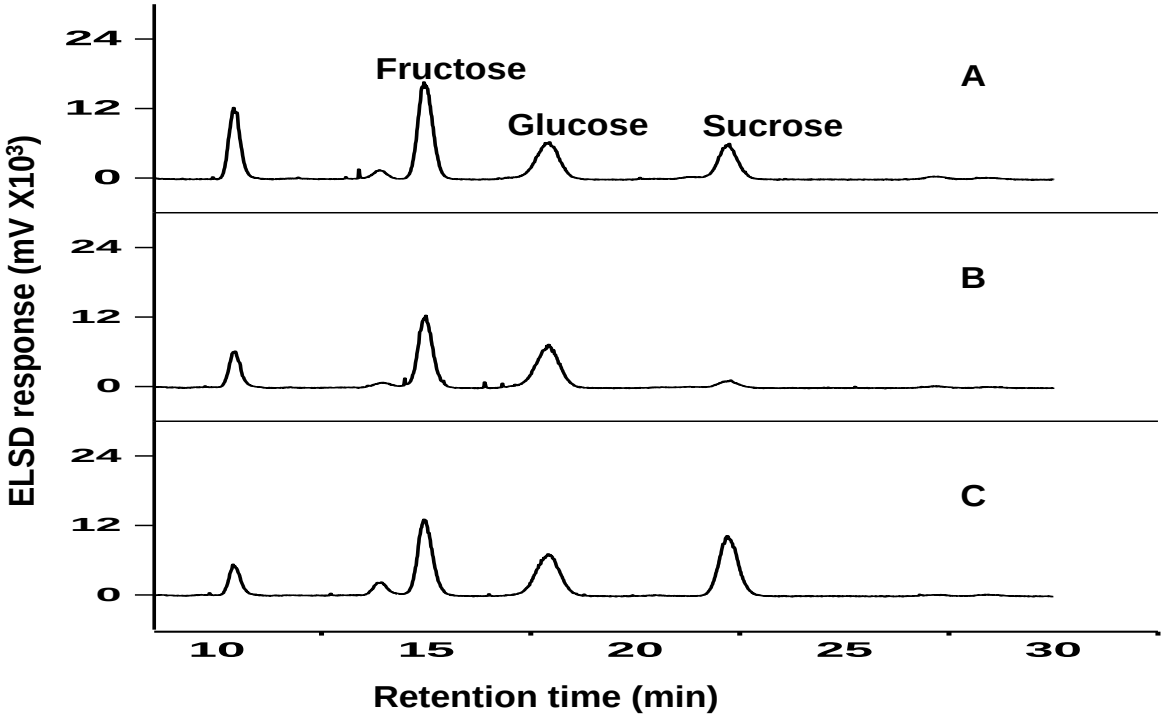


Figure 3-1 HPLC-ELSD chromatogram of sugars identified in (A) dark red, (B) light red and (C) white varieties of *H. sabdariffa.*

3.4.3 Analysis of organic acids in *H. sabdariffa*

H. sabdariffa varieties were found to contain different organic acids as demonstrated in Table 3-3. Malic acid was the main organic acid present in all *H. sabdariffa* varieties, while succinic acid content was highest in the white variety. Jung *et al*. (2013) found only malic acid in the extract of a red variety of *H. sabdariffa*, while in an earlier study oxalic, tartaric, succinic and malic acid were identified and quantified in *H. sabdariffa* calyces (Wong *et al.*, 2002). The result also showed that the white variety contained the highest level of citric acid, while trace amount of oxalic acid were found in both white and light red varieties.

Table 3-1 Physicochemical properties and quantification of sugars and organic acids quantified in three *Hibiscus sabdariffa* varieties.

Properties	Variety		
	Dark red	**Light red**	**White**
Physicochemical properties			
pH	3.16 ± 0.06 [a]	3.08 ± 0.16 [a]	3.07 ± 0.15 [a]
CIE*L*	14.6 ± 0.2 [c]	36.4 ± 0.2 [b]	48.5 ± 0.9 [a]
CIE*a*	34.8 ± 0.3 [a]	24.1 ± 0.2 [b]	nd
CIE*b*	22.4 ± 0.1 [a]	14.2 ± 0.1 [b]	10.8±0.4 [c]
Colour density (CD)	9.60 ± 0.06 [a]	4.70 ± 0.01 [b]	1.10 ± 0.02 [c]
Sugars (mg/g)			
Fructose	16.5 ± 1.7 [a]	11.9 ± 0.9 [b]	15.0 ± 0.9 [ab]
Glucose	22.9 ± 2.7 [a]	18.2 ± 3.1 [ab]	15.3 ± 1.0 [b]
Sucrose	11.7 ± 2.5 [a]	7.3 ± 0.4 [b]	12.7 ± 0.7 [a]
Organic acids (mg/g)			
Oxalic	0.06 ± 0.02	Trace	Trace
Malic	45.59 ± 6.77 [a]	41.72 ± 4.46 [a]	40.68 ± 8.94 [a]
Citric	0.45 ± 0.39 [a]	0.58 ± 0.37 [a]	0.73 ± 0.59 [a]
Succinic	0.80 ± 0.30 [b]	0.85 ± 0.16 [b]	2.27 ± 0.98 [a]
Tartaric	0.11 ± 0.05 [a]	0.11 ± 0.04 [a]	0.06 ± 0.02 [a]

The results are the means of three determinations (n = 3) and values on the same row with different alphabetical letters are significantly different at $p \leq 0.05$. nd: not detected.

Organic acids are known to influence the organoleptic properties, microbial stability as well as mineral bioavailability of plant foods (Hazell and Johnson, 1987). They are also employed in the food industry as acidulants in fruit juices and drinks manufacture. Although different types and quantities of organic acids have been reported (Wong *et al.*, 2002; Ali *et al.*, 2005), all data point to the fact that *H. sabdariffa* extracts are a rich source of organic acids that might find application as additives in the preservation of processed foods. In terms of its winemaking potential, *H. sabdariffa* contains sufficient acids needed for the stability and organoleptic properties of the final product and the adjustment of the pH and acidity in this case may not be needed. However, the presence of malic acid may well imply that malolactic fermentation might be needed to reduce the acidity and add some complexity to the flavour profile of the wine.

3.4.4 Phenolic profile of *H. sabdariffa* varieties

Different classes of polyphenols based on their structures were identified (Figure 3-2 and Table 3-2) in the three varieties of *H. sabdariffa* extracts. These include hibiscus acid and related compounds, phenolic acids, anthocyanins and flavonoids, with the majority of compounds identified having being reported in earlier studies (Rodriguez-Medina *et al.*, 2009; Borrás-Linares *et al.*, 2015). Hibiscus acid detected in the three varieties is a lactone of hydroxycitric acid and it is an α-amylase inhibitor (Hansawasdi *et al.*, 2000). Gallic acid and protocatechuic acid were identified in the red varieties, however, in the white variety, gallic acid was not detected. The main hydroxycinnamic acid in *H. sabdariffa* was the 3-*O*-caffeolquinic acid (3-CQA).

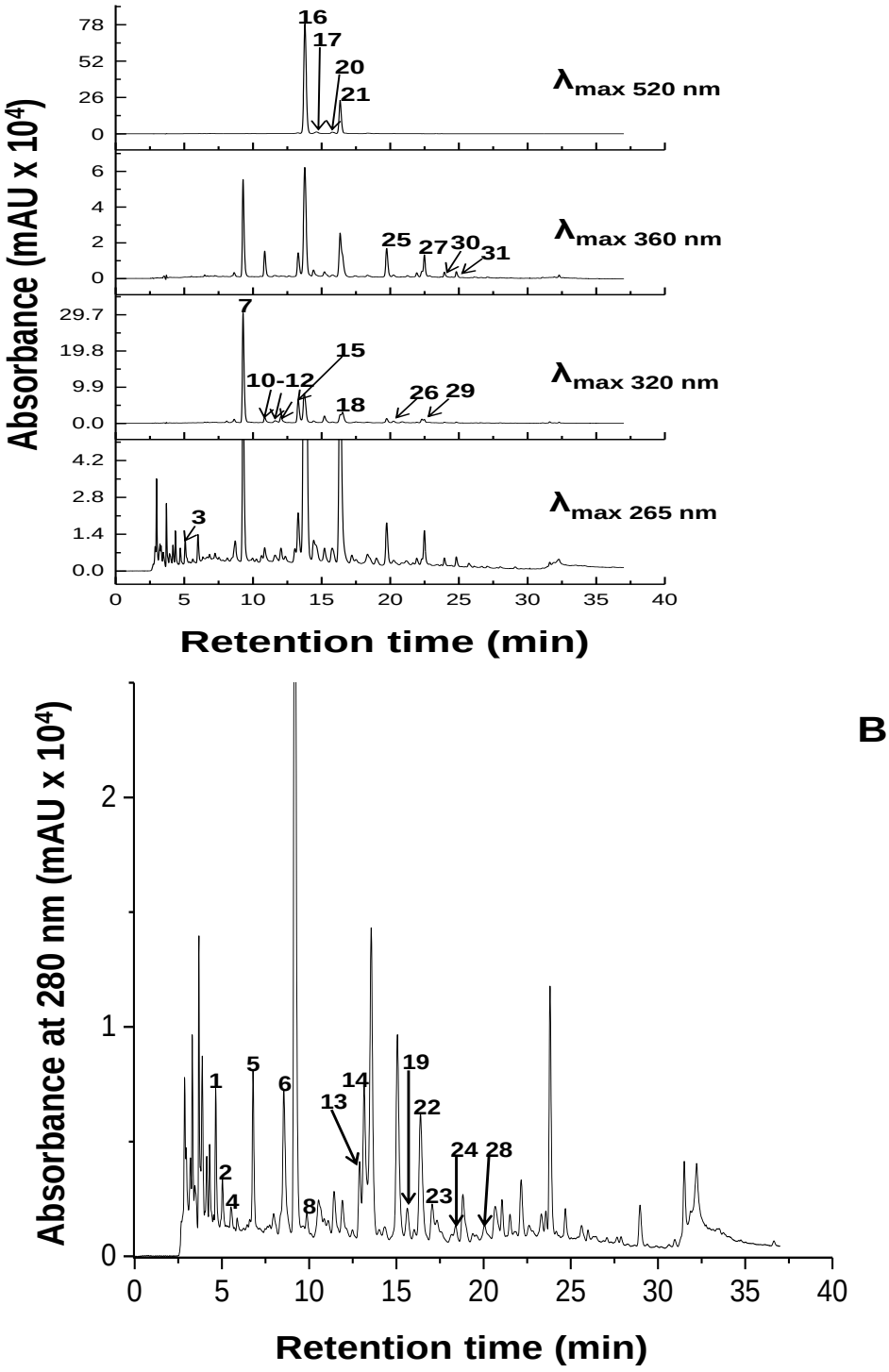


Figure 3-2 Identified phenolic compounds from (A) dark red and (B) white *H. sabdariffa* extracts analysed by HPLC-PDA. The peak numbering corresponds to those listed in Table 3-2.

Peak 15 was identified as 5-CQA with the use of a commercial standard, while peak 10 having similar UV spectrum and m/z ratio was tentatively identified as 4-CQA from existing patterns reported in the literature on *H. sabdariffa* caffeolquinic acids (Ramirez-Rodrigues *et al.*, 2011; Borrás-Linares *et al.*, 2015). The data from the LC-MS analysis also showed the existence of five isomers of chlorogenic acid in the white variety. The presence of free caffeic acid was detected in the three varieties and confirmed with the use of a standard (m/z ratio of 179). The total content of hydroxycinnamic acid in the three varieties ranged from 243 to 844 mg/100 g. Flavonols detected in *H. sabdariffa* extracts were myricetin 3-arabinogalactoside (M3A), quercetin 3-sambubioside (Q3S), rutin and quercetin 3-*O*-glucoside.

The white variety recorded the highest amount of rutin, while in the light red variety the level of Q3S was higher than M3A. Quercetin in the free form was also detected in all the extracts, but it was below the limit of quantification in this study (1.2 mg/L). These flavonols have earlier been in identified in published studies on *H. sabdariffa* polyphenols (Rodriguez-Medina *et al.*, 2009; Ramirez-Rodrigues *et al.*, 2012). As expected, DS and CS in the ratio 3:1 were found to be the most abundant anthocyanins in the red varieties of *H. sabdariffa*, while delphinidin 3-*O*-glucoside and cyanidin 3-*O*-glucoside were tentatively identified in the red extracts. The results of *H. sabdariffa* anthocyanins agree with other published data which also reported DS and CS as the main pigment in the red variety (Segura-Carretero *et al.*, 2008; Sindi *et al.*, 2014). In this study, flavan-3-ols were only identified in the white variety. Catechin (peak 13) was identified with the use of an authentic standard, while peaks 19, 23 24 and 28 were tentatively identified as flavan-3-ols from their UV-visible spectra which showed maximum absorption at 280 nm.

Table 3-2 Identification and quantitation of polyphenols in *H. sabdariffa* varieties according to their retention time, UV- Vis and mass spectral characteristics.

Compound	HPLC-PDA			LC-MS data	dark red variety (mg/100g)	light red variety (mg/100g)	white variety (mg/100g)
	peak no	retention time	λmax	m/z (+ /-)			
hibiscus acid glucoside[a]	1	4.3	256	351	25.8 ± 4.0 [a]	18.1 ± 2.2 [b]	21.7 ± 1.4 [ab]
hibiscus acid[a]	2	4.6	265	189	21.8 ± 4.5 [ab]	24.9 ± 2.9 [a]	17.8 ± 0.3 [b]
gallic acid[c]	3	6.0	271	169	23.2 ± 3.1 [a]	15.9 ± 4.7 [b]	nd
unidentified	4	6.8	282		27.7 ± 0.8 [a]	13.3 ± 0.6 [b]	6.9 ± 2.4 [c]
unidentified	5	7.4	283		nd	nd	8.1 ± 1.4
protocatechuic acid[c]	6	8.8	262	153	16.4 ± 4.2 [a]	17.9 ± 1.5 [a]	5.9 ± 0.7 [b]
unidentified	8	9.86	282		nd	nd	8.7 ± 1.8
unidentified	9	10.53	273		nd	nd	11.1 ± 2.6
Total Hydroxybenzoic					**115 ± 12 [a]**	**90 ± 7 [b]**	**80 ± 6 [b]**
3-*O*-caffeoylquinic acid[c]	7	9.2	324	353	319 ± 22 [b]	381 ± 30 [a]	64 ± 10 [c]
4-*O*-caffeoylquinic acid[a]	10	10.8	324	353	18 ± 2 [a]	12 ± 6 [a]	nd
cis/trans caftaric acid[b]	11	11.6	309		57 ± 12 [a]	66 ± 4 [a]	36 ± 4 [b]
cis/trans caftaric acid[b]	12	12.05	309		193 ± 32 [b]	291 ± 34 [a]	27 ± 6 [c]
chlorogenic acid isomer[a]	14	13.15	325	353	nd	nd	32 ± 7 [c]
5-*O*-caffeoylquinic acid[c]	15	13.31	324	353	69.5 ± 2.3 [a]	41.2 ± 5.4 [b]	34.7 ± 7.2 [b]
caffeic acid[c]	18	15.22	323	179	29.8 ± 4.5 [a]	19.7 ± 2.5 [b]	25.3 ± 4.1 [a]
chlorogenic acid isomer[a]	22	16.37	330	353	nd	nd	13.5 ± 1.9
cinamic acid	26	20.2	322		9.5 ± 0.6 [b]	13.5 ± 1.9 [a]	
coumaric acid[b]	27	20.8	316		7.8 ± 0.7 [a]	8.7 ± 2.2 [a]	5.8 ± 1.5 [a]
ferulic acid[b]	29	22.3	329		11.4 ± 1.6 [a]	11.4 ± 2.2 [a]	5.9 ± 1.3 [b]
Total hydroxycinnamic acids					**714 ± 57 [b]**	**844 ± 7 [a]**	**244 ± 32 [c]**
myricetin 3-arabinogalactoside[a]	25	19.7	354	611	28.5 ± 1.8 [a]	19.4 ± 3.3 [b]	9.1 ± 2.6 [c]
quercetin 3-sambubioside[a]	27	22.4	349	595	20.9 ± 0.9 [b]	34.1 ± 3.1 [a]	12.2 ± 2.9 [c]
rutin[c]	30	23.9	348	463	10.7 ± 0.7 [a]	8.2 ± 0.5 [b]	30.3 ± 3.7 [b]
quercetin 3-*O*-glucoside[c].	31	24.8	348	609	9.9 ± 0.4 [a]	10 ± 0.3 [a]	10.5 ± 1.2 [a]
Total Flavonols					**70 ± 2 [a]**	**72 ± 5 [a]**	**62 ± 5 [a]**
delphinidin 3-*O*-sambubioside[c]	16	13.8	526	597	2116 ± 216 [a]	535 ± 37 [b]	nd
delphinidin 3-*O*-glucoside[b]	17	14.6	526		76 ± 8 [a]	38 ± 1 [b]	nd
cyanidin 3-*O*-glucoside[a]	20	15.79	526		24.1 ± 3.9 [a]	18.6 ± 1.3 [a]	nd
cyanidin 3-*O*-sambubioside[c]	21	16.3	517	581	517 ± 42 [a]	136 ± 19 [b]	nd
Total Anthocyanins					**2732 ± 260 [a]**	**727 ± 55 [b]**	
catechin[b]	13	12.88	279		nd	nd	18.0 ± 4.2
unidentified	19	15.60	285		nd	nd	29.8 ± 1.4
unidentified	23	17.04	271		nd	nd	32.4 ± 3.3
unidentified	24	18.80	279		nd	nd	23.2 ± 2.3
unidentified	28	21.51	279		nd	nd	26.9 ± 3.3
Total Flavan-3-ols							**130 ± 10**
Total phenolics (HPLC) mg/100g					**3632 ± 235 [a]**	**1733 ± 74 [b]**	**516 ± 50 [c]**
Total phenolics from Folin's method					**3801 ± 195 [a]**	**2260 ± 190 [b]**	**994 ± 150 [c]**

[a] compounds tentatively identified by LC-MS data and/or from available information from the literature. [b] compounds were identified by authentic standards, retention time and spiking. [c] compounds identified by authentic standards, retention time, spiking and confirmed with LC-MS analysis. nd: not detected. Values with non-italic similar letters within rows are not significantly different $p \leq 0.05$ ($n = 9$). Some compounds could not be identified in the LCMS due to their low concentrations in the extracts.

In summary, the major phenolic compounds available in *H. sabdariffa* varieties are the anthocyanins (dark and light red only), the hydroxycinnamic acids available in the three varieties and flavan-3-ols only in the white.

3.4.5 Total phenolic content in *H. sabdariffa*

The dark red variety had the highest level of phenolics (38 mg/g) while the white variety had the least amount (9.9 mg/g) as seen in Table 3-2. Christian *et al.* (2009) reported a phenolic content (at the pre-flowering stage) of 23 mg/g and 12 mg/g for dark and white varieties of *H. sabdariffa* respectively. The levels in the light red variety remained stable around 16 mg/g throughout the stages of plant maturity. It is important to state that different levels of total phenols in *H. sabdariffa* have been reported in earlier studies and these variations can be explained by factors such as geographical origin, variety, time of harvesting and extraction conditions.

3.4.6 Seasonal variation of phytochemicals and simple sugars in dark red *H. sabdariffa*

The production of secondary metabolites in plants is important, particularly with respect to its organoleptic properties and health benefit (Ripoll *et al.*, 2014). These secondary metabolites such as polyphenols are reported to accumulate in plants that have been subjected to various forms of stress such as drought, temperature (heat or cold) and other environmental conditions. (Akula and Ravishankar, 2011; Duda *et al.*, 2015). Thus far, there has been no reported study on the impact of seasonal variations on the levels of phytochemicals in *H. sabdariffa.* The results (Table 3-3) in this study showed that CS content was significantly higher ($p = 0.0025$) during the dry season compared to the wet season.

Table 3-3. Content of main bioactive compounds in dark red *H. sabdariffa* grown in two different seasons.

Compound	HPLC-PDA			LC-MS data	dark red *H. sabdariffa*	
	peak no	retention time	Λmax	m/z (+ /-)	dry season (mg/100g)	wet season (mg/100g)
gallic acid	3	6	271	169	23.2 ± 3.1 [b]	34.5 ± 2.5 [a]
3-*O*-caffeoylquinic acid	7	9.2	324	353	319 ± 22 [b]	490 ± 44 [a]
caffeic acid	18	15.22	323	179	29.8 ± 4.5 [a]	35.4 ± 2.6 [a]
myricetin 3-arabinogalactoside	25	19.7	354	611	28.5 ± 1.8 [b]	34.9 ± 1.0 [a]
quercetin 3-sambubioside	27	22.4	349	595	20.9 ± 0.9 [a]	23.8 ± 2.1 [a]
delphinidin 3-*O*-sambubioside	16	13.8	526	597	2116 ± 216 [a]	1610 ± 467 [a]
cyanidin 3-*O*-sambubioside	21	16.3	517	581	517 ± 42 [a]	306 ± 26 [b]
Total Anthocyanins					2632 ± 257 [a]	1916 ± 492 [b]
Total phenolics from Folin's method mg/100g					**3801 ± 195** [a]	**3604 ± 87** [a]

Values with similar letters within row are not significantly different at $p \leq 0.05$ (n = 3).

Table 3-4 Content of organic acids and sugars in dark red *H. sabdariffa* grown in two different seasons.

Properties	Season	
	Dry	Wet
Sugars (mg/g)		
Fructose	16.5 ± 1.7 [a]	9.3 ± 0.2 [b]
Glucose	22.9 ± 2.7 [a]	12.9 ± 1.7 [b]
Sucrose	11.7 ± 2.5 [a]	11.6 ± 2.5 [a]
Organic acids (mg/g)		
Oxalic	0.06 ± 0.02	Trace
Malic	45.59 ± 6.77 [a]	12.23 ± 3.44 [b]
Citric	0.45 ± 0.39 [b]	27.65 ± 4.39 [a]
Succinic	0.80 ± 0.30 [a]	0.57 ± 0.13 [a]
Tartaric	0.11 ± 0.05 [a]	0.18 ± 0.01 [a]

Values with similar letters within row are not significantly different at $p \leq 0.05$ (n = 3).

In addition, the total anthocyanin content was significantly higher ($p \leq 0.05$) for planting done within the dry season. This is justifiable since anthocyanins are the main phenolic compounds present in *H. sabdariffa* calyces and it is expected that the plant in responding to the harsh conditions of the dry season in Nigeria would synthesise more of the main phenolic compound (anthocyanin) in response to water stress for its protection. Anthocyanin accumulation in plants is generally up-regulated by various environmental stresses, such as drought, UV, blue light, high intensity light, wounding, pathogen attack and nutrient deficiency (Kassim *et al.*, 2009; Akula and Ravishankar, 2011). In grapevines (*Vitis vinifera*), anthocyanin biosynthesis was strongly up-regulated in berries grown under drought conditions (water stressed) compared to the control cultivated under irrigated (Castellarin *et al.*, 2007). Furthermore, Carey *et al.* (2010) reported an increase in total phenolic content of lettuce during water stress and a decrease in response to re-watering of plants.

Although the general hypothesis is that stress conditions increases the levels of polyphenols in plants, there are some exceptions to this trend, as seen in this study where the levels of 3CQA, gallic acid and M3A were higher during the wet season. For example, a study evaluated the impact of water-deficit stress and cold stress treatment on some polyphenols in hartworn plants and reported that the polyphenols behaved differently (increase and decrease) to both stress conditions (Kirakosyan *et al.*, 2004). The authors (Kirakosyan and co-workers) suggested that in some instances, the combination of both stress treatment might be needed to bring about the desired levels of some of these plant metabolites.

The data on seasonal variation on the content of organic acid (Table 3-4) suggests that overall the content of simple sugars and organic acids were higher during the dry season. Fructose, glucose and malic were significantly higher ($p \leq 0.05$) for planting done in the

dry season compared to the rainy season, while citric acid was higher during the wet season. This result agrees with data obtained with tomatoes that showed higher content of sugars, malic acid, ascorbic and citric acid when cultivated under water stress compared to the control (Nahar and Gretzmacher, 2002). Similarly, in Braeburn apples fruit, total soluble solids were higher under deficit irrigation compared to the watered (control) apple fruit (Mills *et al.*, 1996). Although most studies report the increase in sugar content in fruit and vegetables exposed to water stress, the results are not that clear with organic acids. For instance, organic acids in peach, pear and tomatoes increased after water stress, however the opposite trend was observed in grapes and nectarines (Ripoll *et al.*, 2014). It would therefore be a case of plants responding differently to the production of organic acids when under water stress. Some of the reasons thought to be responsible for the increase in the contents of these compounds during stress include active solute accumulation or breakdown of starch within the plant (Balibrea *et al.*, 2003; Hummel *et al.*, 2010).

3.5 Conclusion

Extracts processed under identical conditions from three varieties of *H. sabdariffa* (dark red, light red and white) were analysed for their phytochemical and physicochemical properties. The results of the analyses showed that *H. sabdariffa* extracts contained a myriad of phenolic compounds, with the dark red variety being superior in polyphenolic content in comparison to the other varieties. Furthermore, three additional isomers of chlorogenic acids were tentatively identified in the white variety. With regards to the organic acids and sugars in *H. sabdariffa* extracts, malic acid was present in the highest amounts, while fructose, glucose and sucrose occurred in various proportions in the three varieties. Data on the impact of seasonal variation on the *H. sabdariffa* constituents

showed that anthocyanins and soluble solids content were higher during the dry season (water stress condition) than the wet season. Future studies could involve evaluating the impact of seasonal variations on *H. sabdariffa* volatiles.

The findings from this experiment provides a platform for comparison between compositional data on physicochemical properties, phytochemical profiles of *H. sabdariffa* varieties from Nigeria and other geographical regions of the world.

Chapter 4 *In vitro* inhibitory activities of *H. sabdariffa* varieties on α–amylase and α–glucosidase

4.1 Abstract

An approach to reducing postprandial hyperglycemia is the inhibition of carbohydrate-hydrolyzing enzymes in the digestive system. Consequently these assays are vital for identifying inhibitors with potential to reduce the post-prandial glycaemic response. Many publications on phenolics as potential inhibitors report widely varying assay conditions leading to variable estimates of inhibition. On this basis, the assay for *in vitro* α-amylase inhibition was optimised taking into consideration critical steps such as the importance of removing certain polyphenols after the enzymic reaction when using 3, 5-dinitrosalicylic acid since they interfere with this reagent. There was a significant effect ($p \leq 0.05$) on acarbose IC_{50} values when working outside optimal conditions. Under this optimal condition, *H. sabdariffa* extracts were tested for their inhibition of α–amylase and only a very weak inhibition for the three varieties was found despite other reports in the literature. Nevertheless, *H. sabdariffa* extracts inhibited α–glucosidase (maltase) in a dose-dependent manner, with the dark red variety exhibiting the lowest IC_{50} value (4.35 ± 0.07 mg/mL dried powder). Together delphinidin 3-*O*-sambubioside, cyanidin 3-*O*-sambubioside and 3-*O*-caffeolyquinic acid accounted for 65 % of this activity. Put together, the result from this experiment suggests that it is very unlikely that this is one of the mechanisms by which *H. sabdariffa* exerts its anti-diabetic properties reported in folk medicine.

4.2 Introduction

The reduction of postprandial hyperglycaemia is the mainstay in the treatment of type 2 diabetes (Bhandari *et al.*, 2008). Glucosidase inhibitors are commonly prescribed to diabetics to reduce postprandial hyperglycemia induced by the digestion of starch in the small intestine. The target of these drugs used to control blood glucose levels is the inhibition of α-amylase and α- glucosidase (glucosidases) found in the intestinal tract that are critical for the digestion of carbohydrates (Yilmazer-Musa *et al.*, 2012). However, these drugs, such as acarbose and miglitol, are often reported to produce side effects such as diarrhoea and other intestinal disturbances, with corresponding intestinal pain and flatulence (Chiasson *et al.*, 2002). The consumption of natural carbohydrate-digesting enzyme inhibitors derived from plant-based foods offers an attractive strategy to control postprandial hyperglycemia due to their affordability and low incidence of major undesirable side effects. Polyphenolic fractions from plants have in recent years attracted interest for their demonstrated potential in the inhibition of α-amylase and α-glucosidase activities, allowing for tighter control of blood glucose (Yilmazer-Musa *et al.*, 2012). To assess the potency of an inhibitor, *in vitro* assays involving the inhibition of α-amylase and α-glucosidase are performed, usually under different assay conditions which translate to high variability in published results on the IC_{50} values of potential inhibitors. These conditions include the source and concentration of enzyme and substrate, buffers, pH and the detection method amongst others (Acker and Auld, 2014). The most common detection method used for the *in vitro* α–amylase inhibition is the DNS method for detection of reducing sugars. The presence of a free carbonyl group (C=O) in reducing sugars enables them to participate in an oxidation-reduction reaction with 3,5-dinitrosalicylic acid (DNS). However, due to the reducing potential of some polyphenols, it is possible that they could interfere

with the development of the colour and consequently the results of the assay. Therefore, there is the need to optimise critical steps involved in this assay to ease comparison of results.

In traditional medicine, extracts of *H. sabdariffa* have been used in various countries to treat a wide variety of diseases (Hirunpanich *et al.*, 2006; Lans, 2006; Gonzalez-Stuart, 2011). Some scientific reports give credence to its use in folk medicine, in the treatment of health conditions like hypertension, hyperlipidemia and recently type 2 diabetes (Mozaffari-Khosravi *et al.*, 2009; Yang *et al.*, 2009; Hopkins *et al.*, 2013; Patel, 2014). However, there are only a few studies reporting the inhibition of α-amylase and α-glucosidases by extracts of *H. sabdariffa* (Hansawasdi *et al.*, 2000; Adisakwattana *et al.*, 2012; Ademiluyi and Oboh, 2013). Although, hibiscus acid and its 6-methyl ester have been identified as active principles for the inhibition of α-amylase, no compound in *H. sabdariffa* has been linked to the inhibition of α-glucosidases (Hansawasdi *et al.*, 2000). This warrants further investigation in order to facilitate the formulation of test meals containing bioactive compounds in clinical trials. Hence, the objectives of this research were two-fold: (1) Optimisation of the critical steps, showing the conditions required to assess α-amylase inhibition, using DNS as the detection method; (2) To assess the inhibitory potential of *H. sabdariffa* varieties on carbohydrate-digesting enzymes.

4.3 Materials and methods

4.3.1 Reagents and standards

3,5-Dinitrosalicylic acid (DNS), potassium sodium tartrate, chromatographically purified human salivary α-amylase type IX-A, intestinal acetone powder from rat, glucose assay reagent, maltose, sodium mono and dibasic phosphate, epigallocatechin

gallate (EGCG), quercetin, amylose and amylopectin from potato were all purchased from Sigma-Aldrich. Co., Ltd., Dorset, UK. Phloridzin, quercetin-3-*O*-glucoside and luteolin were purchased from Extrasynthase, Genay, France. Gallic acid was obtained from Alfa Aesar, Lancashire, UK. Instant green tea was a gift from Nestle Research Center, Lausanne, Switzerland. Oasis MAX cartridge 1 mL (30 mg) and 3 mL (60 mg) were purchased from Waters Corporation Ltd., Milford, MA, U.S.A. The DNS reagent was prepared by adding to 12 mL water, 20 mL of 96 mM DNS in water and 5.3 M sodium potassium tartrate solution (12 g in 8 mL of 2 M sodium hydroxide). All the reagents were of the highest purity and standards were ≥ 98 %.

4.3.2 Enzyme concentration and reaction time

Enzyme concentration and reaction time were determined by using different enzyme concentrations (0.5, 1.0, 1.5 and 2.0 U/mL) (where 1 "Sigma –defined" unit will liberate 1.0 mg of maltose from starch in 3 minutes at pH 6.9 at 20°C). The enzyme preparation on this basis contained 276 Sigma-units per mg protein by Bradford assay. The assay mixtures were incubated for different times (0, 3, 6, 9, 12 and 15 min) and the linearity plots of absorbance at 540 nm versus time was assessed.

4.3.3 Determination of *Km* and *Vmax*

The kinetic parameters *Km* (substrate concentration at which half the enzyme's active sites are occupied by substrate) and *Vmax* which measures the enzyme maximum rate were determined by using a chosen enzyme concentration and incubation times giving linear rates of reaction. The substrate concentrations ranged from 0 to 1 mg/mL in the final assay volume. Maltose standard curve was obtained by adding 1 mL of the DNS reagent to a total volume of 500 µL of different maltose concentrations (0-2 mM) and then heated (100 °C for 10 min). The absorbance was recorded at 540 nm in a

PHERAstar FS microplate reader (BMG Labtech, Inc., Cary, NC, USA), and the amount of maltose produced was calculated against the standard curve. The Lineweaver-Burk plot was used to calculate *Km* and *Vmax*

4.3.4 Effect of polyphenols on colour reagent

The effect of polyphenols on the DNS reagent was determined by adding 1 mL DNS to an assay mixture containing 450 µL phosphate buffer saline (PBS, 0.01 M, pH 6.9) and 50 µL of different concentrations (0-1 mM) of gallic acid, ferulic acid, EGCG and phlorizin The absorbance was then measured as described in section 4.3.3.

4.3.5 Retention efficiency of solid phase extraction (SPE) cartridges by HPLC-PDA

HPLC analysis for efficiency of retention of polyphenols by the Oasis MAX SPE cartridge was carried out with EGCG as an example using a UFLCXR Shimadzu system (Shimadzu, Japan) consisting of binary pump, a photodiode array with multiple wavelength SPD-20A and a LC-20AD solvent delivery module coupled with an online unit degasser DGU-20A3/A5 and a thermostat autosampler/injector unit SIL-20A (C). The column used was Phenomenex Gemini C18 column (5 µm, 250 mm x 4.6 mm) with a flow rate of 1 mL/min, column temperature set at 35 ˚C with an injection volume of 10 µL and detection at 280 nm. A two phase gradient system consisting of water (millipore grade) with 0.1% triflouroacetic acid (HPLC grade) as mobile phase A and acetonitrile containing 0.1 % triflouroacetic acid as mobile phase B. The gradient conditions were identical to that reported in section 2.5.

4.3.6 α-Amylase inhibition assay

The assay contained 200 µL each of substrate (amylose or amylopectin) and enzyme, 50 µL PBS and 50 µl of inhibitor of different concentrations. For the control assay, the

inhibitor was replaced by an equal volume of PBS. Amylose and amylopectin solutions (2.5 mg/mL) were prepared in water by heating at 90 °C on a hot plate for 15 min. A second stock solution of amylopectin was prepared at 0.925 mg/mL. Human salivary α-amylase solution (1.25 U/mL) was prepared in PBS. The enzyme solution and the assay mixture containing the inhibitor, PBS and substrate were pre-incubated at 37 °C in a water bath for 10 min and the reaction was started by adding the enzyme to the assay solution. The reaction was carried out at 37 °C for 10 min with salivary α-amylase at 0.5 U/mL, substrate at 1 mg/mL and varying concentrations of the inhibitor up to 1 mM (depending on solubility). The reaction was stopped by placing the samples in a water bath (GLS Aqua 12 plus) at 100 °C for 10 min where no further reaction occurred, transferred to ice to cool down to room temperature and centrifuged for 5 min. The sample obtained was used for SPE to remove polyphenols before adding DNS reagent solution. To the resulting sample, 1 mL of the DNS reagent was added and heated at 100 °C for 10 min. After cooling to room temperature, 250 µL from each sample was placed in a 96 well plate (Nunc A/S., Roskilde, Denmark) and the absorbance was recorded at 540 nm.

4.3.7 Inhibition of α-glucosidase

The assay conditions for enzyme concentration, linearity range, *Km* and *Vmax* was optimised by Nyambe (2016). The assay contained 200 µL each of substrate (maltose, final concentration of 3 mM) and enzyme, 50 µL sodium phosphate buffer (10 mM pH 7.0) and 50 µL of potential inhibitor (*H. sabdariffa* extracts, roselle wine or compounds) at different concentrations. For the control assay, the inhibitor was replaced by an equal volume of the buffer. The enzyme source was an acetone protein extract from rat intestine (10 mg/mL), prepared in sodium phosphate buffer (10 mM pH 7.0) to give a concentration of 4 mg/mL in the assay. The enzyme stock solution and the assay mixture containing the inhibitor, PBS and substrate were pre-incubated at 37 °C in a water bath for 10 min and

the reaction was started by adding the enzyme to the assay solution. The reaction was carried out at 37 °C for 20 min with various concentrations of the inhibitor up to 6 mg/mL. The reaction was stopped by placing the samples in a water bath at 100 °C for 10 min, transferred to ice to cool down to room temperature and centrifuged for 5 min. The sample obtained was used for SPE, carried out using Oasis MAX cartridges to remove polyphenols before adding the hexokinase reagent. The resulting sample (50 µL) was added to 250 µL of hexokinase reagent, placed in a 96 well plate and the absorbance recorded at 340 nm. The rate of enzyme inhibition was calculated as a percentage of the control (without inhibitor).

4.4 Results

4.4.1 Effect of enzyme and substrate concentration on inhibition

The IC_{50} of an inhibitor is dependent on the assay conditions such as enzyme concentration, substrate type, reaction duration, temperature and pH. Although the conditions of pH and temperature in most of the published studies on α-amylase inhibition have been standardized to 37 °C and 6.9, respectively, there is a wide variability in the conditions of other parameters. To demonstrate the impact of enzyme and substrate concentration on the inhibition constant, acarbose a well-known α-amylase inhibitor, was tested under two different assay conditions. Concentrations of 0.5 and 3.0 U/mL of enzyme were chosen to conduct this experiment; where 0.5 represents the optimal linear range of enzyme activity (Figure 4-1) and 3.0, a concentration that demonstrates the effect of excess enzyme. The experiment was conducted on both amylose and amylopectin and in addition ECCG, quercetin, luteolin and green tea were also tested in this experiment as they proved to be potent inhibitors of α-amylase. The two different enzyme concentrations had an effect ($p \leq 0.05$) on the apparent IC_{50} value exhibited by acarbose

and was more pronounced when amylopectin was used as substrate. The IC_{50} value of acarbose under non-optimal conditions was 7-8 fold higher than that obtained under optimal conditions for both substrates. When the concentration of amylopectin was reduced from 1 mg/mL to 0.37 mg/mL to give the same ratio of *km* value versus concentration in comparison to amylose, there was an apparent increase in the inhibitory activity of acarbose, and it was observed for green tea (lower IC_{50}) (Table 4-1).

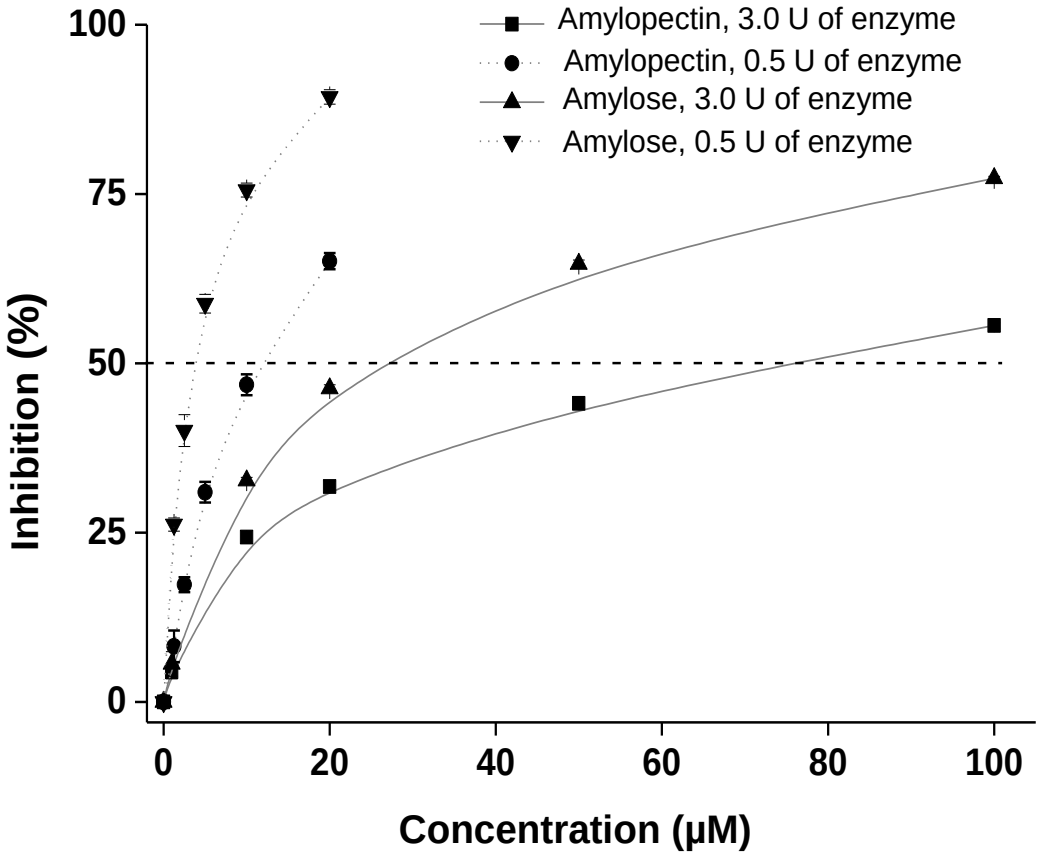


Figure 4-1 The inhibition of α-amylase by acarbose using amylose and amylopectin at 1 mg/mL. Data points are expressed as mean ± SE (n = 3).

Table 4-1 Experimental IC_{50} values of acarbose, selected polyphenols and green tea extract.

inhibitor	substrate		
	amylose (1 mg/mL)	**amylopectin (1 mg/mL)**	**amylopectin (0.37 mg/mL)**
Acarbose	3.5 ± 0.3	10 ± 1	7.6 ± 0.8
EGCG	5.3 ± 0.6	60 ± 2	24 ± 4
Quercetin	19.8 ± 0.3	83 ± 7	22 ± 1
Luteolin	26.3 ± 0.6	75 ± 1	42 ± 9
green tea	8.9 ± 0.1	60 ± 2	25 ± 1

The IC_{50} value of the tested pure compounds is expressed in µM. For green tea, IC_{50} values are expressed in µg/mL.

4.4.2 Kinetic studies on amylose and amylopectin

The time dependence assessed for different concentrations of enzyme was linear for up to 15 min for amylose and up to 12 min for amylopectin using 0.5 U of enzyme as depicted in Figure 4-2. Therefore, 10 min and 0.5 U of enzyme were chosen as the optimum assay conditions to obtain the kinetic parameters, with 1 mg/mL substrate concentration. Using the Lineweaver-Burk plots the values obtained were: amylose, *Km* = 12.9 mg/mL and *Vmax* = 1.67 mmol/min per mg of protein; amylopectin, *Km* = 4.8 mg/mL and *Vmax* = 0.67 mmol/min per mg of protein (Figure 4-3).

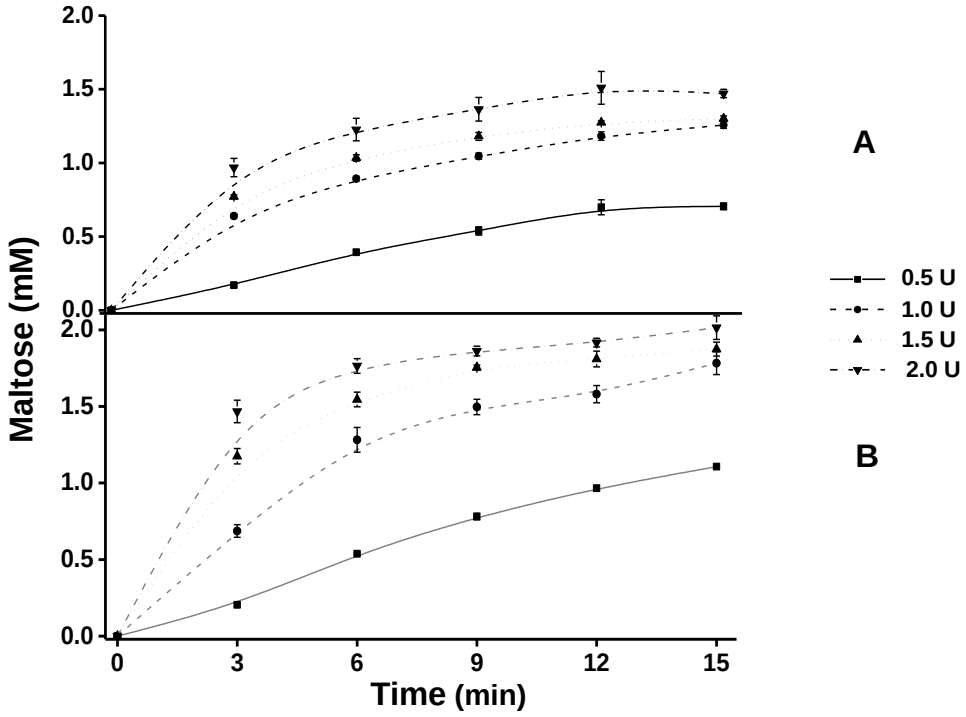


Figure 4-2 Time-dependence and linearity curve of salivary α-amylase hydrolysis of (A) amylose and (B) amylopectin. The hydrolysis was measured by the amount of maltose produced by different concentrations of enzyme. Data points are expressed as mean ± SE (n=3).

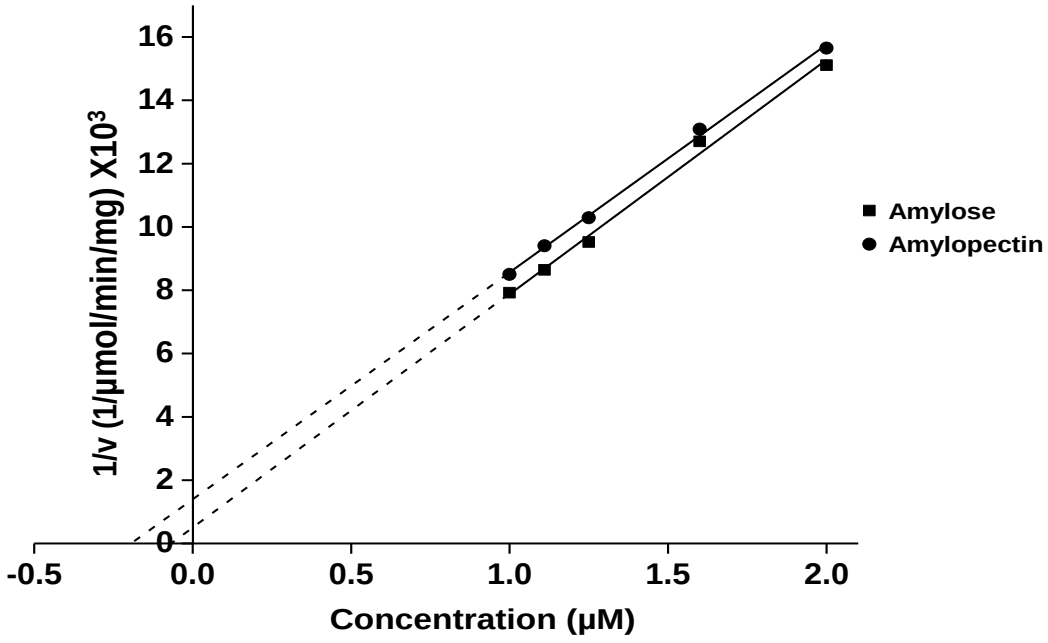


Figure 4-3 Lineweaver-Burk plot for action of salivary α-amylase on amylose and amylopectin.

4.4.3 Interference of polyphenols with DNS reagent

The use of the DNS reagent is one of the most widely reported methods to quantify the content of reducing sugars and it has been largely applied to measure the inhibition of α-amylase activity by many compounds including polyphenols. Three different classes of polyphenols with different reduction potentials were tested to corroborate this fact (Figure 4-4). Significant differences ($p \leq 0.05$) were found between EGCG, gallic acid and phlorizin. EGCG caused the major interference with the DNS reagent in a dose-dependent manner, followed by gallic acid and phlorizin. The extent of the interference roughly correlates with the number of OH groups (Figure 4-5) in the chemical structure of the polyphenol, which also partially predicts their reduction potential (Rice-Evans *et al.*, 1996). While this relationship may not hold for all polyphenols, the removal of polyphenols should be considered in pre-tests involving the DNS reagent. This is something that, to our knowledge, has been ignored in many published studies and may account for the variation in the reported inhibition of α-amylase by EGCG, since EGCG interacts very strongly with the DNS reagent. Ignoring this contribution would decrease the apparent inhibition, i.e. raise IC_{50} and Ki values.

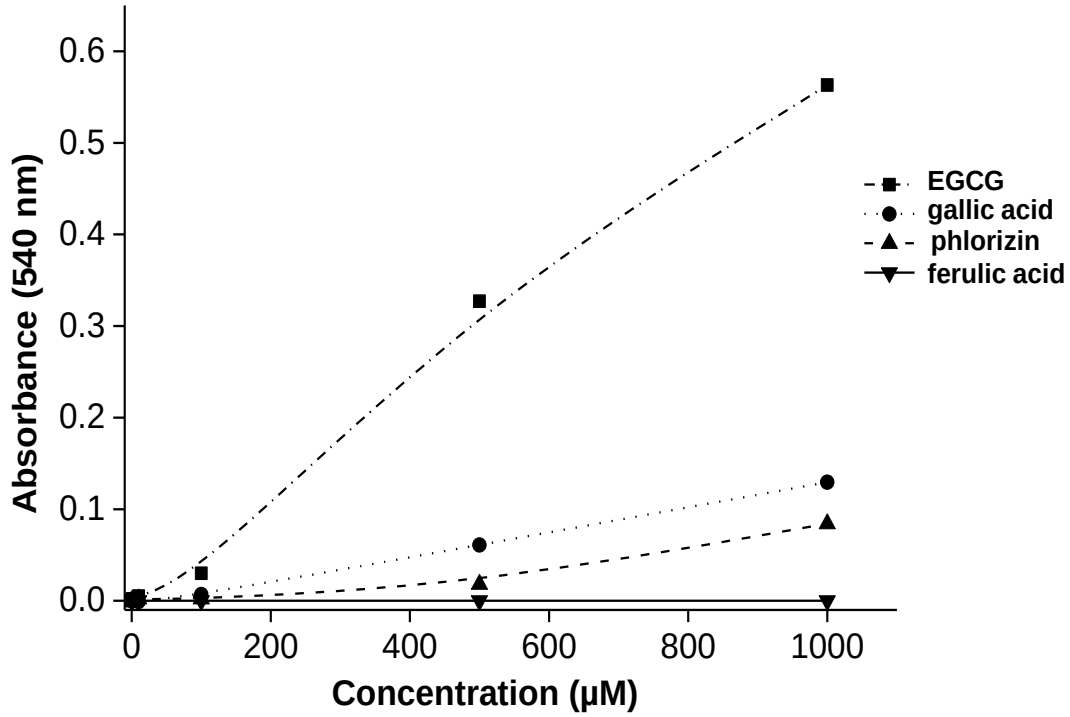


Figure 4-4 The reaction of selected polyphenols with DNS reagent. Data points are expressed as mean ± SE (n=3).

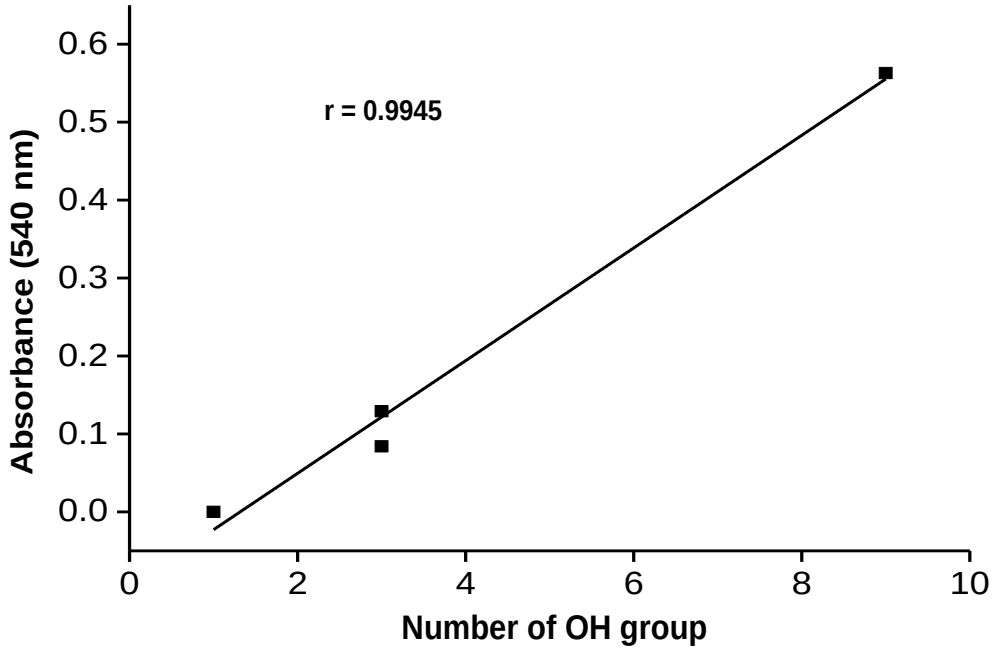


Figure 4-5 Pearson correlation coefficient of linear regression between the number of OH groups and interference with DNS reagent.

4.4.4 Inhibitory effect of selected polyphenols on salivary α-amylase activity

Assays under optimal conditions of enzyme concentration and incubation time were carried out to test the inhibitory activity of selected polyphenols using amylose and amylopectin as substrate. Polyphenols were removed from the reaction solution using SPE and the efficiency of removal is demonstrated in Figure 4-6 using EGCG as an example. The same procedure was carried out with quercetin and luteolin with the same removal efficiency. It is important to note that the retention capacity of the SPE sorbent mass is dependent on the concentration of the analyte, hence the appropriate sorbent mass must be chosen before carrying out SPE to prevent breakthrough of the analyte (polyphenol).

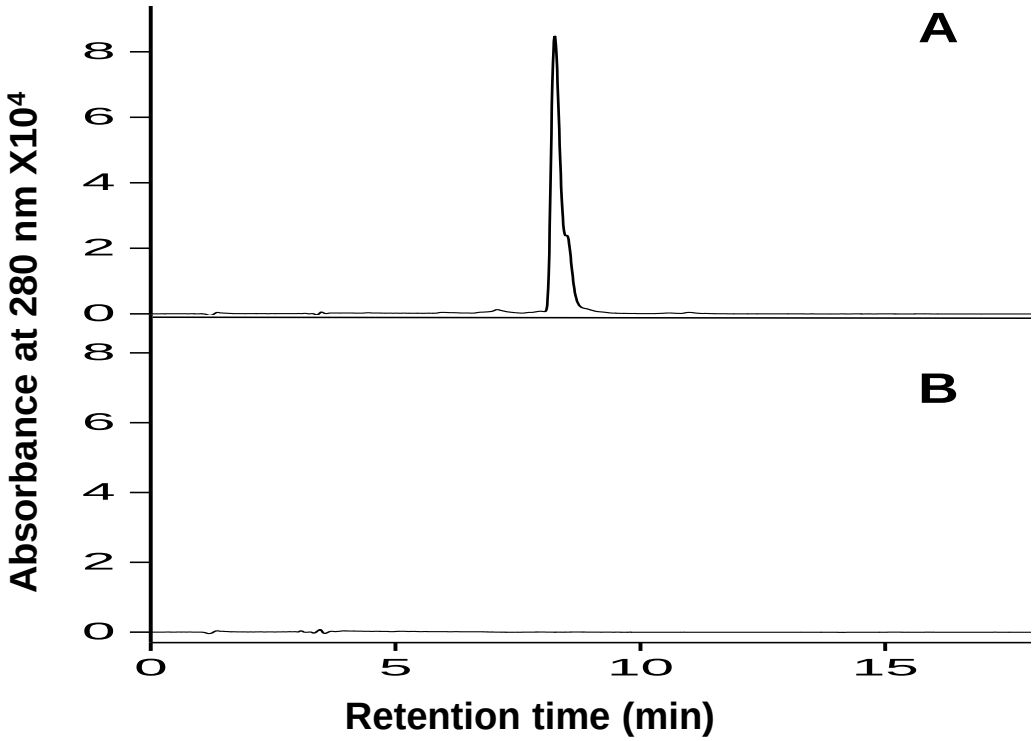


Figure 4-6 HPLC chromatogram of EGCG (A) before removal of EGCG using SPE and (B) after SPE. The removal of EGCG was > 99 %.

All of the tested polyphenols showed dose-dependent inhibition of α-amylase activity on both substrates and, therefore, IC_{50} values could be calculated (Figure 4-7). The inhibitory activity of quercetin, EGCG and luteolin was higher when amylose was used as substrate. EGCG showed the highest inhibition with maximum inhibition at 20 µM and no significant difference ($p \geq 0.05$) was observed above that concentration. For quercetin and luteolin, the highest inhibition was observed at the highest concentration tested (100 µM) owing to limits in solubility, showing significant difference ($p \leq 0.05$) among the tested concentrations. No differences ($p \geq 0.05$) were observed between the three tested polyphenols at a concentration of 100 µM using amylose as substrate. With amylopectin as the substrate, IC_{50} values were higher. The differences in the inhibition behaviour of the polyphenols on α-amylase between amylose and amylopectin could be related to the differences in the affinity (*Km*) for each type of substrate, hence the need to calculate the Ki (Figure 4.8) which, for competitive inhibition, represents the dissociation constant of the enzyme–inhibitor complex independently of the substrate employed. There was no significant difference ($p \geq 0.05$) between Ki values for amylose (0.28 ± 0.64 µM) and amylopectin (4.50 ± 4.53 µM).

4.4.5 Inhibition of α-amylase and α-glucosidase activities by *H. sabdariffa* extracts

Prior to the enzyme inhibition assays, sugars were removed from the samples by SPE to prevent any interference with the assay. The efficiency of sugar removal (Figure 4-9) was confirmed by analysing the sample after SPE under conditions described in chapter 2 section 2.8. The three varieties of *H. sabdariffa* extracts exhibited negligible or no inhibition of human α-amylase activity. The highest inhibition value obtained was only 8 % with the dark red variety even at 6 mg/mL.

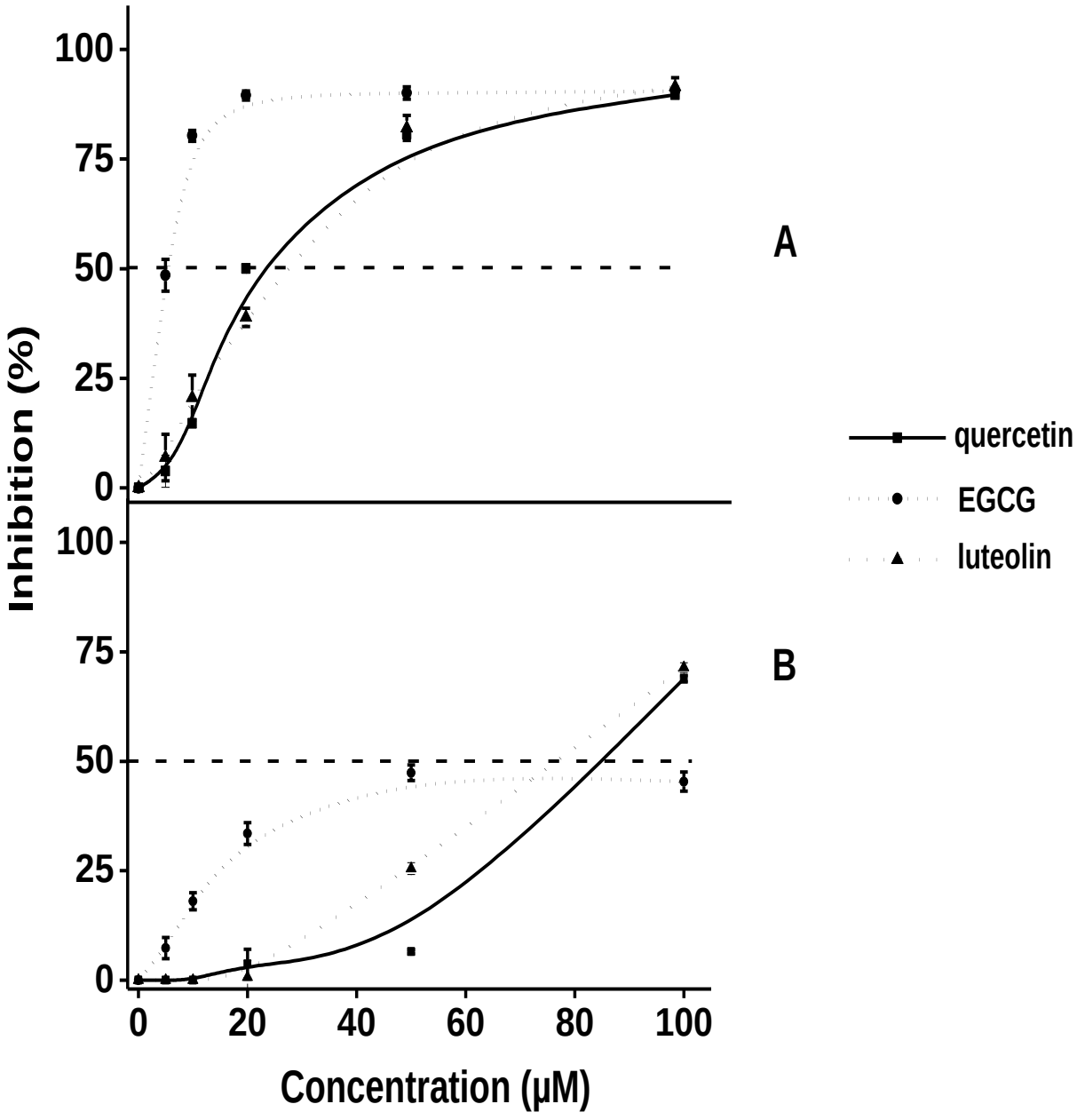


Figure 4-7 Inhibition of α-amylase by selected polyphenols using amylose (A) and (B) amylopectin as substrate. IC_{50} is indicated by the dotted line. Data points are expressed as mean ± SE (n = 3).

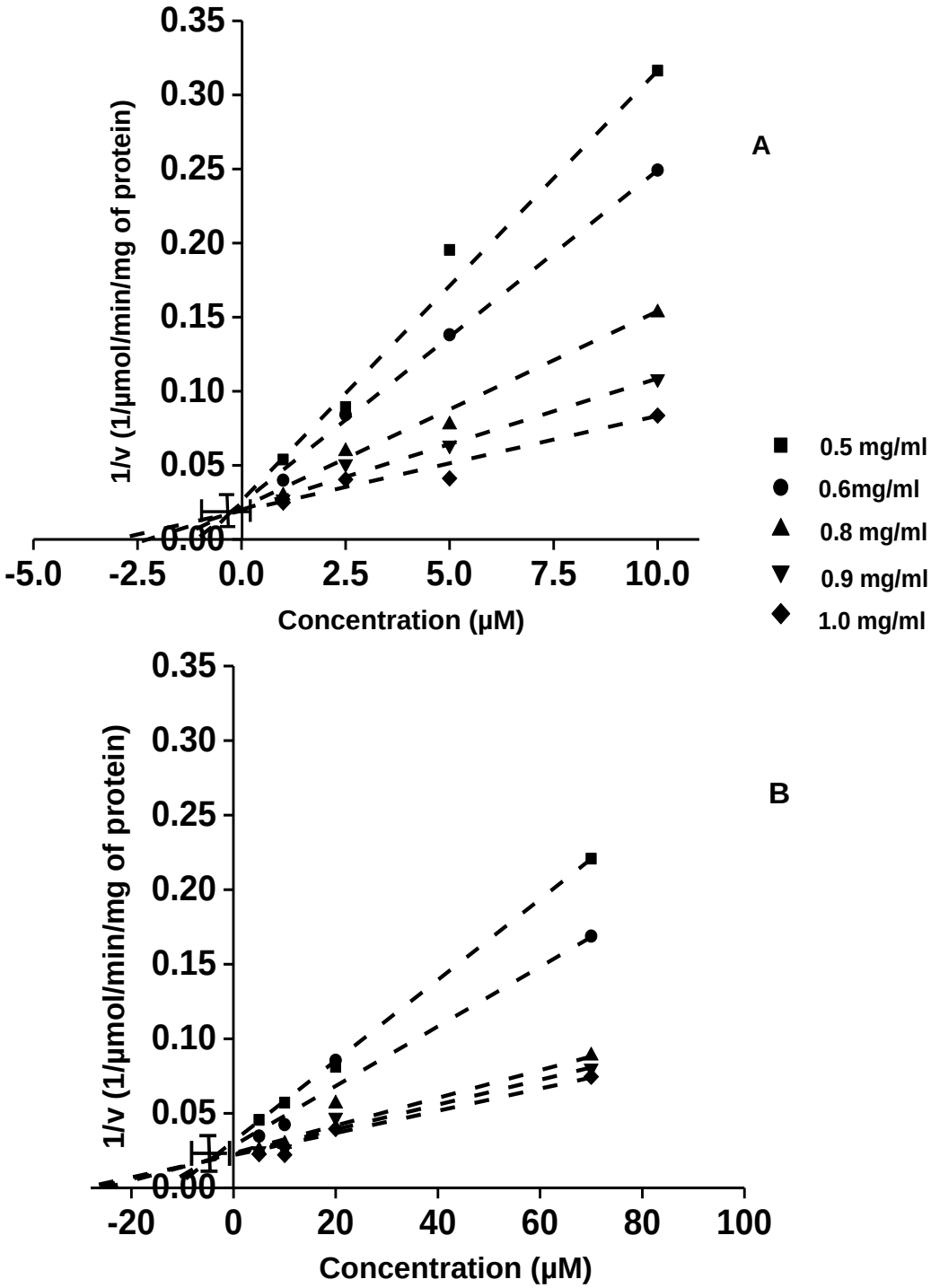


Figure 4-8 Dixon plot showing the kinetic analysis of EGCG against human salivary α-amylase on (A) amylose and (B) amylopectin. The intercept value represents –Ki. Data points are expressed as mean ± SE (n = 3).

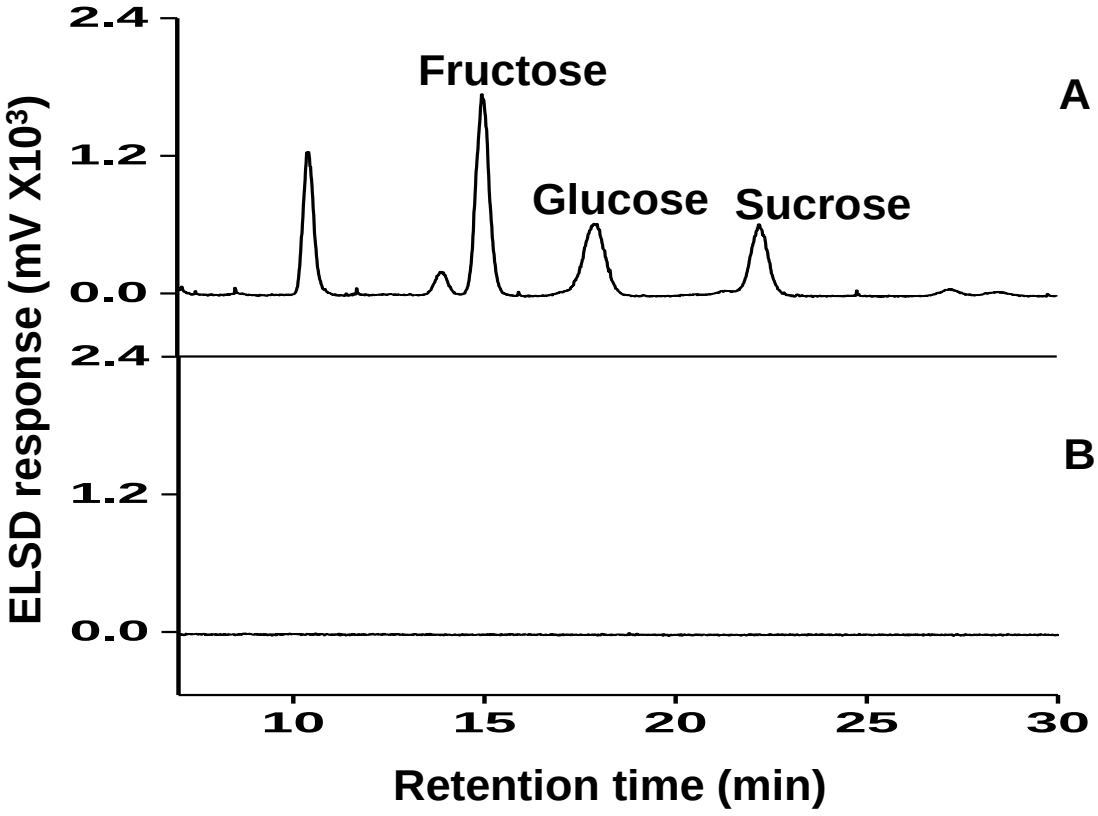


Figure 4-9 HPLC chromatogram of *H. sabdariffa* extract (A) before removal of sugars by SPE and (B) after SPE. The efficiency of sugar removal was > 99 %.

On the other hand, *H. sabdariffa* extracts inhibited α-glucosidase in a dose-dependent manner as depicted in Figure 4-10, except for the white variety where the inhibition was very low even at the highest concentration tested (6 mg/mL). The IC_{50} values of the dark and light red variety were 4.35 ± 0.07 and 5.90 ± 0.14 mg/mL respectively.

4.4.6 Identification of bioactive compounds

To identify the bioactive compounds responsible for α-glucosidase inhibition, delphinidin 3-*O*-sambubioside (DS), cyanidin 3-*O*-sambubioside (CS) and 3-*O*-caffeolyquinic (3-CQA) were tested individually and/or in combination at the concentrations equivalent to

their presence in the dark red variety that gave the IC_{50} value (≈ 4 mg/mL of extract).The result presented in Figure 4-11 shows that DS and CS together provided about half of the inhibition, while the combination of DS, CS and 3-CQA, accounted for 65 % of the total inhibition. In addition, CS proved to be a more potent inhibitor than DS as reflected in their IC_{50} values of 543 and 756 µM respectively.

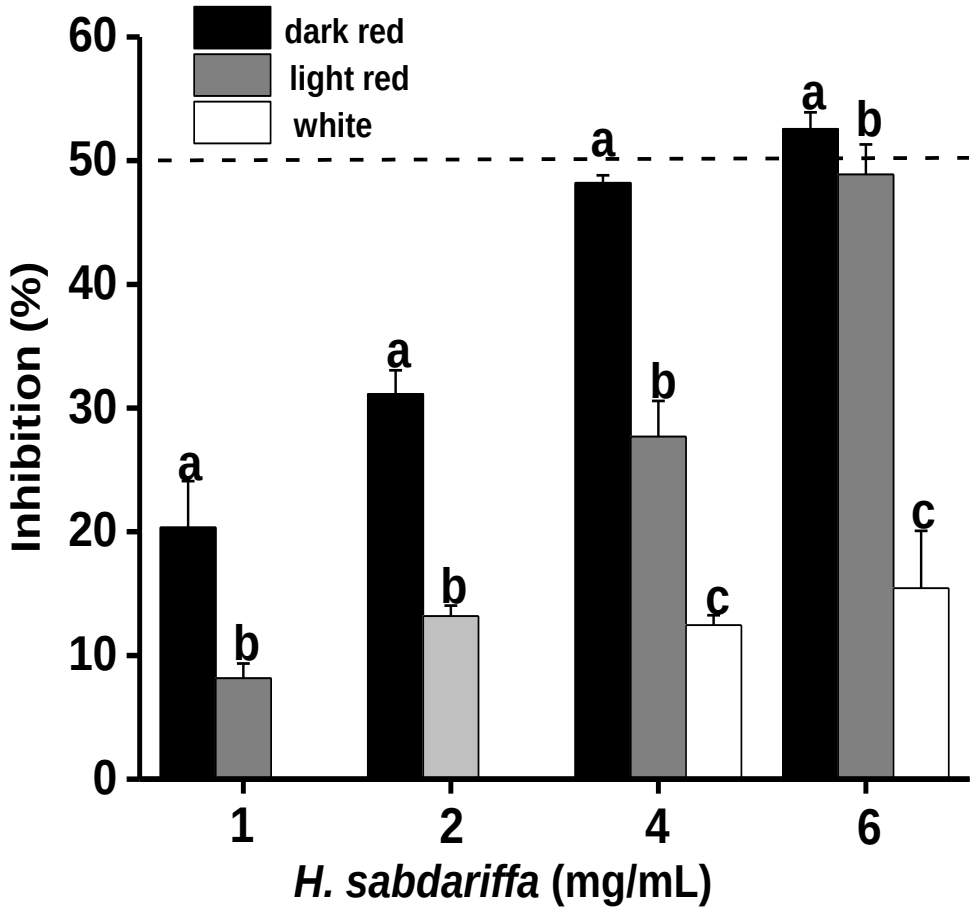


Figure 4-10 Dose-dependent inhibition of α–glucosidase by extracts of *H. sabdariffa* varieties. The results are expressed as mean ± SD (n = 3) and values with different letters are significantly different at $p \leq 0.05$. IC_{50} is indicated by the dotted line.

4.5 Discussion

There exists a wide variation in published studies on the inhibition of α-amylase using pure polyphenols. This is even observed with acarbose, with wide variations in the IC_{50} values. The IC_{50} value of a polyphenol is driven by the type and concentration of enzyme and substrate, and by the inhibitory mechanism (competitive, uncompetitive or non-competitive). The result (Figure 4-1) demonstrates the importance of enzyme concentration on the final inhibition constant. In general when the enzyme concentration is low, it is less problematic than too high concentrations. In the latter, the enzyme converts the substrate instantly, possibly during the mixing and starting procedure and the implication is that the reaction will already be finished at the onset of recording and no inhibition can be observed.

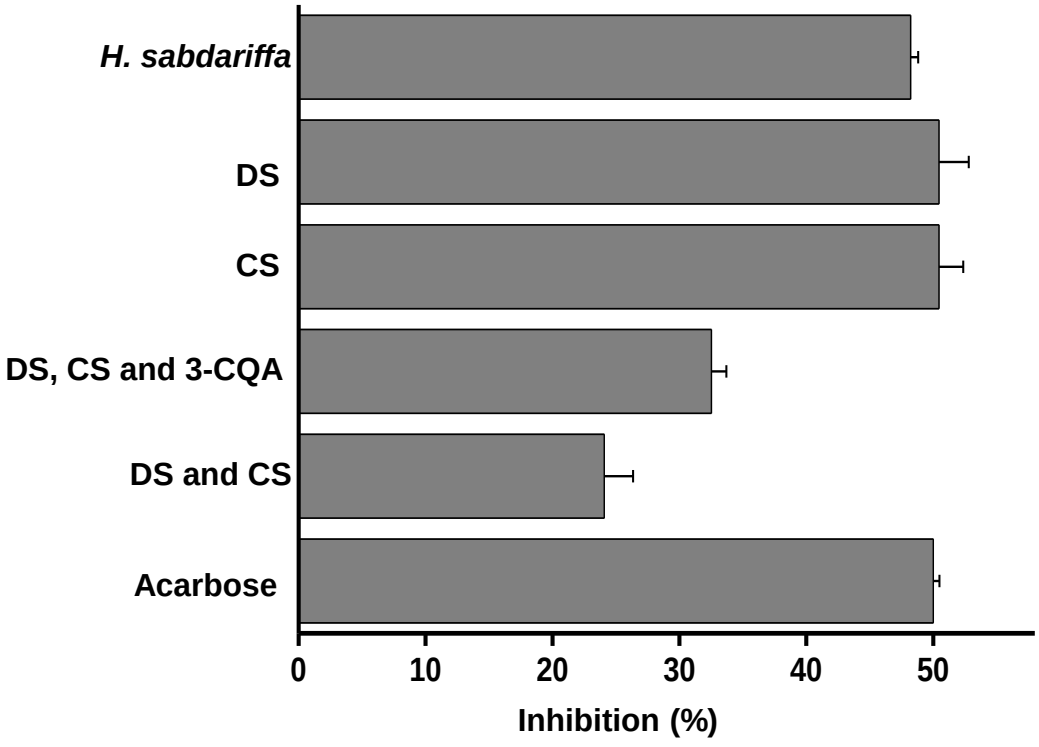


Figure 4-11 Effect of polyphenols in dark red *H. sabdariffa* extract on α–glucosidase inhibition. Compound mixtures were tested at their respective concentrations in the 4 mg/mL samples. Acarbose IC_{50} (0.4 µM) was used as a positive control, DS IC_{50} (756 µM), CS IC_{50} (543 µM) and H. *sabdarifa* at 4mg/mL. The results are expressed as means ± SD (n = 3).

Regarding the reaction time, although there is no general rule, it is important that it must be within the area of the initial linear progression of the reaction as the following nonlinear phase will yield erroneous results (Bisswanger, 2014). Furthermore, the importance of determining the kinetic parameters *Km* and *Vmax* before measuring inhibition constants is critical in enzymatic assays (Figure 4-4). Due to the varying catalytic efficiency, results obtained for the same enzyme but with different substrates and concentration will never be the same and will affect the apparent potency of an inhibitor. For example, the IC_{50} value for EGCG in this study was ≈ 5 and ≈ 60 µM for amylose and amylopectin respectively (substrate concentration = 1 mg/mL) as seen in Table 4-1. Hence the need for optimisation of these parameters since they are critical to the interpretation of correct and comparable IC_{50} values(Acker and Auld, 2014). Most of the research regarding the inhibition of α-amylase activity by polyphenols has been carried out using an enzyme from porcine pancreas which possesses a 14 % difference in the amino acid composition compared to that of human origin (Brayer *et al.*, 1995) and data on the effect of polyphenols on human α-amylase is much more limited when compared to porcine which may account for discrepancies in inhibition results in the literature (Lo Piparo *et al.*, 2008). DNS is used as a detection reagent for the measurement of reducing sugars. The results show that certain redox-active compounds participate in the reaction involving DNS (Figure 4-5) where EGCG with the highest number of OH groups gave the greatest effect and ferulic acid did not react. This implies that a potential inhibitor needs to be tested for any possible interference because any small change in absorbance units entails a major impact on the final inhibition value. This may explain the discrepancy between data, where EGCG was estimated to be less potent: values of 1.5 mM using the DNS reagent as detection method (Koh *et al.*, 2010) and 2.3 mM using Nelson-Somogyi were obtained (Miao *et al.*, 2014).

In this study, the anti-diabetic potential of *H. sabdariffa* extracts used in traditional medicine for the treatment of several diseases was investigated. The inhibition of porcine α-amylase had been reported in previous studies where IC_{50} values of 187.9 and 90.5 µg/mL were obtained for the red and white varieties respectively (Ademiluyi and Oboh, 2013). It was therefore surprising that little or no inhibition of human amylase was found even at the highest concentration (6 mg/mL). It is possible that the conditions under which the assay was performed could explain this conflicting result, because assay conditions greatly influence (e.g. excess enzyme, incubation time) measured IC_{50} values leading to incorrect values (Acker and Auld, 2014; Nyambe-Silavwe *et al.*, 2015). Since there was no data on the kinetics of the reaction (*Km* and *Vmax*, linearity range and enzyme activity), it is difficult to ascertain whether the assay was performed under optimal conditions. Furthermore, the source of the enzyme used in the reported study (porcine) against human salivary amylase which was used in this research might also be responsible for the contradictory results. Regarding α-glucosidase inhibition, the result from this research (IC_{50} = 4 mg/mL) when compared to the data from the same authors (Ademiluyi and Oboh, 2013), where IC_{50} values were 25.2 µg/mL and 47.4 µg/mL for red and white respectively is rather high. This variation could be linked to the reaction time (5 min) and the type of substrate (synthetic source) used for the assay in the referred study. The reaction time seems insufficient to plot the reaction velocity and determine the enzyme activity and these parameters are needed for determining optimal assay conditions. Furthermore, as earlier mentioned (section 4.5) due to the enzyme affinity for the substrate, results using the same enzyme source, but with different substrates, will hardly be comparable (Bisswanger, 2014).

Although the inhibition of rat intestinal α-glucosidase in red and white *H. sabdariffa* varieties have been published, the compounds responsible for the effect have not been

identified (Adisakwattana *et al.*, 2012; Ademiluyi and Oboh, 2013). This study reports for the first time the active principles (DS, CS and 3-CQA) responsible for this activity

4.6 Conclusion

Currently the improvement of glucose homeostasis by reducing intestinal absorption of dietary glucose by alternatives to acarbose through the inhibition of carbohydrate digesting enzymes is of increasing interest. The existing literature (Nyambe-Silavwe *et al.*, 2015) reveals wide variations in assay conditions used for α-amylase inhibition by polyphenols, hence the need for an optimised assay, which is reported in this study. Potential functional foods in the future could use this parameter as an indicator of acarbose-like activity of the constituent polyphenols. With respect to the treatment of diabetes using *H. sabdariffa*, it is possible that there are other mechanisms beyond the scope of this work, through which *H. sabdariffa* may exert its anti-diabetic properties. For instance, Peng *et al.* (2011) analysed the composition of *H. sabdariffa* extract and tested its protective effect on a type II diabetic rat model. The result demonstrated that treatment with *H. sabdariffa* (200 mg/kg) reduced hyperglycaemia and hyperinsulinemia. Furthermore, decreased levels in serum triacylglycerol, cholesterol and the ratio of low density lipoprotein (LDL) to high density lipoprotein (HDL) (all important biomarkers in the pathogenesis of diabetes mellitus) were observed. The treatment with *H. sabdariffa* extract also showed improved insulin resistance in the diabetic rat model. This finding suggests that *H. sabdariffa* might still have potential use, other than the inhibition of carbohydrases in the treatment of diabetes and deserves further investigation.

Chapter 5 The effect of fermentation temperature on the physicochemical properties, phytochemical profile and bioactivity of roselle wine

5.1 Abstract

This study reports the changes in the physicochemical, phytochemical and aroma composition of *Hibiscus sabdariffa* (roselle) wine fermented at 20 ˚C and 30 ˚C and kept at these temperature conditions for 40 days. The changes in colour were measured using both spectrophotometric and colorimetric methods, while the phytochemical and aroma compounds were analysed by HPLC and GC-MS respectively. On day 40, wine colour at both temperatures showed lower colorimetric (L* C* H*) and higher colour density (CD) readings than day 0. The main change in phytochemical composition observed after fermentation was the hydrolysis of 3-*O*-caffeolquinic acid and the concomitant increase of caffeic acid irrespective of fermentation temperature. Although organic acid composition followed the same pattern, acetic acid production was higher at 30 ˚C (≈ 33 %). The major difference in the wines occurred in the α–glucosidase inhibition where wine at 20 ˚C was more potent, and in the aroma profiles, with more fruity aromas at 20 ˚C (ethyl octanoate) and flowery notes (2-phenylethanol) at 30 ˚C at the end of the fermentation.

5.2 Introduction

There are a number of studies that demonstrate the possibility of using other fruits and other plant sources such as pomegranate, raspberry, blueberry, elderberry, cacao, gabiroba, kiwi, mango and orange for the production of wine (Rupasinghe and Clegg, 2007; Duarte *et al.*, 2010; Mena *et al.*, 2012; Johnson and Gonzalez de Mejia, 2012).

The manufacture of wines from other plant sources uses the same processing steps applicable to grape wine, which results in significant changes in the physicochemical, phytochemical and aroma attributes of the raw material. The key steps being the fermentation and ageing conditions, yeast species as well as clarification/fining methods. Firstly, fermentation results in the extraction of phenolic compounds from grape skins which have a major influence on the colour as well as the organoleptic properties of wine. Furthermore, the temperature of fermentation is critical as it determines the extraction efficiency and the proceeding reactions that take place during the early moments of fermentation (enzymatic oxidations and polymerizations), which ultimately has a major impact on the final product quality (Gil-Muñoz *et al.*, 1999; Bautista-Ortín *et al.*, 2004). These reactions might also involve the transformation of native phenolic substances into secondary metabolites that might possess a different biological activity from the parent compound (Ginjom *et al.*, 2011). Secondly, the fermentation process is the main activity by which yeasts make a positive contribution to wine aroma and flavour of wine. They accomplish this by several mechanisms: (i) utilising the raw material (must or juice) constituents, (ii) producing ethanol and other solvents that help to extract flavour components from the solids in the must, (iii) producing enzymes that transform neutral compounds present in the must into flavour active compounds, (iv) synthesising a range of flavour-active, secondary metabolites (e.g. acids, alcohols, esters, polyols, aldehydes,

ketones, volatile sulphur compounds), and (v) autolytic degradation of dead yeast cells (Fleet, 2003).

After fermentation, the wine is generally turbid and fining may be carried out to stabilise the colour and remove undesirable flavours (Section 1.3.3.7). One such fining agent is bentonite which is commonly used in the wine industry to remove proteins that might cause haze in wines. However, this process also brings about the reduction in some phenolic compounds (responsible for colour, bitterness and astringency) (Gómez-Plaza *et al.*, 2000b). Although, wine has been produced from *H. sabdariffa* calyx (roselle wine) the resultant changes in the phytochemical composition and subsequent bioactivity during fermentation has not been investigated (Alobo and Offonry, 2009; Ifie *et al.*, 2012). Furthermore, while the main aroma compounds present in *H. sabdariffa* drinks have been identified (Ramírez-Rodrigues *et al.*, 2012), the fate of these compounds during roselle wine manufacture is unknown. Since fermentation conditions are critical factors influencing wine quality, a study on the impact of this processing step on the phytochemicals and subsequent bioactivity on starch digestion during roselle wine manufacture is needed to establish quality parameters needed for the development and commercialisation of a functional product. The objective of this experiment was to study the impact of two different fermentation temperatures (20 and 30 °C) on the colour attributes, phytochemical composition and aroma profile of roselle wines alongside its effect on the resultant bioactivity.

5.3 Materials and methods

5.3.1 Juice preparation and amelioration

H. sabdariffa calyces were sorted, cleaned to remove extraneous material and washed under cold running tap water. The extraction of roselle juice was done using distilled

water at 30 °C for 1 h and the ratio of calyces to water was 1:35 (w/v). The juice was then pasteurised by placing the vessels containing the juice in incubators programmed at 50 °C for 30 min. After pasteurisation, sodium metabisulphite (60 mg/L) was added to arrest the activities of unwanted microorganisms. Juice amelioration was performed by addition of brewing sugar (glucose) to raise the soluble solids of the must to 20˚ Brix, while yeast nutrient (1 g/L) from Young Home Brew, (Bilston, UK) was introduced into the must according to the manufacturer's instruction.

5.3.2 Fermentation of roselle juice

The fermentation of roselle juice was carried out in 20 L fermentation vats with glass stoppers filled with sodium metabisulphite (100 mg/L) and placed inside incubators (Sanyo- MIR -153, Japan) programmed at 20 and 30 ˚C. Furthermore, the fermentation was allowed to proceed in contact with the calyces (similar to skin contact in grape wine). Dried wine yeast *S. cerevisiae* (1 g/L) from Abbey Brew, (Leeds, UK), re-activated by addition of distilled water (50 mL) at 40 ˚C for 30 min, was added to the vats to initiate the primary fermentation process which was considered finished (cessation of bubbles) on day 8. Wines were then racked (filtering of yeast lees and calyces), transferred into secondary fermentation vats and kept at the stated temperature conditions until day 40. Samples (50 mL) were taken after stirring the must on the days assigned for the different analysis as reflected in the respective tables and figures. The fermentation at each temperature condition was performed in duplicate and independent sampling and analysis from each fermenting vat was conducted in triplicate (n = 6).

5.3.3 Wine analysis during fermentation

Physicochemical analysis, identification and quantification of polyphenols and total phenols in roselle wine were determined according to the methods described in chapter 2

sections 2.4 2.5, 2.6, 2.7, and 2.8 respectively. Simple sugars and organic acids (malic, lactic, acetic, citric and succinic) were analysed under the conditions reported in chapter 2 sections 2.9 and 2.10 respectively. Furthermore, the analysis of roselle wine volatiles was performed under the conditions optimised in chapter 2 section 2.10. Physicochemical properties and organic acid evolution during fermentation were determined on days 0, 3, 8, 21 and 40. To assess the impact of fermentation temperature on yeast activity, sugar consumption analysis was done on the first four days and in addition, days 5, 8 and 40. The changes in the concentration of phenolic compounds and total phenolic content were monitored on days 0, 1, 2, 3, 8, 21 and 40. While α-glucosidase inhibition was evaluated on days 0, 8, 21 and 40. The aroma compounds in roselle wine were analysed at the end of the experiment (day 40).

5.3.4 Impact of fining on roselle wine colour and phenolic compounds

To assess the effect of fining on colour indices and phenolic compounds, bentonite at three different concentrations (240, 480 and 720 mg/L) commonly applied in the wine industry was employed for the experiment. Wine samples (25 mL) on day 60 prior to bottling were collected from a batch and were treated with bentonite prepared by dissolving in warm distilled water. The mixture was then allowed to swell for 48 hours before adding it to the wine samples. The samples were vortexed transferred to 25 mL glass tubes and kept in the dark to protect against light for 72 hrs (Waterhouse and Ebeler, 1998). The fining was done in triplicate after which samples were centrifuged (2500 g;10 min) and filtered using a Whatman no 1 paper and the analysis on specific phenolic compounds, total phenolics and colour parameters were determined as mentioned in section 5.2.3.

5.3.5 Statistical analysis

Statistical analysis was performed by one-way analysis of variance using the Statistical Analysis System (SAS) version 9.4 software. Significant differences were assessed with Least Significant Difference (LSD) test ($p \leq 0.05$).

5.4 Results and discussion

5.4.1 pH, titratable acidity and sugar consumption kinetics

The changes in pH and titratable acidity are presented in Table 5-1. The pH values on the final day (day 40) were ≈ 3.0 and 3.1 at 20 °C and 30 °C respectively and were not significantly different ($p \geq 0.05$). TA levels increased in the course of fermentation at both temperatures and this can be associated with the production of α-ketoglutaric and succinic acids in the glyceropyruvic pathway during fermentation. In the early stages of fermentation, the yeast by-products tend to be pyruvic acid and glycerol, but not ethanol; pyruvic acid then goes on to form secondary products like α-ketoglutaric acid, succinic acid, diacetyl, and acetoin (Whiting, 1976). Concerning yeast activity, the rate of consumption of reducing sugars was faster at 30 °C than at 20 °C up until day 3 (Figure 5-1). This is possibly due to the delay in attaining maximal population (longer lag phase) at 20 °C compared to 30 °C. Thereafter, sugar consumption proceeded faster at 20 °C (longer stationary phase) until the end of fermentation. The same pattern was observed in another study (Torija *et al.*, 2003b), where between fermentation temperatures of 15 to 35 °C, yeast cells attained maximal population size much slower at the lower temperatures, thereafter remaining constant throughout fermentation resulting in higher alcohol.

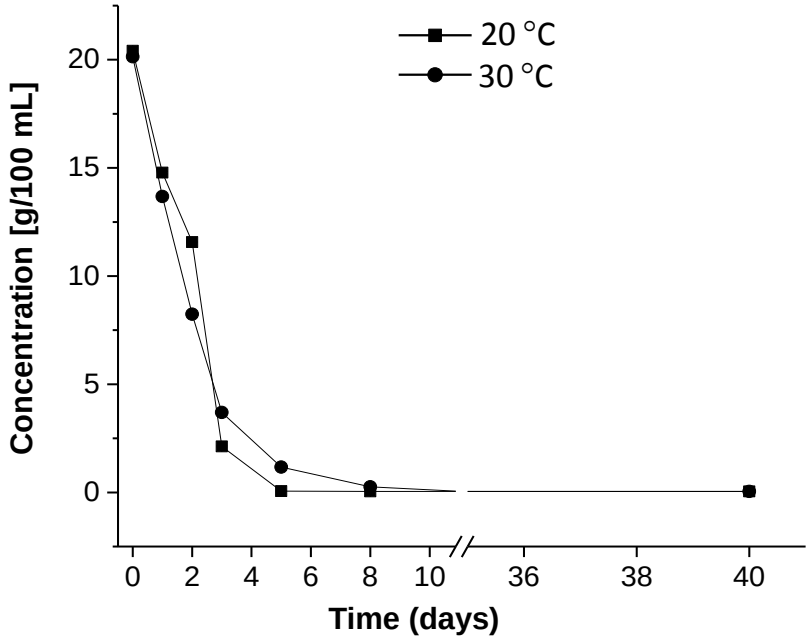


Figure 5-1 Sugar consumption kinetics during fermentation of roselle wine. The results are presented as mean ± SD. (n = 6).

5.4.2 Wine colour

A firm principle in oenology is that a good red wine should be "rich in colour" (Somers and Evans, 1974). Wine colour is derived from a complex chain of reactions controlled by factors such as the type and amount of flavonoids within the raw material, their extraction efficiencies during fermentation and their stability in the course of ageing (Ristic *et al.*, 2007).

Table 5-1 Changes in physicochemical properties during fermentation of roselle wines.

attribute /fermentation temperature (°C)	changes in physicochemical attributes during fermentation of day 0	day 3	day 8	day 21	day 40
pH					
20	3.09 ±	2.96 ±	2.97 ±	2.97 ±	3.00 ± 0.04
30	3.09± 0.04	2.98 ±	3.06 ±	3.06 ±	3.07 ± 0.02
Titratable acidity (g/L)					
20	2.13 ±	5.6 ± 0.01	5.4 ± 0.04	5.74 ±	5.74 ± 0.16
30	2.13 ±	5.7 ± 0.04	5.5 ± 0.16	5.40 ±	5.45 ± 0.17
Colour density					
20	13.4 ± 3.3	31.3 ± 2.2	21.8 ± 0.5	23.5± 0.1	21.8 ±0.2 [a]
30	13.4 ± 3.3	25.0 ± 0.4	17.1 ± 0.1	19.6 ± 0.1	18.9 ± 0.1 [b]
Hue tint					
20	0.35 ±	0.29 ±	0.33 ±	0.34± 0.03	0.36 ±0.02 [b]
30	0.35 ±	0.33 ±	0.35 ±	0.37± 0.01	0.39 ± 0.01[a]
Polymeric anthocyanin					
20	6.6 ± 1.5	5.5 ± 1.4	9.6 ± 0.5	11.7 ± 0.2	12.7 ± 0.8 [a]
30	6.6 ± 1.5	8.2 ± 0.2	11.0 ± 0.3	11.8 ± 0.2	11.5 ± 0.1 [b]
CIE*L*					
20	28.6 ± 3.3	19.4 ± 0.1	23.7 ± 0.1	17.5 ± 0.2	16.7 ± 0.1 [b]
30	28.6 ± 3.3	22.2 ± 0.2	23.8 ± 0.1	20.6 ± 0.3	19.6 ± 0.2 [a]
Chroma (C)					
20	54.9 ± 3.1	51.5 ± 0.3	54.8 ± 0.2	47.3 ± 0.3	45.6 ± 0.1 [b]
30	54.9 ± 3.1	53.6 ± 0.2	53.8 ± 0.1	50.1 ± 0.1	48.5 ± 0.1 [a]
Hue (H)					
20	38.4 ± 2.8	35.2 ± 0.3	38.7 ± 0.4	33.2 ± 0.4	32.8 ± 0.4 [b]
30	38.4 ± 2.8	38 ± 0.34	38.0 ± 0.2	36.3 ± 0.5	35.1 ± 0.6 [a]

Mean values ± standard deviation. Day 0 represents the average in vats prior to fermentation and values with different letters on the same column indicate statistically significant differences at $p \leq 0.05$.

During skin contact fermentation, flavan-3-ols and tannins needed for the stability of wine colour continue to increase their concentration and it has been found that tannins continue to be extracted after anthocyanin levels reach the maximum (Ivanova *et al.*, 2011). Hence, the contact time is also critical for obtaining wines with good colour intensity and stability as longer maceration time has been shown to produce wines with higher anthocyanin content and colour density (Gómez-Plaza *et al.*, 2002). The evolution of colour indices in roselle wine is shown in Table 5-1. There was a significant decrease ($p \leq 0.05$) in L∗ C∗ and H* values from the colorimetric analyses of roselle wine on day 40 against day 0 at both temperatures conditions. The data from the tintometer implies that the wine colour was darker and more saturated on day 40, which can be directly linked to the polymerization of anthocyanins. Furthermore, the spectrophotometric data on wine colour parameters are also presented in Table 5-1. In red young wines, the colour density is mainly due to monomeric anthocyanins and a direct correlation between anthocyanins content and colour density of wines has been reported (Mazza *et al.*, 1999). However, as wine matures, the colour is highly dependent on more stable polymeric compounds formed through polymerization with other flavonoids and tannins (Mazza *et al.*, 1999; Atanasova *et al.*, 2002). The levels of delphinidin 3-*O*-sambubioside (DS), cyanidin 3-*O*-sambubioside (CS) and polymeric anthocyanins recorded on day 0 justifies why CD readings were least for the wines at both temperatures. Thereafter, the increase observed is mainly due to the increased extraction of these anthocyanins and the formation of some polymerised anthocyanins occurring simultaneously during fermentation. The CD values were highest on day 3 and this is similar with published data on wine colour density where maximum values were attained between day 2 and 3 after the onset of fermentation (Reynolds *et al.*, 2001).

For the hue tint (browning index), the values increased (excluding day 0) for both temperatures as fermentation progressed due to polymerization of anthocyanins. The degradation of anthocyanins during pasteurisation of the juice might explain the contradiction observed on day 0. In blue-berry wines, hue readings increased after high temperature short time (HTST) pasteurisation and the same effect was also observed after pasteurisation of orange juice (Rommel *et al.*, 1992; Lee and Coates, 2003). Moreover, the decrease in individual anthocyanins (Table 5-2) after attaining maximum levels and the concomitant increase in polymeric anthocyanins agrees with previous studies on colour evolution during wine processing (Alcalde-Eon *et al.*, 2006).

5.4.3 Main phenolic compounds of roselle wine

The changes in the concentration of selected phenolic compounds during fermentation are shown in Table 5-2. These compounds were chosen on the basis of their relative amounts found in the extracts (Table 3-2). As anticipated, the results showed increases in quantities of phenolic compounds as fermentation progressed as contact between the liquid and solid phase (fermenting must and calyces) provides for more extraction of phenolics from the solid calyces into the fermenting must. DS and CS contents peaked on day 3 at 20 °C, while their concentrations were highest on day 2 for wines fermented at 30 °C. The subsequent decrease observed in the anthocyanin content after day 8 is a combination of polymerization and oxidation reactions, adsorption by yeast cells/fixation on solid portions, and enzymatic activity by microbial glucosidases (Mena *et al.*, 2012). Gallic acid levels increased steadily from the onset achieving highest concentrations on day 21 at both temperatures. The increase in gallic acid can be the result of increased extraction and also the breakdown of hydrolysable tannins arising from yeast activity. Perhaps the major transformation in phenolic compounds (irrespective of fermentation temperature) was the rapid decrease in 3CQA levels. The reduction was followed by the

simultaneous increase in caffeic acid, indicating the hydrolysis of 3CQA into caffeic and quinic acids. By day 40, 3CQA content had dropped by over 80 % from their initial contents, while caffeic acid levels increased by ≈ 84 %. This occurrence could be significant as caffeic acid has been shown to be a more potent inhibitor of both α-amylase and α-glucosidase than chlorogenic acid *in vitro* (Oboh *et al.*, 2015). Furthermore, while caffeic acid is readily absorbed into the bloodstream, most of the chlorogenic acid consumed reaches the colon intact where the quinic acid moiety is cleaved off by the action of the colonic microbiota to release caffeic acid (Plumb *et al.*, 1999). Myricetin 3-arabinogalactoside (M3A) and quercetin 3-sambubioside (Q3S) concentrations peaked around day 3 which was followed by a gradual decrease as fermentation progressed at both temperatures. Their reduction can be attributed to hydrolysis, oxidation and precipitation reactions occurring during the winemaking process (Ginjom *et al.*, 2011). Altogether, the data on compositional changes in phenolic compounds in roselle wine showed that fermentation temperature had no major impact on the phytochemical profiles of the wine. In summary, the proportion of DS, CS, M3A, and Q3S decreased after achieving a maximum concentration, while GA content increased as fermentation progressed. Moreover, the increase in caffeic acid levels occasioned by fermentation makes roselle wine a rich source of this bioavailable compound (Oboh *et al.*, 2015).

Table 5-2 Changes in phenolic compounds during fermentation of roselle wine.

compound/fermentation temperature (°C)	content in mg/L during days of fermentation						
	day 0	day 1	day 2	day 3	day 8	day 21	day 40
gallic acid							
20	4.3 ± 0.2	7.3 ± 0.5	8.2 ± 0.7	8.4 ± 0.1	9.2 ± 0.1	9.6 ± 0.1	9.6 ± 0.04 [a]
30	4.3 ± 0.2	6.9 ± 0.3	8.3 ± 0.2	8.6 ± 0.1	9.2 ± 0.1	9.9 ± 0.4	9.6 ± 0.10 [a]
3-*O*-caffeoylquinic acid							
20	81 ± 9	80.3 ± 2.5	60.3 ± 2.3	48.3 ± 7.2	24 ± 0.1	16.1 ± 0.2	8.4 ± 0.6 [a]
30	81 ± 9	51.3 ± 0.4	33.2 ± 3.5	24.5 ± 4.4	9.9 ± 1.4	6.6 ± 0.1	5.0 ± 0.4 [b]
Caffeic acid							
20	15.2 ± 0.7	54.0 ± 1.8	56.3 ± 0.8	62.0 ± 1.6	73.0 ± 1.4	91.0 ± 3.7	93.0 ± 4.7 [a]
30	15.2 ± 0.7	52.7 ± 2.0	63.2 ± 1.3	66.0 ± 0.8	70.5 ± 2.2	108.0 ± 0.5	98.1 ± 1.7 [a]
DS							
20	243 ± 29	413 ± 17	428 ± 13	452 ± 6	407 ± 3	350 ± 2	318 ± 4 [a]
30	243 ± 29	392 ± 18	400 ± 13	381 ± 10	344 ± 20	290 ± 2	266 ± 2 [b]
CS							
20	61 ± 8	106 ± 5	116 ± 3	129 ± 2	119 ± 2	113 ± 7	112 ± 12 [a]
30	61 ± 8	110 ± 4	116 ± 2	112 ± 2	102 ± 3	97 ± 13	82 ± 3 [a]
M3A							
20	4.4 ± 0.5	5.7 ± 0.2	7.1 ± 0.3	7.3 ± 0.7	5.9 ± 0.2	6.4 ± 0.5	5.8 ± 0.4 [a]
30	4.4 ± 0.5	5.2 ± 0.3	6.6 ± 0.3	6.4 ± 0.4	6.2 ± 0.3	5.5 ± 0.4	4.4 ± 0.2 [a]
Q3S							
20	3.4 ± 0.3	4.3 ± 0.1	5.4 ± 0.1	5.3 ± 0.5	4.4 ± 0.1	4.8 ± 0.8	5.3 ± 0.1 [a]
30	3.4 ± 0.3	3.9 ± 0.1	5.2 ± 0.1	5.3 ± 0.1	5.2 ± 0.2	4.2 ± 0.1	4.9 ± 0.3 [a]
TPC (Folin's)							
20	743 ± 34	888 ± 12	872 ± 76	962 ± 20	1130 ± 60	1332 ± 40	1363 ± 44 [a]
30	743 ± 34	864 ± 90	886 ± 79	1080 ± 35	1103 ± 30	1270 ± 30	1260 ± 13 [b]

Mean values ± standard deviation. Day 0 represents the average in vats prior to fermentation and values with different letters on the same column indicate statistically significant differences at $p \leq 0.05$. ($n = 6$).

5.4.4 Phenolic content and α-glucosidase inhibition of roselle wine

Phenolic compounds apart from their health benefits are known to influence wine colour, astringency and overall quality of wines (Lopez-Velez *et al.*, 2003). The final level in wine is impacted by the variety, fermentation temperature and other processing conditions. The soluble phenolic content in roselle wine increased from 743 mg/L (day 0) to 1260 and 1363 mg/L on the final day for wines fermented at 30 and 20 °C respectively. In comparison with wine from other fruit sources, the concentration in young roselle wine is within the range of 971 - 1753 mg/L found in fruit wines processed from cranberry, raspberry and elderberry (Rupasinghe and Clegg, 2007).

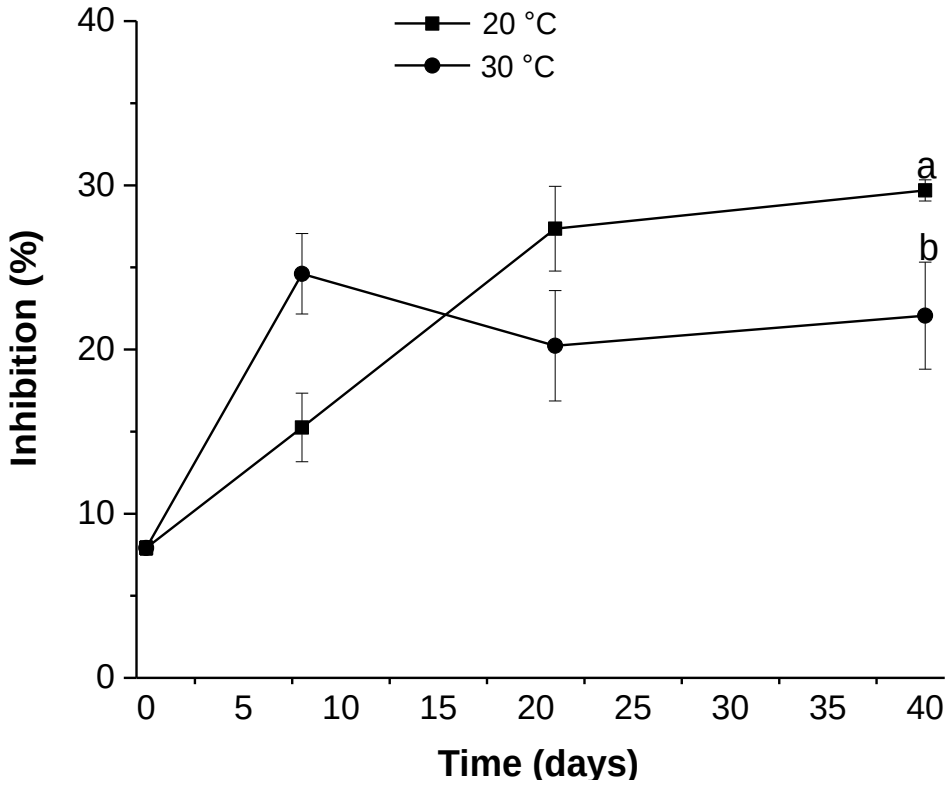


Figure 5-2 Impact of fermentation temperature on α–glucosidase inhibition of roselle wine. The results are expressed as mean ± SD. (n = 6). The different letters indicate significant difference between fermentation temperatures at $p \leq 0.05$.

With respect to the inhibition of α-glucosidase by roselle wine phenolics, this activity increased as fermentation progressed in wine fermented at 20 °C, while at 30 °C, the inhibition increased from day 0 to 8, thereafter declining and remaining constant until day 40 (Figure 5-2). In blueberry wines, fermentation at room temperature (20 - 22°C) and 4 °C showed no difference in their inhibition of α-glucosidases (Johnson *et al.*, 2011). A possible explanation to this occurrence in this experiment is that the secondary compounds derived from anthocyanin breakdown (polymeric compounds after day 8) at 20 °C were better inhibitors than the compounds produced at 30 °C. Future studies could aim at isolating and identifying these compounds by SPE and LC-MS respectively (Sun *et al.*, 2006).

5.4.5 Organic acids profile

The changes in the organic acid profiles of roselle wine during fermentation are shown in Figure 5-3 (A & B). In general as fermentation progresses, yeast cells make adjustments physiologically to the changing medium and the organic acid content is generally affected (Main *et al.*, 2007). For instance, the production of lactic acid by *S. cerevisiae* during fermentation was reported to be favoured in the absence of thiamine in the medium (Whiting, 1976). Malic acid levels in wines at both fermentation temperatures increased from day 0 to day 3. This may be the direct result of the extraction of the organic acid from the calyces into the medium as fermentation progressed. Thereafter, the levels of malic acid reduced minimally possibly arising from yeast and bacterial activities and reached a concentration of ≈ 1.65 g/L on day 40 at both temperature conditions. The apparent increase observed on day 21 occurred after the racking (removal of calyces from yeast lees) of wines, and can be linked to increased solute concentration. Generally, the standard wine yeast *S. cerevisiae* is not able to degrade malic acid efficiently as it does not possess an active malate transport system (Redzepovic *et al.*, 2003). Furthermore, the

production of lactic acid registered on day 3 signifies the activity of lactic acid bacteria in the fermenting medium. The levels increased for both fermentation temperatures although a slight decrease was observed on day 40 for the wines fermented at 20 °C, which could be from the synthesis (esterification) of ethyl lactate. Although wine yeast produces small amounts of acetic acid as metabolites during fermentation, elevated amounts in wine are detrimental to wine quality and are mainly due to the oxidation of ethanol by acetic acid bacteria (Swiegers *et al.*, 2005). In this study, higher amounts of acetic acid were found at 30 °C and this agrees with an earlier study that showed increased acetic acid production as fermentation temperature increased from 18 to 33 °C (Liu *et al.*, 2014). Nevertheless in this work, the levels of acetic acid (0.02-0.04 g/L) are within the permitted levels of up to 1.5 g/L for red wine. One of the major organic acid metabolites produced by yeast which contributes to the salty-bitter acid taste of wines, is succinic acid and there were higher levels (1.18 g/L) produced in wine fermented at 30 °C. In a similar study, succinic acid production was 0.92 and 0.89 g/L for fermentations conducted at 30 and 20 °C on grape wine respectively (Torija *et al.*, 2003b). Citric acid levels in wine can fluctuate as it is both synthesised by yeast cells and later taken in and used up for other metabolic processes such as the production of flavour compounds (diacetyl) (Whiting, 1976). In this study, the final concentrations of citric acid were 0.03 and 0.04 mg/L for wines at 20 and 30 °C respectively. In general, while fermentation temperature did not produce any significant effect ($p \geq 0.05$) in final (day 40) malic acid content, the concentration of the other organic acids were higher at 30 °C and significantly different ($p \leq 0.05$) from wines fermented at 20 °C.

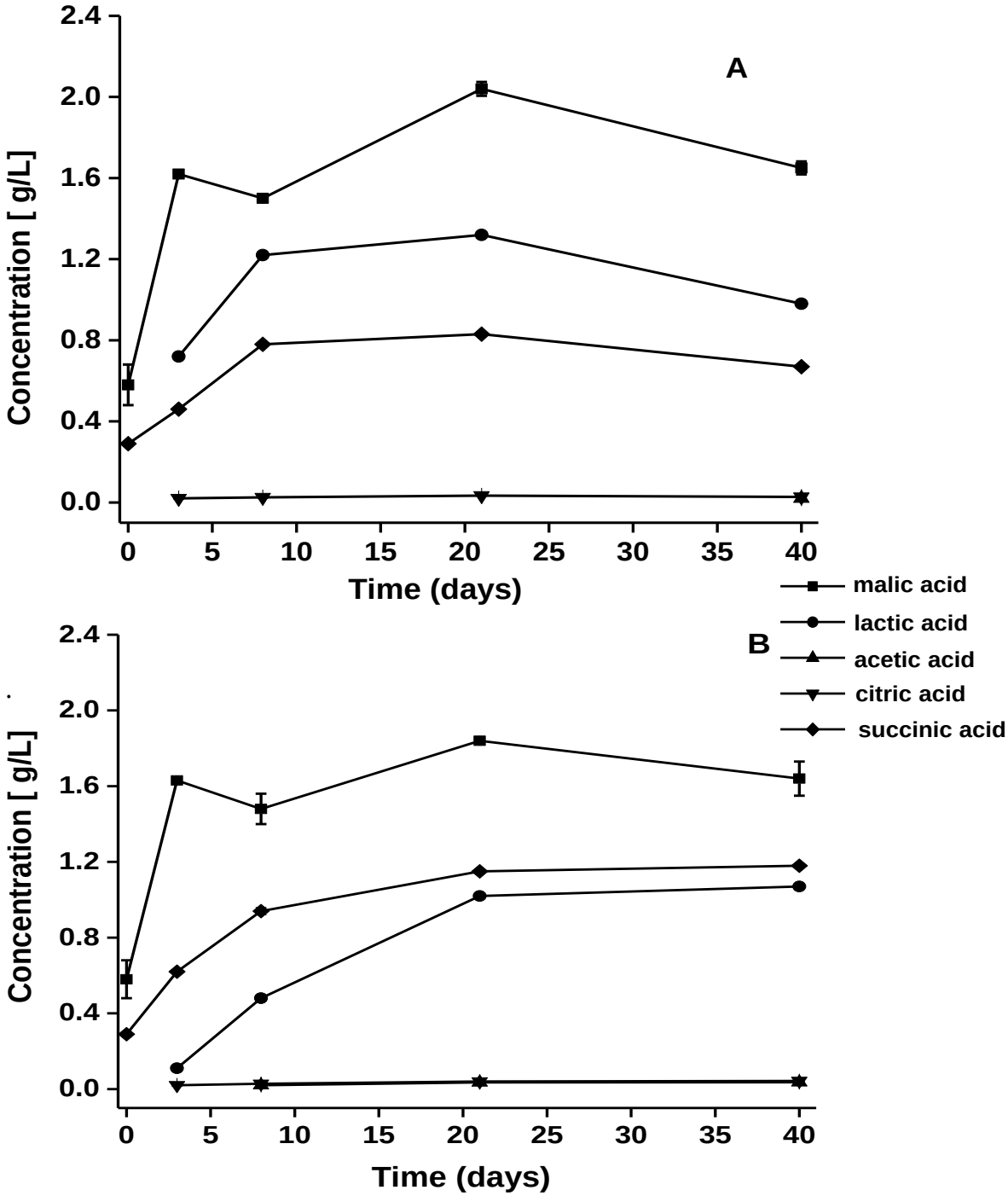


Figure 5-3 Evolution of organic acid during fermentation of roselle wine at (A) 20 °C and (B) 30 °C. The results are expressed as mean ± SD (n = 6).

5.4.6 Volatile composition of roselle wine

Wine aroma is a combination of volatiles derived from the raw material (grape or other plant source), fermentative aromas generated by the yeast (alcoholic fermentation), bacteria (malolactic fermentation), and volatiles generated during ageing of wines (Swiegers *et al.*, 2005; Antalick *et al.*, 2015). Some of the main fermentative volatiles/aromas synthesised by yeast include the higher alcohols, fatty acids, acetate and ethyl esters, amongst others (Pretorius and Lambrechts, 2000). The production of higher alcohols occurs via amino acid metabolism or by reduction of related aldehydes (Ehrlich mechanism) during yeast fermentation, while esters are mainly formed from lipid and acetyl-CoA metabolism (Figure 1-6) (Swiegers *et al.*, 2005). In this study, the major aroma compounds quantified on day 40 as depicted in Figure 5-4 were the fatty acids (hexanoic, octanoic and decanoic) and their ethyl esters, ethyl acetate, isoamyl alcohol, 2-phenyl ethanol and 1-hexanol.

The pre-fermentation volatiles detected in roselle musts that persisted throughout fermentation were 1-hexanol and eugenol (Figure 5-4). In addition, linalool detected in the must had undergone esterification to produce ethyl linalyl ether which was tentatively confirmed by comparing the spectra with that of ethyl linalool standard and published data on the qualifying ions. These three compounds had previously been identified in *H. sabdariffa* extracts (Ramírez-Rodrigues *et al.*, 2012). The concentrations of yeast-derived volatile compounds at the end of alcoholic fermentation are presented in Table 5-3 and revealed that higher levels of ethyl esters responsible for fruity aromas were produced at 20 °C relative to 30 °C. Ethyl hexanoate, ethyl octanoate and ethyl decanoate were the main aroma esters synthesised by yeast during fermentation with odour active values (OAV) ≥ 1, with the concentrations being $\approx$ 2-fold higher at 20 °C than at 30 °C.

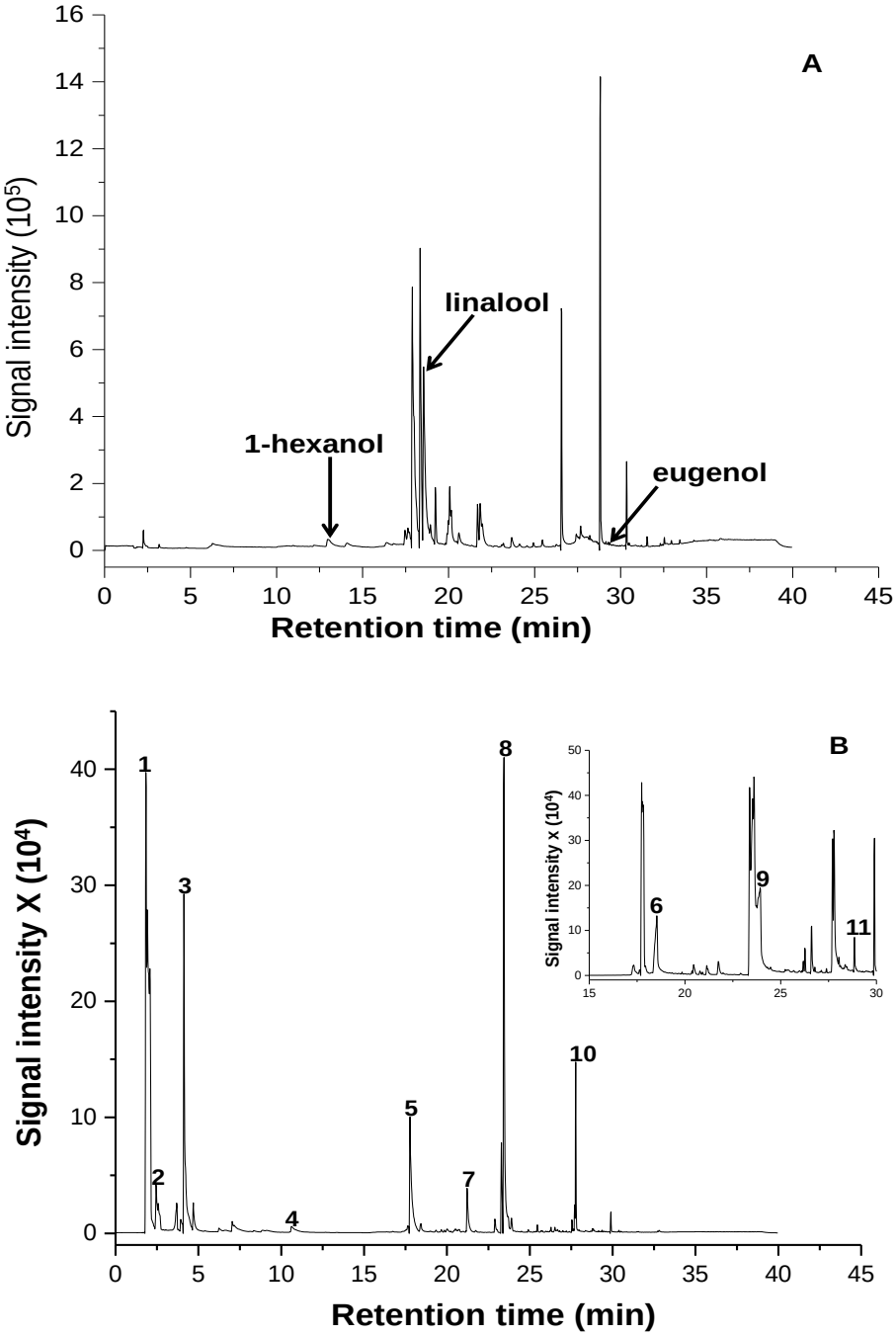


Figure 5-4 GC-MS chromatogram of (A) volatile compounds in roselle juice and (B) volatile compounds in roselle wine after alcoholic fermentation. (1) ethanol, (2) ethyl acetate, (3) isoamyl alcohol, (4) 1-hexanol, (5) ethyl hexanoate, (6) hexanoic acid, (7) 2-phenyl ethanol, (8) ethyl octanoate, (9) octanoic acid, (10) ethyl decanoate and (11) decanoic acid. The chromatogram inserted amplifies the volatiles detected at low concentrations in the wine.

On the other hand, higher amounts of higher alcohols mainly responsible for flowery and fusel notes in wines were synthesised at 30 °C. Three medium chain volatile fatty acids were investigated in this study (hexanoic, octanoic and decanoic acids) because of their known contribution to wine flavour, as well as being the main precursors for ethyl ester biosynthesis (Gallart *et al.*, 1997). These volatiles presented higher concentrations at 20 °C than at 30 °C; hence it is not surprising that higher amounts of their corresponding ethyl esters were formed at 20 °C. This result is consistent with published data on grape wines where fermentation at 13 °C resulted in higher synthesis of fusel alcohol acetate esters, fatty acids and their corresponding ethyl esters, compared to fermentation at 25 °C which favoured the production of fusel alcohols and increased volatile acidity (Beltran *et al.*, 2008). Furthermore, the higher levels of fruity esters obtained at lower temperatures has been reported to be due to greater stability of volatile compounds and reduced evaporative losses, whereas higher temperatures tend to suppress ester accumulation by favouring hydrolysis. With respect to ethyl acetate and diethyl succinate, the higher contents found at 30 °C can be linked to higher lactic acid bacteria activity at that temperature (Pozo-Bayón *et al.*, 2005).

Although the data on the influence of fermentation temperature on volatiles monitored in this research is in accordance with some published results (Torija *et al.*, 2003a; Molina *et al.*, 2007), it is important to state that the concentrations of these volatiles in wine is not totally dependent on temperature. Additional variables such as the raw material, aeration of fermenting medium, amino acid composition and yeast strain have also been proven to influence the content of these volatiles in wine (Pretorius and Lambrechts, 2000; Molina *et al.*, 2007; Saerens *et al.*, 2008). Therefore, it is important to consider all these factors when comparing data between studies involving wine.

Table 5-3 Content of volatile compounds in roselle wine fermented at 20 °C and 30 °C determined by HS-SPME-GCMS.

volatile compound	manufacturer[c]	qualifying ions	aroma descriptors	threshold concentration (ug/L)	concentration (ug/L)		OAV	
					20 °C	30 °C	20 °C	30 °C
ethanol (% v/v)	Sigma-Aldrich	**45**; 43; 74			11.53 ± 1.04 [a]	10.32 ± 1.38 [a]		
2-phenylethanol	Sigma-Aldrich	**91**; 92; 122	flowery,	10,000	527 ± 63 [b]	1163 ± 114 [a]	0.05	0.12
isoamyl alcohol	Sigma-Aldrich	**71**; 43; 55	fusel oil,	30,000	130539 ± 21943 [b]	209777 ± 7645 [a]	7	4.4
1-Hexanol	Alfa Aesar	**56**; 43; 69	green, grass	4000	248 ± 103 [a]	258 ± 60 [a]	0.06	0.06
ethyl acetate	Sigma-Aldrich	**43**; 61; 70	nail polish,	7500	156 ± 32 [b]	371 ± 27 [a]	0.02	0.04
ethyl hexanoate	Sigma-Aldrich	**88**; 99; 145	fruity,	5	3487 ± 363 [a]	1644 ± 90 [b]	697	328
ethyl octanoate	Alfa Aesar	**88**; 101; 172	fruity,	2	1339 ± 135 [a]	740 ± 33 [b]	669	369
ethyl decanoate	Sigma-Aldrich	**88**; 101; 200	pleasant,	200	966 ± 95 [a]	639 ± 53 [b]	4.8	3.1
hexanoic acid	Sigma-Aldrich	**73**; 60; 87	cheese,	8000	104 ± 20 [a]	65 ± 2 [b]	0.01	0.008
octanoic acid	Alfa Aesar	**73**; 60; 101	rancid, fatty	8800	99 ± 10 [a]	80 ± 7 [b]	0.01	0.008
decanoic acid	Sigma-Aldrich	**73**; 60; 129	fatty, rancid	6000	71 ± 7 [a]	61 ± 6 [b]	0.01	0.01
diethyl succinate[d]	Alfa Aesar	**101**; 129; 55	wine		53.92 ± 5.91 [a]	62.74 ± 11.13 [a]		
isoamyl acetate[d]	Sigma-Aldrich	**70**; 41; 88	fruity,		69.07 ±12.72 [a]	51.19 ± 7.59 [b]		
benzaldehye[d]	Sigma-Aldrich	**105**; 77; 51	bitter,		0.38 ± 0.06 [b]	0.61 ± 0.09 [a]		
eugenol[d]	Alfa-Aesar	**164**; 149; 103	spicy, clove		1.25 ± 0.08 [b]	2.07 ± 0.16 [a]		
ethyl dodecanoate[e]		**88**; 101; 228	soapy,		231 ± 36 [a]	112 ± 25 [b]		
2-phenyl acetate[f]		**104**; 91; 65	flowery,		7.08 ± 1.35 [a]	1.14 ± 0.11 [b]		
ethyl linalyl ether[f]		**99**; 71; 43	flowery		0.53 ± 0.03	NQ		

The values are the mean ± SD. (n = 6) Numbers bolded were used as the quantitative ion for aroma compounds; Data on threshold concentration are as presented by (Siebert *et al.*, 2005) . [c] Manufacturers: Sigma-Aldrich.Co., Ltd., Dors
Alfa Aesar, Lancashire, UK. [d] Compounds measured by semi-quantification; relative peak areas (compound: internal standard) were used to calculate means and standard deviation. [e] Quantified as ethyl decanoate equivalent. [f] Compo
tentatively identified. NQ: detected but could not be quantified because of the low MS signal at the quantitation ion.

5.4.7 The effect of fining on phenolic compounds and colour parameters of roselle wine

The use of bentonite, a clay material, is universally accepted for the adsorption of proteins from wines, primarily due to the cation exchange action of these clays. Furthermore, theoretically, bentonite effects include electrostatic interaction with all compounds bearing or carrying a positive net charge at wine pH such as anthocyanins, which may result in colour loss (Gómez-Plaza *et al.*, 2000a). The result of bentonite treatment on selected phenolics in roselle wine is presented in Table 5-4. The results showed that fining with bentonite had a diminishing effect ($p \leq 0.05$) on mainly the anthocyanins, with the impact being more pronounced as the concentration of bentonite increased. This pattern agrees with previous studies on the effect of bentonite on grape wine anthocyanins (Stankovic *et al.*, 2012; González-Neves *et al.*, 2014) The adsorption of anthocyanins by bentonite has been described as being identical to what is obtained in the adsorption of these compounds by yeast cells, with the degree of adsorption increasing with the polarity of the anthocyanins (González-Neves *et al.*, 2014). Therefore, it is justifiable that DS being more polar than CS was more affected by bentonite. Furthermore, we can infer that the reduction in anthocyanin concentration might have occasioned the reduction in the total phenolic content.

The colour parameters CD and the L* values (Figure 5-5) significantly ($p \leq 0.05$) affected by bentonite treatment and this can also be linked to the reductions in DS and CS concentrations. In a similar study, wines treated with bentonite after fermentation had lower CD than the untreated (Castillo-Sánchez *et al.*, 2006). On the contrary, when bentonite was added to wines in the pre-fermentative stage, it produced an increase in colour density and this was attributed to the ability of the fining agent to eliminate solids and yeast lees which get fixed to anthocyanins during fermentation (Gómez-Plaza *et al.*,

2000a). In future, it would be worth investigating which treatment procedure enhances the colour properties of roselle wine as a means of improving the overall quality of the product.

Table 5-4 Effect of bentonite treatment on phenolic compounds in roselle wine.

Compound	Control (Without bentonite content in mg/L)	Bentonite treatment (mg/L)		
		240	480	720
Gallic acid	9.9 ± 0.16^{b}	10.1 ± 0.05^{a}	10.1 ± 0.03^{a}	10.1 ± 0.03^{a}
Caffeic acid	68.7 ± 0.1^{a}	70.5 ± 2.9^{ab}	68.50 ± 0.02^{b}	68.40 ± 0.06^{c}
M3A	4.5 ± 0.01^{a}	4.5 ± 0.01^{a}	4.5 ± 0.01^{a}	4.5 ± 0.01^{a}
Q3S	3.9 ± 0.02^{a}	4.0 ± 0.14^{a}	3.8 ± 0.01^{a}	3.9 ± 0.17^{a}
DS	195.4 ± 0.4^{a}	191.3 ± 0.5^{b}	186.6 ± 0.2^{c}	179.6 ± 1.5^{d}
CS	64.9 ± 0.12^{a}	62.2 ± 2.20^{ab}	59.9 ± 1.02^{b}	54.9 ± 2.6^{c}
TPC (Folin's)	1158.65 ± 8.65^{a}	1125.32 ± 8.83^{b}	1135.85 ± 17.31^{bc}	1114.21 ± 9.28^{c}

Mean values ± standard deviation (n = 3). Values with the same letters on the same row are not significantly different $p \leq 0.05$.

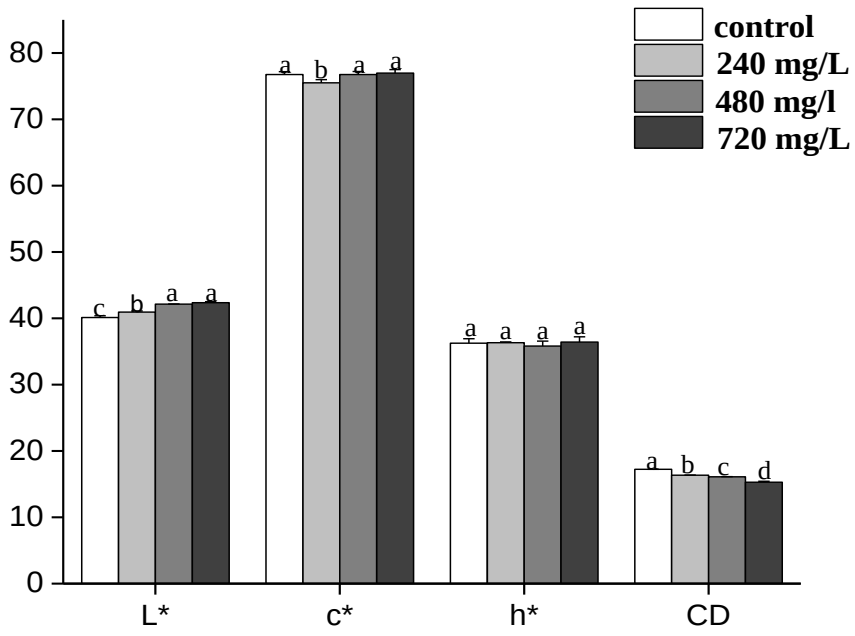


Figure 5-5 The effect of bentonite treatment on colour parameters in roselle wine. Values with the different letters denote significant difference $p \leq 0.05$.

5.5 Conclusion

The aim of this study was to show the impact of fermentation temperature on the physicochemical/phytochemical profiles and aroma attributes of roselle wines. The result show that the fermentation method employed (contact with calyces) had a positive effect in enhancing the colour of the wine, and this style of fermentation provides an alternative to reducing colour degradation previously reported during roselle wine manufacture (Alobo and Offonry, 2009). This may be associated with the increased extraction of compounds (flavonols and tannins) necessary for wine colour stability during fermentation and subsequent ageing. Whilst fermentation temperature had no major effect on physicochemical and phytochemical composition in the wines, it however did produce different aroma attributes at the temperatures investigated. This work clearly demonstrates that fermentation temperature has a major impact on the final product quality. This powerful tool alongside the yeast strain employed for fermentation can be used to modulate the colour, aroma and flavour attributes of roselle wine to meet consumer preferences.

Chapter 6 The effect of ageing conditions on the physicochemical properties, phytochemical profile and bioactivity of roselle wine

6.1 Abstract

The effect of temperature (6, 15 and 30 °C) during ageing on the colour, phytochemical composition and bioactivity of roselle wine was investigated over a period of 12 months. At the end of ageing, wines stored at 6 °C had the highest colour density (CD) and lowest polymeric anthocyanins. Although the concentration of most of the individual phenolic compounds analysed decreased with ageing, the anthocyanins suffered the greatest degradation. The degradation of anthocyanins were seen to follow first-order kinetics, with wine aged at 30 °C having the highest degradation rate constant. However, the decrease in phenolic compounds did not adversely affect the bioactivity of roselle wines, as the inhibition of α-glucosidase remained relatively stable throughout ageing. Regarding the changes in organic acids, there was a gradual decrease in malic and succinic acid content with final concentrations being significantly less ($p \leq 0.05$) than initial conditions, while acetic acid levels increased significantly ($p \leq 0.05$), within the first four months, thereafter remaining stable throughout ageing. Although ageing temperature did not produce an obvious trend in concentrations of most of the volatiles analysed, its impact was clearly demonstrated in the final concentrations of diethyl succinate (vinous), which increased with higher ageing temperature. In summary, the final concentrations of anthocyanins and diethyl succinate were the major compounds influenced by ageing temperature.

6.2 Introduction

The final sensorial quality of a wine is the result of multiple interactions between all the chemical components and specific environmental factors such as the ageing temperature (Styger *et al.*, 2011). The changes in the phenolic compounds of wines during the ageing process are very complex because of a wide variety of factors ranging from the fermentation style employed to the ageing style and vessel etc. Notwithstanding, decreases in flavonoids and anthocyanins compounds are commonly observed with the rate of degradation being more pronounced with the anthocyanins in red wines (Nagel and Wulf, 1979; Sun and Spranger, 2005). One of the obvious effects of anthocyanin degradation during ageing is the wine colour. Initially, red wine may deepen in colour especially during fermentation, because of the rapid extraction of monomeric anthocyanins. However, the intensity gradually fades away as the tint takes on a ruby and then a brick red colour. This, as earlier mentioned (section 1.3.4) is due to the formation of new stable polymeric compounds, brought about by a series of complex reactions which ultimately lead to a decrease in the colour density (CD) and a shift in the maximum absorption spectra from 520 to 420 nm (Somers and Pocock, 2015).

During ageing, the wine acquires aromatic complexity resulting from different phenomena such as: esterification/hydrolysis reactions, redox reactions, spontaneous clarification, CO_2 elimination, slow and continuous diffusion of oxygen through wood pores (oak ageing) and transference of tannins and aromatic substances, mostly terpenes, from wood into the wine. This results in the loss of the characteristic aromas linked to the grape variety (or the raw material), and modifications of the volatiles formed during alcoholic fermentation, which ultimately leads to the formation of new aromas characteristic of older wines (Câmara *et al.*, 2006). Some of the reported modifications in volatile composition include the reduction in concentrations of fusel alcohol acetates and

ethyl esters of straight–chain medium fatty acids, while the levels of the branched chain fatty acid ethyl esters are more or less stable or even increase during aging (Sumby *et al.*, 2010). In the case of higher alcohols, the changes that occur are not clear, while some researchers have reported increases in the concentration of higher alcohols, the reduction in specific alcohols has also been observed in some published studies (Câmara *et al.*, 2006; Boido *et al.*, 2009). These compounds (major and minor higher alcohols) account for about 50 % of aroma compounds, with isoamyl alcohol being a major contributor to the flavour of wines. Regarding fatty acids, it is generally accepted that increases in their concentrations occur from the hydrolysis of esters, notwithstanding the reduction in their concentrations has also been reported (Câmara *et al.*, 2006; Garde-Cerdán *et al.*, 2008). The organic acids also participate in modifying the flavour and aroma of wine by reacting with ethanol and other alcohols to produce esters. The concentration during ageing of succinic acid has been investigated with decreases generally observed and linked to the formation of diethyl succinate (Wang *et al.*, 2015). In addition, some wines can undergo the secondary fermentation known as malolactic fermentation (MLF) which involves the decarboxylation of L-malic acid into L-lactic acid by lactic acid bacteria (LAB) and the production of other metabolites that impact on the sensorial qualities of the wine. Citric acid can also be metabolised by LAB and when it occurs, the most important significance is the production of diacetyl which contributes to the characteristic buttery aroma in wines (du Toit *et al.*, 2011). In the previous experiment (chapter 5), fermentation temperature (20 and 30 °C) did not produce any difference in the phytochemical composition; however it did influence the inhibition of α-glucosidase activity and the aroma profile. Therefore, it was of interest to study how ageing conditions would impact on the phenolic profile and inhibition of α-glucosidase potential as well as the stability of the aroma compounds. The information generated in this experiment could then be used to establish

quality control measures and provide valuable information on the best practices during ageing. In the light of the above, the objective of this experiment was to evaluate the impact of ageing temperatures on roselle wine colour, phytochemicals and the subsequent bioactivity.

6.3 Materials and methods

6.3.1 Ageing/storage conditions

Following the end of fermentation, wines were racked for the second time and degassed using a vacuum chamber (pilot plant of John Fraser and Son Ltd) operated for 30 min. The wines were then divided into two groups: (A) consisted of wines fermented at 20 °C, and (B) wines fermented at 30 °C. The wines were transferred into standard green wine bottles (750 mL) previously sterilised (distilled water at 100 °C for 15 min) and rinsed with a (500 mL) 200 ppm sodium metabisulphite solution. Three temperature conditions (6, 15 and 30 °C) were used for ageing, which lasted for 12 months. Duplicate bottles were prepared for each ageing temperature, bringing the total number of bottles to 12 for the whole experiment. The bottles were placed in slanting positions in incubators (Sanyo-MIR-153, Japan) programmed at the three ageing temperatures. The temperature of 15 °C was chosen to represent the normal ageing temperature (control) for wines, while 6 and 30 °C were chosen as low and high temperature ageing respectively.

6.3.2 Wine analysis during ageing

Physicochemical analysis, identification and quantification of polyphenols and total phenols on roselle wine were determined according to the methods described in chapter 2 sections 2.3 & 2.4, 2.5 to 2.7, and 2.8 respectively. In addition, simple sugars and organic acids were analysed under the conditions reported in chapter 2 sections 2.8 and 2.9 respectively. Furthermore, the analysis of roselle wine volatiles was performed under

the conditions optimised in chapter 2 section 2.10, while the enzyme inhibition assay was performed as described in chapter 4 section 4.2.7. Colour measurements were recorded on months 2, 4, 8 and 12. The changes in phenolic compounds were monitored on months 1, 2, 3, 4, 6, 8 and 12, while the effect of ageing conditions on organic acids, total phenolic content and α-glucosidase inhibition was evaluated on months 4, 8, and 12. Roselle wine volatiles were analysed at the end of the ageing period. Wine samples (5 mL) were taken from each bottle kept at the ageing temperature and the analysis were all performed in triplicates (n = 6).

6.4 Results and discussion

6.4.1 Wine colour during ageing

The temperature of wine storage plays a vital role in wine pigment degradation and polymerisation and it is a primary environmental factor that influences changes in the colour characteristics of red wine (Gómez-Plaza *et al.*, 2000b; Somers and Pocock, 2015). Furthermore, the duration of storage also influences wine colour and this is because most of the changes occurring during storage are time dependent (Dallas and Laureano, 1994). These two factors together with the pH, SO_2, and alcohol content affect the physicochemical equilibria and structure of wine pigment and are responsible for subtle alteration in colour during ageing (Dallas and Laureano, 1994). In this experiment, there was a steady increase in hue and polymeric anthocyanins (PPA) colour readings as ageing progressed with wines stored at 30 °C having the highest measurements (Table 6-1). The increase in these colour indices occurred concurrently with a steady decline in colour (CD) values and at the end of ageing, there was a significant difference ($p \leq 0.05$) between the initial (after fermentation) and final readings for all ageing conditions and also between ageing temperatures. This is consistent with results obtained by other researchers

who have reported the steady increase in polymeric anthocyanins and hue tint during ageing of wines (Dallas and Laureano, 1994; Gao *et al.*, 1997; Gómez-Plaza *et al.*, 1999; Gómez-Plaza *et al.*, 2000b). The decrease in colour density as demonstrated in this experiment was also observed in two separate studies with grape wines stored for 12 and 24 months as well as in raspberry wines aged for 6 months (Rommel *et al.*, 1990; Gómez-Plaza *et al.*, 2002; Castillo-Sánchez *et al.*, 2008). In contrast, in another study CD remained relatively constant during ageing, despite the reduction in monomeric anthocyanins (García-Falcón *et al.*, 2007). In such instances, it has been suggested that the monomeric anthocyanins are polymerised (rather than lost through degradation) and these polymerised anthocyanins contribute to the colour of wine. Furthermore, the formation of reddish brown pigments during ageing that absorb at 420 nm add to the colour density (A420 nm + A520 nm) and might offset any reduction in colour readings at 520 nm. The data in this experiment also showed that ageing temperature had a pronounced effect on the final colour measurements investigated. Regarding the effect of ageing temperature, wines aged at 30 °C had the lowest CD and highest polymeric anthocyanin. This agrees with other published studies that demonstrate the same trend (lower CD and higher polymeric anthocyanins) with higher ageing temperature (Dallas and Laureano, 1994; Gómez-Plaza *et al.*, 2000b).

Table 6-1 Changes in wine colour attributes during bottle ageing at different temperatures.

fermentation temperature	colour indices/ageing temperature (°C)	changes during ageing of roselle wine				
		after fermentation	month 2	month 4	month 8	month 12
20 °C	colour density					
	6	21.8 ± 0.2 $^{\alpha}$	20.16 ± 0.23	20.25 ± 0.28	17.76 ± 0.62	16.76 ± 0.03 $^{a, \beta}$
	15	21.8 ± 0.2 $^{\alpha}$	19.15 ± 0.13	18.05 ± 0.25	15.30 ± 0.15	14.31 ± 0.02 $^{b, \beta}$
	30	21.8 ± 0.2 $^{\alpha}$	17.47 ± 1.00	13.59 ± 0.07	10.48 ± 0.37	9.54 ± 0.58 $^{c, \beta}$
	hue tint					
	6	0.36 ± 0.02 $^{\alpha}$	0.35 ± 0.01	0.41 ± 0.01	0.42 ± 0.10	0.43 ± 0.01 $^{c, \beta}$
	15	0.36 ± 0.02 $^{\alpha}$	0.37 ± 0.00	0.45 ± 0.01	0.51 ± 0.13	0.55 ± 0.01 $^{b, \beta}$
	30	0.36 ± 0.02 $^{\alpha}$	0.42 ± 0.00	0.58 ± 0.00	0.61 ± 0.02	0.68 ± 0.02 $^{a, \beta}$
polymeric anthocyanin (%)						
	6	12.7 ± 0.8 $^{\alpha}$	22.17 ± 0.39	20.78 ± 0.34	31.40 ± 0.60	32.30 ± 5.06 $^{c, \beta}$
	15	12.7 ± 0.8 $^{\alpha}$	27.58 ± 0.25	31.18 ± 0.22	47.95 ± 5.60	63.40 ±1.64 $^{b, \beta}$
	30	12.7 ± 0.8 $^{\alpha}$	33.18 ± 1.64	53.44 ± 0.96	67.14 ± 0.13	96.75 ± 4.02 $^{a, \beta}$
30 °C	colour density					
	6	18.9 ± 0.1 $^{\alpha}$	17.53 ± 0.15	16.78 ± 0.07	15.70 ± 0.34	14.78 ± 0.26 $^{a, \beta}$
	15	18.9 ± 0.1 $^{\alpha}$	17.19 ± 0.08	15.89 ± 0.36	14.45 ± 0.30	13.00 ± 0.14 $^{b, \beta}$
	30	18.9 ± 0.1 $^{\alpha}$	14.81 ± 0.28	10.78 ± 0.78	9.18 ± 0.19	7.45 ± 0.89 $^{c, \beta}$
	hue tint					
	6	0.39 ± 0.01 $^{\alpha}$	0.41 ± 0.01	0.43 ± 0.01	0.45 ± 0.01	0.49 ± 0.02 $^{c, \beta}$
	15	0.39 ± 0.01 $^{\alpha}$	0.44 ± 0.01	0.49 ± 0.01	0.53 ± 0.01	0.56 ± 0.01 $^{b, \beta}$
	30	0.39 ± 0.01 $^{\alpha}$	0.50 ± 0.01	0.60 ± 0.04	0.64 ± 0.02	0.76 ± 0.02 $^{a, \beta}$
polymeric anthocyanin (%)						
	6	11.5 ± 0.1 $^{\alpha}$	18.98 ± 0.21	18.25 ± 0.27	25.72 ± 1.82	34.66 ± 0.67 $^{c, \beta}$
	15	11.5 ± 0.1 $^{\alpha}$	23.40 ± 2.26	29.70 ± 0.57	47.31 ± 2.83	55.04 ± 3.08 $^{b, \beta}$
	30	11.5 ± 0.1 $^{\alpha}$	39.46 ± 2.68	53.79 ± 0.39	69.49 ± 1.45	84.76 ± 8.00 $^{a, \beta}$

Values with different letters and Greek alphabet are significantly different between ageing temperatures and months respectively $p \leq 0.05$ ($n = 6$).

6.4.2 Evolution of phenolic compounds during wine ageing

Phenolic compounds are involved in the changes that take place during wine ageing as they are known to influence the colour, astringency, bitterness, oxidation level, and clarity of wines (del Alamo Sanza *et al.*, 2004). The results presented (Tables 6-2 & 6-3) showed that irrespective of ageing conditions, there was a gradual decrease in the concentrations of phenolic compounds apart from gallic acid. Monagas *et al.* (2005) analysed wines from three different grape varieties (Carbenet Sauvignon, Tempranillo and Graciano) and observed significant decreases in flavanols (glycosides) and anthocyanins content after 26 months of bottle ageing. There was however, no significant change in the final concentrations of total hydroxybenzoic acids although a slight increase in gallic acid concentration was seen in Granciano and Carbernet Sauvignon wines. In this study, the increase in gallic acid may be the outcome of the breakdown of hydrolysable tannins as observed in wines processed from pomegranate (Mena *et al.*, 2012). For hydroxycinnamic acids, Monagas *et al.* (2005) found an increase in caffeic acid, attributing the increase to be from the hydrolysis of caftaric acid. The increase in caffeic acid during bottle ageing of grape wines has also been reported by some authors (Puértolas *et al.*, 2010; Ginjom *et al.*, 2011). In contrast, Gutiérrez *et al.* (2005) found that the concentration of caffeic acid increased within the first three months of ageing, thereafter reducing following 9 months of ageing.

Table 6-2 Changes in phenolic compounds during ageing at different temperatures for wines fermented at 20 °C.

compound/ageing temperature (°C)	content in mg/L during months of ageing							
	after fermentation	1	2	3	4	6	8	12
gallic acid								
6	9.6 ± 0.04 $^{\alpha}$	9.6 ± 0.1	10.14 ± 1.01	10.17 ± 0.05	10.35 ± 0.10	10.65 ± 0.63	10.35 ± 0.09	10.80 ± 0.23 $^{b, \alpha}$
15	9.6 ± 0.04 $^{\alpha}$	10.0 ± 0.1	10.28 ± 1.03	10.23 ± 0.07	10.30 ± 0.10	10.89 ± 0.85	10.34 ± 0.23	10.34 ± 0.23 $^{b, \alpha}$
30	9.6 ± 0.04 $^{\alpha}$	10.2 ± 0.3	10.88 ± 1.12	10.70 ± 0.14	11.21 ± 0.12	13.55 ± 0.34	14.30 ± 0.51	12.38 ± 0.35 $^{a, \beta}$
caffeic acid								
6	93.0 ± 4.7 $^{\alpha}$	88.32 ± 8.86	83.9 ± 1.36	83.1 ± 7.0	81.40 ± 2.72	81.04±1.91	68.79 ± 1.58	60.04 ± 1.52 $^{a, \beta}$
15	93.0 ± 4.7 $^{\alpha}$	92.09 ± 5.30	80.6 ± 3.21	78.3 ± 0.1	78.21 ± 0.30	82.23 ± 2.31	68.27 ± 3.30	61.27 ± 1.11 $^{a, \beta}$
30	93.0 ± 4.7 $^{\alpha}$	91.09 ±13.06	78.05 ± 1.41	75.12 ± 1.51	73.09 ± 1.40	74.16 ± 2.42	67.68 ± 0.48	45.84 ± 0.78 $^{b, \beta}$
DS								
6	318 ± 4 $^{\alpha}$	295.10 ± 6.11	269.00 ± 3.49	252.00 ± 5.7	235.50 ± 3.54	212.54±4.19	187.32±7.98	158.7 ± 20.5 $^{a, \beta}$
15	318 ± 4 $^{\alpha}$	265.8 ± 11.30	222.4 ± 3.2	160.8 ± 2.32	139.32 ± 9.96	76.62 ± 8.50	42.8 ± 5.03	23.24 ± 4.08 $^{b, \beta}$
30	318 ± 4 $^{\alpha}$	174.3±5.55	87.4 ± 1.4	43.4 ± 2.05	28.89 ± 2.29	12.59 ± 0.30	9.84 ± 0.47	5.63 ± 0.20 $^{b, \beta}$
CS								
6	112 ± 12 $^{\alpha}$	85.36 ± 1.80	83.5 ± 1.5	73.0 ± 0.54	72.85 ± 0.58	65.11 ± 1.04	59.51 ± 2.86	47.85 ± 4.92 $^{a, \beta}$
15	112 ± 12 $^{\alpha}$	80.09 ± 5.30	68.5 ± 1.62	50.15 ± 0.98	39.59 ± 2.16	29.60 ± 0.78	15.51 ± 2.45	8.27 ± 0.34 $^{b, \beta}$
30	112 ± 12 $^{\alpha}$	56.18 ± 8.16	30.2 ± 0.4	14.1 ± 0.35	10.05 ± 0.95	5.01 ± 0.13	4.21 ± 0.27	2.41 ± 0.15 $^{b, \beta}$
M3A								
6	5.8 ± 0.4 $^{\alpha}$	5.53 ± 0.29	5.5 ± 1.26	5.35 ± 0.1	5.48 ± 0.31	5.22 ± 0.08	4.43 ± 0.37	4.32 ± 1.69 $^{a, \beta}$
15	5.8 ± 0.4 $^{\alpha}$	5.59 ± 0.43	5.53 ± 1.30	5.2 ± 0.16	5.10 ± 0.29	4.67 ± 0.04	3.70 ± 0.12	3.35 ± 1.20 $^{ab, \beta}$
30	5.8 ± 0.4 $^{\alpha}$	5.70 ± 0.49	5.01 ± 1.31	4.77 ± 0.18	4.31 ± 0.19	3.80 ± 0.08	3.37 ± 0.04	2.76 ± 0.05 $^{b, \beta}$
Q3S								
6	5.3 ± 0.1 $^{\alpha}$	4.38 ± 0.18	4.70 ± 1.76	4.57 ± 0.17	4.43 ± 0.1	4.28 ± 0.09	3.58 ± 0.04	3.87 ± 0.44 $^{a, \beta}$
15	5.3 ± 0.1 $^{\alpha}$	4.62 ± 0.61	4.80 ± 1.80	4.56 ± 0.16	4.27 ± 0.54	3.95 ± 0.66	3.19 ± 0.32	2.44 ± 0.08 $^{b, \beta}$
30	5.3 ± 0.1 $^{\alpha}$	5.60 ± 0.60	4.70 ± 1.51	4.81 ± 0.06	4.36 ± 0.58	2.90 ± 0.02	2.47 ± 0.06	2.42 ± 0.15 $^{c, \beta}$

Values with different letters and Greek alphabet are significantly different between ageing temperatures and months respectively $p \leq 0.05$ ($n = 6$).

Table 6-3 Changes in phenolic compounds during ageing at different temperatures for wines fermented at 30 °C.

compound/ageing temperature (°C)	content in mg/L during months of ageing							
	after fermentation	1	2	3	4	6	8	12
gallic acid								
6	9.6 ± 0.10 $^{\alpha}$	10.01 ± 0.08	10.15 ± 0.03	10.09 ± 0.01	10.65 ± 0.21	10.58 ± 0.23	12.72 ± 0.63	11.47 ± 1.00 $^{c, \beta}$
15	9.6 ± 0.10 $^{\alpha}$	10.37 ± 0.16	10.38 ± 0.35	10.38 ± 0.05	11.04 ± 0.13	10.57 ± 0.11	12.71 ± 0.14	13.47 ± 0.94 $^{b, \beta}$
30	9.6 ± 0.10 $^{\alpha}$	10.82 ± 0.87	10.96 ± 0.11	10.96 ± 0.91	11.78 ±0.10	12.57 ± 1.45	13.56 ± 0.48	15.70 ± 1.18 $^{a, \beta}$
caffeic acid								
6	98.1 ± 1.7 $^{\alpha}$	80.83 ± 8.04	76.5 ± 3.38	74.4 ± 1.43	73.02 ± 1.23	74.06 ± 2.29	75.40 ± 3.13	53.89 ± 0.58 $^{b, \beta}$
15	98.1 ± 1.7 $^{\alpha}$	85.45 ± 10.45	71.14 ± 0.26	74.56 ± 0.31	74.60 ± 0.74	74.37 ± 1.36	82.59 ±5.99	57.83 ± 0.11 $^{a, \beta}$
30	98.1 ± 1.7 $^{\alpha}$	88.9 ± 11.8	69.51 ± 0.43	68.82 ± 2.55	66.5 ± 1.72	68.50 ± 3.66	71.97 ± 1.02	44.96 ± 1.44 $^{c, \beta}$
DS								
6	266 ± 2 $^{\alpha}$	228.8 ± 4.7	218.3 ±3.75	189.8 ± 4.5	176.02 ± 2.95	150.63 ± 3.91	142.62 ± 2.73	123.05 ± 4.12 $^{a, \beta}$
15	266 ± 2 $^{\alpha}$	205.60 ± 3.52	183.83 ± 5.01	121.76 ± 3.11	92.51 ± 0.90	62.14 ± 2.32	48.88 ± 4.22	35.77 ± 2.77 $^{b, \beta}$
30	266 ± 2 $^{\alpha}$	117.2 ± 1.16	56.77 ± 1.22	28.31 ± 3.58	17.63 ± 0.91	9.38 ± 0.75	7.67 ± 0.51	6.21 ± 0.30 $^{c, \beta}$
CS								
6	82 ± 3$^{\alpha}$	74.9 ± 2.5	67.27 ± 1.66	61.23 ± 2.05	57.62 ± 1.55	52.03 ± 7.76	46.50 ± 1.65	38.72 ± 3.90 $^{a, \beta}$
15	82 ± 3$^{\alpha}$	64.9 ± 1.12	53.05 ± 1.31	40.14 ± 3.86	32.74 ± 1.52	22.97 ± 1.64	19.16 ±1.28	10.52 ± 1.44 $^{b, \beta}$
30	82 ± 3$^{\alpha}$	39.16 ± 0.73	19.83 ± 0.72	10.24 ± 1.33	6.88 ± 0.46	3.98 ± 0.31	3.48 ± 0.32	3.02 ± 0.02 $^{c, \beta}$
M3A								
6	5.3 ± 0.1 $^{\alpha}$	4.38 ± 0.18	4.70 ± 1.76	4.57 ± 0.17	4.43 ± 0.1	4.28 ± 0.09	3.58 ± 0.04	3.87 ± 0.44 $^{a, \beta}$
15	5.3 ± 0.1 $^{\alpha}$	4.62 ± 0.61	4.80 ± 1.80	4.56 ± 0.16	4.27 ± 0.54	3.95 ± 0.66	3.19 ± 0.32	2.44 ± 0.08 $^{b, \beta}$
30	5.3 ± 0.1 $^{\alpha}$	5.60 ± 0.60	4.70 ± 1.51	4.81 ± 0.06	4.36 ± 0.58	2.90 ± 0.02	2.47 ± 0.06	2.42 ± 0.15 $^{c, \beta}$
Q3S								
6	4.15 ± 0.14 $^{\alpha}$	4.22 ± 0.12	4.77 ± 0.71	4.10 ± 0.30	3.72 ± 1.27	3.93 ± 0.29	3.50 ± 0.31	2.92 ± 0.04 $^{a, \beta}$
15	4.12 ± 0.13 $^{\alpha}$	4.57 ± 0.16	4.43 ± 1.12	4.02 ± 0.15	3.92 ± 0.47	3.59 ± 0.32	3.18 ± 0.05	2.88 ± 0.02 $^{a, \beta}$
30	4.21 ± 0.21 $^{\alpha}$	4.79 ± 0.40	4.54 ± 0.68	3.63 ± 0.16	3.58 ± 0.71	2.61 ± 0.39	2.59 ± 0.05	2.22 ± 0.02 $^{b, \beta}$

Values with different letters and Greek alphabet are significantly different between ageing temperatures and months respectively $p \leq 0.05$ ($n = 6$).

In this study, caffeic acid concentration which increased rapidly after fermentation (Table 5-2) dropped significantly after ageing at the temperature conditions investigated The reduction in caffeic acid could be because they undergo polymerization reactions, and are involved in the formation of new stable polymeric pigments through condensation reactions with anthocyanins (Gómez-Plaza *et al.*, 2000b). These so called "new pigments" are believed to be responsible for maintaining and stabilizing colour intensity in aged wines (Sun and Spranger, 2005; González-Neves *et al.*, 2014; Somers and Pocock, 2015). Furthermore, the reduction in concentration of the glycosylated flavanols (M3A and Q3S) as seen in this research is most likely due to hydrolysis, which transforms these compounds to their corresponding aglycones (Alén-Ruiz *et al.*, 2009). As anticipated, the content of anthocyanins underwent the largest decrease and by the end of ageing, the concentration of monomeric anthocyanins (DS & CS) had dropped by over 90 % in wines stored at 30 °C, while wines kept at 6 °C retained about half of their starting concentrations. The degradation reactions of the anthocyanins followed first order kinetics (Figure 6-1), which agrees with previous works on grape wine (Dallas *et al.*, 1996; Mateus and de Freitas, 2001). The reaction rate constants (k) determined by calculating the slopes after linear regressions of the graphs ln C = k (t) (where C is the concentration of the anthocyanins and t is the period of ageing) showed that the degradation rate constants were higher with increasing ageing temperatures (Table 6-4). The half-life (the time taken for the concentration of the individual anthocyanin to reach half of its value), calculated using the mathematical equation ($T_{1/2}$) = ln (2)/k, suggests that overall CS had a longer half-life than DS and this is in agreement with a previous study on anthocyanins in *H. sabdariffa,* showing DS to be less stable than CS (Gradinaru *et al.*, 2003). The disappearance of monomeric anthocyanins and the formation of polymeric compounds in aged wines which are difficult to separate using HPLC, can be

seen with the characteristic hump with detection at 520 nm (Figure 6-2). Furthermore, peaks X and Y were tentatively identified as delphinidin and cyanidin (aglycones) by their m/z ratio of 304 and 288 respectively from LC-MS analysis.

6.4.3 Changes in phenolic content and α-glucosidase inhibition during ageing of wine

An important activity of polyphenols is the inhibition of digestive enzymes, especially carbohydrate-hydrolyzing enzymes such as α-amylase and α-glucosidase (McDougall *et al.*, 2005).

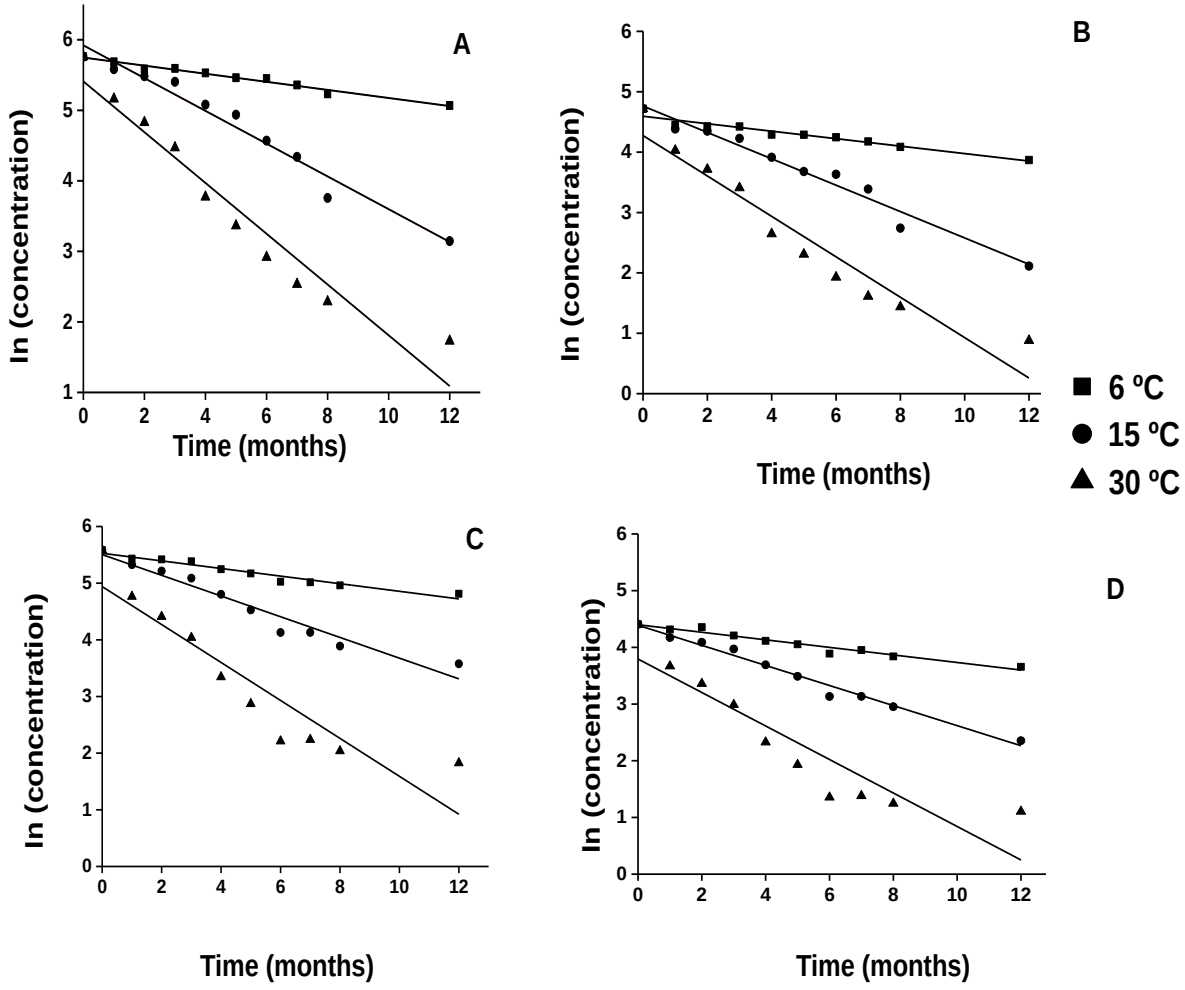


Figure 6-1 First-order kinetic plots for anthocyanins in roselle wines A and B (fermented at 20 °C) are the plots for DS and CS respectively, while C and D (fermented at 30 °C) are the plots for DS and CS respectively.

Table 6-4 Degradation rate constants (k; month-1), half-life ($T_{1/2}$; month) of roselle wine anthocyanins (DS and CS) aged at different ageing temperatures.

		anthocyanin					
		DS			CS		
fermentation temperature	ageing temperature (°C)	k	r^2	$T_{1/2}$ (months)	k	r^2	$T_{1/2}$ (months)
20 °C	6	-0.057	0.979	12.07	-0.062	0.939	11.21
	15	-0.232	0.968	2.98	-0.218	0.970	3.22
	30	-0.360	0.940	1.92	-0.335	0.927	2.07
		anthocyanin					
		DS			CS		
	ageing temperature (°C)	k	r^2	$T_{1/2}$ (months)	k	r^2	$T_{1/2}$ (months)
30 °C	6	-0.067	0.947	10.33	-0.067	0.954	10.35
	15	-0.183	0.948	3.79	-0.177	0.983	3.92
	30	-0.335	0.857	2.07	-0.295	0.837	2.35

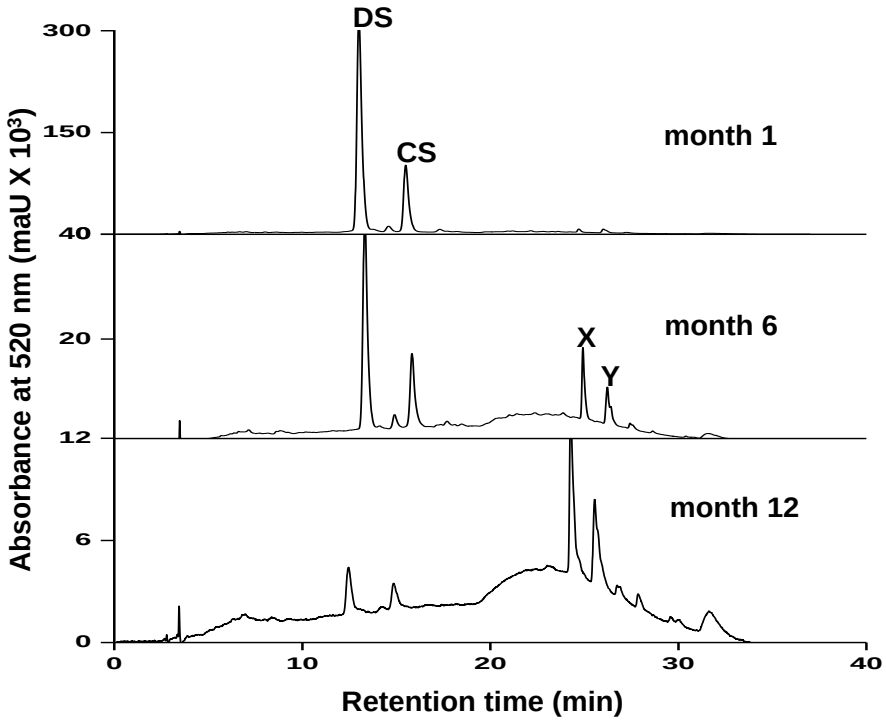


Figure 6-2 HPLC chromatogram of anthocyanins for roselle wine fermented at 20 °C and aged at 30 °C.

This activity has been discussed extensively in Section 1.16 and chapter 4. The total phenolic content (TPC) dropped by the second month at all ageing conditions, thereafter the values fluctuated till the end of ageing (Table 6-5). The reduction in total phenolic content could be due to the decrease in anthocyanin content as reported in other studies (Holzwarth *et al.*, 2012). This pattern (decrease in TPC) has been also been observed during ageing of grape wines and is thought to be attributed to the transformation of phenolic compounds into condensed forms that possess slightly different chemical properties and reactivities (Section 2.7) towards the Folin–Ciocalteu reagent (Garde-Cerdán *et al.*, 2008). This reduction in TPC did not impact on the bioactivity of roselle wine as the inhibition of α-glucosidase remained stable and was not significantly different ($p \geq 0.05$) from the initial value after 12 months of ageing at each of the temperatures investigated (Figure 6-3). In roselle beverage processed with the addition of stevia (artificial sweetener) and citric acid, the inhibition of α-glucosidase reduced with storage time (Pérez-Ramírez *et al.*, 2015). Although the concentrations of DS and CS (thought to be responsible for the inhibition in chapter 4) reduced with ageing, it is possible that the new compounds (aglycones and polymeric compounds) formed during ageing (Figure 6-2), were able to inhibit the enzyme effectively as the parent anthocyanins (DS and CS) and consequently maintained the bioactivity of the product. Fermentation and ageing during wine processing is known to cause changes in the anthocyanin profile and content, allowing for new pigments to be generated in the process (Ginjom *et al.*, 2011; Johnson *et al.*, 2013).

Table 6-5 Changes in the total phenolic content during ageing of wines.

fermentation temperature		content in mg/L during months of ageing				
	ageing temperature (°C)	after fermentation	2	4	8	12
20 °C	6	1363 ± 44 $^{\alpha}$	837 ± 20	890 ± 63	891 ± 28	1042 ± 200 $^{a, \beta}$
	15	1363 ± 44 $^{\alpha}$	933 ± 33	840 ± 17	819 ± 52	1022 ± 15 $^{a, \beta}$
	30	1363 ± 44 $^{\alpha}$	959 ± 50	884 ± 14	822 ± 62	960 ± 26 $^{a, \beta}$
		content in mg/L during months of ageing				
	ageing temperature (°C)	after fermentation	2	4	8	12
30 °C	6	1260 ± 13 $^{\alpha}$	774 ± 40	914 ± 10	854 ± 18	847 ± 34 $^{b, \beta}$
	15	1260 ± 13 $^{\alpha}$	986 ± 27	919 ± 25	818 ± 18	922 ± 12 $^{a, \beta}$
	30	1260 ± 13 $^{\alpha}$	827 ± 63	919 ± 38	691 ± 33	899 ± 86 $^{a, \beta}$

Values with different letters and Greek alphabet are significantly different between ageing temperatures and months respectively at $p \leq 0.05$ (n = 6).

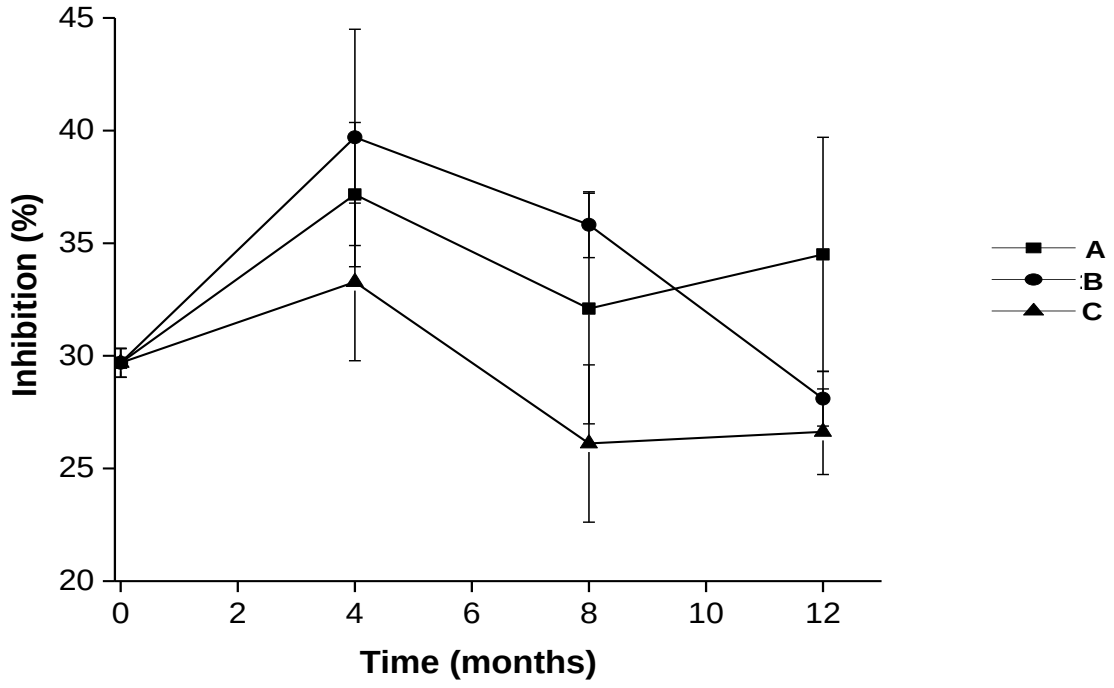


Figure 6-3 Impact of ageing temperature on α–glucosidase inhibition of roselle wines aged at (A) 6°C, (B) 15 °C and (C) 30 °C. The results are expressed as mean ± SD (n = 3). Final inhibition values were not significantly different ($p \geq 0.05$) between temperatures as well as initial values.

6.4.4 Evolution of organic acids during ageing of wine

The organic acids have a major role in the final wine quality (section 1.3.2.4) as they influence the pH, balance of flavour and chemical stability of the wine (Niu *et al.*, 2012). During alcoholic fermentation, yeast activity predominates and influences the content of organic acids in wine. However, during ageing and when secondary fermentation occurs, LAB is generally implicated (du Toit *et al.*, 2011). The decrease in malic acid content (Figures 6-4 & 6-5) and the concomitant increase in lactic acid within the first four months of ageing suggest the occurrence of MLF in the wines. However, since this activity occurred alongside a rise in acetic acid concentration, it can be assumed that some lactic acid was produced through the phosphoketolase pathway (Figure 6-6). This involves the dissimilation of residual simple sugars, xylose and arabinose by LAB during their growth phase resulting in the synthesis of D-lactic acid, acetic acid, and CO_2 as by-products (Lonvaud-Funel, 1999; Bauer and Dicks, 2004). Furthermore, the depletion of citric acid within the first four months of ageing confirms the activity of LAB. Citric acid metabolism by LAB (Figure 6-7) leads to the formation of lactic acid, acetoin, oxaloacetate and acetic acids (Shimazu *et al.*, 1985). Chen and Liu (2016) reported the depletion of citric acid after malolactic fermentation in lychee wine. Similarly, Nielsen and Richelieu (1999) showed that LAB consumed both malic and citric acid during malolatic fermentation of grape wine and although the content of acetic acid increased after malolactic fermentation in both studies, the increase in acetic acid was more pronounced in this experiment. This is probably because MLF was conducted by the indigenous LAB and not pure starter cultures as in the case of the cited studies.

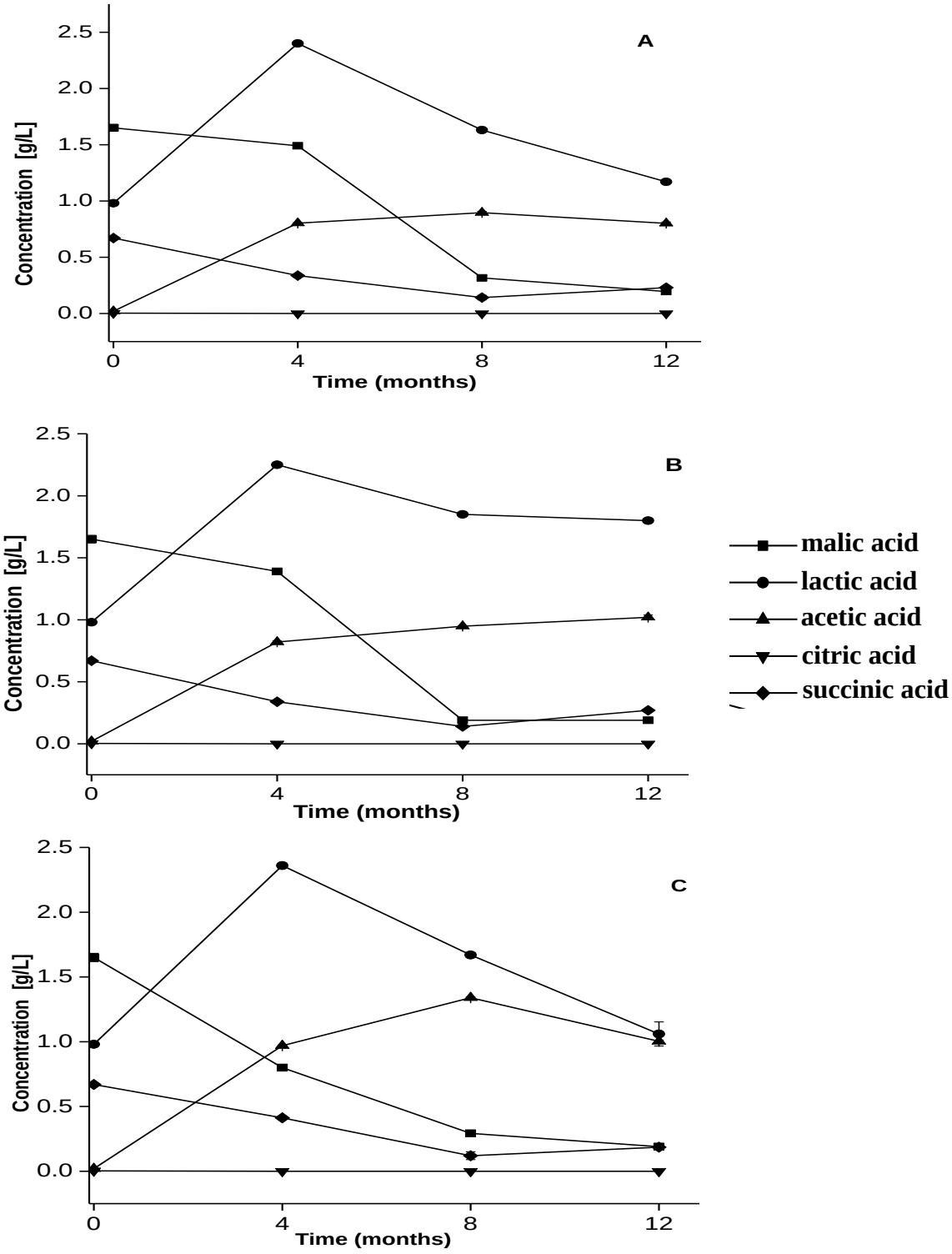


Figure 6-4 Evolution of organic acids of wines during ageing of wines fermented at 20 °C and aged at (A) 6 °C, (B) 15 °C and (C) 30 °C. The results are presented as mean ± SD (n = 6).

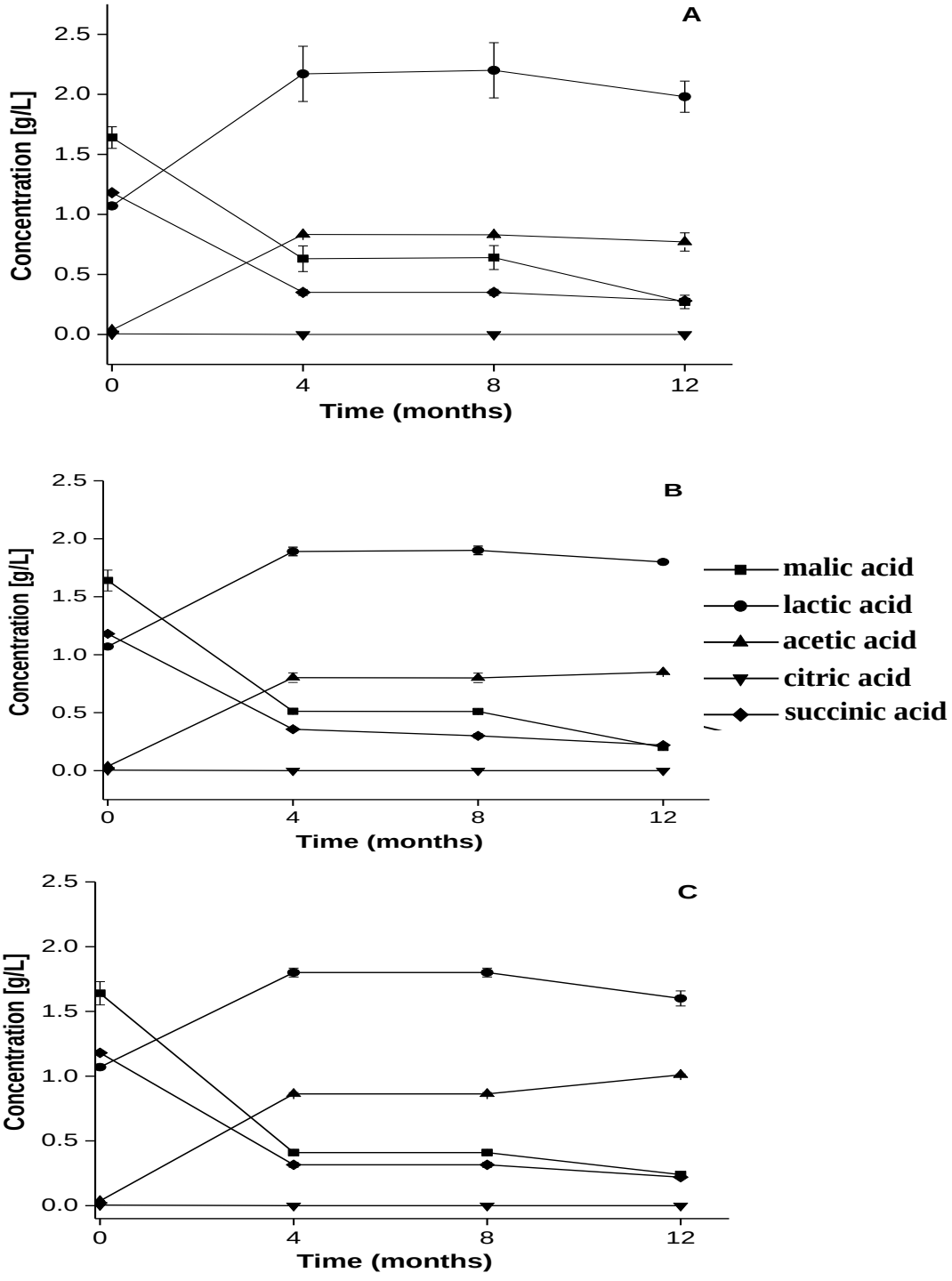


Figure 6-5 Evolution of organic acids of wines during ageing of wines fermented at 30 °C and aged at (A) 6 °C, (B) 15 °C and (C) 30 °C. The results are presented as mean ± SD (n = 6).

The subsequent decrease in lactic acid content observed at all ageing conditions could be the outcome of the synthesis of esters (ethyl lactate, isoamyl lactate etc.) and polymerisation with anthocyanins, which has been reported during ageing of grape wines (Alcalde-Eon *et al.*, 2006). Succinic acid has a high rate of esterification when compared with other organic acids (Lamikanra, 1997). In this study, succinic acid content reduced significantly ($p \leq 0.05$) in the course of ageing and this can be attributed to the production of diethyl succinate in wine.

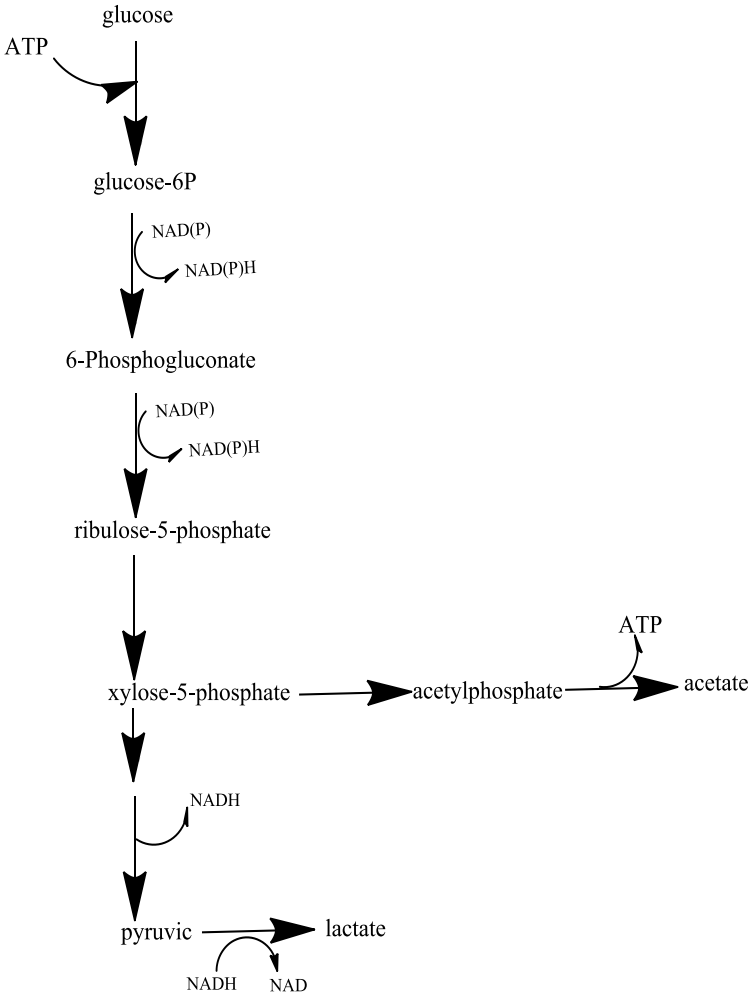


Figure 6-6 Phosphoketolase pathway (pentose phosphate pathway) by LAB.

This result (reduction in succinic acid) agrees with published data on the content of succinic acid after 18 months of bottle ageing of wines (Chung *et al.*, 2008). At the end of ageing, the final concentrations of acetic acid (850 - 1100 mg/L) is still within the permitted levels for red wines of 1400 mg/L (Maicas *et al.*, 1999; Zoecklein *et al.*, 1999).

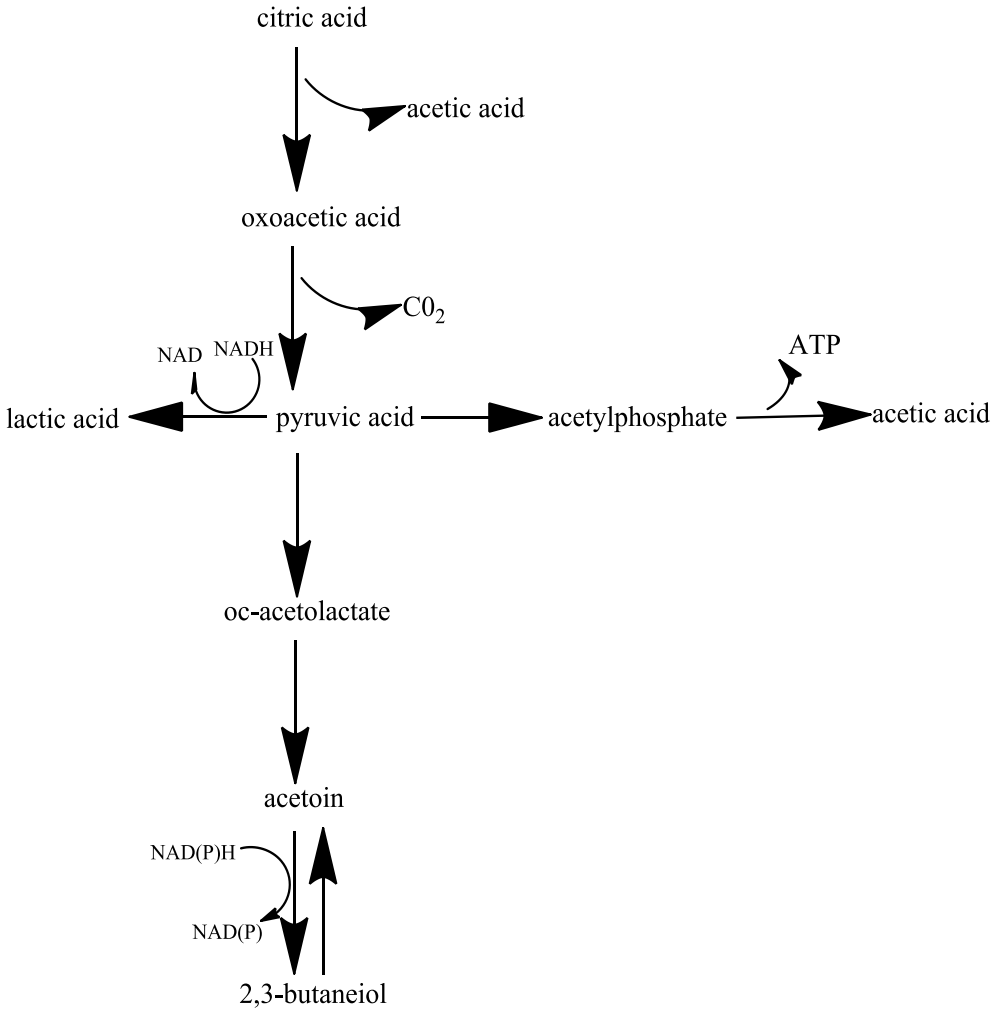
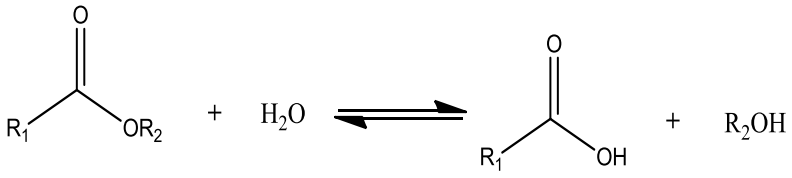


Figure 6-7 Main metabolic pathway for citric acid metabolism by LAB.

6.4.5 Volatile composition of roselle wine after ageing

After the significant modifications in volatile composition during fermentation, chemical constituents generally react slowly during ageing to move to their equilibrium. In this study, the impact of ageing on the concentration of higher alcohols in roselle wines produced different patterns between wine groups (Tables 6-6 & 6-7). In wines fermented at 20 °C, the levels of 1-hexanol and 2-phenylethanol remained relatively stable, while there was a twofold increase in the concentration of isoamyl alcohol. Although hydrolysis of isoamyl acetate (Figure 6-8) was observed after ageing and may account for some increase in isoamyl alcohol content (precursor for the synthesis of isoamyl acetate), it is unlikely that this occurrence alone would have produced the magnitude of increase observed. It is common for wine after alcoholic fermentation to contain residual amounts of amino acid which could be transformed into their corresponding alcohols as mentioned in Section 5.3.6 by the Ehrlich mechanism (Figure 6-9) (de Revel *et al.*, 1999; Pozo-Bayón *et al.*, 2005).



Isoamyl acetate Acetic acid Isoamyl alcohol

Figure 6-8 Hydrolysis of isoamyl acetate.

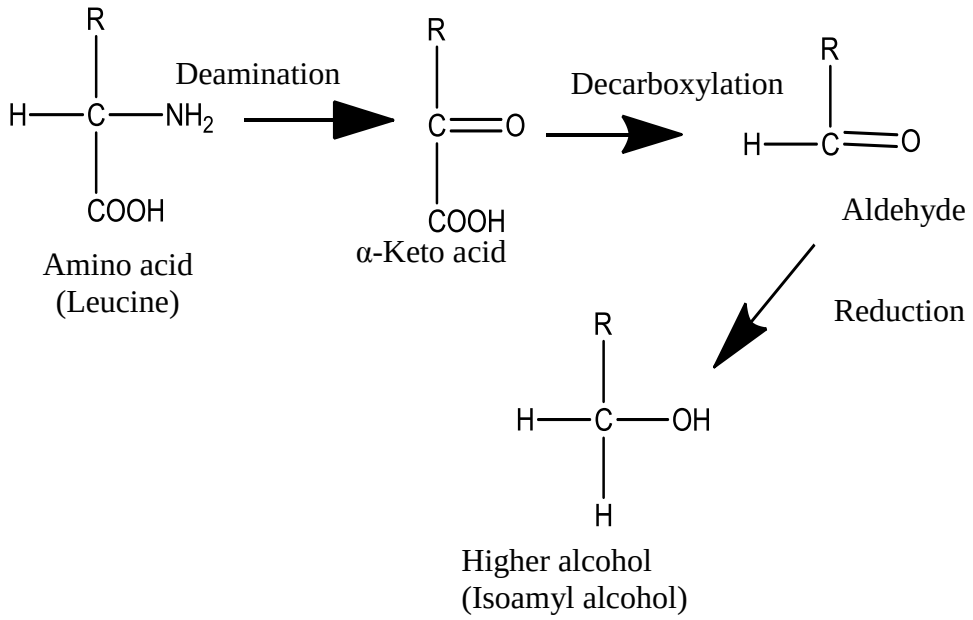


Figure 6-9 Ehrlich pathway for the synthesis of higher alcohols.

Câmara *et al.* (2006) observed an increase in the concentrations of higher alcohols (isoamyl alcohol and 2–phenylethanol) in Madeira after 11 and 25 years of storage in oak barrels. On the contrary, some authors report the relative stability of these compounds during ageing as observed with 2-phenylethanol concentration in wines fermented at 20 °C in this study (Pérez-Prieto *et al.*, 2003; Fang and Qian, 2006).

Table 6-6 Content of volatile compounds in roselle wine fermented at 20 °C after ageing for 12 months.

volatile compound (ug/L)	qualifying ions	after fermentation	ageing temperatures		
			6 °C	15 °C	30 °C
2-phenylethanol	**91**; 92; 122	527 ± 63 [a]	521 ± 52 [a]	564 ± 140 [a]	483 ± 45 [a]
isoamyl alcohol	**71**; 43; 55	130539 ± 21943 [b]	269347 ± 23174 [a]	295046 ± 64401 [a]	252548 ± 7622 [a]
1-hexanol	**56**; 43; 69	248 ± 103 [a]	149 ± 34 [b]	153 ± 79 [b]	103 ± 33 [b]
ethyl acetate	**43**; 61; 70	156 ± 32 [a]	184 ± 45 [a]	226 ± 126 [a]	227 ±11 [a]
ethyl hexanoate	**88**; 99; 145	3487 ± 363[a]	1703 ± 199[b]	1718 ± 459 [b]	1634 ± 339 [b]
ethyl octanoate	**88**; 101; 172	1339 ± 135 [a]	731 ± 105 [b]	803 ± 163 [b]	707 ± 31 [b]
ethyl decanoate	**88**; 101; 200	966 ± 95 [a]	610 ± 81 [b]	607 ± 121 [b]	554 ± 36 [b]
diethyl succinate[a]	**101**; 129; 55	0.003 ± 0.000 [d]	0.24 ± 0.03 [c]	0.40 ± 0.09 [b]	0.77 ± 0.13 [a]
ethyl lactate[a]	**45;** 73;43	0.13 ± 0.04 [b]	0.16 ± 0.04 [ab]	0.21 ± 0.06 [a]	0.19 ± 0.03 [a]
isoamyl acetate[a]	**70**; 41; 88	0.69 ± 0.01 [a]	0.15 ± 0.02 [b]	0.18 ± 0.03 [b]	0.15 ± 0.01 [b]
methyl butyrate[a]	**43**;71;88	0.010 + 0.001 [c]	0.023 ± 0.003 [b]	0.035 ± 0.010 [a]	0.034 ± 0.004 [a]
eugenol[a]	**164**; 149; 103	0.013 ± 0.008 [a]	0.002 ± 0.001 [b]	0.002 ± 0.001 [b]	0.002 ± 0.001 [b]
2-phenyl acetate[a]	**104**; 91; 65	0.07 ± 0.01 [a]	0.007 ± 0.001 [b]	0.007 ± 0.002 [b]	0.006 ± 0.001 [b]
acetoin[a]	**45;** 43; 88	0.046 + 0.038 [b]	0.26 ± 0.18 [a]	0.23 ± 0.19 [a]	0.21 ± 0.17 [a]

The values are the mean ± SD. Numbers bolded were used as the quantitative ion for aroma compounds; [a] Compounds measured by semi-quantification; relative peak areas (compound: internal standard) were used to calculate means and standard deviation .Values with different letters on the same row are significantly different $p \leq 0.05$ (n = 6).

Table 6-7 Content of volatile compounds in roselle wine fermented at 30 °C after ageing for 12 months.

volatile compound (ug/L)	qualifying ions	after fermentation	ageing temperatures		
			6 °C	15 °C	30 °C
2-phenylethanol	**91**; 92; 122	1163 ± 114 [a]	533 ± 53 [b]	532 ± 78 [b]	598 ± 138 [b]
isoamyl alcohol	**71**; 43; 55	209777 ± 7645 [a]	170420 ± 7818 [b]	162220 ± 12224 [c]	187982 ± 20140 [c]
1-hexanol	**56**; 43; 69	258 ± 60 [a]	80.0 ± 7.8 [b]	80.1 ± 3.9 [b]	82.3 ± 16.1 [b]
ethyl acetate	**43**; 61; 70	371 ± 27 [a]	228 ± 39 [c]	293 ± 50 [b]	359 ± 30 [a]
ethyl hexanoate	**88**; 99; 145	1644 ± 90 [a]	1340 ± 64 [b]	1318 ±32 [b]	1334 ± 69 [b]
ethyl octanoate	**88**; 101; 172	740 ± 33 [a]	643 ± 58 [b]	624 ± 44 [b]	698 ± 51 [ab]
ethyl decanoate	**88**; 101; 200	639 ± 53 [a]	527 ± 30 [b]	500 ± 47 [b]	510 ± 36 [b]
diethyl succinate[a]	**101**; 129; 55	0.016 ± 0.001 [c]	0.028 ± 0.003 [c]	0.09 ± 0.010 [b]	0.54 ± 0.088 [a]
ethyl lactate[a]	**45;** 73; 43	0.022 ± 0.011 [c]	0.433 ± 0.081 [ab]	0.405 ± 0.052 [b]	0.441 ± 0.162 [a]
isoamyl acetate[a]	**70**; 41; 88	0.51 ± 0.07 [a]	0.12 ± 0.02 [b]	0.10 ± 0.02 [b]	0.12 ± 0.00 [b]
methyl butyrate[a]	**43;**71; 88	0.040 ± 0.003 [b]	0.031 ± 0.001 [c]	0.038 ± 0.006 [bc]	0.054 ± 0.008 [a]
eugenol[a]	**164**; 149; 103	0.02 ± 0.001 [a]	0.002 ± 0.000 [b]	0.003 ± 0.000 [c]	0.008 ± 0.000 [c]
2-phenyl acetate[a]	**104**; 91; 65	0.014 ± 0.000 [a]	0.012 ± 0.000 [b]	0.010 ± 0.000 [c]	0.012 ± 0.000 [b]
acetoin[a]	**45;** 43; 88	0.04 ± 0.01 [c]	0.12 ± 0.03 [b]	0.12 ± 0.04 [b]	0.18 ± 0.07 [a]

The values are the mean ± SD. Numbers bolded were used as the quantitative ion for aroma compounds; [a] Compounds measured by semi-quantification; relative peak areas (compound: internal standard) were used to calculate means and standard deviation .Values with different letters on the same row are significantly different $p \leq 0.05$ (n = 6).

With wines fermented at 30 °C, the concentration of isoamyl alcohol remained relatively stable, while significant decreases in the concentration of 2-phenylethanol and 1-hexanol were observed irrespective of ageing temperature. Cañas *et al.* (2008) reported a slight decrease in 2-phenylethanol content in Tempranillo wines after spontaneous malolactic fermentation and suggested the synthesis of 2-phenylethyl lactate as a possible explanation for the decrease. In addition, some authors affirm that certain strains of LAB are able to adsorb some volatile compounds provoking decreases in their final concentrations (Maicas *et al.*, 1999; Boido *et al.*, 2009). The results in this study also revealed decreases in fusel alcohol esters and ethyl esters with the reductions in ethyl esters being more pronounced in wines fermented at 20 °C. In the course of alcoholic fermentation, esters are generally formed in excess concentrations above their equilibrium, which governs the extent of hydrolysis (law of mass action) during ageing (Pérez-Prieto *et al.*, 2003). It is possible that these volatiles were synthesised in excess during fermentation at 20 °C and the subsequent reductions mainly due to chemical equilibrium adjustment. Moreover, the decrease in the contents of these esters during ageing has also been mentioned by other researchers (Ortega-Heras *et al.*, 2004; Câmara *et al.*, 2006). Another characteristic of the bouquet of aged wines reported in the literature is the increase in the content of esters of diprotic acids (Chisholm *et al.*, 1995; Pérez-Prieto *et al.*, 2003). Hence, it was not surprising that the concentrations of ethyl lactate and diethyl succinate (esters of diprotic acids) increased at the end of ageing in this experiment. When an increase in ethyl lactate is observed, it is generally associated with malic acid metabolism by LAB. The only volatile compound analysed in this study affected by ageing temperature was diethyl succinate and the results showed that the concentrations of diethyl succinate increased with higher ageing temperature.

In a previous study, wines aged at 23 °C had higher levels of diethyl succinate than their counterparts aged at 5 °C (Garde-Cerdán *et al.*, 2008). Diethyl succinate arises from esterification of succinic acid (by-product of α-ketoglutaric by both yeast and bacteria metabolism) and increase in levels of this ester is generally observed as ageing progresses (Simpson, 1978; Gonzalez-viñas *et al.*, 1996; Pérez-Coello *et al.*, 2003). Acetoin is produced from the reduction of diacetyl in the citric acid metabolism pathway by LAB and its concentration increased significantly ($p = 0.016$) after the ageing period. Hernández-Orte *et al.* (2009) found acetoin concentration to be higher in wines that had undergone MLF compared to the control (those without MLF) after four months of ageing. This compound alongside diacetyl contribute to the buttery flavour and body of wines (Styger *et al.*, 2011). Eugenol was the only terpernoid investigated in this study and the levels in wines decreased after ageing which might be due to esterification reactions. In summary, aroma compounds in roselle wine stored in bottles for 12 months showed decreases in both fusel alcohol acetates and ethyl esters of fatty acids, while increases occurred in the concentrations of esters of diprotic acids. The final amounts of fusel alcohols were more influenced by post fermentation conditions, esterification/hydrolysis equilibrium and possibly modulation by the LAB population. In addition, higher ageing temperature resulted in increased synthesis of diethyl succinate.

6.5 Conclusion

The impact of ageing temperatures (6, 15 and 30 °C) on the colour, phytochemical and bioactivity of roselle wine was investigated over 12 months. The data suggests that roselle wine colour, phenolic profile and subsequent bioactivity can be best maintained under low temperature (6 - 15 °C) ageing/storage conditions. However when it comes to the aroma/flavour profile, more studies are needed, particularly when malic acid remains

after alcoholic fermentation and becomes a source of nutrient for LAB. The introduction of a pure LAB strain into the must after alcoholic fermentation might be needed to facilitate rapid malic acid degradation. This might involve identifying strains of the LAB that adapt well to the conditions in roselle wine and contribute positively to the flavour/aroma profile. One approach could be to identify, isolate and culture LAB that survive after alcoholic fermentation in roselle wine, use them to conduct malolactic fermentation trials and then assess their impact on the overall aroma profile of the wine. This warrants further investigation and could be the focus of future research. Finally, the decision of the acceptability of a product rests entirely on consumer preferences and sensory analysis is needed to generate the necessary feedback/tools which can be re-introduced into the production process and ultimately lead to the commercial success of the product.

Chapter 7 Sensory evaluation of roselle wine

7.1 Abstract

The aim of this study was to rate the quality attributes of roselle wine aged under three different temperatures (6, 15 and 30 °C) for a period of 12 months. A 60 member panel (male and female) consisting of expert and non-expert wine drinkers were recruited for the analysis. A Williams balanced incomplete block design (BIBD) was used for the study design and the rating of the wine samples was based on a modified UC Davis quality assessment rating of red wines. The Durbin test followed by a multiple comparison test based on the statistics of student-t-distribution with holm procedure on p value was applied to the wine quality sensory data. There was no significant difference ($p \geq 0.05$) in the rating of the quality attributes of the wine samples irrespective of fermentation and ageing temperatures. The data from the sensory evaluation suggests that the ageing duration (12 months) was not sufficient for panel members to effectively differentiate between the wine samples. Furthermore, a descriptive sensory analysis involving the use of a trained panel is needed to generate aroma and flavour descriptors of the wine and how these descriptors influence the perceived quality attributes of the wine.

7.2 Introduction

The nature of product quality is considered to be difficult to understand and in the case of wine quality, with its quasi-aesthetic character and relationship to personal taste, it is particularly hard to pinpoint (Charters and Pettigrew, 2005). Indeed the quality of wine is difficult to define, as it is a multi-faceted construct, lacking a uniform and generally accepted definition (Hopfer *et al.*, 2015). Therefore, the term complex is regularly applied as a descriptor of the perceived characteristics of a wine and those involved in the study of wine quality, talk about perceived quality and how various populations differ in their wine quality perception (Charters and Pettigrew, 2007). According to Sáenz-Navajas *et al* (2013), the quality dimensions of wine could be split into two types. The first comprises of external qualities, relating to issues beyond the physical and organoleptic properties of the wine. These include factors like the reputation, region, advertising, brand, price, bottling and labelling. The other category, which is much larger, relates only to what is experienced when the wine is consumed – the organoleptic nature of the product. These include balance, complexity, length (the persistence of the taste after the wine has been swallowed), personality or distinctiveness, intensity of flavour and purity (Amerine and Roessler, 1976).

For wine quality evaluation, there exists a commonly used evaluation scheme developed at the University of California at Davis. In this method, points are assigned in sensory categories such as appearance, colour, aroma and bouquet, volatile acidity, total acidity, sweetness, body, flavour, bitterness, general quality. While the scoring card might not be fully applicable to wines of equal or high quality, it can be used to screen out wines that have obvious defects (Winiarski *et al.*, 1997). Although, there have been a few published sensory studies on roselle wine, these studies have mainly focused on the acceptability of the wine, which is not synonymous with product quality. Since the UC-Davis score card

is a tool designed to identify product defects, the information generated could be used to identify aspects of the wine's intrinsic qualities and how these qualities are influenced by processing conditions. Furthermore, while it was observed that fermentation and ageing conditions resulted in significant differences in the instrumental colour measurement and aroma attributes of roselle wine, it was of interest to investigate whether a sensory panel will also be able to differentiate between the wine sets.

7.3 Wine qualitative assessment

Tasting area: Tasting rooms should be at least well ventilated not only for the comfort of the panel, but also to prevent odour accumulation. Tasting stations must be physically isolated (with separate tasting booths) to prevent taster interaction as comments made during the sessions can influence the perception of panellist. In addition, water or at best dry unsalted bread or crackers should be provided for palate cleansing. (Jackson, 2009).

Number of samples: In general, there is no accepted number of samples appropriate for a tasting session. When samples are similar, only few wines should be tasted. If samples are different, large number of wines can be evaluated. However, six tends to be the limit when jointly comparing wines. (Jackson, 2009).

Temperature: White wines are recommended to be served at cool temperatures (8 – 12 °C), whereas red wines are typically served at room temperature of between 18 to 22 °C. This range enhances the fragrance and diminishes perceived bitterness and astringency. Ideally, wines are maintained at the temperature of serving several hours before serving. (Jackson, 2009).

Sample volume: The volume of each sample should be the same. The volume which depends on the purpose of the tasting should range between 35 -70 mL. In cases where

only simple evaluation is required, a sample volume of 35 mL is adequate. (Jackson, 2009).

Number of panellist: Up to a point, the larger the number of tasters, the greater is the probability of obtaining valid data. However, a few panel members can be acceptable if they are known to be skilled and consistent (Lawless and Heymann, 1999). This minimises cost and statistical analysis.

Information provided: The general details of what is expected from the study should be presented to the panellist. The information could be given verbally or in print form prior to the tasting of the samples and is dependent on the level of direction required as well as the purpose of the study.

Wine glasses: Wine glasses must have specific characteristics appropriate for critical wine tasting. It must be crystal clear and uncoloured. The features of the glass required for wine tasting has been incorporated into the International Standard Organisation (ISO) wine tasting glass (Figure 7-1). The broad base and sloped sides allows for viewing and swirling of the sample, while the narrow mouth is designed to concentrate aromatics by the wine. (Jackson, 2009).

Timing: Tastings are commonly done in the late morning, when people are generally alert. However, when it is not feasible it could be done in the late afternoon or mid-evening (Jackson, 2009).

Figure 7-1 ISO official wine tasting glass.

7.4 Materials and methods

7.4.1 Ethical approval

At the start of this research, the initial plan of the sensory study was to perform a descriptive sensory analysis on roselle wine using a trained panel. In this regard, an ethical application was to the Faculty Research Ethics Committee (see appendix). This kind of sensory analysis (descriptive) on wine provides information on the principal sensory attributes that a wine possesses in order to improve the correlation between sensory descriptions and chemical analysis. Furthermore, the data generated from descriptive sensory analysis of wine has been used to corroborate the influence of the environmental characteristics, the inoculation with different yeast strain, possibility of carrying out

malolactic fermentation, the storage time or the different enological or ageing treatments on the wine flavour or aromatic profile (Falqué *et al.*, 2004). In this analysis, the sensorial properties of the wine are described using vocabulary terms found in the wine aroma wheel (Figure 7-2) and the intensity of specific aroma and flavour notes in the wine samples are rated against a reference standard for that particular note.

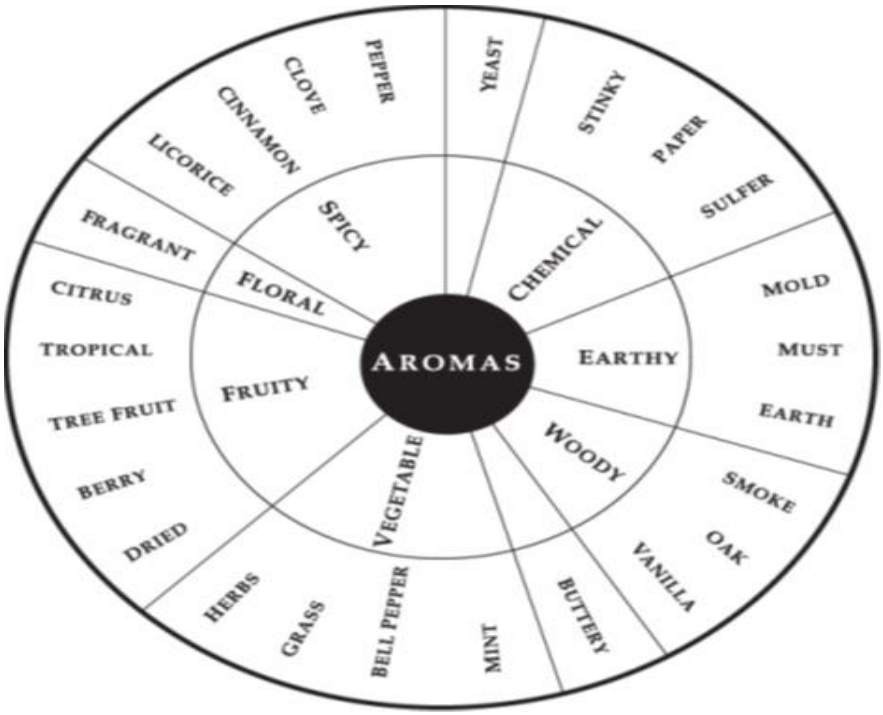


Figure 7-2 Wine aroma wheel. (Noble *et al.*, 1987)

Due to the limitation of the time frame work of this research, the approved ethical application for this research (MEEC 14-021) had to be amended and the descriptive analysis planned for was replaced with a sensory evaluation on roselle wine quality attributes using the UC Davis scoring card.

7.4.2 Objective of the study.

The purpose of the amended study was to determine whether roselle wine samples fermented and aged under different conditions would indeed be perceived as different by a sensory panel.

7.4.3 Design of the study

A total of 60 people comprising of males and females mainly from the University of Leeds were recruited for the sensory analysis. The panel had both regular wine consumers /experts as well as frequent consumers who were all trained on how to rate the different wine samples. The tasting room consisted of individual booths with the front of each booth opening onto the preparation room as shown in Figure 7-3. The wines bottles were transferred to the sensory stations 2 hrs prior to the commencement of sensory analysis to allow for equilibration with the room temperature. The wine tasting procedure used was adapted from the published work of (Jackson, 2009). Wine samples (40 mL) were carefully measured into ISO wine tasting glasses and covered with a petri dish to avoid the escape of volatile components for about 30 min before serving. This was to give time for the wine to "breathe". The participants were trained with the use of a visual aid on the sampling techniques (Table 7-1) to be employed for the sensory evaluation. They however did not have prior knowledge of the samples to be evaluated. Each panellist was given three wine samples served in glasses coded with a three-digit number after signing the consent forms. They were also presented with unsalted crackers and water in between samples to rinse the palate. The order of presentation of the wine samples was completely randomised using the William balanced incomplete block design (Williams 1949). The rating of the quality attributes of each wine samples was based on a modified Davis model quality assessment scoring sheet (Table 7-2) and the tasting took place over two

consecutive days. On each of the days, there were two sessions (11 am to 1 pm and 2 pm – 4 pm) with equal number (30) of panellist attending each day.

A

B

Figure 7-3 (A) sample preparation room and (B) tasting booths.

Table 7-1 Instructions for rating of roselle wine quality attribute.

Attributes	Instructions For Rating The Quality Attributes
Appearance and colour	Pick up the glass as shown in the picture. Raise it up to about 15 cm and tilt the glass forward by 45 degrees. Take a look at the content of the glass and rate the appearance and colour
Aroma and bouquet	Swirl the glass slightly and raise it up, placing your nose close to the glass and take a sniff for about 1 - 2 s, then rate the aroma and bouquet
Taste and flavour	Take a sip, keep the wine in your mouth for 1 - 2 s, before swallowing and rate the taste and flavour
Balance	Take a sip, keep the wine in your mouth for 3 - 5 s and rate the balance (sweetness at the tip and bitterness at the back)
Aroma development/duration	Bring the wine to your nose and sniff the wine for a short duration (1 – 2 s) and then a longer duration (4 - 5 s) then rate the aroma development and duration
Finish (taste and flavour)	Take a sip of wine; keep the wine in your mouth for 4 - 5 s before swallowing
Overall quality	Rate the overall quality based on your impression of the previous attributes

7.4.4 Statistical analysis

The data collected from the sensory evaluation was processed using the R.3.30 Pairwise Multiple Comparison of Mean Ranks Package (PMCMR). The Durbin test followed by a multiple comparison test based on the statistics of student-t-distribution with holm procedure on p value was applied to the wine quality sensory data. The holm correction shows that the test has a prescribed level of significance protection against error of the first kind for any combination of true hypotheses (Holm, 1979).

Table 7-2 General score sheet modified from the Davis model (Jackson, 2009).

Attributes	Scores	Categories	Description
Appearance and colour	1	Poor/unsatisfactory	Dull or slight off-colour
	2	Good	Bright with Characteristic colour as expected
	3	Superior or Excellent	Brilliant with characteristic colour
Aroma and bouquet	1	Faulty	Clear expression of an off-odour
	2	Poor/Off character	Marginal expression of an off-odour
	3	Acceptable	Absence of characteristic varietal-regional stylistic fragrance or bouquet but with no unpleasant off-odours
	4	Good	Mild to standard varietal-regional stylistic fragrance or bouquet
	5	Superior	Varietal-regional stylistic fragrance or bouquet which is distinctive and complex
	6	Exceptional	Rich, complex traditional fragrance or refined lingering aged bouquet
Taste and flavour	1	Faulty	Off tastes or off-odours so marked as to make the wine distinctly unpleasant
	2	Poor/unsatisfactory	Absence of varietal, regional or stylistic taste and flavour characteristics
	3	Good	Presence of distinctive varietal, regional, or stylistic taste and flavour characteristics
	4	Exceptional	Superior varietal, regional or stylistic taste and flavour characteristics
Balance	1	Poor/unsatisfactory	Acid/sweetness ratio inharmonious, excessively bitter and astringent
	2	Good	Acid/sweetness ratio adequate, moderate bitter and astringent
	3	Exceptional	Acid/sweetness ratio invigorating, smooth mouth feel
Aroma development/duration	1	Poor/unsatisfactory	Fragrance simple, does not develop, of short duration
	2	Standard/Satisfactory	Fragrance typical, develops in complexity, does not fade during tasting
	3	Superior	Fragrance improves with intensity and/or character, last throughout the tasting
	4	Exceptional	Rich fragrance, improves in intensity and character, long lasting
Finish (taste and flavour)	1	Poor/unsatisfactory	Little lingering flavour in the mouth, excessive astringency and bitterness
	2	Good	Moderate lingering flavour in the mouth, fresh aftertaste
	3	Exceptional	Prolonged flavour in the mouth (≥10 to 15s), subtle, refined after-sensations
Overall quality	1	Poor/Unacceptable	Distinctly off-character
	2	Good	Acceptable representation of traditional aspects of the type
	3	Superior	Clearly better than the majority of the wine types
	4	Exceptional	So nearly perfect in all sensory qualities as to be a memorable experience

7.5 Results and discussion

The data on roselle wine quality attributes rating by the sensory panel is presented in (Table 7-3). Although the anthocyanin and colour analysis from the HPLC (Tables 6-2 & 6-3) and spectrophotometric analysis (Table 6-1) respectively showed significant differences between ageing temperatures, the same could not be said of the data from the sensory panel, as there was no significant difference ($p \geq 0.05$) in the rating of the appearance and colour of the wine samples. Notwithstanding, the data does seem to suggest that wines aged at 6 and 15 °C were preferred by the panellists as they had better mean values. In terms of the aroma attributes of roselle wine, the chemical analysis by GC-MS revealed that diethyl succinate (vinous) was the aroma compound that was most influenced by ageing conditions (Table 6-6 & 6-7). However, this did not produce any significant difference ($p \geq 0.05$) in the rating of the aroma attribute by panellists. The taste of wine has to do with the sensations of sweetness, sourness, bitterness and astringency. Sweetness is usually the most rapidly detected taste sensations perceived at the tip of the tongue. Sourness is also detected rapidly and at the side of the tongue, while the perception of bitterness is detected later at the back and central portions of the tongue. The acids (organic and volatile acids) and the phenolic compounds present in wines are the main components that influence the flavour and taste of wines. In this study, there was no significant difference ($p \geq 0.05$) between roselle wines, which suggests that fermentation and ageing temperatures did not influence the rating of the flavour attribute of the wines.

Table 7-3 Quality rating of roselle wine by sensory panel.

	Rating of quality attributes					
Wine attribute/sample	A6	A15	A30	B6	B15	B30
Appearance and colour	2.33	2.23	1.96	2.20	2.33	2.03
Aroma and bouquet	3.60	3.50	3.16	3.36	3.66	2.86
Taste and flavour	2.53	2.53	2.26	2.26	2.63	2.36
Balance	1.76	1.93	1.53	1.73	1.83	1.76
Aroma development/duration	2.16	2.20	1.86	1.90	2.16	2.06
Finish	2.00	2.10	1.76	1.76	2.03	1.86
Overall quality	1.93	1.93	1.73	1.76	2.13	1.66
Total score	16.38	16.43	14.30	15.00	16.80	14.63

The letter A represents wine fermented at 20 °C, while B represents wines fermented at 30 °C. The numbers 6, 15 and 30 refer to the ageing temperature. The data represents the mean scores of the quality rating by the panellists. Statistical differences were not found between wine samples at $p \leq 0.05$.

The balance of wine which has to do with the ratio of acidity to sweetness is closely related with the flavour and taste of wine. The result suggests that ageing temperature did not produce any significant difference ($p \geq 0.05$) in the balance attributes of the wine samples. The finish or persistence of wines has to do with the lingering aromatic sensations in the mouth. The duration of the lingering sensation is dependent on the wine characteristics and may last for a few seconds to several minutes. The finish tends to be fleeting and only compounds that persist and escape from the saliva/mucus are likely to be detected by the consumers. The results showed no significant difference ($p \geq 0.05$) in the finish of the wine samples. In a study on the persistence of aroma compounds in Chardonnay wines, the fruity volatile compounds (ethyl esters) were more easily dissipated than volatiles derived from oak ageing (vanillin, β-damascenone) and the

higher alcohols (isoamyl alcohol and 2-phenylethanol) (Buettner, 2004). In this study, it is difficult to predict whether the final levels of higher alcohols in the wine samples could have influenced the ratings of the finish in the wine samples, since ageing temperatures did not produce an obvious pattern in the concentrations of these volatile compounds.

According to (Amerine and Roessler, 1976), the overall quality of wines is easier to detect than define. The different aspects of regional and varietal characteristics; along with the development, (duration and complexity of the fragrance), and the uniqueness of the tasting experience and the taster perceptive acuity, all sum up the overall quality of the wine. The data on the overall quality rating of roselle wine revealed that there was no significant difference ($p \geq 0.05$) between ageing temperatures as well as fermentation temperature.

To compare the findings on the quality attributes of roselle wine with other published studies on grape wine would be a very daunting exercise as the concept of wine quality still remains a variable subject. Furthermore, it is known that individuals vary for many reasons in their evaluations of food and beverages (Brien *et al.*, 1987). For instance, in a study where the quality attributes of 27 California wines were rated by experts, consumer and a trained group, the results showed a broad range of liking and variability and inconsistency, as some wines rated as low in quality by experts were preferred by some consumers (Hopfer and Heymann, 2014). Nevertheless, it is possible to make comparison between studies that have examined the impact of ageing temperature on the rating of the quality and sensorial attributes of wine. For example, the effect of two ageing temperatures (12 and 22 °C for 320 days) on the aroma profile of red Sangiovese wines was evaluated by a sensory panel and in most of the aroma and taste attributes, there were no significant differences ($p \geq 0.05$) (Castellari *et al.*, 2001). However in another study, using a rating scale of 1-10, the impact of ageing temperature (5 °C and commercial

conditions) on the overall quality of wines aged for 12 and 24 months was investigated by a sensory panel (Pérez-Coello *et al.*, 2003). The results showed that the mean scores for wines aged at 5 °C were 5.9 and 5.25 for 12 and 24 months respectively, while for wines aged under commercial conditions, the mean scores were 5.87 and 3.75 respectively. The data also showed a statistical difference ($p \leq 0.05$) between wine samples at 24 months but not at 12 months. The data obtained by Coello and co-workers is similar to what was observed in this study (12 month ageing), as the wines that were aged at 6 and 15 °C were considered better for overall quality than wines aged at 30 °C. Furthermore, it is likely that the duration of ageing in this study (365 days) and that of Castellari and co-workers (320 days) were not sufficient enough for panellist to effectively differentiate between the wine samples.

7.6 Conclusion

The quality attributes of roselle wines fermented and aged under different processing conditions were evaluated by a 60 member sensory panel. The data on roselle wine quality ratings showed that neither fermentation nor ageing temperature showed significant effect ($P \geq 0.05$) on the overall quality of roselle wines. On the other hand, the average mean scores for colour, aroma and overall quality does seem to suggest that wines aged at 6 and 15 °C were rated better than wines aged at 30 °C. There is the possibility that if the ageing time was increased, it might have been easier for the sensory panel to differentiate between the wine samples. This line of reasoning (time-dependency versus sensory attributes) comes as a recommendation for future experiments. In addition, a descriptive sensory analysis on roselle wine is still needed to provide more insight on the aroma attributes (floral, vegetative, earthy, etc.) of the wine. The information generated from the analysis can then be linked to the volatile compounds that might be responsible for the

aroma notes and the transformation they undergo during wine processing. The feedback received from panel members varied, while some rated the wines very high in quality, others did not, possibly due to their own concept of grape wines. This is expected as most of the consumers had never consumed hibiscus wine. As a continuation to this work and to provide more information on panellists knowledge of wine and how that might have influenced the rating of the wine, a wine survey on consumer attitude and knowledge of wine has been launched. The aim of the survey is to place consumers into segments or groups and observe how the different groups rated the wine samples.

Chapter 8 Summary and future perspectives

8.1 Purpose of the investigation and novelty

H. sabdariffa has emerged as a competitive target for phytochemical studies due to its reported use in folk medicine for the treatment of various health diseases. While its use in the treatment of hypertension does appear to have been validated by a number of scientific papers, its anti-diabetic properties is yet to be fully investigated. To examine this, the assay conditions for the *in vitro* inhibition of both α-amylase and α-glucosidase were optimised to test if the reported anti-diabetic properties of *H. sabdariffa* could be partly through the inhibition of α-glucosidase (maltase) and α-amylase. The inhibition of these enzymes was modest, even though the inhibition of the latter has been reported in the literature, and so we can conclude that this mechanism is unlikely to be responsible for the health effects of Hibiscus extracts. It is possible that the conditions under which previous assays were performed could explain this conflicting result, because assay conditions greatly influence (e.g. excess enzyme, incubation time) measured IC_{50} values leading to incorrect values (Acker and Auld, 2014; Nyambe-Silavwe *et al.*, 2015). In the previous literature, no data on the kinetics of the reaction were mentioned (*Km* and *Vmax*, linearity range and enzyme activity), therefore it is difficult to ascertain whether the assays were performed under optimal conditions. However, it is possible that there could be other mechanisms beyond the scope of this work through which *H. sabdariffa* exerts its anti-diabetic property (Peng *et al.*, 2011). These studies could involve the use of type II diabetic rat models, cell studies, and possibly measuring blood glucose response in human subjects after consumption of hibiscus based diet.

Based on the ascribed health benefits, functional foods have been developed from the calyces, the majority being non-alcoholic beverages and tea. In order to diversify the

application of its functional properties, its wine-making potential was explored. In this context, this research work stands out from other studies on *H. sabdariffa* extracts or wine for the following reasons:

Firstly, while there are published studies on the phenolic profile of *H. sabdariffa* varieties, there are no known study on the variations in the content of organic acids and sugars in *H. sabdariffa* varieties. Furthermore, this study provides information on the variations in the content of these phytochemicals brought about by seasonal changes in Nigeria. The experiment in Chapter 3 provides a global map on the phytochemical constituents in *H. sabdariffa* varieties.

Secondly, while hibiscus acid and its 6-methyl ester have been identified as active principles for the inhibition of α-amylase, no compound in *H. sabdariffa* has been linked to the inhibition of α-glucosidases. The compounds were identified and reported in this research in Chapter 4.

Finally, while some studies have demonstrated the possibility of making wine from *H. sabdariffa* calyx (roselle wine), the resultant changes in the phytochemical composition and subsequent bioactivity during fermentation and subsequent ageing has not been previously investigated. In addition the volatile compounds in roselle wine have never been reported. Chapters 5 & 6 deal extensively with these changes and report for the first time the implications to the overall quality of the wine.

The manufacture of wholesome foods from *H. sabdariffa* may entail either a single or multiple processing techniques which could alter the phenolic profile, and consequently the bioactivity of the final product. Prior to the commencement of this study, there was only one published study that had investigated the changes in phytochemical and aroma attributes of hibiscus drinks processed using thermal and non-thermal (Dense Phase Carbon-dioxide) techniques. Therefore, it was of interest to investigate in-depth the

possible changes that could occur in the phytochemical composition and aroma attributes during wine-making from *H. sabdariffa*. To achieve this, three varieties of *H. sabdariffa* (dark, light and white) were screened for their phytochemical and physicochemical properties with the aim of selecting the ideal variety for wine production. Results of the phenolic composition of hibiscus varieties revealed that dark red *H. sabdariffa* contained the highest levels of anthocyanins, and flavonols, while the white variety alone contained flavan 3-ols. Furthermore, glucose was the main reducing sugar, while malic acid was the major organic acid in the three varieties. Regarding the physicochemical properties, the pH values of the extracts were ≈ 3.1, while colour density was highest for the dark red. In addition, the dark red variety was also the most potent in the inhibition of α-glucosidase and for the very first time the compounds (delphindin 3-*O*-sambubioside, cyanidin 3-*O*-sambubioside and 3-*O*-cafeolyquinic acid) responsible for the inhibition were identified. Based on these results (phenolic content and bioactivity), the dark red variety was chosen for wine production.

8.1.1 Wine production

The final quality of wine is dependent on a number of factors which include the fermentation temperature and style, yeast strain, ageing conditions, fining treatments etc. Since it was practically impossible within the time-frame to evaluate the impact of all processing factors on roselle wine quality, only the impact of fermentation and ageing temperatures were studied. This is because the volatiles identified in wines are usually dominated by fermentation products and during ageing these volatiles are subsequently modified resulting in significant changes in the volatile composition of the final product. Fermentation was conducted at two fermentation temperatures (20 and 30 °C) and changes in the physicochemical, and phytochemical properties, as well as carbohydrase inhibition, were monitored over 40 days. On day 40, wine colour at both fermentation

temperatures showed lower colourimetric (L* C* H*) and higher colour density (CD) readings than day 0. While there was no major difference in the physicochemical nor phenolic profiles of the wine, differences occurred in the inhibition of α-glucosidase and in the aroma profile. The inhibition of α-glucosidase was slightly more potent at 20 °C. For the aroma, more fruity notes were detected at 20 °C while there were more flowery notes at 30°C. From a technological point of view, this information could be useful during roselle wine processing to alter the aroma profile to meet consumer's preferences. In terms of the bioactivity of Hibiscus extracts, one of the specific hypotheses of this research was that fermentation would transform the phenolic compounds in roselle juice into more bioavailable forms. The transformation of 3-CQA into caffeic acid during fermentation supports our hypothesis as caffeic acid is more easily absorbed in the human body than chlorogenic acid (Olthof *et al.*, 2001).

After alcoholic fermentation, wines may be subjected to malolatic fermentation, depending on the wine style. However, in this study, wines were not inoculated with lactic acid bacteria, but transferred into bottles to commence the ageing process conducted at three ageing temperatures (6, 15 and 30 °C) for a period of 12 months. Although the concentration of most of the individual phenolic compounds analysed decreased with ageing, the anthocyanins suffered the greatest degradation. The degradation of anthocyanins were seen to follow first-order kinetics, with wine aged at 30 °C having the highest degradation rate constant. However, this did not affect the bioactivity of roselle wine as there was no significant change ($p \geq 0.05$) in the inhibition of α-glucosidase at all storage conditions when compared to the initial inhibition value of the juice prior to fermentation. The implication here is that the polymeric anthocyanins formed during ageing were as potent in the inhibition of α-glucosidase as the monomeric anthocyanins. Consequently, it is clear that processing of *H. sabdariffa* into wine has no negative impact

on the inhibition of α-glucosidase as inhibition results were even higher in wines aged at 6 °C than the juice. Regarding the changes in organic acids, there was a gradual decrease in malic and succinic acid content with final concentrations being significantly less ($p \leq 0.05$) than initial conditions, while acetic acid levels increased significantly ($p \leq 0.05$), within the first four months, thereafter remaining stable throughout ageing. Although ageing temperature did not produce an obvious trend in concentrations of most of the volatiles analysed, its impact was clearly demonstrated in the final concentrations of diethyl succinate, which increased with higher ageing temperature. In summary, the final concentrations of anthocyanins and diethyl succinate were the major compounds influenced by ageing temperature.

The data from the sensory evaluation showed that there was no significant difference ($p \geq 0.05$) in the quality attributes of the wine samples irrespective of fermentation and ageing temperatures. Taking into consideration that the sensory panel was made up of different categories of wine consumers, a possible explanation as to why statistically significant differences were not found between the wine samples (after 12 months of ageing) could be due to the level of expertise of the panel group.

8.1.2 Direction of future studies on *H. sabdariffa* extracts and wine

This study provides a detailed map of *H. sabdariffa* phytochemicals and their transformation during wine-making. However, with the knowledge acquired during this research work and from the benefit of hindsight, there are some limitations in this study. Although the identification of the varieties of *H. sabdariffa* analysed in this study was based on a previous study, a detailed genetic analysis and DNA barcoding of all Hibiscus varieties available in Nigeria is needed to assist future research involving the characterization of phytochemicals in *H. sabdariffa*. Regarding roselle wine, there are

still some aspects on the wine production that could be investigated. The following come as recommendation for future studies.

Raw material (fresh versus dried)

Ramírez-Rodrigues *et al*. (2011) analysed the volatiles in fresh and dried hibiscus and reported that there were more flowery aromas in fresh hibiscus calyces, while dried hibiscus had more green and herbaceous notes. The nature of the raw material could be exploited to produce roselle wines with different aroma profiles.

Must composition and yeast species

A number of studies have shown that the levels of volatile compounds in wine biochemically related to the yeast amino acid (higher alcohols) metabolism are related to the variety of grape from which the wine was processed (Hernández-Orte *et al.*, 2002). Furthermore, the content of volatile compounds in wine is also dependent on the yeast species and strains used for fermentation (section 5.4.6). Torija *et al*. (2003) compared the production of volatiles between *S. cerevisiae* and *S. bayanus*, and reported that the latter produced higher levels of 2-phenyl ethanol (flowery note) than the former. Studies on the influence of must composition of roselle juice and the impact of fermentation conducted by different yeast species/strains could be the direction of future research.

Skin contact fermentation (time contact)

During skin contact fermentation, increase in the herbaceous notes and astringency in wines has been observed which can either have a positive or negative impact on the aroma characteristics of the product depending on the wine style (Selli *et al.*, 2006). In addition, skin contact fermentation could also result in bitter flavour and excess colour, hence the process must be carefully controlled (Palomo *et al.*, 2006). An investigation on the impact

of skin-contact time during fermentation on the aroma and flavour quality attributes is needed to determine optimum conditions of this processing parameter.

Malolactic fermentation and addition of oak chips

The benefits of MLF include the reduction in wine acidity, addition of flavour and bacterial stability of the product. While the addition of oaks has been shown to reduce or mask herbaceous notes in grape wine (Gómez García-Carpintero *et al.*, 2012). In this study, oak chips were deliberately avoided since they contain phenolic and volatile compounds that may well influence the final outcome (phenolic and volatile composition) of this research. These processes could be applied to roselle wine and the impact on its quality attributes of the resultant wine investigated.

8.1.3 Health and economic benefit of roselle wine

Hibiscus sabdariffa has emerged as a competitive target for the development of functional foods due to its high content of bioactive phytochemicals. One of such functional foods is roselle wine which is currently being marketed in Jamaica, Thailand, West Indies and Vietnam under different brand names. However, in developing countries of the world, where the plant is readily available, its economic potential is yet to be fully exploited.

In Nigeria, there is a growing demand for wine as both men and women increasingly adopt wine as their favourite drink, especially at public functions and indoor celebrations. In 2014, the wine industry in Nigeria was valued at 300 million USD per annum and this value is expected to increase over the next five years. On the other hand, 1 kg of dried roselle calyces sells for about 4 USD or less in some developing countries of Sub-Saharan Africa. Therefore, harnessing the potential of *H. sabdariffa* for wine making offers immense benefits to the economy of Nigeria by improving the livelihood of the farmers and stimulating the growth of the small and medium scale enterprises. To minimize the

cost of the raw materials, brewing sugar can be replaced with sugar cane molasses (*Saccharum officinarum*) which, although locally grown in Nigeria, was not used in this study because they have been reported to contain polyphenols (Singh *et al.*, 2015). Thereafter, pilot plants can be set up through collaboration with the relevant governmental agencies for optimization of the process conditions, which can then be scaled up and the product exported to other parts of the world. In conclusion, this research is the first detailed study on roselle phytochemicals and their transformation when processed into wine. It is hoped that the findings of this research will stimulate future studies on the wine processing, which ultimately will be of benefit both to the scientific community and the food industry.

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Appendix

UNIVERSITY OF LEEDS RESEARCH ETHICS COMMITTEE APPLICATION FORM [1]

UNIVERSITY OF LEEDS

Please read each question carefully, taking note of instructions and completing all parts. If a question is not applicable please indicate so. The superscripted numbers refer to sections of the guidance notes, available at www.leeds.ac.uk/ethics. Where a question asks for information which you have previously provided in answer to another question, please just refer to your earlier answer rather than repeating information.

Research ethics training courses: http://www.sddu.leeds.ac.uk/sddu-research-ethics-courses.html

To help us process your application enter the following reference numbers, if known and if applicable:

Ethics reference number:	
Grant reference and/ or student number:	200518354

PART A: Summary

A.1 Which <u>Faculty Research Ethics Committee</u> would you like to consider this application?[2]

- ○ Arts and PVAC (PVAR)
- ○ Biological Sciences (BIOSCI)
- ○ ESSL/ Environment/ LUBS (AREA)
- ○ MaPS and Engineering (MEEC)
- ○ Medicine and Health (Please specify a subcommittee):
- ○ Leeds Dental Institute (DREC)
- ○ Health Sciences/ LIGHT/ LIMM
- ◉ School of Healthcare (SHREC)
- ○ Medical and Dental Educational Research (EdREC)
- ○ Institute of Psychological Sciences (IPSREC)

A.2 Title of the research[3]

The effect of processing conditions on the aroma profile of wines from *Hibiscus sabdariffa*

A.3 Principal investigator's contact details[4]	
Name (Title, first name, surname)	Mr Idolo Ifie
Position	PhD Student
Department/ School/ Institute	School of Food Science and Nutrition
Faculty	Faculty of Mathematics and Physical Sciences

Work address (including postcode)	University of Leeds LS2 9JT
Telephone number	07831017428
University of Leeds email address	fsii@leeds.ac.uk

A.4 Purpose of the research:[5] (Tick as appropriate)

- ☐ Research
- ☐ Educational qualification: ***Please specify:*** **_PhD**
- ☐ Educational Research & Evaluation[6]
- ☐ Medical Audit or Health Service Evaluation[7]
- ☐ Other

A.5 Select from the list below to describe your research: (You may select more than one)

- ☐ Research on or with human participants
- ☐ Research with has potential significant environmental impact.[8] If yes, please give details:

  _____

- ☐ Research working with data of human participants
- ☐ New data collected by questionnaires/interviews
- ☐ New data collected by qualitative methods
- ☐ New data collected from observing individuals or populations
- ☐ Research working with aggregated or population data
- ☐ Research using already published data or data in the public domain
- ☐ Research working with human tissue samples[9]

A.6 Will the research involve any of the following:[10] (You may select more than one)

If your research involves any of the following an application must be made to the National Research Ethics Service (NRES) via IRAS ***www.myresearchproject.org.uk*** as NHS ethical approval will be required. There is no need to complete any more of this form. Contact ***governance-ethics@leeds.ac.uk*** for advice.

☐ Patients and users of the NHS (including NHS patients treated in the private sector)[11]

☐ Individuals identified as potential participants because of their status as relatives or carers of patients and users of the NHS

☐ Research involving adults in Scotland, Wales or England who lack the capacity to consent for themselves[12]

☐ A prison or a young offender institution in England and Wales (and is health related)[14]

☐ Clinical trial of a medicinal product or medical device[15]

☐ Access to data, organs or other bodily material of past and present NHS patients[9]

☐ Use of human tissue (including non-NHS sources) where the collection is not covered by a Human Tissue Authority licence[9]

☐ Foetal material and IVF involving NHS patients

☐ The recently deceased under NHS care

☐ None of the above

You must inform the Research Ethics Administrator of your NRES number and approval date once approval has been obtained.

If the University of Leeds is not the Lead Institution, or approval has been granted elsewhere (e.g. NHS) then you should contact the local Research Ethics Committee for guidance. The UoL Ethics Committee need to be assured that any relevant local ethical issues have been addressed.

A.7 Will the research involve NHS staff recruited as potential research participants (by virtue of their professional role) or NHS premises/ facilities?

☐ Yes ☐ No

If yes, ethical approval must be sought from the University of Leeds. Please note that NHS R&D approval is needed in addition, and can be applied for concurrently: *www.myresearchproject.org.uk*.

Contact governance-ethics@leeds.ac.uk for advice.

A.8 Will the participants be from any of the following groups? (Tick as appropriate)

- ☐ Children under 16[16]
- ☐ Adults with learning disabilities[12]
- ☐ Adults with other forms of mental incapacity or mental illness
- ☐ Adults in emergency situations
- ☐ Prisoners or young offenders[14]
- ☐ Those who could be considered to have a particularly dependent relationship with the investigator, eg members of staff, students[17]
- ☐ Other vulnerable groups
- ☐ No participants from any of the above groups

Please justify the inclusion of the above groups, explaining why the research cannot be conducted on non vulnerable groups.

The research could include students and staff that are all fully registered with the university from the School of Food Science and Nutrition. Mainly staff, Masters and PhD students will be invited to participate. Since the research is in the food science field, students will feel more attracted to take part in the project. However, to ensure students are not coerced we will recruit them by two means.

1- Through a notice posted in the School rather than individual solicitations. The notice will be displayed as a general invitation to participate in the project. Such a notice will also direct anyone interested in the study to pick up an information sheet from the School Administration Office.

2- Invitation via an e-mail from the School secretary to staff, Masters and PhD students. Before sending that inter-department e-mail, we will request an approval from the University Secretary. This is to comply with the university policy about the use of computer systems. The e-mail will include the information sheet and the contact details of the principal investigator to be contacted in case of willingness to get involved.

A Criminal Record Bureau (CRB) check will be needed for researchers working with children or vulnerable adults (see *www.crb.gov.uk* and *http://store.leeds.ac.uk/browse/extra_info.asp?modid=1&prodid=2162&deptid=34&compid=1&prodvarid=0&catid=243*)

A.9 Give a short summary of the research[18]

This section must be completed in **language comprehensible to the lay person**. Do not simply reproduce or refer to the protocol, although the protocol can also be submitted to provide any technical information that you think the ethics committee may require. This section should cover the main parts of the proposal.

In the case of wines from grapes or other fruit sources, the acceptability of the product is dependent on its aroma or its bouquet. Though wine has been produced from Hibiscus and consumed in some parts of the world, there is little scientific information on its aroma attributes. Consequently, this work seeks to identify, through a trained sensory panel, the aroma 'finger print' of hibiscus wine. The sensory panel will be trained using reference standard samples containing substances which they will be told to identify and rate their concentrations in the hibiscus wine sample.

Participants will be asked to sniff and taste the wine and savour its aroma and rate the wine aroma based on the standard reference sample provided. The result obtained will be analysed and used for further aroma analysis using an electronic nose which acts more like an artificial nose followed by the Gas Chromatography-Mass Spectrometery which quantifies these aroma compounds. The results from the sensory analysis will provide clear information on the aroma compounds and terms that can be associated with hibiscus wine.

A.10 What are the main ethical issues with the research and how will these be addressed?[19]

Indicate any issues on which you would welcome advice from the ethics committee.

This research work seeks to classify hibiscus wine based on its aroma profile. There is so far no detailed published article using standard wine descriptive terms on the aroma profile of hibiscus wine. Major ethical issues in such tests could be the following:

- Voluntary participation:

Taking place in the research will be completely voluntary and the assessors can withdraw at any stage of the study if they wish to without giving any reason. People will be invited to take a part in this study via e-mail and via notice boards. They are not deceived or coerced into participating in this research.

- Informed consent:

Consent forms will be provided for each participant at each test period and they all will be asked to sign these forms. All volunteers taking part in the study will be fully informed about the study and the ingredients of the samples will be explained to them during the test.

- Confidentiality:

Personal information that is obtained from the assessors will be kept confidential and will not be shared with anyone.

- Risk of harm:

In the research, reference standard samples prepared in a base wine or 12 % acohol solution will be provided to the sensory panel. They will be told to sniff the wine and taste and identify the correct aroma term present in the different reference samples using descriptions contained in the wine aroma wheel. The wine aroma wheel contains the information of different aroma terms used to describe a wine product. Following the correct identification of the reference samples, they are given the hibiscus wine sample and asked to sniff and taste the wine and identify the correct terms to describe the wine aroma. They will also be asked to rate the concentrations of these aroma attributes. Participants are requested to sip and taste the wine, as the study is concerned with aroma and flavour which has to do with the human olfactory and taste system. The quantity of wine samples given to participate poses no threat of one getting drunk. Therefore the study poses no significant risk to the participants. The amount of wine that will be sniffed and tasted by the participants is shown in the fig below and they are not expected to consume all of it.

The sessions will be divided into two parts. Firstly, the panel will be trained using the reference samples to see if they can properly identify the correct aroma terms associated with each standard reference. Upon proper identification of the reference standard sample, they will then be trained to identify and rate the concentration for similar aroma compounds found in the hibiscus wine sample. A session will not exceed an hour to allow for maximum attention. Also the testing conditions will be optimized in our sensory laboratory. All samples will be stored at appropriate temperatures. A total of 12-15 hours spread over 2 months will be used for training and Hibiscus wine assessment.

- Anonymisation of data:

Two kinds of data will be collected during the test:

1- Personal information of the participant such as name, age, gender and contact details.
2- The assessor's response to the consistency difference within the samples.

The data will be kept in a secure file in the principal investigator's office. When compiling data, participants will be referred to by a code number. The list associating subjects with code numbers will be locked in separate location.

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PART B: About the research team

B.1 To be completed by students only[20]	
Qualification working towards (eg Masters, PhD)	PhD
Supervisor's name (Title, first name, surname)	Dr Peter Ho
Department/ School/ Institute	Food Science and Nutrition
Faculty	Faculty of Mathematics and Physical sciences
Work address (including postcode)	LS2 9JT
Supervisor's telephone number	0113 3438380
Supervisor's email address	P.Ho@leeds.ac.uk
Module name and number (if applicable)	

B.2 Other members of the research team (eg co-investigators, co-supervisors) [21]	
Name (Title, first name, surname)	Dr. Lisa Marshall
Position	Lecturer in Food Chemistry
Department/ School/ Institute	School of Food Science and Nutrition
Faculty	Faculty of Mathematics and Physical Sciences
Work address (including postcode)	Leeds University LS2 9JT
Telephone number	0113 343 1952
Email address	L.J.marshall@leeds.ac.uk

Name (Title, first name, surname)	Professor Gary Williamson
Position	Lecturer in Food Science
Department/ School/ Institute	School of Food Science and Nutrition
Faculty	Faculty of Mathematics and Physical Sciences
Work address (including postcode)	Leeds University LS2 9JT
Telephone number	(0)113 3430358
Email address	G.Williamson@leeds.ac.uk

Part C: The research

C.1 What are the aims of the study?[22] (Must be in language comprehensible to a lay person.)

The Project Aims and Objectives:

The project seeks to evaluate the impact of fermentation and wine ageing temperatures on the aroma profile of Hibiscus wine. The aroma attributes of the wine will be investigated by a trained sensory panel that will seek to differentiate between hibiscus wine samples that have been processed under different conditions. Further objectives from the sensory studies include:

- Developing a lexicon of wine terms appropriately describing hibiscus wine.

Correlate results from sensory analysis with results obtained from the other methods for wine aroma such as the electronic nose and gas chromatography.

It is hoped we could establish certain correlations between results from the sensory panel and results of aroma detection from other instrumentation methods. Such knowledge will be extremely useful in establishing quality parameters in the processing of hibiscus wine to inhibit aroma and flavour deterioration during hibiscus wine processing.

C.2 Describe the design of the research. Qualitative methods as well as quantitative methods should be included. (Must be in language comprehensible to a lay person.)

It is important that the study can provide information about the aims that it intends to address. If a study cannot answer the questions/ add to the knowledge base that it intends to, due to the way that it is designed, then wasting participants' time could be an ethical issue.

In the research as mentioned before there will be instrumental and sensory procedures.

1. Sensory Test Design; Hibiscus wine samples will be sniffed and tasted in order to identify using the wine aroma wheel the correct aroma descriptions of hibiscus wine. Prior to participants being given the wine samples, they will be trained using reference standard wine samples prepared with compounds which describe different wine aromas. Following successful identification of these reference samples and their associated wine aroma term, they will then be given the hibiscus wine samples to identify and rate the aroma attributes of the wine based on the reference standard sample. The wine evaluation sheet which will be properly explained to the participants is attached to this application.

2 Instrumental test design: For the quantitative instrumental tests, an electronic nose and a Gas Chromatography-Mass Spectrophotometer will be used to obtain results about the aroma profile. The results from the sensory panel will provide a lead to the instrumental methods that will be used in analysing hibiscus wine

C.3 What will participants be asked to do in the study?[23] *(e.g. number of visits, time, travel required, interviews)*

The participants will be asked to participate in the training and evaluation sessions with every session not exceeding an hour. During the training sessions, participants will be asked to sniff reference samples and during the test sessions they will be asked to sniff and test a set of (3) hibiscus wine samples processed under different conditions and identify the aroma compounds present in the wine based on the training on the reference wine samples. For instance if a wine is floral (aroma attributes of a flower) a standard solution of wine samples containing compounds that possess floral notes will be given to the participants and they will be asked to rate the intensity of the floral notes present in the hibiscus wine samples. There will be a rating form which will be used to score the aroma attributes of the different wine samples.

C.4 Does the research involve an international collaborator or research conducted overseas:[24]

(Tick as appropriate)

☐ Yes ☐ No

If yes, describe any ethical review procedures that you will need to comply with in that country:

Describe the measures you have taken to comply with these:

Include copies of any ethical approval letters/ certificates with your application.

C.5 Proposed study dates and duration

Research start date (DD/MM/YY): As soon as ethical permission is obtained.
Research end date (DD/MM/YY):30/09/2016

Fieldwork start date (DD/MM/YY): _____ Fieldwork end date (DD/MM/YY): _____

C.6. Where will the research be undertaken? (i.e. in the street, on UoL premises, in schools)[25]

The research will be held in the Food Tech Lab, Food Process Lab and Food Sensory Analysis Lab, in the School of Food Science and Nutrition, University of Leeds where food for consumption is normally prepared.

RECRUITMENT & CONSENT PROCESSES

How participants are recruited is important to ensure that they are not induced or coerced into participation. The way participants are identified may have a bearing on whether the results can be generalised. Explain each point and give details for subgroups separately if appropriate.

C.7 How will potential participants in the study be:

(i) Identified?Participants will be given different identification members. They will preferably be people who are regular and moderate drinkers of wine and acquainted with wine flavour and aroma terms. It is possible that many will be from the Food Science and Nutrition School students and staff.

+

(ii) Approached?

Notice board and invitation e-mail will be used as a mean of participation. For those who are willing to take part at the study, a meeting to discuss more about the study will be arranged.

(iii) recruited?[26]

Aims and objectives will be explained to each participant. They will be also informed about the nature of the test and the reason of the test as well as explaining about how the data will be used. In addition, they will be asked to sign a consent form for the study.

C.8 Will you be excluding any groups of people, and if so what is the rationale for that?[27]

Excluding certain groups of people, intentionally or unintentionally may be unethical in some circumstances. It may be wholly appropriate to exclude groups of people in other cases. Anyone who self-assesses themselves as ‘’healthy’’ will be included. The exception will be those who are under the age of 18.

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C.9 How many participants will be recruited and how was the number decided upon?[28]

It is important to ensure that enough participants are recruited to be able to answer the aims of the research.

As the first stage of the research involves the training, 12 participants will be recruited and for the evaluation stage a minimum of 9 participants is needed in order to achieve meaningful results for statistical studies. This will allow for a 25% drop-out from the study. Results will be statistically analysed for the average and standard deviation. This is typical for this type of study as reflected in an article published by Heymann *et al*. 2012. (Heymann, H.; Machado, B.; Torri, L.; Robinson, A. L. How many judges should one use for descriptive analysis? J. Sensory Stud. 2012, 27, 111−122).

If you have a formal power calculation please replicate it here.

Remember to include all advertising material (posters, emails etc) as part of your application

C10 Will the research involve any element of deception?[29]

If yes, please describe why this is necessary and whether participants will be informed at the end of the study.

No. Participants will be clearly told the aim of the research and what is expected of them. They are also given consent forms which they are told to fill before commencing the training. They are also free to opt out of the study whenever they want to.

C.11 Will informed consent be obtained from the research participants?[30]

☐ Yes ☐ No

If yes, give details of how it will be done. Give details of any particular steps to provide information (in addition to a written information sheet) e.g. videos, interactive material. If you are not going to be obtaining informed consent you will need to justify this.

Written consent form will be supplied to each assessor for each session to the participants. Aims and objectives and the procedure of the test will be clearly given to inform the assessors about the test.

If participants are to be recruited from any of potentially vulnerable groups, give details of extra steps taken to assure their protection. Describe any arrangements to be made for obtaining consent from a legal representative.

Copies of any written consent form, written information and all other explanatory material should accompany this application. The information sheet should make explicit that participants can withdrawn from the research at any time, if the research design permits.

Sample information sheets and consent forms are available from the University ethical review webpage at http://researchsupport.leeds.ac.uk/index.php/academic_staff/good_practice/planning_your_research_project-1/approaching_and_recruiting_participants-1.

C.12 Describe whether participants will be able to withdraw from the study, and up to what point (eg if data is to be anonymised). If withdrawal is not possible, explain why not.

Each assessor will be totally free to withdraw from the test without giving any reason at any time.

C.13 How long will the participant have to decide whether to take part in the research?[31]

It may be appropriate to recruit participants on the spot for low risk research; however consideration is usually necessary for riskier projects.

The notification of the sensory test will be done at least two weeks beforehand. This period of time will give participants an opportunity to decide whether to enter the study or not. Participants will be free to change their mind at any stage.

C.14 What arrangements have been made for participants who might not adequately understand verbal explanations or written information, or who have special communication needs?[32] *(e.g. translation, use of interpreters etc. It is important that groups of people are not excluded due to language barriers or disabilities, where assistance can be given.)*

All the materials will be given in simple language in English.

C.15 Will individual or group interviews/ questionnaires discuss any topics or issues that might be sensitive, embarrassing or upsetting, or is it possible that criminal or other disclosures requiring action could take place during the study (e.g. during interviews or group discussions)?[33] *The information sheet should explain under what circumstances action may be taken.*

☐ Yes ☐ No *If yes, give details of procedures in place to deal with these issues.*

C.16 Will individual research participants receive any payments, fees, reimbursement of expenses or any other incentives or benefits for taking part in this research?[34]

☐ Yes ☐ No

If Yes, please describe the amount, number and size of incentives and on what basis this was decided.

They will be compensated for the time spent during the training and wine assessment sessions. Participants who complete both training and wine assessment sessions will receive a £10 voucher.

RISKS OF THE STUDY

C.17 What are the potential benefits and/ or risks for research participants?[35]

The research will be routine involving a simple sensory analysis. To our knowledge it involves no risk to researchers and to participants. The concentrations of the reference samples

prepared possess no risk to the participants.

Should any unexpected emergency occur during the test, immediate first aid help which is available in food Science building will be administered. For serious cases32222 will be called.

C.18 Does the research involve any risks to the researchers themselves, or people not directly involved in the research? *Eg lone working*[36]

☐ Yes ☐ No

If yes, please describe: _____

Is a risk assessment necessary for this research?

NB: Risk assessments are a University requirement for all fieldwork taking place off campus. For guidance contact your Faculty Health and Safety Manager or visit **http://www.leeds.ac.uk/safety/fieldwork/index.htm**.

☐ Yes ☐ No If yes, please include a copy of your risk assessment form with your application.

DATA ISSUES

C.19 Will the research involve any of the following activities at any stage (including identification of potential research participants)? (Tick as appropriate)

☐ Examination of personal records by those who would not normally have access

☐ Access to research data on individuals by people from outside the research team

☐ Electronic transfer of data

☐ Sharing data with other organisations

☐ Exporting data outside the European Union

☐ Use of personal addresses, postcodes, faxes, e-mails or telephone numbers

☐ Publication of direct quotations from respondents

☐ Publication of data that might allow identification of individuals to be identified

☐ Use of audio/visual recording devices

☐ FLASH memory or other portable storage devices

Storage of personal data on or including any of the following:

☐ Manual files

☐ Home or other personal computers

☐ Private company computers

☐ Laptop computers

C.20. How will the research team ensure confidentiality and security of personal data? E.g. anonymisation

procedures, secure storage and coding of data.[37] You may wish to refer to the data protection and research webpage.

All the data will be held secured and confidential within the school building. Data that is transferred computers will be anonymous pooled results. No individual and personal data will be released in any form at any time.

C.21 For how long will data from the study be stored? Please explain why this length of time has been chosen.[38]

RCUK guidance states that data should normally be preserved and accessible for ten years, but for some projects it may be 20 years or longer.

Students: It would be reasonable to retain data for at least 2 years after publication or three years after the end of data collection, whichever is longer.

4 years, 0 months

CONFLICTS OF INTEREST

C.22 Will any of the researchers or their institutions receive any other benefits or incentives for taking part in this research over and above normal salary or the costs of undertaking the research?[39]

☐ **Yes** ☐ **No**

If yes, indicate how much and on what basis this has been decided

_____

_____

C.23 Is there scope for any other conflict of interest?[40] For example will the research funder have control of publication of research findings?

☐ Yes ☐ No ***If yes, please explain***

_____

C.24 Does the research involves external funding? (Tick as appropriate)

☐ Yes ☐ No ***If yes, what is the source of this funding?***

_____

PART D: Declarations

Declaration by Chief Investigators

1. The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.
2. I undertake to abide by the University's ethical and health & safety guidelines, and the ethical principles underlying good practice guidelines appropriate to my discipline.
3. If the research is approved I undertake to adhere to the study protocol, the terms of this application and any conditions set out by the Research Ethics Committee.
4. I undertake to seek an ethical opinion from the REC before implementing substantial amendments to the protocol.
5. I undertake to submit progress reports if required.
6. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer.
7. I understand that research records/ data may be subject to inspection for audit purposes if required in future.
8. I understand that personal data about me as a researcher in this application will be held by the relevant RECs and that this will be managed according to the principles established in the Data Protection Act.
9. I understand that the Ethics Committee may choose to audit this project at any point after approval.

Sharing information for training purposes: Optional – please tick as appropriate:

☐ I would be content for members of other Research Ethics Committees to have access to the information in the application in confidence for training purposes. All personal identifiers and references to researchers, funders and research units would be removed.

Principal Investigator 

Signature of Principal Investigator: .....
(This needs to be an actual signature rather than just typed. Electronic signatures are acceptable).....

Print name: IDOLO IFIE Date:.....(dd/mm/yyyy): 25/02/2015

Supervisor of student research: I have read, edited and agree with the form above.

Supervisor's signature: .....  ..... (This needs to be an actual signature rather ɜt typed. Electronic signatures are acceptable).....

Print name: .Peter Ho Date: .....(dd/mm/yyyy): 25/02/2015

.....

Please submit your form **by email** to J.M.Blaikie@leeds.ac.uk or if you are in the Faculty of Medicine and Health FMHUniEthics@leeds.ac.uk. **Remember to include any supporting material** such as your participant information sheet, consent form, interview questions and recruitment material with your application.

Checklist:

- ❑ I have used layman's terms to describe my research (applications are reviewed by lay members of the committee as well).
- ❑ I have answered all the questions on the form, including those with several parts (refer to the guidance if you're not sure how to answer a question or how much detail is required)
- ❑ I have included any relevant supplementary materials such as

Recruitment material (posters, emails etc)

Sample participant information sheet

Sample consent form. Include different versions for different groups of participants eg for children and adults.

- ❑ If I am not going to be using participant information sheets or consent forms I have explained why not and how informed consent will be otherwise obtained.
- ❑ If you are a student have you discussed your application with your supervisor and are they satisfied that you have completed the form correctly? (This will speed up your application).
- ❑ I have submitted a signed copy of my application. (If you are a student your supervisor also needs to sign the form).