Identification, characterization and application of a natural food colourant from *Hibiscus sabdariffa*

Submitted in complete fulfilment for the Degree of Master of Applied Sciences (Food Science and Technology) in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa

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July 2016

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REFERENCE DECLARATION

I, Shivon Sipahli – 20902315 and Dr J J Mellem do hereby declare that in respect of the following dissertation:

Title: **Characterisation, identification and application of a natural food colourant from *H. sabdariffa***

1. As far as we ascertain:

a) no other similar dissertation exists;

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AUTHORS DECLARATION

This study presents original work by the author. It has not been submitted in any form to another academic institution. Where use was made of the work of others, it has been duly acknowledged in the text. The research described in this dissertation was carried out in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, South Africa, under the supervision of **Dr J J Mellem and Dr V. Mohanlall**.

Student's signature

OUTPUTS

PUBLICATIONS

Title: Stability and degradation kinetics of crude anthocyanin extracts from *H. sabdariffa*

Journal: Food Science and Technology (Campinas)

Manuscript number: CTA – 2016 – 0142

Status: Accepted

DOI: http://dx.doi.org/10.1590/1678-457X.14216

CONFERENCE OUTPUTS

SAAFoST 2015 – 21st Biannual International Congress and Exhibition (September 2015)

Poster presentation – Stability and degradation kinetics of crude anthocyanin extracts from *H. sabdariffa*

IUFoST 2016 – 18th World Congress of Food Science and Technology (August 2016)

Poster presentation – Stability and degradation kinetics of crude anthocyanin extracts from *H. sabdariffa*

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ACKNOWLEDGEMENTS

A special thank you to the following people for their contribution to this research:

- My supervisor and co-supervisor, Dr J J Mellem and Dr V Mohanlall for their invaluable knowledge, guidance and encouragement,
- Professor H Baijnath for collection of plant material and general assistance,
- My parents, family and friends for their continuous support and encouragement,
- The staff and students at the Department of Biotechnology and Food Technology, Durban University of Technology for their general support and encouragement,
- The Department of Biotechnology and Food Technology (DUT), ADA and Bertie Levenstein and National Research Foundation (NRF) for their financial support.

ABSTRACT

Hibiscus sabdariffa is an under-utilised plant that has been reported to have great potential in the pharmaceutical and nutraceutical industries. The vibrant red pigment indicates a source of anthocyanins that could be produced into a food colourant with additional nutritional benefits however stability is a hindering factor. The crude anthocyanins were extracted from dried calyces by means of four different acidified ethanol and methanol solvent systems to determine the maximum crude anthocyanin yield. The crude extracts were analysed under the following parameters; heat, light, pH stability and degradation kinetics, which included thermal degradation and DPPH radical scavenging ability. Two synthetic colourants were analysed based on the stability parameters; heat, light and pH and compared with the natural *H. sabdariffa* crude extracts. Each of the four crude extracts were analysed for the total phenolic content using Folin Ciocalteu's method. The DPPH and FRAP assays were used to determine the radical scavenging activity of the extract with the highest yield. The identification and quantification of the crude anthocyanins were carried out using HPLC-DAD. The highest crude anthocyanin yield of 19.92% was observed by HCl acidified ethanol extract Acetic acid/water/methanol extract produced the lowest yield of 8.72%. The stability results showed that pigment retention of samples heated at 80˚C had a greater decrease over time than those heated at 50˚C. The pH stability of samples incubated for 7 days indicated that crude anthocyanins degraded slower at acidic pH, which is in keeping with reported literature therefore this extract, should be added to foods with lower pH. Light stability showed slower degradation in dark incubated samples resulting in 84% pigment retention after a 10 day period. Synthetic colourants proved to be superior, as they had showed better stability than the natural colourant under the same conditions. Half-life of thermally treated samples showed a decrease upon heating, colour was also affected as

samples became dull and murky. DPPH of thermal treated samples showed a decline in radical scavenging activity from 70 to 85˚C and thereafter an increase was observed between 85 and 90˚C, this could be due to the release of degradation products that have antioxidant capability. Solvent systems did not have an effect on the total phenolic content of crude extracts as no significant difference was observed by each of the *H. sabdariffa* crude extracts contained an average of 54.67 mg/ml GAE. The radical scavenging ability assessed by the DPPH and FRAP assays showed 53.75% and 57.51% radical scavenging ability respectively. Although the synthetic colourants showed better stability, a natural food colourant from *H. sabdariffa* can still be beneficial as it has potential to be applied into foods that contain low pH such as jelly and yoghurt. The additional benefits that natural food colourants possess aid in the marketability of the product.

1. INTRODUCTION AND LITERATURE REVIEW

Colour can be described as one of the most important aspects of a food product. It determines the way in which consumers perceive the product. A growing trend among consumers is healthy natural food products free from synthetic additives.

Natural pigments are those pigments that are freely found in nature. They can be extracted from a variety of fruit, vegetables and flowers. Plant pigments are compounds that exhibit colours such as lycopene that gives tomatoes its red colour and chlorophyll that gives leaves its green colour. Not only do natural plant pigments impart various colours they can also have advantageous benefits to human health.

Anthocyanins are regarded as one of the most important pigments found in higher plants. They are a group of plant pigments that are responsible for the red, orange, blue and violet colours of plants. They are also known to have high solubility, are high in antioxidants and have potential health benefits. Thus, anthocyanins could be the ideal as a natural plant pigment.

Hibiscus sabdariffa is an herbaceous shrub that is cultivated in tropical and subtropical climates. The plant is grown widely in South Africa as well as other African countries. In a commercial sense, *H. sabdariffa* is regarded as an under-utilised plant. Research indicates that the plant has great untapped potential as it has been reported to impart both pharmaceutical and nutritional benefits.

The calyces of *H. sabdariffa* are vibrant red in colour due to the high quantity of anthocyanins present. The calyces are also a source of vitamins, minerals and pectin.

Traditionally, dried calyces were used for the production of beverages, while fresh calyces were produced into products such as jams and jellies. In traditional medicine, the calyces were used to treat ailments from constipation to heart ailments.

1.1. Scope of Study

With the increasing concern about the safety of synthetic colourants, research on natural colourants are becoming of more significance (Wang et al., 2013). *Hibiscus sabdariffa* is an underutilized crop that has great pharmaceutical and nutritional benefits. The plant has an abundant source of anthocyanins that are responsible for the vibrant red colour however, stability limits their usage in industrial applications. Therefore, the aim of this study was to develop a food colourant from *H. sabdariffa* and to identify the major anthocyanins.

The aim of this study was achieved by conducting the following objectives:

- Extract anthocyanins from *H. sabdariffa* using 4 different solvent systems to determine the most viable extractant
- Determine the stability and total phenolic content of the crude extracts of the 4 solvent systems, i.e. pH, temperature, light and degradation kinetics
- Identify and quantify the major anthocyanins of *H. sabdariffa* by HPLC-DAD
- To determine antioxidant potential of the major anthocyanins by FRAP and DPPH assays
- To apply the food colourant to an appropriate food

1.2. Colour

1.2.1. Colour perception

Colour can be defined as a perception that is expressed by the response to a narrow span of the electromagnetic spectrum emitted by light sources. Light, on the other hand, is colourless however, colour cannot exist on its own but rather it has the ability only to

exist in the mind of the viewer. The colour that is viewed is dependent upon light and therefore the light source. Light is composed of different wavelength radiations. Visible light is described as the most important component with regard to colour appreciation. The wavelength radiation of the visible spectrum ranges between 380 and 750 nm. It is reported that all light that can be perceived by the human eye is related to light radiation (Delgado-Vargas and Paredes-Lopez, 2002).

1.2.2. Measurement

Colorimetry is the technique that is used to measure light. It can be described as the science of colour measurement. Four systems are largely used to assess a colour. Munsell, an old visual colour ordering system. CIE XY2 or the International Commission on Illumination was developed in 1931, this system was the first mathematical system developed to quantify and standardise colour measurement. In 1942, the Hunter LAB system was published. This system was developed to communicate better to an observed colour to the numerical values by applying the colour opponent theory of colour perception. The Hunter LAB system has also been used commonly in the food industry. An improved version of Hunter LAB was developed in 1976, the Hunter CIELAB (Delgado-Vargas and Paredes-Lopez, 2002, Ahmadiani, 2012).

The principle attributes which are measured by any of the colorimetry systems include, hues, lightness and saturation. Hue refers to the quality that is identified with a colour such as red, green or blue. Lightness is a concept that relates to darkness and lightness of the colour by taking into consideration the light source. Saturation is another important attribute as it relates to the clarity or purity of the colour. This attribute also describes the intensity of the hue (Ahmadiani, 2012, Delgado-Vargas and Paredes-Lopez, 2002).

1.3. Pigments

Pigments are described as chemical compounds that are able to absorb light within the visible region in the wavelength range. There are three categories of pigments natural, synthetic and inorganic. Natural pigments are found in nature, they are extracted from living organisms like plants, fungi and microorganisms. Synthetic pigments are man-made; they are produced within the confines of a laboratory. Inorganic pigments are pigments which can be found in nature or are able to be reproduced by synthesis (Hendry and Houghton, 1996, Delgado-Vargas and Paredes-Lopez, 2002).

1.3.1. What are food colourants?

A colour additive or food colourant can be defined as any dye, pigment or substance that has the ability to impart colour to a food, drink or pharmaceutical product. Colourants also include any chemical that results in the formation of a colour due to a chemical reaction (Amchova et al., 2015). Human beings are attracted to items that are visually appealing, therefore, colour can be recognised as one of the most significant attributes of a food. Not only does it make the food appealing to the consumer, it is also related to safety and quality, e.g. if a fruit or vegetable is supposed to be red or green, it should not be brown while meat should be red rather than green.

Colour can indicate factors such as spoilage, poor quality and essentially poor processing. Colourants are added to food products to enhance the existing colour that could possibly be lost during production or the overall shelf life of the product. (Delgado-Vargas and Paredes-Lopez, 2002, Giusti and Wrolstad, 2003, Downham and Collins, 2000, Carocho and Ferreira, 2013).

Colourants added to food is not a new concept it has been in practice since early civilisation. As far back as 1500 BC, Egyptians used natural extracts and even wine for the colouring of candies to improve their consumer appeal. Natural colourants were used such as Saffron to provide both colour and flavour to a product. In the 19th century, inorganic pigments such as lead chromate and copper sulphate were used in candies as well as sauerkraut (Delgado-Vargas and Paredes-Lopez, 2002, Downham and Collins, 2000, Yousuf et al., 2015, Wang et al., 2013).

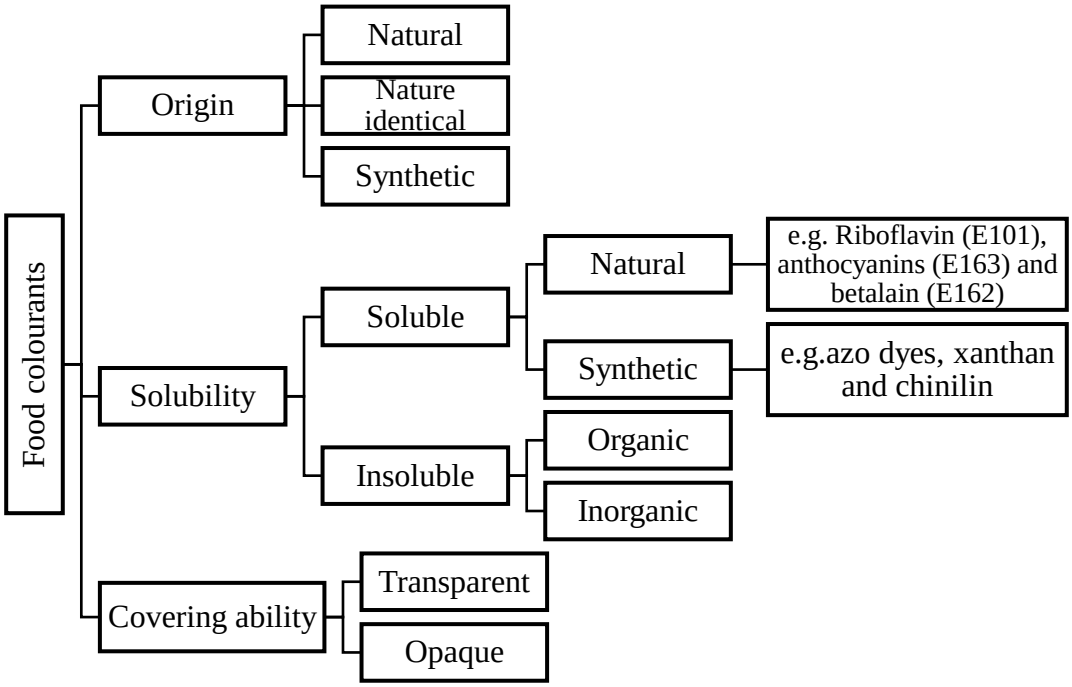

Figure 1.1: Criteria used to categorise food colourants (Amchova et al., 2015)

There are three main categories that food colourants can be divided into origin, solubility and covering ability. Figure 1.1 depicts the criteria used to categorise food colourants accurately. Soluble dyes are divided into natural and synthetic. Natural soluble dyes are of natural origin. They are described as having poor stability. Synthetic soluble dyes are produced by chemical synthesis. They were originally produced from coal tar. These dyes do not impart a flavour and are regarded as more stable when compared to soluble natural dyes. Insoluble dyes are called pigments. These pigments are either organic or

inorganic and cannot be dissolved in common solvents. Inorganic pigments do not have a variety of colours while organic pigments are usually found in lacquer form with a wide array of colours (Amchova et al., 2015).

1.3.2. Synthetic colourants

In the recent years, synthetic colourants have made consumers apprehensive about their use in food products with regard to their association with human health issues. Azo dyes, a group of synthetic colourants that consist of bright colours, have been identified to possibly exhibit toxic effects in food products. According to Buchweitz et al. (2012), azo dyes were linked to the increase of Attention Deficit/ Hyperactivity Disorder in children. Allergenicity and carcinogenicity have also been a major issue with respect to the use and disposal of these colourants (Sharma et al., 2012, Giusti and Wrolstad, 2003).

Table 1.1 shows the effect of synthetic food colourants on the metabolism of mammals (including human beings) when administered orally (Amchova et al., 2015).

Table 1.1: Metabolism of synthetic food colourants after oral administration (Amchova et al., 2015)

Synthetic food colourant	Description	Absorption	Excretion
E124 Ponceau 4R	Red, water soluble anionic monoazo dye	limited	90% by faeces, 25 – 35% unaffected, majority as products resulting in any tissue from azo reduction in the GIT
E127 Erythrosine	Red xanthan dye	< 1%	80 – 100% unaffected by faeces
E129 Allura red	Red, water soluble anionic monoazo dye	limited	Predominately by faeces, 29% unaffected, rest as products from azo reduction in the GIT

A demand has been created for the successful substitution of a synthetic red food colourant (Giusti and Wrolstad, 2003). Synthetic colourants that are currently being used in foods include Ponceau 4R, Carmoisine Red, Allura Red and Solvent Red.

1.3.3. Natural colourants

Natural food colourants are described as any dye that has been extracted from plant, animal or mineral that has the capability to colour food, drugs or cosmetics. There are a variety of sources where these natural food colourants can be obtained from including; seeds, fruits, vegetable, leaves, algae and insects (Sharma, 2014).

There are four main categories of natural food colourant. Yellow, orange and red shades can be attributed to carotenoids, greens are associated with chlorophylls, reds to betanins and the red, blue and purple shades are because of anthocyanins.

There are 26 natural colours currently used in the food industry and 28 that can be applied in the pharmaceutical and cosmetic industries. The most commonly used natural colours include curcumin from turmeric which exhibits a yellow pigment, betalain from beetroot juice which is a reddish pink colour, anthocyanins from red cabbage producing the purple hue and chlorophyll present in spinach that provides the green colour. The use of natural colourants has become something of a marketing trend essentially due to concerns of safety of synthetic colourants and the possible health benefits associated with natural colourants (Sharma, 2014, Rodriguez-Amaya, 2016, Chapman, 2011).

Natural food colourants have been classified into three main classes. Natural colours that are regarded as the original natural colourants as they are refined forms of chlorophyll, carotene and flavonoids. During cooking and processing, browning colours are produced

such as the caramelisation of sugar and baking. These colourants are not of direct importance in foods. The third class is additives. These are food additives are based on anthocyanins derived from plant sources such as red grapes and beetroot (Sharma, 2014). There are many benefits to the use of natural food colourants, some of which include; (Sharma, 2014);

i. Protective role against lethal photo-oxidation
ii. Inhibition of mutagenesis
iii. Boosted immune system
iv. Inhibition of tumour development

Although natural colourants have many positive attributes including their nutritional and pharmacological benefits. A disadvantage that limited their application includes the cost of production and their low stability. Consumers are trying to avoid products containing synthetic food colourants as they consider natural food colourants to be less harmless (Attia et al., 2013, Gerardi et al., 2015).

1.4. Regulations of food colourants

South Africa follows the Food, Cosmetic and Disinfectant Act of 1972 (Act No. 54 of 197). This act includes chlorophyll, beetroot red, betanin, curcumin, carotenoids; annatto and beta-carotene, xanthophyll; canthaxanthin and lutein and anthocyanins. The acts state that anthocyanins are to be prepared using physical methods from plants (Magnuson et al., 2013, Sharma, 2014).

All colour additives are regulated under federal authorities to ensure that foods are safe for consumption and accurately labelled. The history of food regulation began with the

voluntary certification programme developed by the Food and Drug Act of 1906 in the United States where it regulated the addition of colours to food products. Mandatory certification was thereafter imposed for the food products, pharmaceutical and cosmetics by the Federal Food, Drug and Cosmetic (FD & C) Act of 1938. Further amendments were made to the law in 1960. The law stated that any colour additives should be on the approved list compiled by the Federal Food and Drug Administration.

There are two groups of additives; those exempt from certification, namely natural colours and certified colourants referred to as artificial colours. Each country has a regulatory system in place for scientific evaluation and approval of food additives, food ingredients and food contact substances.

1.5. Phenolics

Phenolic compounds are a group of compounds that contains at least one aromatic ring bearing one or more hydroxyl group. Polyphenols are the broadest class of secondary metabolites and their distribution being the most extensive. It has been estimated that 10 000 to 20 000 secondary metabolites exist with approximately 20% of the carbon fixed by photosynthesis is channelled into the phenylpropanoid pathway, thus generating the majority of the naturally occurring phenolic compounds such as flavonoids (Pereira et al., 2009, Ezekiel et al., 2013, Salazar-González et al., 2012).

Phenolic compounds are generated from two metabolic pathways, namely the shikimic acid pathway where phenylpropanoids are formed and acetic acid pathway where the main products are the simple phenol. Plant phenolic compounds are synthesised through the phenylpropanoid pathway. However, the combination of both pathways results in the

formation of flavonoids, the most abundant group of phenolic compounds found in nature (Giada, 2013).

It is possible for phenolic compounds to be found in free form in plant tissue although this occurrence is not very common. In free form, the compound has reported to be toxic. When bound, the toxic effect is removed (Giada, 2013).

Phenolic compounds are vital to the physiology and cellular metabolism of plants as they are involved in a number of plant function. Some of the plant functions include the organoleptic properties such as colour, aroma, taste and astringency, structure, pollination and resistance to pests and predators (Giada, 2013).

1.5.1. Flavonoids

Flavonoids can be described as polyphenolic secondary metabolites that are synthesised by the polypropanoid pathway with phenylalanine activation molecules, which has been illustrated by figure 1.2 (Janićijević et al., 2007, Giada, 2013).

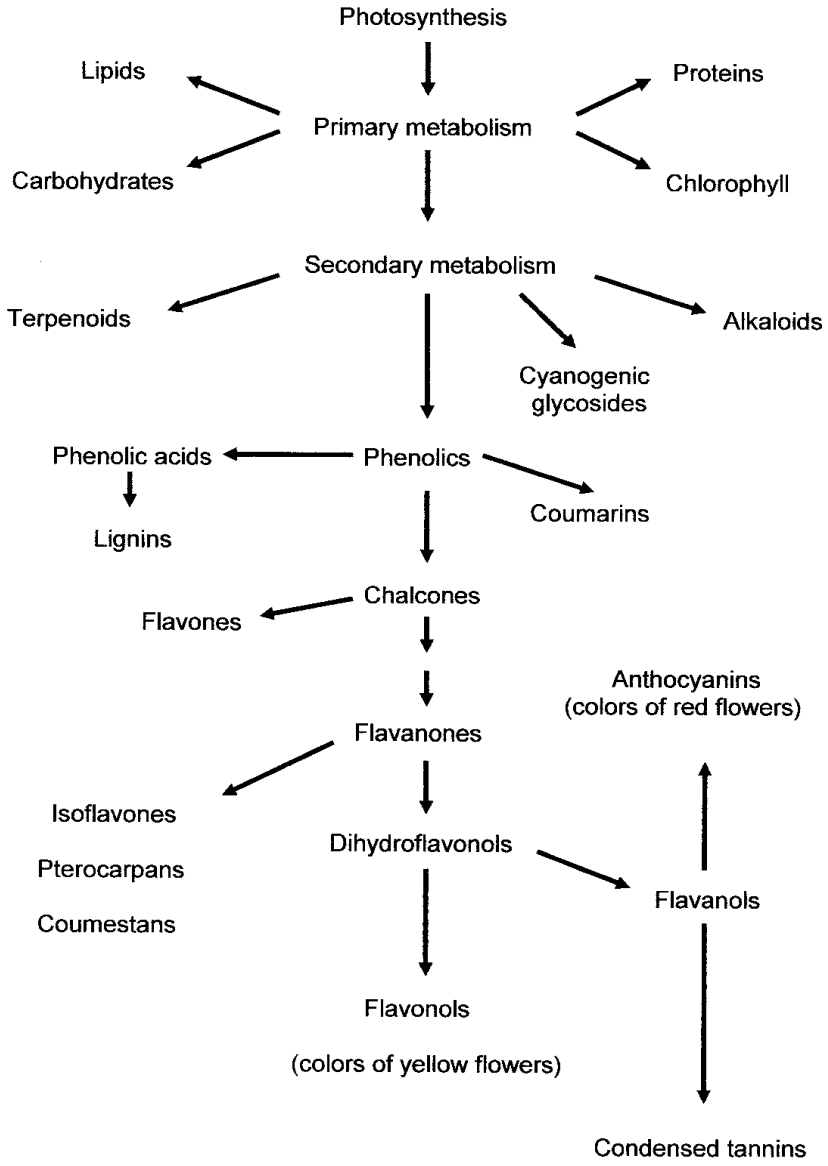

Figure 1.2: Phytochemical pathway for major secondary metabolites (Giada, 2013)

Flavonoids are divided into 13 subclasses that are differentiated by the degree of hydroxylation and the presence of a $C_2 – C_3$ double bond in the heterocycling pyrone ring. The most important groups of flavonoids are flavonols, flavanols, flavones, isoflavones, anthocyanidins and flavanones. Not all flavonoids depict a colour, some are colourless like flavononones while others exhibit a colour such as anthocyanidins (Giada, 2013).

Since flavonoids are potent antioxidants, they are regarded as being an important aspect of human nutrition. The antioxidant activity that are possessed by flavonoids are related to the presence of hydroxyl groups in the 3' and 4' position of the B ring (Giada, 2013).

1.6. Anthocyanins

Anthocyanins are defined as one of the most significant water-soluble vacuole plant pigments found in the flavonoid category. They can be found in almost all higher plants (i.e. angiosperms or flowering plants) and in some cases mosses and ferns but are not found in lower plants such as liverwort and algae. The non-toxic pigment are responsible for the array of attractive colours such as orange, pink, red, purple and blue that are depicted in fruits, flowers, leaves and storage organs (Andres-Bello et al., 2013, Wang et al., 2013).

More than 500 different anthocyanins and 23 anthocyanidins exist in nature. However, there are six most common anthocyanins found in vascular plants or higher plants, namely, delphinidin, malvidin, cyanidin, pelargonidin, peonidin and petunidin (Castañeda-Ovando et al., 2009). Anthocyanidins are available singly in certain fruit such as cyanidin in apples and figs or delphinidin in eggplant and pomegranate.

1.6.1. Anthocyanin chemistry

The basic structure of anthocyanins are made up anthocyanidins, as illustrated by figure 1.3. Anthocyanidins consist of aromatic rings (A), which are bound to a heterocyclic ring (C) that contains oxygen that is bonded with carbon – carbon to a third aromatic ring (B) (Castañeda-Ovando et al., 2009).

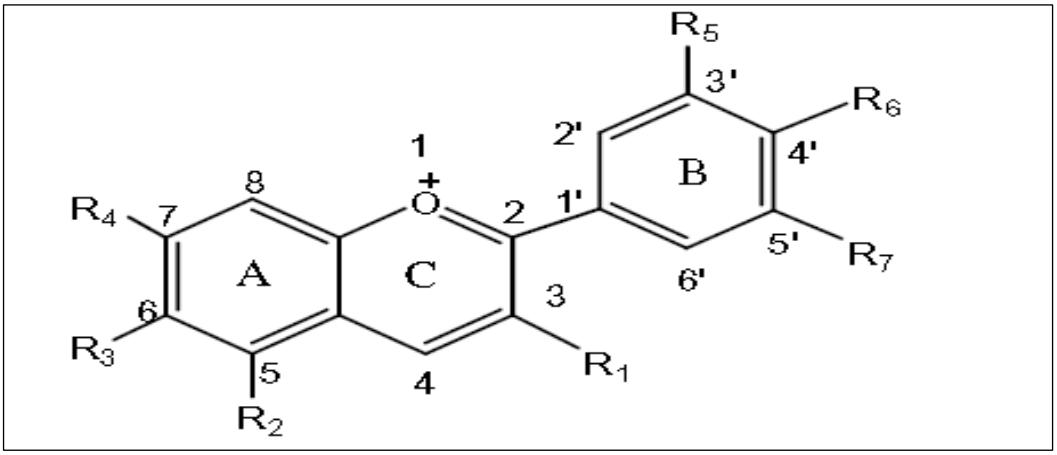

Figure 1.3: Basic structure of an anthocyanidin pigment; Rx could be H, OH or OCH_3 depending on the pigment (Castañeda-Ovando et al., 2009)

When an anthocyanidin is found in glycoside form i.e. when bonded to a sugar moiety, the structure is known as an anthocyanin. Anthocyanins usually have a molecular weight ranging from 400 to 1200 g/mol (Pereira et al., 2009, Yousuf et al., 2015, Prabhu and Bhute, 2012, William, 2014, Ignat et al., 2011).

Anthocyanins have been found to have the same biosynthetic origin as other natural flavonoid compounds. They are based on a C15 skeleton with a chromane ring bearing a second aromatic ring in position 2 i.e. $C_6 – C_3 – C_6$, with one or more sugar molecules bonded at a different hydroxylated positon of the basic structure. Anthocyanins are substituted glycosides of the salt of phenyl-2-benzopyrilium or anthocyanidin. However, unlike flavonoids, anthocyanins are able to absorb visible light and thus impart a variety of colours. These various colours are formed from the basic $C_6 – C_3 – C_6$ anthocyanin structure because of the chemical combination with glycosides and/or acyl groups as well as its interactions with other molecule and conditions of the media. (Markakis, 1982, Hendry and Houghton, 1996, Delgado-Vargas et al., 2000, Prabhu and Bhute, 2012, Yousuf et al., 2015).

The three non-methylated anthocyanidins, cyanidin, delphinidin and pelargonidin are found in about 80% of pigmented leaves, 69% of fruit and 50% of flowers. The differences in anthocyanidins are created due to the number and position of hydroxyl groups and/or the methyl ether group and the sugar molecule. From the 17 structures of anthocyanidin, combinations containing sugar molecules were formed which resulted in the anthocyanin compounds. Therefore, the number of sugar molecule can be described as a method to classify an anthocyanin structurally. When sugar diversity and possible structural points of glycosylation are taken into consideration, the number of possible compounds are greatly increased. The order of sugar occurrence in natural anthocyanins are glucose, rhamnose, xylose, galactose, arabinose and fructose. Ester bonds between sugars and organics acids have been seen in the anthocyanin structure that are referred to as acylated anthocyanins. The most common acyl groups in nature include coumaric, caffeic, ferulic, *ρ*-hydroxybenzoic, synaptic, malonic, acetic, succinic, oxalic and malic. However, the colour that the anthocyanin impart is due to the substation of hydroxyl and methoxyl groups. An increasing number of hydroxyl groups have been reported to deepen colour toward blue while the increasing number of methoxyl groups has the tendency to become more redder (Delgado-Vargas et al., 2000, Fernandes et al., 2014).

1.6.2. Distribution

The vibrant, attractive colour ranging from red to blue are a result of the anthocyanins that are found in plants. There are six most common anthocyanins found in nature. These anthocyanins can be found either singly in a plant or as a combination of two or more.

Fruit such as apple, cherry and fig contain cyanidin and eggplant and pomegranate contains delphinidin while fruit such as cranberry contain cyanidin and peondin. Some fruit like grapes contains more than two anthocyanins (Delgado-Vargas et al., 2000).

Anthocyanins are vacuolar pigments, this means that within this organelle the presence of membrane bound bodies called anthocyanoplast have been proposed and these structures are being formed while pigment synthesis is in operation, they then disperse to produce a completely pigmented vacuole (Delgado-Vargas et al., 2000).

1.6.3. Anthocyanin biosynthesis

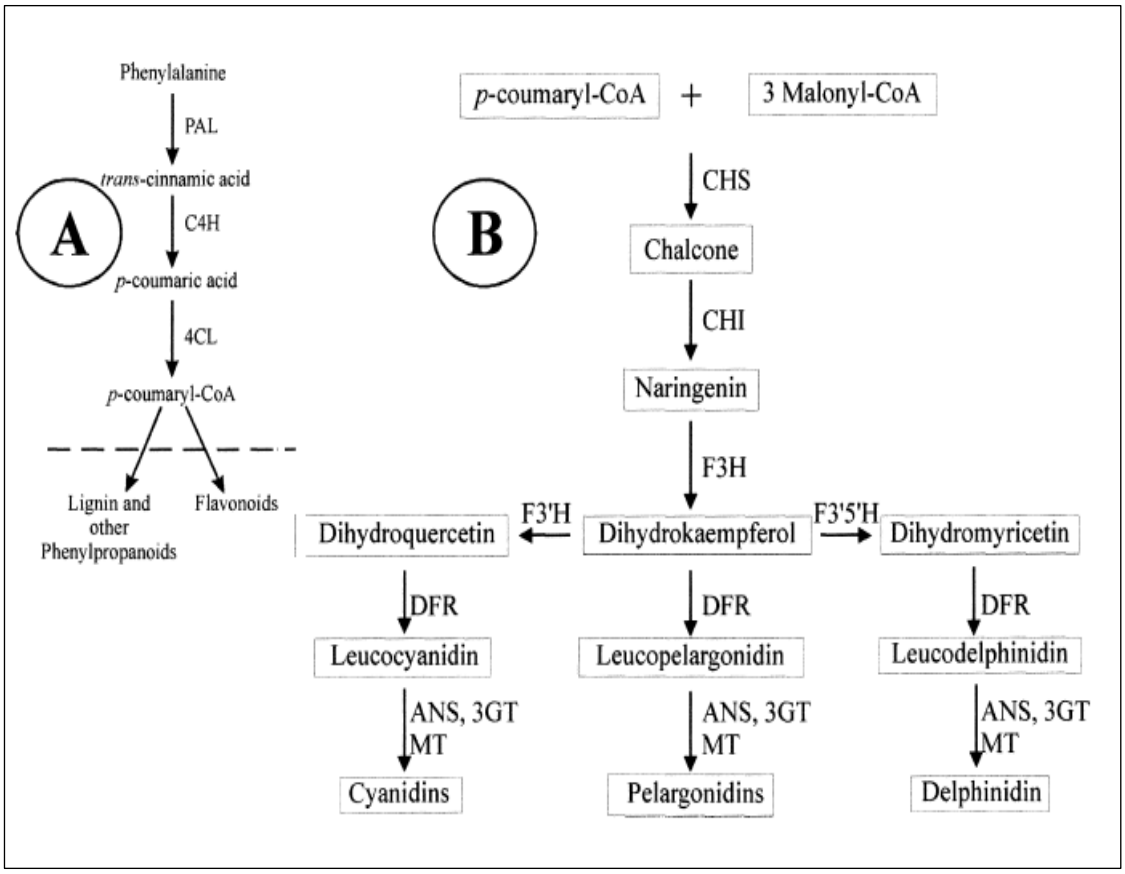

Figure 1.4: Anthocyanin biosynthesis pathway showing A – General phenylpropanoid metabolism and B – specific steps of anthocyanin biosynthesis (Delgado-Vargas et al., 2000)

The anthocyanin biosynthesis pathway can be divided into 2 main parts (Figure 1.4). Part A depicts the precursors of the general phenylpropanoid metabolism while B shows the specific steps toward flavonoid biosynthesis (Delgado-Vargas et al., 2000).

As seen in figure 1.4 A, phenylalanine is converted to *ρ*-coumarl-CoA by three enzymes, phenylalanine ammonia-lyase (Andres-Bello et al.), cinnamate-4-hydroxylase (C4H) and 4-coumaryl-CoA ligase (4CL). The main precursor of flavonoids, lignin and other phenylpropanoid is *ρ*-coumarl-CoA (Jaakola, 2013, Nilsen, 2010).

Figure 1.4 B now shows the specifics steps used in the biosynthesis of anthocyanins. Chalcone synthase (CHS) can be considered the key enzyme in flavonoid synthesis. CHS catalyses the condensation of three molecules of malonyl-CoA with 4-coumaryl-CoA to form the intermediate chalcone.

The chalcone demonstrates a stereospecific isomerisation to naringenin by the enzyme chalcone isomerase (Kerio et al.). The common precursor to flavonoids and isoflavonoids is narigenin. The narigenin flavanone is thereafter converted to dihydrokaempferol flavone by a dioxygenase or a monooxygenase. Dihydrokaempferol is converted to leucoanthocyanidin by dihydroflavonol-4-reductase (DFR), which according to the plant source is dependent on NADPH and NAD. Leucoanthocyanidins are transformed into the coloured anthocyanidins. This conversion involved an oxidation and dehydration step, the associated enzyme activity is referred to as the anthocyanidin synthase (Azima et al.). Thereafter, an anthocyanidin – anthocyanin conversion takes place by means of a glycosylation reaction that employs the use of the 3-*O*-glucosyl-transferase (3GT) enzyme. Glycosyl transferases have shown a noticeable specificity with regard to substrate, position and sugar to be transferred. Hydroxylation and methylation occurs at different stages of flavonoid biosynthesis. Anthocyanin methylation is a late step, the isolated flavonoid *O*-methyltransferases (MT) have shown great specificity, therefore, multiple methylations require multiple enzymes. On the other hand, it has to be proposed that acyl transferases show high specificity and in some cases

acylation must precede glycosylation in the acylation of the anthocyanins (Delgado-Vargas et al., 2000, Jaakola, 2013).

1.7. *Hibiscus sabdariffa*

Hibiscus sabdariffa is an annual herbaceous shrub from the *Malvaceae* family. Within the *Malvaceae* family, there are known to be 73 different genera and approximately 1000 species in total. The plant is largely grown in tropical and subtropical climates; the warm and humid conditions provide the ideal temperature for optimal growth. Cold areas are not suitable for cultivation as the plant is susceptible to frost and mist.

However, the plant can also withstand a wide range of soil conditions making cultivation simpler. One of the common names for *Hibiscus sabdariffa* is Roselle however demographically there are variations such as, Guinea Sorrel or Bissap in Senegal, Karkadé in North Africa, Roselle or Sorrel in Asia and flora of Jamaica in Central America. This is an ideal crop for developing countries as it is reasonably simple to cultivate (Da-Costa-Rocha et al., 2014, Cid-Ortega and Guerrero-Beltrán, 2015, William, 2014).

H. sabdariffa grows to approximately 3.5 to 4.0 m tall with serrated leaves that are between 7.5 and 12.5 cm long. They have smooth cylindrical dark green to red stems and deep penetrating taproots. The calyx contains five large sepals with an epicalyx (collar). The calyces are a bright vibrant red in colour and have a distinct taste that can be described as sour and astringent (Mahadevan et al., 2009, Perry and Greenwood, 1981, Obouayeba et al., 2014, Mungole and Chaturvedi, 2011). The plant is not just cultivated

for its calyces, the leaves, stems and seeds are valuable components as well. The leaves, calyces and stem are depicted in figure 1.5.

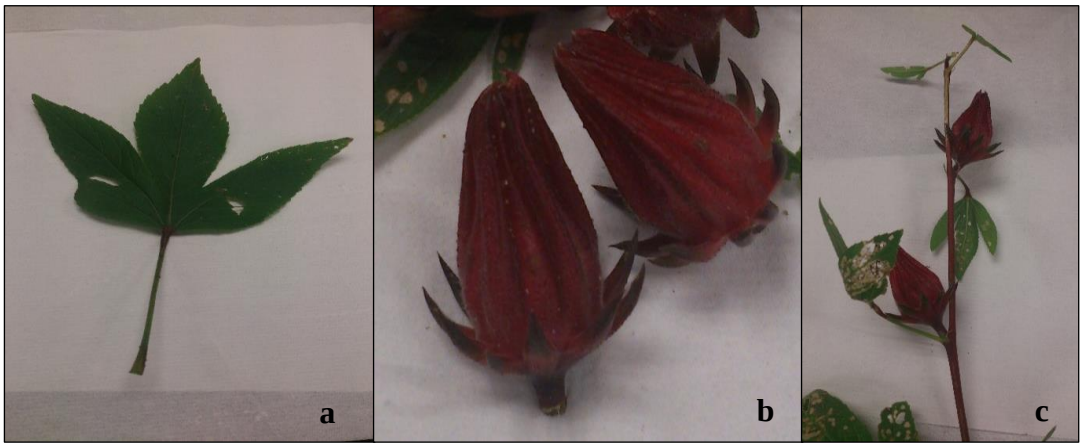

Figure 1.5**: Components of the *H. sabdariffa* plant [a – Leaf; b – Calyces; c – Calyces on the stem]**

1.7.1. Nutritional value

The nutritional composition of *Hibiscus sabdariffa* calyces was reported by various studies however Da-Costa-Rocha et al. (2014) reported the proximate nutritional composition findings that can be observed in table 1.2.

Table 1.2: Proximate nutritional composition of *H. sabdariffa* calyces (Da-Costa-Rocha et al., 2014)

Proximate composition	Calyces* (per 100 g)
Protein	1.9 g
Fat	0.1 g
Carbohydrates	12.3 g
Fibre	2.3 g
Ascorbic acid	14.0 mg
β-carotene	300.0 µg
Calcium	1.7 mg
Iron	57.0 mg

*Dry weight

1.7.2. Traditional uses

Hibiscus sabdariffa is defined as an underutilised plant however for many years it has been used in household across the world. The calyces are used either fresh or dry to produce beverages such as tea and fermented drinks, jam, jellies and candies (Mahadevan et al., 2009, Patel, 2014, Perry and Greenwood, 1981, Wyk and Wink, 2004, Ieri et al., 2011, Puro et al., 2014). In some parts of the world, dried calyces were prepared as tea to be taken for medicinal purposes. Ailments such as constipation, heart ailments, hypertension, urinary tract infections, appetite loss and phlegm of the respiratory tract have been thought to be alleviated.

The plant is also incorporated into ointments to treat skin conditions (Mahadevan et al., 2009, Patel, 2014, Perry and Greenwood, 1981, Wyk and Wink, 2004).

1.7.3. Bioactive profile

In pharmacological context, organic acids, anthocyanins, polysaccharides and flavonoids make up the main constituents of *H. sabdariffa* (Da-Costa-Rocha et al., 2014).

1.7.4. Organic acids

An elevated percentage of organic acids are detected from *H. sabdariffa* extracts. The major organic compounds include citric acid, hydroxycitric acid, hibiscus acid, malic acid and tartaric acid. Oxalic and ascorbic acid are classified as minor compounds.

The amount of ascorbic acid in *H. sabdariffa* varies between fresh and dried calyces as they have been reported to contain 6.7 – 14 mg/100g and 260 – 280 mg/100 respectively. The ascorbic acid amounts can be affected by factors such as the variety, genetics, environment, ecology and harvest conditions (Da-Costa-Rocha et al., 2014, Puro et al., 2014, Salazar-González et al., 2012).

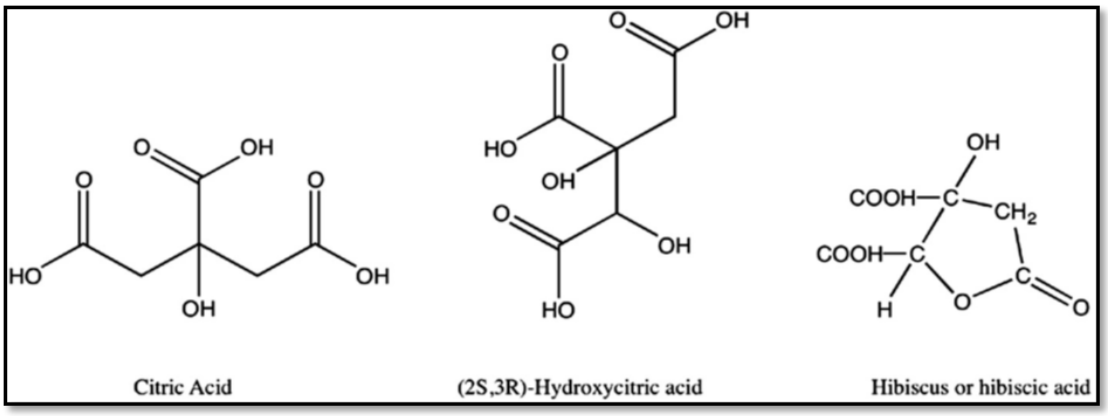

Figure 1.6: Chemical structures of citric acid and its derivatives; hydroxycitric and hibiscus acid found in *H. sabdariffa*

1.7.4.1. Hydroxycitric acid

Hydroxycitric acid is a derived from citric acid (figure 1.6), an additional hydroxyl group is added to citric acid. Furthermore, there are four stereoisomers associated with this acid with their lactone forms namely (2S, 3S), (2R, 3R), (2S, 3R), (2R, 3S). The principal organic acid found in *Hibiscus sabdariffa* calyces is the (2S, 3R) – hydroxycitric acid (Da-Costa-Rocha et al., 2014).

1.7.4.2. Hibiscus acid

Hibiscus acid (figure 1.6) is the lactone form of (+) – allo – hydroxycitric acid. It is composed of the citric acid compound with an additional hydroxyl group at the second

carbon. It also contains two diastereomers because of the existence of two chiral centres in the molecule (Da-Costa-Rocha et al., 2014) .

1.7.5. Mucilage, pectin and polysaccharides

The calyces of *H. sabdariffa* contains between 15 – 28% mucilage while the pectin content ranges from 2 to 4%. Polysaccharide compounds that are detected in calyces were arabinose, galactose, glucose and rhamnose with smaller amounts of galacturonic acid, glucuronic acid, mantose and xylose (Da-Costa-Rocha et al., 2014).

1.7.6. Volatile compounds

The distinctive aroma of *Hibiscus sabdariffa* can be attributed to its volatile compounds. Approximately thirty-two volatile compounds have been identified from calyces. The compounds were divided into 5 main categories aldehydes, alcohols, ketones, terpenes and acids.

Additionally, seven aromatic compounds were identified; hexanal, 3-octanone, 1-octen-3one, nonanal, 2,4-nonadienal (*E,E*) and geranylaceone (Da-Costa-Rocha et al., 2014).

1.7.7. Total phenolic content

Phenolic compounds are secondary plant metabolites that are found universally in fruit, vegetables and flowers, they are important in the sensory and nutritional properties of a plant as well as have been considered to have high free radical scavenging capacity. It has been indicated that phenolic compounds in plants rather than vitamin and minerals are becoming popular as potential agents for the prevention and treatment of much oxidative stress related diseases (Fu et al., 2011, Lapornik et al., 2005).

1.7.8. Antioxidant ability

Anthocyanins are becoming popular not only as a natural food colourant but also because of their antioxidant potential associated with them (Awika et al., 2004, Kim et al., 2009). Strong antioxidants activity which can prevent the oxidation of ascorbic acid protects against free-radicals and has the ability of oxidative enzymes (Bridle and Timberlake, 1997). Anthocyanins and other phenolic compounds can acts as antioxidants due to their ability to donate hydrogen to highly reactive radicals thus preventing further radical formation. The antioxidant potential is dependent on the number and arrangement of the hydroxyl group, extent of the structural conjugation and the presence of electron - donating and electron – withdrawing substituents in the aromatic ring (Lapornik et al., 2005). Most natural antioxidants tend to be multifunctional, there are two key mechanisms employed, hydrogen atom transfer and single electron transfer (Fu et al., 2011, Payne et al., 2013). This study utilises two assays namely, the ferric reducing ability (FRAP) and 2,2-dipheny-2-picrylhydrazyl (DPPH) assays.

1.8. Pharmacological activities

1.8.1. Antimicrobial activity

Hibiscus sabdariffa extracts have demonstrated antimicrobial activity in various bacteria including methicillin-resistant bacteria like *Staphylococcus aureus*, cariogenic bacteria such as *Streptococcus mutans* that can be found in the oral cavity and bacteria that contaminate meat from the *Campylobacter* species. The inhibitory effects were not affected by heating temperature (Da-Costa-Rocha et al., 2014).

1.8.2. Anti – inflammatory activity

According to Da-Costa-Rocha et al. (2014), *H. sabdariffa* extracts exhibit anti-inflammatory effects by impairing cyclooxygenase-2 induction by down-regulating JNK and p38 MAPK. A clinical study has been conducted to support the anti-inflammatory activity, they reported that the ingestion of water extract decreased plasma monocyte chemoattractant protein MCP-1 concentration, which is a biomarker that is used to analyse the inflammatory disease.

1.8.3. Anticancer activity

Studies reveal that *Hibiscus sabdariffa* extracts have had a positive outcome on cancer cells. A compound, protocatechuic acid, demonstrated defensive effects against cytotoxicity and genotoxicity of hepatocytes induced by *tert*-butyl-hydroperoxide (*t*-BHP), through the inhibitory action on DNA repair synthesis caused by *t*-BHP and shows radical quenching effects. The extract also showed inhibition towards cancer cells that cause skin tumours and leukaemia (Da-Costa-Rocha et al., 2014, Patel, 2014).

1.8.4. Antidiabetic activity

Diabetes mellitus is an endocrine and metabolic disorder that is characterised by chronic hyperglycaemia, dyslipidaemia and protein metabolism that is the result of defects in regulation of insulin secretion and/or insulin action. Studies report that *H. sabdariffa* extracts have shown pancreatic α-amylase inhibition. Another study suggested that hibiscus acid inhibited pancreatic α-amylase and intestinal α-glucosidase enzymes. The enzymes α-amylase and α-glucosidase digest carbohydrates thereby significantly decreasing the blood glucose after a meal (Da-Costa-Rocha et al., 2014, Patel, 2014).

1.9. Toxicology

H. sabdariffa has been used traditionally for many years in both food and medicine and is considered safe. Findings from toxicological studies have suggested that dosages up to 200 mg/kg should be safe for human consumption. The metabolic fate of specific anthocyanins has been reported however further studies are still required. Cyanidin chloride metabolites were not detected in rats or *in vitro* with intestinal microorganisms. Pelargonidin breaks down to *p*-hydroxyphenyl lactic acid and another product that is believed to be phloroglucinol. Delphinidin that was administered intragastrically has been reported to produce an unidentified metabolite in urine. Malvidin glycosides produce a number of metabolites that were identified in urine such a syringic acid. Cyanidin and delphinidin reported no mutagenicity when analysed by the Ames test. Anthocyanins are not genotoxic. However, consideration needs to be taken as certain plant extracts may react with chemical drugs that can affect the consumer (Da-Costa-Rocha et al., 2014, Patel, 2014, Delgado-Vargas and Paredes-Lopez, 2002).

1.10. Stability studies

There are however, limitations accompanying the use of anthocyanins as a food colourant thereby restricting their applications. Processing, formulation and storage conditions affect the stability of natural food colourants (Giusti and Wrolstad, 2003). Temperature, pH and light are among the factors that contribute to the instability of natural pigments. Chemically the colour of the anthocyanin is controlled by its resonating structure thereby having an effect on its stability. The stability is directly associated to the number of hydroxyl groups present and inversely related to the number of methoxyl groups. Glycosylation levels also play a role in anthocyanin stability as glycosides are more stable than monoglycosides, however, browning occurs in the

presence of additional sugar molecule (Francis, 1989). One of the most influential aspects of the stability of anthocyanins is pH because the colour is required to be consistent however; anthocyanins react differently at different pH. In an acid solution, anthocyanins appear red, violet and purple while in a neutral and alkaline solution they can be blue. In most cases however anthocyanins are incorporated into acidic conditions (Bridle and Timberlake, 1997).

1.10.1. Effect of pH

The colour of an anthocyanin is dependent on the pH. The anthocyanidin stability is affected by the B substituents and the presence of additional hydroxyl and methoxyl groups, which decrease the aglycone stability in neutral media. In contrast with aglycone, monogycoside and diglycoside derivatives are more stable under neutral pH conditions. This behaviour is due to the sugar molecules that avoid degradation of instable intermediaries into phenolic acid and aldehydes. Anthocyanin colour becomes paler as the pH increases.

At pH 1, the flavylium cation, which is red in colour, is the prime species and contributes to the purple and red colours. Between pH 2 and 4, the quinoidal blue species are predominant. At pH between 5 and 6, there are only two colourless species that can be observed, carbinol pseudobase and a chalcone respectively. Greater than pH 7, the anthocyanins are degraded depending on the substituent group (Andres-Bello et al., 2013, Castañeda-Ovando et al., 2009, Ahmadiani, 2012).

1.10.2. Effect of temperature

Thermal processing including pasteurisation, sterilisation or concentration have the ability to degrade anthocyanins thus causing loss of colour. Anthocyanins become paler

when heated due to the equilibrium between the four anthocyanin species shift in the direction of colourless carbinol base and chalcone form. The relationship between temperature and anthocyanin degradation rate can be described as logarithmic. The proposed mechanism for degradation describes that higher temperature favours the transition of the unstable chalcone form while the open C ring of the chalcone is degraded further to a brown product (Wang et al., 2013, He, 2008).

1.10.3. Effect of light

Intrinsic light absorption properties cause anthocyanin pigments to absorb energy efficiently from photons. It has been reported that acylated anthocyanins are more stable that non-acylated anthocyanins. The second sugar moiety on the C-5 position as well as the co-existence of the other polyhydroxylated flavonoids in the solution also stabilises anthocyanin molecule. A possible solution to minimise instability and preserve anthocyanins could be to use opaque packaging material which would block out incoming light (Ahmadiani, 2012).

1.10.4. Effect of enzymes

Enzymes are naturally occurring in plants. Two of the most common enzymes, polyphenol oxidase (PPO) and peroxidase (POD), are usually released from cells during extraction. However when they are activated they have the ability to accelerate degradation. Enzymatic browning can occur rapidly after release leading to loss of colour. It has been reported that PPO is unable to metabolise anthocyanins directly. Instead, it generates quinones from phenolic acids that subsequently reacts with anthocyanins from condensation or coupled oxidation. In the presence of hydrogen peroxide (H_2O_2), POD has the ability to oxidized phenolic acids to their corresponding

quinone, which polymerise with anthocyanins to increase colour degradation and browning. Both the flavylium cation forms and the hydrated forms can be adduct with quinones. The hydrated forms are however more reactive. Anthocyanins can be preserved from the effects of these enzymes by the prompt inactivation by the temperature used during extraction and the addition of sulphur dioxide (He, 2008).

Co-pigmentation, self-association and acylationCo-pigmentation is the occurrence where the pigments and other colourless organic compounds or metallic ions form molecular or complex associations thereby creating a change or an adjustment in the intensity of a colour. This is a very important occurrence for natural food colours as they assist with improving the stability of the given colour. A number of interactions can be responsible for the formation of co-pigments. Intramolecular interactions are based on the interaction between the pigment and an organic acid, aromatic acyl group or a flavonoid that is covalently linked to an anthocyanin chromophore.

Intermolecular bonds, on the other hand, include colourless flavonoids or other phenolic compounds through weak hydrophobic forces with anthocyanins (Sari et al., 2012, Castañeda-Ovando et al., 2009). It has been established that anthocyanins at high concentrations do not follow Beer's law. Beer's law states that the concentration of a chemical is directly proportional to the absorbance of a solution. The reason for this is that at high concentrations the anthocyanin molecules are closer together; this causes the molecules to create a stacking effect due to self-association thereby enhancing the absorption. The stacking effect was believed to protect anthocyanidins from nucleophilic attack of the water molecules and other reactants (He, 2008).

Another type of stacking effect occurs with intramolecular co-pigmentation. When the acyl group in acylated anthocyanins interact with the aglycone it is referred to as intramolecular co-pigmentation. Acylation, particularly with cinnamic acid, will result in a bathochromic shift meaning the colour will change from a reddish to a bluish hue thus leaving the parent anthocyanins with greater stability (He, 2008).

1.10.5. Influence of ascorbic acid, oxygen and peroxide

Ascorbic acid is classified as an antioxidant, it can be found in fruit juices either naturally or as an antioxidant additive for the prevention of browning and for the improvement of the nutritional value. However when combined with anthocyanins, they can accelerate the rate at which degradation occurs. The level of ascorbic acid and phenolic compounds found in fruit have shown to relate to the above-mentioned degradation. Oxygen has also been known to accelerate the decomposition of anthocyanin.

Since oxygen is regarded as the ultimate electron acceptor, it has the ability to participate both directly and indirectly in a number of mechanism related to anthocyanin degradation. Hydrogen peroxide is known to be the most commonly used packaging sterilant in the food industry. H_2O_2 however is a rather reactive form of oxygen, which consequently causes decomposition to anthocyanins (He, 2008, Andres-Bello et al., 2013, Yousuf et al., 2015, Hernández-Herrero and Frutos, 2015).

1.11. Methodological aspects

1.11.1. Extraction

Anthocyanins have aromatic rings containing polar substituent groups i.e. hydroxyl, carboxyl and methoxyl as well as glycoside residues which produce a polar molecule. They are therefore more soluble in water than in non-polar solvents, however, depending on the conditions of the medium used anthocyanins can be soluble in ether at a pH where the molecule was unionised. These traits aid the extraction and separation of anthocyanin compounds. There are several methods used for the extraction of anthocyanins, the selection of a method would depend on the cost and the type of plant material (Delgado-Vargas et al., 2000, Yousuf et al., 2015).

1.11.1.1. Solvent extraction

Solvent extraction is the most popularly used method. Solvent systems that are most preferred are acidified media such as HCl and methanol that allow the aromatic acyl acid and linkage remain relatively stable. However, weak acids such acetic and formic acid have been suggested to stabilise aliphatic dicarboxylic acyl groups such as malonic, malic and oxalic acid.

Extraction can be enhanced by agitation or stirring during incubation. The extract can be filtered from the extractant and concentrated by rotary evaporation (Delgado-Vargas et al., 2000, Rodriguez-Amaya, 2016, Yousuf et al., 2015).

1.11.1.2. Ultrafiltration and nano-filtration

Ultrafiltration and nano-filtration are methods that can be used to avoid thermal degradation of anthocyanins. These methods may be used for their concentration which gives concentrate of similar quantity as the initial extract (Yousuf et al., 2015).

1.11.1.3. Aqueous two-phase extraction (ATPE)

Aqueous two-phase extraction is used for both the extraction and isolation of natural products from crude extracts. This method is effective and versatile (Yousuf et al., 2015).

1.11.1.4. Supercritical fluid extraction (SFE)

Supercritical fluid extraction is potentially an alternative method to organic extraction. It can be highly beneficial as it is rapid and automatic. SPE methods are selective and they do not require the use of large quantities of toxic solvent. Another advantage is the absence of light and air during extraction and hence there is a reduction in the degradation process during extraction as compared to conventional extraction techniques (Yousuf et al., 2015).

1.11.1.5. Microwave-assisted extraction (MAE)

The energy from the microwaves give rise to molecular movements and rotation of liquids with a permanent dipole. This in turn gives rise to rapid heating of the material. MAE leads to improved efficiency, low solvent consumption and reduced extraction times (Yousuf et al., 2015).

1.11.1.6. Ultrasound-assisted extraction (UAE)

This method is another potential alternative to other time consuming and comparatively low efficiency conventional solvent extraction methods (Yousuf et al., 2015).

1.11.2. Rotary evaporation

The rotary evaporator is a piece of equipment that is used to remove the extraction solvent from the sample thereby resulting in a concentrated sample. The sample is evenly distributed across the inner surface of the vessel in a thin layer thereby decreasing the volume. Increased temperature and decreased pressure is used to achieve the desired concentration. Vacuum conditions are used as the decreased pressure above the bulk liquid will result in the lowering of the boiling points of the sample (William, 2014).

1.11.3. Purification

1.11.3.1. Solid phase extraction (SPE)

SPE is a purification method that uses C18 or Sephadex cartridges. Anthocyanins are bonded strongly through its hydroxyl group, separation from other compounds are carried out by increasing polarity with different solvents (Salazar-González et al., 2012).

1.11.4. Separation

Separation of anthocyanin compounds can be done utilising three methods, namely, Thin Layer Chromatography (TLC), High Performance Thin Layer Chromatography (HPTLC) and column chromatography. TLC and HPTLC use a similar mechanism to separate compounds thereby tentatively identifying the compounds by comparing the retention factors of the standard to the sample. However, HPTLC differs as the method allows for

better separation in a shorter period. The selection of the appropriate mobile phase and stationary phase are the most important factors. Column chromatography employs a glassy column filled with various adsorbents. This method is not often used because of the long analysis times. Compounds eluted would have to undergo TLC and HPTLC thereafter to determine the compound (Kucharska and Grabka, 2010).

1.11.5. Spectroscopy

1.11.5.1. Spectrophotometry

The isolated pigments have been studied by UV – visible spectroscopy. All flavonoids have shown high absorbance in the wavelength range between 250 and 270 nm (UV region). Anthocyanins have intense absorption in the 520 to 560 nm (visible region) range. It has been suggested that UV absorption could be assigned to ring A while visible light to the pyran and ring B (Delgado-Vargas et al., 2000).

1.11.5.2. Liquid chromatography

Liquid chromatography is commonly applied for separation, identification and quantification of food colourants.

High performance ion chromatography, reverse-phase liquid chromatography and ion-pair chromatography are selected as the methods that are preferred as they provide high resolution, sensitivity and selectivity (Delgado-Vargas et al., 2000, Mazdeh et al., 2016).

1.11.6. NMR

NMR with mass spectrometry conclusively identifies anthocyanin compounds. Proton NMR is used to study the self-association of molecule and carbon – 13 NMR can be used

to express the sequence, position and configuration of sugar residues in flavonoid glycosides (Delgado-Vargas et al., 2000).

2. MATERIALS AND METHODS

2.1. Methodology overview

Hibiscus sabdariffa was harvested during May 2014; the plant was thereafter prepared for storage. Figure 2.1 briefly outlines the experimental procedures. The calyces were removed and dried, after which they were stored in amber bottles. Anthocyanins were extracted by means of solvent extraction using acidified ethanol and methanol. The crude extracts were then analysed to determine the most viable extraction medium. Experimentation included stability studies, analysis of total phenolic content and radical scavenging ability and identification and quantification of the specific anthocyanins. The final red food colourant was applied to the appropriate product.

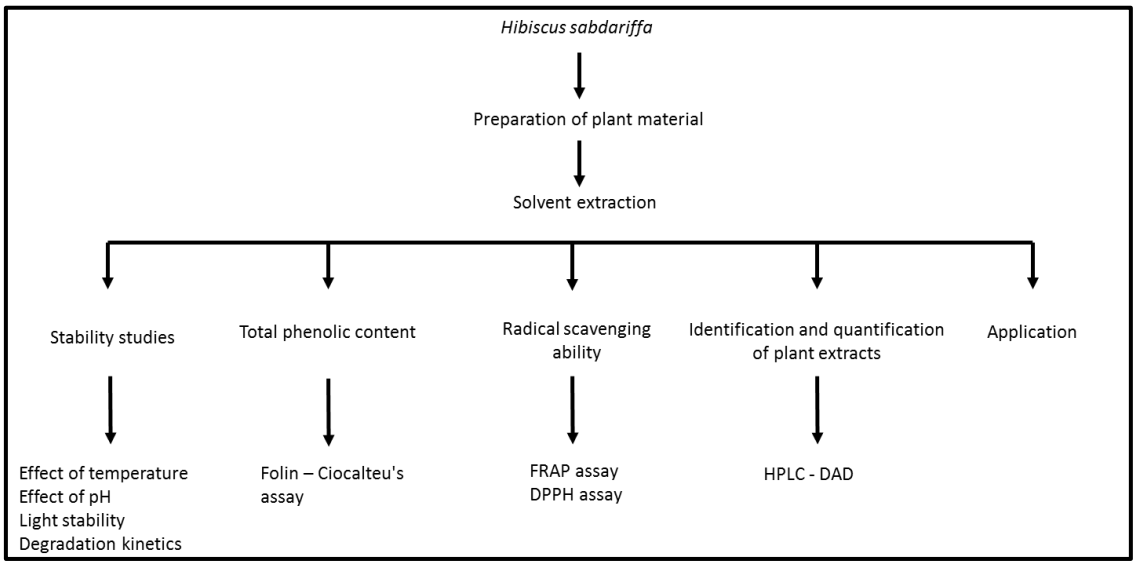

Figure 2.1: Overview of experimental procedures

2.2. Preparation of plant material

Plant taxonomist Prof H. Baijnath collected H. sabdariffa from different locations in and around Durban, Kwazulu Natal, South Africa during May 2014. Voucher specimen was

deposited at the Ward Herbarium (UDW) – (University of Kwazulu Natal, Durban, South Africa – Voucher number: S. Sipahli 1). The fresh *H. sabdariffa* calyces were removed and dried at 40˚C for 48 h in a convection oven, once thoroughly dried, the material was stored in Amber Schott bottles at room temperature until required.

2.3. Pigment extraction

Four different solvents systems were used for the extraction of the crude pigments, hydrochloric acid (37%): ethanol (1:99, v: v), formic acid (85%): methanol (3:97, v: v), citric acid (1 M): methanol (3:97, v: v) and methanol: acetic acid: water (25:1:24 v: v: v). According to the method by Amr and Al-Tamimi (2007) with minor modifications. Two grams of dried calyces were added to 100 mL of the given solvent system. The flasks were then incubated at 25˚C at 150 rpm for 2 hours after which the supernatant was filtered and the residue re-extracted once more. The combined supernatants were concentrated using a rotary evaporator (Buchi RE Rotoevaporator including a Buchi 461 water bath) at 35/40˚C at 60 rpm until approximately 95% of the solvent was evaporated.

2.4. Stability studies

2.4.1. Effect of temperature

Heat stability of crude extracts and synthetic colourants were determined according to the method by Amr and Al-Tamimi (2007) with minor modifications. Briefly, an aliquot of 0.1 ml of extract was made up to 25 mL with 0.1 M (pH 3.5) citrate phosphate buffer and samples heated at 50˚C and 80˚C respectively for 6 hours.

The absorbance was read hourly at 520 nm using a UV/Visible spectrophotometer (Varion Cary IE). Percentage retention of anthocyanins was calculated using the following equation (Selim et al., 2008);

$$Pigment\ retention\ \ (\%) = \frac{\text{Absorbance after heating}}{\text{Absorbance before heating}} X\ 100 \quad \text{.....Equ 1}$$

2.4.2. Effect of pH

The method by Selim et al. (2008) was used with adjustments to determine the influence of pH on crude pigment and synthetic colourant stability. Buffers ranging from pH 1 to pH 9 were prepared according to DeLloyd (2000). A 2 mL aliquot of crude extract was made up to 20 mL with desired buffer. Test tubes containing the samples were covered with aluminium foil to prevent exposure to light and stored at 25˚C for 2 weeks. The absorbance was read at 520 nm after 0, 2, 7 and 14 days, pigment retention was calculated using Equation 1.

2.4.3. Light stability

Light stability of the 4 extracts and synthetic colourants were analysed according to method set out by Amr and Al-Tamimi (2007). Two sample sets of extracts were prepared in equal concentrations in citrate phosphate buffer (pH 3.5) in screw top test tubes. One set of samples were incubated under fluorescent light at room temperature while the second set of samples were incubated under dark conditions at room temperature. Absorbance of both sets of samples were read at 520 nm after 0, 6 hours, and thereafter every 48 hours for 10 days. Pigment retention was then calculated using Equation 1.

2.4.4. Degradation kinetics

2.4.4.1. Preparation of solution

A solution consisting of 0.05 mg/mL of crude extract was added to 0.1 M citric acid - sodium citric buffer (pH 3.0) was prepared.

2.4.4.2. Thermal stability

Thermal stability of crude extracts were determined using the method by Li et al. (2014) with modifications. Briefly, 0.05 mg/mL of extract was made up to 7 mL with 0.1 M citric acid-sodium citrate (pH 3.0). The test tubes were covered with aluminium foil to prevent light exposure. Test tubes were heated from 75 to 85˚C at 5˚C increments for an hour each. Thereafter test tubes were cooled to ± 25˚C for 15 minutes and absorbance was read at 527 nm.

The reaction rate constant (k) and half-life ($t_{1/2}$) was calculated using the following equations;

$$k = \frac{-\ln(\frac{Ct}{Co})}{t} \quad \text{.....Equ 2}$$

Where C_t is the anthocyanin content after heating and C_o is the anthocyanin content before heating and t is time (h).

$$t_{(1/2)} = -\ln 0.5 \times k^{-1} \quad \text{.....Equ 3}$$

2.4.4.3. DPPH radical scavenging assay

The radical scavenging ability of the thermally treated crude extracts were analysed using the method by Li et al. (2014). A 2 mL aliquot of 1 mM DPPH (prepared in methanol) was added to thermal treated samples. Test tubes were shaken vigorously and stored in the dark for 30 minutes at room temperature (25˚C) after which the absorbance was read at 517 nm. The control was carried out by replacing the extract with ethanol. Radical scavenging ability was calculated using the following equation:

$$DPPH\ radical\ scavenging\ ability\ (\%) = \frac{1 - \text{Sample absorbance}}{1 - \text{Control absorbance}} X\ 100$$ ……Equ 4

2.5. Total phenolic content

Folin-Ciocalteu's method, determined the total phenolic content of the crude extracts. Briefly, 0.05 mL of extract and 0.45 mL distilled water were mixed with 2.5 mL of 1:10 2 N Folin-Ciocalteu's phenolic reagent (Sigma Aldrich). Thereafter 2 mL of 7.5% (w/v) sodium carbonate was added. Samples were heated at 50˚C for 5 minutes after which absorbance was read at 760 nm with a UV/vis spectrophotometer (Varion Cary IE). Total phenolic content was estimated according to a Gallic acid standard curve (20 – 100 mg/mL), results were expressed as milligrams of Gallic acid equivalent (GAE) per 100 g of dry weight (Kim et al., 2009).

2.6. Radical scavenging ability

Radical scavenging ability was carried out on the extract that displayed the best overall stability.

2.6.1. Ferric Reducing Ability of Plasma (FRAP)

The FRAP reagent was freshly prepared by adding 300 mM acetic acid buffer, 2,4,6-tripyridyl-s-triazine (TPZT) solution and $FeCl_3$ at a ratio of 10:1:1. An aliquot of 100 μL was added to 3 mL FRAP reagent, the sample was then mixed adequately with a vortex. The samples were placed in a water bath at 37˚C for 4 minutes. Absorbance was read at 593nm after 0 and 4 minutes and the procedure repeated for the standard (ascorbic acid) analysed at 100 μM to 1000 μM (Benzie and Strain, 1999).

$$FRAP\ value\ of\ sample\ = \frac{\text{Change in Abs of sample from 0 to 4 minutes}}{\text{Change in Abs of standard from 0 to 4 minutes}} X\ FRAP\ value\ of\ standard$$ …Equ 5

2.6.2. DPPH

Radical scavenging ability was also carried out by the DPPH assay on the suitable *Hibiscus sabdariffa* extract. A 2.9 mL aliquot of 0.1 mM DPPH solution prepared in methanol was added to 100 μL of sample and vortexed. Samples were thereafter incubated in a dark cupboard at room temperature for 30 minutes. Absorbance was read at 517 nm after 0 and 30 minutes and the procedure repeated for the standard (rutin) analysed at 1000 μM (Kim et al., 2009).

2.7. Identification and Quantification

2.7.1. HPLC-DAD

Four major anthocyanins were identified and quantified from the crude extracts according to the method by Ruiz et al. (2013) with modifications. A Shimadzu HPLC system equipped with a diode array detector, column heater and automatic injection. Chromographic separation was carried out on a C18 column (LiChroCART 250 x 4 mm,

5um) at 40˚C. Two solvents were prepared i.e. solvent A water/acetonitrile/formic acid (87:3:10% v/v/v) and solvent B water/acetonitrile/formic acid (40:50:10% v/v/v). The flow rate was 0.4 mL/min and injection volume was 10 µL. The gradient program was set from 6% of solvent B and increase exponentially over the 22 minute runtime. PDA detection wavelength was recorded at 520 nm. Identification and quantification were carried out by comparing retention values of the standards. Standards, delphinidin chloride, malvidin chloride, cyanidin chloride and pelargonidin chloride, were reconstituted with mobile phase A and concentrations of 20, 40, 60, 80 and 100 µg/mL were used.

2.8. Application

A clear jelly was prepare by adding together 67.13 g of sugar, 20 g castor sugar, 9 g gelatine, 1.75 g fumaric acid, 0.7 g trisodium citrate, 0.53 g sodium chloride and 0.07 g ascorbic acid. To the dry ingredients, 250 mL of boiling water was added until all of the powders are dissolved; thereafter 250 mL of cold water was added. The colourants were added at the following concentrations, 27, 9 and 5 µg/mL. The jelly was stored at 4˚C for approximately 24 hours.

2.9. Data analysis

Statistical analysis was performed by means of one-way analysis of variance, ANOVA (GraphPad Prism), followed by Turkey's test for multiple comparisons. Data values are expressed as a mean ± standard deviation (n = 3).

3. RESULTS

3.1. Pigment extraction

The extraction yield of each solvent system was calculated after approximately 90% of the given solvent had been removed by rotary evaporation. Extraction yield ranged between approximately 9 and 20%. Ethanol acidified HCl proved to extract the highest yield of crude anthocyanins of 19.92% (table 3.1). HCl extracts were significantly different to acetic acid, formic acid and citric acid extracts with mean differences ranging between 2.16 and 12.39%.

Table 3.1: Yields of *H. sabdariffa* anthocyanins with different extraction solvent systems

Solvent system	Yield (%)
HCl (37%)+ Ethanol	**19.92 ± 0.31**
Formic acid (85%) + Methanol	**17.76 ±0.53**
Citric acid (1M) + Methanol	**13.02 ±0.12**
Acetic acid + water + Methanol	**8.74 ±1.25**

Values represent mean ± standard deviation of replicate readings (n = 3).

3.2. Stability studies

3.2.1. Heat stability

Pigment retention for each heat treatment temperature was calculated hourly for a period of 6 hours. The extracts from *Hibiscus sabdariffa* showed an increase followed by a decrease in pigment retention at one point of another during both heat treatment temperatures (Figure 3.1). The 50˚C heat treatment temperature displayed an increase of pigment retention in HCl samples displayed at 4 hours of incubation while acetic acid and citric acid sample showed an increased at 3 hours.

Pigment retention of formic acid samples decrease constantly over the treatment period. An increase of pigment retention was seen after just 2 hours in HCl and acetic acid samples when treated at 80˚C while the formic acid sample showed an increase on the sixth hour of incubation. The citric acid sample displayed an initial rapid decrease and thereafter a slower decrease in pigment retention over the treatment period.

When heated at 50˚C for a period of 6 hours, formic acid samples showed the greatest pigment loss of 87%. While citric acid and acetic acid containing solvent systems showed the greatest pigment retention of 52% and 58% respectively. HCl samples were only able to retain 35% of it pigment even though this sample showed the highest amount of pigment at the 1 hour interval. Acetic acid containing solvent system showed 61% pigment retention after 6 hours when treated at 80˚C. Citric acid samples lost 92% of its pigment. Acetic acid samples displayed the best pigment retention over the both heat treatment temperatures. Synthetic colourants, allura red and ponceau appeared to retain pigments as no significant difference could be observed over the incubation period for both 80˚C and 50˚Cheat treatment temperatures.

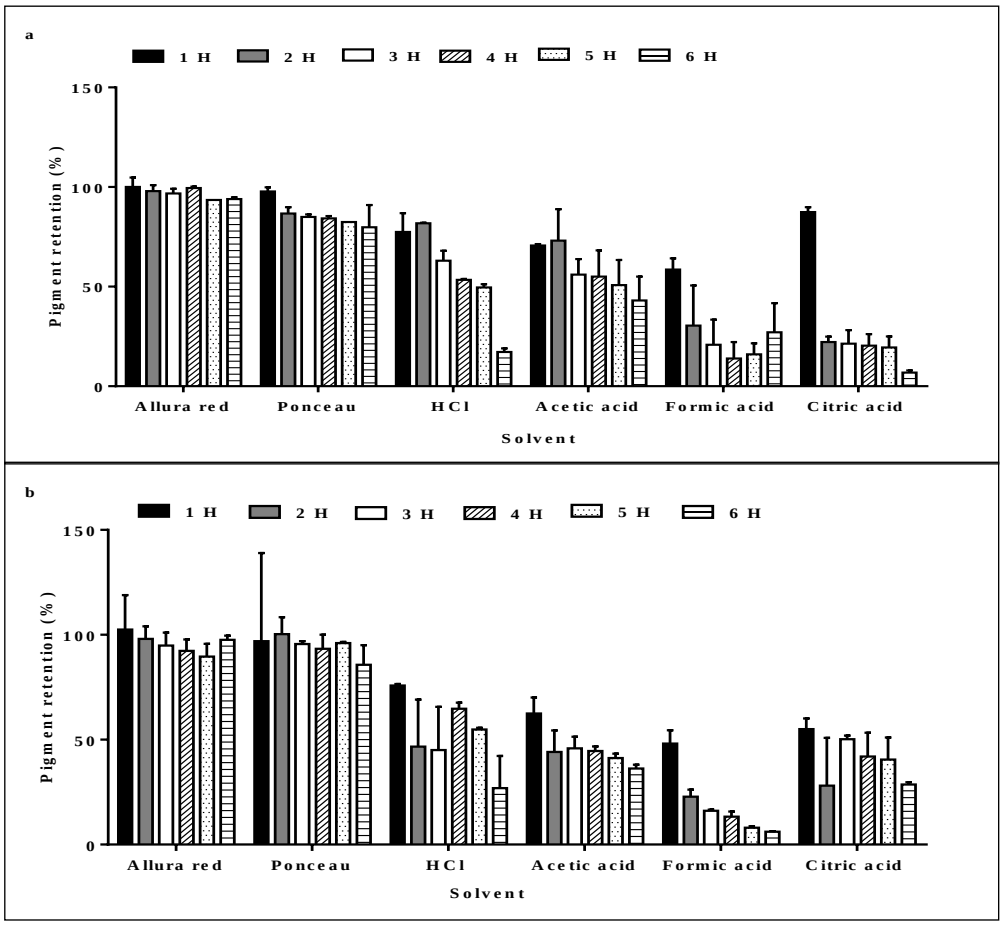

Figure 3.1: Effect of heat on stability of *H. sabdariffa* anthocyanins extracted by different solvent systems [a – 80˚Cand b – 50˚C]. Values represent mean ± standard deviation of replicate readings (n = 3).

3.2.2. pH stability

Natural and synthetic colourants were analysed for their stability against a pH range (pH 1 – 8). An overall view of Figure 3.2 shows that the synthetic colourants, allura red and ponceau had the greatest and most stable pigment retention over the 14 day incubation period. The pH did not affect the stability of the colourant. The natural extracts however did not show a similar trend. It was observed that better pigment retention was obtained at acidic pH (pH 1 – 3). The decline of pigment retention was not rapid therefore, results are only depicted for 48 hour, 7 and 14 days.

HCl extracts were the most stable over time at pH 1, 6 and 7 as there was 33, 30 and 24% difference in pigment retention respectively between 48 hour and 14 days. The maximum difference of pigment retention in acetic acid extracts was 58%. Samples that showed the least difference in degradation were noted at pH 1, 4 and 8 as the difference calculated between 48 hours and 14 days were 21, 29 and 26% respectively.

Formic acid extracts showed better difference in stability between 48 hour and 14 days at pH 4, 6 and 8 as differences observed were 14, 24 and 17% respectively. However, the pigment retention of the extract at pH 1 showed a 78% difference in pigment retention. It could also be noted that there was 36% of the pigments retained after the first 48 hours of incubation.

Citric acid pigment retention was relatively high when analysed at 48 hours however, pigments retained after 14 days were low. It was noted that pH 3 showed the best difference in pigment retention between 48 hours and 14 days at 23% however pigments that remained in the other samples were between 32 and 67%.

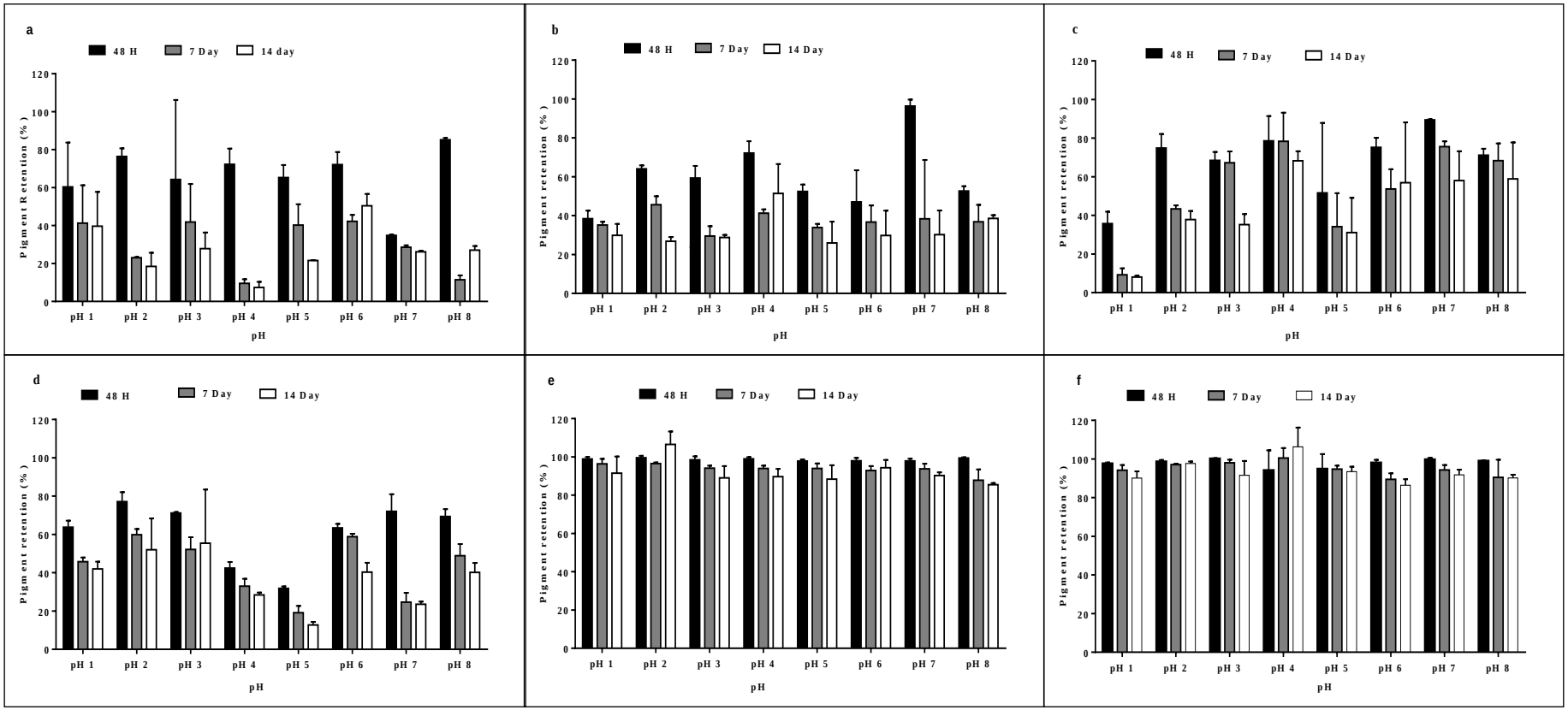

Figure 3.2: Effect of pH on stability of *H. sabdariffa* anthocyanins extracted by different solvent system [a – HCl and ethanol; b – Acetic acid and Methanol; c – Formic acid and methanol; d – Citric acid and methanol; e – Allura red and f – Ponceau]. Values represent mean ± standard deviation of replicate readings (n = 3).

3.2.3. Light stability

Extracts were treated under dark and light conditions for 10 days, pigment retention was analysed every 48 hours for the incubation period (figure 3.3). A brief overview of the results obtained over the 10 day period showed that synthetic pigments had high pigment retention ranging from 110 – 98% under light conditions and 107 – 88% under dark conditions. Natural pigments had a lower pigment retention range as compared to the synthetic pigments. An overall range for light treated extracts were between 99 and 39% and the dark conditions ranged between 96 and 42%. The synthetic pigments, allura red and ponceau do not show a great decrease in pigment retention over time. The colour remained stable; there was no sign of fading over the incubation period. Among the natural pigments, the HCl (97 – 68%) and acetic acid (89 – 71%) extracts had the best dark storage stability as pigment retention declined only slightly over time. While HCl (98 – 66%) and formic acid (99 – 69%) extracts had the best pigment retention amongst the extracts stored under light conditions. Citric acid extracts showed 58% and 40% differences in pigment retention under light and dark storage respectively. Colour of the extracts faded over time. Acetic acid, formic acid and citric acid extracts lost the vibrancy of their colour by the 10^{th} day.

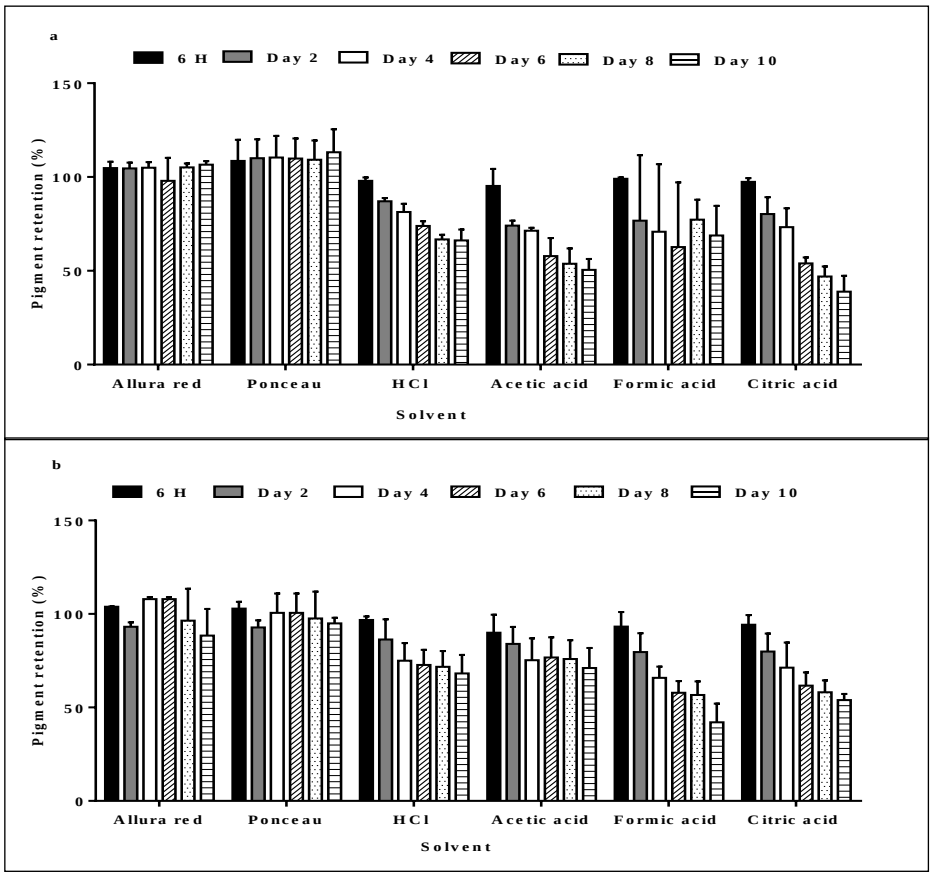

Figure 3.3: Effect of light on stability of *H. sabdariffa* anthocyanins extracted by different solvent system [a – Light conditions; b – Dark conditions]. Each column represents mean ± SD (n=3).

3.2.4. Degradation Kinetics

3.2.4.1. Thermal stability

The four extracts were tested for their thermal stability after which the rate constant in order to calculate the half-life of the extracts at each temperature treatment as depicted in table 3.2. Overall, the half-life ranged between 10 to 26 hours between the solvent systems. HCl containing solvent system showed a slow decrease in half-life, a 22% decrease was observed between 70 and 85˚C. Formic acid samples decreased by 35% between 70 and 80 ˚C however an 11% increase in half-life was observed at 85˚C.

Citric acid samples followed a similar trend as formic acid samples. Between 70 and 80˚C, half-life decreased by 44% which was followed by an increase of 60% at 85˚C. A

gradual decrease in half-life was seen in acetic acid containing solvent systems followed by an increase of 23% between 80 and 85˚C. The colour of these extracts changed over the heating period from the vibrant red to a dull brown. Acetic acid and HCl extracts changed colour slightly however the red colour was retained. Formic acid and citric acid samples lost most of its red pigment and was replaced by a dull brown colour.

Table 3.2: Rate constant (k) and half-life (t1/2) for thermal degradation of *H. sabdariffa* anthocyanins extracted by different solvent systems

Solvent system	Temperature (˚C)	k	$t_{1/2}$ (h)
HCl + EtOH	70	0.00071 ± 0,00034	22.85 ± 3.76
	75	0.00045 ± 0,00003	18.32 ± 8.64
	80	0.00042 ± 0,00005	18.87 ± 8.90
	85	0.00044 ± 0,00005	18.44 ± 8.69
Formic acid + MeOH	70	0.00096 ± 0,00010	26.27 ± 0.34
	75	0.00062 ± 0,00070	17.58 ± 9.27
	80	0.00055 ± 0,00050	17.15 ± 6.50
	85	0.00072 ± 0,00056	19.28 ± 9.09
Citric acid + MeOH	70	0.00109 ± 0,00075	17.55 ± 0.15
	75	0.00109 ± 0,00075	11.91 ± 5.62
	80	0.00129 ± 0,00027	10.17 ± 0.79
	85	0.00121 ± 0,00081	15.55 ± 0.37
Acetic acid + water + MeOH	70	0.00097 ± 0,00060	18.74 ± 2.02
	75	0.00065 ± 0,00008	16.57 ± 0.47
	80	0.00071 ± 0,00044	13.34 ± 3.27
	85	0.00049 ± 0,00042	15.69 ± 0.10

Values represent mean ± standard deviation of replicate readings (n = 3).

3.2.4.2. DPPH radical scavenging ability

Thermally treated samples were analysed for their antioxidant potential by DPPH assay. Figure 3.4 shows that the solvents used in extraction did not have an effect on the radical scavenging activity of anthocyanins greatly. Radical scavenging activity ranged between 81 and 85%. A trend that could be noted was an increase in activity at 85 ˚C by 1 – 2% and thereafter a decrease at 90˚C.

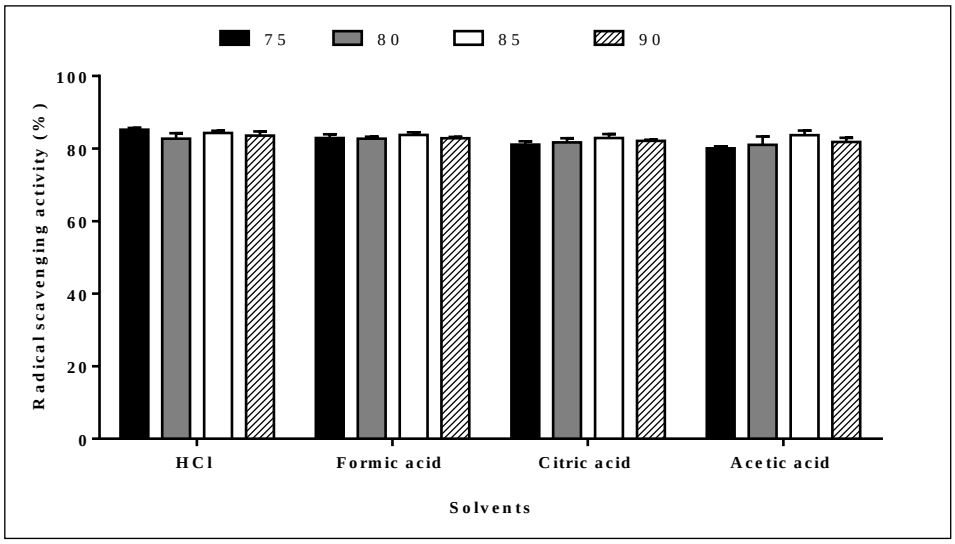

Figure 3.4: DPPH radical scavenging activity of *H. sabdariffa* anthocyanins subjected to thermal treatment at 70, 75, 80, 85 and 90˚Crespectively. Values represent mean ± standard deviation of replicate readings (n = 3).

3.3. Total Phenol Content

Total phenolic content of crude anthocyanins were determine be the Folin-Ciocalteu's method. Extraction solvents did not have an effect on the phenolic content (table 3.3). Total phenolic content ranged from 54.42 – 55.03 mg/100g GAE.

Table 3.3: Total phenolic content of each of *H. sabdariffa* crude extracts extracted by different solvent systems

Solvent	mg/g GAE
HCl	54.42 ± 0.23
Acetic acid	55.03 ± 0.04
Formic acid	54.51 ± 0.04
Citric acid	54.72 ± 0.12

Values represent mean ± standard deviation of replicate readings (n = 3).

3.4. Radical scavenging ability

3.4.1. Ferric Reducing Ability of Plasma

The FRAP assay was used to determine the radical scavenging ability of crude anthocyanins of *Hibiscus sabdariffa* extracted by HCl acidified ethanol. Ascorbic acid was employed as the positive control showed 98.3 mM radical scavenging ability while the *H. sabdariffa* extract showed 41.41 mM radical scavenging ability from 100 µL of sample.

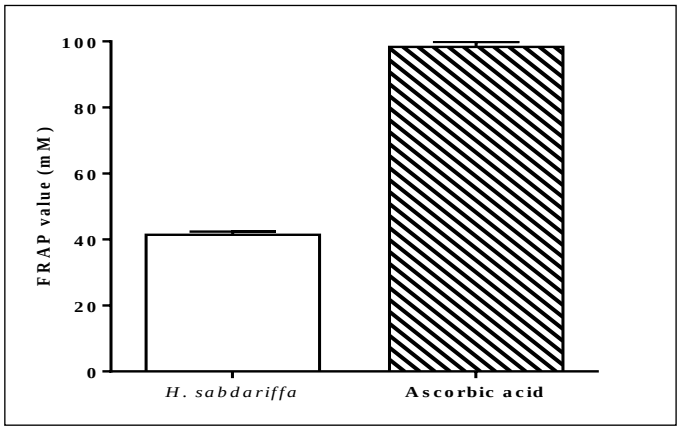

Figure 3.5: Radical scavenging ability of *H. sabdariffa* extracted by HCl solvent system determined by the FRAP assay. Values represent mean ± standard deviation of replicate readings (n = 3).

3.4.2. DPPH

The HCl extract was analysed for its radical scavenging ability, which was carried out by the DPPH assay. The positive control, rutin, showed 98.50% radical scavenging activity and *Hibiscus sabdariffa* extracted by HCl and ethanol showed 53.72% radical scavenging activity.

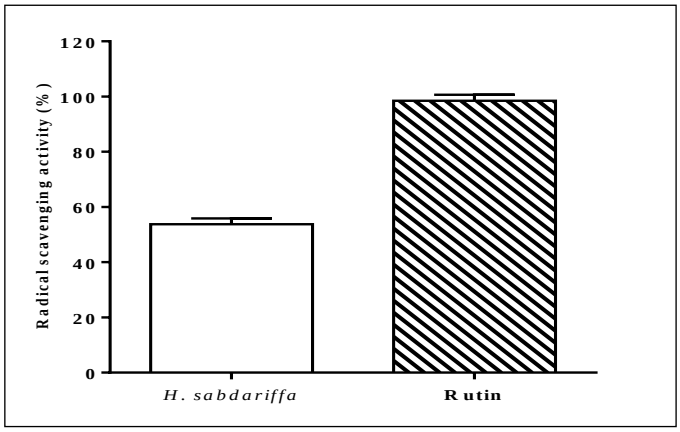

Figure 3.6: Radical scavenging ability of *H. sabdariffa* extracted by HCl solvent system determined by the DPPH assay. Values represent mean ± standard deviation of replicate readings (n = 3).

3.5. Identification and quantification

Crude anthocyanins from *H. sabdariffa* were identified and quantified by HPLC – DAD; data was viewed at 520 nm. Table 3.4 shows the amount of anthocyanidin found in each of the crude extracts. Retention times were used to determine these quantities (table 3.) Figure 3.7 depicts the chromatogram showing the presence of the four compounds in the crude anthocyanin extract. HCl extract detected all four standards, acetic extract detected delphinidin and cyanidin while formic acid and citric acid were not able to detect pelargonidin. Formic acid and citric acid extracts detected a high amount of delphinidin, 415 and 402 mg/100g respectively while HCl detected the lowest quantity.

The HCl extract contained the highest amount of cyanidin 77.06 mg/100 g while acetic acid contained the least amount of 20 mg/100 g. Peaks were also visible at 330 nm indicating that they are acylated.

Table 3.4: Identification and quantification of *H. sabdariffa* anthocyanins extracted by different solvent systems

Solvent	Delphinidin chloride (µg/mL)	Malvidin chloride (µg/mL)	Cyanidin chloride (µg/mL)	Pelargonidin chloride (µg/mL)
HCl	68.78 ± 0.63	12.18 ± 0.15	77.06 ± 2.50	12.22 ± 1.51
Acetic acid	116.32 ± 0.13	ND	19.57 ± 1.46	ND
Formic acid	415.42 ± 3.60	16.48 ± 1.47	76.99 ± 0.77	ND
Citric acid	402,.7 ± 2.49	15.45 ± 0.10	61.63 ± 0.45	ND

Values represent mean ± standard deviation of replicate readings (n = 3). ND – Anthocyanin was not detected

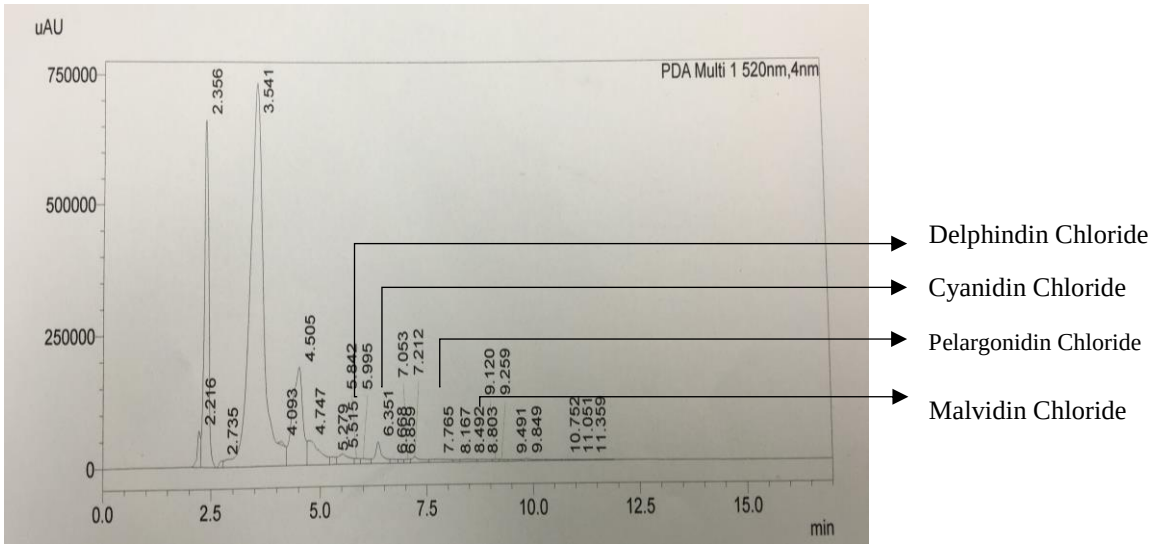

Figure 3.7: Chromatogram of crude extract from *H. sabdariffa*

Table 3.5: Retention times of the anthocyanidin standards

Standard	Retention time
Delphinidin chloride	5.408 ± 0.008
Malvidin chloride	8.350 ± 0.006
Cyanidin chloride	6.500 ± 0.008
Pelargonidin chloride	7.615 ± 0.006

Values represent mean ± standard deviation of replicate readings (n = 2)

3.6. Application

The HCl extract was applied to a clear jelly. The dispersed uniformly into the clear jelly. Varying concentrations shows that the colour intensity can be adjusted as per the requirement of the product. There were no visual changes to the product during storage, the jelly was monitored for 14 days after setting and there were no changes observed.

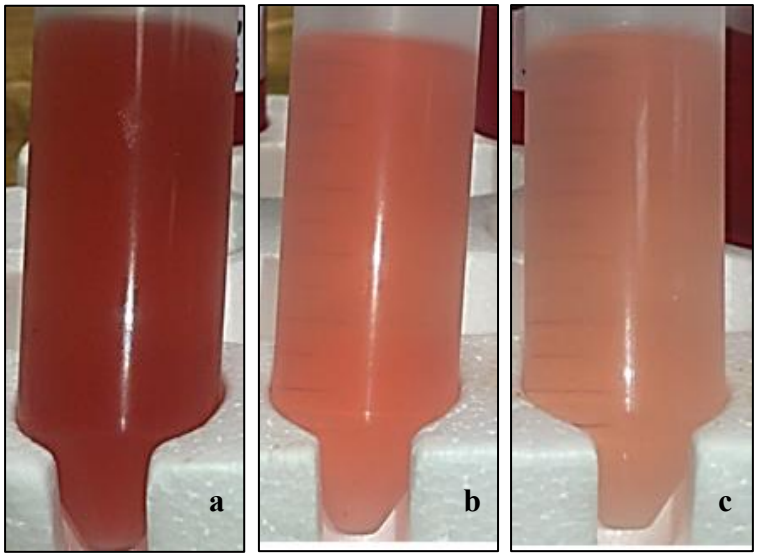

Figure 3.8: Natural food colourant from *H. sabdariffa* added to clear jelly at different concentrations [a – 27 µg/ml; b – 9 µg/ml and c – 5µg/ml].

4. DISCUSSION

Anthocyanins consist of aromatic rings that contain polar groups, hydroxyl, carboxyl and methoxyl, and glycoside residue which together result in a polar molecule (Delgado-Vargas et al., 2000). It is for this reason that the pigments can easily be extracted by polar solvents such as methanol, ethanol and water. The addition of acid to the polar solvent stabilises the flavylium cation which retains the red colour at low pH (Kerio et al., 2012). The acidified ethanol and methanol was used as opposed to water to increase the efficiency of anthocyanin extraction, as the use of a low pH solvent has been shown to stabilise the anthocyanins during the extraction process. (Selim et al., 2008, Rein, 2005). Selim et al. (2008) reported that HCl in ethanol was more efficient than citric acid while formic acid in methanol was the most efficient of the four solvents, namely HCl/ ethanol, formic acid/ methanol, citric acid/ methanol and methanol/ acetic acid/ water. In the food industry yield is the most important factor next to safety therefore although methanol is an efficient solvent for extraction, it is toxic and should not be applied in the food industry. Alcohol based solvents are desirable as they have lower boiling points than water and therefore will cause less damage to the pigments as lower temperatures may be used for concentration (Amr and Al-Tamimi, 2007). HCl acidified ethanol should thus be considered for extraction of anthocyanins, as the solvents are acceptable for the food industry as well as producing as high yield.

Anthocyanins are regarded as unstable pigments thereby making them difficult to be used in application. This study looked at the stability of crude anthocyanins under heat, pH and light conditions. Degradation kinetics was also performed to determine the thermal stability and DPPH radical scavenging ability. Common synthetic colourants that are used food products were also subjected to the parameters.

The four extracts and two synthetic colourants were heat treated at 80 and 50˚C for 6 hours respectively. There was not a vast difference observed in retention between the 80 and 50˚C treatments, this could imply that most of the pigments were degraded at 50˚C and no further degradation could occur (Amr and Al-Tamimi, 2007). Increased pigment retention at certain time intervals could indicate the release of degradation products thereby affecting the absorbance (Yue and Xu, 2008). Comparatively there was significant ($p < 0.05$) difference in retention between 1 and 6 hours by HCl extracts. Acetic acid and citric acid extracts showed a slight, however steady decrease of pigment retention over time at both 50 and 80˚C heat treatment. Pigment retention of formic acid extract showed to degrade the most at both temperature treatments as significant ($p < 0.05$) difference was observed over the time interval.

Acetic acid solvent system showed the most consistency of pigment retention at both temperatures. The rate of anthocyanin degradation when heated increases because of reacting molecules that come closer when the extract is concentrated (Kirca et al., 2007). Colour changes were also noted upon heating. In all of the samples, the colour decreased in intensity. A bleaching effect can be noted because the equilibrium is changing toward the uncoloured forms. The flavonoid structure is open to form a chalcone (an aromatic ketone and an enone that forms the central core of the variety of important biological compounds) which is further degraded to form brown products (Francis, 1989). According to Bordignon-Luiz et al. (2007) there can be a substantial anthocyanin colour degradation when the extract is heated. Heat stability could possibly be enhanced by increasing the anthocyanin concentration, removal of oxygen and he inactivation of enzymes. Methoxylation of the acyl moiety provides improvement to the structural integrity towards heat treatment (Patras et al., 2010, Hellstrom et al., 2013).

The anthocyanin extracts were treated to buffers ranging from pH 1 to pH 9. Anthocyanins are known to favour acidic conditions (Selim et al., 2008). Higher pigment retention values could be observed at pH 1 and 2 and this was also where the anthocyanin colour was most intense and vibrant among this pH range. The results that were obtained were in keeping with this statement as most of the results are at their highest pigment retention at lower pH as seen in figure 3.2. However, Ozela et al. (2007) indicated that pigments were more stable at pH 5 and 6 than 4. This could also explain the reason for pigment retention in HCl containing extracts at 48 h Pigment retention of HCl and citric acid acidified solvents had greater significant ($p < 0.05$) difference between 24 h and 7 day across the pH spectrum (pH 1 – 9) as compared to formic acid and acetic acid acidified solvents. However, the general increasing trend of degradation confirms that crude pigments from *Hibiscus sabdariffa* are more stable at lower pH values. Investigation of anthocyanins from grapes, purple carrots and purple and red sweet potato at pH 3 revealed that greater stability could be achieved at low pH and temperature. Another study conducted on purple sweet potato extracts at pH 2 – 7 reported that anthocyanins were more stable at pH 2 – 4 than at sub-acidic pH of 5 – 6 (Andres-Bello et al., 2013). The colour stability of anthocyanins can be dependent on pH and the anthocyanin structure (Cabrita et al., 2000, Kirca et al., 2007). Colour changes occurred in acetic acid and formic acid containing solvent systems after 7 days of incubation at room temperature. Brown precipitate was found in samples at pH 6 – 9 after 7 days of incubation. The colour changes of anthocyanins are more significant in the alkaline region because of their instability.

A reason for low pigment retention can be explained by the chemistry of anthocyanins, the red flavylium cation concentration in acid medium and its potential interaction with existing co-pigments affect the adsorption properties of anthocyanin solutions thereby

creating a much more stable extract as well as protecting the colour attribute of the solution (Oancea and Drăghici, 2013).

Light stability of anthocyanins is an important aspect as it aids in storage conditions. HCl samples appeared to be the most stable as the least amount of pigment degradation over time, pigment retention ranged from 97 – 66% in light treated samples while dark treated samples ranged from 96 – 68%. Significant ($p < 0.05$) decrease was observed over 6 h and 10 days in acetic acid and citric acid light treated samples however, acetic acid showed no significant ($p < 0.05$) decrease in dark treated samples. According to Amr and Al-Tamimi (2007) their samples retained 84% of the pigment which was treated to dark conditions for 10 days. Various observations were reported with regard to pigment retention, the main reasons for variation include the type of plant source and therefore the structure if the individual anthocyanins that make up the extract. Frimpong et al. (2014) reported that protecting anthocyanins from direct light would assist in the maintenance of the desired red colour. The authors also stated that amber bottle had better anthocyanin stability to light when compared with translucent bottles.

Synthetic colourants, allura red and ponceau have shown better stability when subjected to the stability parameters, heat, pH and light, as compared to the natural *Hibiscus sabdariffa* colourant. There are many disadvantages however associated with the use of these synthetic colourants. One of the main disadvantages is the health concerns that are attached to synthetic colourants as a whole and the consumers' perceptions thereafter. Natural food colourants are thereby increasing in their demand due to their pharmaceutical and nutraceutical properties that are associated with them. However, there are positive attributes of synthetic colourants, which make them appealing for

industrial purposes such as their ability to impart a more intense colour, greater stability and are cost effective. Ultimately a products' success will depend on the consumer and their perceptions as in some cases a product comprising of natural components will outweigh one with synthetic additives (Delgado-Vargas et al., 2000, Wissgott and Bortlik, 1996).

Thermal stability was determined by half-life calculations. Half-life ($t_{1/2}$) can be expressed as the time that is required for 50% of the sample to degrade (Hou et al., 2013). The higher the heating temperature, the smaller the half-life values have been reported in previous studies thereby showing that temperature has a significant role in thermal degradation, which is depicted in Table 2.2 (Li et al., 2014, Kirca and Cemeroglu, 2003). The rate constant (k) shows that the thermal degradation of *Hibiscus sabdariffa* follows the first order kinetic model as a linear regression line is produced when k is plotted against temperature (Verbeyst et al., 2010, Kara and Erçelebi, 2013). A study conducted on Urmu mulberry that was treated at temperature ranging from 60 – 80˚C reported a decrease in half-life as the temperature increase (Kara and Erçelebi, 2013). The temperature also influenced the colour of the samples. Colour changes occurred from vibrant red to dull brown in citric acid and formic acid samples. Acetic acid and HCl samples became dull and murky however, the red colour was retained. Bordignon-Luiz et al. (2007) also reported colour changes of samples as temperature increased. The appearance of brown pigment is associated with thermal degradation as chalcones were formed and further degraded (Reyes and Cisneros-Zevallos, 2007, Francis, 1989). This could be a reason for the increased half-life values that are seen in formic acid, citric acid and acetic acid samples when heated at 85˚C. Kopjar et al. (2009) reported that the

degradation effect on natural pigments upon heating could be reduced by the addition of phenolic compounds such as catechol and catechin.

The protocol of scavenging DPPH is based on the reduction of DPPH ethanol solution in the presence of a hydrogen donating antioxidant, the reaction therefore resulting in the formation of the non-radical form DPPH-H (Li et al., 2014). DPPH radical scavenging activity was quantified in terms of percentage of a pre-formed free radical by antioxidants in each of the samples. Anthocyanins did not experience extreme degradation however, a very slight increase in radical scavenging ability at 85˚Cwas noted. Yue and Xu (2008), noted an increase in radical scavenging activity at 80 and 120˚C (Hou et al., 2013). This could be due to the release of degradation products during heating that had antioxidant capability. A study conducted on anthocyanins extracted from litchi observed that the antioxidant activity was heightened when the temperature was increased up to 45˚C thereby suggesting that antioxidant activity could be maintained with controlled temperature, this could be due to an effected created by the combination of non-enzymatic reactions and anthocyanin stability (Ruenroengklin et al., 2008).

The total phenolic content was determined by Folin-Ciocalteu's method. The mechanism employs the transfer of electrons from phenolic compounds to the Folin-Ciocalteu's reagent in alkaline medium (Fu et al., 2011). The total phenol content of the extracts ranged from 54.42 – 55.03 mg/g GAE. Although each of the extraction solvents were different, the plant extracted was the same, therefore there was no significant difference observed between the four solvents.

No significant ($p < 0.05$) difference was observed by formic acid acidified solvent in relation to HCl acidified solvent. The results that were obtained were in keeping with literature as fruit such as apple, litchi and navel oranges were reported to have similar total phenolic content values (Fu et al., 2011). Sindi et al. (2014), reported to have lower phenolic content from *Hibiscus sabdariffa* anthocyanins extracted from methanol while Sirag et al. (2014), found 41 mg/g GAE total phenolic content. Abou-Arab et al. (2011), assessed the total phenolic content of *H. sabdariffa* extracted by HCl acidified ethanol, the authors found 42.00 mg/g GAE.

The ferric reducing ability of plasma (FRAP) assay utilises the single electron transfer mechanism and is based on the ability of antioxidants to rapidly reduce from ferric (III) ions to ferrous (II) which is blue in colour by the present antioxidants in the sample (Fu et al., 2011, Payne et al., 2013). A study carried out by Azima et al. (2014), looked at the radical scavenging ability of *Garcinia mangostana* (purple mangosteen) peels, *Syzigium cumini* (java plum) and *Clitoria ternatea* (Asian pigeonwings) The authors found that these plants contained 79.37 mM/g, 25.66 mM/g and 13.32 mM/g radical scavenging ability respectively. These figures are higher and lower than what was found in this study.

The DPPH assay also makes use of the single electron transfer mechanism. DPPH is considered a stable radical because of the delocalisation of the additional electron as that the molecule does not dimerise which is done by most other free radicals. The deep violet colour that is associated with the reaction is due to this delocalisation. When DPPH solution is added with a substance that is able to donate a hydrogen atom, the reduced form is developed and the violet colour becomes yellow (Sirag et al., 2014, Kedare and

Singh, 2011). Slightly higher results were found from carrot juice, 57.8% inhibition and *Hibiscus sabdariffa*, 60.38%, extracted by ethanol (Wootton-Beard et al., 2011, Jung et al., 2013).

The anthocyanins were identified and quantified by HPLC-DAD. Four anthocyanin standards, delphinidin chloride, malvidin chloride, cyanidin chloride and pelargonidin chloride were evaluated. Identification was determined by comparing retention times of the standards while quantification was calculated from the calibration curves. Yue and Xu (2008) reported that delphinidin and cyanidin contribute to approximately 80% of the total anthocyanins present in a plant. The results obtained from *H. sabdariffa* extracts indicates that these compounds occupy between 85 and 100% of the total anthocyanins. Delphinidin and cyanidin are known to contribute largely to the bioactive compound of anthocyanins and have higher bioavailability as compared to the other anthocyanins (Yue and Xu, 2008). Absorption peaks appeared at 330 nm of the UV-vis characteristics of the major anthocyanins, which indicated that the anthocyanins are acylated. Acylated anthocyanins suggest that the anthocyanins are mono- or di- acylated forms. A high amount of acylated anthocyanins show high stability thereby confirming the industrial use as a natural food colourant (Fan et al., 2008).

H. sabdariffa has widely been used in application at household level however; its industrial use is limited. The calyces have been used to produce and/or colour product such as sauces, jam, jelly, marmalade and soft drinks (Salazar-Gonzalez et al., 2012, Mungole and Chaturvedi, 2011, Mohamed et al., 2012). Jelly made an ideal candidate for the application of the natural food colourant due to its acidity being between pH 3 and 4.

5. CONCLUSIONS

The highest crude anthocyanin yield was seen by HCl acidified ethanol. Ethanol is favoured by the food industry rather than methanol as methanol is regarded as toxic if consumed. Alcohol is better for extraction as it has a lower boiling point thereby lowering the risk of damage due to heating. The HCl extract seemed to be a common factor in the stability studies.

Upon heating at 80 and 50˚C, acetic acid extract showed the most consistency over time. Overall, there was not a large difference of pigment retention at both heat treatments thereby suggesting that most of the degradation occurred at the 50˚C heat treatment.

It has been established that anthocyanins have better stability at acidic pH. Pigment retention was favoured by the HCl and citric acid extracts. When subjected to light storage, HCl extract showed the least amount of pigment degradation over time.

The synthetic colourants, allura red and ponceau fared far better than the natural colourants as they obtained high pigment retention over all of the stability parameters that they were compared with. The advantage that natural food colourants carry however is the ability to fill the consumers' requirement for a natural food colourant.

Degradation kinetics of thermal stability showed that the half-life of anthocyanins are relatively low. The radical scavenging ability of the thermally treated samples showed that high temperatures release free radicals upon degradation resulting in an increase in antioxidant ability.

The FRAP and DPPH assays proved that crude anthocyanins from *Hibiscus sabdariffa* have an average radical scavenging ability. All four of the standards, delphinidin chloride, malvidin chloride, cyanidin chloride and pelargonidin chloride, were detected in the HCl extract. The highest amount of anthocyanins were delphinidin and cyanidin, these two compounds proved to occupy between 85 and 100% of the total anthocyanins quantified in each of the extracts.

The *H. sabdariffa* food colourant extracted by HCl acidified ethanol was stable in a clear jelly. This application reiterates that this food colourant is stable at acidic pH, able to withstand refrigeration and room temperature and can withstand the presence of light.

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