EXTRACTION, CHARACTERISATION AND APPLICATION OF BETALAINS FROM CACTUS PEAR, BEETROOT AND AMARANTH

By

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Declaration

I, Vuyisa Ntombiyolwazi Sigwela, declare that this submitted dissertation in the fulfilment of MSc Consumer Science at the University of Free State, Bloemfontein, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution. I further cede copyright of this thesis in favour of the University of the Free State.

Signature

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This thesis is dedicated to all the women who have been circumstantially denied access to good, quality, education…

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Amaranth

Antioxidant capacity

Antioxidants

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Beetroot

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Betaxanthins

Cactus Pear

Colourants

Colouring Foods

Natural colourants

Opuntia ficus-indica

Opuntia robusta

Pigments

Product development

Abbreviations

Abbreviation	Description
DPPH	2,2-diphenyl-1-picrylhydrazyl
ARC	Agricultural Research Council
ANOVA	Analysis of Variance
AA	Ascorbic acid
Bc	Betacyanins
Bx	Betaxanthins
°Brix	Degrees Brix
dH_20	Distilled water
EtOH	Ethanol
EMEA	Europe Middle East and Africa
EFFL	European Food and Feed Law
E	European number
EU	European Union
FDA	Food and Drug Administration
FPs	Functional Products
GRAS	Generally Regarded as Safe
G	gram
HTST	High temperature short time
MeOH	Methanol
Mg	milligram
mg CE/g	Milligram catechin equivalent/gram
mg GAE/g	milligram gallic acid equivalent / gram
mg/100 g DW	milligram/ dry weight
µl	Microlitres
O.	*Opuntia*
%	Percentage
KCl	Potassium chloride
pH	potential Hydrogen
$C_2H_3Na_2$	Sodium acetate
Rf	ratio of distance
Spp.	species
TSS	Total soluble solids
UV	Ultraviolet
USA	United States of America
H_20	Water
a_w	Water activity
WHO	World Health Organisation

CHAPTER 1

1.1 Introduction

The colouring of foodstuffs is an ancient practice that can be traced back to as early as 400 BC when Egyptians were colouring candy and wine. In the late 1800s, many different synthetic colourants were available on the market (Downham & Collins, 2000). Sometimes these colourants were used for decorative purposes. Unfortunately, colourants were also used as a disguise to cover low-quality foods (Downham & Collins, 2000; Roy et al., 2004).

Food colourant history further ascertains that the food industry has come a long way: from people consuming toxic colourants in the late 1800s; to first regulating them in 1906, and implementing stricter food laws in 1938 (Downham & Collins 2000). Also, in the 1900s, there were more than 700 colourants used in the industry, most of which were hazardous to human health (Burrows, 2009). Nowadays, if there is a one in nineteen billion chance of a food colourant having carcinogenic effects, it is removed from production (Downham & Collins, 2000; Burrows, 2009).

Both natural and synthetic food additives are used in the food industry. Some colourants, mainly natural ones, enhance the taste of foodstuffs and are a pre-requisite for safety (Martins et al., 2016). Subsequently, there is a growing interest in the use of natural colourants as opposed to synthetic ones (Nunes, 2014).

The addition of food colouring increases the aesthetic value of foods, improves their quality and taste, increases nutritional value and warrants easy product identification. Beyond that, current consumers are also impressed with the medicinal benefits and low to zero toxicity that comes with natural colourant consumption (Chaitanya, 2014).

There are three main natural pigments used in the colouring of food, namely carotenoids, flavonoids and betalains. Betacyanins (from betalains) and anthocyanins (from flavonoids) are the primary red colourants, and both colourants are soluble in water (Howitt & Pogson, 2006). During the development of new natural colourants, anthocyanins have been biotechnologically tested and applied to foods for their tinctorial abilities. Carotenoids, which are isoprenoid derivatives, are also vastly used in the food industry for food enrichment and colouring (Shimada et al., 2005;

Brockington et al., 2011). The focus of this study is betalains, which are only found in one group of angiosperms, the Caryophyllales order. These pigments are red and yellow and derived from tyrosine (Polturak et al., 2016).

The red to yellow-pigmented plant derivatives are found in various sources, including (1) red and yellow beetroot (*Beta vulgaris* L. ssp. vulgaris), (2) red pitahaya (*Hylocereus* sp.), which is highly comparable to and can even replace beetroot as a colourant, (3) amaranth (*Amaranthus*) and (4) cactus pear (*Opuntia* species) as well as (5) red-purple, purple, yellow-orange and white stem coloured Swiss chard, all of which are shown in Figure 1.1 (Kugler et al., 2004).

Cactus pear is a very old plant, with species that are spineless or with spines (very prickly). Some of these species are used as ornamental plants in private properties, nurseries and landscapes; hence, they can be grown for commercial use (Salem-Fnayou et al., 2014). The oval-berry cactus pear fruit is a multifunctional nutraceutical that is available in different colours (Piga, 2004). The cactus pear fruit can be consumed fresh or in its processed forms, such as in jams, juices and chutneys (Du Toit 2013; Sáenz et al., 2013). Cactus pear leaves are called cladodes that form part of the green stem and can also be eaten in their fresh state when they are young (Sáenz, 2000; Jaramillo-Flores et al., 2003).

Cactus pear fruits and betalains have been chosen as the focus of this study, for the following reasons: (1) carotenoids are soluble in oil, which makes their water-solubility very low, a significant disadvantage to technological processing. Yellow betaxanthins (water-soluble) can then be used instead of carotenoids. (2) Anthocyanins have a weaker colour strength than betacyanins. (3) Betalains do not have any toxic effects in humans (Castellar et al., 2006; Azeredo 2009).

Another aspect included in this study is Colouring Foods. These are foods that have an inherent ability to impart colour to other foodstuffs and, depending on their extraction method, can be classified under natural colours or Colouring Foods. An example is beetroot; beetroot is already an approved natural colourant, yet beetroot juice (depending on its extraction method) can be classified under natural colourants or Colouring Foods (Reinhart, 2014; Lehto et al., 2017).

Chapter two gives a broader detail of anthocyanins, carotenoids, beetroot, amaranth, cactus pear, as well as Colouring Foods and their classification.

Figure 1. 1: Betalain-containing plants. A-beetroot; B-amaranth; C-cactus pear; D-*Hylocereus* sp; D Red Pitahaya; E-coloured Swiss chard (Kugler et al., 2004; Gengatharan et al., 2015)

1.2 Aims and objectives of the study

The development of natural food colourants which possess colouring properties comparable to approved industry colourants and still possess healthful benefits is a breakthrough for both the food industry and consumer market. To meet that demand, the objectives of the study were to:

1. report on the history of synthetic food colourants, their harmful side-effects, as well as legal advancements that have taken place over the years. This would be achieved through highlighting the shortcomings of the food industry, ancient colourants and giving a brief review of natural and synthetic food colourants to show a possible positive future in food colouring;
2. have an in-depth discussion on different natural, nature-identical, Colouring Foods, inorganic and synthetic food colourants, as well as their sources and application to food products;
3. find methods that ensure inexpensive, time-saving, non-toxic and safe betalain extraction. This will be conducted with beetroot and three cactus pear cultivars, which derive from three different coloured cactus pears. The main aim of the chapter will be to test the effectiveness of the project, the ways in which it can be carried out, and chapters that follow will include elaborate analysis. Importantly, this phase took place in 2016, while the rest of the tests were done in 2017 and 2018;

4. compare different extraction methods between one beetroot cultivar and eight different cactus pear cultivars, which are found in four different coloured fruits. The extraction method which works best, i.e., highest betalain content, will be chosen and chapters that follow will be based on it;
5. investigate the properties of the three different plants: beetroot, amaranth and cactus pear. Betalains possess antioxidant properties and finding their capacity in the different plants will be beneficial, as it would be a breakthrough to finding colourants that possess healthful benefits to the end-user. The total soluble solids (TSS) and thin-layer chromatography (TLC) will also be tested for assessment of their sweetness and determination of betalain presence. Colourants will be classified according to the standards of Colouring Foods;
6. add the extracted betalain pigments to different food products; this would aid in food colouring and product development. The stability of coloured foodstuffs will also be tested over a period of ten days through colour parameter analysis; and
7. give a summative report on the findings of the project, through highlighting the best extraction methods, the properties that have been found in the plants, as well as successes and shortcomings from applying the colourants to different food products. Added to this are recommendations for future studies.

The experimental outlay of the study is summarized in the diagrammatic illustration in Figure 1.2. The diagram represents the three main divisions of the project: (1) extraction; (2) properties of extracts; as well as (3) application of betalains from cactus pear, beetroot and amaranth. Notably, three plants are used in the study, namely, cactus pear, beetroot and amaranth, of which the last two are already approved natural colourants. As such, the already approved colourants are both used as standards. Beetroot is used in all experiments and amaranth in property analysis in Chapter 5 and application in polony in Chapter 6.

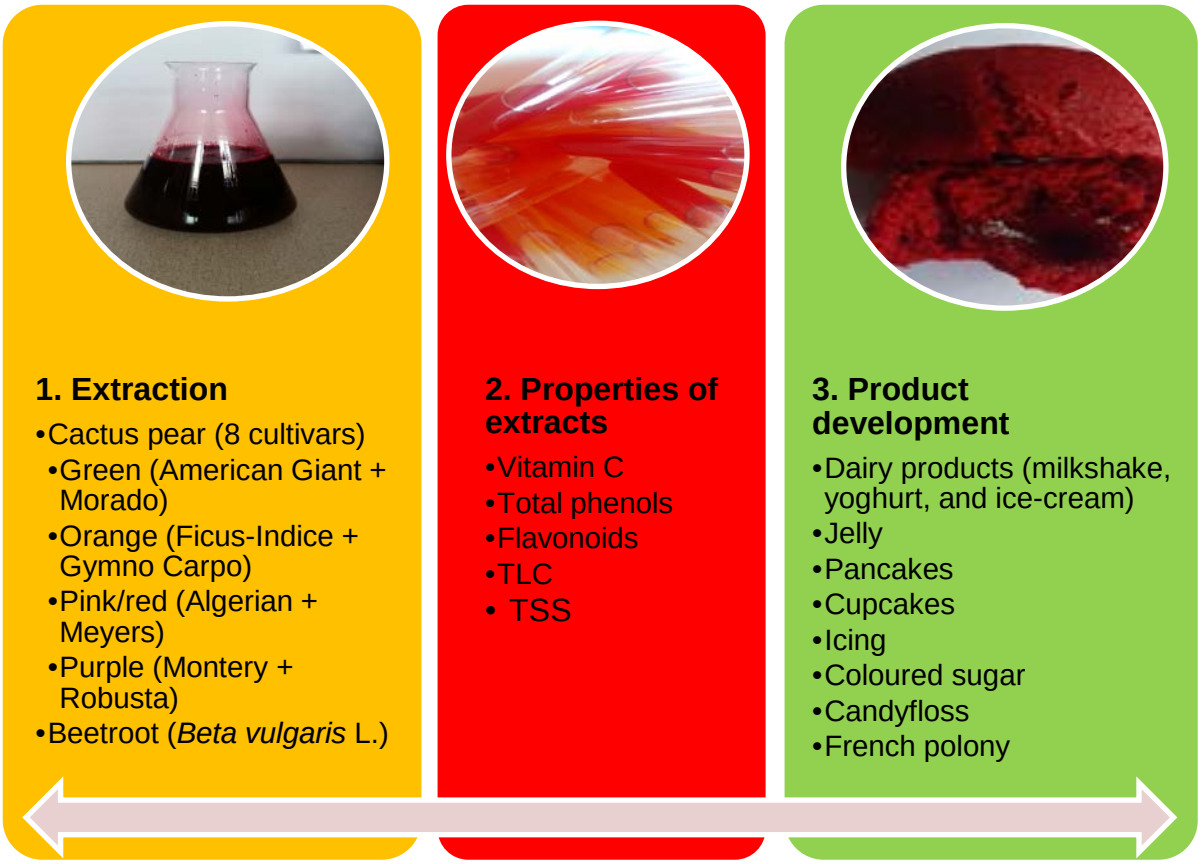

Figure 1. 2: Diagrammatic layout of the study aims of the experimental outlay of the study

CHAPTER 2

Literature review

Abstract

According to Europe, Middle East, and Africa (EMEA) Mintel Food and Drink Trends in 2018, consumers require explicit disclosure on food and drink labels. The requirements go hand in hand with the preference for nutritionally-dense foods that are pleasing to the eye and prepared under safe conditions (Gengatharan et al., 2015; Martins et al., 2016). The preference for explicit disclosure includes information on how, as well as where, products are grown and produced, and could result in interactive, reliable relationships between customers and the food industry.

The food industry is, therefore, presented with an opportunity to conduct research on various colourants that are available in the market, the countries that consume them, their healthful and detrimental effects, as well as which ones can be used in the food industry going forward. This chapter gives an understanding of consumer perceptions, some of the different colourants that exist, their characterisation, as well as application in foods. It further shows the need for the industry to shift to natural colourants by drawing particular attention to the benefits of natural plant pigments, primarily betalains.

2.1 Food colourant influence on consumers

Before consumers eat foodstuffs, they have preconceived perceptions of their organoleptic properties. In fact, the colour of utensils, packaging, crockery and the food itself, may exert more influence on the perceived taste of food than its actual taste (Burrows, 2009; Spence, 2018). Beverage-research has proven that the psyche of people informs them of the expected flavour of food even before they consume it, and if the taste and colour do not correspond, the food product is not accepted. A relevant example is that of clear Pepsi and other beverages, which were manufactured with a transparent colour that does not coincide with the flavour, and those beverages were not well accepted (Downham & Collins, 2000; Lehto et al., 2017).

Consumers desire food which is pleasing to the eye; factors, such as air, light and temperature (during food processing) may alter the colour of food products (Herbach et al., 2006). Food colourants bridge this gap as they create enticing food products (Sarıkaya et al., 2012). Thus, the main aim of adding colourants to foods is to provide food products with desirable colour or maintain an initially desirable colour, which could have been altered or lost during processing, transportation or storage (Msagti, 2013).

The process of choosing food products includes the application of the sense of sight, taste, smell and texture, and the ultimate choice of what buyers eat is greatly influenced by what they see (colour) (Cejudo-Bastante et al., 2014). The quality and acceptability of foodstuffs can be graded according to colour. In fact, the final colour of foodstuffs is a result of the concentration of colour pigments in the plant source that is used as a colourant or in the artificial colourant used (Schwartz et al., 2008; Rodriguez-Amaya, 2016).

Food scientists, law regulators, business enterprises, as well as consumers, determine the pace it will take to manufacture new and safer food additives, including food colourants (Carocho et al., 2014). Currently, over 2 500 food additives are used in the manufacture of high quality and attractive food, making it easy for consumers to be persuaded to purchase foodstuffs (Carocho et al., 2014). There are 39 (17 synthetic) approved food colourants in the European Union (EU), while in the United States of America (USA), there are 36 permitted colourants (only nine synthetic) (Lehto et al., 2017). These colourant guideline developments are remarkable as they reflect a 56.4% and 75.0% natural colourant use in the EU and USA, respectively.

2.2 Colourants in the food industry

According to the Food and Drug Administration (FDA, 2016), food colourants are "any dye, pigment, or substance, which when added or applied to a food, drug or cosmetic, or the human body, is capable (alone or through reactions with other substances) of imparting colour". FDA guidelines further state that colourants fall into two categories: ones that need certification (usually synthetic colourants, such as Yellow No. 6); and ones exempt from certification (usually natural, such as vegetable juice and annatto) (Simon et al., 2017).

Certified colours form part of two categories: dyes or lakes. Dyes are water-soluble; and are available in granulated, water or powder form. They can also be applied in dairy, baked confectionery and many more products. Lake colourants are insoluble in water, yet dispersible in oil, therefore readily applicable to fat-based products. They colour products, such as beverages, cakes and confections (FDA, 2014).

Certified food products have E (European) numbers, which are found in food packaging and used as means of identifying food additives (Haen, 2014). Some of these E-labelled colourants are natural colourants, such as beetroot red. Notably, Colouring Foods do not have E numbers (Lehto et al., 2017).

Table 2.1 comprises the acceptable colourants in the EU and USA. The EU and the USA differ in some of their permitted food colourants, yet both accept the same Colouring Foods. Countries in South America and Africa accept the colourants recommended by the Joint Expert Committee for Food Additives (JECFA).

Table 2.1: Corresponding approved colours in the EU and the U.S. Permitted use of the lake forms and the US attribution of subject to certification are also indicated (Lehto et al., 2017)

	EU		**USA**		
E number	Name	Lake permitted	Name (common name)	Lake permitted	Subject to batch certification
E 100	Curcumin	Yes	Tumeric	NM	NM
E 101	Riboflavins S (including riboflavin-5'-phosphate)	Yes	Turmeric oleoresin	NM	NM
E 102	Tartrazine	Yes	Riboflavin	NM	NM
E 104	Quinoline Yellow	Yes	FD&C Yellow No. 5 (Tartrazine	Yes	Yes
E 110	Sunset Yellow FCF/Orange Yellow S	Yes	FD&C Yellow No. 6 (Sunset Yellow FCF) Orange B	Yes	Yes
E 120	Cochineal, carminic acid, carmines	Yes	Cochineal extract, carmine	Yes	Yes
E 122	Azorubin, carmoisine	Yes	NM	NM	NM
E 123	Amaranth	Yes	NM	NM	NM
E 124	Ponceau 4R, Cochineal Red A	Yes	NM	NM	NM
E 127	Erythrosine	Yes	FD & C Red No. 3 (Erythrosine)	NM	Yes
E 129	Allura Red AC	Yes	FD&C Red No. 40 (Allura Red AC)	Yes	Yes

			Citrus Red No. 2		Yes
E 131	Patent Blue V	Yes	NM	NM	NM
E 132	Indigotine, Indigo carmine	Yes	FD&C Blue No. 2 (Indigotine)	Yes	Yes
E 133	Brilliant FCF	Yes	FD&C Blue No. 1 (Brilliant Blue FCF)	Yes	Yes
E 140	Chlorophylls and chlorophyllins	Yes	NM	NM	NM
E 141	Copper complexes of chlorophylls, chlorophyllins	Yes	Sodium copper chlorophyllin	NM	NM
E 142	Green S	Yes	FD&C Green No. 3 (Fast Green FCF	Yes	Yes
E 150 a-d	Plain caramel, caustic sulphite caramel, ammonia caramel, sulphite ammonia caramel	Yes	Caramel	NM	NM
E 151	Brilliant Black PN	Yes	NM	NM	NM
E 153	Vegetable carbon	Yes	NM	NM	NM
E 155	Brown HT	Yes	NM	NM	NM
E 160a	Carotenes	NM	β-Carotene Carrot oil	NM	NM
E 160b	Annatto, bixin, norbixin	NM	Annatto extract	NM	NM
E 160c	Paprika extract, capsanthin, capsorubin	NM	Paprika, paprika oleoresin	NM	NM
E 160d	Lycopene	NM	Tomato lycopene extract; tomato lycopene concentrate	NM	NM
E 160e	β-Apo-8'-carotenal	NM	β-Apo-8'-carotenal	NM	NM
E 161b	Lutein	NM		NM	NM
E 161g[a]	Canthaxanthin	Yes	Canthaxanthin (not synthetic)	NM	NM
E 162	Beetroot Red, betanin	NM	Dehydrated beets (beetroot powder)	NM	NM
E 163	Anthocyanins	Yes	Grape colour extract Grape skin extract [d]	NM	NM
E 170	Calcium carbonate	NM		NM	NM
E 171	Titanium dioxide	NM	Titanium dioxide	NM	NM
E 555 and E 171[b]	Potassium aluminium silicate (mica) and titanium oxide	NM	Mica-based pearlescent pigments	NM	NM
E 172	Iron oxide and hydroxides	NM	Synthetic iron oxide	NM	NM

E 173	Aluminium	NM	NA	NM	NM
E 175	Silver	NM	NA	NM	NM
E 174	Gold	NM	NA	NM	NM
E 180	Litholrubin BK	Yes	NA	NM	NM
E 579[c]	Ferrous gluconate	NM	Ferrous gluconate	NM	NM
E 585[c]	Ferrous lactate	NM	Ferrous lactate colour fixative for ripe olives	NM	NM
Colouring food	Vegetable juice	NM	Vegetable juice	NM	NM
Colouring food	Fruit juice	NM	Fruit juice	NM	NM
Colouring food	Saffron	NM	Saffron	NM	NM
Colouring food	Spirulina extract	NM	Spirulina extract	NM	NM

NM: not mentioned in table. a Only for medicinal products. b Potassium aluminium silicate, i.e., mica (E 555), is an approved carrier for titanium dioxide (E 171), iron oxides and hydroxides (E 172). Mica platelets can be coated with E 171 or E 172 to form pearlescent pigments. In the US, only coating with titanium dioxide is permitted. c Other food additives in the EU. d. In USA, calcium carbonate is listed as a food substance affirmed as generally recognised as safe (GRAS).

The difference in acceptable food colourants sometimes makes it hard to trade food between countries (European Commission, 2013; Lehto et al., 2017). Figure 2.1 shows the usage of natural, artificial and colouring food usage according to the 2016 Mintel Global New Products Database (GNPD). Figure 2.1 shows that Africa and the Middle East had the lowest use of natural colours, whereas Europe and Latin America had the highest. Europe had the lowest artificial colourant usage, while Latin America had the highest. Europe also led in the Colouring Food usage, reaching up to 14%, whereas other parts of the world were significantly lower (4% or less).

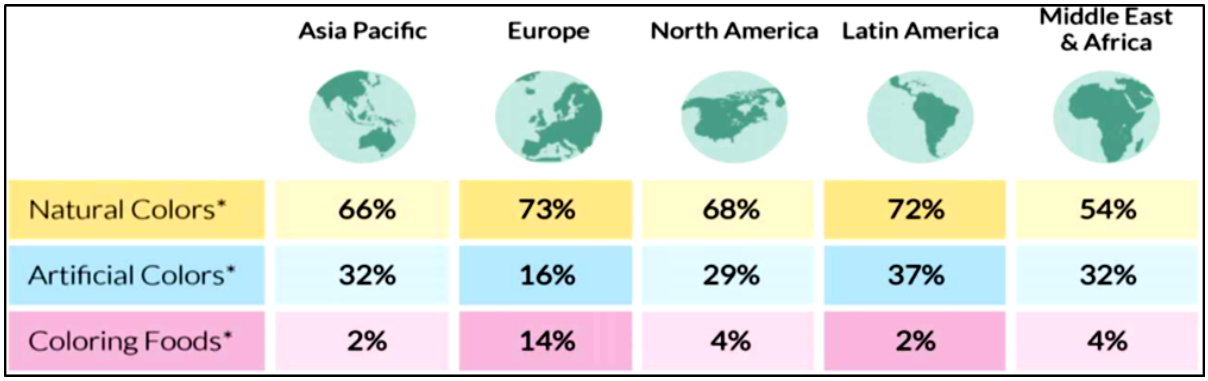

Figure 2.1: Food colourant usage around the world (September 2015 to August 2016) Mintel GNPD, 2016 as cited in Simon et al. (2017)

2.3 Classification of food colourants

According to Msagati (2013), there are two methods used to classify colourants: (1) according to the chemical structure, which identifies the functional groups responsible for imparting colour; and (2), as natural or synthetic colourants. Scholars, such as Mortensen (2006) and Aberoumand (2011), only classify colourants according to their natural or synthetic state. Similar to the analysis of Msagati (2013), Schwartz et al. (2008) state that colourants are any chemical, either natural or synthetic, that can impart colour. Kumar and Sinha (2004) state that there are several ways in which natural colourants can be classified, mostly dependent on their chemical structure and use. The final intensity of colourants is also a result of the chemical components of their natural source.

There are various ways in which colours can be classified; their classification in the current study borrows from that of Msagati (2013) and Gengatharan et al. (2015) broken down in Table 2.2 and further deliberated in different segments (2.2 and 2.3) of the chapter. The European Commission (2013) further distinguishes the dissimilarity between natural food colourants (extracted from plants, animals and minerals) and Colouring Foods (foods with a natural ability to colour food). Although sources may be the same, the production process of colourants determines whether they fall under natural colourants or Colouring Foods (Simon et al., 2017).

Table 2.2 is numbered according to the classification of sections in the chapter. It is also numbered according to the order of discussion: 2.2 of the chapter entails that of natural or synthetic colourants, whereas 2.3 entails discussions of chemical classification:

Table 2.2: Classification of colourants (Msagati, 2013; Gengatharan et al., 2015)

2.2 Natural or synthetic	**2.3 Chemical structure**
2.2.1 Nature-identical *B-carotene, flavonoids, etc.*	2.3.1 Flavonoids *Found in fruits and vegetables*
2.2.2 Natural *Plants* (in which betalains are found)*, algae, insects, etc.*	2.3.2 Indigoids *Found in beetroot*
2.2.3 Inorganic *Titanium dioxide, silver and gold*	2.3.3 Betalains *Found in beetroot, amaranth and cactus pear*
2.2.4 Synthetic or artificial *Azo dyes, quinolone, xanthenes, etc.*	2.3.4 Carotenoids *Found in tomatoes and oranges*

2.3.1 Nature-identical colourants

These are human-made colourants which are found in nature. For instance, lycopene is naturally found in tomatoes, yet it has a nature-identical version that contains colouring abilities. The development of these colourants is aesthetically and pharmacologically beneficial to consumers (Downham & Collins, 2000; Mortensen, 2006). Examples of other nature-identical colourants from Downham and Collins (2000), Breithaupt (2004), Mortensen (2006) and European Union (2008) are:

(1) β-carotene (E 160a), which is mostly used in the consumer market and is also a natural colourant;

(2) β-apo-89-carotenal (E 160e), which is red-orange, oil-soluble, and principally comprises of trans-isomers. It also contains vitamin A and is commercially used in conjunction with β-carotene;

(3) ethyl ester of β-apo-89 carotene acid (E 160f), which is yellow-orange and contains vitamin A. It can be used alone or in conjunction with other carotenoids and xanthophylls for colouring the feed of poultry;

(4) riboflavin and riboflavin-5'-phosphate:

· Riboflavin (E 101) (i), also known as lactoflavin, is a yellow-orange and yellow-crystalline powder that has a slight odour. The colour quickly fades as a result of light sensitivity.

· Riboflavin-5'-phosphate (E 101 (ii), also known as riboflavin-5'-phosphate sodium yellow is a yellow-orange crystalline, hygroscopic powder with a slight odour.

(5) canthaxanthins (E 161g) are chemical colourants. These are orange-pink and naturally found in salmon, shrimp and flamingos.

2.3.2 Natural colourants

Natural colourants are produced from sources which naturally occur in nature: algae, insects, cyanobacteria, fungi and plants. They can be extracted and concentrated, using water or low levels of alcohol for hydrophilic colourants and organic solvents for hydrophobic ones (Mortensen, 2006; Aberoumand, 2011). Some colourants can be harmful; as such, they have usage restrictions, purity specifications and maximum permitted levels that correlate with other food additives (Scotter, 2011b). Likewise, consumer perception advocates the safety of natural colourants, yet they can also

have intolerance and allergic reactions against them; thus, the need exists to set consumption levels for them (Martins et al., 2016).

Colourants that are produced by tampering with natural products or organisms are also regarded as natural: caramel, vegetable-carbon, and chlorophyllin-copper (Mortensen, 2006).

Caramel is produced during the controlled catalytic heat treatment of some carbohydrates, which results in a reddish-brown to brown-black colourant (Msagati, 2013). The heating occurs alone or with the assistance of acids that can be used in food, salts, and salts with alkalis or just alkalis. They are also produced from commercially available and nutritive sweeteners that can be used in food. They comprise of fructose, dextrose (glucose), invert sugar, sucrose, malt syrup, molasses, and starch hydrolysates as well as fractions of it. Caramel is a natural liquid or solid colourant that is generally brown-black in colour and is a multifaceted mixture of compounds. There are four main groups of caramel:

(1) plain caramel, caustic caramel (E 150a): carbohydrates are heated with or without acids or alkalis;

(2) caustic sulphite caramel (E 150b): carbohydrates are heated with sulphite compounds in conjunction with or without acids or alkalis;

(3) ammonia caramel (E 150c): carbohydrates are heated in the presence of ammonium compounds, with or without acids or alkalis; and

(4) sulphite ammonia caramel (E 150d): carbohydrates are heated in the presence of both sulphite and ammonium compounds, with or without acids or alkalis (Scotter, 2011a).

Vegetable-carbon (E 153), also known as carbon black, lamp black and carbon ash, are produced during the carbonization of vegetable materials, such as cellulose residues, wood, coconut, peat and other shells (Miranda-Bermudez et al., 2012). It is an odourless and tasteless black powder that is insoluble in water and organic solvents. It is widely used as a colourant in confectionery products (Downham & Collins, 2000; European Union, 2008).

Chlorophyllin (Cu-Chl) (E 141 (ii), also known as sodium copper chlorophyllin, potassium copper chlorophyllin and CI Natural Green 5, is a dark green/blue to black

powder when extracted (European Union, 2008; Code of Federal Regulations, 2017). It is a product that is derived from chlorophyll in edible parts of nettle, grass and lucerne (Tumolo & Lanfer-Marquez, 2012). It may be used as a food colourant and possesses health-promoting qualities relating to its antioxidant, antimutagenic and anticarcinogenic properties (Azeredo, 2009; Code of Federal Regulations, 2017).

Natural colourants are generally expensive; they may also be unstable during processing, and that could tempt food manufacturers to mix natural colourants with synthetic ones. Most natural colourants are derived from plant material, and in instances of climate instabilities, such as drought and excess rain, there may be significant crop losses. Resultantly, there would be shortages of natural colourants and a further increase in food prices. Natural colourants also have fewer colour parameters. Therefore, food colourants might be limited to natural colours, such as red from beetroot (Rayner, 2007 as cited in Wrolstad & Culver 2012; Rodriguez-Amaya, 2016).

2.3.2.1 Insects

Lac and cochineal are colouring insects that are commercially applied to a variety of products (Mortensen, 2006). Lac insect *Kerria lacca* (Kerr), shown in Figure 2.2(a) is found in plants, such as usum (*Schleichera oleosa*), palas (*Butea monosperma*) and ber (*Zizyphus mauritiana*). It secretes body fluid, namely resin, which can be used as a food colourant, as shown in Figure 2.2(b). Purified lac is deep orange-red and shows potential to be used as a colourant in jams, meat products, noodles and beverages (Mohanta et al., 2013; Srivastava et al., 2013).

Cochineal (E 120), the insect is well known as cochineals or cochinilla, is found on the cactus pear plant, with the scientific name *Dactylopius coccus*. Female cochineal insects produce carmine (dark red colour of unprocessed pigments) or carmic acid (colouring property of carmine), a red thick watery substance in colour (Scheinvar, 1995; Dapson, 2007; Sabatino et al., 2012). When the colourant is used commercially, it is extracted with water or ethanol and then dried. Its main colouring property is carmic acid (Dapson, 2007; Scotter 2011a; Ledwaba et al., 2012).

Depending on colourant formulation, cochineal (Figure 2.3 a and b) can produce pink, orange and purple hues (Ahmad et al., 2012). There have been reported cases of

allergic reactions upon consumption of the colourant. Thus, more research is required to eliminate the health danger from the colourant. Both carmine and carmic acid can be applied as food colourants in soft drinks, dairy products, edible ice, and desserts. However, carmine can be applied to more products than carmic acid: fish, baked goods; coatings; and more. Moreover, their properties and appearance differ slightly: carmine is a red-dark, friable, solid or powder. On the other hand, carmic acid, which is soluble in water, is a red to orange powder or dark red liquid (Smith & Hong-Shum, 2003; Azeredo, 2009; Dufossé, 2014).

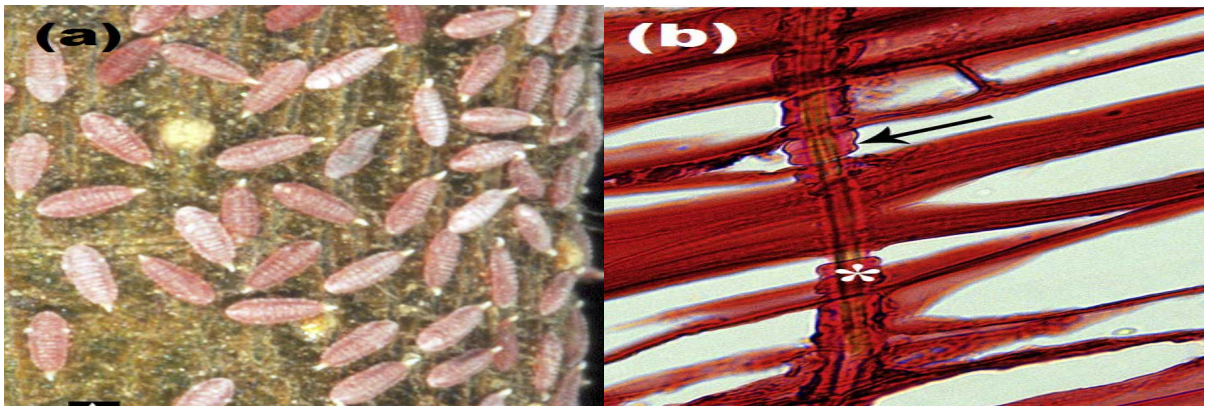

Figure 2. 2: (a) Live lac insect (b) salivary sheath where the arrow is pointed, scraped to be used as a colourant (Ahmad et al., 2012)

Figure 2. 3: Cochineal insect in fruit and cladode of *Opuntia* species (a) Sigwela (2016) (b) Dufossé (2014)

2.3.2.2 Algae

Microalgae are microorganisms that use solar energy to grow via photosynthesis. These microalgae include *Spirulina* (*Arthrospira platensis*) and cyanobacteria that can both be industrialized (Markou & Georgakakis, 2011). The application of Functional

Products (FPs) containing *Spirulina platensis* provides health benefits to the consumer as it contains antioxidant properties. As it is blue-green colour, it is used as a food colourant. Sensory evaluations confirm that its odour, texture and taste are also acceptable to consumers (Baky et al., 2015). On the other hand, other microalgae, including *Spirulina platensis,* can be used in the nutraceutical, pharmaceutical, aquaculture and food industries. In foods, it can be applied to jellies; ice cream; and juice (Mosulishvili et al., 2002; Begum et al., 2015).

Experiments conducted by Gouveia et al. (2008), where 1 and 3% of the microalgae *Isochrysis galbana* were added to biscuits, proved that microalgae could be used as a colourant in biscuit manufacture (Figure 2.4 a). Results of the experiment showed that the colourant is stable; the overall texture is food-friendly, and the polyunsaturated fatty acids which are found in the microalgae, prove it to be a valuable food colouring alternative.

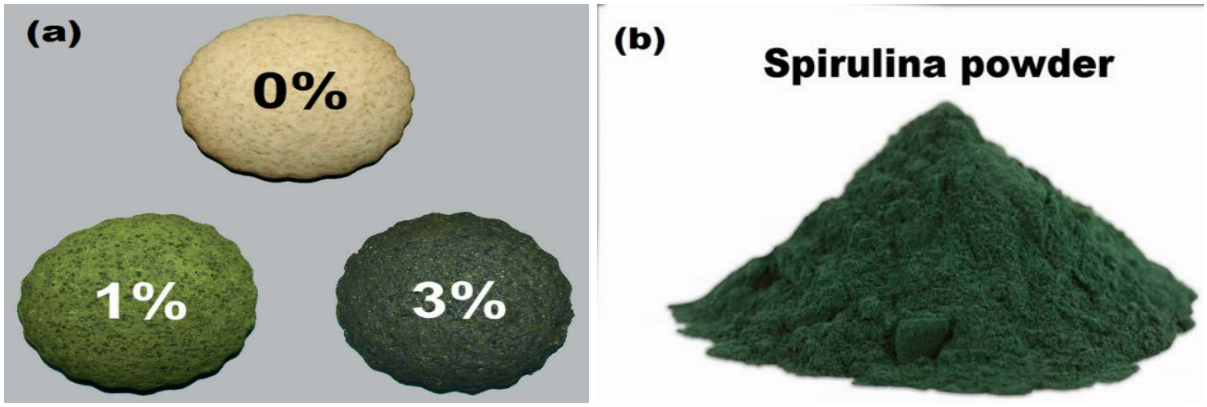

Figure 2. 4: Biscuits with different incorporation levels (1 and 3%) of *Isochrysis galbana* biomass and (b) spirulina powder from *Spirulina platensis* (Gouveia et al. 2008; Priyadarshani & Rath, 2012)

2.3.2.3 Cyanobacteria

Cyanobacteria, otherwise known as ascyanophyceae, are blue to green in colour; these are prokaryotic organisms that are already used in human food products (Singh et al., 2005). Manufactured cyanobacteria have a high content of protein and carbohydrates. Applying them as food colourants would be of great benefit, because of their vitamin density, anti-viral and anti-fungal properties (Thajuddin & Subramanian 2005; Markou & Georgakakis, 2011).

Different genera of filamentous cyanobacteria can be used as food colourants; the first example is the *Arthrosporic* spp. that are used in the food industry as a colourant (Mortensen 2006; Miklaszewska et al., 2012). Second is *Arthrospira platensis* (Eriksen, 2008) and third is *Spirulina maxima* and fourth *Spirulina arthrospira* which are safe for human and animal consumption. They can also be used as food colourants (Figure 2.4 a) and powder, as shown in Figure 2.4 (b) (Shimamatsu, 2004).

2.3.2.4 Fungi

The application of filamentous fungi in food products seems possible because the fungi have raw materials, which are easily accessible and can be chemically structured to suit the desired colour and form of application. In fact, a variety of colours can be formulated through the manipulation of fungi (Dufosse et al., 2014; Torres et al., 2016). The production of microorganisms occurs via fermentation of a mixture of liquid mediums, and other substrates (Santos-Ebinuma et al., 2016), the use of fungi is advantageous because it is not affected by the climate.

A study conducted by Mapari et al. (2006) showed that ascomycetous fungi could be used in food as colourants. In the study, ascomycetous fungi were compared to other natural colourants, such as annatto and cochineal. It was found that the fungi can produce red and yellow colourants in variable spectrums. Figure 2.5, by Dufosse et al. (2014), shows that extracts from filamentous fungi can be used as colourants in food products. They are also easy to collect because they are readily available in nature.

Figure 2. 5: Filamentous fungi extract at different stages of production (Dufosse et al., 2014)

Lycopene from the fungi *Blakeslea trispora* is a GRAS food product and can be used to colour a variety of foodstuffs. Examples of these include dairy products, baked

goods, breakfast cereals, puddings, gelatines and sauces (Mortensen, 2006). *Monascus* spp. are filamentous fungi that are well known for producing red mould rice (RMR), seen in Figure 2.6, that is very popular in Asian countries. Amongst their various uses, it can be used as a food colourant that is applicable to fish and meat (Downham & Collins, 2000; Shao et al., 2014; Chen et al., 2015). A study by Kumari et al. (2009) proved that the red mould rice (RMR) is safe for consumption and can lower cholesterol.

Figure 2. 6: The traditional process of red mould rice (RMR) production. ZENG, a kind of wooden rice steamer (Chen et al., 2015)

2.3.2.5 Colouring Foods

Colouring Foods are foodstuffs that have an inherent ability to colour food; thus, they do not need to be labelled as colourants as they fall under food ingredients that have colouring properties (European Commission, 2013; Reinhart, 2014). The practice of colouring food products with food is the most natural way of imparting colour to food (Carle & Schweiggert, 2016).

The EU has published guidelines that determine whether a pigment falls in the colouring food category or that of Colouring Foods. Table 2.2 highlights that Colouring Foods do not have E numbers. For example, vegetable juice, fruit juice, saffron and spirulina extract are accepted in the EU and USA, making it easy to trade food products coloured with Colouring Foods between countries (Lehto et al., 2017). Products have also been successfully coloured with Colouring Foods. In a poster delivered by Rodríguez et al. (March 2017), at the International Congress on Cactus Pear and Cochineal in Chile, it was indicated that freeze-dried yellow-orange Colouring Foods from *O. ficus-indica* could be used as food colourants for yogurt and

ice cream, and the colourant was stable. Wine gums which were made from purple cactus pear Colouring Food is shown in Figure 2.7.

Figure 2. 7: Jelly sweets coloured with purple cactus pear (Sáenz et al., 2013)

Figure 2.8 (a-d) shows an example of food products that are coloured with Colouring Food. Swart et al. (2016) used green beans, butternut, carrots, sweetcorn, cabbage, cauliflower, and beetroot to colour vegetable-based potato chips. Interestingly, the chips were treated under high temperatures, such as deep frying at 200°C for 7 minutes and the colour of the chips remained stable.

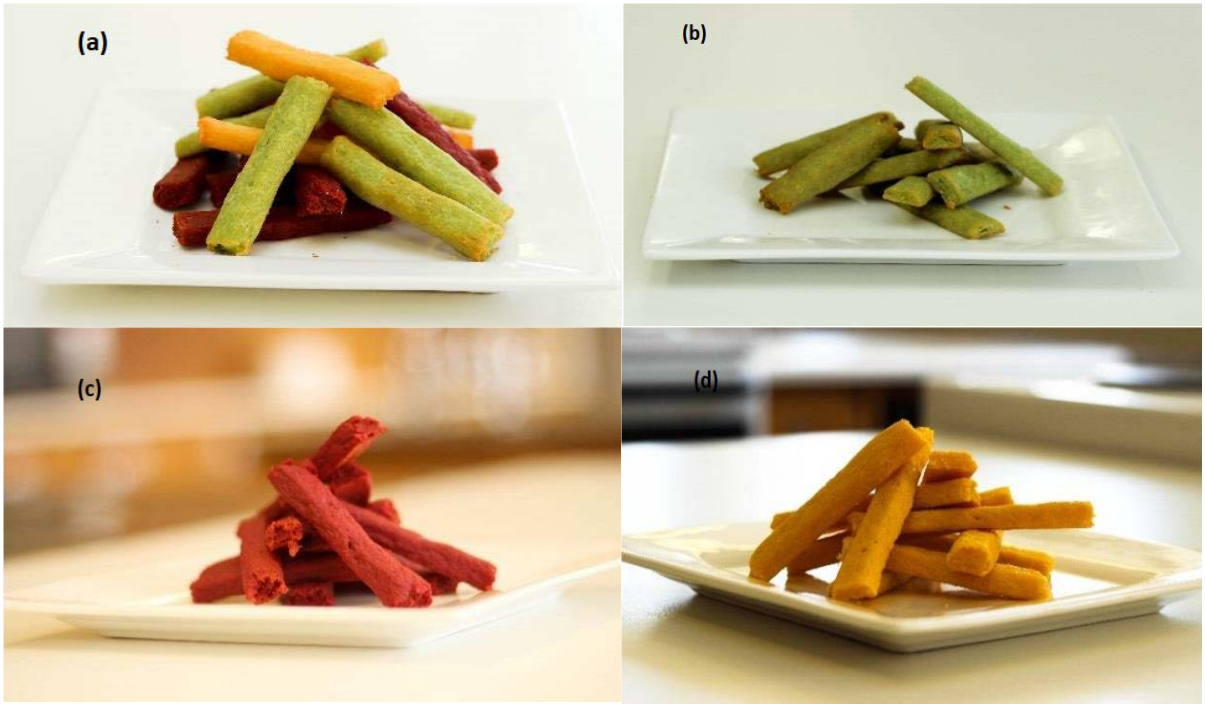

Figure 2. 8: (a-d): Vegetable-based chips naturally coloured with vegetable juice or pulp (Swart et al., 2016)

2.3.2.6 Plants

Most natural plant-derived colourants are extracted from *Magnoliophyta* flowering plants (Mortensen, 2006). These plants have phytochemicals which play a role in the production of colour in food products. They are regarded as safe, possess health-promoting qualities and fall under the category of functional foods. Such plants include various *Opuntia* species, beets and amaranth. The plants are good sources of ascorbic acid, as well as antioxidant compounds phenolics and betalains (Dantas et al., 2015).

2.3.2.6.1 Cactus pear

Cactus pear, also known as prickly pear, belongs to the Cactaceae family. Research on this fruit had been side-lined until 1980 when it was realised that it could be of unlimited use in the food industry (Piga 2004; Kunyanga et al., 2009). One of the greatest attributes of cactus pear is its ability to grow in semi-arid regions and thrive under minimally irrigated soil, where other plants would not be able to survive (Sáenz et al., 2013). Likewise, this plant is temperature-resistant and grows freely, even with the challenge of climate change (Rai et al., 2011). Its ability to grow and spread fast has led it to be labelled as a weed or an invasive species in various countries. People, especially those from impoverished environments, use it in various ways because both animals and humans consume it. The benefits of various cultivars are highlighted in Table 2.3 (Ledwaba et al., 2012; Shackleton et al., 2017).

Cactus pear is popularly known as the royal fruit of the desert and forms part of about 1 600 species in 130 genera and segmented into three small groups, namely, *Opuntioideae, Pereskioideae* and *Cactoideae*. *Opuntiodeae*, the most widespread of these three segmented groups, is found in most countries and has more than 300 species (Rai et al., 2011). The fruit is an excellent nutraceutical that plays a critical role in the production of natural medicines, cosmetics, and colouring of food products (Piga, 2004; Aragona et al., 2017).

The cactus pear species, deliberated in this study, are classified as follows:

Order: Caryophyllales

Suborder: Portulacineae

Family: Cactaceae

Subfamily: Opuntioideae

Genus: *Opuntia*

Subgenus: *Opuntia*

Species: *ficus-indica* and *robusta* (L.) Mill., Gard. Dict. Abr. ed. 8. No. 2. 1768 (Scheinvar, 1995)

Table 2. 3: Cactus biodiversity and their major uses (Compiled from: Teixeira et al., 2000; Budinsky et al., 2001; Obón et al., 2009; Rai et al., 2001)

Name of species	**Part of plant**	**Useful as**
Selenicereus grandiflorus	Whole plant	Hedge plants
Trichocereus pachanoi	Pads or cladodes	Urinary tract infection, diuretic, hallucinogenic drug
Saguaro cactus	Stem	Enhance milk flow in mother
Peyote cactus	Fruit stem	Neurasthenia
Opuntia ficus-indica	Whole plant, flowers	Good source of milk-clot enzymes, red dye, cicatrizant, jams, pickle, waterproofing paints, reduce side-effects of hangover
Opuntia stricta	Fruit	Yoghurt and soft drink food colourant
Opuntia robusta	Fruit	Fights against diabetes mellitus
Caralluma fimbriata	Stem	Suppress appetite neuroprotective effects
Hoodia	Stem	Enhance and provide energy

2.3.2.6.1.1 Usage of cactus pear in different countries

The domestication of *Opuntia* cultivars for human consumption can be traced back to more than 8000 years ago in Mexico, where the fruit played a positive role in the agricultural economy of the country (Sáenz, 2000). Mexico is the biggest cactus producer, with a harvest of 300 000 tonnes of the fruit on about 70 000 hectares (ha) of land per annum, thus, enabling Mexico to export the fruit in bulk. The young fresh stems, known as nopalitos, are a staple low-cost vegetable (Flores-Valdez et al., 1995 as cited in Basile 2001; Sáenz, 2000; Snyman, 2014).

Cactus pear is largely found and forms part of the diet in America and Mediterranean countries (Rami´Rez-Moreno et al., 2011). The USA produces 4 000 tonnes of the fruit a year, Argentina 7 500 tonnes, and Chile 8 000 tonnes (Basile, 2001). In Chile, it is usually eaten as fresh fruit, and produced into juice, vinegar, candies and jams (Sáenz et al., 2013).

Opuntia stricta species is used for fencing in Brazil. Its water retention properties also allow it to be used as animal fodder (Ferreira et al., 2012; Dantas et al., 2015).

Italy is one of the biggest cactus pear producers, where 3000 ha of the land produces around 70 000 tonnes of fruit. Unlike Mexico, nopalitos are not eaten in Italy (Basile, 2001). People from India and their livestock consume it fresh and use it as a medicinal plant (Rai et al., 2011).

Cactus pear is also found in parts of Africa, such as Uganda, Tanzania, Kenya, Egypt, Morocco, Tunisia and South Africa (Piga, 2004; Yahia, 2011; Shackleton et al., 2017). The water-retention properties of the plant are beneficial during dry seasons, as cattle feed on it (Rai et al., 2011). Ethiopians harvest the fruits of freely growing wild cactus for consumption. In 2015 Belay (2015) reported that Ethiopia had more than 16000 ha of invasive cactus pear.

2.3.2.6.1.2 Cactus pear in South Africa

Opuntia ficus indica is a foreign plant that was introduced to South Africa (SA), particularly the Eastern Cape, towards the end of the nineteenth century. It spread very quickly and became popular in the rural communities, where it was used for animal feed in the Winterberg (Van Sittert, 2002). The country produces around 15 000 tonnes of cactus pear fruits per annum, on 1 500 hectares of land (Basile, 2001).

Opuntia ficus-indica and *O*. *sticta* are the fastest growing cactus fruits in many African countries, including SA (Githure et al., 1999). South Africa has more than 40 spineless cactus pear cultivars, mainly from two species, i.e., *O. ficus-indica* and *O. robusta,* and breeds one of the most varied genetic collections of *Opuntia* spp. (Mashope, 2007; Ledwaba et al., 2012). *Opuntia ficus-indica* fresh produce is exported by commercial farmers and sold informally on the side of the road in Limpopo while flruit from the prickly *O*. ficus-indica is sold on road sides in the Eastern Cape (Sáenz et al., 2013).

Introduced to SA in the 1930s, *O. stricta* is regarded as an invasive weed in many parts of the country. Of the different places where *O*. *stricta* in invading fast is the Kruger National Park, one of the largest game reserves in Africa (Hoffmann et al., 1999; Foxcroft et al., 2004). It grows quickly in the area as it has invaded around 30 000 ha in Skukuza alone (Lotter & Hoffmann, 1998).

Water is undeniably becoming a scarce resource and it is vital to plant crops that thrive under minimal water requirements. A study conducted by De Wit et al. (2010) confirms that *Opuntia* spp. cultivars can thrive in these climate changes and include cultivars, such as Meyers (red to pink fruit), Roedtan (orange fruit), Gymno Carpo (orange fruit) and Robusta (purple fruit) all of which, except Roedtan, are used in the current study.

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2.3.2.6.1.3 Roots

Cactus pear has a wide, shallow, fleshy, lateral root system that enables it to absorb water quickly, so that it can be used more efficiently (Snyman, 2014). The roots make up about 9% of the plant and its xeromorphic features enable it to survive for prolonged periods in arid regions and thrive in shallow soils. Water use may differ according to the different cultivars, soil type and management thereof. Cactus pear roots have the potential to be used as animal feed in times of need (Scheinvar, 1995 Dubrovsky, et al., 1998; Ramakatane, 2003; Snyman, 2014).

2.3.2.6.1.4 Cladodes

Small mature cladodes are 30-40 cm long, whereas larger ones are 70-80 cm long, both with a width of 18-25 cm (Scheinvar, 1995). Cladodes are good sources of pectin, minerals and mucilage (Habibi et al., 2005). Moreover, they are a good source of dietary fiber (soluble and insoluble), and the advantage of processing it is that it has excellent water retention properties (Sáenz et al., 2012). Their moisture content is 92%, protein 1-2% and fiber 4-6% (Jaramillo-Flores et al., 2003). Both *O. ficus-indica* and *O. robusta* young cladodes are also valuable vegetables for human consumption and can be used for animal fodder (Sáenz, 2013).

Nopalitos, the soft young stems of cactus, are eaten in their fresh state. They are good sources of fibre and may be effective for medical use (Sáenz, 2000). When consumed frequently, their antioxidant activity destabilizes oxidative injury (Butera et al., 2002). They are also high in proteins, amino acids, as well as vitamins (Jana, 2012). The chemical composition of cactus pear cladodes changes as it grows (Rai et al., 2011).

Cladodes are used to combat diseases, such as diabetes, bronchial asthma, burns and indigestion (Zhao et al., 2007).

2.3.2.6.1.5 Fruit

The fruit of most cactus pear cultivars is lengthy and oval, with a thick pericarp, a lot of hard seeds, as well as a juicy pulp (Nerd et al., 1991). Cactus pear fruit is mostly consumed fresh because of its quality and taste (Sáenz, 2013). It has a higher vitamin C content than regularly consumed fruits, such as apples and apricots (Kuti, 2004). It is also useful in the food industry, because of its constituent antioxidants, minerals, taurine and betalains (Moβhammer et al., 2006b; Khatabi et al., 2013). The fruit contains polyphenols, which are more prevalent in red cactus pear. Some red cultivars also possess inherent colouring properties (Castellanos-Santiago & Yahia, 2008; Nunes, 2014).

The *O. robusta* species has a big, round, dark purple fruit that is not consumed fresh because of its unacceptable taste (raw potato-like). Examples of cultivar names are Robusta and Monterey (Stintzing et al., 2005).

The *O*. *ficus-indica* species have some of the most domesticated cultivars of cactus pear that is important in many agricultural economic countries (Snyman, 2014). These cultivars consist of 48% peel, ±45% pulp and ±7% seeds. The edible pulp contains 84-90% water and 12.8-14.6% sugar, with a pH of 5.3 – 7.1. Its low acidity and high sugar content are the reasons for its delicious flavour. The fruit is consumed in its fresh state, which comes with added benefits, as it is loaded with polyphenols and antioxidants (Bouzoubaâ et al., 2016). Table 2.4 showcases the technological, chemical, mineral and amino acid composition of cactus pear fruit.

Table 2.4: Main technological parameters: chemical and mineral composition of cactus-pear pulp Piga (2004)

Cactus pear fruit pulp technological parameters	
Technological Parameters	**Range**
Pulp (%)	43-57
Seeds (%)	2-10
Peel (%)	33-55
pH	5.3-7.1
Acidity (% of citric acid)	0.05-0.18
°Brix	12-17
Total solids	10-16.20
Chemical Composition of the Pulp	**Range**
Moisture (%)	84-90
Protein (%)	0.2 – 1.60
Fat (%)	0.09-0.70
Fibre (%)	0.02-3.10
Ash (%)	0.3-10
Total sugars (%)	10-17
Vitamin C ($mg \cdot 100g^{-1}$)	1-41
Minerals	**Range**
Ca ($mg \cdot 100g^{-1}$)	12.8-59
Mg ($mg \cdot 100g^{-1}$)	16.1-98.4
Fe ($mg \cdot 100g^{-1}$)	0.4-1.5
Na ($mg \cdot 100g^{-1}$)	0.6-1.1
K ($mg \cdot 100g^{-1}$)	90-217
P as PO_4 ($mg \cdot 100g^{-1}$)	15-32.8
Amino acids	**Maximum Content (mg/L)**
Proline	1 768.7
Glutamine	574.6
Taurine	572.1
Serine	217.5
Alanine	96.6
Glutamic acid	83.0
Methionine	76.9
Lysine	53.3

In a study conducted by Bouzoubaâ et al. (2016), it was concluded that the fruit could be used as a good source of colourants, which cover a variety of hues for example, yellow and red pigments. Du Toit et al. (2018) found that specific antioxidants were found in different coloured fruits, depending on their colour. According to Khatabi et al. (2013), there are more polyphenols in red cultivars than yellow ones. Both betalains and polyphenols are good sources of antioxidants. Morado is light green to white and is very well-liked for its exquisite taste. Gymno Carpo is yellow to orange and very sweet. Algerian is red to pink fruit with a delicate sweet taste, which is mainly exported and has recently gained popularity in the local South African market (Ledwaba et al., 2012).

According to Cardador-Martínez et al. (2011), cactus pear with light-green, yellow and brown peels have more antiradical activity and Trolox equivalent antioxidant capacity (TEAC) than cultivars with purple peels. Purple cultivars are a source of betalains, which are antioxidants and pigments that are like that of *Beta vulgaris* (beetroot), which is already in use in the food industry (Sáenz et al., 2004; Moßhammer et al. (2006b); Du Toit et al. (2018) also stated that purple (*O. robusta* cv Robusta) and orange (*O. ficus-indica* cv Ficus-Indice) cactus pear fruit cultivars contain high levels of betalains and antioxidant activity.

Khatabi et al. (2013) note that red cultivars have higher polyphenol and betalain contents than yellow cultivars. Both betacyanins and anthocyanins produce red to purple colour hues in comparison to orange colours, which are produced from red betalains and yellow colours from betaxanthins. Anthocyanins lose their colouring ability at pH 2 and betalains are stable from pH 3-7 (Stintzing & Carle, 2004). *Opuntia ficus-indica* cultivars which produce yellow-orange cactus pear fruit could be used as a raw material source of yellow-orange colouring for foods. Betaxanthins furnish the fruit with the yellow-orange colouring and are water-soluble (Stintzing et al., 2005; Herbach et al., 2006).

2.3.6.2 Beetroot

Beta vulgaris L. is a red commercially used beetroot cultivar that is also known as beet (beetroot) red (E 162) or betanin. E 162 is a highly concentrated colourant. The main colouring constituent of red beetroot betalains is betacyanins. Betacyanins comprise of betanins, which make up 75-95% of betacyanins (Delgado-Vargas et al., 2000;

Smith & Hong-Shum 2003; Scotter, 2011a). Betanin (betanidin-5-O-β-glucoside), which falls under natural red colourants (E 162), is the most common betacyanin in plants; this includes *Opuntia* species and beetroot. The pigment is a colourant in cosmetics and pharmaceutical products. In comparison, betaxanthins are yellow-orange pigments which are mainly vulgaxanthins. Beet red (E 162) is a commercial food colourant that can add colour to baked goods, salad dressings, desserts, meat and poultry, dairy products and more (example in Figure 2.9); it can be applied as a liquid or dried product. Moreover, it is very similar to the colourants obtained from amaranth, carmine and carminic acid (Delgado-Vargas et al., 2000; Smith & Hong-Shum 2003; Sivakumar et al., 2009).

As with other plants that are found in the Caryophyllales order, betalains, obtained from red beetroot can replace anthocyanins as colourants *Beta vulgaris* L. contains enough betalains to serve as a very successful food colourant and antioxidant (Suganyadevi et al., 2010).

The betalains found in beetroot are made up of both betacyanins and betaxanthins, known as vulgaxanthin 1 and 2 (Figure 2.10). Heat degrades beet-red colour strength however, vitamin C helps to stabilise it (Koul et al., 2002; Smith & Hong-Shum, 2003; Cardoso-Ugarte et al., 2014). In India, betalains from beetroot aid in colouring sweet products, such as Sandesh and sweet meats (Roy et al., 2004).

Figure 2.9: Pancake and ice-cream coloured with *Beta vulgaris* (Chilson, 2017)

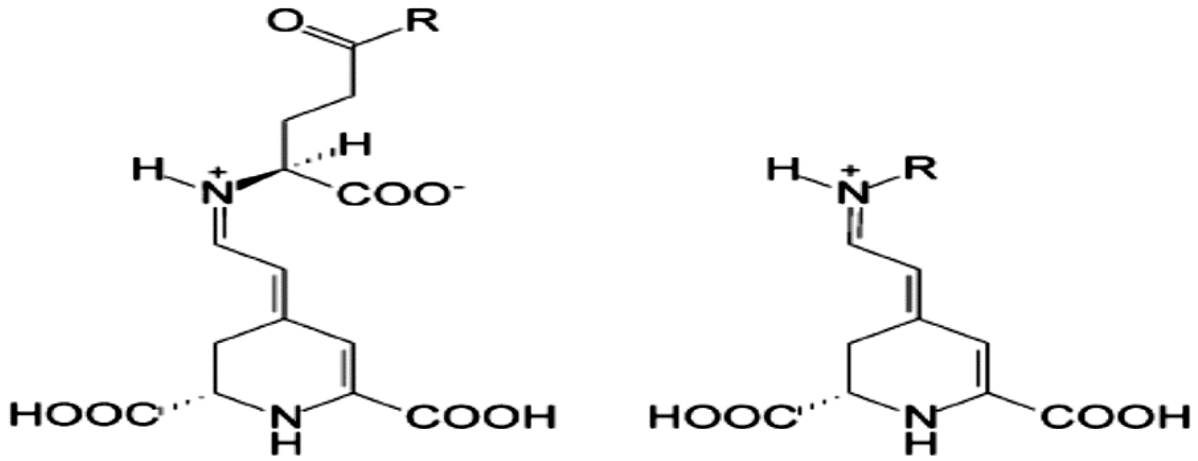


Figure 2.10: Structures of betaxanthins occurring in the hairy roots of *Beta vulgaris* (Böhm & Mäck, 2004)

Vulgaxanthin 1: R= NH2
Vulgaxanthin 2: R= OH

2.3.6.3 Amaranth

The amaranth (*Amaranthus* spp.) plant from the Amaranthaceae family consists of almost 60 species, which have been cultivated from as early as 6 700 BC. This makes amaranth one of the oldest food crops in the world. It can thrive in arid regions and is consumed as a vegetable in various parts of the world (Department of Agriculture, Forestry and Fisheries, 2014). One of the red amaranth cultivars, *Amaranthus tricolour* L. (Figure 2.11) species also contains betalains. The crop was previously known as a source of betacyanins and has recently been found to be a source of betaxanthins as well (Biswas et al., 2013).

Betacyanins are be found in the Gangetic family of *Amaranthus*; it is water-soluble and can thus be extracted using water. After extraction and drying, it presents itself as a reddish-brown powder or granule. It is closely related to carmine acid and betanin; thus, the three colourants can be used interchangeably. This vegetable is a food colourant (E 123, food red 9) that is applied in confectionery products, soft drinks, fish roe decorations, coatings and more. Its light and heat stability (stable even up to 105 °C) makes it ideal to use (Smith & Hong-Shum 2003).

Figure 2.11: Amaranthus *tricolour* leaves (Ebert et al., 2011)

2.3.3 Inorganic colourants

Examples of inorganic colourants are titanium dioxide (the whitest pigment known to man), silver and gold. The gold colourant coats the exterior of sugary confectionary and liqueurs. Silver, on the other hand, is used as a coating of products, particularly of chocolate (Mortensen, 2006; Liu & Dong, 2012).

2.3.4 Synthetic colourants

Synthetic colourants are chemically fabricated colourants that are not found in nature, but their molecules produce colour (Mortensen, 2006; Msagati, 2013). These food colourants are very useful in food manufacturing, as they make food attractive, are stable, and their functionality is widely spread from coating to colouring foodstuffs (Wang et al., 2014). Moreover, they are used to colour sweets, food products and beverages throughout the world, all of which are mostly consumed by children in high volumes (Stevens et al., 2015).

Synthetic-colourant consumption can be harmful and has been proven to have many undesirable side-effects. The medium to long-term side-effects includes allergic reactions, behavioural and cognitive effects (Martins et al., 2016). According to Masone and Chanforan (2015), and Honma (2015), other side-effects include DNA and liver damage, bone marrow damage, and attention deficit hyperactivity disorder (ADHD) among children.

According to Msagati (2013), there are five main groups of synthetic colourants:

1. azo dyes, e.g., tartrazine and carmoisine, which are used in the colouring of food, cosmetics and medication. Even low dosages of these colourants can alter the functionality of important organs, such as the liver (Amin et al., 2010);

2. quinoline (E 104), e.g., quinolone yellow: lemon yellow has good light, acid, and heat stability;
3. triarylmethanes (E 127): examples are Green S and Patent Blue. Colourings are dark blue-green and can be applied to baked products, nuts and vegetables (Smith & Hong-Shum, 2003; Dossi et al., 2007);
4. xanthenes, e.g., Erythrosine: bright pink to red, is insoluble below pH 5 and is applied in cocktail and candied cherries. Erythrosine may also be added to processed meats, such as polony; and
5. indigoid, e.g., indigo carmine (E 132): is royal blue and has very low heat and acid stability (Downham & Collins, 2000; Msagati, 2013).

2.4 Chemical structure of colourants

2.4.1 Flavonoids

Flavonoids are phenolic compounds that are synthesized from the phenylalanine amino acid and can be extracted from plants. They can be divided into the following categories: anthocyanins; flavanonols; flavanols; flavanones; flavonols; flavones and isoflavone, which are explained in Table 2.5 (Yao et al., 2004). These pigments produce a variety of colours in flower petals and can be found in fruits and vegetables, such as apples, broccoli, citrus fruits, grapes, red peppers, and yellow onions. The consumption of flavonoid-containing foods is beneficial to health as their daily intake may protect against a variety of diseases, such as viral infections and rheumatic diseases. Some flavonoids have high antioxidant levels and protect against cancer. (Balasundram et al., 2006; Simpson et al., 2012). They are generally soluble in water and have lower colour intensity than anthocyanins or anthocyanidins (Msagati, 2013).

Anthocyanins (E 163) are flavonoids that are different in colour: blue; magenta purple; violet and red. Most of its red hues are found in flowers, leaves, stems, fruits, and roots. Although anthocyanins can be used as natural colourants, their final colour is greatly influenced by pH. For instance, anthocyanins are red in acidic solutions, yet the colour fades when the pH rises (Yao et al., 2004; Scotter, 2015; Chung et al., 2017). According to El Kouari et al. (2015), anthocyaniodins are the central atoms that are responsible for giving colour to foodstuffs. In Figure 2.12 is the chemical structure of anthocyanins, and cyanidin (central atom) is R1 OH and R2 H.

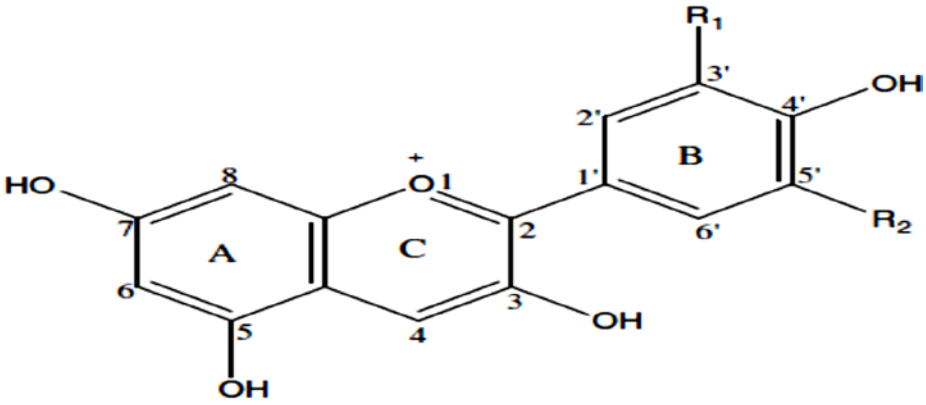


Figure 2. 12: Chemical structure of anthocyanins (Mortensen, 2006)

Table 2.5: Main subgroups of flavonoids, individual compounds and food sources (Yao et al., 2004)

Subgroup	Colour	Representative flavonoids	Food sources	Comment
Anthocyanins	Blue, red, violet	Cyanidin	Fruits and flowers	Natural dyes
Flavanols	Colourless	Catechins, gallocatechin, epicatechin, epigallocatechin, gallate	Apples, hops, tea, beer	Astringent taste
NM	Yellow	Procyanidin	Wine, fruit juice	NM
NM	NM	Theaflavins	Black tea	NM
Flavanones	Colourless	Hesperidin	Citrus fruits	Bitter taste
NM	Pale (almost colourless)	Naringenin, eriodictyol	Cumin, oranges, grapefruits, peppermint	NM
NM	Yellow	Neohesperidin	NM	NM
Flavones	Pale yellow	Apigenin, chrysin, luteolin	Herbs, cereals, fruits, parsley, thyme	Bitter taste
NM	NM	Diosmetin, luteolin	Vegetables, flowers	NM
Flavonols	Pale yellow	Isorhamnetin, kaempferol, quercetin, myricetin, rutin	Onions, cherries, apples, broccoli, kale, tomatoes, berries, tea, red wine, tartary and buckwheat	NM
Flavanonols	NM	Taxifolin	Lemon, Aurantium	NM
Isoflavones	Colourless	Daizein, genistein, glycitein, formononetin	Legumes (e.g. soybeans)	NM

NM: not mentioned in the table

2.4.2 Indigoids

Indigoids (E 132), with many names, such as indigo carmine, Blue no.2, food blue 1 and indigotine, are very old blue colourants. They were discovered in 2 000 BC, making them part of the oldest colourants used by humans that are still used to this day, more specifically in the food, clothing and pharmaceutical industries (Hendry & Houghton, 1996; Smith & Hong-Shum, 2003). It is evident that they were used in the olden days as they are still found in ancient paintings, yarns, writings and textiles. Indigoid colourants were mainly found in woad plants (*Isatis tinctoria* L.) and indigo plants (*Indigofera tinctoria* L.) (Karsli-Ceppioglu & Yurdun, 2012).

According to Msagati (2013), the main source of indigoid is beetroot. Likewise, Hendry and Houghton (1996) noted that the indigoid structure, shown in Figure 2.13, is related to betalains, which are explained in 2.2.3.

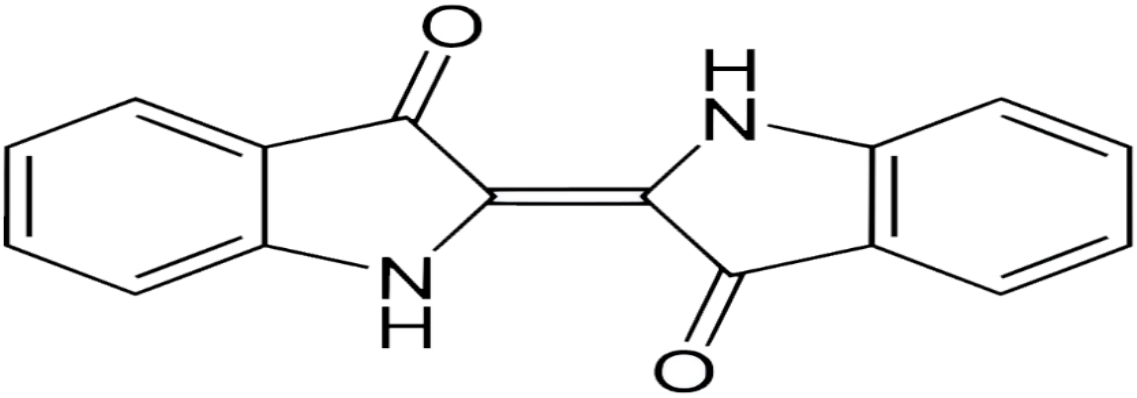


Figure 2. 13: Chemical structure of indigoid (Hendry & Houghton, 1996; Sassatelli et al., 2004)

2.4.3 Betalains

Betalains are plant-based, nitrogen-containing pigments that are divided into two mutually exclusive groups: yellow-orange betaxanthins; and red-violet betacyanins (Esatbeyoglu et al., 2015). The pigment structure and genetic make-up of yellow betaxanthins and red-purple betacyanins are very similar, making them difficult to separate; hence they are often used as they naturally occur (Chandrasekhar & Raghavarao, 2015). The distinctness in pigment subgroups is due to their structural difference, which is caused by the presence or absence of glycosylation. As shown in Figure 2.13, betaxanthins do not contain any glycosylation group, whereas either glycosylation or acyglycosylation are found in betacyanins (Polturak et al., 2016).

There are about 75 categorized betalains from ±17 families, 42 betacyanins and 32 betaxanthins (Khan & Giridhar, 2015). Betalain pigments are similar to anthocyanins; they virtually replace anthocyanins in plants, yet no species has been found to produce both pigments. Anthocyanins are derived from phenylalanine (Davies, 2015; Moghe et al., 2018), whereas the biosynthetic pathway in Figure 2.13 shows that betalains are tyrosine derivatives (Polturak et al., 2016). They are natively found in plants of the Caryophyllales order and bring out the bright colours in their flowers, stems, leaves and fruit. These plants include beetroot, amaranth, cactus pear, Swiss chard and quinoa (Azeredo, 2009; Gandía-Herrero et al., 2010; Polturak et al., 2016).

In its biosynthetic pathway, tyrosine is converted to L-3, 4-dihydroxyphenylaylanin (L-DOPA) through an unknown enzyme. The L-DOPA is converted to betalamic acid, a yellow compound. The formation of betacyanins is caused by the condensation of betalamic acid and cyclo-DOPA. When cyclo-DOPA is not available, betalamic acid condenses with other amine groups to form betaxanthins (Hatlestad & Lloyd, 2015).

Betalain pigments are non-toxic, have a bioavailability that is greater than that of many flavonoids and is more stable than anthocyanins (Tanaka et al., 2008; Suganyadevi et al., 2010). They are valuable substitutes in instances where people may be sensitive to artificial colourants; an example is that they are used to dye children's clothing (Sivakumar et al., 2009). In the food industry betalains are used to colour dairy, meat and frozen dessert. Commercially used betalains are found in beetroot, and the Cactaceae and Amaranthaceae families (Pavokovic et al., 2011).

2.4.3.1 Medicinal properties of betalains

Betalains have antimicrobial, antiviral and antioxidant properties (Tanabtabzadeh et al., 2017) and their medicinal properties include:

- protection against oxidative stress and antioxidants (Stafford, 1994; Strack et al., 2003);
- antioxidants and anti-inflammatory properties that prevent liver damage and fight against cancer properties (Piga, 2004; Georgiev et al., 2010); and
- betaxanthins from *Opuntia ficus-indica* L. can be used to fight against inflammatory bowel disease (IBD) (Tesoriere et al., 2014).

2.4.3.2 Classification of betalains

Table 2.6 shows the classification of betalains. Betanins are the most prevalent betacyanin pigments in red beetroot and purple cactus pear cultivars. They have the potential to be used as colourants in the pharmaceutical and food industries (Albano et al., 2015; Esatbeyoglu et al., 2015). The yellow betaxanthins are colourants with high potential for use in the food industry, which are found in yellow cactus pear fruit (Fernández-López et al., 2018). Vulgaxanthin I is the most prevalent betacyanin pigment, as shown in Figure 2.14. It is one of the pigments that are responsible for the red colour in betacyanins and can be found in beetroot, and it is the most widely used betalain pigment (Sawicki et al., 2016; Tanabtabzadeh et al., 2017).

Table 2.6: Betalain groups (Strack et al., 2003; Pavokovi and Krsnik-Rasol., 2011) as cited in Gengatharan et al., 2015).

Betacyanins	**Betaxanthins**
Betanin group	*Amino acid derived conjugate group*
• Phyllocactin	• Portulacaxanthin II
• 2'-apiosyl-phyllocactin	• Portulacaxanthin III
• 2'-betanin	• Tryptophan-betaxanthin
• Hylocerenin	• Tyrosine-betaxanthin
Amaranthin group	*Amine derived conjugate group*
• Amaranthin	• 3-methoxytyroamine-betaxanthin
Gomphrenin group	
• Gomphrenin class I, II, III	
• Betaninidin 6-O-sophorosides derives	

2.4.3.3 Betalain synthesis

The first step to betalain synthesis is tyrosine hydroxylation to DOPA, thus forming betalamic acid and ultimately betaxanthins. Another pathway moves from the DOPA to betanidin (found in *Beta vulgaris*). Alternatively, the pathway can follow the trend from DOPA betalamic acid and betanidin or cyclo-DOPA to cDOPA 5-0-glucoside betanin. This process is illustrated in Figure 2.14.

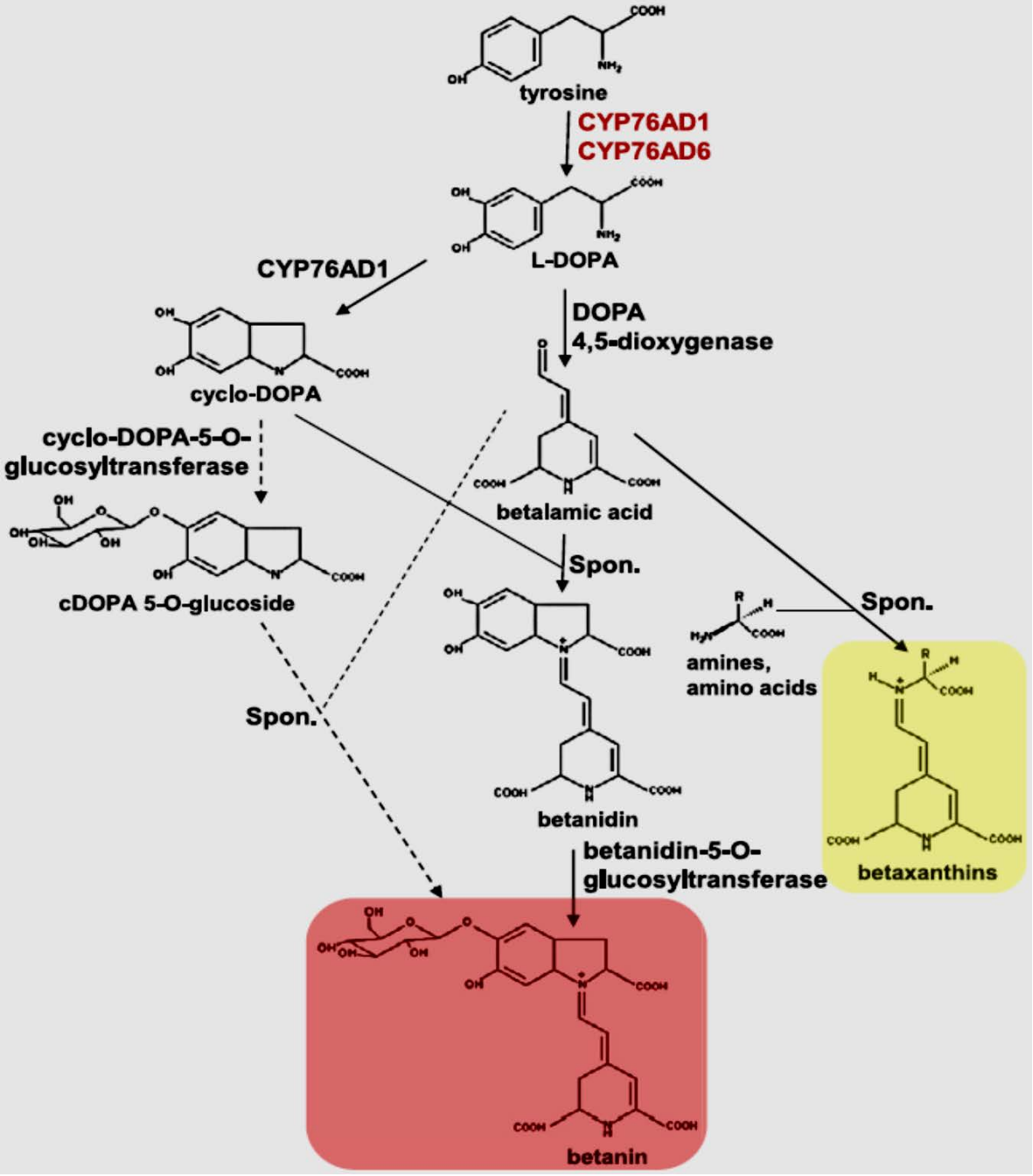


Figure 2. 14: The betalain biosynthetic pathway (Polturak et al., 2016)

2.4.4 Carotenoids

Carotenoids are fat-soluble colourants that fall under two colourant groups; natural and nature-identical (Msagati, 2013). Their biosynthesis takes place in algae, bacteria, fungi and plants. The colours derived from carotenoids include yellow, orange and red plant pigments found in raw carrots, tomatoes and oranges (Jaswir et al., 2011; Scotter, 2011a). Based on their chemical structure, carotenoids are divided into two

key classes (Jaswir et al., 2011; Scotter, 2011a; Msagati, 2013; Mezzomo & Ferreira, 2016):

(1) carotenes, which comprise of carbon and hydrogen. Examples are β-carotene (the main component of carotenoids), α-carotene and lycopene; and

(2) xanthophylls, which constitute of carbon, hydrogen, and oxygen. Examples are lutein, β-cryptoxanthin, zeaxanthin, astaxanthin, and fucoxanthin.

Carotenoids are commercially used as food colourants, feed additives, animal feed supplements, nutrient supplements and as nutraceuticals in the pharmaceutical industry (Mezzomo & Ferreira, 2016).

2.5 Conclusion

Food consumption is an interactive activity that involves, among others, the sense of sight. In fact, sight is the very first interaction that consumers have with food and plays a significant role in determining whether the prospective consumer will ultimately buy food. After the examination of food colourants and their components, how sight and colour interrelate, the benefits of natural colourants; the shift to natural colourants is necessary.

As with the demand of consumers, the food industry is looking for more natural and healthier food colouring alternatives. These include colourants, such as betalains from cactus pear, beetroot and amaranth as they offer more robust benefits to end-users. The most important aspect of natural colourants is their healthful benefits, while the proven antioxidant properties of betalains is an advantageous bonus.

CHAPTER 3

Extraction, property analysis, and application of betalain pigment extracts from beetroot and three cactus pear cultivars

Abstract

Betalain pigment extracts comprise of red and yellow betalain pigment extracts that can be found in various beetroot and cactus pear fruit cultivars. These betalain pigment extracts carry a vast array of nutritional benefits and have the potential to be used as colourants. Thus, the examination of betalain pigment extracts that carry nutritional benefits and tinctorial abilities is worth exploring.

In the current chapter, a variety of extraction methods were tested with the intention to find time-saving, high betalain yielding extraction methods that can be used for both beetroot and cactus pear fruit cultivars. The experimentation to find the best extraction methods included physical, chemical, and different temperature tests. Evaluations were done after each method to detect the effect of extraction method on betalain characteristics from beetroot (red/purple) and three cactus pear fruit (Algerian (pink/red), Gymno Carpo (orange) and Robusta (purple) cultivars. The purpose of this was to find the best methods for betalain extraction.

The stability of the betalain pigment extracts obtained from the beetroot and cactus pear fruit were analysed over a period of seven days, at different pH levels and the thermal stability was tested. The antioxidant capacity of betalain pigment extracts was tested to indicate the nutritional capacity of betalain pigment extracts. Lastly, the extracted betalain pigments were applied to food products (fruit juice and low-fat plain yoghurt) to assess the capacity of betalain pigment extracts to influence the colour and taste over time (ten days and four weeks).

Results showed that the best physical maceration tool was a liquidiser as liquidised samples (with or without AA) gave higher results than diced ones. The best chemical extraction was at 50% ethanol (EtOH), and smaller particle sizes produced higher betalain yields. The best time for stove-top extraction was 180 seconds, while microwave extraction with and without ascorbic acid (AA) was dependent on the pre-treatment of the beetroot. Beetroot and cactus pear fruit from the Robusta cultivar

produced the highest betalain yields and were more heat-stable. Beetroot and Robusta cactus pear fruit produced the highest betalain yields.

Betalain pigment extracts from beetroot coloured the juice wine-red and the yoghurt dark pink. Betalain pigment extracts from the Robusta cactus pear fruit cultivar coloured the juice a very dark red to purple colour, and yoghurt was coloured pink. Gymno Carpo cactus pear fruit (orange) coloured the juice a light orange colour and the yoghurt a very light orange colour. The Algerian cactus pear fruit (red) coloured the juice a dark orange colour and the yoghurt a very light orange colour. Thus, red/purple betalain sources (beetroot and Robusta cactus pear fruit) produced higher betalain yields, showed more stability and imparted more colour to food products.

3.1 Introduction

The use of natural colourants in functional food products provides healthful and tinctorial value to the food products (Gengatharan et al., 2015). Betalain pigment extracts offer such characteristics; these are yellow and red to purple betalain pigment extracts that are found in plants of the Caryophyllales order such as purple and yellow beetroot, as well as various cactus pear fruit cultivars and amaranth flowers (Stintzing et al., 2002; Gengatharan et al., 2015).

Betalain pigment extracts from the *O. ficus-indica* cactus pear fruit cultivars have the potential to be used as colourants in the cosmetic, pharmaceutical and food industries. In the food industry, betalain pigment extracts can be used as multifunctional natural colourants that increase health as they contain phenolic compounds. Cactus pear fruits with yellow pulp can be used to colour food products yellow. Likewise, the purple pulped fruits can impart a pink to dark red colour to food products (Cejudo-Bastante et al., 2014). Beetroot is an approved food colourant used in various pink or red food products. Moreover, both beetroot and cactus pear are bioactive compounds that are rich in antioxidants (Khan, 2016b).

The betalain colouring strength is said to be affected by intrinsic and extrinsic factors. The intrinsic factors refer to the betalain source, and the extrinsic factors refer to the effect of air and light on betalain pigment extracts (Herbach et al., 2006; Celli & Brooks, 2017). Therefore, the exploration of different extraction methods, the comparison of these methods in accordance to betalain yield, quality, and source, as well as

assessing their stability and colouring ability in food products would give an indication of intrinsic and extrinsic factors that have an influence on total betalain yields.

The aim of this chapter was to find extraction methods that are inexpensive, time-saving, non-toxic and safe. The chapter consists of preliminary methods, evaluations, and results, which were conducted and analysed in 2016.

This chapter precedes three chapters (Chapter 4-6) which go into a more in-depth analysis of the three main aspects of the study: extraction, property analysis, and application of betalain pigment extract to food products. It further entails all these aspects and provides a primary understanding of how methods would be conducted in the chapters to follow.

3.2 Materials and methods

This section consists of preliminary experiments where betalains were extracted, characterised, and applied to food products. Evaluations of the methods included quantitative evaluations, quality analysis, stability tests and food applications. Two plants were used for analysis: cactus pear, and beetroot. Beetroot was used as a control, as it was readily available, and is an approved colourant in the food industry (beetroot red/E 162). Beetroot was used in all the experiments that were conducted in the chapter.

The cactus pear fruit cultivars were more difficult to obtain and were only included in the study for evaluation purposes once the extraction methods were established. The cactus pear fruit included in the study were three cactus pear cultivars: round-shaped purple Robusta fruit, as well as oval pink/red Algerian and orange Gymno Carpo fruit. These cultivars were selected for the varying orange to purple colours to test if betalain pigment extracts can be found in different coloured cactus pear fruit and to evaluate their yield, quality, and stability characteristics.

3.2.1 Sample collection

3.2.1.1 Beetroot

Red beetroot (*Beta vulgaris* L.) of the Detroit Red cultivar was bought from a local supermarket in Bloemfontein, SA in January 2016. The samples were washed in

potable water, placed in sealable plastic bags, and kept for a maximum of seven days at 4°C before use.

3.2.1.2 Cactus pear

Three cactus pear fruit cultivars were harvested during the summer season of January 2016. These cultivars were collected from an experimental orchard at Waterkloof farm, which is located in Bloemfontein, Free State Province, SA. The cultivars were chosen to compare the different colours: Robusta (purple); Algerian (pink/red); and Gymno Carpo (orange). After harvest, cactus pear fruits were kept in sealable plastic bags and stored at -20°C until used (no longer than one month).

All fruits were harvested at their optimum ripening stage, 50% colour-break stage, when colours of the fruit were fully developed and the fruit was not overripe. The fruit was collected at this stage, as Dantas et al. (2015) stated that the content of betacyanins increases with the maturation of the fruit. Moreover, Sáenz et al. (2013) added that the fruit does not ripen after harvesting, which was the motivation for the fruit to be collected at the optimum ripening stage.

3.2.2 Methods

This section gives an outlay of all the preliminary tests that were conducted on beetroot and cactus pear fruits. The tests were done in 2016, and their overview is shown in Table 3.1. Experiments were first conducted on beetroot since it was abundantly available in local supermarkets.

Some extraction methods from beetroot extraction were not conducted in the cactus pear preliminary phases; a shortage of cactus pear fruits caused this.

All extractions took place under minimal air and light; this was achieved by eliminating artificial light, switching off air conditioners, and enclosing samples in airtight containers. The intentional limitation of air and light was done because Herbach et al. (2006) highlighted that exposure to air and light caused betalain deterioration.

Table 3. 1: Outline and overview of betalain extraction, property analysis and food application methods of beetroot and three cactus pear cultivars

Method	Beetroot	Cactus pear
Extraction		
Physical extraction	✓	✓
• Sample sizes: 0.5 x 0.5 cm^3 or 0.1 x 0.1 cm^3 or liquidized	✓	**X**
• Agitator equipment (magnetic stirrer, ultrasonic bath, and liquidiser)	✓	✓
Chemical extraction		
• EtOH	✓	**X**
• MeOH	✓	**X**
Extraction at various temperatures		
• Heating at different temperatures	✓	**X**
• Microwave extraction	✓	**X**
Microwave-assisted extraction with variations in		
• Heating time	✓	**X**
• Addition of AA	✓	**X**
• Before and after freeze-drying	✓	**X**
• Different pre-treatments	✓	**X**
Stability		
• Betalain quality stability	✓	✓
• Stability of samples at different pH levels		
• Thermal stability	✓	✓
Application of betalain pigment extracts to food products		
• Apple juice	✓	✓
• Yoghurt	✓	✓

✓: method conducted; **x**: method not conducted
All extractions were done with dH_20
Some methods were not applied in cactus pear cultivars due to shortage of samples during sample analysis

3.2.3 Physical extraction methods on beetroot and cactus pear fruit

3.2.3.1 The effect of sample size on betalain quality of beetroot samples

Samples of beetroot were divided into different sizes to test which size samples would produce the highest quality betalain pigment extracts. First, they were diced into 0.5 x 0.5 cm^3 pieces with a mechanical chopper. Secondly, the samples were cut into very small pieces (0.1 x 0.1 cm^3), using a semi-automatic Life Changing Products multi-purpose cold meat slicer. Thirdly the samples were liquidised using a Safeway hand liquidiser. Extraction of the betalain pigment extracts from the samples was accomplished by placing 100 g samples in 100 ml dH_20 in a glass cylinder for an hour. After extraction, the samples were strained through a 0.5 mm mesh-size stainless steel sieve. There was no agitation or change in samples until they were strained and the remaining liquid analysed.

The betalain quality was determined by using a Genesys 10 VIS UV-light spectrophotometer at 480 nm for betaxanthins (Bx) and 530 nm for betacyanins (Bc), betalain totals were assessed on liquid samples. Total betalain pigment extracts were determined by calculating the total Bx and Bc values.

Total betalain content was determined, according to the formulae reported by Stintzing et al. (2003):

Bx = calculation of betaxanthins at 480 nm
Bc = calculation of betacyanins at 530 nm

$$\boldsymbol{Bc}\left(\frac{\boldsymbol{mg}}{\boldsymbol{100\ g}}\right) = \frac{\boldsymbol{A\ 530 \times DF \times MW \times 1000}}{\in \times l}$$

A = Absorbance
DF = Dilution factor
MW = Molecular weight of betanin 550 g mol^{-1}
$\in$ = Molar extinction coefficients 60 000 L mol^{-1} cm in H_2O
I = Path length of cuvette = 1 cm

$$\boldsymbol{Bx}\left(\frac{\boldsymbol{mg}}{\boldsymbol{100\ g}}\right) = \frac{\boldsymbol{A\ 480 \times DF \times MW \times 1000}}{\in \times l}$$

A = Absorbance
DF = Dilution factor
MW = Molecular weight of betanin 380 g mol^{-1}
$\in$ = Molar extinction coefficients 48 000 L mol^{-1} cm in H_2O
I = Path length of cuvette = 1 cm

3.2.3.2 The effect of agitator extraction equipment on betalain quality of fresh and freeze-dried beetroot samples

Beetroot samples were diced into 0.1 x 0.1 cm^3 sample size and divided into two different groups for the following experiment. One half was freeze-dried at -20°C for

72 hours using the Labconco FreeZone® Cascade Benchtop Freeze Dry System, before the extraction procedure, and the other half was not freeze-dried (fresh samples). For the freeze-dried and the fresh beetroot extraction, 100 g of diced samples were mixed with 100 ml of distilled water (dH_20) for each process of physical extraction with different equipment. The betalain was extracted from the samples using three different agitator equipment, namely the UMC5 ultrasonic bath, a Freed Electric magnetic stirrer, and a Safeway stick blender (liquidiser).

In the first extraction process, 100 g of diced beetroot was mixed with 100 ml dH_20 in a glass cylinder, the mixture was covered with parafilm and kept on the ultrasonic bath for 10 min. In the second extraction, 100 g diced beetroot was mixed with 100 ml dH_20 in a glass cylinder; the mixture was covered with parafilm and placed in a magnetic stirrer without any heat for 10 min. In the third extraction, 100 g of diced beetroot was mixed with 100 ml dH_20, mixed in a glass jar, and liquidised with a stick blender until the sample was crushed to its finest particle sizes for 3 min.

For the evaluation of betalain pigment extracts after all three of the extraction methods, the extracted pigment was filtered through a 0.5 mm mesh size sieve. The extraction efficiency was determined by measuring the betalain yield, using a volumetric cylinder to measure yield volume. Betalain quality was determined using a Genesys 10 VIS UV-light spectrophotometer at 480 nm (Bx) and 530 nm (Bc), betalain totals were assessed on liquid samples. Total betalain pigment extracts were determined by calculating the total Bx and Bc values (Stintzing et al., 2003).

The colour strength and stability of the beetroot pigment extracts was determined by conducting colour analysis over three weeks. The samples were extracted from freeze-dried samples with distilled water and different apparatus: liquidiser; magnetic stirrer; and an ultrasonic bath. A Minolta Chroma Meter was used. The colour stability of the three maceration equipment methods was tested after one, two and three weeks. The Chroma meter expresses colour as follows:

There are three CIELAB (also known as CIE L* a* b*) colour space coordinates that indicate colour:

- L* indicates lightness of the colour, it is black at 0, whereas it shows white at 100;

- a* is an indication of green when values are negative and red when they are positive; and
- b* values are negative when blue and positive when yellow (HunterLab, 2007).

3.2.3.3 The effect of physical extraction methods on betalain quality of cactus pear fruit samples

Betalain pigment extracts from cactus pear fruit samples included purple Robusta, red/pink Algerian, as well as orange Gymno Carpo.

Cactus pears contain seeds; therefore the samples were liquidised and strained to remove the seeds. One hundred grams of cactus pear samples were mixed with 100 ml dH_20, mixed in a glass jar. Betalain quality was determined by using a Genesys 10 VIS UV-light spectrophotometer at 480 nm (Bx) and 530 nm (Bc), the tests were conducted on liquid samples. Total betalains were determined by calculating the total Bx and Bc values.

3.2.4 The effect of chemical extraction mediums on betalain quality of beetroot samples

Betalain pigment extracts are water-soluble pigments that can be extracted with water and solvents with low alcohol levels. The addition of 20-50% EtOH or MeOH has been proven to assist in maximum betalain extraction (Aberoumand, 2011; Pitelli, 1981 as cited in Ravichandran, 2013). Therefore, 10-50% EtOH and 10-50% MeOH was used to extract betalain pigment extracts from beetroot.

Preliminary tests to determine the most efficient chemical extraction solvent and pre-treatments were undertaken using Ethanol (EtOH). The most effective pre-treatment method was selected from the Ethanol tests and applied on Methanol tests.

Ethanol (EtOH) was used to test for the best physical agitator equipment (liquidiser, ultrasonic bath and magnetic stirrer) at 10%, 20%, 30%, 40% and 50% concentrations. Extraction with methanol (MeOH) was only conducted at 50% concentration, using the liquidiser as the only equipment as it was shown to be the most effective method. The ratio in the chemical extractions was 1:1 (w/v), i.e., 100 g of the 0.1 x 0.1 cm^3 diced or liquidized beetroot sample in 100 ml of the solvent (Ravichandran et al., 2013; Sanchez-Gonzalez et al., 2013).

The evaluation of chemical extraction methods was only done on beetroot due to shortage of cactus pear fruits during the time of extraction.

The extraction efficiency was determined by measuring the betalain yield, using a volumetric cylinder to measure yield volume. Betalain quality was determined using a Genesys 10 VIS UV-light spectrophotometer at 480 nm (Bx) and 530 nm (Bc), betalain totals were assessed on liquid samples. Total betalain pigment extracts were determined by calculating the total Bx and Bc values (Stintzing et al., 2003).

3.2.5 The effect of various temperature treatments on betalain quality of beetroot samples

3.2.5.1 Extraction of betalains in a water bath at different temperatures

One hundred grams of fresh diced sample material was added to 100 ml of water and heated in a Grant Y22 water bath, at temperatures of 5, 25 or 40 °C. After the temperature treatment, the samples were liquidised, centrifuged, and taken for evaluation of betalain pigment extracts.

The betalain quality was determined by using a Genesys 10 VIS UV-light spectrophotometer at 480 nm (Bx) and 530 nm (Bc) on liquid samples. Total betalain pigment extracts were determined by calculating the total Bx and Bc values.

3.2.5.2 Extraction of betalains with variations in heat treatments

Beetroot samples were cut into 0.1 x 0.1 cm^2 cubes, and freeze-dried at -20°C for 72 hours using the Labconco FreeZone® Cascade Benchtop Freeze Dry System. One hundred grams of freeze-dried beetroot samples were exposed to three heat treatments: No heat, microwave heat and stovetop heat. All the extractions were done with a 50% EtOH as extraction medium: 50 ml dH_20 and 50 ml EtOH.

(i) No heat freeze-dried beetroot samples were either diced (0.1 x 0.1 cm^3) or liquidised using a stick blender. For extraction, the samples were liquidised in 100 ml dH_20, using a stick blender for ±3 min.

Microwave-heat (100 g of 0.1 x 0.1 cm^3) diced freeze-dried samples were placed in microwave-safe dishes and heated at medium (50%) heat setting, using a Defy microwave (1000 W). The heating time was 10, 20 and 30 s. After the heat treatment, samples were liquidised in 100 ml dH_20, using a stick blender for ±3 min.

(ii) Stovetop heated (100 g of 0.1 x 0.1 cm^3) diced freeze-dried samples were placed in a stainless steel 5.5 mm thick-base double boiler with 10 ml dH_20. The samples

were heated to ± 80°C, on setting 3, for ±10 minutes, and frequently stirred with a wooden spoon. Different sample batches were heated for 60, 120 and 180 s with 90 ml dH_20, making the total added dH_20 100 ml (Ravichandran et al., 2013).
The betalain quality was determined by using a Genesys 10 VIS UV-light spectrophotometer at 480 nm (Bx) and 530 nm (Bc) on liquid samples. Total betalain pigment extracts were determined by calculating the total Bx and Bc values.

3.2.5.3 Microwave-assisted extraction of beetroot betalains with variations in heating periods, with and without the addition of ascorbic acid

One hundred grams of 0.1 x 0.1 cm^3 beetroot samples were placed in microwave-safe dishes and heated at medium (50%) heat setting, using a Defy microwave (1000 W**)** The heating time was 10, 20 and 30 s. After the heat treatment, samples were liquidised in 100 ml dH_20, using a stick blender for ±3 min. The extracted samples were immediately placed in sealable containers and cooled in ice-cold water to stop further cooking (Cardoso-Ugarte et al., 2014). For betalain extraction, the samples were liquidised and homogenised with a stick blender (Stintzing et al., 2005) and centrifuged at 9000 rpm at 4°C for 15 min.

Han et al. (1998) reported that the addition of ascorbic acid before the microwave-assisted heating of pigment samples resulted in higher pigment retention. Cardoso-Ugarte et al. (2014) also stated that microwave-assisted extraction leads to high betalain yields. In this test, the method of Cardoso-Ugarte et al. (2014) was used with minor modifications; 5 ml of a 5% AA solution was added to 100 g of diced beetroot samples (0.1 x 0.1 cm^3) before heating them in microwave-safe dishes in the microwave at medium (50%) setting, using a Defy microwave (1000 W) oven for 10, 20 and 30 s (Cardoso-Ugarte et al. (2014).
After the heat treatment, the cubes of beetroot samples were liquidised in 100 ml dH_20, using a stick blender for ±3 min. The liquidised samples were immediately placed in sealable containers and cooled in ice-cold water after their set cooking time; to stop further cooking.
In both tests, samples were centrifuged at 9000 rpm at 4°C for 15 min to obtain a clear sample for the spectrophotometer. The betalain quality was determined by using a Genesys 10 VIS UV-light spectrophotometer at 480 nm (Bx) and 530 nm (Bc) on liquid

samples. Total betalain pigment extracts were determined by calculating the total Bx and Bc values.

3.2.5.4 Microwave-assisted extraction of betalains from beetroot samples before and after freeze-drying

In the test, the effect of freeze-drying in conjunction with microwave-assisted heat treatments was tested to determine extraction efficiency. The test was conducted in two batches.

(i) Freeze-drying before microwave heat treatments: In the first batch, beetroot samples were cut into 0.1 x 0.1 cm2 cubes, and freeze-dried at -20°C for 72 hours using the Labconco FreeZone® Cascade Benchtop Freeze Dry System. One hundred grams of the freeze-dried samples were mixed with 100 ml dH_20. The samples were heated in the microwave oven for 10, 20, or 30 s; at 50% heat-setting. All tests were conducted in triplicate.

Samples were placed in sealable containers and placed in ice-cold water to prevent further cooking. For extraction, the samples were liquidised and homogenised with a stick blender and centrifuged at 9000 rpm at 4°C for 15 min. Samples were centrifuged to obtain a clear sample for the spectrophotometer.

(ii) Microwave heat treatments before freeze-drying: The second batch of samples were heated in the microwave before freeze-drying. Thus, the 0.1 x 0.1 cm^2 diced samples were heated for 10, 20, or 30 s; at 50% heat-setting, triplicate. Samples were placed in sealable containers and placed in ice-cold water to prevent further cooking. The samples were frozen at 18°C for 24 hours and subsequently freeze-dried at -20°C for 72 hours using the Labconco FreeZone® Cascade Benchtop Freeze Dry System. After freeze-drying, 100 g of freeze-dried samples were mixed with 100 ml dH_20. For extraction, the samples were liquidised and homogenized with a stick blender and centrifuged at 9000 rpm at 4°C for 15 min.

The betalain quality was determined by using a Genesys 10 VIS UV-light spectrophotometer at 480 nm (Bx) and 530 nm (Bc) on liquid samples. Total betalain pigment extracts were determined by calculating the total Bx and Bc values.

3.2.5.5 Microwave extraction of betalains with and without AA addition

Four extraction techniques were applied in the microwave oven for 10 s, two of the methods were extraction with AA and the other two without AA. This was done to test the impact of AA in the microwave when samples were heated for 10 s.

(i) 100 g of diced (0.1 x 0.1 cm^2) beetroot sample was mixed with 100 ml dH_20 and liquidised.

(ii) 100 g of diced (0.1 x 0.1 cm^2) beetroot sample was mixed with 100 ml dH_20, liquidised, and mixed with AA.

(iii) 100 g of diced (0.1 x 0.1 cm^2) beetroot sample was mixed with 100 ml dH_20.

(iv) 100 g of diced (0.1 x 0.1 cm^2) beetroot sample was mixed with 100 ml dH_20 and AA.

The samples were individually heated in the microwave for 10 s. The liquidised samples were centrifuged, and the diced samples were strained.

The Bc and Bx value were determined by using a Genesys 10 VIS UV-light spectrophotometer at 480 nm (Bx) and 530 nm (Bc) on liquid samples. Total betalain pigment extracts were determined by calculating the total Bx and Bc values.

3.2.5.6 Microwave extraction of betalains with variation in pre-treatment of samples

This test was conducted on beetroot to compare different pre-treatment methods on samples. All samples were heated in the microwave-oven at 50% setting (medium) for 10 s.

Four pre-treatments were tested:

(i) 100 g diced (0.1 x 0.1 cm^2) beetroot samples;

(ii) 100 g diced (0.1 x 0.1 cm^2) beetroot samples were mixed with 100 ml dH_20 and liquidised.;

(iii) 5 ml of a 5% AA solution was added to 100 g of diced (0.1 x 0.1 cm^2) beetroot samples and

(iv) 5 ml of a 5% AA solution was added to 100 g of diced (0.1 x 0.1 cm^2) beetroot samples and was mixed with 100 ml dH_20 and liquidised.

The samples were frozen at -18°C for 24 hours and subsequently freeze-dried at -20°C for 72 hours using the Labconco FreeZone® Cascade Benchtop Freeze Dry

System. For extraction, the diced samples were mixed with 100 ml dH_20, and all the samples were liquidised and homogenised with a stick blender and centrifuged at 9000 rpm at 4°C for 15 min. Samples were centrifuged to obtain a clear sample for the spectrophotometer.

The betalain quality was determined by using a Genesys 10 VIS UV-light spectrophotometer at 480 nm (Bx) and 530 nm (Bc) on liquid samples. Total betalain pigment extracts were determined by calculating the total Bx and Bc values.

3.2.6 Stability of betalain pigment extracts

Stability testing is an essential part of betalain production as it determines which conditions are suitable for pigment application. Delgado-Vargas et al. (2000) indicated that the determination of pigment stability could be tested on pH; temperature; light; water activity (a_w); and oxygen. Thus, the stability of betalain pigment extracts can be affected by factors such as heat, light, and air. Therefore, the stability of betalain yield and quality over time, the stability of betalain pigment extracts at different pH levels and the thermal stability were analysed in this study. The stability tests were conducted on beetroot samples and in a separate experiment on cactus pear (Robusta) fruit. Robusta fruit was selected for the stability study as both beetroot and Robusta are red-purple and would be comparable to the colour template (Figure 3.1) used in the assessment of stability tests. All pigment extractions took place under minimal air and light; both measures ensured longer betalain stability.

For stability tests on beetroot, the following pre-treatments were conducted on beetroot samples:

Treatment A: 100 g liquidized sample added to 100 ml dH_20 (1:1 dilution)

Treatment B: AA was added to 100 ml dH_20 100 g liquidised samples and microwave-heated for 10 s and freeze-dried.

Treatment C: AA was added to 100 ml dH_20 100 g liquidised samples and microwave-heated for 10 s, no freeze-drying.

Treatment D: 100 g diced (0.1 x 0.1 cm^2) sample added to 100 ml dH_20 and microwave-heated for 10 s, no freeze-drying, no added AA.

Treatment E: 100 g diced (0.1 x 0.1 cm^2) sample added to 50% EtOH and microwave-heated for 10 seconds, no freeze-drying, no added AA.

For stability tests on cactus pear fruit the following pre-treatments were conducted:
Treatment A: 100 g liquidized sample added to 100 ml dH_20 (1:1 dilution)
Treatment B:100 g liquidized sample added to 100 ml dH_20 (1:1 dilution) and microwave-heated for 10 s
Treatment C: AA was added to 100 g liquidized sample added to 100 ml dH_20 (1:1 dilution) and microwave-heated for 10 s
Treatment D: 100 g liquidized sample added to 50 ml dH_20 and 50 ml EtOH
Treatment E: 100 g liquidized sample added to 50 ml dH_20 and 50 ml MeOH

3.2.6.1 Stability of betalain quality over time

The betalain quality of beetroot and cactus pear cultivars, Gymno Carpo, Algerian and Robusta betalain pigment extracts, were measured on day one and again after seven days. The Bx and Bc absorbance was measured using a UV-VIS spectrophotometer, DrionTm AquaMate 8000 model, at 480 and 530 nm (Castellar et al., 2003; Agrawal, 2013; Ravichandran, 2013). Total betalain pigment extracts were determined by calculating the total Bx and Bc values.

3.2.6.2 Stability of betalain quality at different pH levels

The stability of betalain pigment extracts at different pH levels was tested on beetroot and cactus pears (Gymno Carpo, Algerian and Robusta) at two different pH values; this was done by adjusting the extracts to two different pH levels (1 or 4.5). Five millilitres of the extract was treated with 1 ml sodium acetate ($C_2H_3NaO_2$) to obtain a pH of 1.0, while 1 ml of potassium chloride (KCl) was added to 5 ml of extract to obtain a pH of 4.5. The solution was left for 1 hour, and the colour change (if any) was visually observed by comparing original samples with those that had chemicals added, using the colour template in Figure 3.1.

Figure 3. 1: Red colour template (Sunderberg, 2014)

3.2.6.3 Stability of betalain at various temperatures

The thermal stability of the beetroot and cactus pear cultivars (Gymno Carpo, Algerian and Robusta) extracts was tested at 25, 50, 80 and 90°C. One millilitre sample was diluted with 50 ml dH_20 and placed in a Grant Y22 water bath for 15 min (Merin et al*.,* 1987). The stability was determined through visual observation of pigment colour change.

3.2.7 Antioxidant properties of betalain extracts

Thin-layer chromatography (TLC) was used to evaluate specific antioxidant properties of the different extracts. These include Rf values for the extracts from the different samples, namely, beetroot, Algerian, Gymno Carpo and Robusta; to test for the antioxidant activity as well as to determine the presence of phenolic compounds including flavonoids. Silica plates (silica gel 60; 200 microns) were used. The TLC plates were used to obtain individual pigments (Bc and Bx) and to determine which reagent provided the best separation. Ten milligrams of the dried product was dissolved in 70 Methanol: 20 Ethyl acetate: 5 Acetic acid: 5 dH_20 to obtain individual pigments, where the stationary phase is non-polar, and the mobile phase (solvent) is polar. A small quantity of each pigment was applied several times to a plate, allowing the sample to dry. Once the sample is dry, the plate was developed at an incline in a beaker with a few millilitres of a mobile phase in the bottom of the beaker. Each plate was allowed to develop until the mobile phase was drawn ≈ 8 cm up the plate by way of capillary action.

i. Rf values were determined first to indicate the concentrations of the pigments
ii. Antioxidant activity was determined by using the DPPH (2.2-diphenyl-1-picrylhydrazyl) method, where a yellow colour change is indicative of antioxidant activity.
iii. Phenolic compounds (flavonoids) were determined using $FeCl_3$, where a grey colour change is indicative of phenolic compounds.

3.2.8 Application of betalain pigment extracts in food products

For pigment extraction, 100 ml dH_20 was combined with 100 g of individual samples of beetroot and cactus pear fruit (cultivars Gymno Carpo, Algerian and Robusta) and freeze-dried. The extracted betalain pigments were freeze-dried at -20°C for 72 hours using the Labconco FreeZone® Cascade Benchtop Freeze Dry System and crushed

to obtain the pigment powder. Beetroot and cactus pear fruit (cultivars Gymno Carpo, Algerian and Robusta) betalain pigment extracts were used to colour store-bought apple juice and low-fat yoghurt. Apple juice (Ceres) and low-fat yoghurt (Nutri-day) was obtained from a local supermarket in Bloemfontein, South Africa.

In the colouring of juice, 10 g of powdered betalain extracts from beetroot and the three cactus pear cultivars (Gymno Carpo, Algerian and Robusta) was added to 200 ml of apple juice.

To colour yoghurt, 200 g of low-fat Nutriday yoghurt was coloured using 5 g powdered betalain extracts from the beetroot and the three cactus pear cultivars (Gymno Carpo, Algerian and Robusta). The products were hand-mixed (the yoghurt was stored at 4°C).

A colour display was compiled of the actual colours of control and coloured samples of apple juice and low-fat yoghurt on the first day of the colour application. Three batches of each coloured addition were prepared.

Colour strength and stability was tested using a Minolta Chroma Meter, L*, a* and b* colour reading tests were conducted. The tests were done in triplicate.

The colour stability of juice was done once a week for a period of four weeks and that of yoghurt on day one and ten days after the betalain application.

3.2.9 Analytical design

Beetroot samples of the red Detroit cultivar were bought from local supermarkets whereas cactus pears were picked from an experimental orchard. The orchard has a total of 42 cactus pear cultivars, each with two replications. The two replications each have five trees, which makes a total of 10 trees for each cultivar (420 in total). The fruits were picked on a random basis and were randomly mixed during the extraction process.

The experimental analysis of raw scores of data was analysed on Microsoft Excel. Samples were done in triplicate, and a simplified analysis was used, where mean values were used. This process was applied because this part of the project was a preliminary study.

Standard error bars were used in the analysis of L*, a* and b* colour readings, betalain yield of cactus pear cultivars, varying temperature extraction to analyse the substantial difference in the variables.

3.3 Results and discussion

3.3.1 Evaluation of physical extraction methods on beetroot and cactus pear fruit

3.3.1.1 The effect of sample size on betalain quality of beetroot samples

Table 3.2 represents beetroot samples that were analysed after being diced or liquidised into different sample sizes. The samples that were diced into 0.5 x 0.5 cm^3 produced the least betalain pigment extracts (422.125 mg/g). The samples that were diced into 0.1 x 0.1 cm^3 pieces produced the second-highest betalain yields (472.511 mg/g), and the liquidised samples yielded the highest betalain pigment extracts (656.104 mg/g).

The results indicated that the smaller the sample size, the higher the betalain yield. Therefore, betalain yield is dependent on particle size. Based on the betalain yields, samples hereafter will be cut into 0.1 x 0.1 cm^3 sizes or liquidised before extraction.

Table 3. 2: The effect of sample size on betalain quality of beetroot samples

Sample size	**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**
Diced (0.5 x 0.5 cm^3)	132.611	289.514	422.125
Diced (0.1 x 0.1 cm^3)	135.178	337.333	472.511
Liquidised	183.410	472.694	656.104

100 g Beetroot samples extracted with 100 ml dH20. Tests were done in triplicate and the mean values are presented
Bx = betaxanthins; Bc = betacyanins

3.3.1.2 The effect of agitator extraction equipment on betalain quality of fresh and freeze-dried beetroot samples

The evaluation of the freshly-cut beetroot samples is presented in Table 3.3. The liquidiser extracted the highest yield of liquid (227 ml), whereas the ultrasonic bath and magnetic stirrer extracted 197 ml and 185 ml respectively. The Bx, Bc and total betalain yield values of betalains extracted using the liquidiser are more than ten times higher than that of ultrasonic bath and magnetic stirrer.

Interestingly, the samples which were extracted without agitation in Table 3.2, produced higher betalain yields than the ones which were extracted with the assistance of an ultrasonic bath or magnetic stirrer. This may have been caused by

agitation time (10 minutes), compared to the samples which were extracted in a cylinder without agitation for an hour. Therefore, extraction time and tool had an impact on total betalain yield.

Thus, the extraction tool (liquidiser), which transformed the samples into micro-fine sizes, extracted higher yield and quality betalain pigment extracts from the samples.

Table 3. 3: The effect of agitator extraction equipment on betalain quality of fresh beetroot samples

Treatment	Yield (ml)	Bx (mg/g)	Bc (mg/g)	Total (mg/g)
Liquidiser	227	201.483	302.041	503.525
Ultrasonic bath	197	19.999	27.958	47.957
Magnetic stirrer	185	18.893	26.889	45.782

100 g beetroot samples extracted with 100 ml dH20. Tests were done in triplicate and the mean values are presented

Bx = betaxanthins; Bc = betacyanins

The evaluation of agitator equipment analyses on freeze-dried samples is presented in Table 3.4. Results showed that the samples which were freeze-dried before extraction and then liquidised had the highest yield (214 ml) compared to the other agitator equipment. The ultrasonic bath yielded 195 ml and the magnetic stirrer 187 ml. Moreover, the total betalains produced by the liquidiser was higher than total betalains from a magnetic stirrer and ultrasonic bath. In fact, the total betalains from the liquidiser were 754.523 mg/g, whereas the ultrasonic bath (192.683 mg/g) magnetic stirrer (210.787 mg/g) were more than three times lower than liquidiser total betalains.

The total betalain pigment extracts obtained from fresh samples (Table 3.4) was lower for all three treatments. Therefore, the samples that were freeze-dried before extraction yielded the highest total betalain pigment extracts.

Table 3. 4: The effect of agitator extraction equipment on betalain quality of freeze-dried beetroot samples

Treatment	Yield (ml)	Bx (mg/g)	Bc (mg/g)	Total (mg/g)
Liquidiser	214	314.523	440.000	754.523
Ultrasonic bath	195	80.850	111.833	192.683
Magnetic stirrer	187	83.524	127.264	210.787

100 g beetroot samples extracted with 100 ml dH20. Tests were done in triplicate and the mean values are presented

Bx = betaxanthins; Bc = betacyanins

In conclusion, the comparison of Table 3.3 and 3.4 shows that the total yield of samples from the liquidiser was higher than that of an ultrasonic bath and magnetic stirrer. Secondly, the liquidiser extracted the highest quality betalain pigment extracts. Thirdly, the sample from the ultrasonic bath was more than three times less than that of the liquidiser. Fourth, freeze-dried samples yielded higher betalains than samples which were not freeze-dried. Lastly, in both freeze-dried and non-freeze-dried samples, betacyanins were higher than betaxanthin levels.

After the analysis of Table 3.3 and 3.4, the L*, a*, and b* colour strength of freeze-dried (after extraction) samples were tested over a period of three weeks. Evaluation of colour strength of physically extracted freeze-dried beetroot samples

In Figure 3.2, the L* (lightness) value of the liquidised samples remained steady throughout the weeks. Ultrasonic bath and magnetic stirrer readings were similar and higher than that of liquidised readings and constant from week one to week two, with a slight decrease from week two to week three. Thus, the liquidised samples were more stable than the ones from the ultrasonic bath and magnetic stirrer.

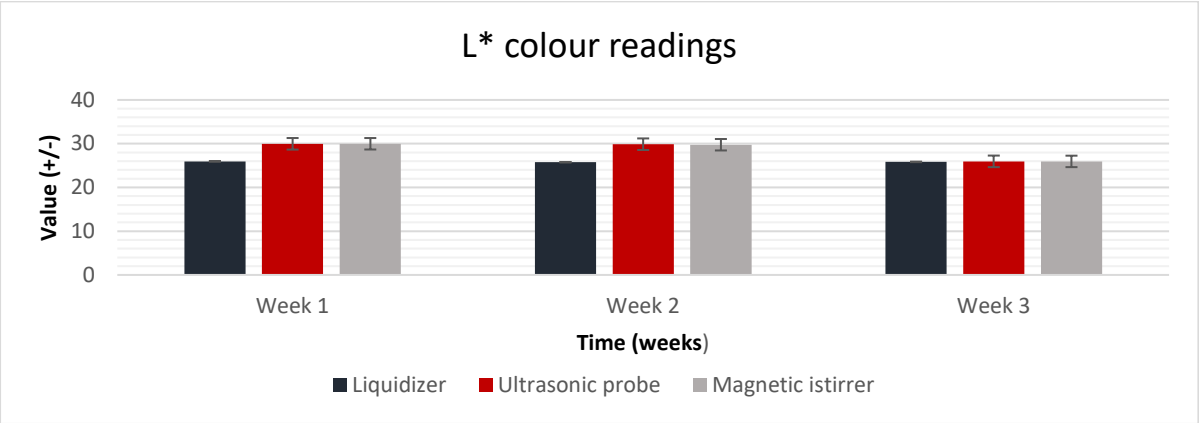


Figure 3. 2: L* colour readings of the beetroot extract
Bars representing average values of triplicate analysis with error bars indicating standard deviations

In Figure 3.3, all a* readings were positive values, which indicated a red shade in the samples. There was a small gradual decrease in liquidiser and ultrasonic bath samples, whereas the magnetic stirrer samples had a substantial decrease from week one to two while remaining steady from week two to three.

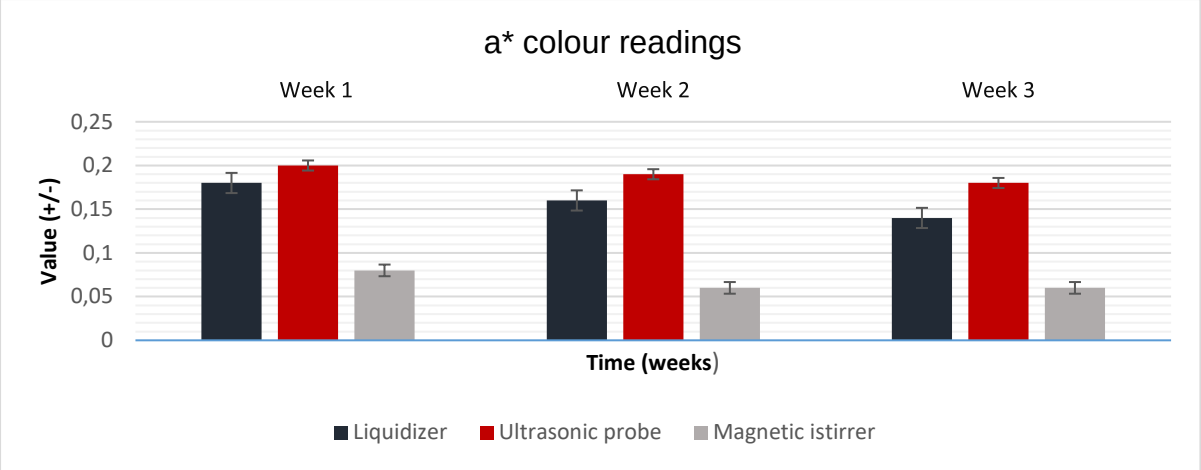


Figure 3. 3: a* colour readings of the beetroot extract
Bars representing average values of triplicate analysis with error bars indicating standard deviations

In Figure 3.4, the b* values showed a positive colour value, which reflected a yellow colour. The liquidiser and ultrasonic bath samples showed a gradual decrease from week one to three. The magnetic stirrer extract had a slight decrease from week one to two and remained steady from week two to three.

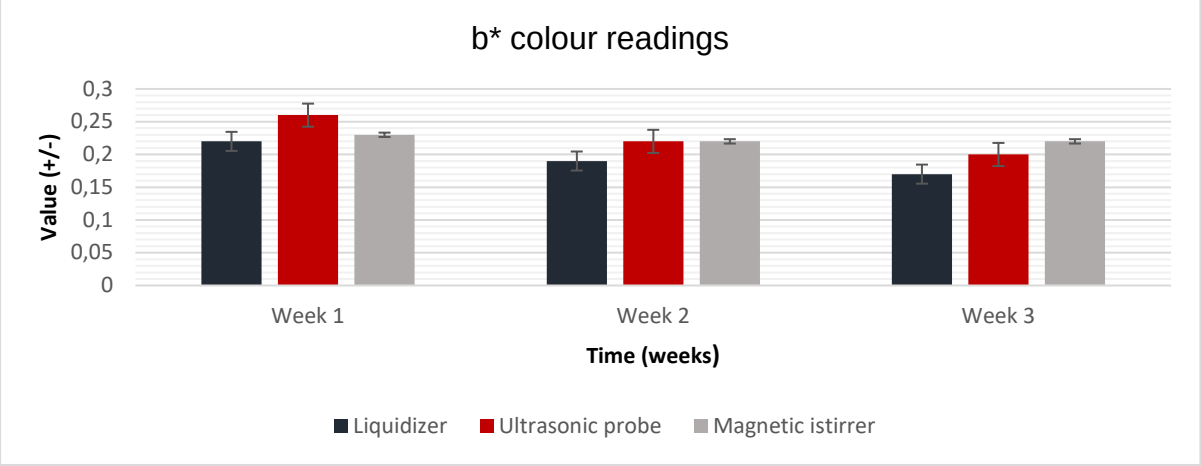


Figure 3. 4: b* colour readings of the beetroot extract
Bars representing average values of triplicate analysis with error bars indicating standard deviations

The data showed that liquidised samples were the most pigmented and stable in the L*, a*, b* results. Thus, it was deducted that the liquidiser was the best agitator equipment (Figure 3.2-4).

3.3.1.3 The effect of physical extraction methods on betalain quality of cactus pear fruit samples

Cactus pear extractions were done on Robusta (purple), Algerian (pink/red) and Gymno-Carpo (orange) cactus pear fruit cultivars, using the best physical extraction method established in freshly-cut samples, a liquidiser as the agitator equipment. The results revealed (Figure 3.5) that the Robusta cultivar (303.208 mg/g) contained higher total betalain pigment extracts than the other two cultivars namely orange Gymno Carpo (39.479 mg/g) and red Algerian (122.479 mg/g). Robusta was followed by Algerian, making Gymno Carpo the lowest betalains-containing cultivar. From these results, it could be deduced that betalain pigment extracts from red fruit contained more total betalains than those obtained from the orange fruit. Also, the darker shade of red the cultivar, which is due to higher betacyanin content, caused higher total betalains. As such, cultivar Robusta is purple or wine red and is the highest betalain containing cultivar out of the three cactus pear cultivars.

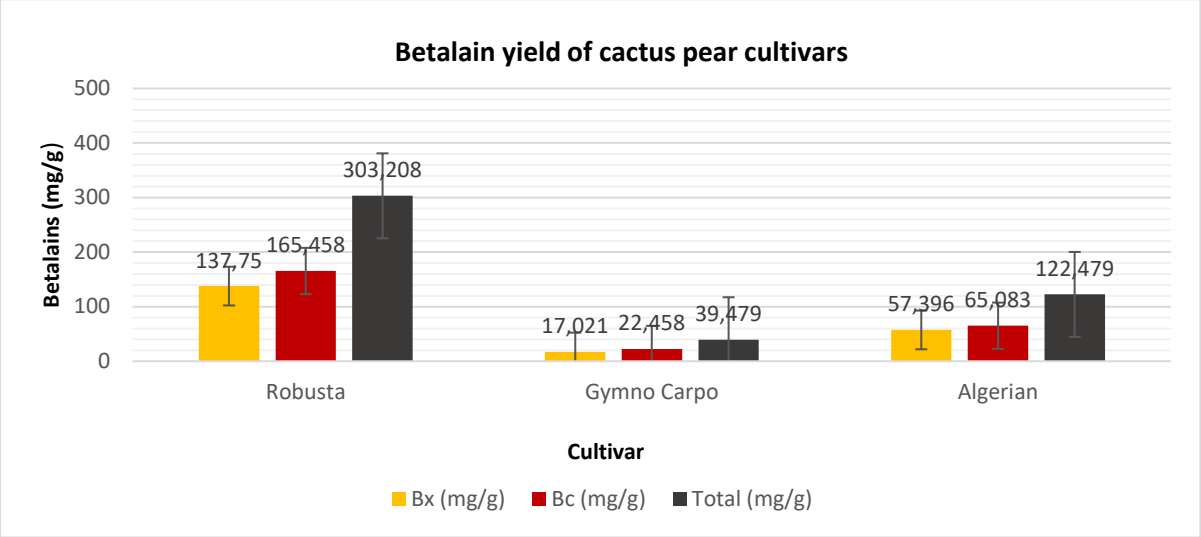


Figure 3. 5: Betalain quality of liquidised cactus pear samples
Bars representing average values of triplicate analysis with error bars indicating standard deviations

3.3.2 The effect of chemical extraction mediums on betalain quality of beetroot samples

EtOH extraction results are indicated in Table 3.5 and showed that the ultrasonic bath extraction yielded the lowest total betalain pigment extracts (37.832 mg/g) at 10% EtOH. As with other extraction equipment, the total betalain extraction increased with

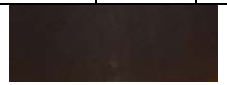
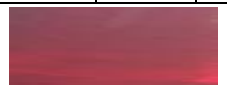
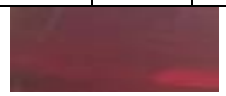
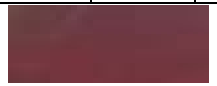
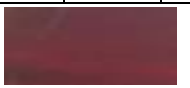
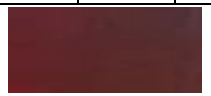
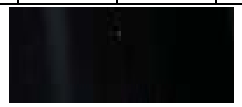
EtOH concentration increase and was optimal at 50%. Moreover, the observed colour of ultrasonic bath extracted samples were lighter when compared to other equipment. The second-highest total betalains-yielding extraction tool was the magnetic stirrer (Table 3.5), and as with the ultrasonic bath, the total betalains values increased with the increase of EtOH concentration. The colour of the magnetic stirrer extracted betalain pigment extracts looked darker than those extracts of the ultrasonic bath, yet lighter than those of the liquidiser. Notably, the pigment darkness also increased as the EtOH concentration increased.

The liquidiser crushed beetroot into small particles which enabled it to produce higher pigment volume (216-218 ml) than the other equipment. It also yielded more than twice as much total betalain pigment extracts (423.77-728.842 mg/g) than the other equipment. The colour of the material was also much darker: dark wine-red to purple hues. In fact, pictorial illustrations in Table 3.4 show betalain pigment extracts that look almost black, especially at 50% extraction.

After EtOH extraction, 10-50% MeOH extraction was conducted. This extraction was done with the assistance of a liquidiser, as it was selected after the EtOH extraction tests after the results had already proven that it was the best betalain yield-producing maceration equipment. It was observed that total betalains were higher in EtOH concentrations 10%, 20%, 30% and 40%. Using MeOH, the total betalains were only extracted at 50% concentration. Therefore, of all the MeOH samples which were extracted with a liquidiser at 10-50% MeOH levels, the highest extraction was at 50%. Consequently, it was decided that EtOH was the better solvent as it produced better results at different concentration levels. Thus, the best chemical extraction method was using EtOH as solvent at 50% concentration and a liquidizer to macerate the samples.

These results agreed with Ravichandran et al. (2013), who extracted pre-freeze-dried beetroot at different EtOH levels and found that maximum betalain extraction was at 50% EtOH. It could then be deduced that, whether freeze-dried or not, 50% EtOH extraction yielded high betalain contents.

Table 3. 5: 10-50% EtOH physical extraction of beetroot using a liquidiser, ultrasonic bath, and magnetic stirrer and 10-50% MeOH extraction using a liquidiser

Ethanol (EtOH) extraction																		
	10% EtOH				**20% EtOH**				**30% EtOH**				**40% EtOH**				**50% EtOH**			
Method of extraction	**(ml)**	**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**	**(ml)**	**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**	**(ml)**	**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**	**(ml)**	**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**	**(ml)**	**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**
Liquidiser	218	157.636	266.139	423.775	217	242.334	366.208	608.542	218	194.211	336.569	530.780	217	228.006	443.056	671.061	216	296.022	432.819	728.842
Sample pigmentation																				
Ultrasonic bath	193	16.443	21.389	37.832	184	23.742	33.917	57.658	197	68.444	96.250	164.694	197	38.072	77.764	115.836	195	100.955	143.305	244.261
Sample pigmentation																				
Magnetic stirrer	185	18.394	26.431	44.825	195	37.431	53.167	90.597	186	66.199	99.153	165.352	185	42.350	67.222	109.572	191	128.868	162.250	291.118
Sample pigmentation																				
Methanol (MeOH) extraction																				
	10% MeOH				**20% MeOH**				**30% MeOH**				**40% MeOH**				**50% MeOH**			
Treatment		**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**		**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**		**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**		**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**		**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**
Liquidiser		85.876	71.597	157.474		69.407	94.111	163.518		70.262	88.917	159.179		71.867	335.340	407.214		314.523	440.000	754.523
Sample pigmentation																				

All samples tested in triplicate and mean values are displayed in the table. Bc = betacyanins; Bx = betaxanthin

3.3.3 The effect of various temperature treatments on betalain quality of beetroot samples

Betalain pigment extracts can be extracted under different temperature conditions, with the assistance of distilled water (Pitelli, 1981 as cited in Ravichandran, 2013). In the current section, betalain strength was tested with betalain pigment extracts that were extracted under low temperatures, such as 5 °C and higher temperatures up to ±80 ˚C, with the assistance of a microwave oven and stovetop.

3.3.3.1 Extraction of betalains in a water bath at different temperatures

In Figure 3.6, the beetroot samples were exposed to temperatures of 5, 25 and 40 °C in a water bath. The betaxanthin (11.229 mg/g) values at 5°C were lower than betacyanin (28.875 mg/g) values. The differences in betaxanthin and betacyanin values were even higher at 25 °C extraction: 14.117 mg/g for betaxanthin and 46.292 mg/g for betacyanin. However, only a slight difference was noticed between betaxanthin and betacyanin yields at 40 °C extraction. The betaxanthin yield at this temperature was also higher than that of betacyanins with 17.967 mg/g (betaxanthins) and 15.125 mg/g (betacyanins), respectively. Thus, the results showed that optimal betalain extractions took place at 25 °C, followed by 5°C with the lowest extraction taking place at 40 °C.

These results were contrary to a study conducted by Roy et al. (2004), where the optimal betalain extraction for beetroot was 40 ˚C. In fact, 40 °C in the present study was the lowest betalain extraction temperature.

The optimal extraction temperature (25 °C) had an energy-saving advantage since the water did not need to be heated before extraction.

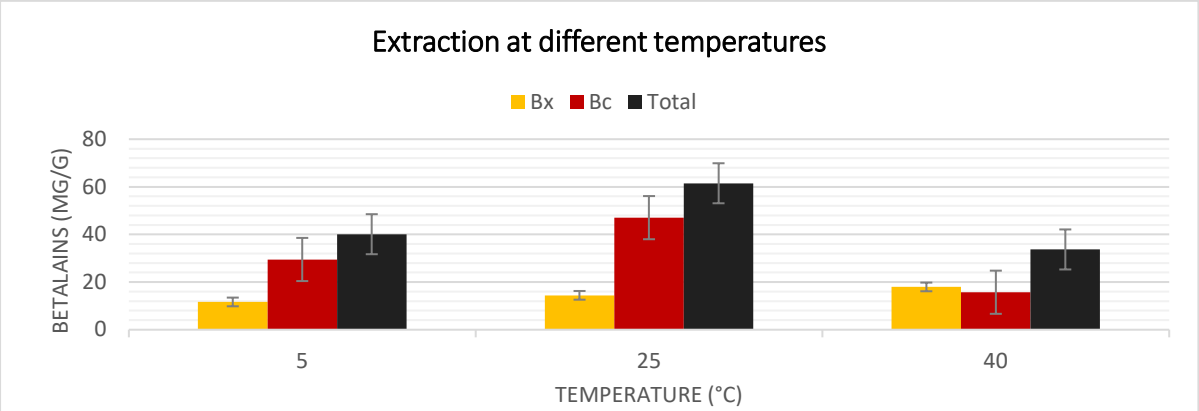


Figure 3. 6: Extraction of betalains in a water bath at different temperatures
Bc = betacyanins; Bx = betaxanthins; Bars representing average values of triplicate analysis with error bars indicating standard deviations

3.3.3.2 Extraction of betalains with variations in heat treatments

Heat treatments using the microwave and stovetop extraction methods of betalain pigment extracts lead to betalain degradation (Herbach, 2006). However, during the betacyanin extraction of dragon fruit (*Hylocerious polyrhizus*) peel, which was pre-treated with Bonomyl and liquid nitrogen, the resultsshowed the highest extraction yield at 100 °C for 5 min in a pH 5 citric acid solution. Furthermore, the roasting of betalain samples for 5 min by Ravichandran (2013) also led to a 7% betaxanthin increase. However, boiling samples for 60, 120 and 180 s led to betalain yield decrease.

The extraction of betalains using ethanol as extraction medium extracted using no heat, exposed to microwave heat (10 s, 20 s, and 30 s) and stovetop heat (60 s, 120 s and 180 s) is observed in Table 3.6. The stove-top heated samples had the highest total betalains. There were no significant differences in total betalain yield for stove-top heated samples at 60, 120 and 180 s. Nevertheless, the total yields at 180 seconds extraction produced the highest amounts of betalain at 1 475.656 mg/g for the extraction and the lowest at 60 s at 1 433.526 mg/g. Notably, the Bc results were higher than Bx under all conditions.

Table 3. 6: Extraction of betalains using ethanol as extraction medium with different heat treatments

Treatment	Bx (mg/g)	Bc (mg/g)	Total (mg/g)
No heat, cut in cubes	352.596	907.042	1 259.638
No heat, liquidised	379.225	990.001	1 369.225
Microwave (10 s)	370.883	1 060.583	1 431.466
Microwave (20 s)	337.838	917.583	1 255.421
Microwave (30 s)	356.767	984.500	1 341.267
Stove-top (60 s)	446.279	987.250	1 433.529
Stove-top (120 s)	460.075	999.625	1 459.700
Stove-top (180 s)	472.331	1 003.325	1 475.656

Tests done in triplicate; mean values presented
Bc = betacyanins; Bx = betaxanthins

3.3.3.3 Microwave-oven assisted extraction of beetroot betalains with variations in heating periods, with and without the addition of ascorbic acid

Comparable to this current study is research conducted by Cardoso-Ugarte et al. (2014), where diced, freeze-dried red (*Beta vulgaris*), beetroot samples were placed

in a microwave oven at 400 watts for up to 160 s, and ascorbic acid (AA) added. A 100% duty cycle yielded the highest betaxanthin extraction at 140-150 s and betacyanins at 190-120 s. The addition of heat can increase the betalain yields of samples. Thus, although heat has been reported to add to betalain degradation, it may, in fact, aid to obtain a higher concentration of total betalains. According to literature, betalain extractions were more efficient with the assistance of the microwave oven, and the addition of ascorbic acid (AA). Han et al. (1998) reported that the addition of ascorbic acid before microwave-assisted heating of pigment samples resulted in pigment retention. Experiments conducted by Cardoso-Ugarte et al. (2014) also showed that the addition of ascorbic acid to samples leads to high betalain extraction. Moßhammer et al. (2006a) added 0.1 % of ascorbic acid before heating yellow-orange cactus pears and reported that the betacyanin and betaxanthins stability increased.

The addition of ascorbic acid to microwave heated betalain pigment extracts also prevented quick degradation of betalain pigment extracts and caused rapid extraction of the pigment (Han et al., 1998). Ascorbic acid addition in the current research may be incomparable to that of Cardoso-Ugarte et al. (2014), as their microwave oven was set at 400 watts, while the heating time and temperatures varied. Ravichandran et al. (2013) agreed that the pre-treatment of beetroot had an impact on the betalain stability thereof. According to Ravichandran et al. (2013), beetroot samples were first freeze-dried before any treatment. Thus, the addition of ascorbic acid, the effect of freeze-drying and combinations of different pre-treatments were tested in this study and results are shown in Tables 3.7, 3.8, 3.9 and 3.10.

Table 3. 7: Microwave-assisted extraction of betalains with variations in heating periods, with and without the addition of AA

	No added AA				Added AA			
Treatment	**dH_20**	**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**	**dH_20 + AA**	**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**
Microwave (10 s)		154.21	402.79	557.00		213.29	329.87	543.17
Microwave (20 s)		174.88	383.99	558.88		231.12	350.21	581.44
Microwave (30 s)		183.22	413.17	596.40		172.03	322.79	494.82

Tests done in triplicate; mean values presented in the table

It was observed in Table 3.7 that the highest total betalains were found at 30 s without AA (596.401 mg/g). The 10 s (557.004 mg/g) and 20 s (558.880 mg/g) extractions without AA produced very similar results. The colour of the extracted samples was dark purple, which could reflect high betacyanin presence. Betacyanin values were high at all heating exposure periods without AA: 402.790 mg/g, 383.998 mg/g and 413.174 mg/g for 10, 20 and 30-s of heat exposure periods respectively. However, in Table 3.7 the highest total betalains in added AA samples were found at 20-s heat exposure (581.440 mg/g) and the lowest at 30 s heat exposure (494.826 mg/g). The colour of extracted samples was lighter than that of samples which did not have AA; thus, the addition of AA resulted in lighter shades of the pigment.

In conclusion for the microwave-assisted test at different temperature with and without AA (Table 3.7 and Table 3.8), the samples with and without AA had higher betacyanin yields than betaxanthins, and the colour of the samples without AA was darker than those with AA. The addition of AA altered the colour of the samples, and at 10 s and 20 s (in certain conditions) increased total betalains, possibly by aiding in betalain stability during the heating process. The total betalain yields were at their highest at 20 s extraction (581.440 mg/g), and at their lowest at 30-s extraction (494.826 mg/g).

In conclusion for microwave-assisted extraction with and without AA, the total betalain yields were comparable in 10 and 20 s extractions. However, vast differences can be seen in 30 s extractions, where betalain samples with added AA (Table 3.6) were lower than samples without AA (Table 3.6). The colour of the samples that were extracted with ascorbic acid (AA) was not as dark as the ones without AA. The colour of samples without ascorbic acid was wine red, while the samples with AA were scarlet red (this is in reference to the red colour guide in Figure 3.1).

3.3.3.4 Microwave-assisted extraction of betalains before and after freeze-drying

The data from Table 3.8 showed that samples which were microwave heated before freeze-drying produced higher betalain yields (almost double) than samples which were freeze-dried before microwave-heating. Samples which were freeze-dried before microwave heating produced the highest betalain pigment extracts at 10 s (321.793 mg/g); the lowest yields were at 30 s extraction (135.543 mg/g). Highest yields were found at the 30 s extraction (627.443 mg/g) for samples microwave heated before freeze-drying.

Table 3. 8: Microwave-assisted extraction of betalains before and after freeze-drying

	Freeze-drying before microwave heat treatments			Microwave heat treatments before freeze-drying		
Treatment	Bx (mg/g)	Bc (mg/g)	Total (mg/g)	Bx (mg/g)	Bc (mg/g)	Total (mg/g)
Microwave (10 s)	183.834	137.959	321.793	164.802	440.305	605.107
Microwave (20 s)	70.476	104.958	175.434	169.721	449.472	619.193
Microwave (30 s)	61.600	73.943	135.543	167.582	459.861	627.443

Tests done in triplicate; mean values presented in the table
Bc = betacyanins; Bx = betaxanthins

3.3.3.5 Microwave assisted extraction of betalains with and without AA

In test 6, the effect of the addition of AA, with microwave-assisted extractions at 10 s were tested. The time of extractions was standardized at 10 s.

Table 3. 9: Microwave-assisted extraction of betalains with and without AA additions

Treatment	Bx (mg/g)	Bc (mg/g)	Total (mg/g
10 s in microwave + liquidised only	378.813	260.792	639.605
10 s in microwave + liquidised + AA	167.688	330.917	498.605
10 s in microwave + diced (0.1 x 0.1 cm^2)	296.479	280.042	576.521
10 s in microwave + diced (0.1 x 0.1 cm^2) + AA	170.063	163.708	333.771

Tests done in triplicate; mean values presented in the table. Bc = betacyanins; Bx = betaxanthins

The general trend in Table 3.9 was that samples which had added AA produced lower betalain yields than ones without AA. Liquidised samples also produced higher betalain results than diced samples. In fact, the highest yields were found in liquidised samples which were heated without AA (639.605 mg/g). The lowest contents were found in diced samples which had AA (333.771 mg/g).

The results show that AA may not lead to higher betalain extraction at 10 s extraction. Therefore, the contributing factors were time and the addition of AA, with the AA results not showing the ability to increase betalain strength.

3.3.3.6 Microwave-assisted extraction of betalains with variation in pre-treatment of samples

This test was conducted on beetroot to compare different pre-treatments of samples. All samples were heated in the microwave-oven at 50% setting (medium) for 10 s.

Values in Table 3.10 showed that liquidised samples yielded higher amounts of total betalain pigment extracts than diced beetroot samples. Therefore this is evidence that increasing the total surface area yielded higher betalain quality. In fact, the results of liquidised beetroot are more than double that of diced samples.

The microwave-assisted extractions showed better results when liquidised. Interestingly, liquidised samples without AA (848.71 mg/g) produced higher total betalains than samples that had added AA (597.147 mg/g).

Thus, the most efficient extraction at various temperature treatments was 10 s extraction when samples were liquidised.

Table 3. 10: Microwave-assisted extraction of betalains with variations in pre-treatments

Pre-treatment of samples	**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**
10 s in microwave + AA + liquidized	230.786	366.361	597.147
10 s in microwave + AA + diced (0.1 x 0.1 cm ^{2})	130.900	193.264	324.164
10 s in microwave diced + liquidized	336.447	512.264	848.711
10 s in microwave+ diced (0.1 x 0.1 cm ^{2})	137.316	208.084	345.400

Tests done in triplicate; mean values presented in the table. Bc = betacyanins; Bx = betaxanthins

3.3.4 Stability of betalain pigment extracts

Table 3.12 demonstrates a comparison of betalain pigment extracts that were extracted from beetroot samples under different conditions and analysed after different time frames. The first group of betalain pigment extracts were analysed immediately after extraction. The second group was betalain pigment extracts that were extracted and kept in the refrigerator at 4°C for seven days and subsequently analysed on the seventh day. The purpose of the experiment was to test the stability of betalain pigment extracts, pH and heat after one and seven days, to indicate the best stability conditions between the two different testing times.

Values for treatment A (dH_20 (1:1) dilution) on day one was 29.02% higher than that of betalain pigment extracts analysed after seven days. The pH stability of samples

analysed after the different times was similar: at pH 1, all samples changed to a darker colour, which indicated that they were unstable. All samples at pH 4.5 were stable and did not change in colour, proving what was stated by Stintzing & Carle (2004), that betalain pigment extracts are stable between pH 3.5-7. The degradation of heated samples was very similar; the colour of samples decreased with increased heat.

The results for treatment B was poor (93.179 mg/g); thus the test was not repeated after 24 hours. For treatment C samples, the total sum of betalain pigment extracts that were analysed after one day (175.969 mg/g) was almost half as that of samples which were analysed after seven days (268.385 mg/g). This may be due to degradation of betalain pigment extracts during storage. The samples were kept at 4 °C, and although the keeping temperature was low, it might still not be adequate for betalain stability. The pH of samples was similar: unstable (darker scarlet red than control) at pH 1 and stable at pH 4.5. Thermal stability results were also similar; notably, samples that contained AA had a lighter shade of red (scarlet red). Although the pigmentation became lighter with increased heat, the initial colour of samples did not change. Accordingly, samples changed from scarlet red to very light red.

Treatments D and E, in Table 3.12 differed, the general trend between all these samples was that they were unstable at pH 1, yet stable at pH 4.5. The trend is similar to that of samples of treatment A where samples changed from wine red to brick red. In the case of AA added samples (treatments B and C), the betalain pigment extracts became lighter: from scarlet red to very light scarlet red. Even samples which were extracted with ethanol changed from brick red to very light brick red. The conclusion would then resonate with that of Reynoso et al. (2007), who stated that the addition of 1% acetic acid or ascorbic acid (AA) assists in the stability of betalain pigment extracts.

Betalain pigment extracts that were kept in the refrigerator for seven days had a stronger, earthy-like aroma, which could be caused by geosmin reactions in beetroot (Lu et al., 2003). The samples were covered in black plastic bags in a refrigerator, where the light was switched off, so as to diminish light permeability and enhance betalain stability. Additionally, the colour of the supernatant was also darker than that of freshly liquidised sample.

Table 3. 11: Stability of beetroot betalainswith different pre-treatments analysed after 1 day and 7 days

		DAY 1										DAY 7									
		Betalain quantification			pH stability			Thermal stability				Betalain quantification			pH stability			Thermal stability			
	Treatment	Bx (mg/g)	Bc (mg/g)	Total (mg/g)	Initial pH	Colour change at pH 1	Colour change at pH4.5	25 ˚C	50 ˚C	80 ˚C	90 ˚C	Bx (mg/g)	Bc (mg/g)	Total (mg/g)	Initial pH	Colour change at pH 1	Colour change at pH4.5	25 °C	50 ˚C	80 ˚C	90 ˚C
Treatment A	Liquidised, +: dH_20	67.696	148.500	216.196	5.320	Unstable dark red	Stable 4.51	Wine red	Wine red	Brick red	Very light Brick red	49.408	104.042	153.450	5.55	Unstable dark red	Stable 3.57	Wine red	Wine red	Brick red	Very light Brick red
B	Liquidised + dH_20H +AA + M10 s + FD	31.762	61.417	93.179	4.730	Unstable Dark red	Stable 4.46	Wine red	Light wine red	Brick red	Very light brick red	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
C	Liquidised + dH_20 + AA + M10 s (no FD)	89.940	178.445	268.385	5.900	Unstable dark red	Stable 4.45	Scarlet red	Light scarlet red	Very light scarlet red	Very light scarlet red	60.317	115.653	175.969	5.46	Unstable dark red	Stable 4.68	Scarlet red	Scarlet red	Light scarlet red	Very light Scarlet red
D	Diced + dH_20 + M10 s + No AA, no FD	28.768	60.653	89.421	5.420	Unstable Dark red	Stable 4.45	Wine red	Wine red	Sangria red	Brick red	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM
E	Diced + 50% EtOH + M10 s No AA, no FD	39.676	81.583	121.260	5.630	Unstable Dark red	Stable 4.45	Wine red	Wine red	Sangria red	Brick red	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM

Tests done in triplicate; mean values presented in the table
All samples 100 g, M10 s indicates microwave heating for 10 s, AA implicates Ascorbic acid added, FD for Freeze-dried
NM: Not Measured
Bc = betacyanins; Bx = betaxanthins

Table 3.4 demonstrates a comparison of betalain pigment extracts that were extracted from cactus pear fruit cultivar Robusta samples under different conditions and analysed after different time frames. Thermal and pH stability of differently pre-treated Robusta samples were conducted in order to test betalain stability under different treatment conditions. Moreover, Table 3.11 precedes Table 3.12, where beetroot betalain extract stability was also tested against various conditions, and the two betalain sources were compared against each other.

The second point of comparison was pH stability and the results were similar to those found for beetroot: unstable at pH at 1, and stable at pH 4.5. Thermal stability also showed similar results to beetroot samples: samples had a wine-red control and ended up to be a light peach colour at 90 °C. The colour change was different from that of beetroot, which turned brick red (close to brown).

Microwave-extracted samples showed that samples with AA had lower betalain yields than samples without AA. These findings are contradictory to those of Cardoso et al. (2014), who stated that the addition of AA adds to betalain stability. However, it is important to note that the extraction time was 10 s. A longer extraction time may be required for more conducive results for betalain extraction. Moreover, experiments by Cardoso et al. (2014) were conducted on beetroot, and the current experiment was on cactus pear.

Table 3.12 further shows that thermal stability tests showed better stability of samples without AA: the control of samples that contained AA was scarlet red, while those without AA were wine red. After maximum heating at 90 °C, samples containing AA were a very light peach colour, while those without AA were a light peach colour. Notably, beetroot did not turn peach in colour when heated; instead, it turned a brick red (close to brown) colour.

In the comparison of EtOH and MeOH extraction data, ethanol showed the highest betalain yield. In fact, EtOH extraction yielded the highest betalain yield of all the treatments in Table 3.12. In pH stability tests, all samples were unstable at pH 1 and stable at pH 4.5. In thermal stability tests, both MeOH and EtOH extracted samples had a wine-red control and ended up a light peach colour at 90 °C.

Table 3. 12: Stability of Robusta betalains with different pre-treatments

		Betalain yield			pH stability			Thermal stability			
	Treatment	**Bx (mg/g)**	**Bc (mg/g)**	**Total (mg/g)**	**Initial pH**	**Colour change at pH 1**	**Colour change at pH 4,5**	**25 ˚C**	**50 ˚C**	**80 ˚C**	**90 ˚C**
A	100 ml dH_20	102.667	148.195	250.861	4.91	Dark purple unstable	4.49 Stable	Wine red	Pink	Peach	Light peach
B	100 ml dH_20 + 10 s	106.089	152.319	258.408	4.57	Dark purple unstable	4.52 Stable	Wine red	Pink	Peach	Light peach
C	100 ml dH_20 + 10 s + AA	60.423	87.083	147.507	4.61	Dark purple unstable	4.5 Stable	Scarlet red	Dark peach	Light peach	Very light peach
D	50 ml dH_20 + 50 ml MeOH	99.031	146.514	245.545	4.75	Dark purple unstable	4.51 Stable	Wine red	Pink	Peach	Light peach
E	50 ml dH_20 + 50 ml EtOH	119.243	172.028	291.271	4.76	Dark purple unstable	4.5 stable	Wine red	Pink	Peach	Light peach

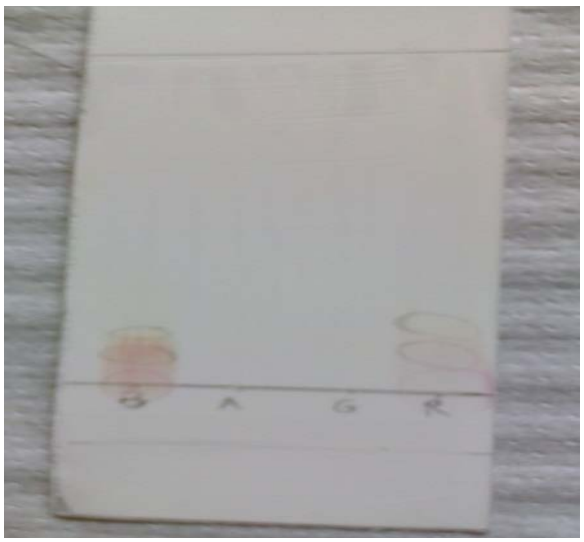
Tests done in triplicate; mean values presented in the table
Bc = betacyanins; Bx = betaxanthins
100 g liquidised sample used

3.3.5 Antioxidant properties

Constituents that are responsible for the pigmentation of plant material contain antioxidants (Schwartz et al., 2008). The betalain content that was found in beetroot or cactus pear differed according to cultivar (varieties) and were depended on different parts, such as the peel of the fruit and the colour of the root or fruit which were analysed. The categorised analysis demonstrated that outer layers, including the peel of the fruit, contain the highest betalain content. In addition, the antioxidant capacity of betalain-carrying fruit correlates with the betalain capacity thereof (Du Toit et al., 2015; Sawicki et al., 2016).

The TLC plates (Figure 3.7) were used to obtain individual pigments (Bc and Bx) and to determine which reagent provided the best separation.

Figure 3. 7: TLC plate



Lane 1 (A) Algerian, Lane 2 (B) Beetroot, Lane 3 (G) Gymno Carpo, Lane 4 (R) Robusta

Figure 3. 8: Antioxidant activity

Figure 3. 9: Phenolic compounds

i. Rf values were determined first to indicate the concentrations of the pigments. The separation of individual pigments and calculation of Rf values are shown in the calculations below:

Robusta

$$= \frac{distance\ moved\ by\ spot(cm)}{distance\ moved\ by\ solvent\ (cm)}$$

$$= \frac{0.6}{6.3}$$

=0.0952

=0.0635

Beetroot

$$= \frac{distance\ moved\ by\ spot(cm)}{distance\ moved\ by\ solvent\ (cm)}$$

$$= \frac{0.4}{6.3}$$

=0.0635

Algerian

$$= \frac{distance\ moved\ by\ spot(cm)}{distance\ moved\ by\ solvent\ (cm)}$$

$$= \frac{0.7}{6.3}$$

=0.1111

Gymno Carpo

$$= \frac{distance\ moved\ by\ spot(cm)}{distance\ moved\ by\ solvent\ (cm)}$$

$$= \frac{0.6}{6.3}$$

=0.0952

Thus, the results showed that the intensity of beetroot and Robusta was prominent and indicated higher content of betacyanins. However, the Rf values results indicated higher levels of Bx in Algerian and Gymno Carpo.

ii. Antioxidant activity

Figure 3.8 showed that all the samples (beetroot, Algerian, Gymno Carpo and Robusta) contained antioxidants. The antioxidant levels differed among the different cultivars. In Figure 3.8, the highest values were observed for beetroot (lane 1) and Robusta (lane 4). Very little activity was observed for Algerian and Gymno Carpo.

iii. Test for phenolic compounds (flavonoids)

In Figure 3.9 it was observed that the beetroot (lane 1), Gymno Carpo (lane 3) and Robusta (lane 4) had much higher phenolics levels than Algerian (lane 2). In fact, the values and intensities for Beetroot, Gymno Carpo and Robusta were very similar.

3.3.6 Application of betalain pigment extracts in food products

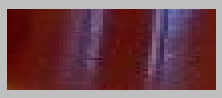
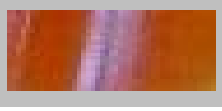
The application possibilities of using betalain extracts from beetroot (Table 3.10) and cactus pear were evaluated by their addition to store-bought apple juice and low-fat plain yoghurt. The colour of the products was evaluated on day one as well as after ten days of refrigerated storage for yoghurt and four weeks for juice.

According to the results in Table 3.13, the juice colour was darker for all cultivars, especially for Robusta. The Algerian cultivar was a deep orange and beetroot a dark red colour. The most pigmented yoghurt was the one coloured with beetroot. The Gymno Carpo samples were light orange, almost white.

The colour of the juice changed very quickly after the betalain extracts were dispensed into it. The apple juice with beetroot betalain extracts had a very earthy aroma; on the other hand, the juice with Robusta betalain extracts did not have a strong aroma; however, the taste was like that of the apple juice with beetroot betalain extracts. The colour of juice with Robusta betalain extracts was much darker than that of apple juice with beetroot betalain extracts. The Algerian coloured apple juice was not sufficiently pigmented and looked more dark orange than pink/red. Gymno Carpo betalain extracts produced a yellowish colour, which did not have a specific taste.

For coloured yoghurt samples, the beetroot betalain extracts produced a very dark-pink colourant, much darker than any yoghurt which is already available in the industry. Contrastingly, Robusta-coloured yoghurt was a light pink, very similar to that which is already available in the industry. All the other yoghurts were very light, no distinguishable/noticeable colour, while the control (uncoloured yoghurt) was white.

Table 3. 13: Colour display of apple juice and low-fat yoghurt coloured with freeze-dried betalain pigment extracts

Cultivar	Juice	Low-fat yoghurt
Control		
Robusta		
Beetroot		
Gymno Carpo		
Algerian		

Background coulour changed to better accommodate the colour of samples

After adding the betalain extracts to the juice and the low-fat yoghurt, L*, a* and b* colour reading tests were conducted. Colour was determined on coloured apple juice over four weeks (Figure 3.10 - 3.12), while the colour readings of low-fat yoghurt were done on day one and ten after the addition of betalain extracts (Figure 3.13 – 3.15).

In Figures 3.10 – 3.12the colour stability of control and coloured apple juice are observed. There was stability in the lightness in coloured apple juice in the first three weeks. In a* readings, all the samples showed redness in them, with Gymno Carpo showing a noticeable difference between week one and four. There was also a decreasing trend for all cultivars from week one to four. The b* samples all showed yellowness, with a general decline in all cultivar colours from week one to four (Figure 3.10).

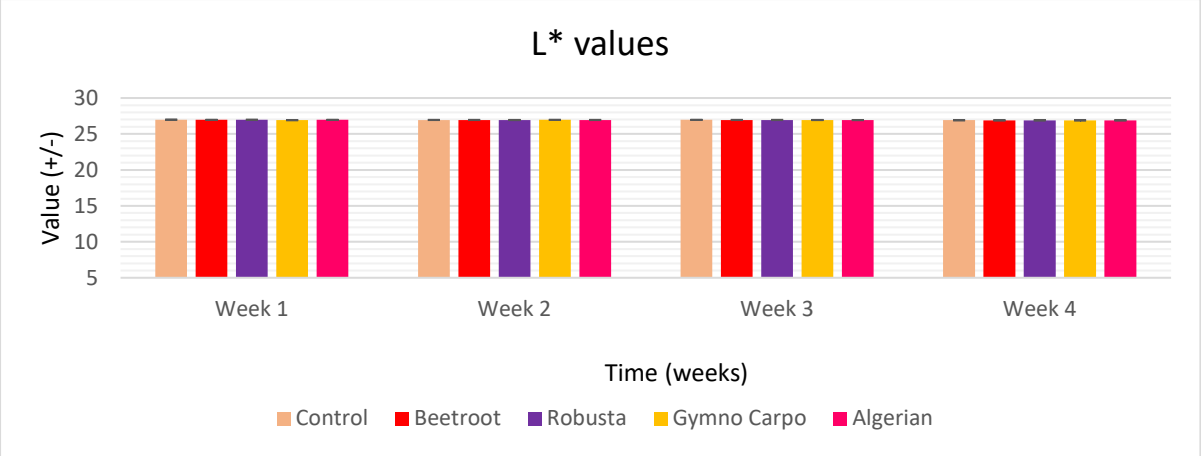


Figure 3. 10: L* juice colour readings of betalain pigment extracts
Bars representing average values of triplicate analysis with error bars indicating standard deviations

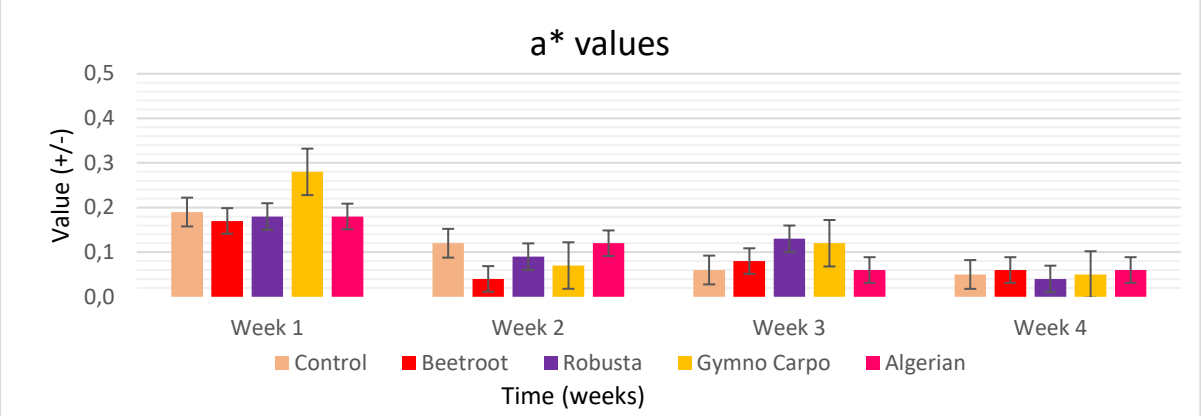


Figure 3. 11: a* juice colour readings of betalain pigment extracts
Bars representing average values of triplicate analysis with error bars indicating standard deviations

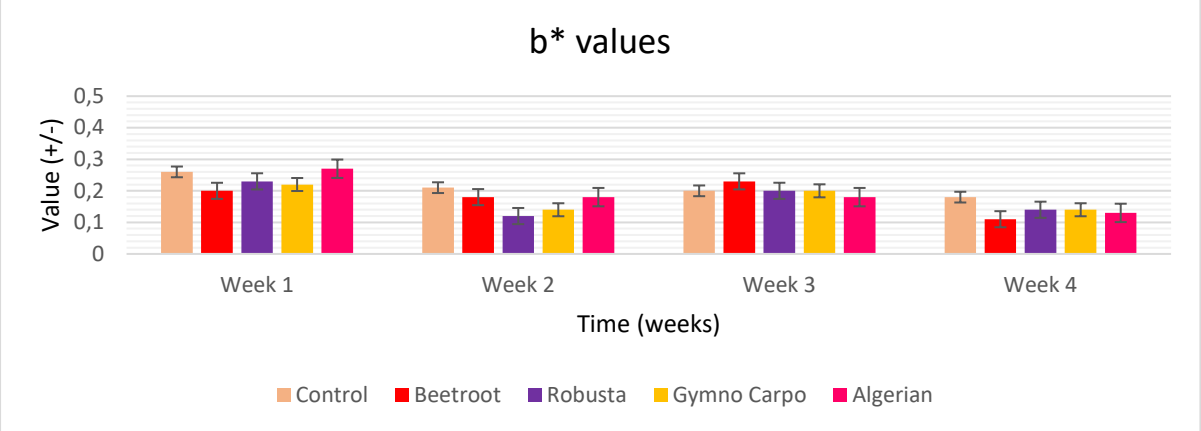


Figure 3. 12: b* juice colour readings of betalain pigment extracts
Bars representing average values of triplicate analysis with error bars indicating standard deviations

The L*, a* and b* colour readings of all produced yoghurts are shown in Figure 3.13-3.15, where the analysis was done on day one and ten. The lightness (L*) of all samples (Figure 3.14) seemed steady. The a* value of all samples was red initially

and showed a decline from day one to ten, mainly observed in Figure 3.15. Gymno-Carpo showed the highest a* value in day one. On the other hand, the b* indicated a yellow colour and showed the highest value in Algerian (day one), and showed a decline from day one to ten in all the cultivars.

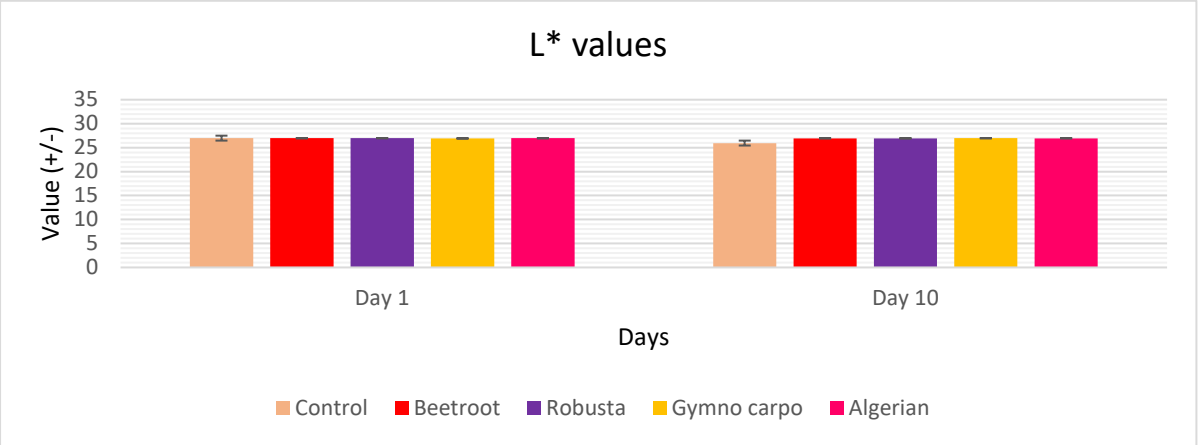


Figure 3. 13: L* yoghurt colour readings of betalain pigment extracts
Bars representing average values of triplicate analysis with error bars indicating standard deviations

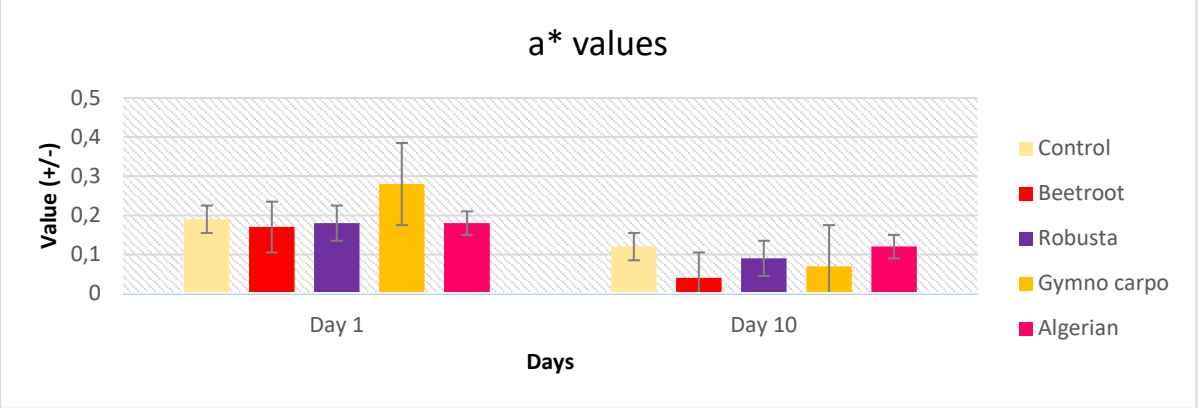


Figure 3. 14: a* yoghurt colour readings of betalain pigment extracts
Bars representing average values of triplicate analysis with error bars indicating standard deviations

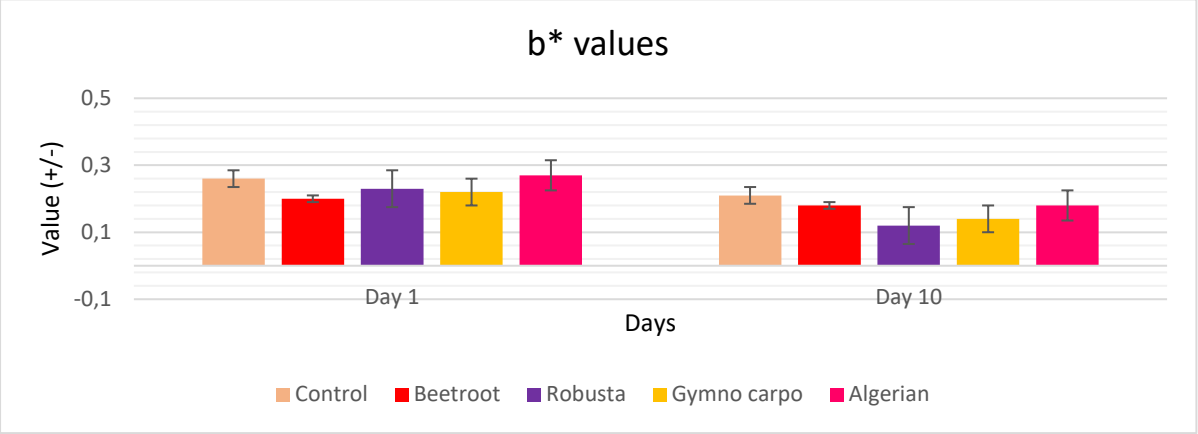


Figure 3. 15: b* yoghurt colour readings of betalain pigment extracts
Bars representing average values of triplicate analysis with error bars indicating standard deviations

3.4 Conclusion

Betalain pigment extracts can be found in various beetroot and cactus pear cultivars, these betalain pigment extracts offer healthful and aesthetically pleasing food products to end-users. The first and one of the most crucial processes in betalain extraction is the way in which betalain sources are handled. This is because total extractable betalain pigment extracts are highly dependent on both intrinsic and extrinsic factors. This includes the type of extraction tool used, the pre-preparation methods that were applied, the temperature during extraction, environment (air and light) as well as the chemicals that were used in the process.

The extraction tools (magnetic stirrer, ultrasonic bath, and liquidiser) all gave different betalain results upon extraction. The contributing factors to the extraction tool results were, among others, the particle sizes of the samples upon extraction. For example, when samples were cut into 0.1 x 0.1 cm^2 piece-sizes gave better results (higher betalain yields) than samples that were cut into 0.5 x 0.5 cm^2 sizes. Moreover, the liquidizer gave the highest betalain yields because it transformed samples into very fine particles and an increased total surface area. Therefore, size played a significant role in total betalains, in fact, the smaller the particle sizes produced higher betalain yields.

Based on the outcomes of the extraction methods, total betalain yield is dependent on the extraction tool and extraction method (physical, chemical, and heat). For chemical extraction, the best was ethanol (EtOH) and methanol (MeOH) extraction was at 50% chemical extraction.
The best temperature for extraction for microwave extraction with or without AA was mostly 10 or 20 s depending on the pre-treatments of the betalain extracts. Adding AA to samples altered the original colour of betalain pigments to a lighter shade of red. The best temperature for stove-top extraction was 180 s, which meant that increased time exposure to stove-top extraction gave higher betalain results.
Freeze-dried samples gave higher betalain values than samples that were not freeze-dried. The samples were also easily applied to food products (juice and yoghurt).

Both beetroot and Robusta are red/purple in colour, which means that colour (an intrinsic factor) may have more influence on betalain yield, stability, and food

application than plant source (beetroot or cactus pear). Moreover, extrinsic factors such as extraction methods also have an impact on total betalain yield.

The liquidiser was chosen as the only extraction tool that would be used going forward. Since freeze-dried samples gave high betalain results, it was no longer used as it took a long time to first freeze-dry samples and then extract them. Cactus pear samples also did not freeze-dry well (mostly remained sticky). Moreover, some of the betalain results from freeze-dried samples were comparable to that of EtOH extraction.

3.5 Recommendation

In subsequent chapters, the extraction methods will be repeated on eight different cactus pear cultivars and beetroot to determine the effect of extraction methods on betalain quality.

CHAPTER 4

Extraction of betalains from beetroot and eight cactus pear cultivars

Abstract

Betalains are red and yellow pigments which can be found in red beetroot and various cactus pear cultivars. The current chapter aimed to use different extraction methods to extract betalains from red beetroot and eight cactus pear cultivars, as well as find the most cost-effective and healthful extraction method. The cactus pear cultivars were selected from four different colours: green, orange, pink/red and purple. Chemical extraction was the first to be explored: betalain pigments were extracted with water, 10-50% ethanol (EtOH) and 10-50 % methanol (MeOH) separately. In the second investigation, pigments were heat-extracted with stove-top extraction at 60, 120 and 180 seconds. Another heat extraction method involved heating pigment extracts in the microwave for 10, 20 and 30 seconds; some batches had ascorbic acid (AA) added to them and some not.

After extraction, the pH, thermal and UV-light stability of the samples were investigated. The pH stability tests were accomplished through observing the colour change of betalain pigments at pH 1 and 4.5. Thermal stability was accomplished by extracting samples at 25 °C and heating them to 50, 80, and 90 °C for 15 min, and observing the colour change after each heating temperature. The UV-light stability was done through analysing betalain contents of selected betalain pigment extracts before and after UV-light exposure for 15 min.

Beetroot and cactus pear fruit peels from the cultivars American Giant, Morado, Ficus-Indice, Meyers, Monterey and Robusta have the same colour pulp and peel, thus produce the greatest plant material for extraction, without altering the colour of the pigment. The cultivars, Algerian and Gymno Carpo, have a fruit pulp colour that is different from the colour of their peels, and the removal of the peel from thee pulp reduces total extracted pigment colour.

The extraction methods did not have a significant effect on total betalain content. However, the interaction between cultivar and extraction method was significant for

betalain content. This means that for certain extraction methods, certain cultivars gave better results.

The most heat-stable pigments were the red/purple pigment sources from beetroot and cactus pear (Robusta and Monterey). In pH stability, all pigments were stable at pH 4.5 and unstable at pH 1. The UV-light results showed that sample colours and betalain contents did not show substantial changes before and after UV-light exposure.

4.1 Introduction

Betalains are food colourants that can be found in about 17 families of the Caryophyllales order. Depending on their extraction method, they can be labelled as natural colourants or Colouring Foods, as highlighted in Table 2.1 of Chapter 2. Table 2.1 further shows that Colouring Foods do not need certification, as they are regarded as safe. The Colouring Foods are also referred to as “fruit *or* vegetable juice” (Lehto et al., 2017), which means that beetroot (an approved colourant) can be a Colouring Food, depending on its extraction processes. Therefore, the safety of food remains dependent on processing technologies and the need for lasting as well as food-safe betalain processing techniques remains a necessity (Khan, 2016b).

Pigment extraction is one of the most critical steps in betalain production (Ngamwonglumlert et al., 2017). In order to meet food safety demands and explore stable colourant-producing extraction methods, various non-conventional extraction methods have been developed. These extraction processes include pressurized liquid extraction, microwave-assisted extraction and maceration (Celli & Brooks. 2017; Miguel, 2018). The last two methods are both incorporated in the current study. Although the application of heat may cause an initial degradation of betalain extracts, it has been found to ensure colour stability in the long run. The process of High-Temperature Short Time (HTST) is similar to heat application in microwave and stove-top extraction, which will also be conducted in the current chapter. The difference is that the HTST in Dos Santos et al. (2018) was achieved by adding samples in a 120 °C pre-heated oil bath for 80, 100, and 120 s.

Increasing the yield and concentration of extracted betalains are of great economic advantage and can be made possible through better and improved extraction methods

(Roy et al., 2004). Moreover, the stability of betalains may also be influenced by the extraction apparatus (Celli & Brooks. 2017). The combination of methods and apparatus is essential in extracting the highest possible yields. Betalain pigments are stable at a wide pH range and can regenerate after heat treatment (Miguel, 2018).

This chapter aims to compare different extraction methods between one beetroot cultivar and eight different cactus pear cultivars which are found in four different coloured fruits. The extraction method which works best, i.e., highest betalain content, will be chosen and chapters that follow will be based on those findings.

4.2 Materials and methods

4.2.1 Sample collection

One beetroot and eight different cactus pear cultivar samples were bought or collected and kept under conditions as explained in Chapter 3, section 3.2.1.1.

4.2.1.1 Beetroot

Beetroot was bought during the summer of January 2017. An illustration of freshly washed samples is shown in Figure 4.1.

Figure 4. 1: Fresh beetroot

4.2.1.2 Cactus pear

Cactus pear fruits from eight cultivars were harvested at 50% colour-break stage and stored according to the method in section 3.2.1.2, of Chapter 3. The eight fruit cultivars which are found in four different colours were collected from an experimental orchard at Waterkloof farm, Bloemfontein, Free State Province, SA. Green fruit cultivars included (1) American Giant and (2) Morado, orange fruit cultivars included (3) Ficus-Indice and (4) Gymno Carpo, while the pink/red fruit cultivars included (5) Algerian and (6) Meyers. The afore mentioned cultivars were all from the *O. ficus-indica* species.

However, the purple coloured fruit was from the *O. robusta* species and included cultivars (7) Monterey and (8) Robusta. After harvest, cactus pear fruits were kept in sealable plastic bags and stored at -20°C until used (not longer than one month). All eight cactus pear cultivars are shown in Figure 4.2.

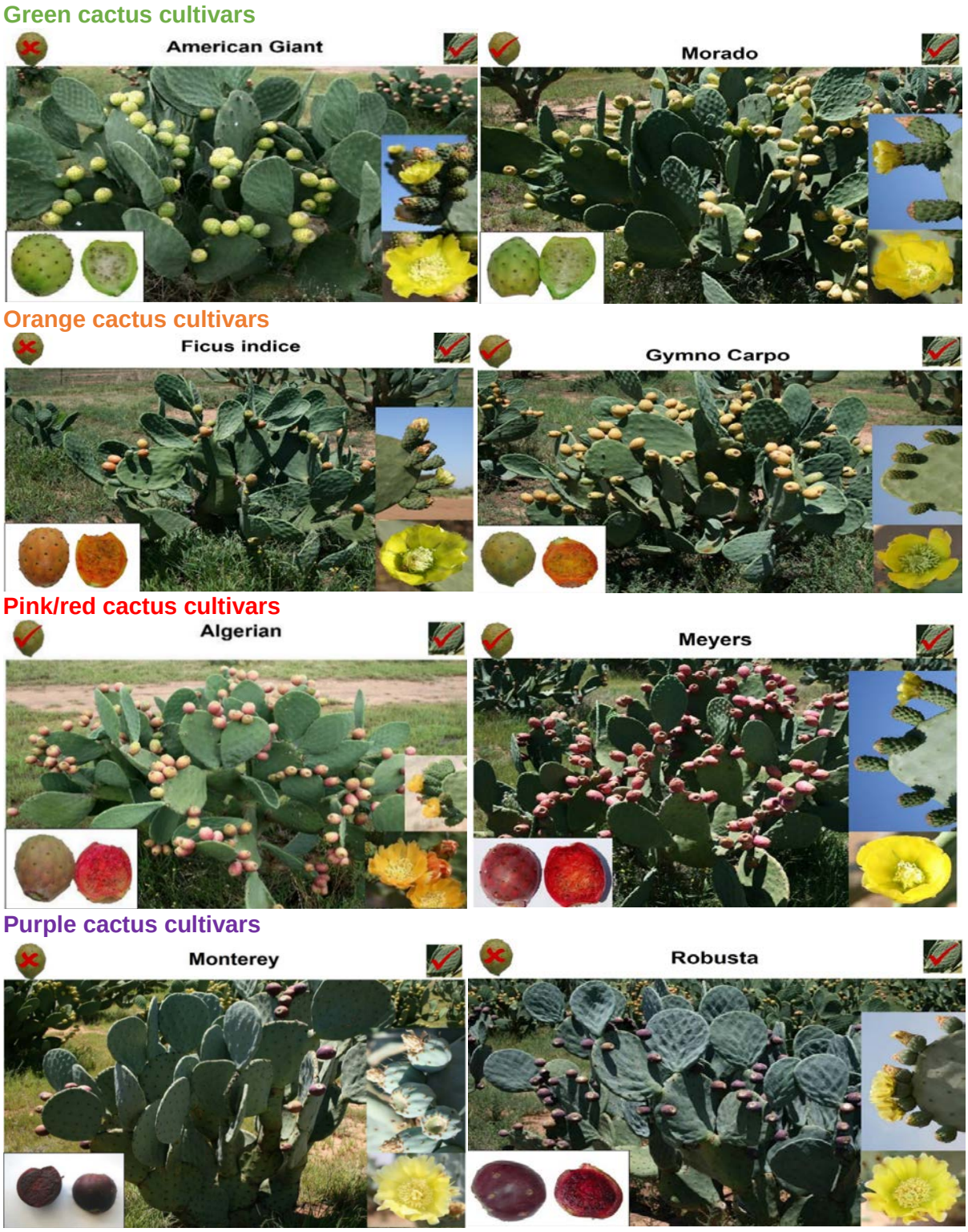


Figure 4. 2: Different cactus cultivars from Waterkloof farm, pictures supplied by Fouché, 2017

4.2.2 Extraction

All samples in the current chapter were extracted with a liquidiser. This is because a liquidiser (with the assistance of dH_20) liquefies and homogenises samples.

Table 4. 1: Extraction methods of beetroot and cactus pear

Method	Beetroot	Cactus pear
Chemical extraction	✓	✓
• dH_20	✓	✓
• MeOH	✓	✓
• EtOH	✓	✓
Heat extraction		
• Microwave extraction	✓	✓
• Microwave assisted extraction	✓	✓
• Stovetop extraction	✓	✓
Stability		
• pH stability	✓	✓
• Thermal stability	✓	✓

✓: Method conducted; **x**: method not conducted

4.2.2.1 Plant material waste during betalain extraction of beetroot and eight cactus pear cultivars

Cactus pear fruit samples were prepared through partial thawing at 4°C to remove excess ice particles. Thereafter, samples were weighed and hand peeled with the cut and tear method (du Toit, 2013), before they were not fully thawed (to prevent water and pigment loss). Beetroot samples were peeled and liquidised. The cactus pear fruit and beetroot were weighed in grams before and after peeling, and the difference in weight was calculated to determine the waste (g).

4.2.2.2 Extraction

For extraction, 100 g of sample was extracted using different chemicals: dH_20; methanol (MeOH); and ethanol (EtOH) solvents or heated in the microwave or on a stovetop. After extraction, the samples were liquidised using a Safeway stick blender,

as this physical extraction method was proven to be the most effective during the 2016 extraction process. The liquid pulp was strained through a 5 mm mash-size sieve, centrifuged and the supernatant used for further tests (Butera et al., 2002). The process of cactus pear fruit extraction will be discussed in full and is shown in a pictorial form in Figures 4.3 - 4.4.

Figure 4. 3: cactus pear cultivars after weighing and before peeling

Figure 4. 4: Fruit before centrifugation

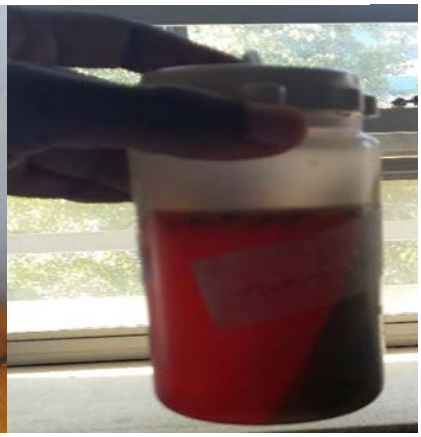


Figure 4. 5: Fruit supernatant

4.2.2.2.1 Water extraction

Chemical extraction was conducted because the addition of water, 20-50% EtOH or MeOH assists in extracting higher betalain yields (Pitelli, 1981 as cited in Ravichandran, 2013; Aberoumand, 2011).

Cactus pear fruit betalains were extracted using a ratio of 1:1, where 100 g of cactus pear or beetroot sample was mixed with 100 ml dH_20 and liquidised for ± 3 min with a Safeway stick blender. The liquidised samples were centrifuged, as explained in section 3.2.2.2 of Chapter 3. An example of the supernatant is shown in Figure 4.5.

The betalain quality was determined through the Genesys 10 VIS UV-light spectrophotometer at 480 nm betaxanthins (Bx) and 530 nm betacyanins (Bc) on liquid samples. Total betalain pigment extracts were determined by calculating the total Bx and Bc values as shown in section 3.2.3.1 of Chapter 3.

4.2.2.2.2 Methanol and ethanol extraction

Beetroot and cactus pear samples were liquidised in the solvents at different concentrations. EtOH and MeOH was tested at 10%, 20%, 30%, 40% and 50% concentrations. The ratio in the chemical extractions was 1:1 (w/v), i.e., 100 g of the liquid sample in 100 ml of the solvent. The colour of EtOH and MeOH extracted samples is shown in Figure 4.6.

The betalain quality was determined through the Genesys 10 VIS UV-light spectrophotometer at 480 nm betaxanthins (Bx) and 530 nm betacyanins (Bc) on liquid samples. Total betalain pigment extracts were determined by calculating the total Bx and Bc values, as shown in section 3.2.3.1 of Chapter 3.

Figure 4. 6: 50% EtOH and 50% MeOH extraction of all samples, except for Gymno Carpo
MeOH extraction in the back row (from left to right): Beetroot, Monterey, Robusta, Meyers, Morado, American Giant, and Ficus-Indice
EtOH extraction in the front row (from left to right): Beetroot, Monterey, Robusta, Meyers, Morado, American Giant, and Ficus-Indice

4.2.2.2.3 Heat extraction

In microwave extractions, 100 g beetroot and cactus pear samples were placed in microwave-safe dishes and heated at medium (50%) heat setting, using a Defy microwave (1000 W). The heating time was 10, 20 and 30 s. In microwave assisted

extraction, the experiment was extended by adding 5 ml of a 5% AA solution to the samples before heating.

For the stovetop extractions, beetroot and cactus pear samples were placed in a stainless steel 5.5 mm thick-base double boiler with 10 ml dH_20. The samples were heated to ± 80°C, on setting 3, for ±10 minutes, and frequently stirred with a wooden spoon. Different sample batches were heated for 60, 120 and 180 s.

After the heat treatments, the samples were liquidised in 100 ml dH20, using a stick blender for ±3 min. The liquidised samples were immediately placed in sealable containers and cooled in ice-cold water after their set cooking time; to stop further cooking. Figure 4.7 showed the colour of extracted Robusta and Monterey pulp when extraction was done by stovetop.

The betalain quality was determined through the Genesys 10 VIS UV-light spectrophotometer at 480 nm betaxanthins (Bx) and 530 nm betacyanins (Bc) on liquid samples. Total betalain pigment extracts were determined by calculating the total Bx and Bc values, as shown in section 3.2.3.1 of Chapter 3.

Figure 4. 7: Boiling stove-top extracted Robusta (left) and Monterey (right)

4.2.3 Stability

4.2.3.1 Betalain content, pH and heat stability

In this section, the stability of betalains against heat, and pH was tested, as explained in section 3.2.6.2, of Chapter 3. All tests were conducted within 24 hours of extraction.

4.2.3.2 UV stability

The UV-light stability test was done through the addition of 100 ml of centrifuged betalain extracts of two orange cactus pear cultivars (Gymno Carpo and Ficus-Indice), two pink/red cactus pear cultivars (Algerian and Meyers), one purple cactus pear cultivar (Robusta) and beetroot were used. The cultivars were chosen to represent every colour of the cactus cultivars available. The green cactus cultivar had been excluded at this point, because of very low (or zero) betalain yields.

One hundred millilitres of the dispensed extracts, in a round, flat, black plastic containers, were kept in the UV light room for 15 min. The samples' L*, a* and b* values were determined, before and after UV light exposure. The L*, a* and b* values were recorded in order to see the impact of UV exposure on the pigments.

4.2.4 Statistical design

The layout of the experiment is a factorial design with nine cultivars (eight cactus pear and one beetroot) and twenty extraction methods. All analyses were done on three samples per cultivar that were extracted individually. The effect of cultivar, extraction method and their interaction on betalain yield were analysed with an analysis of variance (ANOVA) procedure (NCSS 11 Statistical Software 2016). Means were compared with the Tukey-Kramer multiple comparison test at $\alpha = 0.05$ (NCSS 11 Statistical Software 2016).

4.3 Results and discussion

4.3.1 Plant material

The peel colour of cactus pear cultivars such as Gymno Carpo and Algerian is greenish; therefore, it differs from that of the pulp, which is orange and pink/red, respectively. Conversely, cultivars such as American Giant and Robusta have a uniform peel and pulp colour. Colour differences are illustrated in Figure 4.2.

At times, the fruit colour is not uniform due to inferior quality caused by mechanical, chemical, physical, and microbial damage; thus, fruit must be carefully evaluated before use (De Wit, 2006). In addition to the material that is lost during peeling and evaluation, the pulp of the fruit still contains seeds that must be discarded as they are not used during betalain production. As stated before, seeds make up 7% of the fruit

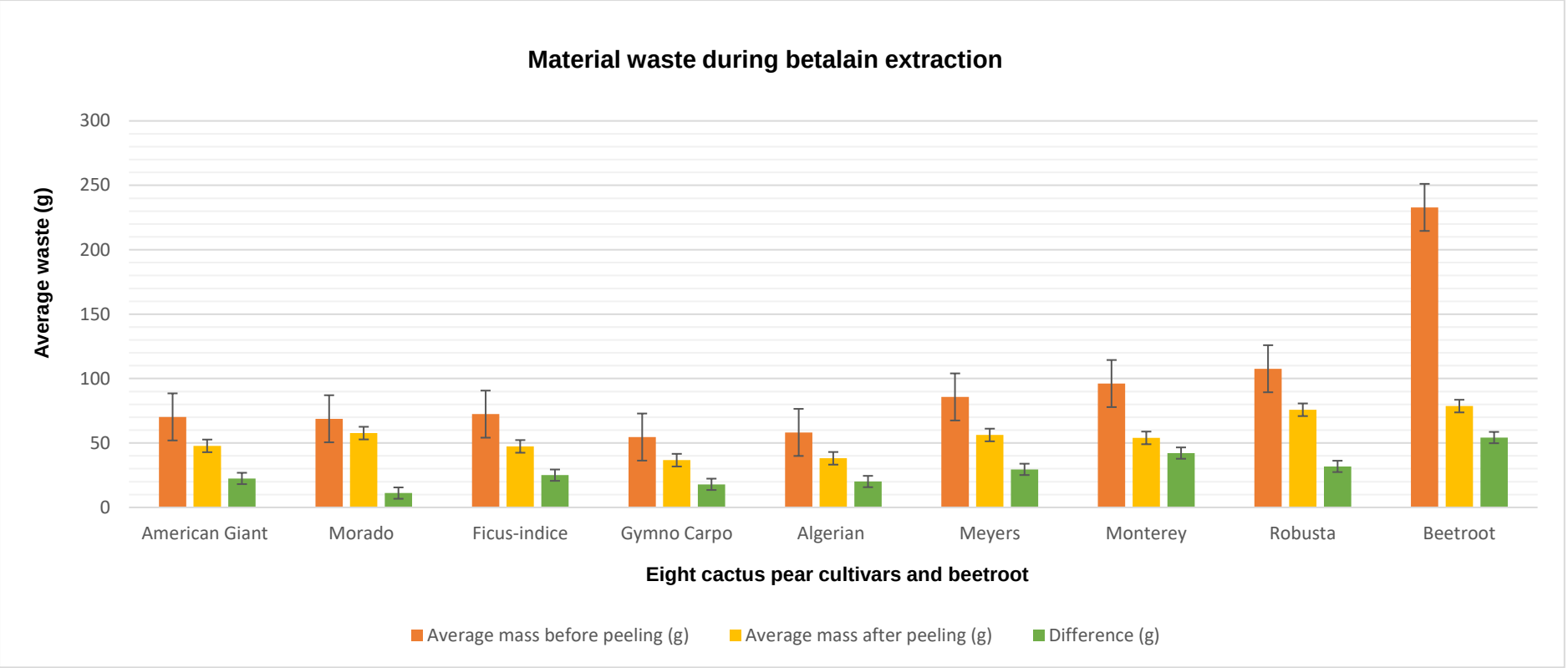


Figure 4. 8: Plant material waste during betalain extraction of beetroot and eight cactus pear cultivars
Bars representing average values of triplicate analysis with error bars indicating standard deviations

(Bouzoubaâ et al., 2016). Accordingly, an estimated 23% to more than 50% of the fruit is lost before processing.

Given these colour differences, all cultivars of the cactus pear fruit were peeled according to the cut and tear method (du Toit, 2013); a method which separates the peel from the pulp and ensuring that contrast colours do not mix. The method described by du Toit (2013) is useful for uniform colour extraction. It also resulted in a lot of fruit material loss, which might be unnecessary in cultivars whose peel is the same colour as the pulp. In the current study, plant material waste, as shown in Figure 4.8, is between 11.12 g (Morado) and 42.18 g (Monterey). The inclusion of fruit peel could increase the pigment concentration, save fruit material and ultimately betalains. To substantiate this, Moßhammer et al. (2006c) stated that cactus pear peels are not usually consumed, and are not included during production. However, the total yield of cactus juice is increased by at least 10% when the whole fruit (including the peel) is used.

Beetroot, on the other hand, was thinly sliced (because of colour uniformity) and does not contain seeds, all of which results in less material loss. However, it is also susceptible to the same microbial, mechanical, and chemical damages as cactus pear, which contribute to the amount of loss during production. The average loss for beetroot was 23%, which is less than most cactus pear cultivars. A study conducted by Sawicki et al. (2016) shows that the betalain concentration of beetroot peel is greater than that of pulp and, thus, it would be beneficial to extract the whole vegetable. In a study where the betalains of four red beetroot cultivars were tested, Kujala et al. (2002) also found that beetroot peel contained more betacyanins and betaxanthins than the pulp.

4.3.2 The effect of cultivar, extraction, and their interaction on betalain content

The results were analysed using analysis of variance (ANOVA), with p-values represented in Table 4.2. The p-values differed for the different tests that were conducted. Cultivar had a significant effect on betalains ($p < 0.001$). Extraction method had no significant effect ($p = 0.884$ for betaxanthins, $p = 0.963$ for betacyanins and $p = 0.965$ for total betalains) on betalains. The interaction between cultivar and extraction method had a significant effect on betalains ($p < 0.001$).

Table 4. 2: The effect of cultivar, extraction method and the interaction between cultivar and extraction method on betalains

Pigment	Cultivar	Extraction method	Cultivar X Extraction method
Bx (mg/g)	$p < 0.001$	$p = 0.884$	$p < 0.001$
Bc (mg/g)	$p < 0.001$	$p = 0.963$	$p < 0.001$
Total (mg/g)	$p < 0.001$	$p = 0.965$	$p < 0.001$

4.3.3 The effect of cactus pear cultivars and beetroot on betalain content

The results in Table 4.3 present the effect of cultivar on betalains ($p < 0.001$). The general trend was that pigments from purple pigment sources had numerically higher betaxanthin, betacyanin, and total betalains than other sources. The total betalains of green cultivars were numerically lower than all cactus cultivars with 12.65 mg/g in American Giant and 7.64 mg/g in Morado. The red cultivars had higher betacyanins values than betaxanthins, and the orange cultivars had higher betaxanthin values than betacyanins.

The pink/red cultivars, Meyers, and Algerian had values in the same range as the orange Gymno Carpo. Thus, the cultivar (no matter the colour) was the determining factor of the total betalain yields.

The green cactus cultivars contained the lowest amount of betaxanthins with 4.92 mg/g in American Giant and 3.56 mg/g in Morado. This made the green cultivars significantly lower than all the other cultivars. The orange Ficus-Indice (76.14 mg/g) value was significantly higher than the orange Gymno-Carpo (47.11 mg/g), an indication that betaxanthins may differ significantly in cultivars of the same colour. In the purple pigments, the cactus pear cultivar Monterey (284.24 mg/g) betaxanthins were significantly higher than Robusta (252.16 mg/g) and beetroot (182.64 mg/g). Thus, the betaxanthin results show that betaxanthins are dependent on cultivar.

The lowest betacyanin values were found in the green cactus cultivar, Morado (4.09 mg/g), and the highest levels were found in Robusta (396.27 mg/g). There were no statistically significant differences between the green, orange, and red/pink cactus pear cultivars. However, significant differences were found between the purple Monterey (289.55 mg/g) and Robusta (396.27 mg/g). The highest betacyanins were

observed in Robusta and beetroot. Thus, the purple colour pigments had the highest betacyanin yields.

The total betalain yields showed that the green Morado (7.64 mg/g) and American Giant (12.65 mg/g) had significantly lower total betalains than all the other cactus pear cultivars. The highest values were found in purple pigments, with Robusta (648.44 mg/g) significantly higher than all the samples.

In conclusion, cactus pear cultivars which have the same colour may differ significantly in their betalain content. Thus, total betalain content is dependent on cactus cultivar rather than colour.

Table 4. 3: The effect of cactus pear cultivars and beetroot on betalain content

Cultivar	Bx (mg/g)	Bc (mg/g)	Total Betalain (mg/g)
American Giant	$4.92^{a} \pm 4.47$	$7.76^{a} \pm 9.13$	$12.65^{a} \pm 13.35$
Morado	$3.56^{a} \pm 2.96$	$4.09^{a} \pm 2.72$	$7.64^{a} \pm 5.26$
Ficus-Indice	$76.14^{c} \pm 37.26$	$15.10^{a} \pm 16.58$	$91.24^{b} \pm 53.36$
Gymno Carpo	$47.11^{b} \pm 24.78$	$8.16^{a} \pm 3.81$	$55.27^{ab} \pm 26.18$
Algerian	$20.84^{ab} \pm 9.47$	$43.13^{a} \pm 87.28$	$63.97^{ab} \pm 88.43$
Meyers	$23.08^{ab} \pm 6.87$	$35.80^{a} \pm 13.05$	$58.89^{ab} \pm 18.63$
Monterey	$284.24^{f} \pm 84.92$	$289.55^{b} \pm 92.21$	$573.79^{c} \pm 165.07$
Robusta	$252.16^{e} \pm 84.71$	$396.27^{c} \pm 147.23$	$648.44^{d} \pm 224.91$
Beetroot	$182.64^{d} \pm 66.69$	$359.28^{c} \pm 143.01$	$541.92^{c} \pm 192.61$
Significance level	$p < 0.001$	$p < 0.001$	$p < 0.001$

Means with different superscripts in the same column differ significantly

4.3.4 The effect of extraction method on betalain content

Table 4.4 demonstrates the effect of the extraction methods on betalain content. Notably, Table 4.2 has already highlighted that there were no significant differences between extraction methods and total betalain yields and Table 4.4 showed that extraction method had no significant effect on betalain content. In fact, the p-value of betaxanthins was $p = 0.884$, betacyanins $p = 0.963$, and total betalains $p = 0.965$. The highest total betalains were observed for water extraction (299.20 mg/g), 50% MeOH extraction (299.24 mg/g), 50% EtOH extraction (266.03 mg/g), and 60-s stove-top extraction (263.52 mg/g) methods (not statistically different).

The betalain content from heat extraction methods was dependent on the heating source and heat-time exposure. For example, microwave heat had a maximum heating time of 30-s; this was with and without AA. However, the highest betalains were found at 10-s heating time; this was the case with AA (213.90 mg/g) or without AA (223.60 mg/g). Contrastingly, the highest betalain content in stove-top extraction was found in 180-s extraction (263.52 mg/g). Therefore, stove-top extraction or more prolonged heating exposure depending on the heating device may yield higher betalain results.

The comparison of betalain yields and extraction methods showed that water is highly comparable to other extraction mediums. It would also be the least expensive way of extraction.

Table 4. 4: The effect of extraction method on betalain content

Extraction Method	**Bx (mg/g)**	**Bc (mg/g)**	**Total Betalains (mg/g)**
Water	129.38 ± 153.74	169.82 ± 238.56	299.20 ± 389.14
10% MeOH Extraction	87.04 ± 107.16	100.78 ± 155.18	187.82 ± 259.33
20% MeOH Extraction	85.39 ± 105.96	105.91 ± 152.59	191.30 ± 255.64
30% MeOH Extraction	110.35 ± 153.52	166.05 ± 223.45	276.41 ± 354.81
40% MeOH Extraction	94.75 ± 114.42	143.05 ± 178.87	237.80 ± 282.65
50% MeOH Extraction	140.49 ± 141.35	158.75 ± 211.46	299.24 ± 346.94
10% EtOH Extraction	94.22 ± 114.23	105.43 ± 138.99	199.58 ± 250.24
20% EtOH Extraction	108.96 ± 124.74	131.32 ± 173.04	240.29 ± 296.09
30% EtOH Extraction	104.43 ± 121.75	142.41 ± 192.77	246.83 ± 311.88
40% EtOH Extraction	111.99 ± 125.72	153.61 ± 206.62	265.60 ± 327.55
50% EtOH Extraction	115.55 ± 130.88	150.49 ± 205.10	266.03 ± 332.93
Microwave 10 s	101.05 ± 124.44	122.54 ± 163.33	223.60 ± 266.83
Microwave 10 s with AA	111.82 ± 142.88	102.09 ± 126.35	213.90 ± 261.55
Microwave 20 s	86.45 ± 79.73	104.86 ± 141.83	191.30 ± 206.11
Microwave 20 s with AA	81.51 ± 78.44	115.06 ± 151.14	196.57 ± 215.71
Microwave 30 s	72.72 ± 70.62	98.75 ± 149.68	171.47 ± 208.89
Microwave 30 s with AA	79.53 ± 82.09	108.68 ± 166.69	188.21 ± 237.64
Stove-top 60 s	86.98 ± 98.06	116.23 ± 163.98	203.21 ± 254.56
Stove-top 120 s	80.70 ± 80.11	121.44 ± 171.44	202.14 ± 244.78
Stove-top 180 s	104.89 ± 108.23	158.63 ± 212.00	263.52 ± 313.43
Significance level	p = 0.884	p = 0.963	p = 0.965

4.3.5 The effect of the interaction between the source (cactus pear cultivars and beetroot) and extraction method on betalain content

The effect of the interaction between betalain source (cactus pear cultivars and beetroot) and extraction method on betalains are observed in Table 4.5.

Water extraction

In Table 4.5, the general trend in the water extraction method was that high yields of betalains were found in purple cactus pear cultivars and beetroot. The green cultivars, American Giant and Morado, produced betalain yields that were significantly lower than all other cactus pear cultivars. The purple pigment sources, Monterey, Robusta, and beetroot, produced betalain yields that were significantly higher than all the other cultivars. The orange cultivars Ficus-Indice and Gymno Carpo sometimes had higher betaxanthins than betacyanins, and vice versa. These results contrast the assumption that cultivars which are inherently orange would have higher levels of the yellow pigment. This was also similar in pink/red cultivars Algerian and Meyers, where betaxanthin levels were sometimes numerically higher than betacyanins.

The highest content of betaxanthins (orange pigment) were found in Robusta (398.15 mg/g), which is a purple cultivar, and the highest amounts of betacyanins were also found in Robusta (669.93 mg/g) (Figure 4.5).

The results also showed vast differences between samples from different cultivars. The green cactus pear cultivars (5.76 mg/g for American Giant and 4.34 mg/g Morado) contained the lowest betalain contents, whereas the purple (838.14 mg/g for Monterey and 1 068.08 mg/g Robusta) cultivars had the highest betalain contents. Beetroot (503.52 mg/g) was significantly lower than Robusta (1068.08 mg/g) and Monterey (838.14 mg/g).

The green cultivars, Morado and American Giant, had the lowest betalain content. Morado significantly differed from all other cultivars and the total calculated betalain amounts were more than fifteen times less than that of all purple samples, Monterey, and Robusta, as well as beetroot.

Both Ficus-Indice and Gymno Carpo, which are orange cultivars, had higher betaxanthin (yellow to orange pigment) values than betacyanins (red to purple

pigment). This may be caused by the colour of the fruits; thus, the yellow to orange fruit had higher betaxanthins. Interestingly, there was a noticeable numerical difference between the total betaxanthin values of the two cultivars; Ficus-Indice had ±36.25% more than that of Gymno Carpo. Gymno Carpo and Ficus-Indice are orange, and their betaxanthin content results would be expected to be higher than that of all other cultivars. However, Monterey and Robusta had significantly higher values, as their betaxanthin content is more than 15 times higher than that of all the other cultivars.

Algerian and Meyers (pink/red cultivars) had higher betacyanin (red to purple pigment) values than betaxanthins; with total betalains being higher in Meyers. The total betalain content of Meyers is higher, ±50% higher than that of Algerian. In fact, there is a significant difference observed in both betaxanthin and betacyanin values.
Purple cactus pear cultivars and beetroot contained the highest total betalain values. Thus, it can be deduced that betalain quality and strength is dependent on the colour of the betalain source, as shown in Table 4.3.

Highly significant differences were observed between the purple samples (beetroot and cactus pear) and all other coloured fruit. For the extraction of betalains with MeOH, as shown in Table 4.5, purple cactus pear cultivars had significantly higher betalain contents. In fact, the betalain content was more than five times higher than that of the other cultivars.

Methanol extraction

The MeOH extraction results in Table 4.5 show that the highest betalain content for American Giant was 20% MeOH, Morado and Robusta at 30% MeOH (also the highest yield among all the cultivars), Meyers at 40% MeOH, Ficus-Indice, Gymno Carpo, Algerian and Monterey at 50% MeOH.

At 10% MeOH extraction, the purple cultivars had betacyanin and betaxanthin values which were significantly higher than all other samples. Beetroot samples had lower leels than purple cactus with a total of 157 mg/g. In fact, beetroot results were vastly different from the purple cactus cultivars until 40% extraction where 407.21 mg/g total betalains were extracted.

With the 20% MeOH extraction, Robusta contained the highest betalain amounts (692.19 mg/g). With the MeOH 30% extraction, very high levels of betacyanins were extracted from Monterey (419.22 mg/g).

The highest betalain contents for different cultivars reached its peak at different MeOH concentrations. Robusta yielded the highest betalain pigment content at 30% MeOH extraction method.

Ethanol extaction

Results for the EtOH extraction of betalains are represented in Table 4.5. Results showed that green cultivars produced betacyanins, betaxanthins, and total betalain contents which were significantly lower than all other cultivars. The purple cultivars (Monterey and Robusta) together with beetroot, had significantly higher yields ($p < 0.001$). The orange cultivars had higher amounts of betaxanthins than betacyanins, while the pink/red cultivars had higher amounts of betacyanins than betaxanthins.

There was a decreasing trend in content from 10-20% EtOH extraction in cultivars Morado and Algerian. After that, betalain yields of both cultivars increased with increased EtOH concentrations.

American Giant and Monterey had the highest betalain content at 20% EtOH extraction, Meyers, and Robusta at 30% EtOH extraction, and Ficus-Indice at 40%.

Interestingly, none of the cactus pear cultivars had their highest betalain yields at 50% EtOH concentration. Results may also differ because Ravichandran et al. (2013) researched on beetroot, and extraction was conducted with 30, 50 and 70% EtOH. The method was most similar to section 4.2.1.3 of the current chapter, where samples had their highest betalain yields at 50% EtOH extraction, even when previously not freeze-dried.

Monterey and Robusta produced significantly higher betalain contents than beetroot and all the other cactus cultivars. Monterey betalain yield was higher at 10-20% EtOH extraction and Robusta at 30-50% EtOH extraction. Also, Monterey mostly had higher betaxanthin values and Robusta betacyanin values. Optimal betalain yields were at different EtOH concentrations for different cultivars. Therefore, optimal extraction was dependent on method and cultivar.

Microwave, and microwave assisted extraction

Microwave heat extraction, which is also known as green extraction, is an environmentally friendly mode of extraction. Higher water volumes can be diffused from samples during the heating period. The extraction time of samples can also be minimised. In fact, even a few seconds (s) (such as with the current research) are enough for extraction. The extraction process can then take place with the assistance of solvents such as EtOH at different concentrations or can even be done without any solvents (Destandau et al., 2013; Michel et al., 2011).

It is also important to note that acidification and heating of plant matter may be required to remove certain matter before the extraction process takes place, thus giving more reason to the heating of samples during extraction (Delgado-Vargas et al., 2000).

In agreement with microwave green extraction of natural products is the INRA Agrosciences National Research Institute, University of Avignon, France Prof. Farid Chemat advocates for the use of green technology during extraction as solvents can be pricy, the process can be time-consuming, allows for rapid extraction which minimises loss (Milestone – Helping Chemists, 2016). This process allows a product such as beetroot to be extracted with minimal to zero solvents using microwave hydro diffusion and gravity.

Table 4.5, illustrates the results of microwave heat extraction, a general betalain yield increase, as the heating time of the extractions increased, was observed, especially in Robusta. Even the green, orange and pink/red cultivars had an increase in betalain content. Also, the total betalain yield of purple cactus pear cultivars and beetroot were significantly higher than for the other cultivars.

The general trend with the microwave extraction method was that purple cactus pear cultivars and beetroot had the highest betalain content, whereas the green cultivar, Morado, had very low betalain levels.

When ascorbic acid (AA) was added to the samples used in the microwave oven method, the colour of the extracted samples changed to a lighter shade of red (scarlet red). On the other hand, microwave-oven heated samples that did not have AA did not change in colour.

At 10 s of microwave extraction, the betalain yield decreased when AA was added to samples such as beetroot and increased in samples such as Monterey and Gymno Carpo. During 10-second extraction method without AA, the highest betalain and betaxanthin content were in Monterey at 656.35 mg/g, and 397.08 mg/g respectively. The highest betacyanin content was found in beetroot at 440.31 mg/g. When AA was added to samples, Monterey had the highest total betalains (777.58 mg/g), betacyanin (319.00 mg/g) and betaxanthins (458.58 mg/g). Contrastingly, Morado had the lowest betalain, betaxanthin (5.13 mg/g and 8.02 mg/g) contents for samples without AA and with AA, respectively) and betacyanin levels (6.88 mg/g and 6.26 mg/g without and with AA, respectively).

At 20 s of extraction without AA, beetroot yielded the highest betalain (619.19 mg/g), and betacyanin (449.47 mg/g) contents. Robusta produced the highest betaxanthin amounts (184.16 mg/g), significantly higher than beetroot (169.72 mg/g) and all other pigments. The orange cultivars Ficus-Indice (166.53 mg/g) and Gymno Carpo (47.38 mg/g) produced higher levels of betaxanthins (orange pigment) than betacyanins (red/purple pigment); of which the betacyanins were 58.82 mg/g for Ficus-Indice and 10.08 mg/g, both much lower than betaxanthin values.

At 20 second extraction with AA, beetroot contained the highest betaxanthins and betacyanin content at 208.60 mg/g and 455 mg/g, respectively. Purple cultivars contained the highest betalain amounts in most extraction procedures. Interestingly, Ficus-Indice (orange cultivar) contained the second-highest betaxanthin yields at 195.92 mg/g, and its total betalain yield was 263.14 mg/g, higher than the purple Monterey cultivar, which yielded 234.41 mg/g.

The 30-second pigment extraction without AA reveals that beetroot contained the highest total betalain (627.44 mg/g) and betacyanins levels (459.86 mg/g) whereas Monterey contained the highest betaxanthins levels. American Giant (green) contained the lowest betaxanthins levels (5.24 mg/g) and Morado (green) the lowest betacyanin levels (2.75 mg/g).

Ficus-Indice and Gymno Carpo with betaxanthin levels of 66.20 mg/g and 44.28 mg/g, respectively, had much higher levels of the yellow pigment than red betacyanins which were 9.17 mg/g and 11.15 mg/g, respectively. The pink/red cultivars had betacyanins levels which were slightly lower than the betaxanthin levels with betaxanthin levels at

21.07 mg/g and 23.99 mg/g for Algerian and Meyers, respectively. The betacyanins contents for Algerian and Meyers are 23.99 mg/g and 23.22 mg/g, respectively.

The different betalain pigments reacted differently to different heating methods. Most samples yielded higher values when AA was added, and total betalain yields differed according to betalain source and applied method.

Stovetop extraction

In Table 4.5, at 60-second extraction, betalain yields of Ficus-Indice and Gymno Carpo had much higher betaxanthin (53.79 mg/g and 37.75 mg/g, respectively) content than betacyanin contents (6.27 mg/g (Ficus-Indice) and 7.33 mg/g (Gymno Carpo). Also, at 60-second extraction, Algerian and Meyers had higher betacyanins (at 19.10 mg/g and 30.86 mg/g, respectively) than betacyanins.

Interestingly, the green cultivars had the lowest values for betacyanin and betaxanthin; the values were closer to the red/pink cultivars than the orange cultivars that were the highest, following the purple samples.

Beetroot produced the highest betalain yields at 60 s (685.06 mg/g) and 120 s (707.85 mg/g) and maintained the same yield at 180 s (707.85 mg/g). Importantly, Robusta yielded the highest betalains at 180 s (791.50 mg/g). Both betalain pigments yielded higher betalain yields with more prolonged heat exposure.

The results showed a general increase in betalain content as heat exposure time was increased from 60 s to 180 s. The significant increase is especially noticeable in purple cactus pear cultivars and beetroot. Interestingly, the highest betalain content was found in beetroot at the 60 and 120 s extraction, and with Robusta at 180 s extraction. This is in contrast with a study conducted by Ravichandran et al. (2013) which showed that the boiling and roasting of beetroot samples deteriorated betalain levels.

Table 4. 5: Mean Betalain content for the extraction method X cultivar interaction

Extraction method	Cultivar	Bx (mg/g)	Bc (mg/g)	Total Betalain (mg/g)
Water	American Giant	2.25^{abcd} ± 0.64	3.51^{a} ± 0.27	5.76^{ab} ± 0.43
	Morado	1.28 ^{ab} ± 0.32	3.06 ^{a} ± 2.61	4.34 ^{a} ± 2.84
	Ficus Indice	80.53 ^{wxyzAA} ± 1.28	13.60 ^{abcd} ± 0.70	94.13 ^{defgh} ± 0.61
	Gymno Carpo	51.55 ^{defghijklmnopqrstuvwxy} ± 0.19	10.08 ^{ab} ± 3.18	61.63 ^{abcdef} ± 3.09
	Algerian	13.90 ^{abcdefghij} ± 4.06	18.03 ^{abcde} ± 5.07	31.93 ^{abcde} ± 9.13
	Meyers	34.54 ^{abcdefghijklmnopqrstuvw} ± 2.14	50.72 ^{abcdefg} ± 1.91	85.27 ^{bcdefgh} ± 1.46
	Monterey	380.72 ^{AQAR} ± 1.85	457.42 ^{AAABACAD} ± 5.72	838.14 ^{AKAL} ± 7.54
	Robusta	398.15 ^{ARAS} ± 35.93	669.93 ^{AG} ± 19.48	1068.08 ^{AM} ± 16.46
	Beetroot	201.48 ^{AFAGAHAI} ± 0.56	302.04 ^{pqrstuv} ± 1.59	503.52 ^{rst} ± 2.14
10% MeOH Extraction	American Giant	2.99 ^{abcd} ± 1.45	3.97 ^{a} ± 1.74	6.97 ^{abc} ± 3.14
	Morado	1.50 ^{abc} ± 0.19	2.14 ^{a} ± 1.47	3.64 ^{a} ± 1.66
	Ficus Indice	59.89 ^{jklmnopqrstuvwxy} ± 0.49	10.24 ^{ab} ± 2.35	70.13 ^{abcdef} ± 2.62
	Gymno Carpo	37.54 ^{abcdefghijklmnopqrstuvwx} ± 2.00	3.36 ^{a} ± 2.61	40.90 ^{abcde} ± 4.61
	Algerian	16.26 ^{abcdefghijklm} ± 4.04	18.94 ^{abcde} ± 5.21	35.20 ^{abcde} ± 9.23
	Meyers	23.96 ^{abcdefghijklmnopqrst} ± 0.67	43.24 ^{abcdefg} ± 0.95	67.19 ^{abcdef} ± 1.56
	Monterey	287.68 ^{AKALAMANAO} ± 8.58	318.08 ^{rstuvw} ± 2.55	605.76 ^{vwxy} ± 6.42
	Robusta	267.67 ^{AJAKALAM} ± 3.21	435.42 ^{AAAB} ± 2.00	703.08 ^{ABACADAEAFAG} ± 3.89
	Beetroot	85.88 ^{xyzAAAB} ± 1.45	71.60 ^{efgh} ± 52.23	157.47 ^{ghi} ± 51.03
20% MeOH Extraction	American Giant	3.74 ^{abcde} ± 1.13	4.74 ^{ab} ± 1.06	8.48 ^{abc} ± 2.09
	Morado	1.50 ^{abc} ± 1.21	4.43 ^{a} ± 2.52	5.93 ^{ab} ± 3.72
	Ficus Indice	64.59 ^{mnopqrstuvwxyz} ± 0.67	6.88 ^{ab} ± 5.16	71.47 ^{abcdef} ± 5.42
	Gymno Carpo	37.54 ^{abcdefghijklmnopqrstuvwx} ± 1.95	4.58 ^{a} ± 2.79	42.12 ^{abcde} ± 4.74
	Algerian	16.68 ^{abcdefghijklm} ± 1.16	35.60 ^{abcdef} ± 3.05	52.28 ^{abcdef} ± 2.18
	Meyers	25.03 ^{abcdefghijklmnopqrst} ± 1.79	48.58 ^{abcdefg} ± 0.92	73.61 ^{abcdef} ± 2.47
	Monterey	287.89 ^{AKALAMANAO} ± 1.03	324.19 ^{stuvwxy} ± 4.13	612.09 ^{wxyz} ± 4.17
	Robusta	262.12 ^{AJAKAL} ± 2.94	430.07 ^{AAAB} ± 6.68	692.19 ^{AAABACADAEAF} ± 3.84
	Beetroot	69.41 ^{pqrstuvwxyz} ± 1.61	94.11 ^{ghi} ± 2.31	163.52 ^{hi} ± 3.61
30% MeOH Extraction	American Giant	1.82 ^{abc} ± 1.45	2.29 ^{a} ± 0.01	4.11 ^{a} ± 1.45
	Morado	4.17 ^{abcdef} ± 1.40	4.58 ^{a} ± 1.83	8.75 ^{abc} ± 3.07
	Ficus Indice	64.38 ^{lmnopqrstuvwxyz} ± 0.81	6.88 ^{ab} ± 0.92	71.26 ^{abcdef} ± 1.72
	Gymno Carpo	39.89 ^{abcdefghijklmnopqrstuvwx} ± 3.21	12.22 ^{abc} ± 4.45	52.11 ^{abcdef} ± 1.60
	Algerian	23.31 ^{abcdefghijklmnopqrst} ± 1.34	419.22 ^{zAA} ± 2.31	442.54 ^{pqr} ± 1.48
	Meyers	24.92 ^{abcdefghijklmnopqrst} ± 0.81	37.43 ^{abcdef} ± 1.91	62.35 ^{abcdef} ± 2.71
	Monterey	325.97 ^{AOAP} ± 57.81	274.85 ^{opqrs} ± 4.13	600.81 ^{uvwx} ± 61.34
	Robusta	438.47 ^{ASAT} ± 43.47	648.08 ^{AG} ± 5.04	1086.56 ^{AM} ± 48.51
	Beetroot	70.26 ^{rstuvwxyz} ± 2.94	88.92 ^{fghi} ± 3.97	159.18 ^{hi} ± 5.37
40% MeOH Extraction	American Giant	1.71 ^{abc} ± 0.49	2.14 ^{a} ± 1.61	3.85 ^{a} ± 1.12
	Morado	3.53 ^{abcde} ± 1.28	4.58 ^{a} ± 1.83	8.11 ^{abc} ± 3.12
	Ficus Indice	76.47 ^{vwxyzAA} ± 0.37	18.03 ^{abcde} ± 1.85	94.49 ^{defgh} ± 2.20
	Gymno Carpo	53.37 ^{fghijklmnopqrstuvwxy} ± 3.83	12.22 ^{abc} ± 3.05	65.59 ^{abcdef} ± 6.28
	Algerian	18.61 ^{abcdefghijklmno} ± 0.01	27.04 ^{abcde} ± 4.51	45.65 ^{abcdef} ± 4.51
	Meyers	29.73 ^{abcdefghijklmnopqrstuv} ± 1.13	64.93 ^{cdefgh} ± 2.76	94.66 ^{defgh} ± 1.64
	Monterey	315.91 ^{AMANAOAP} ± 1.13	363.46 ^{wxy} ± 0.92	679.37 ^{xyzAAABACADAEAF} ± 0.23
	Robusta	281.59 ^{AKALAMANAO} ± 7.22	459.71 ^{AAABACAD} ± 10.54	741.29 ^{AEAFAGAHAI} ± 3.32
	Beetroot	71.87 ^{stuvwxyz} ± 5.04	335.35 ^{tuvwxy} ± 1.06	407.21 ^{nopq} ± 5.32
	American Giant	1.93 ^{abc} ± 1.28	2.44 ^{a} ± 1.74	4.37 ^{a} ± 2.86

	Morado	1.71 ^{abc} ± 1.21	1.38 ^{a} ± 0.92	3.09 ^{a} ± 2.02
	Ficus Indice	82.45 ^{wxyzAA} ± 2.42	15.13 ^{abcd} ± 2.55	97.58 ^{efgh} ± 0.68
	Gymno Carpo	110.79 ^{zAAABAC} ± 96.04	13.44 ^{abcd} ± 0.26	124.24 ^{fgh}± 95.91
50% MeOH	**Algerian**	55.29 ^{hijklmnopqrstuvwxy} ± 0.19	13.29 ^{abcd} ± 0.46	68.58 ^{abcdef} ± 0.63
Extraction	**Meyers**	30.48 ^{abcdefghijklmnopqrstuv} ± 1.28	45.53 ^{abcdefg} ± 4.16	76.01 ^{abcdef} ± 2.89
	Monterey	337.73 ^{APAQ} ± 4.68	361.47 ^{wxy} ± 0.53	699.20 ^{ABACADAEAFAG} ± 5.19
	Robusta	329.50 ^{AOAP} ± 2.57	536.10 ^{AF} ± 0.26	865.59 ^{AL} ± 2.80
	Beetroot	314.52 ^{AMANAOAP} ± 0.49	440.00 ^{AAAB} ± 1.65	754.52 ^{AFAGAHAIAJ} ± 2.07
	American Giant	1.18 ^{ab} ± 0.81	1.83 ^{a} ± 1.38	2.37 ^{a} ± 1.38
	Morado	2.78 ^{abcd} ± 1.30	3.82 ^{a} ± 1.85	6.60 ^{ab} ± 3.12
	Ficus Indice	50.26 ^{bcdefghijklmnopqrstuvwxy} ± 2.09	3.51 ^{a} ± 2.98	53.78 ^{abcdef} ± 0.92
	Gymno Carpo	30.37 ^{abcdefghijklmnopqrstuv} ± 5.19	7.79 ^{ab} ± 2.38	38.16 ^{abcde} ± 7.57
10% EtOH	**Algerian**	21.71 ^{abcdefghijklmnopqr} ± 0.37	28.88 ^{abcde} ± 0.46	50.59 ^{abcdef} ± 0.23
Extraction	**Meyers**	11.87 ^{abcdefghij} ± 0.32	23.22 ^{abcde} ± 3.90	35.09 ^{abcde} ± 4.22
	Monterey	299.23 ^{ALAMANAOAp} ± 3.55	365.29 ^{wxyz} ± 3.21	664.52 ^{xyzAAABACADAE} ± 0.49
	Robusta	272.92 ^{AJAKALAMAN} ± 1.03	248.42 ^{mnop} ± 0.79	521.34 ^{rstu} ± 0.82
	Beetroot	157.64 ^{ACADAEAF} ± 0.93	266.14 ^{opqr} ± 0.70	423.78 ^{opq} ± 1.60
	American Giant	2.78 ^{abcd} ± 0.81	3.67 ^{a} ± 1.83	6.45 ^{ab} ± 2.64
	Morado	1.07 ^{ab} ± 0.49	1.99 ^{a} ± 1.40	3.06 ^{a} ± 1.86
	Ficus Indice	57.64 ^{ijklmnopqrstuvwxy} ± 0.19	6.72 ^{ab} ± 0.70	64.37 ^{abcdef} ± 0.57
	Gymno Carpo	42.46 ^{abcdefghijklmnopqrstuvwx} ± 15.53	5.35 ^{ab} ± 4.36	47.80 ^{abcdef} ± 11.65
20% EtOH	**Algerian**	20.64 ^{abcdefghijklmnopq} ± 0.49	26.89 ^{abcde} ± 0.27	47.53 ^{abcdef} ± 0.71
Extraction	**Meyers**	21.71 ^{abcdefghijklmnopqr} ± 1.77	23.83 ^{abcde} ± 1.83	45.54 ^{abcdef} ± 0.12
	Monterey	317.95 ^{ANAOAP} ± 1.16	372.78 ^{xyz} ± 4.70	690.72 ^{zAAABACADAEAF} ± 5.36
	Robusta	274.10 ^{AJAKALAMAN} ± 28.99	374.46 ^{yz} ± 3.01	648.56 ^{wxyzAAABAC} ± 26.26
	Beetroot	242.33 ^{AIAJAK} ± 1.03	366.21 ^{wxyz} ± 0.92	608.54 ^{vwxy} ± 1.84
	American Giant	2.57 ^{abcd} ± 0.32	3.51 ^{a} ± 1.15	6.08 ^{ab} ± 1.47
	Morado	0.75 ^{a} ± 0.19	2.29 ^{a} ± 2.38	3.04 ^{a} ± 2.57
	Ficus Indice	69.94 ^{qrstuvwxyz} ± 0.32	8.25 ^{ab} ± 0.46	78.19 ^{abcdefg} ± 0.41
	Gymno Carpo	24.38 ^{abcdefghijklmnopqrst} ± 17.65	3.51 ^{a} ± 2.52	27.90 ^{abcde} ± 19.59
30% EtOH	**Algerian**	15.08 ^{abcdefghijkl} ± 1.60	18.03 ^{abcde} ± 3.70	33.11 ^{abcde} ± 4.86
Extraction	**Meyers**	29.62 ^{abcdefghijklmnopqrstuv} ± 2.41	42.78 ^{abcdefg} ± 2.07	72.40 ^{abcdef} ± 1.34
	Monterey	295.38 ^{ALAMANAOAP} ± 5.82	352.00 ^{vwxy} ± 9.92	647.38 ^{wxyzAAABAC} ± 15.71
	Robusta	307.89 ^{ALAMANAOAP} ± 1.34	514.71 ^{AEAF} ± 12.73	822.60 ^{AJAKAL} ± 14.05
	Beetroot	194.21 ^{AFAGAHAI} ± 0.19	336.57 ^{tuvwxy} ± 0.70	530.78 ^{stuv} ± 0.57
	American Giant	1.60 ^{abc} ± 0.01	3.67 ^{a} ± 2.29	5.27 ^{a} ± 2.29
	Morado	2.46 ^{abcd} ± 1.58	3.06 ^{a} ± 2.26	5.52 ^{a} ± 3.82
	Ficus Indice	74.54 ^{uvwxyz} ± 2.77	11.92 ^{abc} ± 0.92	86.46 ^{cdefgh} ± 3.20
	Gymno Carpo	48.55 ^{abcdefghijklmnopqrstuvwx} ± 2.91	5.35 ^{ab} ± 3.90	53.90 ^{abcdef} ± 6.79
40% EtOH	**Algerian**	10.91 ^{abcdefghij} ± 1.70	14.51 ^{abcd} ± 2.35	25.42 ^{abcde} ± 3.90
Extraction	**Meyers**	27.38 ^{abcdefghijklmnopqrstuv} ± 3.12	41.10 ^{abcdefg} ± 1.85	68.48 ^{abcdef} ± 1.52
	Monterey	315.59 ^{AMANAOAP} ± 11.05	345.43 ^{uvwxy} ± 0.70	661.02 ^{xyzAAABACAD} ± 10.89
	Robusta	298.91 ^{ALAMANAOAP} ± 7.23	514.40 ^{AEAF} ± 1.85	813.31 ^{AIAJAKAL} ± 9.05
	Beetroot	228.01 ^{AHAIAJ} ± 0.49	443.06 ^{AAAB} ± 0.70	671.06 ^{xyzAAABACADAE} ± 1.19
	American Giant	3.21 ^{abcde} ± 1.28	3.06 ^{a} ± 1.91	6.26 ^{ab} ± 2.97
	Morado	1.50 ^{abc} ± 1.13	1.68 ^{a} ± 0.95	3.18 ^{a} ± 2.05
50% EtOH	**Ficus Indice**	72.51 ^{tuvwxyz} ± 2.57	6.42 ^{ab} ± 0.01	78.93 ^{abcdefg} ± 2.57
Extraction	**Gymno Carpo**	43.53 ^{abcdefghijklmnopqrstuvwx} ± 2.14	5.96 ^{ab} ± 3.30	49.49 ^{abcdef} ± 5.41
	Algerian	9.73 ^{abcdefghi} ± 1.58	12.53 ^{abc} ± 3.12	22.26 ^{abcde} ± 4.70
	Meyers	25.24 ^{abcdefghijklmnopqrstu} ± 1.45	37.28 ^{abcdef} ± 3.82	62.52 ^{abcdef} ± 5.24

	Monterey	300.09 ^{ALAMANAOAP} ± 6.55	343.14 ^{uvwxy} ± 3.70	643.23 ^{wxyzAAABAC} ± 10.19
	Robusta	288.11 ^{AKALAMANAO} ± 1.70	511.50 ^{ADAEAF} ± 0.79	799.61 ^{AHAIAJAKAL} ± 2.48
	Beetroot	296.02 ^{ALAMANAOAP} ± 0.67	432.82 ^{AAAB} ± 1.06	728.84 ^{ADAEAFAGAH} ± 1.71
Microwave 10 s	**American Giant**	15.40 ^{abcdefghijklm} ± 0.32	24.29 ^{abcde} ± 0.46	39.69 ^{abcde} ± 0.14
	Morado	5.13 ^{abcdefg} ± 2.10	6.88 ^{ab} ± 3.01	12.01 ^{abc} ± 5.11
	Ficus Indice	49.30 ^{abcdefghijklmnopqrstuvwx} ± 2.28	11.15 ^{abc} ± 2.07	60.45 ^{abcdef} ± 1.74
	Gymno Carpo	49.62 ^{abcdefghijklmnopqrstuvwx} ± 2.28	2.90 ^{a} ± 3.47	52.53 ^{abcdef} ± 5.54
	Algerian	26.52 ^{abcdefghijklmnopqrstu} ± 1.61	34.53 ^{abcde} ± 3.25	61.05 ^{abcdef} ± 3.97
	Meyers	15.61 ^{abcdefghijklm} ± 1.58	2.29 ^{a} ± 1.83	17.91 ^{abcde} ± 3.25
	Monterey	397.08 ^{ARAS} ± 29.36	259.26 ^{nopq} ± 5.05	656.35 ^{xyzAAABACAD} ± 26.70
	Robusta	185.98 ^{AFAGAH} ± 1.03	321.29 ^{stuvwxy} ± 7.38	507.27 ^{rst} ± 6.83
	Beetroot	164.80 ^{ADAEAFAG} ± 0.37	440.31 ^{AAAB} ± 0.26	605.11 ^{vwx} ± 0.11
Microwave 10 s with AA	**American Giant**	14.76 ^{abcdefghijk} ± 0.85	23.07 ^{abcde} ± 0.70	37.83 ^{abcde} ± 1.50
	Morado	8.02 ^{abcdefgh} ± 0.64	6.26 ^{ab} ± 1.47	14.28 ^{abc} ± 1.05
	Ficus Indice	52.51 ^{efghijklmnopqrstuvwxy} ± 1.13	11.92 ^{abc} ± 0.46	64.43 ^{abcdef} ± 1.58
	Gymno Carpo	48.02 ^{abcdefghijklmnopqrstuvwx} ± 4.31	8.10 ^{ab} ± 0.70	56.12 ^{abcdef} ± 4.59
	Algerian	27.59 ^{abcdefghijklmnopqrstuv} ± 0.32	29.64 ^{abcde} ± 4.23	57.23 ^{abcdef} ± 4.25
	Meyers	19.04 ^{abcdefghijklmno} ± 0.81	30.86 ^{abcde} ± 3.12	49.90 ^{abcdef} ± 2.40
	Monterey	458.58 ^{AT} ± 0.74	319.00 ^{rstuvwx} ± 1.65	777.58 ^{AGAHAIAJAK} ± 1.23
	Robusta	171.86 ^{ADAEAFAG} ± 32.88	292.72 ^{pqrstu} ± 68.84	464.58 ^{qrs} ± 101.70
	Beetroot	205.97 ^{AFAGAHAI} ± 0.46	197.24 ^{klm} ± 5.21	403.20 ^{nopq} ± 5.55
Microwave 20 s	**American Giant**	8.45 ^{abcdefghi} ± 0.67	8.86 ^{ab} ± 1.47	17.31 ^{abcd} ± 1.69
	Morado	2.25 ^{abcd} ± 3.33	4.43 ^{a} ± 0.70	6.68 ^{ab} ± 2.68
	Ficus Indice	166.51 ^{ADAEAFAG} ± 1.93	58.82 ^{bcdefg} ± 0.95	225.33 ^{ij} ± 2.85
	Gymno Carpo	47.38 ^{abcdefghijklmnopqrstuvwx} ± 1.03	10.08 ^{ab} ± 0.46	57.46 ^{abcdef} ± 1.47
	Algerian	20.32 ^{abcdefghijklmnop} ± 1.34	24.75 ^{abcde} ± 0.79	45.07 ^{abcdef} ± 0.60
	Meyers	17.75 ^{abcdefghijklmn} ± 0.74	28.88 ^{abcde} ± 0.46	46.63 ^{abcdef} ± 1.16
	Monterey	161.49 ^{ADAEAFAG} ± 31.96	169.12 ^{jk} ± 3.30	330.61 ^{klmn} ± 28.70
	Robusta	184.16 ^{AFAGAH} ± 55.87	189.29 ^{kl} ± 50.60	373.45 ^{lmnop} ± 96.20
	Beetroot	169.72 ^{ADAEAFAG} ± 0.56	449.47 ^{AAAB} ± 0.53	619.19 ^{wxyzAA} ± 1.08
Microwave 20 s with AA	**American Giant**	13.05 ^{abcdefghij} ± 2.18	35.75 ^{abcdef} ± 8.34	48.80 ^{abcdef} ± 10.49
	Morado	2.78 ^{abcd} ± 1.52	2.60 ^{a} ± 1.91	5.38 ^{a} ± 3.28
	Ficus Indice	195.92 ^{AFAGAHAI} ± 26.56	67.22 ^{defgh} ± 1.47	263.14 ^{jk} ± 25.91
	Gymno Carpo	41.17 ^{abcdefghijklmnopqrstuvwx} ± 0.74	8.56 ^{ab} ± 0.26	49.73 ^{abcdef} ± 0.48
	Algerian	23.85 ^{abcdefghijklmnopqrst} ± 0.98	26.43 ^{abcde} ± 1.40	50.28 ^{abcdef} ± 0.87
	Meyers	23.10 ^{abcdefghijklmnopqrs} ± 2.31	33.00 ^{abcde} ± 1.21	56.10 ^{abcdef} ± 3.40
	Monterey	99.35 ^{yzAAAB} ± 46.75	135.06 ^{ij} ± 1.61	234.41 ^{ij} ± 45.69
	Robusta	125.77 ^{AAABACAD} ± 30.36	271.64 ^{opqrs} ± 123.03	397.41 ^{mnopq} ± 153.23
	Beetroot	208.60 ^{AGAHAI} ± 2.38	455.28 ^{AAABAC} ± 0.70	663.88 ^{xyzAAABACADAE} ± 2.25
Microwave 30 s	**American Giant**	5.24 ^{abcdefg} ± 0.49	6.88 ^{ab} ± 1.21	12.12 ^{abc} ± 1.13
	Morado	6.10 ^{abcdefgh} ± 1.60	2.75 ^{a} ± 2.55	8.85 ^{abc} ± 3.77
	Ficus Indice	66.20 ^{nopqrstuvwxyz} ± 8.26	9.17 ^{ab} ± 1.21	75.37 ^{abcdef} ± 7.09
	Gymno Carpo	44.28 ^{abcdefghijklmnopqrstuvwx} ± 1.93	11.15 ^{abc} ± 1.85	55.43 ^{abcdef} ± 3.13
	Algerian	21.07 ^{abcdefghijklmnopqr} ± 0.81	23.99 ^{abcde} ± 0.53	45.05 ^{abcdef} ± 0.91
	Meyers	18.82 ^{abcdefghijklmno} ± 2.32	23.22 ^{abcde} ± 1.47	42.04 ^{abcde} ± 3.50
	Monterey	191.43 ^{AFAGAH} ± 9.14	130.01 ^{ij} ± 25.03	321.44 ^{klm} ± 18.54
	Robusta	133.79 ^{ABACADAE} ± 36.23	221.68 ^{klmno} ± 62.58	355.47 ^{lmno} ± 97.53
	Beetroot	167.58 ^{ADAEAFAG} ± 0.19	459.86 ^{AAABACAD} ± 0.53	627.44 ^{wxyzAAAB} ± 0.34
Microwave 30 s with AA	**American Giant**	6.63 ^{abcdefgh} ± 0.67	8.56 ^{ab} ± 0.70	15.19 ^{abcd} ± 1.33
	Morado	8.13 ^{abcdefgh} ± 2.89	9.93 ^{ab} ± 2.69	18.06 ^{abcde} ± 5.52

	Ficus Indice	63.31 ^{klmnopqrstuvwxyz} ± 1.77	12.07 ^{abc} ± 1.47	75.38 ^{abcdef} ± 2.76
	Gymno Carpo	44.17 ^{abcdefghijklmnopqrstuvwx} ± 1.85	10.85 ^{abc} ± 1.15	55.02 ^{abcdef} ± 2.07
	Algerian	14.12 ^{abcdefghijk} ± 0.32	17.57 ^{abcde} ± 0.26	31.69 ^{abcde} ± 0.57
	Meyers	11.44 ^{abcdefghij} ± 1.30	28.42 ^{abcde} ± 1.38	39.86 ^{abcde} ± 0.21
	Monterey	183.30 ^{AFAGAH} ± 5.71	118.25 ^{hij} ± 23.09	301.55 ^{jkl} ± 19.68
	Robusta	181.49 ^{AEAFAGAH} ± 39.99	265.22 ^{opqr} ± 73.19	446.71 ^{pqr} ± 35.70
	Beetroot	203.19 ^{AFAGAHAI} ± 1.21	507.22 ^{ACADAEAF} ± 6.63	710.42 ^{ACADAEAFAG} ± 7.76
Stove-top 60 s	**American Giant**	2.14 ^{abc} ± 0.93	3.51 ^{a} ± 0.95	5.65 ^{ab} ± 1.13
	Morado	3.96 ^{abcde} ± 0.98	3.97 ^{a} ± 1.32	7.93 ^{abc} ± 2.16
	Ficus Indice	53.79 ^{ghijklmnopqrstuvwxy} ± 0.37	6.72 ^{ab} ± 0.27	60.51 ^{abcdef} ± 0.10
	Gymno Carpo	37.75 ^{abcdefghijklmnopqrstuvwx} ± 0.37	7.33 ^{ab} ± 0.01	45.08 ^{abcdef} ± 0.37
	Algerian	14.87 ^{abcdefghijk} ± 1.88	19.10 ^{abcde} ± 3.38	33.96 ^{abcde} ± 5.26
	Meyers	12.94 ^{abcdefghij} ± 3.83	30.86 ^{abcde} ± 3.84	43.80 ^{abcde} ± 7.67
	Monterey	259.98 ^{AJAKAL} ± 2.67	271.33 ^{opqrs} ± 2.00	531.32 ^{stuv} ± 4.19
	Robusta	191.00 ^{AFAGAH} ± 1.03	224.58 ^{lmno} ± 0.92	415.59 ^{opq} ± 0.66
	Beetroot	206.40 ^{AFAGAHAI} ± 0.74	478.65 ^{ABACADAE} ± 1.06	685.06 ^{yzAAABACADAEAF} ± 1.80
Stove-top 120 s	**American Giant**	3.10 ^{abcd} ± 1.13	4.74 ^{ab} ± 2.26	7.84 ^{abc} ± 1.42
	Morado	3.42 ^{abcde} ± 1.21	3.82 ^{a} ± 1.47	7.24 ^{abc} ± 2.69
	Ficus Indice	54.44 ^{ghijklmnopqrstuvwxy} ± 0.37	7.64 ^{ab} ± 0.53	62.07 ^{abcdef} ± 0.90
	Gymno Carpo	50.69 ^{cdefghijklmnopqrstuvwxy} ± 0.56	9.93 ^{ab} ± 0.95	60.62 ^{abcdef} ± 1.40
	Algerian	21.28 ^{abcdefghijklmnopqr} ± 1.34	26.74 ^{abcde} ± 0.70	48.02 ^{abcdef} ± 1.96
	Meyers	27.38 ^{abcdefghijklmnopqrstuv} ± 2.91	37.28 ^{abcdef} ± 3.56	64.66 ^{abcdef} ± 6.13
	Monterey	201.16 ^{AFAGAHAI} ± 1.95	205.18 ^{klmn} ± 0.70	406.34 ^{nopq} ± 2.55
	Robusta	167.37 ^{ADAEAFAG} ± 0.81	287.22 ^{pqrst} ± 3.38	454.59 ^{qrs} ± 3.96
	Beetroot	197.42 ^{AFAGAHAI} ± 0.37	510.43 ^{ADAEAF} ± 0.26	707.85 ^{ACADAEAFAG} ± 0.63
Stove-top 180 s	**American Giant**	3.85 ^{abcde} ± 0.32	4.74 ^{ab} ± 0.70	8.59 ^{abc} ± 0.86
	Morado	9.09 ^{abcdefghi} ± 5.74	8.10 ^{ab} ± 1.85	17.19 ^{abcd} ± 6.39
	Ficus Indice	67.59 ^{opqrstuvwxyz} ± 1.77	9.78 ^{ab} ± 1.15	77.37 ^{abcdef} ± 2.09
	Gymno Carpo	59.14 ^{jklmnopqrstuvwxy} ± 0.49	10.39 ^{ab} ± 0.70	69.53 ^{abcdef} ± 1.17
	Algerian	25.03 ^{abcdefghijklmnopqrst} ± 0.01	26.89 ^{abcde} ± 0.95	51.91 ^{abcdef} ± 0.95
	Meyers	31.12 ^{abcdefghijklmnopqrstuv} ± 3.57	42.62 ^{abcdefg} ± 3.97	73.75 ^{abcdef} ± 7.42
	Monterey	268.32 ^{AJAKALAM} ± 0.49	305.71 ^{qrstuv} ± 2.10	574.03 ^{tuvw} ± 1.90
	Robusta	282.44 ^{AKALAMANAO} ± 0.81	509.06 ^{ACADAEAF} ± 0.70	791.50 ^{AHAIAJAKAL} ± 1.49
	Beetroot	197.42 ^{AFAGAHAI} ± 0.37	510.43 ^{ADAEAF} ± 0.26	707.85 ^{ACADAEAFAG} ± 0.63
Significance level		p < 0.001	p < 0.001	p = < 0.001

Means with different superscripts in the same column differ significantly
The background was alternately changed to highlight the different extraction methods that were conducted

Table 4. 6: Stability of all cactus pear cultivars which were tested within 24 hours

	Betalain content			pH stability			Thermal stability			
Sample	**Bx (mg/g)**	**Bc (mg/g)**	**Total**	**Control**	**pH 1**	**pH 4.5**	**25° (extraction temperature)**	**50°**	**80°**	**90°**
American Giant	2.246	3.514	5.760	Yellow green	Very light	Yellow/ green	Yellow green	Very light	Almost colourless	Colourless
Morado	1.283	3.056	4.339	Yellow / green	Very light	Yellow green	Yellow green	Very light	Almost colourless	Colourless
Ficus-Indice	80.529	13.597	94.126	Orange	Lighter orange	Orange	Orange	Orange	Light orange/ yellow	Very light orange yellow
Gymno Carpo	51.547	10.084	61.631	Orange	Lighter orange	Lighter orange	Orange	Orange	Light orange yellow	Very light orange yellow
Algerian	13.903	18.028	31.931	Dark pink	Dark pink	Dark pink	Pink	Pink	Very light	Colourless
Meyers	34.543	50.722	85.265	Dark pink	Very dark	Dark pink	Pink	Pink	Very light	Colourless
Monterey	380.722	457.417	838.139	Wine red	Very dark	Wine red	Light purple	Light purple	Orange	Yellow-gold
Robusta	398.154	669.930	1068.085	Wine red	Very dark	Wine red	Light wine	Light wine	Orange	Yellow-gold
Beetroot	201.483	302.041	503.525	Wine red	Very dark	Wine red	Wine red	Wine red	Brick	Light-brick

Tests done in triplicate; mean values presented in the table
NM: No tests conducted on samples
Bc = betacyanins; Bx = betaxanthins

4.3.6 Stability tests

Colourant stability plays a critical role in determining the conditions for betalain extraction, as well as the products to which they can be applied. As such, the stability of naturally coloured products may be of concern. For example, natural colourants may need to be used in higher concentrations than synthetic colours; this may affect the sensory evaluation of colourants (Galaffu et al., 2015).

4.3.6.1 pH stability

Table 4.6 depicts stability pH tests. The colour of the pigments changed at pH 1, which reflected instability at pH 1. However, there was little to no colour change at pH 4.5, which reflected the stability of all cactus pear extracts cultivars and for beetroot at pH 4.5. As mentioned before, betalains are stable between pH 3.5 and 7. The findings coincided with that of Agrawal (2013), meaning that the pigments can be applied to a variety of food products.

4.3.6.2 Thermal stability

Table 4.6 shows that purple cultivars are the most heat stable; the orange and green cactus cultivars were colourless after heating the samples to 90 °C. In fact, the green cultivars started losing stability at 50 °C, which was the first heating temperature; American Giant and Morado were almost colourless at 80 °C. The rest of the cultivars were much lighter in colour at 80°C. The orange cultivars kept a bit of colour, even at 90 °C.

4.3.6.3 UV light stability

In UV light stability tests, the green cultivars were not included in the test as the results from all the extraction methods have shown that betalain presence in green cultivars was very low. The orange and pink/red cultivars were included. The purple cultivar was represented by Robusta, together with beetroot. There was no trend in the stability of tested samples, some samples slightly dropped in after UV-light exposure, while others slightly increased.

Table 4. 7: UV-light exposure (betalain results before and after UV-light exposure)

	Before UV-light exposure			After UV-light exposure		
Sample	**Bx (mg/g)**	**Bc (mg/g)**	**Total**	**Bx (mg/g)**	**Bc (mg/g)**	**Total**
Ficus-Indice	41.763	9.610	51.373	40.393	11.087	51.480
Gymno Carpo	30.930	9.497	40.427	29.947	6.863	36.810
Algerian	28.760	25.530	54.290	30.640	27.457	58.097
Meyers	25.367	26.450	51.817	25.027	25.627	50.654
Robusta	19.040	18.977	38.017	18.260	17.620	35.880
Beetroot	20.600	17.600	38.200	18.433	14.910	33.343

The UV-betalain-stability test of the chosen samples showed that there was no major stability difference before and after samples were placed under UV-light. The decrease and increase of betalains which is shown in Figure 4.9, reflects minor decreases and increases of colour readings.

The L* a* b* results which are presented in Figure 4.9 show that there were no significant differences to samples before and after UV-light exposure. Some samples become slightly lower and other slightly higher than they were before UV-light exposure.

For L*, orange (Gymno Carpo) had the highest values, followed by pink (Algerian). In a*, pink had the highest values, followed by purple. For b*, orange had and pink had the highest

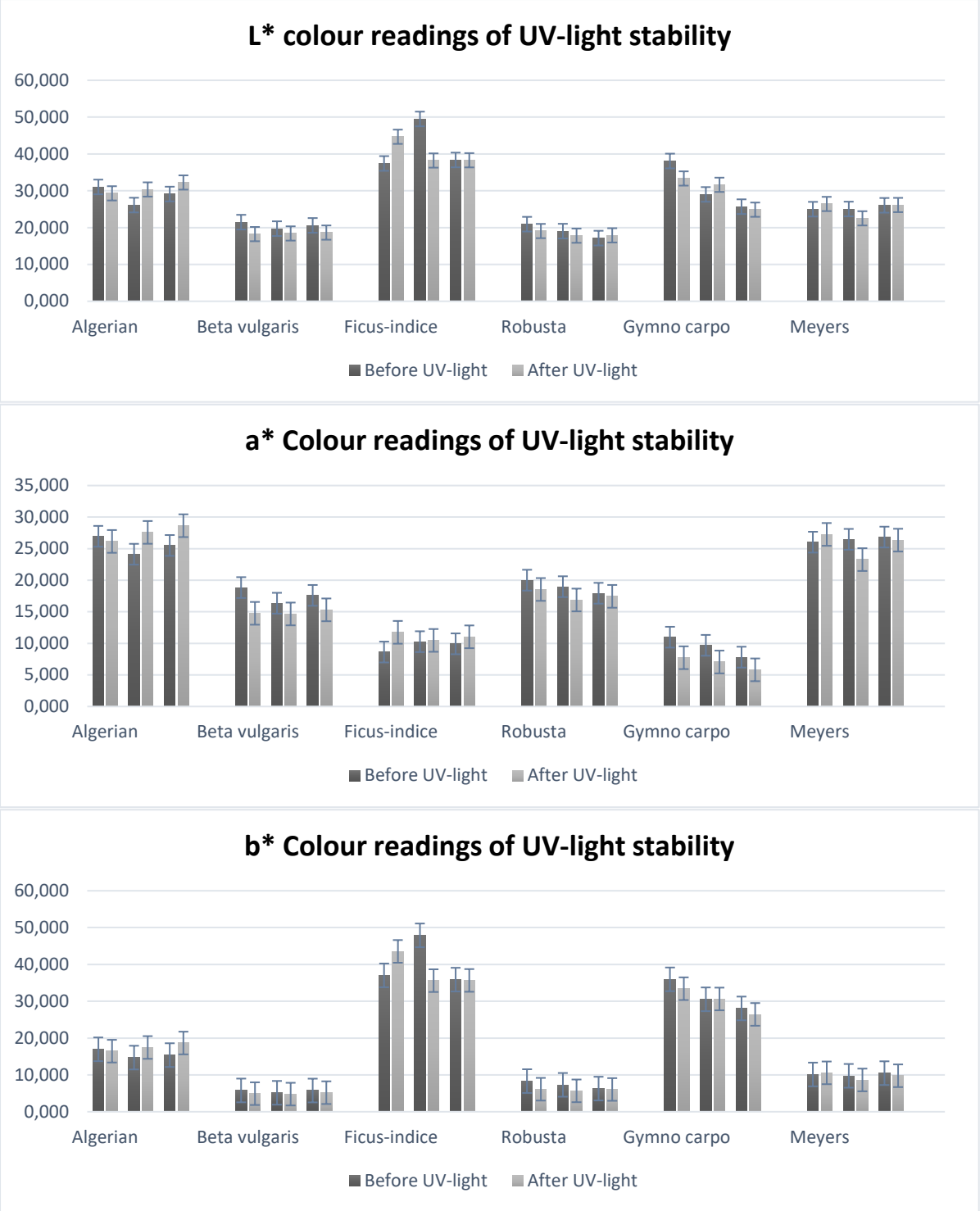


Figure 4. 9: L*, a*, b* values of UV-light stability
Bars representing average values of triplicate analysis with error bars indicating standard deviations

4.4 Conclusion

In this chapter, pink/red, green, purple, and orange cactus pear fruits and beetroot were extracted, and their pH, thermal and UV-light stability tested. Total betalain contents were dependent on the extraction method and pigment source. Green cactus cultivars produced minimal amounts of betalains. The orange cultivars generally contained more betaxanthins and betacyanins, whereas the pink/red cultivars mostly contained more betacyanins than betaxanthins. The purple cactus cultivars contained more betacyanins and betaxanthins than the orange and pink/red cultivars, yet comparable to beetroot.

The highest betalain yields, as per numerical values, were found in water extraction (299.20 mg/g), 50% MeOH extraction (299.24 mg/g), 50% EtOH extraction (266.03 mg/g), and 60-s stove-top extraction (263.52 mg/g). Total betalain yields were dependent on the extraction method and pigment source. Moreover, the highest betalain values were found in purple pigment sources. Therefore, purple pigment sources (even if not cactus) seem to have a more prevalent betalain presence.

Green cactus fruits cannot synthesize yellow and red betalains. Based on this and low betalain yields that were observed throughout the chapter, green cactus pear cultivars were omitted from further analyses. Further experiments were carried out with extracts from beetroot as well as purple, red and orange cactus pears, and purple amaranth (as a control).

The most heat-stable pigments were from red/purple betalain extracts. In pH stability, all pigments were stable at pH 4.5 and unstable at pH 1. The UV-light results showed that sample colours and betalain contents did not have noteworthy changes before and UV-light exposure.

CHAPTER 5

Analysis of betalain extract properties from six cactus pear cultivars, beetroot and amaranth

Abstract

In recent years there has been an increased focus on natural food products and the added benefits they carry. Betalains are nutritious pigments which contain antioxidant properties. Thus, in order to test some of the nutritional benefits of beetroot, six cactus pear cultivars and amaranth vitamin C, total phenols, and flavonoid contents were determined. The thin layer chromatography (TLC) was also tested in order to find the prevalence of betacyanins and betaxanthins in samples. Moreover, extracts from cactus pear, beetroot, and amaranth were classified according to a decision tree which was designed by the EU.

Beetroot samples that were not extracted with the addition of ascorbic acid (AA) had lower phenols, flavonoids, and ascorbic acid than beetroot samples which were extracted with the addition of AA. Amaranth had a significantly higher content of antioxidants than all the other plants.

Results for phenolic compounds showed that there were no significant differences between cactus pear cultivars. However, significant differences were seen between beetroot 1 (Microwave heated without AA) and beetroot 2 (microwave heated with AA) as well as amaranth. In flavonoid compounds, amaranth was significantly higher than all other samples (5.07 mgCE/g). Conversely, the lowest flavonoid compounds were found in beetroot 1 (0.49 mgCE/g). In ascorbic acid, significantly high differences were noticed between amaranth (71.71 mg/100 gDW) and samples from cactus pear and beetroot.

The TLC results showed that Robusta (most vivid in colour), Monterey and Algerian had plates with the most vivid colours, a reflection of the high betacyanin and betaxanthin prevalence in the cultivars. Total soluble solids (TSS) results showed the highest sugar-containing pigment to be Monterey at 13.9, while the lowest was beetroot (5.5).

The classification of betalain pigment extracts as Colouring Foods was achieved through enrichment factor calculation, and the colourant decision tree. The results showed that the betalain pigment extracts could be classified as Colouring Foods.

5.1 Introduction

Betalains are nutritionally-dense pigments which contain colouring abilities and antioxidant properties. The pigments are regarded as functional ingredients for foods which have anti-fat (lipidemic) and anti-cancer properties (Gengatharan et al., 2015). The therapeutic properties contribute to preventing noncommunicable diseases such as diabetes, hypertension, cardiovascular disease, and obesity (Del Socorro Santos Díaz et al., 2017; Neha et al., 2018). These beneficial attributes stem from their high micronutrient presence of flavonoids and ascorbic acid, amongst other nutrients (Khan, 2016b; Antigo et al., 2018).

The cactus pear, amaranth and beetroot plants are good sources of ascorbic acid, antioxidants, and phenolics. Betalains are obtainable from fruit (cactus pear) and vegetables (beetroot and amaranth) and are classified as Colouring Foods (Stich, 2016; Lehto et al., 2017). Colouring Foods are foods with colouring properties which are extracted with the primary intention to add colour to food products (Stich, 2016).
The aim of this chapter was to determine the nutritional benefits of betalain pigments. Thus, the phenolic, flavonol and ascorbic acid presence in beetroot, amaranth and cactus pear were tested. The total soluble solids (TSS) and TLC, tested sugar levels and betalain presence. Betalains were classified according to the EU standards of Colouring Foods.

Amaranth tricolour L. was mentioned in section 2.3.6.3 of Chapter 2, and the experimental analysis was introduced to the project in the current chapter. It was used as a second control as it is currently regarded as an approved colourant in the food industry. This study was the analysis and comparison of the betalain extract properties from six cactus pear cultivars, beetroot and amaranth.

5.2 Materials and methods

5.2.1 Sample collection

5.2.1.1 Beetroot

Beetroot (*Beta vulgaris* L.), Detroit Red cultivar was bought in January 2017, from a local supermarket in Bloemfontein, South Africa. An illustration of similar samples is shown in Figure 4.1.

Beetroot samples were diced into 0.1 x 0.1 cm^3 sample size and divided into two different groups. For beetroot samples, 100 g of was used without the addition of AA. Beetroot 2 samples were prepared by adding 5 ml of a 5% AA solution to 100 g of diced beetroot samples. Both beetroot 1 and 2 samples were placed in microwave-safe dishes and heated for 10 s at medium (50%) heat setting, using a Defy microwave (1000 W**).** For betalain extraction, the samples were liquidised and homogenised with a stick blender (Stintzing et al., 2005) and centrifuged at 9000 rpm at 4°C for 15 min.

The extracted samples were immediately placed in sealable containers and cooled in ice-cold water to stop further cooking (Cardoso-Ugarte et al., 2014). Some of the samples were used in their liquid state (TSS tests and colour classification), and the rest were freeze-dried. The samples that were freeze-dried were frozen at -18°C for 24 hours and subsequently freeze-dried at -20°C for 72 hours using the Labconco FreeZone® Cascade Benchtop Freeze Dry System, and crushed to obtain the pigment powder.

5.2.1.2 Cactus pear

In Chapter 4, it was determined that green fruit did not contain significant amounts of betalains and were therefore left out in the current study. Thus, six cactus pear fruits from three different coloured fruit cultivars; the orange (1) Ficus-Indice and (2) Gymno Carpo; pink/red fruit cultivars (3) Algerian and (4) Meyers, as well as the purple (5) Monterey and (6) Robusta were harvested at 50% colour-break stage. Cactus pear fruits were kept in sealable plastic bags and stored at -20°C until used (less than a month). An example of all six cactus pear cultivars is illustrated in Figure 4.2 of the previous chapter.

The peeled cactus pear fruit was liquidised and passed through a 5 mm mash-size sieve to remove the seeds. For extraction, 100 g of sample was extracted using 100 ml dH_20; and heated in the microwave for 10 s (as described in 5.2.1.1). The samples were liquidised using a Safeway stick blender. The liquid pulp was strained through a 5 mm mash-size sieve, centrifuged and the supernatant frozen at -18°C for 24 hours and subsequently freeze-dried and crushed to obtain the pigment powder used in tests.

5.2.1.3 Amaranth

The purple amaranth (*Amaranthus tricolour* L) leaves were picked in the summer of 2017, from the ARC Vegetable and Ornamental Institute in Roodeplaat, Pretoria, Gauteng Province, SA. Upon picking, the leaves were placed in sealable plastic bags and frozen at -20 °C until used. One hundred grams of leaves were placed in 100 ml dH_20 and heated in the microwave for 10 s (as described in 5.2.1.1). The samples were liquidised using a Safeway stick blender. The liquid pulp was centrifuged and the supernatant frozen at -18°C for 24 hours and subsequently freeze-dried and crushed to obtain the pigment powder used in tests.

5.2.2 Methods overview

Table 5.1 shows a summary of the betalain property tests that were conducted throughout this chapter, and samples that were used in the different analyses.

5.2.2.1. Antioxidants

Antioxidant tests included vitamin C, total phenols and flavonoid contents, done by HPLC on freeze-dried extracts of beetroot 1 and 2, cactus pear (orange Ficus Indice and Gymno Carpo, pink/red Algerian and Meyers, purple Robusta and Monterey), as well as amaranth (as explained in 3.2.5 a).

5.2.2.1.1 Total phenols

The phenolic content of beetroot 1 and 2, six cactus pear cultivars, and amaranth was determined through a method established by Makkr (1999) and modified by Fawole et al. (2009). In a test tube, 50 µl betalain extract which was prepared according to the method in section 4.2.2.2.1 of Chapter 4 was mixed with 450 µl of 50% methanol

Table 5. 1: Tests conducted on betalain extracts from six different cactus pear cultivars, beetroot and amaranth

	Freeze-dried (yes/no)	**Beetroot**	**Beetroot (1)**	**Beetroot (2)**	**Cactus pear**						**Amaranth**
Method					**Ficus-Indice**	**Gymno Carpo**	**Algerian**	**Meyers**	**Monterey**	**Robusta**	
Vitamin C	Yes	**X**	✓	✓	✓	✓	✓	✓	✓	✓	✓
Total phenols	Yes	**X**	✓	✓	✓	✓	✓	✓	✓	✓	✓
Flavonoids	Yes	**X**	✓	✓	✓	✓	✓	✓	✓	✓	✓
TSS °Brix	No	✓	**X**	**X**	✓	✓	✓	✓	✓	✓	**x**
TLC	Yes	**X**	✓	✓	✓	✓	✓	✓	✓	✓	✓
Classification of Colouring Foods	No	**X**	**X**	**X**	**X**	**X**	**x**	**X**	✓	✓	**x**

✓ : method conducted; **x**: method not conducted
Beetroot 1: microwave heated without AA
Beetroot 2: microwave heated without AA

followed by the addition of 500 µl Folin–C and then sodium carbonate (2%) solution after 2 min. The mixture was vortexed, and absorbance read at 725 nm using a UV–visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). Gallic acid standard curve (0.02−0.10 mg/ml) was used, and total phenols were expressed as mg GAE/100 g).

5.2.2.1.2 Flavonoids

The flavonoid content of beetroot 1 and 2, six cactus pear cultivars and amaranth was determined according to an established method by Zhishen et al. (1999). One millilitre of each extract was extracted with 50% methanol (10 ml) and vortexed for 30 s. The mixture was sonicated in an ultrasonic bath for 10 min and centrifuged. Distilled water (1.2 ml) was added to 250 µl of extracted betalain samples and then followed by 75 µl of 5% sodium nitrite. After 5 min, freshly prepared 10% aluminium chloride (150 µl) was added to the mixture, followed by the addition of 500 µl sodium hydroxide after another 5 min, and 775 µl distilled water bringing the final volume to 3 ml. The mixture was vortexed, and absorbance was immediately read using a spectrophotometer at 510 nm.

The calibration curve was plotted using catechin (Sigma-Aldrich, USA) as a standard, and each sample was tested in triplicate. The content was expressed in mg catechin equivalents (CE) per gram DW (Amoo & Van Staden, 2013).

5.2.2.1.3 Ascorbic acid

The ascorbic acid content of beetroot, six cactus pear cultivars and amaranth was determined according to an established method by Moyo et al. (2018), where 1 g of each sample was weighed in a tube. Thereafter, 10 ml of 50% metaphosphoric acid was added to the tube. The samples were then sonicated in an ice-cold water bath for 15 min, centrifuged at 9000 rpm at 4°C for 15 min and filtered through Whatman™ filter paper. The tests were done in the Prominence-*i* HPLC-PDA model system that has a sample cooler LC – 2030C (Shimadzu, Kyoto, Japan).

The samples were chromatographically separated with a C_{18} Luna®column (150 × 4.6 mm, 5 µl), which was kept at 25 °C. Next, an isocratic mobile phase was used, comprising of water (99): acetonitrile (0.9): formic acid (0.1), at a flow rate of 1 ml per

min. The samples were quantified on the calibration curve, which was plotted with L-ascorbic acid dry weight.

5.2.2.2 Total soluble solids (TSS) (° Brix)

TSS ° (Brix) sugars were conducted on beetroot 1 and 2 and six cactus pear cultivars, by using an Atago hand-held refractometer. This was done through squirting two drops of centrifuged samples into the refractometer, which was held against light, and the readings were recorded. The test was done in triplicate for each sample; the mean values were recorded and analysed.

5.2.2.3 Thin layer Chromatography (TLC)

The TLC of beetroot 1 and 2, amaranth and six cactus pear cultivars were done according to the method used by Fawole et al. (2009), where 10 µl of the sample was spotted on TLC plates (Silica gel 60 F_{254}, Merck, Germany). Two plates were developed using methanol and 5% aqueous acetic acid in 50% acetone. The first plate was visualised under short and long wavelengths, whereas the second plate was dipped in a vanillin reagent mixture, which was reported to bind to amines. After development, the plates were dried and viewed under UV light (254, 366 nm low nm for Bx and high nm for Bc). If the sample tests positive for betalains, it verifies that the colouring components of the samples are not from anthocyanins. The quantification of Rf values is shown in the equation below:

$$\text{Rf value} = \frac{distance\ travelled\ by\ solute\ (cm)}{distance\ travelled\ by\ solvent\ (cm)}$$

Quantification of TLC Rf values (retention factor) was calculated for the TLC samples, by using a ruler to measure the distance between the solvent and solute, the calculation is shown below:

5.2.2.4 Classification of Colouring Foods

The classification of colourants was determined according to the decision tree in Figure 5.1; and followed by and the calculation of the threshold value (Reinhart, 2014). There is a classification which standardises the naturalness of food, as not all natural

colourants are edible. With that, the EU guidelines define selective and non-selective, giving guiding principles through the calculation of threshold values and enrichment factors (Stich, 2016).

The extracts can be referred to as vegetable or fruit juice if they can be consumed as food (FDA, 2017). The ratio of pigments to nutritive ingredients determines whether colourants can be classified as Colouring Foods. Threshold values for selective value should be above 6.6; it is the borderline that differentiates between selective and non-selective extraction.

The European Commission (2013) established guidelines which determine whether colourants fall under the group of natural colourants or Colouring Foods. The guidelines include the calculation of the enrichment factor and following the guidelines of the decision tree:

Calculation of the enrichment factor:

$$Fn\ (enrichment\ factor) = \frac{\frac{Cp\ (pigment\ in\ extract)}{Np\ (nutritive\ content\ in\ extract)}}{\frac{Cs\ (pigment\ in\ source)}{Ns\ (nutritive\ content\ in\ source)}}$$

Decision tree

The decision tree is a step-by-step process which determines whether an extract is an additive, whether the EU approves it and if it can be classified as a Colouring Food. The decision tree is elaborated on in Figure 5.1:

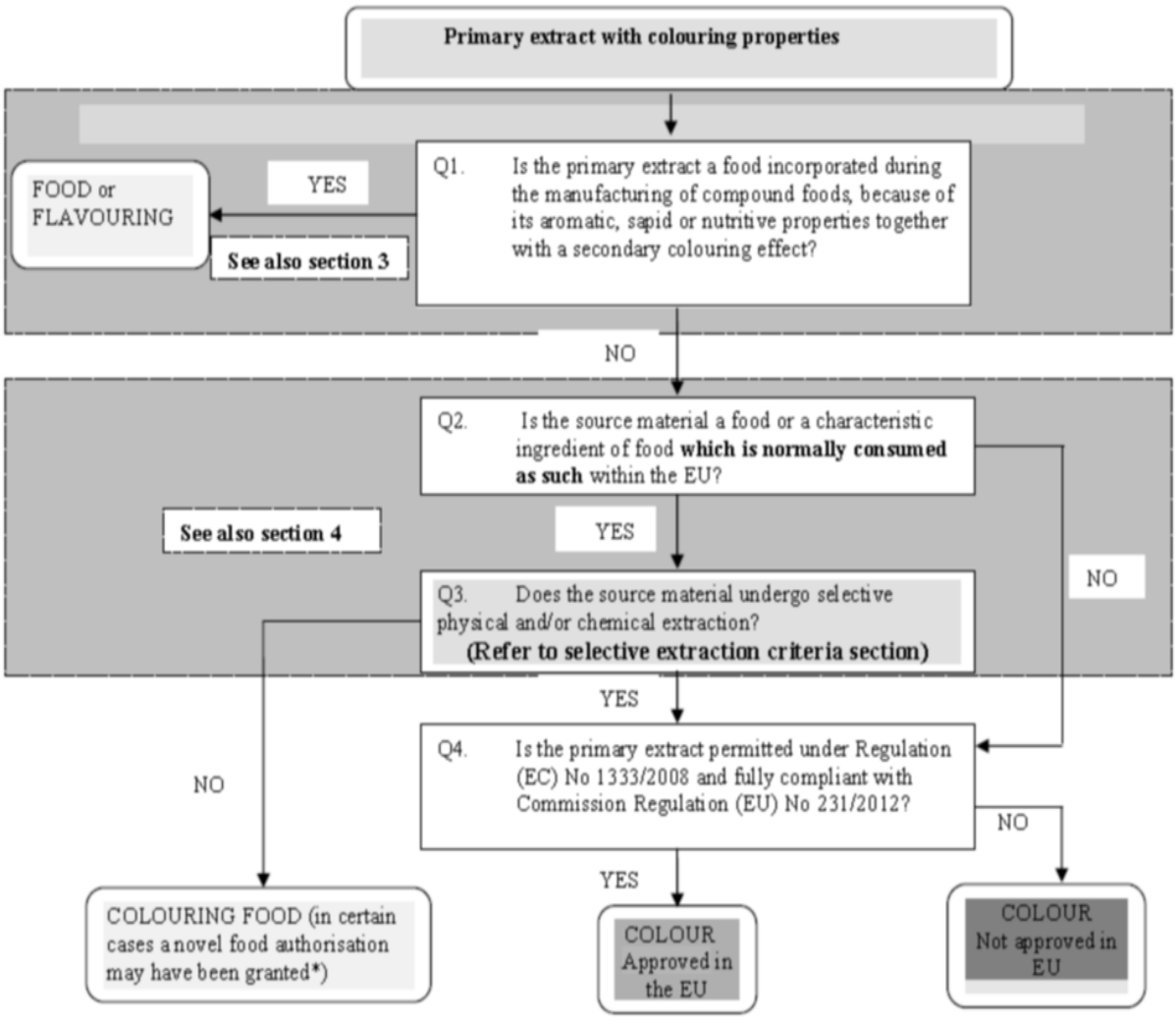


Figure 5. 1: The decision tree for the classification of colourants (Reinhart, 2014)

5.2.3 Statistical analysis:

Antioxidants (ascorbic acid, total phenols and flavonols) were measured on three individual samples from 6 cactus pear cultivars, one amaranth cultivar and one beetroot cultivar. The effect of cultivar on antioxidant content was analysed with one-way analyses of variance (ANOVA) procedure (NCSS 11 Statistical Software 2016). Means were compared with the Tukey-Kramer multiple comparison test at $\alpha = 0.05$ (NCSS 11 Statistical Software 2016). The results for all other analyses are presented as the means of triplicate analyses on three individual samples.

5.3 Results and discussion

5.3.1 Antioxidants

The antioxidant characterisation of betalain extracts from beetroot (heated with and without AA), amaranth and cactus pear (Ficus-Indice, Gymno Carpo, Algerian, Meyers, Monterey and Robusta) is indicated in Table 5.2 and were prevalent, especially for ascorbic acid. Amaranth generally had the highest levels of antioxidants with 71.71 mg/100 g DW for vitamin C, 5.07 mg/ 100 g CE flavonoids, and 34.91 mg GAE/g phenols. The lowest values for ascorbic acid (11.10 mg/100g DW) was found in Gymno Carpo, while low values of 0.49 mg/ 100 g CE flavonoids, as well as 3.18 mg GAE/g phenols, were found in beetroot without AA.

Total phenol results showed that the beetroot samples with AA (29.86 mg GAE/g) and amaranth (34.91 mg GAE/g) were significantly higher than other samples. However, beetroot samples without AA (3.18 mg CE/g) were significantly lower than other samples. There was no significant difference between samples from the orange, pink/red and purple cultivars. The results were 5.35 mg GAE/g, 7.00 mg GAE/ mg GAE/g, 5.38b ± 0.11 mg GAE/g, 5.96b ± 0.20 mg GAE/g, and 6.27 mg GAE/g for Ficus-Indice, Gymno Carpo, Algerian, Meyers, Monterey and Robusta, respectively.

The highest flavonoid levels were generally found in red/purple cultivars and Gymno Carpo from the orange cultivar. Flavonoid levels of the orange cultivars Ficus-Indice and Gymno Carpo differed significantly. In fact, Ficus-Indice showed similar trends to Gymno Carpo and beetroot heated with AA. All the other samples differed significantly. The highest flavonoid numerical values were found in amaranth (5.07 mg CE/g), and lowest in beetroot which did not have AA (0.49 mg CE/g).

The highest ascorbic acid levels were found in amaranth (71.71 mg/100 g DW), which is significantly higher for other samples. It was followed by the orange Ficus-Indice (12.35 mg/100 g DW), which was significantly higher than the orange Gymno Carpo (11.10 mg/100 g).

Du Toit et al. (2015) reported that the colour of the fruit has an impact on the specific antioxidant content. This was further elaborated on in Du Toit et al. (2018), that the highest antioxidant potential was found in purple *O*. Robusta cultivar and orange *O*.

ficus-indica. The ascorbic acid values reported in Du Toit et al. (2015) were higher than the values reported here. In this study, the colour of pigments from cactus pear did not have an impact on antioxidant content results.

According to Ravichandran et al. (2013), extracts of beetroot that have been microwave heated or boiled are high in antioxidants. Those findings, to a certain degree, correlate with the current study, as antioxidants were very high in heated beetroot samples when AA was added.

Amaranth samples contained significantly higher levels of antioxidants compared to all other samples for total phenols, total flavonoids and ascorbic acid. Heat-treated beetroot samples had higher antioxidant values when AA was added to them.

Table 5. 2: The effect of cultivar on antioxidant content

Cultivar	**Total Phenols (mg GAE/g)**	**Total Flavonoids (mg CE/g)**	**Ascorbic acid (mg/100 g DW)**
Ficus-Indice	$5.35^{b} \pm 0.30$	$0.63^{ab} \pm 0.02$	$12.35^{d} \pm 0.08$
Gymno Carpo	$7.00^{b} \pm 0.11$	$3.15^{f} \pm 0.15$	$11.10^{a} \pm 0.19$
Algerian	$5.26^{b} \pm 0.04$	$0.62^{ab} \pm 0.01$	$12.17^{cd} \pm 0.07$
Meyers	$5.38^{b} \pm 0.11s$	$0.69^{b} \pm 0.04$	$12.30^{cd} \pm 0.25$
Monterey	$5.96^{b} \pm 0.20$	$1.39^{c} \pm 0.07$	$11.71^{bc} \pm 0.03$
Robusta	$6.27^{b} \pm 0.55$	$1.62^{d} \pm 0.03$	$11.45^{ab} \pm 0.14$
Beetroot 1	$3.18^{a} \pm 0.03$	$0.49^{a} \pm 0.07$	$11.11^{ab} \pm 0.13$
Beetroot 2	$29.86^{c} \pm 1.40$	$1.84^{e} \pm 0.04$	$12.09^{cd} \pm 0.05$
Amaranth	$34.91^{d} \pm 1.35$	$5.07^{g} \pm 0.05$	$71.71^{e} \pm 0.51$
Significance level	$p < 0.001$	$p < 0.001$	$p < 0.001$

Means with different superscripts in the same column differ significantly
Beetroot 1 (Microwave heated + no AA)
Beetroot 2 (Microwave heated + AA)

5.3.2 TSS (°Brix)

°Brix is an indication of the sugar content in fruit and has a positive influence on the sweet taste and sensory acceptability of food products (Kgatla et al*.,* 2011). Table 5.4 presents an analysis of °Brix, conducted on freshly-centrifuged cactus pear pulp; the °Brix extract had an impact on the final taste of the food products. Monterey (13.9%), with the highest numerical value, does not only impart colour and nutritive value to

food products, but a sweet sensory effect was detected as well. Robusta (10.8%) and beetroot (5.5%) which had the lowest °Brix levels, both do not taste sweet and had low °Brix levels.

Table 5. 3: °Brix for beetroot 1 and six cactus pear cultivars

Cultivar	**Sugar (Glc and Fru) (%)**
Ficus-Indice	13.3
Gymno Carpo	12.2
Algerian	13.1
Meyers	11.9
Monterey	13.9
Robusta	10.8
Beetroot	5.5

Mean values of three replications

5.3.3 TLC

The two figures (Figure 5.2 and 5.3) display bands on the TLC plates from amaranth, cactus pear, as well as beetroot. From Figure 5.2 it can be seen that the two purple (Monterey and Robusta), as well as the red Algerian cultivar, showed similar patterns with the most prominent red (Bc) and yellow (Bx) bands. The other red cultivar, Meyers, showed only Bc (red) bands, while the two orange cultivars had only the Bx pigments. Interestingly, the two beetroot samples (purple) only had the Bc values, while the purple amaranth showed a different (straight, wispy, and oval) pattern where trails of pink/red and yellow were very light.

Figure 5.3 indicates the results of the TLC plates treated with vanillin. Vanillin binds to amines (which are the basic structure of betalains), to verify if the observed bands are true betalains. Of interest are the two orange cultivars, Ficus-Indice, as well as the pink/red Meyers which showed the most prominent bands. Gymno Carpo only showed bands of betaxanthins.

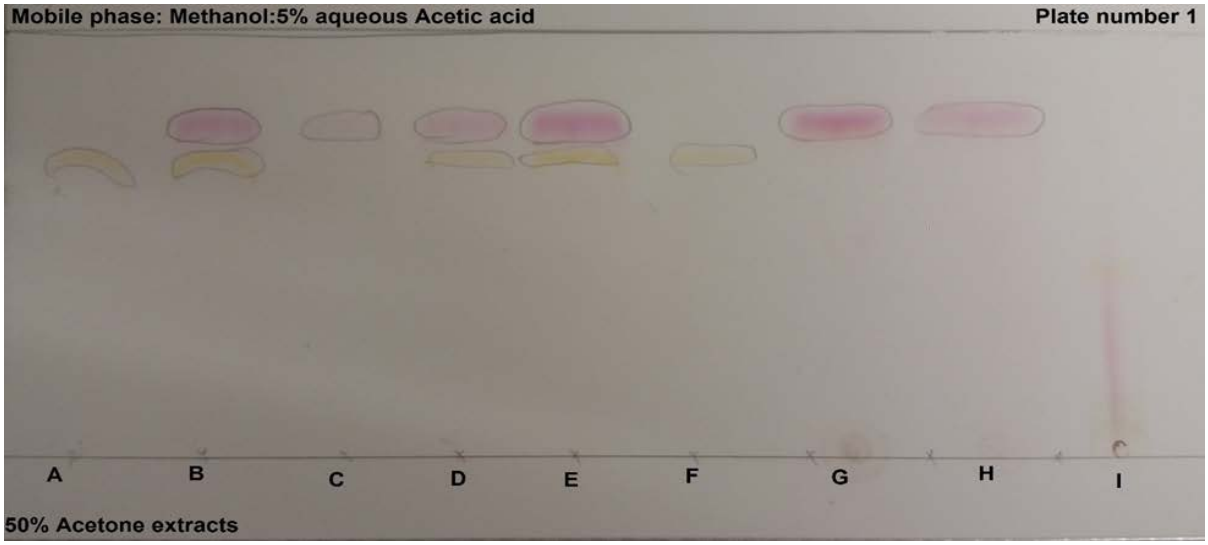


Figure 5. 2: TLC plate visualized under short and long wavelength

A	Ficus Indice	B	Monterey	C	Meyers
D	Algerian	E	Robusta	F	Gymno Carpo
G	Beetroot 1	H	Beetroot 2	I	Amaranth

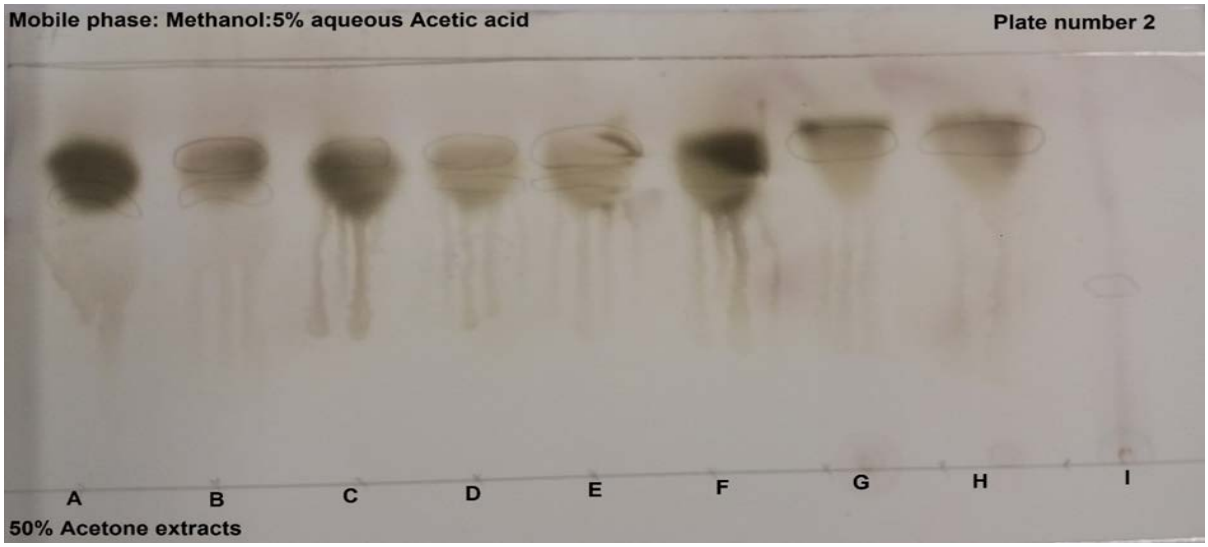


Figure 5. 3: TLC. Vanillin plate

A	Ficus Indice	B	Monterey	C	Meyers
D	Algerian	E	Robusta	F	Gymno Carpo
G	Beetroot 1	H	Beetroot 2	I	Amaranth

The results in Table 5.3, show that Bc (red) pigments were found in the purple pigment sources Monterey, Robusta, both beetroot samples, as well as in the red Algerian and Meyers. However, Bc pigments were absent in the two orange cultivars, Ficus-Indice and Gymno Carpo, as well as Amaranth. Yellow (Bx) pigments were found in the

orange Ficus-Indice and Gymno Carpo, red Algerian, as well as the purple Monterey and Robusta cultivars.

Table 5. 4: Rf value calculation for TLC samples with (Figure 5.3) and without vanillin (Figure 5.3)

		Without vanillin		With vanillin	
TLC code	**Cultivar**	**Bc (mg/g)**	**Bx (mg/g)**	**Bc (mg/g)**	**Bx (mg/g)**
A	Ficus indica	NM	0.70	NM	0.75
B	Monterey	0.78	0.70	0.77	0.71
C	Meyers	0.77	NM	0.76	NM
D	Algerian	0.70	0.78	0.77	0.70
E	Robusta	0.80	0.72	0.80	0.71
F	Gymno Carpo	NM	0.72	NM	0.73
G	Beetroot 1	0.80	NM	0.80	NM
H	Beetroot 2	0.80	NM	0.80	NM
I	Amaranth	NM	NM	NM	NM

Bc betacyanin Rf values; Bx betaxanthins Rf value
NM: Not Measured
Beetroot 1 (Microwave heated + no AA)
Beetroot 2 (Microwave heated + AA)

5.3.4 Classification of colourants according to the decision tree

Figure 5.4 shows the decision tree which was designed according to the EU; the responses (yes or no) are related to the extracted pigments of the current research, and the pathway followed to answer all the questions are indicated in red.

According to the pathway followed in the decision tree, by responding to the questions asked on the diagram, the current betalain colourants can, therefore, be classified as a Colouring Food.

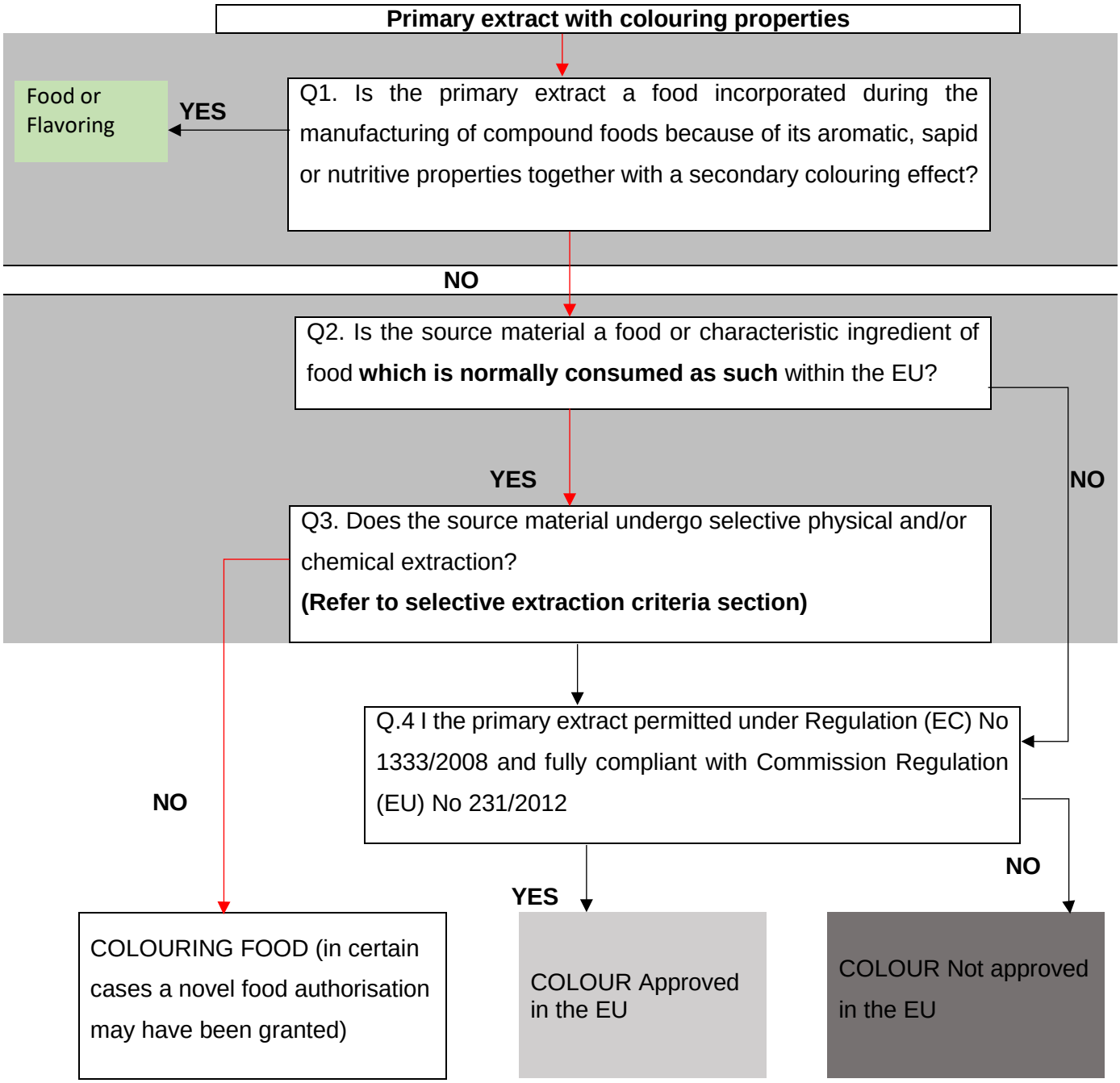


Figure 5. 4: The decision tree for the classification of colourants
Pathway followed is shown in red

In Table 2.1 of Chapter 2, Lehto et al. (2017) stated that vegetable and fruit juice that are classified as Colouring Foods, are not listed as colourants with EU numbers, and are permitted for use in the EU and USA. According to Stich (2016), Colouring Foods are food with colouring properties and are extracted with the intent to add colour to foodstuffs. These Colouring Foods include carrots, spinach and spirulina. Moreover,

these Colouring Foods are described as juices, edible, non-selective, not standardised and have a low colour intensity.

Firstly, the threshold should be above 6.6 to be regarded as non-selective colourants that are Colouring Foods. Both the purple cactus pear cultivars and beetroot are above that prerequisite value (as indicated Table 5.5). Therefore, it can be deduced that beetroot and Robusta can be viewed and classified as Colouring Foods.

Secondly, the extracted pigments should possess primary or secondary colouring effects. For this research, the extracts are primarily used for colouring purposes.

Thirdly, consideration is on whether the pigments are concentrated, such as freeze-dried. Most extracts were not concentrated. The concentration of pigments mainly took place during the analysis of TLC and antioxidants.

Finally, the selectivity or non-selectivity of pigments is dependent on the ratio of pigments to nutritive or aromatic constituents (Reinhart, 2014).

Table 5. 5: Colouring Food classification for beetroot and purple cactus pear cultivar

	Beetroot	**Purple cactus pear fruit (Robusta)**
Cp (pigment in extract)	302.04	669.93
Np (nutritive content in source)	5.50	10.80
Cs (pigment in source)	54.00	77.47
Ns (nutritive content in source)	9.40	9.50
Threshold	7.9	8.9

5.4 Conclusion

Antioxidant presence in pigment sources provides healthful benefits to consumers. In the property analysis of beetroot, six cactus pear cultivars, and amaranth, the betalain extracts had varying contents of vitamin C, phenols, and flavonoids.

Antioxidants were prevalent in amaranth, and beetroot that was heated with AA. The antioxidants in amaranth were significantly higher than all the other samples, with 34.91 mgGAE/g in phenols, 5.07 mg CE/g in flavonols, and 71.71 mg/100g DW.

Beetroot samples that were heated without AA had the lowest numerical value for all antioxidants. Therefore, betalain extracts from amaranth contained the highest betalains than betalain extracts from beetroot and cactus pear. These results show that betalain presence is dependent on the extraction method and betalain source.

The extracted samples can be classified under Colouring Foods. Results further showed that Robusta, Monterey and Algerian fruit had the highest betacyanin and betaxanthin content in the cactus pear cultivars.

CHAPTER 6

Application of betalain extracts as Colouring Foods to food products

Abstract

Colouring Foods have an inherent ability to impart tinctorial properties to food products. They often carry nutritional benefits and may impart taste to food products; the taste is found in geosmin (earthy) in beetroot and sweetness found in the Monterey cactus pear cultivar.

The determination of Colouring Foods shows that the industry can finally break-through in the development of 100% natural colouring products. It would ultimately come up with new accurate, present-day, catchy phrases such as naturally produced and coloured, plant-based colourants, and nutritionally dense colourants.

Three different plants were used during the colouring process (beetroot, cactus pear and amaranth) and use of each plant was dependent on availability as certain cactus pear cultivars, and amaranth was not always available during the colouring sessions. Colouring Foods were extracted according to the method in Chapter 4, section (4.2.2.2.1) and applied in their watery or freeze-dried state. Nine food products were coloured with the watery extract: yoghurt, milkshake, ice-cream, jelly, pancakes, cupcakes, icing, coloured sugar and candyfloss. The tenth, polony, was coloured with freeze-dried pigments because of its high heat (72°C) exposure over a long period (3 hours).

Results showed that dairy products such as milkshake, yoghurt and ice cream were coloured successfully and remained stable even after ten days. Other products where pigment colour was stable included jelly, coloured sugar and icing sugar. Products such as candyfloss were not stable due to high heat exposure and did not remain stable over the ten days.

Robusta and Monterey pigment extracts imparted more colour than other pigment sources. Monterey imparted a pleasantly sweet and fruity flavour to food products. In its freeze-dried state, amaranth powder coloured polony more effectively than Robusta and beetroot.

6.1 Introduction

There is a growing need to replace synthetic colourants with natural ones (Antigo et al., 2018). This is primarily caused by the younger customer base, millennials, who enjoy experimenting with new interactive food products, as well as daring colours and flavours (Mintel, 2018). Accordingly, betalain-containing plants, such as beetroot, add colour to food products (Antigo et al., 2018). The use of natural pigments, such as beetroot, is beneficial because it is affordable and has not been reported to cause any allergic side effects (Neha et al., 2018).

The strength of the colour of colouring pigments is dependent on the concentration of the pigments in the fruit (Rodriguez-Amaya, 2016). According to Delgado-Vargas et al. (2000), adding 50 mg of beetroot colourant (betalain) per kg of product could result in the desired colouring. Betalains are stable at pH 3.5 - 7, therefore, they can impart colour to a variety of foods such as gelatines, desserts, meat products, confectioneries, dry mixes and poultry (Agrawal, 2013). Notably, the stability of betalain pigments is influenced by pH, temperature, time of heat exposure and water activity (a_w) of the food product (Nemzer et al., 2011).

Research proves that most of the natural-colourant qualities are comparable to those of synthetic colourants. Recent studies have proven that colourant instability can be solved by micro or nanoencapsulation (Nunes, 2014). Thus, developing acceptable colours with lawfully permitted colourants, such as betalains (including betanin), quinones, such as cochineal, flavonoids, such as anthocyanins, isoprenoids (carotene), annatto (bixin, norbixin), paprika extract, lutein, canthaxanthin, porphyrins (chlorophylls and chlorophyllins) and copper complexes of these compounds, as well as caramels, curcumin or plant coal (Janiszewska-Turak et al*.,* 2016).

Beetroot and cactus pear can be used interchangeably, as cactus has a broader colour spectrum and more palatable sensory impact than raw beets, which have an earthy (geosmin) flavour. In addition, the hues of both beetroot cactus pear can allow the colourant to be used without any certification (Azeredo, 2009; Stintzing et al., 2001).

The aim of this chapter was to apply the extracted betalain pigments to food products; this aids in food colouring and product development. The stability of coloured foodstuffs will also be tested over a period of 10 days through colour parameter analysis.

6.2 Materials and methods

The food products were coloured with watery betalain extracts, also referred to as vegetable or fruit juice. This is except for polony, which was coloured with freeze-dried extracts. Polony was also the only product where amaranth was added as an additional control colourant. This was done to compare the three purple plant extracts which derive from Robusta (cactus pear), beetroot and amaranth. The three plant extracts were also compared to Erythrosine, a synthetic polony colourant which is used in the food industry and mentioned in section 2.3.4 of Chapter 2.

6.2.1 Methods

Food product formulation was achieved through pigment application in a variety of food products: low-fat and full cream yoghurt; milkshake; ice-cream; jelly; pancakes; cupcakes; icing sugar; coloured sugar; candyfloss; and French polony. Pigments from different betalain sources, namely beetroot, cactus pear, and amaranth, coloured different food products. The list of coloured products and pigment sources is highlighted in Table 6.1.

Table 6. 1: Betalain pigments from beetroot, amaranth and Robusta applied to different food products

	Beetroot (red/purple)	**Cactus pear**						**Amaranth (red/purple)**
Food product		**Ficus-Indice (orange)**	**Gymno Carpo (orange)**	**Algerian (pink/red)**	**Meyers (pink/red)**	**Monterey (red/purple)**	**Robusta (red/purple)**	
Low-fat yoghurt	✓	✓	**X**	**x**	✓	**X**	✓	**x**
Full-cream yoghurt	✓	✓	**X**	**x**	✓	**X**	✓	**x**
Milkshake	✓	**X**	**X**	**x**	**X**	**X**	✓	**x**
Ice cream	✓	✓	**X**	**x**	✓	✓	✓	**x**
Jelly	✓	✓	**X**	**x**	✓	**X**	✓	**x**
Pancakes	✓	✓	✓	✓	✓	**X**	✓	**x**
Cupcakes	✓	**X**	**X**	**x**	**X**	**X**	✓	**x**
Icing sugar	✓	✓	**X**	**x**	**X**	**X**	✓	**x**
Coloured sugar	✓	**x**	**X**	**x**	**X**	✓	✓	**x**
Candyfloss	✓	**x**	**X**	**x**	**X**	✓	✓	**x**
French polony	✓	**x**	**X**	**x**	**X**	**X**	✓	✓

✓ : method conducted; **x**: method not conducted

- French polony was coloured with powered betalain extracts;
- Other samples were coloured with liquid betalain extracts

6.2.1.1 Formulation of food products

6.2.1.1 Yoghurt

Yoghurt was used in the betalain pigment test to observe the effect of low pH and low-temperature storage conditions on betalain stability. The pH of the yoghurt during production was tested and found to be 4.6, which is within the range of betalain pH stability (3-7). Yoghurt is also kept at a low temperature (4°C), which is also suitable for the stability of betalains (Pavokovic et al., 2011). The effect of the fat content was also determined on colouring ability.

Danone Nutriday plain, low-fat and double-cream yoghurts were bought from a local supermarket in Bloemfontein, SA. To colour yoghurt, 1 ml of liquid betalain extracts from beetroot and three cactus pear cultivars (Ficus-Indice, Meyers, and Robusta) was hand-mixed with 200 ml of the yoghurt using a wooden spoon.

The colour stability of the samples was determined by taking photos of the colour on day one and again on day ten in order to make a visual colour comparison. The samples were kept in sealable containers, placed in a box to keep light from coming into the products, and kept at 4°C in a refrigerator. The colour determination formulae are shown below:

Colour determination

In addition to the visual comparison test, the L*, a*, b*, C* and h° values were determined on the day of production and again after ten days using a Minolta CR400 chronometer (Rippoll et al., 2012).

Colour is usually determined according to the method in section 3.2.3.2 of Chapter 3, where the L* values reflect lightness, where 0 indicates black and 100 white. The a* measures redness where +a* is red and –a* is green. The b* reflects yellowness where +b is yellow and –b is blue. The C* measures Chroma saturation index (SI), the purity or saturation of the colour Chroma or saturation index (SI); h° is the hue angle of colour tone and reflects the colour variation in the plane formed by a* and b* coordinates of the sample where: 0° or 360° is red, 90° is yellow, 180° is green, and 270° is blue (Cai et al., 1998; HunterLab, 2007) and was calculated as follows:

Metric chroma: C* = $\sqrt{a^2 + b^2}$

Metric Hue-Angle: h°= $\tan^{-1} \frac{b*}{a*}$

a* and b* chromaicity coordinates in the L*, a*, b* colour space

6.2.1.2 Milkshake

Milkshake was included in order to observe the effect of neutral pH and low-temperature storage conditions on betalain stability. Milkshake is a dairy-based beverage, thus, has a neutral pH, which enables colouring with betalain pigments (Agrawal, 2013). The key ingredients of the manufactured milkshake were ice cream and milk; hence the product was prepared under low temperatures (±4 °C). The milkshake, including the control (strawberry milkshake), was prepared according to the instructions in the manual of the Safeway milkshake maker, with slight modifications. The ingredients consisted of 70 g of vanilla ice cream and 25 ml of Pick ’n Pay full cream milk which was mixed in the milkshake maker.

For the control, 25 g of fresh strawberries were added to the mixture, as per manufacturer instructions.

Since the control was pulpy, centrifuged and uncentrifuged (pulpy) betalain extracts from beetroot and Robusta were added to the milkshake. This was done to compare the colouring of milkshake with strawberries with beetroot and Robusta and whether or not the centrifugation of the samples would have an effect on the final colour.

The milkshake was coloured with 2 ml (centrifuged or uncentrifuged) betalain extract, which was combined with 100 ml milk and stirred into the flavourless milkshake samples for 5 minutes.

The colour stability of the samples was determined by taking photos of the colour on day one and again on day ten in order to make a visual colour comparison. The samples were kept in sealable containers, placed in a box to keep light from coming into the products, and kept at 4°C in a refrigerator.

The L*, a*, b*, C* and h° values were determined on the day of production and again on the tenth day of colouring.

6.2.1.3 Ice-cream

Ice-cream was used in the betalain pigment test to observe the effect of neutral pH and extremely low-temperature storage conditions on betalain stability. Ice cream is a dairy product that has a neutral pH and can easily be coloured using betalains (Stintzing & Carle, 2004). The preparation of ice cream takes place under low temperatures, in fact, the ice cream tank that was used to make the ice-cream was kept in the freezer -18°C for more than 8 hours before production.

All ingredients were kept at 4 °C, according to the Safeway ice cream maker manual.

The ingredients comprised of 100 ml Pick ’n Pay full cream milk, 50 ml Clover whipping cream, and 15 g Hulletts sugar. Milk and sugar were placed in a saucepan and stirred until all ingredients were melted and sticky.

Five millilitres of natural colourant from beetroot and four cactus pear cultivars (Ficus-Indice, Meyers, Monterey, and Robusta) were added to 100 ml ice-cream mixture. The ice cream was made with a Safeway ice-cream maker which was pre-frozen for 8 hours before mixing the ingredients.

The control sample was Dairymaid Farmhouse vanilla ice cream that is already used in the industry.

The colour stability of the samples was determined by taking photos of the colour on day one and again on day ten in order to make a visual colour comparison. The samples were kept in sealable containers, placed in a box to keep light from coming into the products, and kept at -5 °C in a freezer.

The L*, a*, b*, C* and h° values were determined on the day of production and again on the tenth day of production.

6.2.1.4 Jelly

The colouring of jelly was done to observe the effect of gelling agents which have been exposed to high temperature and low pH on betalain stability. Gelling agents are inherently found in fruit pectin, which is used to prepare fruit jelly. Jelly is used to formulate even more food products such as confectionery gels, e.g. jelly babies (Budinsky et al., 2001). Gels, including confectionery gels, are found in various textures and colours; therefore jelly, which can be manipulated into different forms of

food products, was formulated to test if betalain colourants can be used to break into the gel-colouring market.

The jelly was prepared with freshly boiled water at temperatures higher than 90°C (temperatures where most betalains were unstable during thermal stability tests, shown in section 3.2.6 of Chapter 3). During the formulation of jelly, 10 g of clear, unflavoured and uncoloured Sheridans gelatine was added to 100 ml of boiled dH_20 for the control sample. Two millilitres of the pigment was added to the other samples. Colourants that were added during product formulation were from four different betalain sources, namely Meyers, Robusta, Ficus-Indice and beetroot. The colourants added were representative of all the different cultivar-colours used in the study (orange, purple, pink/red and orange).

According to literature, the addition of 1% acetic or ascorbic acid assists in the stability of betalains and it can be used in heated products (Reynoso et al*.,* 1997). Moßhammer (2006a) also reported that the addition of 0.1% of ascorbic acid leads to higher retention of betalains from yellow cultivars.
In accordance with the literature, the pH of the second set of samples was altered by adding 5% of citric acid ($C_6H_8O_7$) after the solution had cooled down and before the gel was formed.

The colour stability and pH of both the sets of samples were determined by taking photos and pH readings of the colour on day one and again on day ten in order to make a visual colour comparison. The samples were kept in sealable containers, placed in a box to keep light from coming into the products, and kept at 4°C in a refrigerator.

The L*; a*; b*, C* and h° values were determined on day one and day ten of production.

6.2.1.5 Pancakes

Pancakes are versatile, generally pan-fried flat cakes that can be used in savoury and sweet dishes. The colouring of pancakes was done to observe the effect of direct stove-top heat and ambient air temperatures on betalain colouring stability.

During the colouring process, 5 ml of liquid extract beetroot and five cactus pear cultivars (Gymno Carpo, Ficus-Indice, Meyers, Algerian, and Robusta) were hand-

mixed into 60 ml of batter and the pancakes shallow-fried at medium-high heat temperature on a stove.

The pancake batter was formulated according to a standard pancake recipe; 250 ml Golden Cloud cake flour; 30 ml Hullets white sugar; 15 ml Royal baking powder; 1 egg; 30 ml Woodenspoon melted margarine and 2.5 ml salt.

The colour stability of the samples was visually determined by taking photos of the colour of the batter, the heated pancake and after cooking was complete. The colour stability visually was tested again on day ten in order to make a visual colour comparison. After day one, each sample was kept in a zip bag, placed in a box to keep light from coming into the products, and kept at 4°C in a refrigerator. The L*, a*, b*, C* and h° values were determined on the day of production and again after ten days.

6.2.1.6 Cupcakes

Cupcakes are available in different colours and flavours. In the current study, red velvet cupcakes were made from two industry pre-mixed formulations (Ina Paarman and Golden Cloud) as well as colouring an established recipe with beetroot and Robusta cactus cultivar.

The cupcakes were formulated with the intention to observe the effect of rapid and high heat (oven temperatures) exposure in enclosed environments in baked products on betalain stability. Cupcakes are oven or microwave baked small cakes. The baking process exposes them to high temperatures in enclosed environments, which also results in rapid and excessive heat exposure. During their cooling period, they are further exposed to room temperature air.

Two controls and betalain two extracts were used in this test. The ingredients are listed with the intention to determine if the ingredients have an effect on the final colour and its stability.

Control 1: Golden Cloud pre-mix red velvet cupcakes. Ingredients: Stabilised wheat product (wheat, gluten), sugar, dextrose monohydrate, chemically modified maize starch, emulsifier, raising agents, cocoa powder, flavouring (milk), salt, and colourant.

Control 2: Ina Paarman pre-mix red velvet cupcakes. Ingredients: sugar, wheat flour (gluten), corn-starch, raising agent, dextrose, cocoa powder, salt, fibre, beetroot powder (contains: beetroot powder juice concentrate, maltodextrin, citric acid), flavouring, colourant.

The standard cupcake batter as per recipe in the Safeway cupcake maker, with slight modifications, comprised of 450 g Golden Cloud self-raising flour, 190 g Pick ’n Pay castor sugar, 160 ml Pick ’n Pay full cream milk, 2 lightly beaten eggs, 125 g (melted and cooled), and 2.5 ml Robertsons vanilla extract.

The samples were prepared by adding 25 ml of colour extract to 100 g of batter:

Beetroot sample: 25% of centrifuged or uncentrifuged (unfiltered) pigment extract into the standard cupcake batter.

Robusta sample: 25% of centrifuged or uncentrifuged (unfiltered) betalain Robusta pigment liquid extract to the standard cupcake batter.

All cupcakes were baked for 6 min in a Safeway cupcake maker.

The colour stability of samples was determined by taking photos of the colour on day one and again on day ten in order to make a visual colour comparison. The samples were kept in zip bags, placed in a box to keep light from coming into the products, and kept at 4°C in a refrigerator.

The L*, a*, b*, C* and h° values were determined on the day of production and again after ten days.

6.2.1.7 Icing

The icing was included in the betalain pigment test to observe the effect of high viscosity pastes on betalain stability. Cupcakes have a decorative topping that enhances the appearance and flavour of baked products.

Ina-Paarman pre-mix icing was used to prepare the control and samples. The icing was prepared using the included Pick ’n Pay icing sugar mixed together with yellow Woodenspoon margarine as stated in Ina Paarman’s recipe, which consists of cupcake and icing pre-mix contents as well as instructions.

The control sample was prepared using two drops of Crimson Pink Moir's synthetic colourant; while in other samples, 5 ml of the prepared colourant from beetroot and two cactus pear cultivars (Ficus-Indice and Robusta) was added. All colourants were first mixed into the icing by hand and later with a Safeway stick blender with 50 g icing.

The colour stability of samples was determined by taking photos of the colour on day one and again on day ten in order to make a visual colour comparison. The samples were kept in sealable containers, placed in a box to keep light from coming into the products, and kept at 4°C in a refrigerator.

The L*, a*, b*, C* and h° values were determined on the day of production and again after ten days.

6.2.1.8 Coloured sugar and Candyfloss

Coloured sugar was included in the betalain pigment test to observe the effect of minimal processing on betalain stability. Coloured sugar crystals can be used in a variety of foods for purposes such as garnishing food and beverages; as such, Huletts has sugar crystals on the market. Coloured sugar is sold as a snack to children and mostly packaged in straws. It is, therefore, suitable that natural pigments colour these sugar crystals. They are formulated and storable at both room and lower temperatures.

For the colouring of these sugar crystals, which will also be used in the processing of candyfloss, three drops of Crimson Pink synthetic colourant was added to 80 g of Huletts granulated white sugar and used as a control. Thereafter, 5 ml of betalain extracts from beetroot and two cactus pear cultivars (Monterey and Robusta) were hand-mixed with 80 g of sugar. The colourants were mixed with the sugar in sealant bags and allowed to dry overnight in the dark.

Candyfloss was included in the betalain pigment test to observe the effect of high and direct heat exposure of food products on betalain stability. Candyfloss is a popular sweet and colourful delicacy, especially among children. It is included in formulations because it is important for child-friendly foods to be coloured with natural colourants as synthetic colourants can contribute to ADHD prevalence (Masone & Chanforan, 2015; Honma, 2015).

Coloured sugar was used to make candyfloss using a Safeway candyfloss maker. Coloured sugar granules were placed in the candyfloss maker and heated for ±5 min before the floss started formulating and collected with skewers.

The colour stability of samples was determined by taking photos of the colour on day one and again on day ten in order to make a visual colour comparison. The samples were kept in sealable containers, placed in a box to keep light from coming into the products, and kept at 4°C in a refrigerator.

6.2.1.9 French polony

French polony was coloured to observe the effect of prolonged moist heat exposure on betalain stability. French polony is a meat product that is cooked at 72 °C for 2 hours.

The control polony sample was formulated with 1.28 kg lean meat, 0.77 kg cold pork back fat emulsion, 0.773 kg ice water, 123 g spice mixture, 53 g salt mixture, 0.09 g Erythrosine (E 127, a synthetic colourant that is used in the polony-making industry). The samples were prepared using respectively 0.9 g: Robusta (cactus pear), amaranth and beetroot colourants. Samples were sealed with polony casings and cooked at 72 ˚C in a polony steam oven for 2 hours.

The colour stability of samples was determined by taking photos of the colour of the colourant solution, polony emulsion with colourant and cooked polony samples on day one and again on day ten of the cooked polony sample in order to make a visual colour comparison. The samples were kept in sealable containers, placed in a box to keep light from coming into the products, and kept at 4°C in a refrigerator.

The L*, a*, b*, C* and h° values were determined on the day of production and again after ten days.

For freeze-drying, 100 g of colourants from Robusta, amaranth and beetroot was added to 100 ml dH_20. The mixtures were frozen for three days at -18°C before freeze-drying.

6.2.3 Statistical analysis

Six individual samples were randomly selected from each treatment of each product type that was manufactured and subjected to colour measurement, as explained above. Due to the complexity of the extraction process, there was only enough pigment available to manufacture one batch per treatment for each product type; thus, serial replicates were used. The effect of treatment on various colour parameters was analysed with one-way analysis of variance and the means compared with the Tukey-Kramer multiple comparison test at $\alpha = 0.05$ (NCSS 11 Statistical Software, 2016).

6.3 Results and discussion

Colour application and colour determination

Upon observation of betalain strength using different extraction methods, stability, and properties, it is evident that it can be added as a colourant to different foods that vary in pH levels (3-7). It can be prepared and kept under different temperature conditions. This is further justified by Albano et al. (2015) who among other scholars, agree that cactus pear fruits have the potential to be used as a food colourant, especially the purple species. In agreement is Merin et al. (1987) who stated that betalain pigments could be stable, even up to 90 °C, depending on betalain concentration. The results of betalain colouring ability correlating with pigment concentration are also mentioned by Rodriguez-Amaya (2016) and explained in Chapter 1 of the current study.

6.3.1 Yoghurt

The control of the samples was low-fat, and double-cream Nutriday yoghurt, pictorial differences of the samples are shown in Table 6.2. The low-fat yoghurt had a whiter shade than the double-cream, which is an off-white to cream colour. Low-fat coloured yoghurt products showed a more intense colour than full-cream coloured samples. The most vivid colour for both yoghurts on both day one and 10 was from Robusta pigments. The low-fat coloured yoghurt was a darker shade of pink, whereas the full-cream was a softer/lighter shade of pink. The trend was the same with the orange Ficus-Indice low-fat samples were slightly more orange than full-cream. They were followed by the pink/red Meyers, which was slightly pinker in low-fat yoghurt than full-cream. Finally, the beetroot samples were a little redder in the low-fat yoghurt than full-cream yoghurt.

Table 6. 2: Pictorial differences of low fat and full cream yoghurt samples

	Day 1		**Day 10**	
Cultivar	Low-fat	Full-cream	Low fat	Full-cream
Control				
Ficus-Indice				
Meyers				
Robusta				
Beetroot				

The colour differences of samples remained the same on day 1 and 10; the yoghurt did not show any spoilage and readings could be done effectively. Also, the colour intensity of samples did not seem to show a colour loss over the time period.

The L*, a*, b*, C*, and h° of low-fat yoghurt (Table 6.3) showed that the lightness (L*) of day one and ten of the samples differed significantly. The a* values of the control, Ficus-Indice and Meyers reflected a greenish colour; Robusta and beetroot showed a more red colour. The b* of all samples reflected yellowness, with the hue and chroma showing a general redness of Robusta and beetroot being different to that of the other samples with a more intense red tone.

Table 6.4 shows the L*, a*, b*, C*, and h° values of the full-cream yoghurt, where the L* values had a darker shade, with the lowest value found in Robusta (71.59) on day one, and 75.92 on day ten. The a* values from the control, Ficus-Indice, Meyers, and beetroot showed yellowness; while Robusta was the only sample which reflected redness. The b* values were also all yellow; with Robusta showing a different hue value.

Table 6. 3: The effect of cultivar on colour parameters of low-fat yoghurt

	Day 1					Day 10				
Sample	**L***	**a***	**b***	**Chroma**	**Hue**	**L***	**a***	**b***	**Chroma**	**Hue**
Control	$81.53^{c} \pm 0.10$	$-3.42^{a} \pm 0.09$	$8.18^{c} \pm 0.01$	$8.86^{c} \pm 0.03$	$-67.32^{c} \pm 0.53$	$75.16^{a} \pm 0.38$	$-2.59^{b} \pm 0.13$	$6.38^{b} \pm 0.23$	$6.88^{a} \pm 0.17$	$-67.89^{c} \pm 1.71$
Ficus-Indice	$90.91^{d} \pm 0.14$	$-3.55^{a} \pm 0.07$	$12.50^{d} \pm 0.53$	$12.99^{d} \pm 0.52$	$-74.11^{b} \pm 0.56$	$93.58^{c} \pm 0.16$	$-3.91^{a} \pm 0.40$	$12.71^{d} \pm 0.42$	$13.31^{b} \pm 0.33$	$-72.88^{b} \pm 2.06$
Meyers	$103.95^{e} \pm 1.31$	$-0.83^{b} \pm 0.03$	$7.57^{c} \pm 0.24$	$7.62^{b} \pm 0.24$	$-83.74^{a} \pm 0.36$	$83.14^{b} \pm 1.01$	$-0.58^{c} \pm 0.02$	$7.32^{c} \pm 0.18$	$7.35^{a} \pm 0.17$	$-85.47^{a} \pm 0.26$
Robusta	$74.99^{a} \pm 0.69$	$20.35^{d} \pm 0.37$	$1.28^{a} \pm 0.03$	$20.39^{e} \pm 0.36$	$3.61^{d} \pm 0.13$	$82.70^{b} \pm 1.35$	$15.18^{e} \pm 0.69$	$4.89^{a} \pm 0.22$	$15.95^{c} \pm 0.72$	$17.86^{d} \pm 0.19$
Beetroot	$78.81^{b} \pm 1.46$	$2.73^{c} \pm 0.07$	$5.91^{b} \pm 0.44$	$6.52^{a} \pm 0.40$	$65.13^{e} \pm 1.64$	$96.03^{d} \pm 0.18$	$0.65^{d} \pm 0.03$	$7.20^{c} \pm 0.34$	$7.23^{a} \pm 0.34$	$84.84^{e} \pm 0.23$
Significance level	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

Means with different superscripts in the same column differ significantly

Table 6. 4: The effect of cultivar on colour parameters of full cream yoghurt

	Day 1					Day 10				
Sample	**L***	**a***	**b***	**Chroma**	**Hue**	**L***	**a***	**b***	**Chroma**	**Hue**
Control	$93.29^{c} \pm 0.02$	$-3.77^{a} \pm 0.03$	$10.80^{c} \pm 0.50$	$11.44^{b} \pm 0.46$	$-70.73^{c} \pm 0.89$	$102.44^{d} \pm 0.77$	$-3.16^{a} \pm 0.59$	$11.77^{bc} \pm 0.87$	$12.19^{bc} \pm 0.98$	$-75.04^{c} \pm 1.81$
Ficus-Indice	$97.61^{d} \pm 0.42$	$-3.29^{b} \pm 0.05$	$15.45^{d} \pm 0.52$	$15.80^{c} \pm 0.50$	$-77.98^{b} \pm 0.44$	$85.06^{b} \pm 0.62$	$-2.97^{a} \pm 0.31$	$13.21^{c} \pm 0.30$	$13.55^{cd} \pm 0.22$	$-77.33^{c} \pm 1.55$
Meyers	$99.09^{d} \pm 0.52$	$-3.50^{ab} \pm 0.02$	$8.99^{b} \pm 0.11$	$9.65^{a} \pm 0.11$	$-68.73^{c} \pm 0.14$	$90.12^{c} \pm 1.35$	$-0.60^{b} \pm 0.05$	$10.16^{b} \pm 0.67$	$10.17^{a} \pm 0.67$	$-86.60^{a} \pm 0.25$
Robusta	$71.59^{a} \pm 1.80$	$14.71^{d} \pm 0.31$	$2.55^{a} \pm 0.33$	$14.93^{c} \pm 0.29$	$9.84^{d} \pm 1.35$	$75.92^{a} \pm 2.30$	$12.58^{c} \pm 0.57$	$6.73^{a} \pm 0.26$	$14.26^{d} \pm 0.62$	$28.14^{d} \pm 0.23$
Beetroot	$79.12^{b} \pm 0.55$	$-0.44^{c} \pm 0.04$	$10.19^{bc} \pm 0.65$	$10.20^{a} \pm 0.64$	$-87.52^{a} \pm 0.27$	$106.14^{d} \pm 1.48$	$-1.51^{b} \pm 0.36$	$11.08^{b} \pm 0.89$	$11.19^{ab} \pm 0.93$	$-82.31^{b} \pm 1.28$
Significance level	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p = 0.000$	$p < 0.001$

Means with different superscripts in the same column differ significantly

6.3.2 Milkshake

The control was a strawberry milkshake, which was prepared according to the manual of the Safeway milkshake maker, and had a berry/pink colour which was imparted by the strawberries in the milkshake. The colour of the control was similar to that of the samples that had been coloured with filtered Robusta and beetroot (Table 6.5). The unfiltered samples of beetroot and Robusta were slightly darker than filtered samples from both plants. All the samples seemed to be colour stable throughout the stability testing time. Unfortunately, the milkshake got spoiled during the keeping time, and the L*, a* and b* colour readings could only be done on day one (Table 6.6). This is an indication of the short shelf-life, and that testing should have been done sooner (maybe day four or five).

Apparent differences were observed between the beetroot and Robusta, as well as between the two colourants and the control.

The L*, a* b*, chroma, and hue of the samples indicated in Table 6.6 show that the L* value of the samples indicated darker colours. The a* values showed a reddish colour of all samples and the b* yellowness in all samples. Notably, the a* (-1.46) and hue (-85.67) values of unfiltered Robusta reflected a darker red colour when compared to other samples.

Table 6. 5: Pictorial differences of milkshake coloured with beetroot and Robusta cactus pear cultivar

Cultivar	**Day 1**
Control: strawberry milkshake	
Robusta filtered 2:100	
Robusta unfiltered 2:100	
Beetroot filtered 2:100	
Beetroot unfiltered 2:100	

Table 6. 6: The effect of cultivar on colour parameters of milkshake

Sample	L*	a*	b*	Chroma	Hue
Control: strawberry milkshake	$82.38^{c} \pm 0.55$	$8.56^{c} \pm 0.41$	$3.25^{c} \pm 0.20$	$9.15^{b} \pm 0.45$	$20.81^{d} \pm 0.23$
Robusta 2:100	$70.51^{a} \pm 0.90$	$10.71^{d} \pm 0.41$	$2.75^{b} \pm 0.10$	$11.05^{c} \pm 0.37$	$14.41^{c} \pm 1.04$
Robusta unfiltered 2:100	$77.77^{b} \pm 0.08$	$-1.46^{a} \pm 0.03$	$19.25^{e} \pm 0.28$	$19.30^{d} \pm 0.28$	$-85.67^{a} \pm 0.04$
Beetroot 2ml:100	$70.22^{a} \pm 1.07$	$7.45^{b} \pm 0.41$	$0.89^{a} \pm 0.07$	$7.51^{a} \pm 0.40$	$6.84^{b} \pm 0.88$
Beetroot: filtered 2:100	$77.93^{b} \pm 0.11$	$8.74^{c} \pm 0.03$	$6.12^{d} \pm 0.02$	$10.67^{c} \pm 0.02$	$35.00^{e} \pm 0.18$
Significance level	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

Means with different superscripts in the same column differ significantly
Day one samples represented on the Table; samples were spoiled by day ten

6.3.3 Ice-cream

The control sample (Farmhouse vanilla ice-cream) had a cream colour. When the orange Ficus-Indice colourant was added to the home-made ice-cream, which was made according to the method in 6.2.1.3, the colour of the ice cream (very light orange) did not differ much from that of the control. The pink/red Algerian colourant imparted a light pink colour to the ice-cream. On the other hand, the red/purple pigments: Monterey, Robusta, and beetroot imparted a rich pink/purple colour, seemingly more intense in beetroot samples (Table 6.7). Notably, the Monterey-coloured samples had a sweet aroma and taste.

Table 6. 7: Pictorial differences of ice cream coloured with beetroot and different cactus pear cultivars

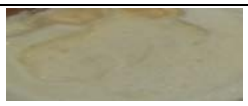
Cultivar	**Day 1**	**Day 10**
Control: farmhouse vanilla ice-cream		
Ficus-Indice		
Meyers		
Monterey		
Robusta		
Beetroot		

Table 6. 8: The effect of cultivar on colour parameters of ice cream

	Day 1					Day 10				
Sample	**L***	**a***	**b***	**Chroma**	**Hue**	**L***	**a***	**b***	**Chroma**	**Hue**
Control: Farmhouse Vanilla ice cream	$63.58^{c} \pm 0.08$	$0.48^{b} \pm 0.02$	$13.23^{d} \pm 1.16$	$13.24^{ab} \pm 1.16$	$87.91^{f} \pm 0.19$	$57.82^{a} \pm 3.07$	$7.39^{c} \pm 0.20$	$3.65^{b} \pm 0.15$	$8.25^{a} \pm 0.20$	$26.28^{d} \pm 1.08$
Ficus-Indice	$78.26^{e} \pm 1.52$	$-2.21^{a} \pm 0.01$	$15.99^{e} \pm 1.01$	$16.14^{bc} \pm 1.00$	$-82.11^{a} \pm 0.47$	$76.09^{c} \pm 3.25$	$-3.41^{a} \pm 0.14$	$25.68^{d} \pm 1.14$	$25.91^{c} \pm 1.14$	$-82.43^{a} \pm 0.20$
Meyers	$74.32^{d} \pm 0.73$	$2.79^{b} \pm 0.17$	$9.93^{c} \pm 0.73$	$10.31^{a} \pm 0.75$	$74.31^{e} \pm 0.28$	$65.54^{b} \pm 1.03$	$2.51^{b} \pm 0.09$	$12.00^{c} \pm 0.35$	$12.26^{b} \pm 0.36$	$78.19^{e} \pm 0.16$
Monterey	$57.39^{b} \pm 2.68$	$17.29^{c} \pm 2.15$	$3.32^{b} \pm 0.13$	$17.61^{cd} \pm 2.11$	$10.97^{c} \pm 1.38$	$57.89^{a} \pm 2.95$	$27.39^{d} \pm 0.20$	$3.65^{b} \pm 0.15$	$27.64^{c} \pm 0.20$	$7.59^{c} \pm 0.32$
Robusta	$43.21^{a} \pm 0.72$	$15.39^{c} \pm 0.48$	$3.80^{b} \pm 0.12$	$15.85^{bc} \pm 0.48$	$13.87^{d} \pm 0.46$	$58.56^{a} \pm 0.99$	$30.41^{e} \pm 1.52$	$4.60^{b} \pm 0.50$	$30.75^{d} \pm 1.58$	$8.59^{c} \pm 0.54$
Beetroot	$59.12^{b} \pm 0.72$	$20.35^{d} \pm 0.91$	$0.83^{a} \pm 0.02$	$20.37^{d} \pm 0.91$	$2.35^{b} \pm 0.12$	$53.00^{a} \pm 1.51$	$26.99^{d} \pm 0.45$	$-1.26^{a} \pm 0.16$	$27.02^{c} \pm 0.45$	$-2.68^{b} \pm 0.34$
Significance level	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

Means with different superscripts in the same column differ significant

With the L*, a*, b*, c*, and h* results (Table 6.8) the lightness (L*) measurement of the samples showed that samples were generally in between: not too light, and not too dark. The darkest samples were obtained with the orange Ficus Indice (78.26) and pink-red Meyers (74.32). An increase in darkness was noticed in Robusta with 43.21 on day one and 58.56 on day ten. With the a* results, Ficus-Indice reflected a yellow colour, and the rest were more red. The b* samples of beetroot were yellow (0.83) on day one and with a blue tint (-1.26) on day ten. It was completely different from all other samples on both days. Notably, the rest of the samples (b*) were yellow. The chroma and hue reflect redness in Monterey, Robusta, and beetroot. The hue angle of the control, Ficus-Indice, and Meyers colourants are higher than that of the red/purple cultivars, reflecting a different shade.

6.3.4 Jelly

Jelly is a popular dessert and a primary ingredient for desserts and confectionary products such as gummy bears. The possibility of colouring jelly with betalains opens opportunities for a variety of products to be produced with natural colourants. Saenz et al. (2017) produced gummy bears from the pulp of purple cactus (pictures of the gummy bears are shown in Figure 2.8 of Chapter 2).

The jelly made with water did not show any observable colour change during the addition of the pigmentation, and the colours were intense and bright. However, the acidified samples showed duller shades (Table 6.9).

Both the acidified and non-acidified jellies remained stable in colour for the period of the ten-day stability-test. It is known that betalains can colour products at pH 3-7; and the pH of all the samples before the addition of colour was within that range.

The L*, a*, b*, chroma, and hue values (Table 6.10) shows that the samples were light in colour as all the L* values were less than 45, not much difference was noticed between day one and ten. The control with acid (-0.69) and Ficus-Indice with acid (-0.80) were greenish on day one and red on day ten; the rest of the samples showed redness. The b* of the non-acidified control sample was more yellowish on day one and more blueish on day ten. The rest of the samples were yellow on both days. The hue of the control changed drastically from day one (80.31) to day ten (-22.53); there were also substantial differences on both day one and ten of the different cultivars. The chroma did not show much difference between day one and ten.

Table 6. 9: Pictorial differences of jelly coloured with beetroot and different cactus pear cultivars

	Day 1				Day 10	
	pH values and colour change of jelly					
Cultivar	**pH of acidified sample**	**Colour of acidified sample**	**pH of non-acidified sample**	**Colour of non-acidified sample**	**Colour of acidified sample**	**Colour of non-acidified sample**
Control	4.30		5.97			
Ficus-Indice	4.16		5.97			
Meyers	4.24		5.90			
Robusta	4.66		5.82			
Beetroot	4.99		6.08			

Table 6. 10: The effect of cultivar and pH on colour parameters of jelly

	Day 1					Day 10				
Sample	**L***	**a***	**b***	**Chroma**	**Hue**	**L***	**a***	**b***	**Chroma**	**Hue**
Control	$34.63^{e} \pm 0.29$	$0.62^{ab} \pm 0.07$	$3.62^{ab} \pm 0.21$	$3.67^{a} \pm 0.21$	$80.31^{e} \pm 1.22$	$31.71^{c} \pm 0.91$	$2.18^{ab} \pm 0.20$	$-0.90^{a} \pm 0.19$	$2.37^{a} \pm 0.12$	$-22.53^{a} \pm 6.21$
Control + acid	$36.31^{e} \pm 2.18$	$-0.69^{a} \pm 0.05$	$8.08^{c} \pm 1.72$	$8.11^{b} \pm 1.71$	$-84.93^{a} \pm 1.38$	$29.78^{bc} \pm 1.10$	$3.88^{cd} \pm 0.71$	$6.97^{e} \pm 0.81$	$8.02^{d} \pm 0.49$	$60.74^{ef} \pm 6.79$
Ficus-Indice	$33.66^{de} \pm 0.60$	$1.85^{b} \pm 0.22$	$13.34^{d} \pm 1.92$	$13.47^{c} \pm 1.91$	$82.02^{e} \pm 1.09$	$29.06^{bc} \pm 0.44$	$3.75^{cd} \pm 0.34$	$11.44^{f} \pm 1.44$	$12.04^{f} \pm 1.46$	$71.79^{f} \pm 1.08$
Ficus-Indice + acid	$41.79^{f} \pm 1.52$	$-0.80^{a} \pm 0.21$	$24.68^{e} \pm 1.82$	$24.70^{d} \pm 1.82$	$-88.15^{a} \pm 0.36$	$28.31^{bc} \pm 0.50$	$1.33^{a} \pm 0.45$	$18.70^{g} \pm 0.71$	$18.75^{g} \pm 0.72$	$85.94^{g} \pm 1.31$
Meyers	$31.30^{d} \pm 0.25$	$11.91^{e} \pm 0.32$	$3.39^{ab} \pm 0.39$	$12.39^{c} \pm 0.25$	$15.90^{c} \pm 2.04$	$28.59^{bc} \pm 1.35$	$5.72^{ef} \pm 0.37$	$2.38^{cd} \pm 0.35$	$6.21^{bc} \pm 0.35$	$22.60^{c} \pm 3.33$
Meyers + acid	$30.50^{cd} \pm 1.07$	$13.29^{e} \pm 0.48$	$7.00^{c} \pm 0.44$	$15.03^{c} \pm 0.58$	$27.77^{d} \pm 1.16$	$28.32^{bc} \pm 1.53$	$6.70^{fg} \pm 0.32$	$7.51^{e} \pm 0.41$	$10.07^{e} \pm 0.28$	$48.26^{d} \pm 2.51$
Robusta	$27.42^{bc} \pm 0.73$	$7.76^{d} \pm 0.18$	$1.83^{a} \pm 0.13$	$7.97^{b} \pm 0.19$	$13.24^{c} \pm 0.75$	$22.31^{a} \pm 0.26$	$7.15^{g} \pm 0.05$	$1.71^{bc} \pm 0.10$	$7.35^{cd} \pm 0.06$	$13.48^{bc} \pm 0.72$
Robusta + acid	$23.99^{a} \pm 1.22$	$5.56^{c} \pm 0.65$	$2.43^{a} \pm 0.55$	$6.07^{ab} \pm 0.81$	$23.45^{d} \pm 2.26$	$23.44^{a} \pm 1.10$	$2.74^{bc} \pm 0.28$	$3.60^{d} \pm 0.52$	$4.53^{b} \pm 0.49$	$52.57^{de} \pm 4.28$
Beetroot	$24.14^{a} \pm 0.95$	$7.80^{d} \pm 0.43$	$0.96^{a} \pm 0.02$	$7.86^{b} \pm 0.43$	$7.03^{b} \pm 0.36$	$27.84^{bc} \pm 3.28$	$2.60^{abc} \pm 0.23$	$0.47^{ab} \pm 0.07$	$2.64^{a} \pm 0.22$	$10.35^{b} \pm 1.97$
Beetroot + acid	$26.80^{ab} \pm 0.64$	$13.62^{e} \pm 1.99$	$5.86^{bc} \pm 0.84$	$14.85^{c} \pm 1.90$	$23.48^{d} \pm 3.86$	$27.66^{b} \pm 0.54$	$5.09^{de} \pm 1.03$	$8.34^{e} \pm 0.33$	$9.80^{e} \pm 0.46$	$58.76^{de} \pm 5.71$
Significance level	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

Means with different superscripts in the same column differ significantly

6.3.5 Pancakes

Results (Table 6.11) showed that on day one, all the coloured batter samples were well coloured; although the higher colour intensity was noticed for samples with beetroot and cactus pear Robusta pigments. Most samples remained stable at initial stages of baking and changed to paler shades with longer heating time. The control samples changed first from a gold/light orange to a pale shade of orange. The Gymno Carpo coloured sample changed from honey-like orange to a pale yellow during the cooking and off-white after the cooking was complete. The Ficus-Indice coloured sample was initially a darker shade of honey-like orange, lost a bit of colour during cooking and was off-white after cooking. The Meyers coloured sample was rose-pink before heating, it changed to very light shades of the pink colour (almost nude), and finally, the colour was a mix of light pink and off-white. The Algerian coloured sample was initially peach/pink, changed to light peach during cooking and finally to off-white after cooking. The beetroot coloured sample was initially a purple/pink colour, changed to a dark pink/red colour, and was finally a light purple colour after cooking was complete. The Robusta coloured sample was dark pink at first, then a lighter shade of the pink was observed during cooking, and finally a dark shade of rose pink after cooking.

In the orange colourants, Ficus-Indice colourant seemed more stable than Gymno Carpo, while in the pink/red, Algerian colourant was more stable than Meyers and in the red/purple. Robusta colourant was more stable than beetroot. The colour of all samples had a lighter shade after ten days, which meant colour was deteriorating during the storage time.

L, a*, b*, chrome and hue tests in Table 6.12 were also done on the pancakes on day one of their manufacture and day ten after being stored in the refrigerator for ten days. The L* values reflected that the samples had medium lightness i.e., not too dark, and not too light. Beetroot and Algerian became lighter from day one (55.74 and 71.20) until day ten (45.20 and 52.49) respectively. The a* values of the control (-0.27) on day one was a bit green, the rest of the samples were red on day one. On day ten, Gymno Carpo and Ficus-Indice colour (orange) shifted from red to more green; also reflected on the hue values. The b* values were all yellow on both day one and ten and maintained stable throughout. The chroma of the orange cultivars was higher than the red colourants.

Table 6. 11: Pictorial differences of pancakes coloured with beetroot and different cactus pear cultivars

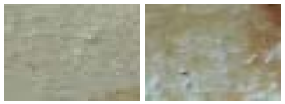
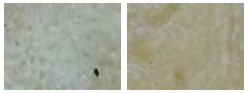
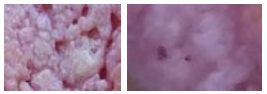
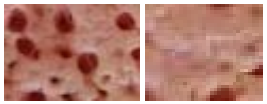
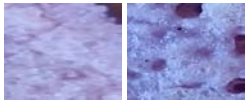
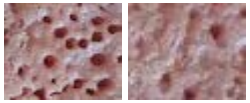
	Day 1			Day 10
Cultivar	**Batter**	**Heated**	**Completed cooking**	**Completed cooking**
Control				
Gymno Carpo				
Ficus-Indice				
Meyers				
Algerian				
Robusta				
Beetroot				

Table 6. 12: The effect of cultivar on colour parameters of pancakes

	Day 1					Day 10				
Sample	**L***	**a***	**b***	**Chroma**	**Hue**	**L***	**a***	**b***	**Chroma**	**Hue**
Control	$68.76^{b} \pm 2.85$	$-0.27^{a} \pm 0.02$	$36.87^{c} \pm 3.99$	$36.87^{bc} \pm 3.99$	$-89.58^{a} \pm 0.02$	$64.63^{b} \pm 2.94$	$-1.80^{a} \pm 0.24$	$33.25^{c} \pm 1.69$	$33.30^{bc} \pm 1.70$	$-86.90^{a} \pm 0.30$
Gymno Carpo	$76.98^{b} \pm 1.68$	$3.64^{b} \pm 0.47$	$42.15^{cd} \pm 2.56$	$42.31^{c} \pm 2.52$	$85.03^{f} \pm 0.88$	$73.44^{b} \pm 2.03$	$-1.49^{a} \pm 0.15$	$38.90^{d} \pm 0.73$	$38.93^{d} \pm 0.73$	$-87.81^{a} \pm 0.23$
Ficus-Indice	$69.10^{b} \pm 0.55$	$2.88^{ab} \pm 0.56$	$43.45^{d} \pm 3.02$	$43.55^{c} \pm 3.03$	$86.21^{f} \pm 0.66$	$65.68^{b} \pm 1.59$	$-1.59^{a} \pm 0.23$	$37.31^{d} \pm 1.07$	$37.34^{cd} \pm 1.07$	$-87.55^{a} \pm 0.36$
Meyers	$76.68^{b} \pm 4.58$	$11.33^{c} \pm 0.66$	$27.00^{b} \pm 1.53$	$29.29^{a} \pm 1.51$	$67.21^{d} \pm 1.36$	$70.46^{b} \pm 1.93$	$9.26^{bc} \pm 1.00$	$23.97^{a} \pm 0.34$	$25.71^{a} \pm 0.67$	$68.90^{c} \pm 1.81$
Algerian	$71.20^{b} \pm 1.42$	$7.84^{c} \pm 0.23$	$27.99^{b} \pm 0.65$	$29.07^{a} \pm 0.66$	$74.36^{e} \pm 0.38$	$52.49^{a} \pm 4.07$	$7.25^{b} \pm 0.35$	$23.88^{a} \pm 1.16$	$24.96^{a} \pm 1.12$	$73.09^{c} \pm 1.04$
Robusta	$56.58^{a} \pm 2.20$	$23.11^{d} \pm 2.50$	$24.51^{b} \pm 1.45$	$33.72^{ab} \pm 2.35$	$46.76^{c} \pm 2.83$	$51.85^{a} \pm 3.61$	$15.22^{d} \pm 3.08$	27.54b $^{\pm}$ 2.20	$31.50^{b} \pm 3.37$	$61.31^{b} \pm 3.25$
Beetroot	$55.74^{a} \pm 5.93$	$21.50^{d} \pm 2.09$	$17.83^{a} \pm 0.97$	$27.97^{a} \pm 1.51$	$39.75^{b} \pm 3.61$	$45.28^{a} \pm 5.73$	$12.93^{cd} \pm 2.99$	$21.93^{a} \pm 0.36$	$25.55^{a} \pm 1.58$	$59.72^{b} \pm 5.87$
Significance level	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

Means with different superscripts in the same column differ significantly

6.3.6 Cupcakes

As the pictorial evidence in Table 6.13 shows, the first control sample Golden cloud pre-mix batter was a darker shade of red (wine red), and the colour remained wine red throughout the keeping period. The beetroot batter was a rose pink/purple shade. The cupcake was more pink than purple on the first day while obtaining a more purple shade on the tenth day.

The second control, Ina Paarman pre-mix batter, was initially a scarlet red colour, but it became darker upon baking, and the colour was lost on the tenth day. The scarlet red may be caused by the addition of acetic acid (as in the ingredient list of Ina Paarman's cake mix). It was seen in Chapter 3 and 4 that samples where extracts were scarlet red, remained scarlet red when ascorbic acid (AA) was added.

The Robusta (filtered and unfiltered) coloured pre-mix batter was a dark pink, while the cupcake had a lighter shade of pink on day one and very light on day ten. Both Robusta and beetroot cupcakes were very light pink in the middle, and both changed drastically in colour in the final product. The duration of the cooking time, as well as being enclosed in a warm environment (oven) with rapidly increasing heat, might have caused this drastic effect. This observation differed to that observed for pancakes which were exposed to air.

All the cupcakes showed a bit of deterioration after the ten-day storage period, especially samples which were coloured with unfiltered beetroot as it had visible beetroot particles. The colour of beetroot and Robusta samples showed the most drastic change in colour when comparing the initial (batter) stage to the final product.

The L*, a*, b*, chroma and hue of the products in Table 6.14 differed significantly; samples were light (L*) on day one, beetroot (48.08 to 67.56) and Robusta (32.30 to 64.54) samples became darker from day one to ten. The a* values of the control samples were significantly higher than samples coloured with beetroot and Robusta (red), and b* values showed that the samples showed yellow pigmentation; the hue and chroma also indicated redness in samples.

On day one, the chroma value of beetroot cupcakes was less intense than the other samples, while after ten days, the chroma values were comparable to the control cupcakes. The intensity of the Robusta cupcakes was higher than the other cupcakes after ten days.

Table 6.13: Pictorial differences of cupcakes coloured with beetroot and different cactus pear cultivars

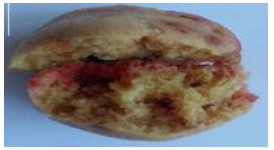
Product name	Day 1		Day 10
Samples	Batter	Baked goods (before storage)	Baked goods (after 10-day storage)
Control 1 (Golden Cloud)			
Control 2 (Ina Paarman)			
Robusta			
Robusta unfiltered			
Beetroot			
Beetroot unfiltered			

Table 6. 14: The effect of cultivar on colour parameters of cupcakes

	Day 1					Day 10				
Sample	**L***	**a***	**b***	**Chroma**	**Hue**	**L***	**a***	**b***	**Chroma**	**Hue**
Control 1 (Golden cloud)	$39.06^{b} \pm 0.70$	$28.92^{d} \pm 0.79$	$9.65^{a} \pm 1.35$	$30.51^{c} \pm 0.32$	$18.47^{a} \pm 2.87$	$35.01^{b} \pm 0.63$	$24.89^{d} \pm 0.22$	$10.79^{a} \pm 0.54$	$27.13^{a} \pm 0.17$	$23.44^{a} \pm 1.17$
Control 2 (Ina Paarman)	$33.25^{ab} \pm 0.77$	$29.87^{d} \pm 0.91$	$16.16^{b} \pm 0.30$	$33.96^{d} \pm 0.94$	$28.43^{b} \pm 0.32$	$28.16^{a} \pm 1.31$	$27.69^{e} \pm 0.57$	$15.57^{a} \pm 0.16$	$31.77^{abc} \pm 0.57$	$29.35^{b} \pm 0.29$
Robusta	$32.30^{a} \pm 0.14$	$25.20^{c} \pm 0.11$	$20.49^{c} \pm 0.20$	$32.48^{cd} \pm 0.21$	$39.11^{c} \pm 0.15$	$64.54^{cd} \pm 0.30$	$0.86^{ab} \pm 0.19$	$36.56^{bc} \pm 5.06$	$36.57^{bc} \pm 5.06$	$88.64^{d} \pm 0.29$
Robusta unfiltered	$51.00^{c} \pm 5.09$	$19.01^{b} \pm 1.81$	$25.02^{d} \pm 1.40$	$31.43^{cd} \pm 2.14$	$52.82^{d} \pm 1.50$	$61.35^{c} \pm 3.26$	$5.11^{c} \pm 1.34$	$37.41^{c} \pm 2.64$	$37.78^{c} \pm 2.41$	$82.10^{c} \pm 2.66$
Beetroot	$48.08^{c} \pm 0.86$	$11.87^{a} \pm 1.52$	$17.16^{b} \pm 0.22$	$20.89^{a} \pm 0.75$	$55.40^{d} \pm 3.64$	$67.56^{d} \pm 1.77$	$0.77^{a} \pm 0.06$	$30.24^{b} \pm 1.32$	$30.25^{ab} \pm 1.32$	$88.54^{d} \pm 0.18$
Beetroot unfiltered	$54.04^{c} \pm 1.89$	$18.14^{b} \pm 0.88$	$18.53^{bc} \pm 1.50$	$25.97^{b} \pm 0.47$	$45.57^{c} \pm 3.69$	$65.56^{cd} \pm 1.84$	$2.88^{b} \pm 1.15$	$30.21^{b} \pm 0.45$	$30.36^{ab} \pm 0.39$	$84.54^{cd} \pm 2.21$
Significance level	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p = 0.001$	$p < 0.001$

Means with different superscripts in the same column differ significantly

6.3.7 Icing

Cupcakes go hand in hand with icing which decorates and adds sweetness to cupcakes. Table 6.15 shows pictorial results of adding colourants to icing. First, the control (Crimson pink) had a very dark colour even though only two drops of the colourant was added to the icing. When Ficus-Indice was added to the icing, the end-product was not very pigmented; it looked close to the original colour without colourant. The beetroot and Robusta were pink, much less pigmented than the control. No icing samples displayed visible colour changes during storage.

The lightness (L*) of the control (34.85) was significantly lower than that of samples coloured with Ficus-Indice (72.83), beetroot, and Robusta (50.25). The a* value of Ficus-Indice (-2.69 on day one and -0.68 on day ten) was significantly lower than other samples, and these values reflected a yellow-green colour. On both day one and day ten, the b* values of the Ficus-Indice coloured sample (31.17 on day one and 29.44 on day ten) indicated higher values than that of the other samples; these high levels may be caused by the orange colour of the cultivar. This difference of Ficus-Indice (orange) compare to other red/pink samples were also evident in the hue values (Table 6.16).

Table 6. 15: Pictorial differences of icing coloured with beetroot and different cactus pear cultivars

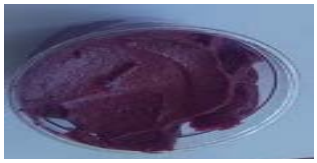
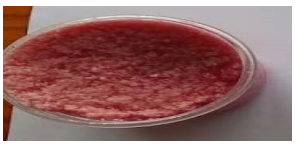
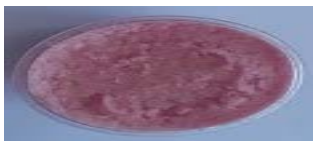
Cultivar	Day 1	Day 10
Control: Crimson pink colourant		
Ficus-Indice		
Robusta		
Beetroot		

Table 6. 16: The effect of cultivar on colour parameters of icing

	Day 1					Day 10				
Sample	**L***	**a***	**b***	**Chroma**	**Hue**	**L***	**a***	**b***	**Chroma**	**Hue**
Control: Crimson pink	$34.85^{a} \pm 1.15$	$38.11^{c} \pm 1.40$	$9.55^{b} \pm 0.84$	$39.29^{c} \pm 1.52$	$14.06^{c} \pm 0.86$	$27.63^{a} \pm 0.05$	$30.76^{c} \pm 0.95$	$8.07^{a} \pm 0.31$	$31.80^{b} \pm 0.99$	14.69 ± 0.10
Ficus-Indice	$72.83^{d} \pm 4.06$	$-2.69^{a} \pm 0.09$	$31.17^{e} \pm 0.44$	$31.29^{b} \pm 0.43$	$-85.07^{a} \pm 0.23$	$72.26^{d} \pm 2.13$	$-0.68^{a} \pm 2.90$	$29.44^{c} \pm 0.95$	$29.54^{b} \pm 0.96$	-28.56 ± 98.35
Beetroot	$65.15^{c} \pm 1.27$	$24.49^{b} \pm 0.29$	$10.88^{c} \pm 0.30$	$26.79^{a} \pm 0.38$	$23.95^{e} \pm 0.33$	$54.78^{c} \pm 2.10$	$21.78^{b} \pm 0.84$	$12.62^{b} \pm 0.31$	$25.18^{a} \pm 0.57$	30.11 ± 1.56
Robusta	$50.25^{b} \pm 0.01$	$24.18^{b} \pm 1.43$	$8.05^{a} \pm 0.39$	$25.48^{a} \pm 1.48$	$18.42^{d} \pm 0.18$	$41.35^{b} \pm 1.96$	$22.48^{b} \pm 1.40$	$7.26^{a} \pm 0.49$	$23.62^{a} \pm 1.47$	17.89 ± 0.54
Significance level	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p = 0.053$

Means with different superscripts in the same column differ significantly

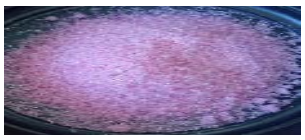
6.3.8 Coloured sugar and candyfloss

The addition of colourants to sugar is a product development method that requires minimal processing. The preparation of candyfloss, which happens under very high, direct heat and air exposure requires more complex processing steps. In Table 6.17, it is observed that all the coloured sugar samples were coloured well, whereas the pigment-coloured samples from Robusta, beetroot and Monterey became faint during candyfloss production.

The control sugar sample, which was coloured with crimson pink, initially changed to a lighter shade of pink after the candyfloss production. The Robusta sample was pink/purple and very light pink/peach after heat exposure; beetroot was a purple shade and became very light brown after heating, whereas the Monterey sample changed from dark pink/purple to a very light shade of pink after heating. The Monterey sample had a pleasant smell with an extra sweet taste to it.

Neha et al. (2018) discovered that beetroot colourants change colour when heated. This is noticeable in the change of colour in pancakes in Table 6.11 and that of cupcakes in Table 6.13. What was more interesting in cupcake production was the change of colour from rose pink (batter), to pink (day 1) and purple (day ten). In candyfloss production (Table 6.17) it was shown that betalain pigments from cactus pear became lighter with heat exposure whereas beetroot changed in colour. It could be concluded that the betalain source influences the stability of the pigment. Thus, the purple cactus pear cultivars Robusta and Monterey have more potential to colour heated products. All the colourants coloured sugar crystals well. It appears that the cactus pear pigments have more potential to be used in candyfloss and that the dosage could be increased.

Table 6. 17: Pictorial differences of coloured sugar and candyfloss coloured with beetroot and different cactus pear cultivars

Cultivar	Coloured sugar	Candyfloss
Control: crimson pink		
Robusta		
Beetroot		
Monterey		

6.3.9 French polony

The colouring of polony is displayed in four different stages in Table 6.18. The first stage is the colourant solution, where the freeze-dried betalain pigments were dissolved in distilled water (dH_20). The second stage is the colour of the batter after addition of colourant. The third stage is the finished product on day one, while the fourth stage represents the finished product after ten days.

The Erythrosine solution (generally used as a colourant) was initially maroon, pink in its emulsified form and remained pink after cooking as well as, even after 10-day storage. Robusta extract was initially purple, turned much lighter during emulsification and cooking. Amaranth was initially maroon (a lighter shade than that of Erythrosine), changed to pink during emulsification, and lighter after cooking. The control sample without added colourant was very light pink and remained so until the cooking was done and after 10 days of storage.

Monterey was not included since the sweeter taste would be unfitting in the salty polony. The colour of the untreated sample (that did not have added colourants) had a similar colour to beetroot and Robusta samples, while the amaranth pigment was more pink than the beetroot and Robusta samples. Zhou et al. (2012) state that 0.1-0.3% of amaranth colourant is of acceptable sensory taste even after 29 days of storage.

It is recommended that the betalain extracts from beetroot, Robusta, and amaranth should be concentrated in order to be able to be used as a colourant in processed meat products.

Regarding the L*, a*, b*, chrome and hue values of French polony the lightness of the samples generally became lower between day one and ten. The a* levels showed that the Erythrosine (20.86 on day on and 20.19 on day ten) is significantly higher than other samples, and remained in stable throughout the keeping time. The colour intensity is similar to the results in Table 6.19, where Erythrosine was more pink than other samples. Interestingly, the b* results of all samples decreased significantly from day one to day ten. The hue and chroma showed redness in all the samples, and a distinct difference in hue and chroma values between the Erythrosine and the other samples visible.

Table 6. 18: French polony formulation

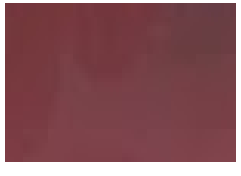
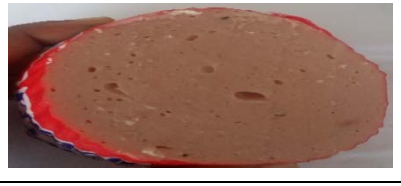
Colourant	**Day 1**			**Day 10**
	Colourant solution	Polony emulsion with colouroant	Cooked polony	Cooked polony
Erythrosine				
Robusta betalain				
Amaranth betalain				
Beetroot betalain				
No colourant added				

Table 6. 19: The effect of cultivar on colour parameters of polony

Sample	L*	a*	b*	Chroma	Hue	L*	a*	b*	Chroma	Hue
Erythrosine	$53.39^{a} \pm 4.05$	$20.86^{b} \pm 1.77$	$57.50^{b} \pm 4.43$	$21.33^{b} \pm 0.73$	$5.08^{a} \pm 0.54$	$43.64^{a} \pm 4.37$	$20.19^{b} \pm 1.54$	$4.15^{a} \pm 0.40$	$20.61^{b} \pm 1.57$	$11.63^{a} \pm 0.81$
Robusta	$56.90^{b} \pm 4.15$	$7.88^{a} \pm 0.71$	$57.45^{b} \pm 4.14$	$7.91^{a} \pm 0.76$	$9.55^{b} \pm 0.68$	$46.68^{ab} \pm 4.87$	$7.32^{a} \pm 0.50$	$7.95^{b} \pm 0.55$	$10.80^{a} \pm 0.71$	$47.36^{b} \pm 1.11$
Amaranth	$54.58^{ab} \pm 4.26$	$8.00^{a} \pm 0.47$	$55.17^{a} \pm 4.27$	$8.36^{a} \pm 0.35$	$9.41^{a} \pm 0.61$	$46.89^{ab} \pm 4.75$	$7.52^{a} \pm 0.51$	$8.75^{c} \pm 0.62$	$11.54^{a} \pm 0.79$	$49.34^{c} \pm 0.80$
Beetroot	$53.72^{ab} \pm 3.37$	$7.54^{a} \pm 0.49$	$54.25^{a} \pm 3.37$	$8.01^{a} \pm 0.55$	$9.73^{a} \pm 0.54$	$50.03^{b} \pm 4.55$	$7.68^{a} \pm 0.52$	$8.45^{c} \pm 0.49$	$11.42^{a} \pm 0.68$	$47.75^{b} \pm 1.03$
Significance level	$p = 0.044$	$p < 0.001$	$p = 0.048$	$p < 0.001$	$p < 0.001$	$p = 0.002$	$p < 0.001$	$p < 0.001$	$p < 0.001$	$p < 0.001$

Means with different superscripts in the same column differ significantly

6.5 Conclusion

When natural colourants are used, the hidden and ambiguous meanings of food colours on food labels may be a thing of the past. The colourant list would include organic, juice as well as natural colourants and food products. In this chapter, food products, except polony, were coloured with fruit and vegetable juice. Thus, Colouring foods which contain antioxidant properties were used as colouring agents.

Betalains imparted colour well in milkshakes, yoghurt and ice cream. Yoghurt and ice cream remained stable for ten days, whereas milkshake was spoilt within a few days of production. Other products where colours were well imparted included jelly, coloured sugar and icing sugar. Thus, dairy products and products which are prepared under low or no heat can be coloured with Colouring foods and remain stable.

Products such as candyfloss and polony were not well coloured. Although candyfloss was coloured with fruit and vegetable juice (Colouring Foods) and polony with freeze-dried samples, both were exposed to high heat. The type of heat each sample were exposed to was different (direct heat for candyfloss and cooking for polony). Therefore, it can be deduced that high heat exposure in Colouring Foods and freeze-fried samples leads to instability in colours. In fact, little or no colour is shown after heat exposure.

Robusta and Monterey cactus pear cultivars imparted more colour than other pigment sources. Additionally, the Monterey cactus pear cultivar had a pleasant sweet/fruity flavour to food products. In their freeze-dried state, amaranth coloured polony better than Robusta and beetroot.

Betalain stability was dependent on the betalain source. For example, the stability of betalains in polony was better when amaranth pigments were used and less

stable with Robusta and beetroot. Also, candyfloss, which was coloured with cactus pear cultivars Monterey and Robusta showed more stability than candyfloss, which was coloured with beetroot. This shows that betalains from red to purple cactus pear cultivars may be more heat stable than betalains from beetroot.

CHAPTER 7

General conclusions

This dissertation has shown that modern-day, knowledgeable and candid consumers have an urgent need for natural, nutritionally-dense colourants. With that need exists endless possibilities to extract and apply natural colourants from insects, bacteria, fungi, algae and plants. Leaders of natural colourant usage are Europe and America, while Asia and Africa are still far behind.

The introduction of natural colourants to other parts of the world can be made more reachable through sourcing plants such as cactus pear, which grow in various parts of the world. The cactus pear plant has multiple uses; thus, reproducing it for colourant usage provides multiple health benefits. All the betalain sources used in the current research have medicinal properties, which are very beneficial to consumers.

The highest yield of betalain pigments was extracted from very small particle sizes, making a liquidiser the best extraction tool for betalain extraction, as it can produce fine particles through liquidation. Moreover, extraction with 50% ethanol, 50% methanol or distilled water produced the highest betalain yields. Different betalain sources produced high levels of betalains during different extraction methods.

The green American Giant and Morado cultivars had traceable amounts of betalains in all extraction methods, and the calculated values may not be from betalains, but rather carotenoids. The orange Ficus-Indice and Gymno Carpo cultivars generally had a higher level of betaxanthins than betacyanins, whereas the pink/red Algerian and Meyers were higher in betacyanins.

Importantly, the highest betalain yields for yellow (betaxanthin), and red/purple (betacyanin) pigments were found in red/purple cultivars (Monterey and Robusta) as well as red cultivars. Thus, a strong association exists between the source betalains and the colour thereof.

More to this, the extraction method did not have a significant effect on betalain content. The interaction between cultivar and extraction method was, however, significant for betalain content, meaning that for specific cultivars, certain extraction methods gave better results.

In the analysis of betalain properties, betalain sources had very high vitamin C levels, with the highest levels found in amaranth. Amaranth also had the highest total phenol and flavonoid levels. The highest Rf values in TLC results (with and without vanillin) were found in Robusta and beetroot, a reflection of higher betacyanin and betaxanthin presence in the samples. Monterey cactus pear (13.9%) had the highest TSS values, whereas beetroot had the lowest (5.5). These results showed that Monterey is much sweeter than beetroot.

According to the decision tree which was established by the EU, betalain extracts which are extracted with water (and deemed as fruit juices) can be classified as Colouring Food.

Betalains were successfully applied in dairy products; such as ice cream, yoghurt and milkshake. The pigments were also coloured and remained stable in jelly, icing sugar and coloured sugar. However, pigments from cactus pear and amaranth lost colour during heat exposure, and ones from beetroot changed colour. Robusta and Monterey showed the potential of remaining stable. The stability and colour strength of the pigments could be intensified through freeze-drying or spray drying.

The future of food colourants aims to provide natural colourants, whose stability is comparable to that of synthetic colourants. That can be achieved through freeze-drying, micro-encapsulation or spry-drying, which produce micro-fine particles. Concentrated pigments could be added to food products, such as cupcakes that are exposed to high heat. Colouring Foods can, however, be added to products, such as ice-cream and jelly. Colouring Foods can also be useful in child-friendly food products.

Stintzing & Carle (2004) stated that plant breeding could aid in improving pigment quality of beetroot. Plant-breeding could also allow easier and quicker ways to peel and de-seed cactus, possibly produce cultivars without seeds, in order to hasten production and minimise oxidation, which decreases betalain content in the fruit.

The constant improvement and development of natural colours have opened a margin for producing 100% natural colourants with medicinal properties. Whether dried or in watery form red and purple colourants have a solid colour hue, depending on what food product to which they are added.

Future recommendations for a study of this magnitude include the incorporation of yellow beetroot (mainly to test betaxanthin presence), combine different colourants (to provide a wide variety of hues), using the whole fruit in order to increase extracted volume, have less waste and add to the nutritional benefits. Moreover, add shelf-life studies longer than ten days, especially for products that are not easily perishable, e.g., ice-cream.

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