AN ANALYTICAL PRE-BREEDING METHOD FOR FLAVONOID SCREENING IN GRAPEFRUIT

by

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Declaration

I Almari MJ van der Loo hereby declare that this dissertation, for the degree Magister Scientiae, which was submitted by me to the University of the Free State, is my own original work and has not previously in its entirety or in part been submitted to any other University. All sources of materials and financial assistance used for this study have been duly acknowledged. I also agree that the University of the Free State has the sole right to the publication of this dissertation

Almari MJ van der Loo

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Dedication

I dedicate this dissertation to my parents Francois and Alida van der Loo, who’s unconditional love and encouragement I have been blessed with my entire life. You have given me the most precious gift in life, by believing in me.

SUMMARY

The citrus industry forms a major part of the agronomic and economic sectors of South Africa, and supports the livelihood of an estimated 1 million South Africans. The citrus industry has a long history in South Africa with the first exports of fruit dating back to 1907. Presently the South African industry consists of 120 commercial citrus varieties, of which grapefruit accounts for 11% of the total citrus orchards planted in the 2015/16 production season. Grapefruit are naturally rich in flavonoids, with naringin as the major flavonoid, best known for its distinct bitterness. Global breeding and selection programmes for new cultivars focus on increased yield, pest and disease resistance, and improved nutritional content. Citrus fruit quality is influenced by several genetic and environmental factors.

The aim of this study was to provide the pre-breeding programme of the Agricultural Research Council (ARC) with a flavonoid screening technique for grapefruit germplasm, which takes into account factors such as variety differences, as well as the fruit location within the tree canopy. The physical and chemical traits as well as the naringin and naringenin content of three grapefruit varieties (Star Ruby, Sweetheart and Marsh) grown and harvested over two seasons (2015 - 2016 and 2016 - 2017), were evaluated.

For determination of the naringin and naringenin content of the fruit juice, a high performance liquid chromatography (HPLC)-UV/Vis method was optimised, using the Accela 600 HPLC system with an Accela UV/Vis Detector. Successful resolution and retention times were achieved using an Accucore C18 (2.3 µm particle size, 50×3 mm i.d) column at 0.818 ml min^{-1} flow rate, with a gradient of acetonitrile:water at a constant temperature of 25°C. The method gave acceptable linearity for both naringin and naringenin with a R2 value of 0.999 in both cases. A limit of detection (LOD) and limit of quantification (LOQ) of 1.77 mg 100 ml^{-1}, 5.35 mg 100 ml^{-1} for naringin and 0.23 mg 100 ml^{-1}, 0.72 mg 100 ml^{-1} for naringenin was obtained. The inter and intra-day repeatability was determined by injecting the standard calibration solutions six times per day over three consecutive days obtaining a relative standard deviation (RSD) (%) consistently lower than 5%.

There were significant varietal differences for fruit size, °brix, pH and naringin content due to season and canopy position. Fruit in different canopy positions varied in physical traits such as fruit mass, circumference, peel mass and segment mass. As for the chemical composition of the fruit, the northern quadrant fruit were highest in °brix content, and also had a high °brix:acid ratio. The effect of quadrant sampling on flavonoid content indicated no significant differences. The interactions between the physical and chemical traits were constant over both seasons.

This study indicated that when screening fruit in a citrus breeding programme for new or improved flavonoid traits, such as the naringin content, the sampling quadrant does not seem to have an effect, but genetic differences and climatic differences between seasons would affect the naringin content. This study demonstrated that the sampling of fruit for determining naringin/naringenin content in grapefruit can be simplified, which is beneficial for screening of a large number of possible parents with regard to naringin/naringenin content in a breeding programme.

Key words: breeding, canopy position, flavonoids, fruit quality, grapefruit, HPLC, naringenin, naringin

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LIST OF ABBREVIATIONS

ARC-TSC	Agricultural Research Council-Tropical and Subtropical Crops
BA ratio	°brix:acid ratio
DAD	Diode array detection
DAFF	Department of Agriculture, Forestry and Fisheries
E	East
FC	Fruit circumference
FM	Fruit mass
HPLC	High performance liquid chromatography
M	Marsh
MT	Metric tons
N	North
NE	Naringenin
NI	Naringin
PC	Principal components
PM	Peel mass
PT	Peel thickness
Q	Quadrant
RSD	Relative standard deviation
S	South
SH	Sweetheart
SM	Segment mass
SR	Star Ruby
TA	Titratable acidity
TSS	Total soluble solids
UV-Vis	Ultra violet and visible light
W	West

Chapter 1

GENERAL INTRODUCTION

Citrus is regarded as a globally important tree fruit crop, with production in over 100 countries on all six continents (Suant, 2000). The citrus fruit trees constitute six genera, of which three are of commercial importance, being *Poncirus* (trifoliate orange), *Fortunella* (Kumquat) and *Citrus* (Saunt, 2000). According to Suant (2000) citrus genera have eight important commercial species: sweet orange (*Citrus sinensis),* mandarin (*Citrus reticulata*), grapefruit (*Citrus paradisi*), pummelo (*Citrus grandis*), lemon (*Citrus limon*), sour lime (*Cirtus aurantifolia*), citron (*Citrus medica*), and sour orange (*Citrus aurantium*). In South Africa only sweet orange, mandarins, grapefruit and lemons are of commercial importance (Bjizet, 2006).

In South Africa the total gross value of agricultural production was made up of animal products (47.2%), horticultural products (28.5%) and field crops (24.3%) for the year ending on 30 June 2016 (2015/16). This represents an increase from the previous year (2014/15) of 4.9%, from R226 162 million to R237 317 million, in farming income (the value of sales and production for other uses, plus the value of changes in inventories). This can be ascribed mainly to increases in income from horticultural and animal products (DAFF, 2017). Horticultural products attained a gross income growth of 15.2% from the previous season (R61 067 million in 2014/15 to R70 340 million in 2015/16). The citrus production specifically reached a 12.4% income growth for the 2015/16 season, representing an amount of R14 817 million, which illustrates the economic importance of citrus (DAFF, 2017).

The South African citrus industry is globally competitive, consisting of 120 commercial citrus varieties (Sikuka, 2017). Grapefruit accounted for 11% of the total citrus orchards planted in the 2015/16 production season (Sikuka, 2017). According to the USDA (2018) report, South Africa is the fourth largest producer of fresh grapefruit in the world, producing 366 000 metric tons (MT) for the 2016/2017 season of which 232 000 MT was exported, making South Africa the largest fresh grapefruit exporter.

The development of the different citrus species is believed to be the result of interspecific crosses, for example, grapefruit is thought to be a cross between an orange (*Citrus sinensis)* and a pomelo (*Citrus grandis*) (Sinclair, 1972; Suant 2000). Furthermore, because grapefruit is not a true biological species, hybridisation within the group is not an option for variety improvement, and all varieties originate as spontaneous mutations, such as Marsh, or from artificial mutation induction by radiation, such as Star Ruby (Sinclair, 1972; Suant 2000).

Grapefruit is a subtropical citrus tree, best known for its large (100 - 150 mm) light lemon to yellow orange coloured fruit. The flesh of grapefruit fruit is divided into 12-14 segments and are greyish to pink in colour (Figure 1.1). The juice of the fruit is known for its distinctive flavour described as a blend of acid, sub-acid bitterness and sweetness (Bijzet, 2006).

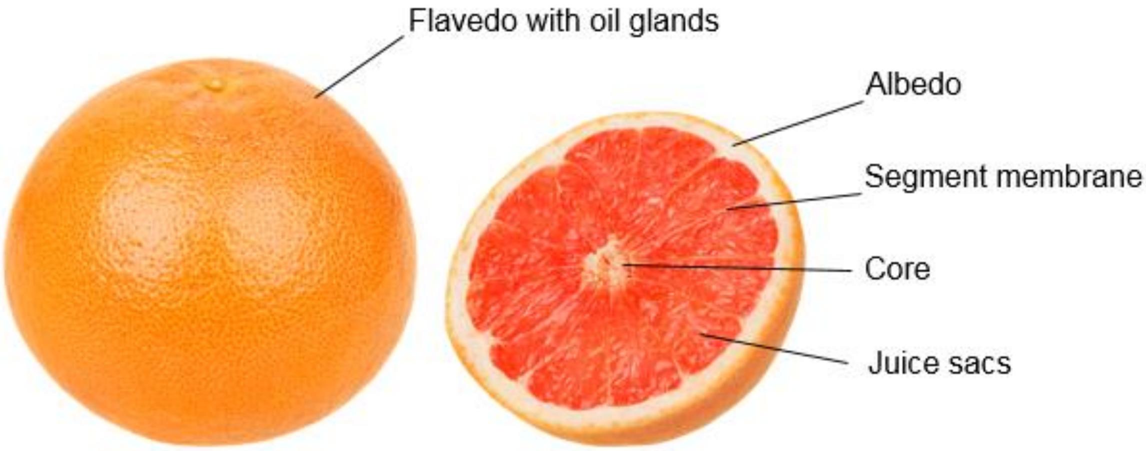

Figure 1.1 A typical grapefruit (*Citrus paradisi*) with a cross section depicting the various parts of the fruit anatomy (Bijzet, 2006)

Currently the public and researchers are becoming more concerned with nutritional security, which is one of the contributing factors to the new interest in grapefruit consumption (Owira and Ojewole, 2010). Grapefruit is rich in vitamins and has a naturally high flavonoid content with antioxidant and free radical scavenging activities (Ferreyra et al., 2012; Khoddami et al., 2013). The major flavonoid, best known for its distinct bitterness, is naringin, and also proven to be useful in activating insulin signalling pathways (Allister et al., 2009).

Plant breeding is an important factor for food and nutritional security. The reasons for developing new cultivars and varieties include amongst others increasing yield, pest and disease resistance, drought tolerance and regional adaptation to different environments and growing conditions (PBWB, 2016). Grapefruit breeding efforts in South Africa has resulted in the development of commercial grapefruit varieties such as Nelspruit Ruby (Nelruby), and Sweetheart (Bjizet, 2006). Plant breeding also provides an effective strategy to increase the nutritional value of food, for example by increasing the levels of health promoting bioactive compounds in fruit and therefore the human diet (Patil et al., 2014).

However, before a breeder can start a specific breeding programme, specific goals need to be set. The breeder must firstly establish the needs of the consumer and producer as well as the deficiencies in the current varieties (Luckett and Halloran, 2005). Extensive knowledge of traits within germplasm is systematically accumulated through horticultural trials as well as breeding programmes. However, a changing world and the ever increasing need for food and nutritional security, calls for identifying the relevant attributes to be incorporated into a new variety. For this, gene banks represent a biorepository of preserved genetic material that is available to be exploited and by definition, pre-breeding refers to all activities designed to identify desirable traits and/or genes from such a gene bank in order to use in a future breeding programme. Through these pre-breeding activities, suitable breeding parents with the desirable attributes are selected, and hybridisation is performed (Luckett and Halloran, 2005). The breeder then progressively selects the progeny of the crosses with desirable traits and removes the undesirable or inferior genotypes. If a new genotype is selected, the worth is compared to that of an existing variety. If the new genotype proves to have value, propagation material is bulked for distribution to farmers and is finally released as a new variety (Luckett and Halloran, 2005).

In a citrus breeding programme, many of the fruit quality traits of suitable breeding parents or of promising new genotypes, are assessed post-harvest. The fruit quality traits of citrus, such as fruit size, juice content and °brix:acid ratio has been shown to be influenced by several factors, such as genetic factors (variety differences), stages of maturity, environmental factors such as climate, soil conditions, cultural practices and fruit location within the tree canopy (Sinclair, 1972; Chen, 1990; Hunlun, 2016). Thus, during the screening phase for breeding parents or promising new genotypes, the breeder needs a reliable screening technique which considers the influencing factors.

The aim of this study was to provide a flavonoid screening technique for grapefruit germplasm, which takes into account factors such as variety differences, as well as fruit location within tree canopy, to be applied in the ARC's pre-breeding programme. To reach this aim, the primary objectives of this study were:

1. The optimisation of an analytical method for the identification and quantification of flavonoids in grapefruit varieties, as the information will provide valuable insights to the Agricultural Research Council's citrus pre-breeding programmes.
2. Evaluation of the effect of varietal, seasonal and canopy position differences on the fruit traits and flavonoid composition of three grapefruit varieties.
3. Determining the interactions and associations between the physical fruit traits and their chemical composition as well as the relationships thereof between the grapefruit varieties, fruit location within tree canopy and seasons.

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Chapter 2

FLAVONOID CONTENT AS PART OF GRAPEFRUIT BREEDING PROGRAMMES: A LITERATURE REVIEW

2.1 INTRODUCTION

The rapid increases in global population and the subsequent food insecurity has recently intensified concerns about global and sustainable nutritional security. Plant breeding efforts towards food security primarily focused on increasing crop yield with significant impact. However, it has become apparent that a paradigm shift regarding breeding objectives towards nutrition, flavour, quality, and enhanced health-promoting properties is crucial to address nutritional deprivation (Patil et al., 2014).

2.2 ORIGIN OF GRAPEFRUIT AND SOUTH AFRICAN PRODUCTION

The grapefruit was first isolated as a distinct species and designated as *Citrus paradisi*, by the botanist James MacFayden in 1830 (Sinclair, 1972). In Jamaica, the name “grapefruit” was used to describe the fruit, since the fruit are mainly born in clusters, and the name has been used since 1814. The definite origin of the grapefruit is not known but, it is thought to be a cross between an orange (*Citrus sinensis)* and a pomelo (*Citrus grandis*). With the close relation to the pomelo, one of the fundamental distinctions between the grapefruit and the pomelo is that grapefruit seeds are polyembrionic and pomelo seeds are monoembryonic (Sinclair, 1972).

Developed in the West Indies in the early 1700s, the grapefruit was first introduced to Florida in the United States of America (USA) in the 1820’s (Kiani & Imam, 2007). The first commercial shipments of grapefruit were from Florida to Philadelphia and New York between 1880 and 1885 (Sinclair, 1972). From merely grown as a curiosity, grapefruit production today has become one of the most economically important in the citrus industry globally. The grapefruit harvest in 2014/2015 yielded 6.3 million tons globally, with the Chinese harvest volumes increasing from 2.9 million tons in 2009 to 4.1 million tons (USDA, 2017). Other export countries included the USA, Mexico and South Africa in 2014/2015 with 826 000, 424 000, and 387 000 tons exported respectively. The largest importers of grapefruit are the European Union, Russia and Japan, accounting for more than 600 000 tons (USDA, 2017).

For the South African 2015/2016 harvest season, there was a significant decline from the 14.2 million cartons in 2015 to 12.4 million cartons in the 2016 season (one carton = 15 kg). This decline can be attributed to the occurrence of drought as well as grapefruit orchards increasingly being replaced by soft citrus and lemons (Ntshangase et al., 2016; USDA, 2017). A South African analysis report of orchard registrations for 2016 indicate a total area of 7 658 ha of grapefruit orchards planted (Edmonds, 2013).

The domestic fresh consumption of grapefruit in South Africa is low, with only 5000 tons being consumed, which could be attributed to grapefruit being an acquired taste (Sikuka, 2017). Of the 366 000 MT of grapefruit produced in South Africa, 129 000 MT is processed to juice of which the bulk is exported to Europe. By-products of commercial juice-extraction are pulp, albedo and peel. Grapefruit oil is extracted from the pulp and used as a flavouring agent in an assortment of soft drinks. Pectin and citric acid from the albedo are used in the food industry to preserve fruits and produce jams and marmalades (Sulieman et al., 2013). Oil extracted from the peel is used in scented fragrances (Arthey and Ashurst, 1995). Lastly, flavonoids such as naringin are also extracted from the peel to be used as a distinctive bittering flavour, for example in tonic water (Sikuka, 2017).

Several commercial grapefruit varieties are grown in South Africa, with Star Ruby being the most planted variety (84%) due to its high global demand (EuroFresh, 2015; Edmonds, 2013). Other varieties include Marsh, Rose, Flame, Nelspruit Ruby (Nelruby), Redheart and Sweetheart. Star Ruby was produced by irradiating seed from the Hudson variety by R. A. Hensz, Texas A & I University, Weslaco, Texas, in 1959 (Suant, 2000). Unlike in other competitive countries, Star Ruby gives good yields of large fruit in South Africa with tree growth being more compact and bushy, with smaller fruit mostly being born at the bottom third of the tree (Suant 2000; Bijzet 2014).

The external appearance of Star Ruby rind colour is a deep pink blush, similar to that of Rio Red, but with a brighter yellow-orange background and the fruit is nearly round (Saunt, 2000). Star Ruby has a thinner rind than Marsh or Rosé, with a smooth and fine texture like that of Flame (Bijzet, 2014). Internally the flesh colour of Ruby is slightly redder than the Hudson grapefruit variety, and is classified as a red grapefruit. The deep red flesh is very juicy and sweet to sour, with a slight bitter after-taste and is as sweet as, or sweeter than, Marsh (Bijzet, 2014; CRI, 2012b). This variety is harvested in South Africa from end March/early April to end August/early September (CRI, 2012b; Sikuka, 2017).

Marsh has two possible origins. Robinson (1933) stated that Marsh originated as a root sprout from a seedy variety, probably Duncan, around 1880 near Lakeland, Florida, while Hume (1926) was of the opinion that it was of seedling origin from around 1850. It was first propagated by nurseryman E.H. Tison in 1886 and later named as ‘Marsh Seedless’ in 1890 by C.M. Marsh, to whom the nursery was sold. It is now more commonly known simply as Marsh (Robinson, 1933). Marsh is a large spreading tree that is vigorous and must be grown in locations that meet its high heat requirement (Bijzet, 2006). As a grapefruit, Marsh is classified as a medium sized fruit with an oblate to spherical shape. Although being called seedless, originally Marsh sometimes contained a few seeds. The fruit has a pale yellow external colour at maturity with a medium-thin rind that is tough and has a smooth even surface (Bijzet, 2014). According to Suant (2000) the flesh is pale yellow and Bijzet (2014) deemed it to be juicy with a sweet to sour flavour with a slight bitter after taste. Production in South Africa is from end March/early April to end June/ early July (CRI, 2012a; Sikuka, 2017)

Sweetheart was developed by the ARC-TSC (Agricultural Research Council-Tropical and Subtropical Crops) from Henderson (ARC#1284) by bud irradiation. The irradiated buds were top worked to existing rootstocks at Mussina research farm, where two selections were made. Sweetheart was selected for its low naringin content, high °brix percentage, lycopene containing flesh and prolific cropping (Personal communication Dr. Z. Bijzet). This variety has an upright tree that produces round fruit, similar in size to Star Ruby and has an external light to medium yellow colour with a medium glossy, semi-smooth rind surface. The seedless fruit and the lower naringin content (compared to Star Ruby), together with high sugar content, instils a pleasant eating experience. Although the internal lycopene (colour pigment) is accentuated in hotter grapefruit production areas, the Sweetheart is deemed a pink cultivar when compared to Star Ruby (Citrogold, 2016).

2.3 FLAVONOIDS

Flavonoids are the major group of phenolic compounds found in citrus fruits (Lv et al., 2015). Flavonoids are bioactive phenolic compounds that are universally present in plants as secondary metabolites, which serve a multitude of biological functions, such as protection against ultraviolet (UV) radiation and phytopathogens by antioxidant activity (Ferreyra et al., 2012; Khoddami et al., 2013). Flavonoids are also able to chelate certain metal cations such as Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Al^{3+} and Mg^{2+} which are involved in ROS formation in the Fenton reaction (Mierziak et al., 2014). Other functions include providing tolerance to aluminium toxicity, nodulation signalling, growth regulation, auxin transportation, male fertility and pigmentation (Ferreyra et al., 2012; Khoddami et al., 2013). More than 6000 flavonoids have already been identified, and the number is expected to increase (Khan, 2017).

Flavonoids are not essential nutrients, but are beneficial to human health due to their antioxidant capacity both in in vivo and in vitro systems (Zhang, 2010). The basic chemical structure of flavonoids is a 15-carbon skeleton consisting of two benzene rings, A and B shown in Figure 2.1, linked via a heterocyclic pyrane C-ring (Zhang, 2010; Kumar and Pandey, 2013).

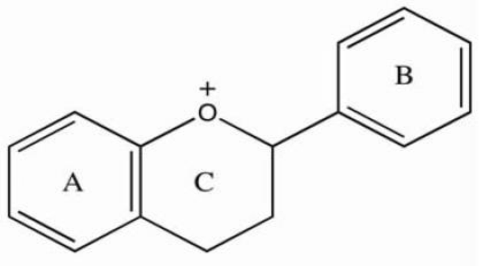

Figure 2.1 Basic chemical structure of flavonoids (Khoddami et al*.,* 2013)

Flavonoid functionality is structure dependant, thus the chemical nature of flavonoids depends on the structural class, degree of hydroxylation, other substitutions and conjunctions, and degree of polymerization. Based on the different levels of oxidation and pattern of substitution of the C-ring, flavonoids are divided into six subgroups namely flavanones, flavonols, flavones, flavanols, anthocyanidins and isoflavonoids, depending on the level of oxidation and pattern of substitution of the C-ring (Zhang, 2010; Kumar and

Pandey, 2013). Within these subgroups, different individual compounds are distinguished based on the pattern of substitution of the A and B benzene rings. The major flavonoids found in grapefruit are flavanones, flavones and flavonols.

Furthermore, flavonoids can occur in different forms in various parts of citrus fruits. First as a glycoside, whereby a sugar molecule is linked to the flavonoid, normally at position 3 or 7; secondly as an aglycone, without the addition of a sugar molecule, and thirdly as a methylated derivative (Zhang, 2010).

Studies on flavonoids by UV spectroscopy have revealed that most flavones and flavonols exhibit two major absorption bands: Band I (320 - 385 nm) represents the B ring absorption, while Band II (250 - 285 nm) corresponds to the A ring absorption. Spectral studies done for flavanone identification have shown that flavanones exhibit a distinctive strong Band II absorption maximum between 270 nm and 295 nm, such as Naringenin 288 nm (Zhang, 2010).

Flavanones (Figure 2.2), account for 98% of the grapefruit's flavonoid content (Zhang, 2010). In grapefruit, flavones are mostly present as glycosides, through the addition of the sugars neohesperidose and rutinose. Neohesperidoside flavanones have a distinct bitter taste, whereas rutinoside flavanones are tasteless.

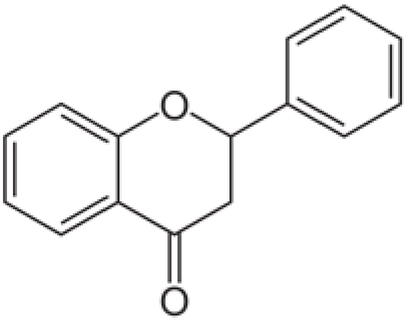

Figure 2.2 Basic structure of flavanones (Ferreyra et al., 2012)

In plant systems, flavonoids are mostly located in the nucleus of mesophyll cells and within the centers of ROS generation (Kumar and Pandey, 2013). Immunolocalisation experiments suggest that flavonoid synthesizing enzymes are bound loosely in a multi-enzyme complex, to the endoplasmic reticulum (Winkel-Shirley, 2001). Other enzymes such as flavonol synthase 1 (Kuhn et al., 2011), as well as chalconesynthase and chalconeisomerase (Saslowsky et al., 2005) were localised in the Arabidopsis nuclei. Some enzymes such as *Antirrhinummajus aureusidinsynthase*, involved in aurone biosynthesis, are localised in the vacuole (Ono et al., 2006). The enzyme flavonoid-3-hydroxylase has recently been located in the tonoplast in the hilum region of the soybean immature seed coat (Toda et al., 2012).

Flavonoids are synthesized via the phenylpropanoid pathway (Figure 2.3), usually as a response to microbial infection (Kumar and Pandey, 2013; Ferreyra et al., 2012). Phenylalanine is transformed to *p*-coumaroyl-CoA (Ferreyra et al., 2012).

Three malonyl-CoA molecules are condensed with *p*-coumaroyl-CoA by the enzyme chalcone synthase, producing naringenin chalcone, which is a chalcone scaffold from which other flavonoids are derived (Ferreyra et al., 2012). Catalysed by the enzyme chalcone isomerase, the naringenin chalcone is converted to naringenin by a stereospecific ring closure isomeration step (Frydman et al., 2004). Hereafter naringenin undergoes two glycosylation steps. The first glycosylation is catalysed by the enzyme 7-*O*-glucosyltransferase, whereby a glucose molecule is attached to the naringenin molecule at position 7, this forms the flavanone-7-*O*-glucoside (naringenin-7-*O*-glucoside). The second glycosylation step is catalysed either by 1-6 rhamnosyltransferase or 1-2 rhamnosyltransferase, producing either tasteless 7-*O*-rutinosides (narirutin) or bitter 7-*O*-neohesperidosides (naringin) respectively. Thus, the position of the rhamnose attachment is the determinant of the bitter flavour of the fruit (Frydman et al., 2004).

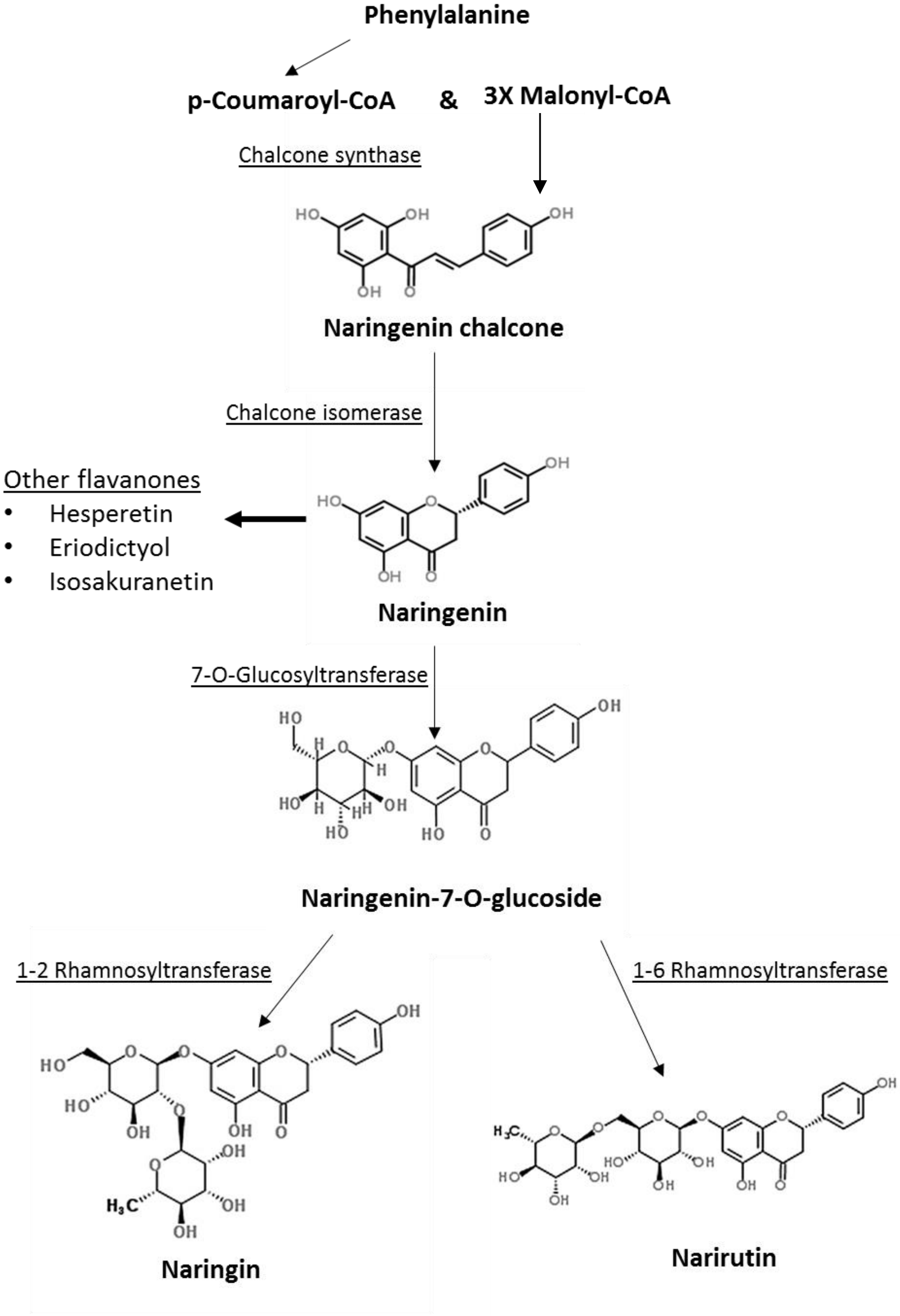

Figure 2.3 Biosynthesis of the flavanones naringin and narirutin (Kumar and Pandey, 2013; Ferreyra et al., 2012)

2.4 NARINGIN

Naringin is the major flavanone found in grapefruit (Ribeiro and Ribeiro, 2008; Zhang, 2010). Naringin (4',5,7-trihydroxy flavanone-7-rhamno-glucoside) with a molecular weight of 580.53458 g mol^{-1}, occurs on average at 17 mg aglycone 100 g^{-1} edible fruit or juice in grapefruit (Peterson et al., 2006). It is water soluble and also the primary bittering component found in the fruit membrane and albedo of grapefruit. The bitterness of naringin was stated by Olsen and Hill (1964) to be detectable when 1 part is dissolved in 50 000 parts water, thus trace amounts as low as 0.07% in grapefruit juice result in a bitter taste, rendering the juice inferior (Zhang et al*.,* 2010; Ribeiro and Ribeiro, 2008).

Naringin is present in grapefruit throughout the entire growth period from the ovary to maturity, however, the levels of naringin in the different tissues differ (Albach et al., 1969; Jourdan et al., 1984). Frydman et al. (2004) investigated the gene *Cm1,2RhaT* encoding the 1,2 rhamnosyltransferase key enzyme in the naringin biosynthesis pathway, and found that the regulation of flavanone accumulation is regulated by gene expression, and that there is a strong likelihood that the same gene is expressed in both leaves and fruit. However, the possibility of a *CM1,2RhaT*-like homologous gene being differently expressed in different citrus tissues should not be ruled out (Frydman et al., 2004)

For pre-breeding purposes, it is important to understand the flavonoid biosynthetic pathway, and be able to screen the germplasm for this complex trait, as it is a suitable target for metabolic engineering and the further development of new varieties where the flavone content of fruit can be managed as desired by plant breeders (Kumar et al., 2013; Ferreyra et al., 2012).

2.5 HEALTH BENEFITS

In recent years, public interest in flavonoids has increased, largely due to their variety of pharmacological activities, especially their antioxidant activity in the mammalian body, therefore flavonoids may also be referred to as “nutraceuticals” (Tapas et al., 2008). Grapefruit are naturally rich in flavonoids with a total flavanone content of 27 mg aglycones per 100 g edible fruit or fruit juice (Peterson et al., 2006). Grapefruit is further a healthy addition to a balanced diet, as it is low in calories and high in dietary fibre, with high concentrations of vitamin C and potassium (Economos and Clay, 1998).

Recent medical research has further added to the enthusiasm around grapefruit, as it is suggested that grapefruit and grapefruit juice could reduce atherosclerotic plaque formation, inhibit breast cancer cell proliferation and mammary tumour genesis as well as having a preventative influence on cardiovascular diseases (Cerda et al., 1994; So et al., 1996; Guthrie et al., 1998; Owira and Ojewole, 2009). Other protective effects of grapefruit flavonoids include anti-ischemic, antioxidant, vasorelaxant and antithrombotic properties (Owira and Ojewole, 2009).

The consumption of grapefruit may also be beneficial to patients with Type 2 Diabetes Mellitus and other degenerative diseases (Muraki et al., 2013). Owira and Ojewole (2009) stated that grapefruit juice has metformin-like effects in the regulation of blood glucose. The dominant flavanone, naringin, in grapefruit, has properties similar to those of insulin. Naringin was proven to decrease microsomal triglyceride transfer protein expression in vitro, thus rendering naringin useful in activating insulin-signalling pathways important for the regulation of hepatocyte lipid metabolism (Allister et al., 2009).

Meiyanto et al., (2012) reported that, due to its high flavonoid concentration, grapefruit could be considered as a valuable natural chemopreventative agent for targeted cancer therapy. Narginin is listed with the following chemopreventative activities; suppression of carcinogenesis, cell cycle regulation, apoptosis, co-chemotherapeutic and antioxidant activities (Kim et al., 2008; Leslie et al., 2008; Adina et al., 2014). Other flavonoids in grapefruit, such as hesperidin with a concentration average of 3 mg aglycone per 100 g edible grapefruit or grapefruit juice, has also exhibited natural chemopreventative effects (Chen et al., 2003; Peterson et al., 2005; Choi et al., 2007).

2.6 HEALTH DETRIMENTS

Hazardous and sometimes fatal consequences of grapefruit-drug interactions have unfortunately been documented (Bailey et al., 1989). The grapefruit-drug interactions are unique in that the cytochrome P450 enzyme CYP3A4, which metabolises over 60% of commonly prescribed drugs, as well as other drug transporter proteins such as P-glycoprotein and organic cation transporter proteins, which are all expressed in the intestines, are involved (Kiani & Imam, 2007; Owira and Ojewole, 2009).

One the earliest evidence of grapefruit-drug interaction was documented during a study conducted by Bailey et al. (1989) when the interactions between the vasodilating/diuretic drugs ethanol and felodipine, a 1,4-dihydropyridine calcium entry blocker, were assessed in 10 patients with untreated borderline hypertension.

During this experiment, felodipine with ethanol treatments, showed felodipine plasma concentrations at least three times higher than what was expected, contradicting previous studies. In addition to this, the patients also had a higher frequency of adverse drug effects, which could all be traced back to the increase in drug bioavailability. A systematic examination for the cause of the increased concentrations, led to the finding that grapefruit juice, used as a flavour enhancer to mask the ethanol taste, could markedly increase the oral bioavailability of a number of medications. Grapefruit juice inhibits the CYP3A4 enzyme of the cytochrome P450 system in the intestinal mucosa, increasing the bioavailability of drugs with a high first pass metabolism (Bailey et al., 1989).

The dietary intake of grapefruit needs to be taken into account and managed if an individual is prescribed cardiovascular or other drugs, as the full extent of grapefruit-drug interactions have not been fully determined yet and could lead to increased bioavailability and subsequent adverse drug reactions.

2.7 CITRUS BREEDING IN SOUTH AFRICA AT THE ARC-TSC

Citrus breeding at the ARC-TSC citrus plant improvement programme, officially started in 1974 when the first project was registered (Sippel et al., 2015). The main breeding goal at that stage was on blackspot resistance and easy peeling of mandarins. Since then the programme has expanded substantially with many different breeding objectives, which are based on the needs of the industry (Breedt et al., 1996). Breeding methods for scion and rootstock breeding employed by the ARC-TSC include conventional, mutation and rootstock breeding as well as various biotechnology techniques (Sippel et al., 2015).

Conventional breeding methods entails applying Mendel’s principles to develop a new individual through sexual crossing from two parents (cultivated lines or varieties) who have expression of the desirable traits between them (Caligari, 2001). Conventional breeding of citrus scion and rootstock cultivars are generally based on controlled crosses (Sippel et al., 2015; Abouzar and Nafiseh, 2016) which is usually done by hand, using tweezers and paintbrushes. After the hybrid fruits have matured, the seed is extracted from the fruit and planted in the nursery. Once the seedlings have attained sufficient size, they are directly planted in the field or as with citrus, grafted onto rootstocks and then planted for evaluation. During this first phase, scion progeny are evaluated only for fruit traits. Once a novel fruit with the desired attributes is identified, the single plant is then multiplied for statistical analysis of horticultural traits including, amongst others, yield, biotic and abiotic stress tolerance, overall growth traits, as well as fruit traits of interest (Sippel et al., 2015; Abouzar and Nafiseh, 2016). At this stage, the interactions of the scion with the rootstocks and environment is also assessed (Bijzet, 2014). Conventional breeding of rootstocks are similar but instead of being planted in the field, the progeny is subjected to various disease screening protocols after which the selected few are grafted with scions and screened for their ability to impart good quality and production to citrus scions (Bijzet, 2014).

The development of new and improved citrus cultivars by conventional methods is a slow and costly process that could take as long as 20-35 years from making the cross to releasing a new cultivar (Bijzet, 2014; Sippel et al., 2015). Other methods such as mutation breeding as well as molecular and biotechnological tools have been implemented to overcome barriers such as sterility, self- and cross-incompatibility and polyembryony. Optimised screening protocols reduce cost of field evaluation by decreasing the number of progeny that is promoted to field trials.

Grapefruit cultivars produced by the ARC-TSC’s induced mutation breeding strategy include Nelruby, Sweetheart and Redheart. Induced mutagenesis offers the opportunity to obtain improved selections of citrus cultivars by altering one or a few negative traits (such as seediness) of an otherwise successful cultivar without changing the rest of its genetic composition (Vardi et al., 2008).

Induced mutagenesis is carried out by irradiating buds with 60Co. The total dose applied to budwood varies between the different citrus varieties. Currently, commercial citrus cultivars and promising lines from the conventional breeding project are being irradiated in order to artificially induce genetic changes in this selected material. Only virus-free material is used in mutation breeding to prevent the possibility of virus mutations in plant material (Sippel et al., 2015).

Biotechnological techniques utilised by the ARC-TSC's citrus breeding projects has allowed new breeding directions to be explored (Sippel et al., 2015). In vitro embryo rescue is one of the techniques used to recover triploid plants from controlled tetraploid and diploid crosses for the development of new scion cultivars with traits such as improved fruit quality, variable ripening seasons and seedlessness (Gmitter, 1994, Sippel et al., 2015). In vitro ovule rescue from sectoral chimeras is used to harness spontaneous mutations in the field. Ovules isolated from mutated sectors are rescued and germinated in vitro and subsequently established in the field for evaluation for any of a number of improved horticultural traits such as rind and flesh colour and texture, maturity date, °brix and TSS:acid ratio (Gmitter, 1994, Sippel et al., 2015). The ploidy (Aleza et al., 2010) of crosses as well as embryo-rescued material is confirmed using flow cytometry. Support from the molecular techniques includes, amongst others, the development of microsatellite (SSR) markers for mandarin genotyping and the establishment of a molecular genotype database for mandarin accession verification (Sippel et al., 2015).

2.8 CITRUS BREEDING FOR FLAVONOID CONTENT

In order to keep pace with the rising global demand, the main goal of plant breeding in the past was to improve crop yield, pest and disease resistance while currently plant breeding efforts tend to focus also on nutritional security (Patil et al., 2014). Modern tools of molecular biology could shed more light on the functions of enzymes, their pathways and the genes controlling them (Mouradov and Spangenberg, 2014). For example, problems relating to the seedless citrus cultivars as well as flavour and eating quality of fruit can be reduced by using genetic engineering techniques to form desired metabolites through modifying the metabolic pathways (Ollitrault et al., 2008).

Research done to investigate the mechanisms involved in the metabolism of monoterpenes, limonoids, flavonoids and carotenoids in citrus has paved the way for new breeding strategies such as DNA marker-based selections. DNA marker-based selection methods are developed and can be applied to the selection of new cultivars enriched with health-promoting substances (Omura and Shimada, 2016). Frydman et al. (2004) isolated and characterised the gene encoding 1,2 rhamnosyltransferase, a key enzyme in the biosynthesis of the bitter flavonoids in citrus (Figure 2.3). The other metabolic genes responsible for the hydroxylation, methylation and glycosylation of flavonoids are still not fully known, yet plant breeding provides an effective strategy to increase the levels of health promoting bioactive compounds in fruit and therefore the human diet (Patil et al., 2014).

2.9 SCREENING GRAPEFRUIT GERMPLASM FOR FLAVONOID CONTENT

According to Panguluri and Kumar (2013) a significant limitation to an effective breeding programme is generating reliable phenotype data which in turn indicates the paramount importance of a convenient, reproducible, reliable and rapid assessment of the phenotype, also called a screening protocol. In a citrus breeding programme, many of the fruit quality traits are assessed post-harvest and are mainly determined by the amounts and relationship between the organic constitutes within the fruit (Sinclair, 1961). However, this relationship and therefore the subsequent expression of the trait can be influenced by the genotype, the environment (climate, soil conditions, cultural practices as well as fruit location within tree canopy) as well as the interaction between genotype and environment such as the stages of fruit maturity (Sinclair, 1961; Chen, 1990; Hunlun, 2016).

Grapefruit varieties have different genetic traits, which mainly influence the micro constituents and consequently the phenolic and flavonoid content and composition thereof (Hunlun, 2016). In an extensive study done by Peterson et al*.* (2006) it was concluded that white grapefruit varieties tend to be slightly, but not significantly, higher in total flavanone content (27 mg 100 ml^{-1}) compared to the pink and red varieties (18 mg 100 ml^{-1}).

Climatic and geographical conditions influence growth and fruit development and will therefore result in distinguishable phytonutrient contents (including flavonoids), and high variability in the phytonutrient composition was found due to variation in the climatic conditions over seasons and geographic locations (Hagen et al., 1966; Albach et al., 1981; Girennavar et al., 2008; Hilal et al., 2008). A study of annual and seasonal changes in naringin of Texas Ruby Red Grapefruit juice over five consecutive seasons by Albach et al. (1981) revealed that naringin concentrations were influenced by geographical location in the same year and seasonal variation during the growing season as well as over crop years.

The contribution of geographical location to the occurrence of variation in bioactive compounds was emphasised by extensive research done on other fruits such as pomegranates (Mditshwa et al., 2013) where different total phenolic contents were observed for pomegranates grown in different regions (elevation and temperature). In this study it was also concluded that altitude affected the biosynthesis of phenols (Mditshwa et al., 2013).

Barry et al. (2000) proved that the variability in juice quality of Valencia sweet orange in Florida was affected by canopy microclimate. According to Dr Barry (personal communication, April 2016), several factors such as genetic traits, climatic and geographical factors as well as fruit location within tree canopy and fruit maturity level should be taken into account when screening grapefruit germplasm for flavonoid content in a breeding programme.

Freeman and Robbertse (2003) illustrated that fruit sampled from various canopy positions and light exposures exhibited pronounced differences in fruit quality that could be attributed to the amount of light and higher temperatures to which different canopy positions are exposed. Fruit from the exterior, more exposed canopy positions and fruit from the upper canopy positions generally have a higher soluble solid content regardless of the geographical exposure to light (Freeman and Robbertse, 2003). Freeman and Robertse (2003) found that fruit quality is also influenced by the geographical direction of light exposure; their study was done in the Southern hemisphere, thus fruit from the southern canopy position had a higher juice content compared to those from the northern positions.

Hemmati et al. (2014) concluded from their study done in the Northern hemisphere, that there was a significant difference in the flavonoid content of four citrus species with regard to the geographical canopy position. The highest amount of naringin tended to be in lemons produced in the northern canopy position of the tree.

Another flavonoid composition factor to consider is fruit maturity. High levels of naringin, and other flavanones, are associated with young tissues, especially young developing leaves and stems where lower levels are associated with more mature and older tissues (Jourdan et al., 1985). Small green 1-month-old grapefruit, with a fruit weight of 0.45 g, was reported to have a naringin concentration of 162.70 µmol g^{-1} and as the fruit ripened, there was a 26-fold decrease in the relative naringin concentration, reported as 6.3 µmol g^{-1}. This decrease in naringin concentration is likely due to the increase in fruit fresh weight, which dilutes the naringin concentration (Jourdan et al., 1985).

The anabolism of naringin (Figure 2.4) occurs as the fruit ripens. Naringin is hydrolysed by the enzyme L-rhamnosidase to produce prunin and L-rhamnose. Prunin is then further hydrolysed to naringenin and D-glucose (Puri et al., 1996). This reduces the overall bitterness of the fruit, with prunin only 33% as bitter as naringin and naringenin being almost tasteless (Puri et al., 1996). Thus the concentration of naringin can be used as an indication of fruit ripeness.

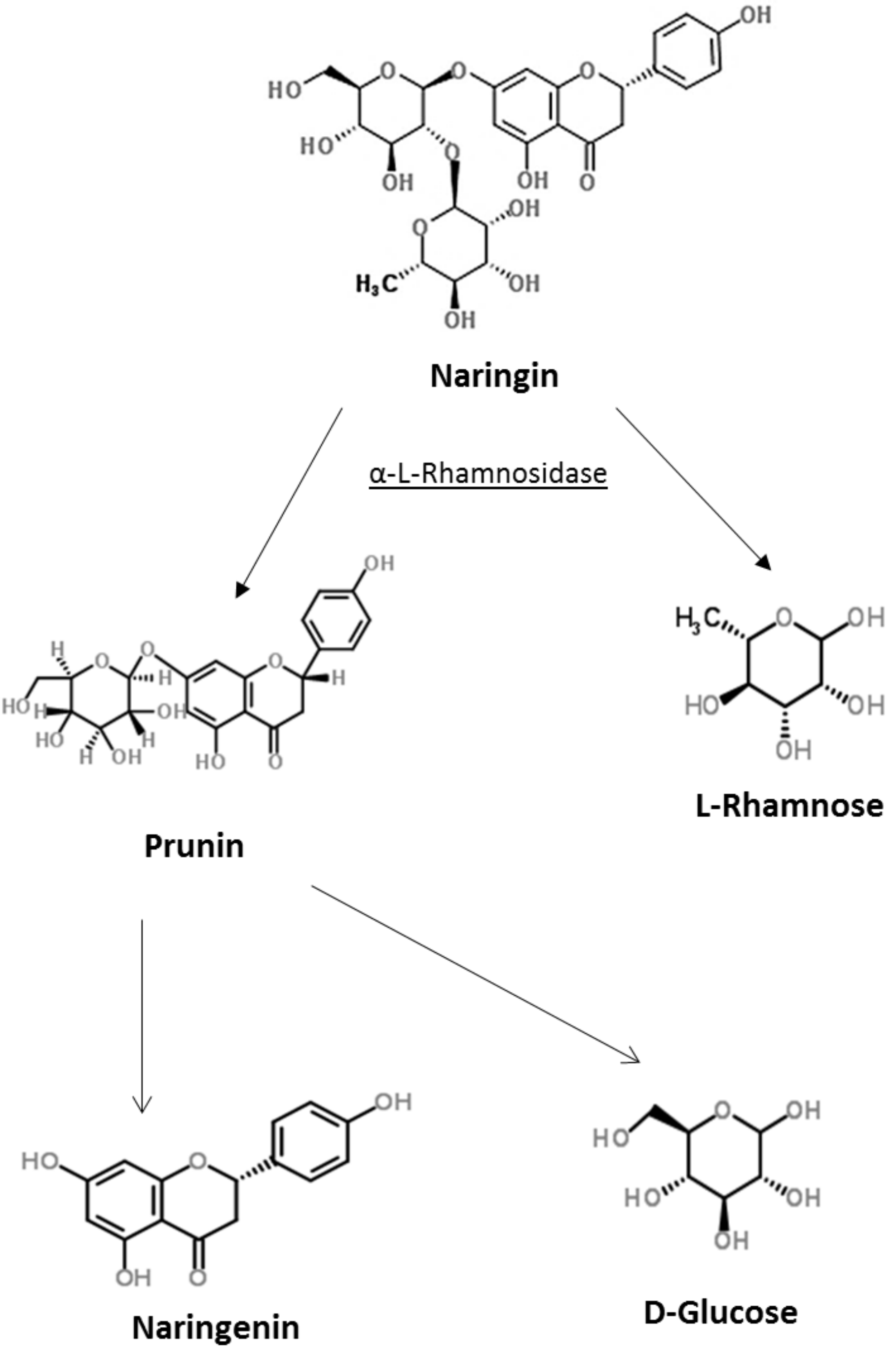

Figure 2.4 Anabolism of the flavanone naringin during fruit ripening

2.10 ANALYSIS OF FLAVONOIDS

There are a number of identification and quantification based methods of flavonoids in various matrices (such as blood, urine, fruit juices) in published investigations (Ribeiro and Ribeiro, 2008). Of these, HPLC and gas chromatography in combination with mass spectrometry were the two most commonly applied methods to quantify these phenolic compounds mainly due to the specificity and accuracy thereof (Khoddami et al., 2013).

HPLC is the most widely applied method, documented in literature, for the quantification of phenolics and specifically for citrus flavonoid classes (Hunlun, 2016). Examples of HPLC methodologies applied for the determination of citrus flavonoids are listed in Table 2.1. This method is also preferred due to cost and time effectiveness, as fruit juice samples' flavonoid profiles can be obtained without prior sample preparation or extraction. Reversed-phase HPLC is the specific method most frequently used for the separation of flavonoids on C-8 and C-18 columns. Flavonoid separation occurs by using relatively polar mobile phases such as methanol, acetonitrile or tetrahydrofuran in combination with acidic aqueous solutions under gradient elution conditions (Hunlun, 2016). Generally, diglycosides elute before monoglycosides, followed by aglycones. This is due to the polarity of the groups of compounds.

For the detection of flavonoid classes found in citrus species, UV-Vis (Ultra Violet and Visible light) or diode array detection (DAD) detectors are mostly used. To identify individual classes of flavonoids, specific wavelengths are used. For example, flavanones have their absorption maximum at 280 - 290 nm, flavones at 304 - 350 nm and flavonols at 352 - 385 nm (Gattuso et al., 2007).

Table 2.1 Examples of HPLC methodologies applied for the determination of citrus flavonoids documented in literature

Variety	Plant part	Sample preparation / Extraction	Column	Solvents	Compounds	Reference
Commercially available grapefruit juice	Juice	Solvent extraction	HSS C18-column (50 mm × 2.1 mm i.d., 1.8 µm)	MeCN/H_2O	Naringin, Narirutin, Naringenin, Hesperidin	VanderMolen et al., 2013
Citrus grandis L. Osbeck	Flavedo and juice	Solvent extraction of freeze dried sample	RP Zorbax SB C18 column (250 mm × 4 mm, 5 µm)	1% Acetic acid:water and 1% acetic acid:acetonitrile	Naringin Neoeriocitrin Acetylnaringin Melitidin Rhoifolin Diosmin O-Triglycosylnaringenin Lucenin-2 Vicenin-2 Apigenin 6-C-glucosyl-7-O-glucoside Lucenin-2 4'-methyl ether Diosmetin 6-C-glucoside Apigenin 6,8-di-C-(sinapolyl) glycoside Apigenin 6,8-di-C-(feryloyl) glycoside Kaempferol 7-O-rhamnosyl 3-O-glucoside	Zhang et al., 2014
42 Species and cultivars of the Citrus genus and those of two Fortunella and one Poncirus species	Flavedo, Albedo, Segment Epidermis, Juice Vesicle	Solvent extraction using a seppak C_{18} cartridge	LiChrospher 100 RP-18, 250 x 4:0 mm-i.d	10mM Phosphoric acid:water/ MeOH	Eriocitrin Neoeriocitrin Narirutin Naringin Hesperidin Neohesperidin Neoponcirin Poncirin Rutin Isorhoifolin Rhoifolin Diosmin Neodiosmin Sinensetin Nobiletin Tangeretin Heptamethoxyflavone	Nogata et al., 2014
Satsuma Clementine Navel Mandarin Valencia	Juice	None	Gemini-NX C18 (3 µm particle size, 110 Ǻ pore size,150 × 4.6 mm ID)	0.2% Acetic acid:water / acetonitrile	Quercetin-3-O-rutinose-7-O-glucoside, ferulic acid-O-hexoside, vicenin-2, naringenin-7-O-rutinose-4'-O-glucoside, narirutin, hesperidin, neoponcerin,	Hunlun, 2016
Jaffa blond oranges (*Citrus sinensis*) Jaffa red Star Ruby (Sunrise) and blond grapefruit (*Citrus paradisi*), and pummelo–blond grapefruit hybrid (*Citrus paradisi* var Jaffa Sweetie)	Juice and pulp	Solvent extraction	Spherisorb ODS1 column	2% Acetic acid:water / acetonitrile	Naringin Hesperidin	Gorinstein et al., 2006
Commercially available grapefruit juice	Juice	None	Lichrosher® 100 RP-18 (5µm particle size, 250 x 4 mm i.d.)	Acetonitrile/ water	Naringin and Naringenin	Ribeiro & Ribeiro, 2008
Hamlin oranges and Valencia oranges (*Citrus sinensis (L.) Osbeck*)	Juice	Solvent extraction using a SPE-C18 cartridge	C-18 column (Phenomenex Luna 5 µ C18, 250 × 4.60 mm 5µ)	Acetonitrile/aqueous acetic acid (1%)	Narirutin Hesperidin Didymin	Dagulo et al., 2010

2.11 CONCLUSIONS

The citrus industry forms a major part of the agronomic and economic sector of South Africa, thus the breeding and selection of new cultivars is necessary in order to keep up with global demand. Most breeding objectives are derived from the needs of a profit driven industry, such as improvement of crop yield, and pest and disease resistance. In the last decade, food security has become an important focus for plant breeders and has in recent times expanded to include nutritional security as a new global objective. This entails selecting cultivars with increased levels of health promoting bioactive compounds (Patil et al., 2014). As the growing awareness of diet linked to health continues, the interest in specifically citrus flavonoids have also increased. This prompts a new challenge in so far as screening potential germplasm towards possible breeding parents to develop the flavonoid levels or compositions beneficial to human health. Thus, this study aims to provide a screening technique for grapefruit germplasm, which takes into account the factors such as variety differences, as well as fruit location within tree canopy, to ultimately be applied in the ARC's pre-breeding programme.

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Chapter 3

OPTIMISATION OF A HPLC METHOD FOR FLAVONOID QUANTIFICATION IN GRAPEFRUIT

3.1 INTRODUCTION

Each type of citrus fruit has its own distinguishing fruit quality traits such as colour and flavour (Schaffer and Anderson, 1994). Grapefruit has a unique flavour described as a blend of acid, sub-acid bitterness and sweetness (Bijzet, 2006). Fruit quality is determined mainly by the amounts and relationship of the organic constituents within the fruit, this includes phenolic compounds such as flavonoids (Sinclair, 1972).

In the case of grapefruit, the characteristic of bitterness is caused by the flavonoid naringin, which is a major flavanone found in grapefruit tissue and fruit juice (Ribeiro and Ribeiro, 2008; Zhang, 2010). Naringin is an end product of the phenylpropanoid pathway, and produced throughout the growth period, with high concentration levels in especially young tissue and small green fruit (Albach et al., 1969; Jourdan et al., 1985). In the fruit, the anabolism of naringin occurs as the fruit ripens. Naringin is hydrolysed to prunin and naringenin, which is almost tasteless (Puri et al., 1996). Furthermore, as the fruit develops and ripens, the concentration of naringin in the fruit juice is also diluted with the other organic constituents in the fruit juice (Jourdan et al., 1985; Kesterson et al., 1953; Frydman et al., 2004).

There are a number of identification and quantification based methods of flavonoids in various matrices (such as blood, urine, fruit juices) (Riberio and Ribeiro, 2008). Of these, HPLC and gas chromatography in combination with mass spectrometry are the two most commonly applied methods to quantify these phenolic compounds, mainly due to the specificity and accuracy thereof (Khoddami et al., 2013).

HPLC is the most widely applied method documented in literature for the quantification of phenolics and specifically for citrus flavonoid classes (Hunlun, 2016). This method is also preferred for cost and time effectiveness, as flavonoid profiles can be obtained for fruit juice samples without prior sample preparation or extraction. This method is extensively used, and typically involves reversed-phase HPLC for the separation of flavonoids on C-8 and C-18 columns. Separation of flavonoids can be achieved using relatively polar mobile phases such as methanol, acetonitrile or tetrahydrofuran in combination with acidic aqueous solutions under gradient elution conditions (Hunlun, 2016).

Furthermore, UV-Vis or diode array detection (DAD) is used to characterise flavonoid classes found in citrus species. Specific wavelengths are used to identify individual classes, for example flavanones have their absorption maximum at 270 - 295 nm, flavones at 304 - 350 nm and flavonols at 352 - 385 nm (Gattuso et al., 2006; Kumar et al., 2014).

The aim of this study was the optimisation of an analytical method for the identification and quantification of flavonoids in grapefruit varieties, as the information will provide valuable insight to the future of the Agricultural Research Council's citrus pre-breeding programmes.

3.2 MATERIALS AND METHODS

3.2.1 Ribeiro and Ribeiro HPLC analysis method

The method applied by Ribeiro and Ribeiro (2007) proved successful for the separation and identification of naringin and naringenin in fresh pressed and commercial grapefruit and orange juice samples.

The sample preparation method of Ribeiro and Ribeiro (2007) was used by fresh pressing juice from grapefruit. Thereafter the juice was centrifuged at 8000 rpm for 15 min. The supernatant of the juice was filtered (number 1 Whatmann filter) and diluted 1:8 with sodium acetate buffer 0.02 M, pH 4.0. The method employed a HPLC Waters 2690 separation module. Separation of analytes was achieved using acetonitrile (Solvent A) and pure water (Solvent B), as mobile phase, with a Merck analytical column, Lichrospher® 100, RP-18 (5 µm particle size, 250 x 4 mm i.d) at 25°C. The following gradient programme was used: 23% A at 0 - 8 min, 23-65% A linear at 8 -15 min, 65 - 70% A linear at 15 -20 min, 70 - 23% A linear at 20 -21 min and 23% A at 21 - 22 min. The flow rate was kept constant at 1 ml min^{-1}, and the injection volume was 20 µl. The photodiode array detector (PAD) was set at 200 - 400 nm wavelength and the chromatograms were recorded at 280 nm. Data acquisition was done with Millennium®32, Waters software (Water Corporation, Milford, Ireland).

3.2.2 Optimising the Ribeiro and Ribeiro HPLC analysis method

The parameters described by Ribeiro and Ribeiro (2007) were used as the starting point, except that an Accucore C18 (2.3 µm particle size, 50×3 mm i.d) column was used after adapting the gradient to the shorter column length. Subsequently, the sample volume, gradient programme and detection wavelength was systematically adjusted until the method gave the best baseline separation for naringin and naringenin in the test samples. Column overload was problematic, therefore different sample dilutions (1:3 and 1:10 juice sample: methanol) and sample volumes (2.7 µl, 2 µl, 1 µl and 0.8 µl) were evaluated. The best separation and resolution were obtained using 0.8 µl undiluted grapefruit juice. The flow rate and gradient programme was adjusted by systematically adjusting the acetonitrile content in the mobile phase until baseline separation in the shortest run time was achieved.

3.2.3 Optimised HPLC analysis method

Chemicals: Deionised water was prepared with a Synergy® UV Millipore system (Merck Millipore, Darmstadt, Germany) and subsequently treated with a SynergyPak (Millipore) water purification system in order to obtain HPLC-grade water. HPLC-grade acetonitrile and methanol were purchased from Anatech (Olivedale, Gauteng, RSA). Naringin (purity >95%), and naringenin (purity >95%) standards were purchased from Sigma-Aldrich (St Louis, MO, USA). The manufacturers reported purity of standards as determined by HPLC.

Apparatus: The flavonoid analysis was performed by an Accela 600 HPLC system (in-line degasser, quaternary solvent delivery pumps, automatic injector with 100 µl loop and column oven) with an Accela UV/Vis Detector (1 cm flow cell). All chromatographic analyses were performed on an Accucore C18 (2.3 µm particle size, 50×3 mm i.d) column. Data acquisition was done with Thermo Scientific ChromQuest™ 5.0 chromatography data system.

Chromatographic conditions: Separation was performed with a constant injection volume of 0.8 µl at 25°C column temperature, with a flow rate of 0.818 ml min^{-1} using HPLC-grade acetonitrile (solvent A) and deionised water (solvent B). A gradient programme was applied as follow: 10 - 20% A at 0.0 - 2.8 min, 20 - 24% A at 2.8 - 3.0min, 24 - 28% A at 3.0 - 3.5 min, 28 - 40% A at 3.5 - 4.0 min and 40 - 60% A at 4.0 - 6 min. The UV/Vis detector was set at 210 - 500 nm wavelengths and the chromatograms were recorded at 283 nm for naringin and 289 nm for naringenin.

Standard solution preparation: Standard stock solution of Naringin (1000 mg l^{-1}) and naringenin (200 mg l^{-1}) were prepared by dissolving 25 mg naringin and 4.9 mg naringenin in 25 ml HPLC-grade methanol. These stock solutions were used to prepare standard dilutions for each compound at concentrations ranging from 100 mg l^{-1} to 400 mg l^{-1} (naringin), and 10 mg l^{-1} to 200 mg l^{-1} (naringenin). These solutions were used to determine the intra- and inter-day repeatability, limit of detection (LOD) and limit of quantification (LOQ).

Plant material: Fruit samples were harvested over two seasons (2015 - 2016 and 2016 - 2017) from a hot-humid area as classified by Barry (1996) with the goodwill of Golden Frontiers Citrus Pty Ltd from their farm at Hectorspruit, Mpumalanga. The GPS coordinates of the grapefruit orchards used were -25,445111, 31,661606. These orchards were planted in December 2006, with a planting distance between trees of 7 m × 3 m, in an east - west orchard orientation. The soil was a shallow Mispah type and fertilisation of these trees was done according to annual soil and leaf analyses.

The fertilisation programme starts July with the foliar application of Low-Biuret Urea (LB-Urea) mixed with the micronutrients zinc and copper, to promote uptake. According to fertiliser guidelines provided by Abercrombie (2006), grapefruit trees of the ages between 8 - 10 years should receive 560 g nitrogen per tree and the recommended concentration of LB-Urea mixed with the micronutrients should be 1000 g 100 l^{-1} but these values are adjusted according to the annual soil and leaf analyses. The grapefruit trees received another fertiliser application during the season (October-November) of CUAN, Kelp and OMNIBOOST-Aerial in the ratios of 5:1:15. CUAN is nitrogen/calcium based fertiliser (28.5 % N and 9.1 % Ca) that helps maintain balanced nutrition throughout the growing season. The calcium provided here is important for root development and fruit quality (Abercrombie, 2006). The OMNIBOOST-Aerial is a leaf application given as a supplement to the normal fertiliser programme especially during times of stress. The average annual rainfall for the Malelane area is 716 mm, but during the 2015/16 season intense drought conditions were experienced in South Africa (Anon 2018). The low rainfall and high temperatures, reduced river levels and the Kwena dam capacity to around 35%. This led to the implementation of irrigation restrictions by the Inkomati-Usuthu Catchment Management Agency (IUCMA) throughout the 2016-2017 season. Irrigators on the main stem were restricted to 20 hours per week, compared to the unrestricted maximum of 120 hours (Anon, 2016).

Sampling: For each of the three varieties, eight sampling trees were identified. The canopy area of each tree was divided into four quadrants: north, east, south and west. Ten fruit for each quadrant data point were sampled at random canopy heights and canopy depths. Each fruit was specifically labelled with respect to the grapefruit variety, sample tree and the canopy quadrant from where it was harvested. The fruit were peeled and stored, at -18°C prior to analysis. At the time of analysis, the sample fruit were thawed and each fruit was individually mechanically juiced.

HPLC analysis: 15 ml of the sample juice was centrifuged at 5700 rpm (centrifuge model Z 206 S, HERMLE, Germany) for 8 min at room temperature, to remove solids from the juice. The juice supernatant was filtered using a 0.2 μm nylon membrane filter (Membrane Solutions, Germany) into 1.5 ml auto sampler vials prior analyses. A volume of 0.8 μl was injected for HPLC analysis in triplicate injections. Chromatograms were recorded as intensity measured (mAU) over time (minutes).

Method validation: The optimised HPLC method was validated by evaluating the linearity of the calibration curves, calculating the limit of detection (LOD) and limit of quantification (LOQ), as well as evaluating the repeatability and reproducibility (intra- and inter-day repeatability) for the standard calibration dilutions.

3.3 RESULTS

The method described by Ribeiro and Ribeiro (2007) used to quantify flavonoids in grapefruit juices, was modified in this study to shorten the run time. Figures 3.1 A and 3.2 A show the recorded chromatograms of the standard solutions with retention times of 3 min and 4 min for naringin and naringenin respectively.

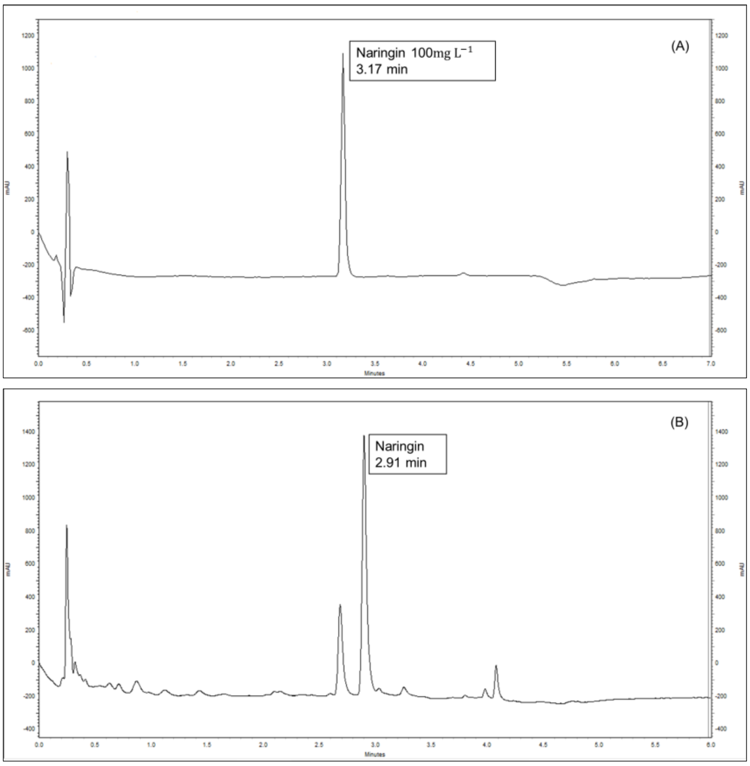

Figure 3.1 HPLC chromatograms recorded at 283 nm of the standard reference naringin solution (A) and the grapefruit juice sample (B)

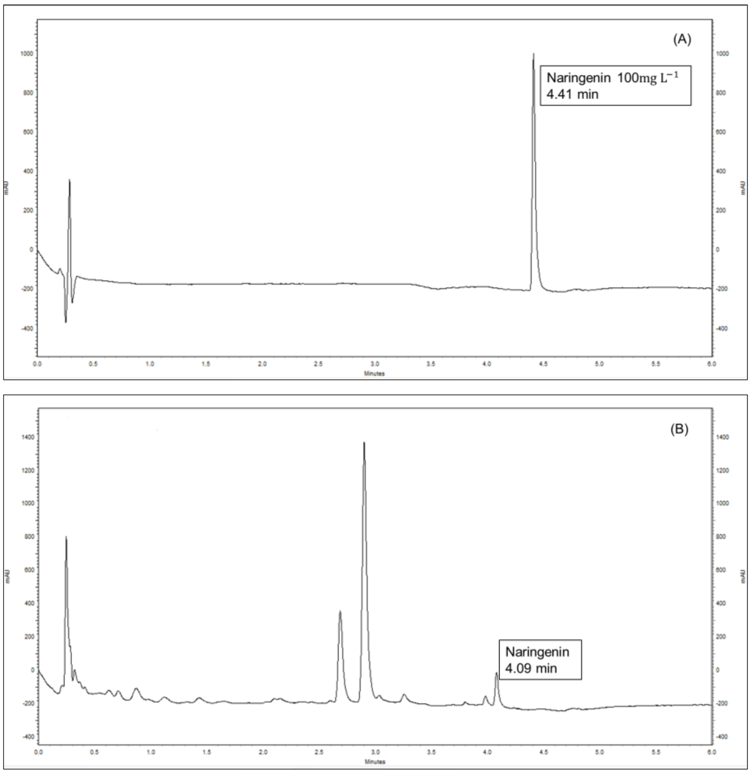

Figure 3.2 HPLC chromatograms recorded at 289 nm of the standard reference naringenin solution (A) and the grapefruit juice sample (B)

Flavanones such as naringenin exhibit a very strong Band II absorption maximum between 270 and 295 nm, and only a shoulder for Band I at 326 and 327 nm (Kumar et al., 2013). The results indicated that the standard reference naringin had maximum absorption at 282 nm, and the naringin peak in the sample had a maximum absorption at 283 nm (Figures 3.3 A and B). This difference in absorption maximum values could be because of interference of substances in the juice sample. In the method described by Ribeiro and Ribeiro (2008) the maxima absorption of naringin was reported as 282.8 nm and another method reported it as 283.6 nm (Sun et al., 2010). For sensitivity, the naringin peak area at 283 nm was used for quantification. The naringenin standard and the naringenin in the juice sample had high absorptions at 288 nm and 289 nm (Figures 3.4 A and B). In the method described by Ribeiro and Ribeiro (2008) the maximum absorption of naringenin was reported as 288.7 nm. For sensitivity the naringenin peak area at 289 nm was used for quantification.

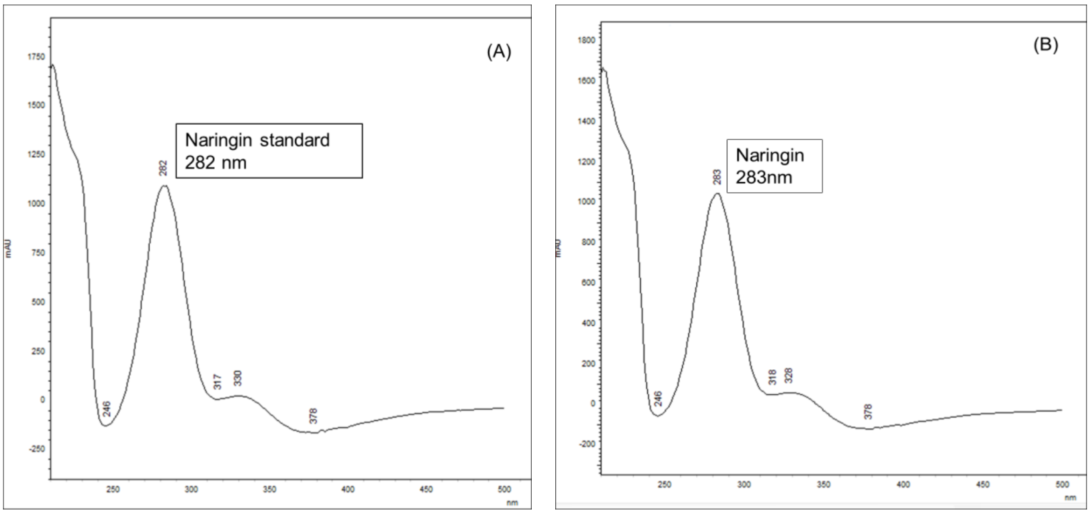

Figure 3.3 UV-Vis spectra of the flavonoid naringin. (A) naringin standard absorption maximum 282 nm, (B) naringin peak in juice sample absorption maximum 283 nm

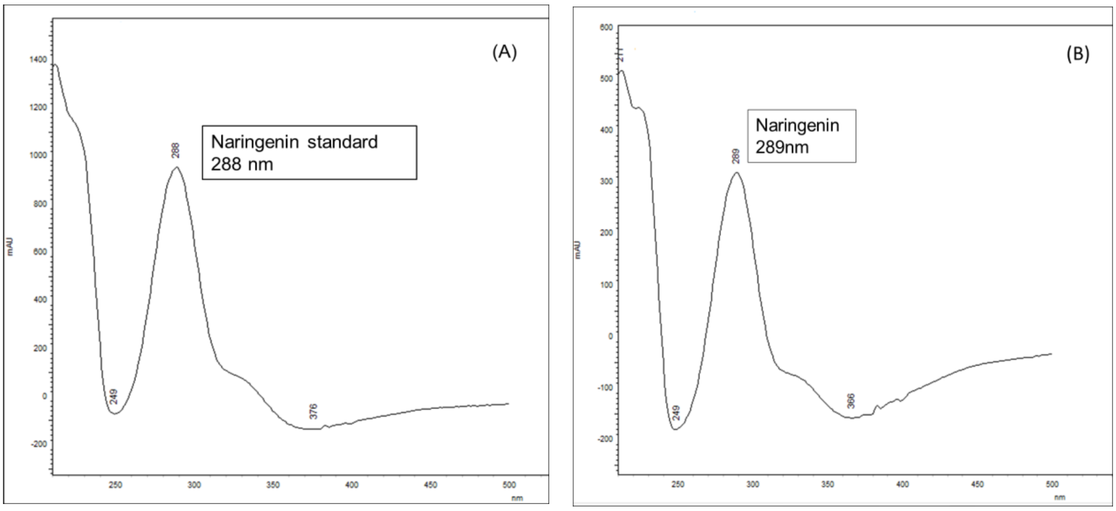

Figure 3.4 UV-Vis spectra of the flavonoid naringenin. (A) naringenin standard absorption maximum 288 nm, (B) naringenin peak in juice sample absorption maximum 289 nm

Linearity: In the linearity range, five different standard solutions were used for both naringin (100 mg l^{-1} to 400 mg l^{-1}) and naringenin (10 mg l^{-1} to 200 mg l^{-1}). Calibration curves were obtained by plotting the peak area versus the prepared concentrations of naringin or naringenin (mg l^{-1}), which resulted in straight lines over the concentration range. The peak area was measured in µAU s^{-1}. Linear regression was done by using the least squares method on the calibration curves' data to test for linearity. The slope, y intercept and correlation coefficients (R^2 and R) were determined and used to quantify the naringin and naringenin content in grapefruit juice. Typical calibration curves were defined as $y = 10051x - 17753$ for naringin and $y = 26904x + 489962$ for naringenin. Naringin and naringenin had a R^2 value of 0.999.

Limit of detection and limit of quantification

The limit of detection (LOD) was defined as the concentration corresponding to the signal detection limit, which was defined as $b + 3s_y$, where b is the y-intercept of the calibration curve, and s_y is the standard deviation of the vertical deviations (Harris, 2007). LOD was calculated as 1.77 mg 100 ml^{-1} for naringin and 0.23 mg 100 ml^{-1} for naringenin. The limit of quantitation (LOQ) was defined as $b + 10s_y$. LOQ was calculated as 5.35 mg 100 ml^{-1} for naringin and 0.72 mg 100 ml^{-1} for naringenin.

Intra and inter-day repeatability

The inter and intra-day repeatability was performed by injecting the standard calibration solutions six times per day over three consecutive days. The percentage relative standard deviation of the peak areas (% RSD) was determined for replicate injections on the same day to obtain the intra-day repeatability given in Table 3.1.

Table 3.1 Intra and inter-day repeatability for analysis of naringenin

Naringenin	Intra-day (n=6/day)			Inter-day (n=3)
Conc. mg l^{-1}	RSD% Day 1	RSD% Day 2	RSD% Day 3	%RSD
10	1.39	0.44	1.04	0.30
20	0.93	0.17	1.14	0.73
40	0.41	0.61	0.51	1.05
80	0.16	1.20	0.38	0.11
160	1.66	1.68	0.84	1.49
196	1.03	0.97	0.41	0.57

RSD = relative standard deviation

Application

Optimal conditions were applied to the quantitative analysis of naringin and naringenin in the juice of three different grapefruit varieties over two harvesting seasons. The overall mean flavonoid content for the three grapefruit varieties are presented in Table 3.2.

Table 3.2 Amount of naringin and naringenin in three grapefruit varieties over two seasons

Grapefruit variety	Contents (mg 100 ml^{-1}, mean ± standard error)	
	Naringin	Naringenin
Sweetheart	36.79 ± 0.76	1.15 ± 0.03
Star Ruby	38.91 ± 0.73	1.31 ± 0.03
Marsh	33.95 ± 0.55	0.98 ±0.02

3.4 DISCUSSION

The method described by Ribeiro and Ribeiro (2007) used to quantify flavonoids in grapefruit juices, was adapted in this study to accommodate the shorter and smaller column. The parameter modifications consequently led to a shortened run time. A slight difference in retention time between the sample compounds and the standard analytes were observed. This retention time difference can be an effect of the sample matrix or pH. The method is robust and the <0.5 min difference in retention time was accepted. Good resolution peaks of both the standard analytes and sample analytes were obtained with this optimised HPLC method. For reliable quantification to be possible, the HPLC method was validated by evaluating the linearity of the calibration curves, calculating the limit of detection (LOD) and limit of quantification (LOQ), as well as evaluating the repeatability and reproducibility (intra- and inter-day repeatability) for the standard calibration dilutions. Acceptable linearity ranges were obtained for both naringin and naringenin calibration curves (R^2 = 0.999 for both). LOD was calculated as 1.77 mg 100 ml^{-1} for naringin and 0.23 mg 100 ml^{-1} for naringenin. The limit of quantitation (LOQ) was defined as $b + 10s_y$. LOQ was calculated as 5.35 mg 100 ml^{-1} for naringin and 0.72 mg 100 ml^{-1} for naringenin. The percentage RSD of the peak area mean values for each day was determined to evaluate the inter-day repeatability. The intra and inter-day repeatability were between 0.22 and 2.07, which is far below the accepted threshold of 5% for all standard compounds. The method was applied to quantify the naringin and naringenin content in the grapefruit samples. The mean concentration (±SD) of naringin and naringenin over all three grapefruit varieties was 36.17 ± 8.34 mg 100 ml^{-1} and 1.06 ± 0.33 mg 100 ml^{-1} respectively, which is consistent with literature values (Gorinstein et al., 2006; Peterson et al., 2006; Ribeiro and Ribeiro, 2007; Zhang, 2010; Van der Molen et al., 2013). Literature indicates white grapefruit varieties to be slightly but not significantly higher in total flavanone content than pink and red varieties (Peterson et al., 2006). However, from Table

3.3, it is seen that the white grapefruit variety namely Marsh was slightly lower in naringin and naringenin content than the pink and red varieties used in this study.

3.5 CONCLUSIONS

A simple, reliable and rapid analytical method for the identification and quantification of naringin and naringenin in grapefruit juice by HPLC-UV/Vis was optimised. This method was used for the determination of naringin and naringenin content in the grapefruit fruit juice samples.

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Chapter 4

THE EFFECT OF VARIETY, SEASON AND CANOPY POSITION ON THE FRUIT TRAITS AND FLAVONOID COMPOSITION OF THREE GRAPEFRUIT VARIETIES

4.1 INTRODUCTION

The grapefruit was first deemed a distinct species and designated as *Citrus paradisi*, by the botanist James MacFayden in 1830 (Sinclair, 1972). The definite origin of the grapefruit is not known but, it is thought to be a cross between an orange (*Citrus sinensis)* and a pummelo (*Citrus grandis*). Grapefruit is a subtropical citrus tree, known for its distinctive flavour described as a blend of acid, sub-acid bitterness and sweetness (Bijzet, 2006). Because of their tropical origin, citrus species' fruit quality varies intensely with climate. Citrus fruit quality also varies among cultivars and stages of maturity (Chen, 1990).

Furthermore, the fruit's organic constituents mainly determine internal fruit quality. These include the total soluble solid (TSS), acid content and the ratio between acid and TSS (Bijzet, 2006). Another contributor to citrus fruit quality is the amount of specific phenolic constituents present in the fruit juice. In grapefruit, the main phenolic component is the flavonoid naringin, and its aglycone naringenin (Gattuso et al., 2007).

Albach et al. (1981) studied the annual and seasonal changes in naringin of Texas Ruby Red grapefruit juice over five consecutive seasons. The study showed that naringin concentrations were subject to seasonal variation. The authors concluded that climatic differences between the crop years influenced the naringin concentrations and that location also influenced the results of some of the crop years. In a study of four citrus species, the highest amount of naringin was found in lemons produced in the northern canopy position of the tree and it was further concluded that there was a significant difference in the flavonoid content of fruit with regard to the geographical canopy position (Hemmati, 2014).

Little is currently known on the variation in phenolic composition of different South African citrus fruit due to varietal and seasonal effects (Hunlun, 2016). Thus, the aim of this study was to evaluate the effect of variety, and seasonal and canopy position differences on the fruit traits and flavonoid composition of three grapefruit varieties.

4.2 MATERIALS AND METHODS

4.2.1 Plant material

For this study, three commercial grapefruit varieties were used, namely, Marsh, Sweetheart and Star Ruby, and fruit were harvested over two seasons (2015-2016 and 2016-2017). These three varieties respectively represented white, pink and red grapefruit. The general fruit traits as well as the naringin concentration are known and well documented for these varieties. To compensate for the possible influence of genetic factors on the fruit traits and other micro constituents and consequently the phenolic and flavonoid content in fruit, the sampling trees of each respective variety was clonal material, and of the same age. Furthermore, the trees were in a commercial orchard and subjected to the same management programme, thus compensating for agro management practices which may influence fruit traits and flavonoid content.

4.2.2 Sampling

Sampling was done as described in Chapter 3 section 3.2.3.

4.2.3 Fruit physical and biochemical traits

Each individual fruit was weighed (Sartorius digital scale, d=0.01 g), the circumference was measured and then each fruit was carefully dissected into pulp and peel. The peel (flavedo and albedo) was weighed and the flavedo thickness was measured with a digital Vernier caliper in mm. Each peeled fruit was weighed and mechanically juiced. The juice sample of each fruit was used for standard (Hardy and Sanderson, 2010) pH, TSS and TA measurements as well as HPLC analysis.

The pH of each juice sample was measured with a standard pH meter after calibration with pH 4.0 and 7.0 buffers. The titratable acidity (TA) of juice samples was determined by titrating 10 ml sample against 0.1562 M sodium hydroxide solution (NaOH) until the end-point of pH 8.2 was reached. The results were expressed as citric acid (% $w.w^{-1}$).

Total soluble solids (expressed as °brix) of juice samples were measured using a hand held refractometer (Atago model PAL^{-1}, Tokyo, Japan). In addition, a °brix:acid ratio was calculated for each sample.

The naringin and naringenin concentration in each fruit was determined by the optimised HPLC method discussed in Chapter 3.

4.2.4 Statistical analyses

The experimental design was a randomised complete (all factors in all blocks) block design with four replications. For each season, the data were analysed as a split plot design with variety as main plot factor and quadrant as subplot factor. The data was subjected to analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.2; SAS Institute Inc, Cary, USA). Observations over time (years) were combined in a split-split-plot analysis of variance with years as sub-subplot factor (Little and Hills, 1972). A Shapiro-Wilk test was performed on the standardised residuals from the model to verify normality (Shapiro and Wilk, 1965). Fisher's least significant difference (LSD) was calculated at the 5% level to compare treatment means (Ott and Longnecker, 1998). A probability level of 5% was considered significant throughout. Data were expressed as means ± standard deviations (SD).

4.3 RESULTS

4.3.1 Analysis of variance for the 2015-2016 season

The ANOVA results for the fruit physical traits measured in the first season (2015-16) are summarised in Table 4.1 and for juice traits in Table 4.2.

Table 4.1 Analysis of variance for fruit traits for the 2015-2016 season

	Fruit circumference (mm)		Fruit mass (g)		Peel mass (g)		Segment mass (g)		Peel thickness (mm)	
	Df	MS	Df	MS	Df	MS	Df	MS	Df	MS
Block	3	3.12	3	2668.03	3	647.54	3	6333.21	3	0.12
Variety	2	0.94	2	9600.45	2	4847.65*	2	23368.31*	2	1.11**
Q	3	1.23	3	10655.47**	3	810.75	3	8445.55***	3	0.02
Q x V	6	1.13	6	2860.95	6	451.50	6	1747.94*	6	0.05**
Error	27	1.05	27	1816.81	27	297.76	27	649.83	27	0.02

*P≤0.05, **P≤0.01, ***P≤0.001

Table 4.2 Analysis of variance for juice traits and naringin and naringenin for the 2015-2016 season

	pH		TA		°brix		°brix:acid		Naringin (mg 100 ml^{-1})		Naringenin (mg 100 ml^{-1})	
	Df	MS	Df	MS	Df	MS	Df	MS	Df	MS	Df	MS
Block	3	0.001	3	0.036	3	1.26	3	1.30	3	88.62	3	0.006
Variety	2	0.082**	2	0.002	2	14.91**	2	26.10**	2	51.02	2	0.500
Quadrant	3	0.024***	3	0.018**	3	0.38**	3	1.22**	3	7.75	3	0.023
Q x V	6	0.002	6	0.003	6	0.14	6	0.92**	6	12.42	6	0.019
Error	27	0.002	27	0.002	27	0.11	27	0.34	27	27.81	27	0.050

P≤0.05, **P≤0.01, ***P≤0.001

There was a significant level of variation between varieties for peel mass, segment mass, peel thickness, pH, °brix as well as the °brix:acid ratio. Between quadrants a significant level of variation was seen between fruit mass, segment mass, pH, TA, °brix, and also the °brix:acid ratio. Only segment mass, peel thickness and °brix:acid showed significant interaction between quadrants and varieties (Q× V) (Tables 4.1 and 4.2).

The tables listed below are of the respective variables with a significant difference between means when analysed against the main plot factors (varieties) and subplot factors (quadrants).

Table 4.3 The effect of quadrants on the fruit mass (g) for the 2015-2016 season

Factor	N	Mean	Std Dev	Grouping
East	12	515.67	60.43	a
North	12	453.08	37.84	ab
South	12	454.05	41.10	b
West	12	485.32	57.91	b

P=0.0032

Table 4.4 The effect of varieties on the peel mass (g) for the 2015-2016 season

Factor	N	Mean	Std Dev	Grouping
Sweetheart	16	149.54	26.47	a
Marsh	16	126.16	18.05	ab
Star Ruby	16	115.52	18.58	b

p= 0.0466

Table 4.5 The effect of varieties on the segment mass (g) for the 2015-2016 season

Factor	N	Mean	Std Dev	Grouping
Star Ruby	16	379.13	48.86	a
Marsh	16	354.60	44.89	ab
Sweetheart	16	304.18	43.21	b

p= 0.0138

Table 4.6 The effect of quadrants on the segment mass (g) for the 2015-2016 season

Factor	N	Mean	Std Dev	Grouping
East	12	376.17	55.23	a
West	12	359.83	57.15	a
North	12	328.29	32.26	b
South	12	319.61	56.02	b

p<0.0001

Table 4.7 The effect of interaction of quadrants and varieties on the segment mass (g) for the 2015-2016 season

Factor	N	Mean	Std Dev	Grouping
East x Star Ruby	4	422.75	46.26	a
West x Marsh	4	390.77	49.98	ab
West x Star Ruby	4	388.02	44.99	abc
East x Marsh	4	383.59	25.89	bc
North x Star Ruby	4	354.06	28.77	bcd
South x Star Ruby	4	351.71	49.63	cde
South x Marsh	4	329.73	28.91	def
East x Sweetheart	4	322.17	38.98	def
North x Sweetheart	4	316.47	37.93	ef
North x Marsh	4	314.32	14.72	fg
West x Sweetheart	4	300.68	21.25	fg
South x Sweetheart	4	277.39	65.74	g

p=0.0353

Table 4.8 The effect of varieties on the peel thickness (mm) for the 2015-2016 season

Factor	N	Mean	Std Dev	Grouping
Sweetheart	16	2.60	0.21	a
Marsh	16	2.34	0.19	b
Star Ruby	16	2.08	0.15	c

p=0.0029

Table 4.9 The effect of the interaction of quadrants and varieties on the peel thickness (mm) for the 2015-2016 season

Factor	N	Mean	Std Dev	Grouping
West x Sweetheart	4	2.74	0.32	a
East x Sweetheart	4	2.57	0.18	ab
South x Sweetheart	4	2.56	0.19	ab
North x Sweetheart	4	2.55	0.14	b
North x Marsh	4	2.45	0.16	bc
West x Marsh	4	2.42	0.06	bc
East x Marsh	4	2.32	0.12	cd
South x Marsh	4	2.19	0.28	de
South x Star Ruby	4	2.16	0.14	de
East x Star Ruby	4	2.15	0.13	de
West x Star Ruby	4	2.05	0.12	ef
North x Star Ruby	4	1.95	0.14	f

p=0.0166

Table 4.10 The effect of varieties on the pH for the 2015-2016 season

Factor	N	Mean	Std Dev	Grouping
Sweetheart	16	3.40	0.07	a
Marsh	16	3.30	0.05	b
Star Ruby	16	3.26	0.07	b

p=0.0099

Table 4.11 The effect of quadrants on pH for the 2015-2016 season

Factor	N	Mean	Std Dev	Grouping
East	12	3.37	0.08	a
North	12	3.33	0.08	b
West	12	3.30	0.08	b
South	12	3.26	0.08	c

p<0.0001

Table 4.12 The effect of quadrants on the titratable acidity (TA) for the 2015-2016 season

Factor	N	Mean	Std Dev	Grouping
South	12	0.10	0.08	a
West	12	0.94	0.09	b
North	12	0.94	0.08	b
East	12	0.89	0.08	c

p=0.0008

Table 4.13 The effect of varieties on °brix for the 2015-2016 season

Factor	N	Mean	Std Dev	Grouping
Sweetheart	16	11.19	0.19	a
Star Ruby	16	9.70	0.56	b
Marsh	16	9.39	0.76	b

p=0.0030

Table 4.14 The effect of quadrants on the °brix for the 2015-2016 season

Factor	N	Mean	Std Dev	Grouping
South	12	10.22	0.87	a
North	12	10.21	0.91	a
West	12	10.10	1.01	ab
East	12	9.84	1.12	b

p=0.0338

Table 4.15 The effect of quadrants on the °brix:acid ratio for the 2015-2016 season

Factor	N	Mean	Std Dev	Grouping
East	12	11.30	1.60	a
North	12	11.10	1.40	a
West	12	10.91	1.40	ab
South	12	10.55	1.35	b

p=0.0256

4.3.2 Analysis of variance for the 2016-2017 season

The fruit variables measured in the second season showed a significant level of variation between varieties for segment mass, pH and °brix. Between quadrants, a significant level of variation was seen between fruit mass, fruit circumference, peel mass, segment mass, °brix, an also the °brix:acid ratio. Interaction between quadrants and varieties (Q× V) was significant only for °brix:acid ratio (Tables 4.16 and 4.17).

Table 4.16 Analysis of variance for fruit traits for the 2016-2017 season

	Fruit circumference (mm)		Fruit mass (g)		Peel mass (g)		Segment mass (g)		Peel thickness (mm)	
	Df	MS	Df	MS	Df	MS	Df	MS	Df	MS
Block	3	3.59	3	2945.72	3	340.05	3	2248.48	3	0.327
Variety	2	2.25	2	23127.57	2	705.63	2	29333.03**	2	0.413
Q	3	6.54**	3	8908.08**	3	623.05**	3	5592.53**	3	0.031
QxV	6	1.94	6	2844.41	6	240.89	6	1546.79	6	0.010
Error	27	1.08	27	1831.01	27	135.24	27	955.86	27	0.015

*P≤0.05, **P≤0.01, ***P≤0.001

Table 4.17 Analysis of variance for juice traits, and naringin and naringenin for the 2016-2017 season

	pH		TA		°brix		°brix:acid		Naringin		Naringenin	
	Df	MS	Df	MS	Df	MS	Df	MS	Df	MS	Df	MS
Block	3	0.015	3	0.105	3	1.14	3	6.03	3	443.04	3	0.332
Variety	2	0.165*	2	0.012	2	13.80**	2	21.63	2	530.99	2	0.231
Q	3	0.002	3	0.006	3	0.55**	3	0.96*	3	89.57	3	0.071
QxV	6	0.001	6	0.001	6	0.40**	6	0.33	6	68.68	6	0.079
Error	27	0.001	27	0.003	27	0.10	27	0.24	27	34.42	27	0.047

P≤0.05, **P≤0.01

The tables listed below are of the respective variables with a significant difference between means when analysed against the main plot factors (varieties) and subplot factors (quadrants).

Table 4.18 The effect of quadrants on fruit mass (g) for the 2016-2017 season

Factor	N	Mean	Std Dev	Grouping
West	12	394.34	81.29	a
East	12	376.71	57.01	ab
North	12	349.80	47.54	bc
South	12	333.27	37.73	c

p= 0.0078

Table 4.19 The effect of quadrants on fruit circumference (cm) for the 2016-2017 season

Factor	N	Mean	Std Dev	Grouping
West	12	29.57	1.62	a
East	12	29.19	1.32	ab
North	12	28.63	1.21	bc
South	12	27.88	1.10	c

p= 0.0027

Table 4.20 The effect of quadrants on peel mass (g) for the 2016-2017 season

Factor	N	Mean	Std Dev	Grouping
West	12	100.58	14.78	a
East	12	93.80	17.31	a
North	12	91.15	13.49	ab
South	12	83.15	17.33	b

p= 0.0099

Table 4.21 The effect of quadrants on segment mass (g) for the 2016-2017 season

Factor	N	Mean	Std Dev	Grouping
Marsh	16	288.05	49.47	a
Star Ruby	16	287.59	47.65	a
Sweetheart	16	213.66	19.45	b

p= 0.0127

Table 4.22 The effect of quadrants on segment mass (g) for the 2016-2017 season

Factor	N	Mean	Std Dev	Grouping
West	12	286.48	69.77	a
East	12	275.17	51.60	ab
North	12	251.59	43.46	bc
South	12	239.15	36.58	c

p=0.0033

Table 4.23 The effect of varieties on °brix for the 2016-2017 season

Factor	N	Mean	Std Dev	Grouping
Sweetheart	16	10.54	0.43	a
Marsh	16	9.11	0.70	b
Star Ruby	16	8.80	0.64	b

p=0.0059

Table 4.24 The effect of quadrants on °brix for the 2016-2017 season

Factor	N	Mean	Std Dev	Grouping
North	12	9.70	0.92	a
South	12	9.53	0.80	a
East	12	9.51	0.98	a
West	12	9.19	1.18	b

p=0.0038

Table 4.25 The effect of interaction of quadrants and variety on °brix for the 2016-2017 season

Factor	N	Mean	Std Dev	Grouping
West x Sweetheart	4	10.67	0.27	a
East x Sweetheart	4	10.60	0.53	a
South x Sweetheart	4	10.54	0.56	a
North x Sweetheart	4	10.35	0.40	a
North x Marsh	4	9.33	0.58	b
West x Marsh	4	9.23	0.85	bc
East x Marsh	4	9.17	0.82	bc
South x Marsh	4	9.12	0.89	bc
South x Star Ruby	4	9.01	0.31	bc
East x Star Ruby	4	8.94	0.44	bc
West x Star Ruby	4	8.82	0.57	c
North x Star Ruby	4	8.03	0.27	d

p=0.0043

4.3.3 Analysis of variance for both seasons combined

Table 4.26 Combined analysis of variance for fruit traits for both seasons

	Fruit Circumference (mm)		Fruit mass (g)		Peel mass (g)		Segment mass (g)		Peel thickness (mm)	
	Df	MS	Df	MS	Df	MS	Df	MS	Df	MS
Block	3	482.67	3	482.67	3	552.12	3	1341.76	3	0.043
Variety	2	31217.97	2	31217.97	2	4607.00*	2	51101.44*	2	1.437**
Q	3	16938.27**	3	16938.27**	3	934.86*	3	13111.20***	3	0.050*
QxV	6	5053.77	6	5053.77	6	588.47	6	2826.32*	6	0.033*
Year	1	309169.72***	1	309169.72***	1	35085.27***	1	164839.14***	1	0.054
Y×V	2	1510.06	2	1510.06	2	946.28*	2	1599.90	2	0.086
Y×Q	3	2625.29	3	2625.29	3	498.95	3	926.88	3	0.003
Y×Q×V	6	651.59	6	651.59	6	103.92	6	468.41	6	0.028
Error	36	1689.37	36	1689.37	36	259.05	36	1285.64	36	0.089

*P≤0.05, **P≤0.01, ***P≤0.001

Table 4.27 Combined analysis of variance for fruit traits and flavonoid concentration for both seasons

	pH		TA		°brix		°brix:acid		Naringin		Naringenin	
	Df	MS	Df	MS	Df	MS	Df	MS	Df	MS	Df	MS
Block	3	0.011	3	0.111	3	1.73	3	5.935	3	179.95	3	0.138
Variety	2	0.240^{**}	2	0.010	2	27.89^{**}	2	45.835	2	406.63^{*}	2	0.703
Q	3	0.016^{***}	3	0.014^{**}	3	0.56^{**}	3	1.287^{*}	3	60.56	3	0.053
QxV	6	0.002	6	0.014	6	0.32^{*}	6	0.726	6	44.17	6	0.071
Year	1	0.116^{***}	1	0.826^{***}	1	8.93^{***}	1	123.48^{***}	1	50.12	1	2.437^{***}
Y×V	2	0.007^{*}	2	0.005	2	0.81^{*}	2	1.891^{*}	2	175.38	2	0.029
Y×Q	3	0.009^{*}	3	0.010	3	0.37	3	0.896	3	36.77	3	0.042
Y×Q×V	6	0.001	6	0.002	6	0.22	6	0.516	6	36.92	6	0.027
Error	36	0.002	36	0.008	36	0.17	36	0.553	36	77.39	36	0.058

$^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$

There was a significant effect of varieties for peel mass, segment mass, peel thickness, pH, °brix and naringin concentration (Tables 4.26 and 4.27). Between quadrants a significant level of variation was seen between fruit mass, peel mass, segment mass, peel thickness, pH, TA, °brix, and also the °brix:acid ratio. The interaction between quadrants and varieties (Q× V) for segment mass, peel thickness and °brix was significant. Between years, a significant level of variation was seen between fruit mass, fruit circumference, peel mass, segment mass, pH, TA, °brix, °brix:acid ratio and naringenin concentration. Interaction between year and variety (Y×V) was significant for fruit circumference, pH, °brix and °brix:acid ratio. There was a significant interaction of year with quadrants (Y×Q) only for pH.

Table 4.28 The effect of seasons on fruit mass (g)

Factor	N	Mean	Std Dev	Grouping
2015_16	48	477.03	55.23	a
2016_17	48	363.53	61.14	b

$p < 0.0001$

Table 4.29 The effect of quadrants on fruit mass (g) for both seasons

Factor	N	Mean	Std Dev	Grouping
East	24	446.19	91.31	a
West	24	439.83	83.21	a
North	24	401.44	67.44	b
South	24	393.66	72.76	b

p= 0.0013

Table 4.30 The effect of varieties on fruit mass (g) for both season

Factor	N	Mean	Std Dev	Grouping
Star Ruby	32	438.94	82.49	a
Marsh	32	437.68	79.26	a
Sweetheart	32	384.22	71.87	b

p= 0.0411

Table 4.31 The effect of season on fruit circumference (cm)

Factor	N	Mean	Std Dev	Grouping
2015_16	48	32.57	1.10	a
2016_17	48	28.82	1.44	b

p< 0.0001

Table 4.32 The effect of season on peel mass (g)

Factor	N	Mean	Std Dev	Grouping
2015_16	48	130.41	25.38	a
2016_17	48	92.17	16.55	b

p< 0.0001

Table 4.33 The effect of season on segment mass (g)

Factor	N	Mean	Std Dev	Grouping
2015_16	48	345.97	54.73	a
2016_17	48	263.10	53.61	b

p< 0.0001

Table 4.34 The effect of quadrants on segment mass (g) for both seasons

Factor	N	Mean	Std Dev	Grouping
East	24	325.67	73.44	a
West	24	323.15	72.75	a
North	24	289.94	54.18	b
South	24	279.38	61.89	b

p< 0.0001

Table 4.35 The effect of seasons on segment mass (g)

Factor	N	Mean	Std Dev	Grouping
2015_16	48	345.97	54.73	a
2016_17	48	263.10	53.61	b

p< 0.0001

Table 4.36 The effect of varieties on segment mass (g) for both seasons

Factor	N	Mean	Std Dev	Grouping
Star Ruby	32	333.36	66.46	a
Marsh	32	321.33	57.47	a
Sweetheart	32	258.92	56.58	b

p= 0.0411

Table 4.37 The effect of varieties on peel thickness (mm) for both seasons

Factor	N	Mean	Std Dev	Grouping
Sweetheart	32	2.57	0.27	a
Marsh	32	2.37	0.21	a
Star Ruby	32	2.15	0.23	b

p= 0.0082

Table 4.38 The effect of seasons on pH

Factor	N	Mean	Std Dev	Grouping
2015_16	48	3.32	0.08	a
2016_17	48	3.25	0.11	b

p< 0.0001

Table 4.39 The effect of quadrants on pH for both seasons

Factor	N	Mean	Std Dev	Grouping
East	24	3.31	0.11	a
North	24	3.29	0.11	a
West	24	3.27	0.09	b
South	24	3.25	0.09	c

p< 0.0001

Table 4.40 The effect of varieties on pH for both seasons

Factor	N	Mean	Std Dev	Grouping
Sweetheart	32	3.38	0.09	a
Marsh	32	3.26	0.05	b
Star Ruby	32	3.21	0.07	b

p=0.0094

Table 4.41 The effect of seasons on titratable acidity (TA)

Factor	N	Mean	Std Dev	Grouping
2016_17	48	1.13	0.14	a
2015_16	48	0.94	0.09	b

p< 0.0001

Table 4.42 The effect of quadrants on on titratable acidity (TA) for both seasons

Factor	N	Mean	Std Dev	Grouping
South	24	1.05	0.13	a
West	24	1.04	0.16	a
North	24	1.04	0.16	a
East	24	0.10	0.15	b

p= 0.0091

Table 4.43 The effect of seasons on °brix

Factor	N	Mean	Std Dev	Grouping
2015_16	48	10.10	0.96	a
2016_17	48	9.48	0.96	b

p< 0.0001

Table 4.44 The effect of varieties on °brix for both seasons

Factor	N	Mean	Std Dev	Grouping
Sweetheart	32	10.87	0.46	a
Marsh	32	9.25	0.73	b
Star Ruby	32	9.25	0.75	b

p=0.0024

Table 4.45 The effect of seasons on °brix:acid ratio

Factor	N	Mean	Std Dev	Grouping
2015_16	48	10.97	1.42	a
2016_17	48	8.70	1.60	b

p< 0.0001

Table 4.46 The effect of varieties on naringin concentration (mg 100ml-1) for both seasons

Factor	N	Mean	Std Dev	Grouping
StarRuby	32	39.99	8.53	a
Marsh	32	32.93	6.68	b
Sweetheart	32	35.59	8.34	ab

P=0.0265

Table 4.47 The effect of season on naringin concentration (mg 100ml-1)

Factor	N	Mean	Std Dev	Grouping
2015_16	48	1.22	0.26	a
2016_17	48	0.90	0.31	b

p< 0.0001

Fruit mass

Significant differences in fruit mass were observed between the quadrants within the tree canopies (Table 4.3 and 4.17). In the first season the eastern quadrant had the highest mean fruit mass (515.67 ± 60.43 g). The fruit harvested in the south and west quadrants were significantly lower in mean fruit mass, with the western quadrant being the lowest

(485.32 ± 59.91 g). In the second season the western quadrant had the highest mean fruit mass (394.34 ± 81.30 g) and the eastern quadrant’s (376.71 ± 57.01 g) mean fruit mass was not significantly different from the western quadrant. The fruit harvested in the north and south quadrants were significantly lower in the second season (p= 0.0013) with regard to mean fruit mass, with the southern quadrant the lowest.

Table 4.28 illustrates the significant seasonal effect on mean fruit mass (p<0.0001). The 2015-16 seasons’ average fruit mass was 477.03 ± 55.23 g and the 2016-17 season average fruit mass was 113.5 g lower (363.53 ± 61.14 g).

Table 4.29 supports the results of Tables 4.1 and 4.17 with a significant difference (p= 0.0013) in mean fruit mass between quadrants. Over seasons, the east and west quadrants produced fruit with a significantly higher fruit mass than the north and south quadrants with east the highest (446.19 ± 91.31 g) and south the lowest (393.66 ± 72.75 g). Significant differences (p= 0.0411) between varieties for mean fruit mass were seen over the two seasons (Table 4.30), with Star Ruby and Marsh both significantly higher than Sweetheart. Star Ruby had the highest fruit mass value (438.94 ± 82.49 g) and Sweetheart had the lowest (384.22 ± 71.87 g).

Fruit circumference

No statistically significant differences in the average fruit circumferences between varieties or quadrants were noted in the first season. In the second season there was a significant difference in mean fruit circumference between quadrants within tree canopies. The fruit from the western quadrant had the largest circumference (29.57 ± 1.62 cm), and the southern quadrant fruit had the smallest (27.89 ± 1.10 cm) (Table 4.18). The main effect of season on fruit circumference was highly significant (p< 0.0001) (Table 4.31). The fruit from the first season had an average circumference of 32.57 ± 1.10 cm and that of season two 28.82 ± 1.44 cm.

Peel mass

In the first season the main effect of varieties on average peel mass was significant (Table 4.2). Sweetheart had the highest peel mass (149.45 ± 26.47 g) and Star Ruby the lowest (115.52 ± 18.58 g). Marsh was not significantly different from either (126.16 ± 18.05 g). In the second season (Table 4.19), quadrants had a significant effect on peel mass. Fruit from the western quadrant had the highest average peel mass (100.58 ± 14.78 g), and the fruit from the southern quadrant the lowest (83.15 ± 17.33 g). The main effect of season was highly significant (Table 4.32), with the first season having the highest average peel mass value (130.41 ± 25.38 g) and the second season the lowest (92.17 ± 16.55 g).

Segment mass

In the first season segment mass was significantly affected by variety (Table 4.5) and quadrant (Table 4.6). The interaction between variety and quadrant was also significant in this season (Table 4.7). The average segment mass of Star Ruby (379.13 ± 48.86 g) was significantly higher than that of Sweetheart (304.18 ± 43.22 g). The segment mass of Marsh was not significantly different from either Star Ruby or Sweetheart (354.60 ± 44.89 g). The effect of tree canopy quadrant was highly significant ($p<0.0001$). The east and west quadrants had a higher average segment mass than that of north and south, with east the highest (376.17 ± 55.23 g) and south the lowest (319.61 ± 56.02 g) (Table 4.6).The interaction between quadrants and varieties (Table 4.7) complements the findings of Tables 4.5 and 4.6. East × Star Ruby (422.75 ± 46.25 g) had the highest segment mass that was significantly higher than east × Sweetheart (322.17 ± 38.98 g) and with the smallest segment mass south × Sweetheart (277.39 ± 65.74 g) (Table 4.7). The segment mass values of the Marsh variety were not significantly different from either Star Ruby of Sweetheart, thus supporting Table 4.6.

In the second season the segment mass was once again affected by variety (Table 4.20) and quadrant (Table 4.21). The average segment mass of Marsh (288.05 ± 49.47 g), and Star Ruby (287.59 ± 47.65 g), was significantly higher than that of Sweetheart (213.66 ± 19.45 g). The main effect of quadrants was more significant than that of varieties ($p=0.0033$), just as in the first season. West and east had a higher segment mass than north and south. West had the highest average segment mass (286.48 ± 69.78 g). South had the lowest (239.15 ± 36.58 g) segment mass, just as in the first season.

Both seasons demonstrated definite differences in mean fruit segment mass with the first season having the highest mean mass of 345.97 ± 54.73 g and the second season 1.3 times lower at 263.10 ± 53.61 g (Table 4.34). The main effect of quadrants was highly significant for both seasons ($p<0.0001$). The overall segment mass of east and west was higher than that of north and south (Table 4.35). Segment mass differences were also significant over the two seasons with Star Ruby the highest (333.36 ± 66.46 g) followed by Marsh, which were both significantly different from that of Sweetheart (258.92 ± 56.58 g).

Peel thickness

In the first season the main effect of varieties on peel thickness was significant (p=0.0029). Sweetheart had the thickest peel (2.60 ± 0.21 mm) followed by Marsh (2.34 ± 0.19 mm) and Star Ruby (2.08 ± 0.15 mm) (Table 4.8). As for the interaction between quadrants and varieties (Table 4.10), a clear difference was observed between the groupings of west × Sweetheart, north × Marsh and south × Star Ruby, but not so much for the grouping of quadrants. No significant mean differences in peel thickness, between varieties or quadrants were seen for the second season.

Over the two seasons, the difference in peel thickness between quadrants, was significant (p=0.0082) (Table 4.36). Sweetheart had the thickest peel (2.57 ± 0.27 mm) followed by Marsh (2.37 ± 0.21 mm), and Star Ruby (2.15 ± 0.23 mm).

pH

The effect of variety and quadrants were highly significant (p=0.0099, p<0.0001) on the fruit juice pH value (Tables 4.11 and 4.12). For season one Sweetheart had the highest pH value (3.40 ± 0.07), followed by Marsh and Star Ruby (3.30 ± 0.05 and 3.26 ± 0.07 respectively). With regards to the quadrants, the fruit harvested from the east quadrant had a higher pH value (3.37± 0.08) than those harvested in the north and west quadrants. The south quadrant fruit had the lowest pH value (3.26 ± 0.08).

No significant mean differences in pH value between varieties or quadrants were noted for the second season.

Season had a significant effect on the fruit pH value (p<0.0001). The first season was significantly higher in average pH (3.31 ± 0.08) than the second season (3.25 ± 0.11) (Table 4.37). Indicated in Table 4.36, the fruit location within tree canopy also had a significant effect on average fruit pH. East and north quadrant fruit were grouped together, east had the highest pH value (3.30 ± 0.12). West and south quadrant fruit were grouped together with south having the lowest pH value (3.25 ± 0.09). Varieties had a slightly less significant effect (p=0.0094) on the overall pH value (Table 38). Sweetheart had the highest pH (3.38 ± 0.09), and grouping together was Marsh and Star Ruby, with Star Ruby having the lowest value (3.20± 0.07).

Titratable acidity

There was a significant difference between the average TA value of fruit from different tree canopy quadrants (Table 4.13). South had the highest TA value (0.99 ± 0.08). West and north grouped together. East had the lowest TA value (0.90 ± 0.08).

No significant mean differences in TA value, between varieties or quadrants were noted for the second season.

The second season had the highest TA value (1.13 ± 0.14) and the first season had a lower value (0.94 ± 0.08) (Table 4.40). No significant differences were seen between varieties over the two seasons, but there was a significant difference between quadrants (Table 4.41). South, west and north were not significantly different, although south had the highest TA value (1.05 ± 0.13). The mean TA from the eastern quadrant was significantly lower than the rest (0.10 ± 0.15).

°Brix

In the first season there was a difference between mean total soluble solids value (measured in °brix) of the grapefruit varieties. Sweetheart's average °brix was significantly higher (11.19 ± 0.19) than that of Star Ruby (9.70 ± 0.56) and Marsh (9.39 ± 0.76) (Table 4.14).

For the second season, mean °brix of Sweetheart was significantly higher (10.54 ± 0.43) than that of the other varieties (Table 4.22). The °brix value in season two was significantly different between quadrants (Table 4.23). Fruit harvested from the north (9.70 ± 0.92) south (9.53 ± 0.80) and east (9.51 ± 0.98) quadrants, were significantly higher in °brix than those harvested from the west quadrant (9.20 ± 1.18). In Table 4.23, all four quadrants of the Sweetheart variety had significantly higher °brix content than the other varieties and their respected quadrants. Within the quadrants of Sweetheart there was no significant difference in mean °brix content. Star Ruby and Marsh had similar °brix content throughout the respective quadrants, except for the western quadrant of Star Ruby, which was significantly lower in °brix than all the other specific quadrants of varieties (8.03 ± 0.27).

°Brix: acid ratio

No significant mean differences in °brix:acid ratio values were observed in the fruit for the two seasons separately, either between varieties or quadrants. Over both seasons there were also no significant mean differences in °brix:acid ratio between varieties or quadrants. The main effect of season was highly significant (Table 4.44). The first season had a °brix:acid ratio 1.26 times higher than the second season (10.96 ± 1.42:1) vs. (8.69 ± 1.60:1).

Flavonoid content (naringin and naringenin)

There were no significant mean differences in naringin and naringenin concentration values in fruit, between varieties or quadrants, for the two seasons separately but over the two seasons there was a significant mean difference in naringin concentrations between varieties (Table 4.45). Star Ruby was the variety with the overall highest naringin concentration (39.99 ± 8.53 mg 100 ml^{-1}) followed by Sweetheart (35.57 ± 8.34 mg 100 ml^{-1}). Marsh overall had a significantly lower naringin concentration than Star Ruby (32.93 ± 6.68 mg 100 ml^{-1}). Naringenin on the other hand, had a 1.3 times higher mean concentration in season one (1.22 ± 0.26 mg 100 ml^{-1}) than in season two (0.90 mg ml^{-1} ± 0.31 mg 100 ml^{-1}) (Table 4.46).

4.4 DISCUSSION

Citrus fruit are of tropical origin and therefore, more than most fruits, citrus fruit quality varies significantly with climate (Sinclair, 1961; Barry, 2000; Bijzet, 2014). In citrus, differences in fruit quality, both chemical composition and physical traits, are related to the position of the fruit on the tree. Studies done on fruit quality with regard to canopy position found that fruit mass, juice content and peel thickness of fruit in the internal part of the tree canopy were significantly higher when compared with external canopy fruit (Fallahi and Moon, 1989). In this study, fruit varied in physical traits such as fruit mass, circumference, peel mass and segment mass between tree canopy positions. For the first season, the eastern quadrant had the highest fruit and segment mass. For the second season the western quadrant had the largest fruit overall, yet not significantly larger than north or east. Overall the eastern quadrant yielded fruit that was larger in size. As for the chemical composition, Barry (2000) reported that warmer canopy positions yielded fruit higher in soluble solids. Cohen (1988) reported that citrus fruit from the warmer exterior or upper part of the tree were more mature and tastier than fruit from cooler, interior or lower parts of the tree. In the southern hemisphere the north western side of the tree canopy is the warmest. Exposed fruit in the northern canopy position was reported to have higher soluble solids and soluble solid to acid ratios (Freeman and Robbertse, 2003). For the first season of this study, the eastern quadrant had the highest pH value and °brix:acid ratio. Fruit from the west and north quadrants did not show significant differences in pH value or °brix:acid ratio. For the second season the northern quadrant fruit had the highest °brix content. Overall the northern quadrant fruit were highest in °brix content, and also had a high °brix:acid ratio. No significant differences were noted between tree canopy position and flavonoid content.

Citrus species and varieties have different genetic traits, which mainly influence the micro constituents and consequently the phenolic and flavonoid content and composition thereof (Hunlun, 2016). In the current study, differences were noted in the overall fruit size, as well as chemical composition of fruit. Overall, Marsh and Star Ruby yielded larger fruit. The Sweetheart variety yielded fruit with characteristically higher °brix content and pH value. In a study done by Peterson et al*.* (2008) it was concluded that white grapefruit varieties tend to be slightly, but not significantly, higher in total flavanone content (27 mg 100 ml^{-1}) compared to the pink and red varieties (18 mg 100 ml^{-1}). In the current study the red variety, Star Ruby, had a slightly higher naringin content than Marsh (white variety) and Sweetheart (pink variety). This could be ascribed to fruit maturity. As the fruit ripens and matures, the levels of naringin in fruit juice lowers, mainly by means of dilution, and to a lesser extent, by means of enzymatic anabolism (Jourdan et al., 1985; Puri et al., 1996).

Climatic conditions can vary from season to season, causing high variability in chemical composition and physical traits of citrus fruit cultivated in the same region over different seasons. In this study, differences were noted in the overall fruit size, as well as chemical composition of fruit over seasons. Fruit from the second season tended to be smaller, with a lower pH value, °brix content, °brix:acid ratio as well as a lower naringenin concentration. Citrus fruit quality is strongly influenced by water stress. The effect of water stress is dependent on the duration and phenological stage of the fruit in which the stress occurs. Fruit physical traits, such as peel thickness, fruit size and set, and juice yield are all affected by water stress (Levy et al., 1979). Stress during the third stage of development in citrus fruit growth, increased peel thickness, soluble solid content and acid content while, during the first and second growth period, it decreased these parameters (Cruse et al., 1982; Ginestar and Castle, 1996). From the seasonal decrease in fruit size, soluble solid content, °brix:acid ratio as well as a lower naringenin content, it is suggested that this could be an effect of the drought experienced in 2016, when the fruit were in the first and second stage growth period.

4.5 CONCLUSIONS

In a breeding programme where fruit are sampled for the screening of physical and chemical traits, factors that could have an impact on the selection of sample fruit need to be taken into account. From this study it was shown that there are some significant differences in traits between grapefruit varieties as well as fruit canopy position and climate. Genetic and environmental factors can interact (Kuriyama et al., 1981). Thus, from the differences noted in this study it is proposed that further research be devoted to determine the possible interactions and associations between the physical traits and chemical content, as well as the relationships thereof between the grapefruit varieties, fruit canopy position and seasons.

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Chapter 5

THE RELATIONSHIP BETWEEN FLAVONOID CONTENT AND FRUIT TRAITS OF THREE SELECTED GRAPEFRUIT VARIETIES

5.1 INTRODUCTION

For any breeding programme there are specific breeding goals, which are usually industry related. In the past, the goals were mainly focused on improved crop yield, pest and disease resistance, in order to keep pace with the rising global demand. With research done on health benefits of fruit in the human diet and the global concern for nutritional security, the nutritional value of fruit has become an important focus point in some breeding programmes (Patil et al., 2014).

Citrus is an abundantly grown fruit crop and during 2016/17, 99.62% was produced in 15 countries on different continents (USDA-FAS, 2017). It also has a relatively high phenolic content compared to other edible plants, and is thus an important source of bioactive compounds. Biomedical research has indicated that the consumption of citrus bioactive compounds, which include flavonoids, carotenoids and ascorbic acid, are linked with lower risk of many diseases such as cancer and cardiovascular disease due to its antioxidant capacity (Cerda et al., 1994; So et al., 1996; Guthrie and Carroll, 1998; Owira et al., 2009). Research done on the consumption of grapefruit, suggested that bioactive compounds in grapefruit may be beneficial to patients with Type 2 Diabetes Mellitus and other degenerative diseases (Muraki et al., 2013).

Breeding efforts in the citrus industry has delivered a variety of citrus species, each producing fruit known for their distinct colour, flavour, and nutritional content (Schaffer and Anderson, 1994). These specific fruit quality traits are mainly determined by the amounts and relationship between the organic components within the fruit (Sinclair, 1961).

The organic constituents of citrus fruit are influenced by many factors, these include genetic factors such as variety differences and stages of maturity, as well as environmental factors such as climate, soil conditions and cultural practices (Sinclair, 1961; Chen, 1990; Hunlun, 2016). In a study done in the Northern hemsiphere by Hemmati et al. (2014), there were significant differences in the flavonoid content of mandarin, orange, orange and sour lemon fruit relative to each other and to the geographical canopy position. Hemmati et al. (2014) reported that the highest amount of naringin tended to be in lemons produced in the northern canopy position of the tree. Water stress is a significant environmental factor to consider. Depending on the duration and phenological stage of the fruit, water stress can affect the physical quality traits of a fruit such as peel thickness, fruit size and set, and juice yield (Levy et al., 1979).

All these factors, genetic and environmental, have some form of interaction. This makes analysis of fruit quality complicated (Kuriyama et al., 1981). The aim of this chapter was to investigate the interactions and associations between the physical traits and chemical composition, as well as the relationships thereof, between the grapefruit varieties and fruit location within the tree canopy and seasons.

5.2 MATERIALS AND METHODS

Sampling

Fruit samples from three different grapefruit varieties (Marsh, Sweetheart and Star Ruby) were harvested over two seasons (2015-2016 and 2016-2017), as discussed in Chapter 3.

Fruit traits

Each individual fruit’s traits were measured and recorded as described in Chapter 4.

Flavonoid content

The naringin and naringenin concentration in each fruit was determined by the optimised HPLC method discussed in Chapter 3.

Statistical procedure

The trials were conducted in 2015/16 and 2016/17. The experimental design was randomised complete (all factors in all blocks) block design with four replications. For each year, the data were analysed as a split plot design with cultivar as main plot factor and quadrant as subplot factor. Pearson Product Moment Correlations were performed using Correlation Procedure (PROC CORR) of SAS software (Version 9.2; SAS Institute Inc, Cary, USA). Principal component analysis was conducted for each year separately and years combined to investigate the relationship between the factors (years, varieties and quadrants) and variables, using XLSTAT (Version 2015.1.03.15485, Addinsoft, Paris).

5.3 RESULTS

5.3.1 Pearson product moment correlation coefficients

The correlation coefficients were calculated for all the variables over all three varieties for the 2015-16 season (Table 5.1), the 2016-17 season (Table 5.2) and for all the variables over all three varieties over both seasons (Table 5.3).

Significant positive correlations were displayed for 2015-16 (Table 5.1) between segment mass and fruit mass ($r=0.82$, $p<0.0001$), pH and °brix: acid ratio ($r=0.83$, $p<0.0001$), naringin and naringenin ($r= 0.74$, $p<0.0001$) and between °brix and °brix: acid ratio ($r=0.82$ $p<0.0001$). Negative correlations were displayed between pH and titratable acidity ($r=-0.63$, $p<0.0001$) and between segment mass and °brix (-0.67, $p<0.0001$).

As seen in Table 5.2 positive correlations for the second season (2016-17) were displayed between fruit mass and fruit circumference ($r = 0.90$, $p<0.0001$), fruit mass and segment mass ($r=0.97$, $p<0.0001$), fruit circumference and peel mass ($r = 0.76$, $p<0.0001$), fruit circumference and segment mass ($r = 0.79$, $p<0.0001$), pH and °brix: acid ratio ($r = 0.89$, $p<0.0001$).

Table 5.1 Pearson correlation coefficients between variables for the 2015-2016 season (n=48)

Variables	FM	FC	PM	SM	PT	pH	TA	°brix	BA ratio	NI	NE
FM	1.00	0.11	0.24	**0.82**	-0.09	-0.06	-0.25	-0.55	-0.24	0.01	-0.17
FC	0.11	1.00	0.02	0.11	-0.04	0.12	-0.30	-0.08	0.19	-0.03	-0.12
PM	0.24	0.02	1.00	-0.17	0.46	0.40	-0.08	0.32	0.31	-0.02	-0.20
SM	**0.82**	0.11	-0.17	1.00	-0.29	-0.15	-0.33	**-0.67**	-0.28	0.12	-0.03
PT	-0.09	-0.04	0.46	-0.29	1.00	0.54	-0.01	0.50	0.43	0.05	-0.23
pH	-0.06	0.12	0.40	-0.15	0.54	1.00	**-0.63**	0.43	**0.83**	0.05	-0.14
TA	-0.25	-0.30	-0.08	-0.33	-0.01	**-0.63**	1.00	0.22	-0.59	-0.08	0.11
°brix	-0.55	-0.08	0.32	**-0.67**	0.50	0.43	0.22	1.00	**0.65**	0.12	0.13
BA ratio	-0.24	0.19	0.31	-0.28	0.43	**0.83**	-0.59	**0.65**	1.00	0.16	0.01
NI	0.01	-0.03	-0.02	0.12	0.05	0.05	-0.08	0.12	0.16	1.00	**0.74**
NE	-0.17	-0.12	-0.20	-0.03	-0.23	-0.14	0.11	0.13	0.01	**0.74**	1.00

Values in bold have a correlation value of $r > 0.60$ and are significant at $p \leq 0.0001$

FM = fruit mass, FC = fruit circumference, PM = peel mass,SM = segment mass, PT = peel thickness, TA = titratable acidity, BA ratio = °brix: acid ratio, NI = naringin, NE = naringenin

Table 5.2 Pearson correlation coefficients between variables for the 2016-2017 season (n=48)

Variables	FM	FC	PM	SM	PT	pH	TA	°brix	BA ratio	NI	NE
FM	1.00	**0.90**	0.52	**0.97**	-0.09	-0.33	-0.03	-0.51	-0.35	-0.05	-0.19
FC	**0.90**	1.00	**0.76**	**0.79**	0.06	-0.20	0.13	-0.29	-0.33	-0.12	-0.16
PM	0.52	**0.76**	1.00	0.32	0.44	0.16	0.23	0.13	-0.10	0.02	0.08
SM	**0.97**	**0.79**	0.32	1.00	-0.19	-0.40	-0.09	**-0.60**	-0.37	-0.05	-0.23
PT	-0.09	0.06	0.44	-0.19	1.00	0.14	0.38	0.28	-0.10	0.32	0.33
pH	-0.33	-0.20	0.16	-0.40	0.14	1.00	-0.54	0.59	**0.89**	-0.41	-0.29
TA	-0.03	0.13	0.23	-0.09	0.38	-0.54	1.00	0.14	**-0.74**	0.38	0.49
°brix	-0.51	-0.29	0.13	**-0.60**	0.28	0.59	0.14	1.00	0.54	-0.15	0.11
BA ratio	-0.35	-0.33	-0.10	-0.37	-0.10	**0.89**	**-0.74**	0.54	1.00	-0.39	-0.32
NI	-0.05	-0.12	0.02	-0.05	0.32	-0.41	0.38	-0.15	-0.39	1.00	**0.85**
NE	-0.19	-0.16	0.08	-0.23	0.33	-0.29	0.49	0.11	-0.32	**0.85**	1.00

Values in bold have a correlation value of $r > 0.60$ and are significant at $p \leq 0.0001$

FM = fruit mass, FC = fruit circumference, PM = peel mass,SM = segment mass, PT = peel thickness, TA = titratable acidity, BA ratio = °brix: acid ratio, NI = naringin, NE = naringenin

Table 5.3 Overall Pearson correlation coefficients for 2015-2016 and 2016-2017 seasons (n=96)

Variables	**FM**	**FC**	**PM**	**SM**	**PT**	**pH**	**TA**	**°brix**	**BA ratio**	**NI**	**NE**
FM	1.00	**0.81**	**0.65**	**0.93**	-0.12	0.10	-0.51	-0.14	0.25	0.04	0.23
FC	**0.81**	1.00	**0.70**	**0.72**	-0.06	0.25	-0.53	0.15	0.45	0.02	0.33
PM	**0.65**	**0.70**	1.00	0.42	0.27	0.43	-0.38	0.38	0.48	0.06	0.28
SM	**0.93**	**0.72**	0.42	1.00	-0.24	0.00	-0.50	-0.29	0.16	0.06	0.20
PT	-0.12	-0.06	0.27	-0.24	1.00	0.26	0.23	0.34	0.06	0.22	0.03
pH	0.10	0.25	0.43	0.00	0.26	1.00	**-0.63**	0.57	**0.86**	-0.22	-0.02
TA	-0.51	-0.53	-0.38	-0.50	0.23	**-0.63**	1.00	-0.07	**-0.80**	0.14	-0.07
°brix	-0.14	0.15	0.38	-0.29	0.34	0.57	-0.07	1.00	**0.64**	0.64	0.25
BA ratio	0.25	0.45	0.48	0.48	0.16	**0.86**	**-0.80**	**0.64**	1.00	-0.11	0.17
NI	0.04	0.02	0.06	0.06	0.22	-0.22	0.14	-0.02	-0.11	1.00	**0.74**
NE	0.23	0.33	0.28	0.20	0.03	-0.02	-0.07	0.25	0.17	**0.74**	1.00

Values in bold have a correlation value of $r > 0.60$ and are significant at $p \leq 0.0001$

FM = fruit mass, FC = fruit circumference, PM = peel mass,SM = segment mass, PT = peel thickness, TA = titratable acidity, BA ratio = °brix: acid ratio, NI = naringin, NE = naringenin

As in the first season, strong correlation was again evident between naringin and naringenin (r= 0.85, p<0.0001). Strong negative correlations were displayed between segment mass and °brix (r=-0.60, p<0.0001) and between titratable acidity and °brix: acid ratio (-0.74, p<0.0001).

Table 5.3 represents the correlation coefficients of all the variables over all three varieties over both seasons (2015-16 and 2016-17) combined. Strong positive correlations were displayed between fruit mass and fruit circumference (r= 0.81, p<0.0001), fruit mass and peel mass (r= 0.65, p<0.0001), fruit mass and segment mass (r= 0.93, p<0.0001), fruit circumference and peel mass (r= 0.696, p<0.0001), fruit circumference and segment mass (r= 0.72, p<0.0001), pH and °brix: acid ratio (r= 0.86, p<0.0001) as well as between °brix and °brix: acid ratio (r= 0.635, p<0.0001). The flavonoids naringin and naringenin (r= 0.74, p<0.0001) also again displayed a significant correlation. Negative correlations were displayed between pH and titratable acidity (r=-0.63, p<0.0001) and between titratable acidity and °brix: acid ratio (-0.80, p<0.0001).

5.3.2 Principal component analysis

Associations between the traits of the grapefruit varieties, fruit location within tree canopy, seasons and fruit traits were evaluated using PCA.

The number of the principal components versus its corresponding eigenvalues (obtained from the correlation matrix) for the 2015-16 season is displayed in a scree plot (Figure 5.1) and was used to select the number of components to use. Choosing only eigenvalues larger than one led to three principal components, accounting for 83.12% of the total variability. As seen from Figure 5.1 neither the cumulative variability of F1 and F2 (64%) nor F1 and F3 (62%) complied with the minimum percentage of variation (70%) explained. Both biplots are therefore displayed in Figure 5.2 and discussed, taking the squared cosines values (Table 5.4) into account.

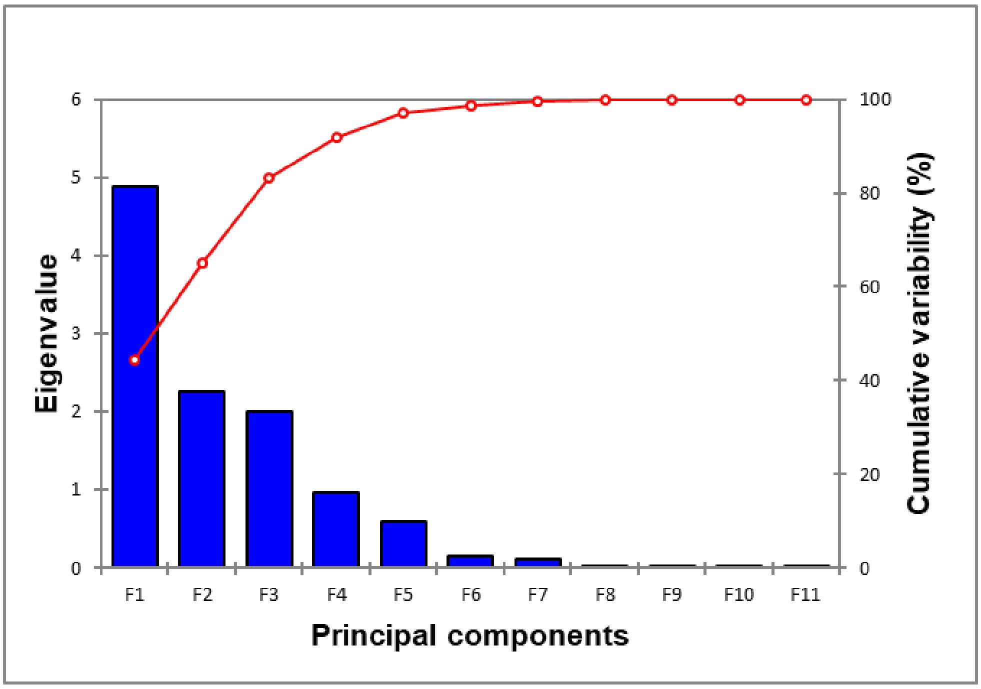

Figure 5.1 Scree plot of the 2015-16 season's principal components versus its corresponding eigenvalues

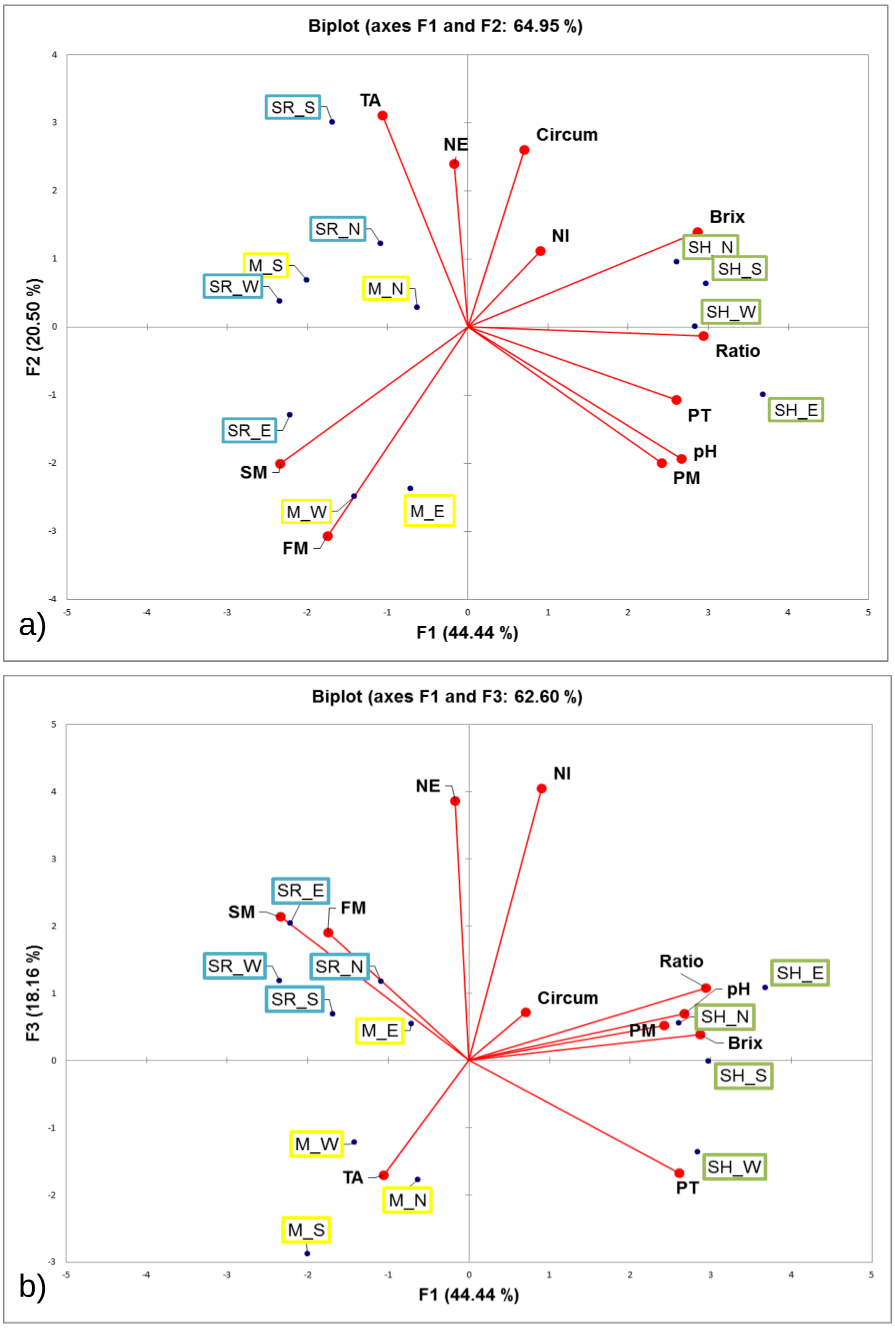

Figure 5.2 PCA biplot of associations between variety and quadrants based on fruit traits and flavonoid concentration for 2015-2016 with a) axes F1 and F2 and b) axes F1 and F3

FM = fruit mass, Circum = fruit circumference, PM = peel mass, PT = peel thickness, SM = segment mass, TA = titratable acidity, Ratio = °brix: acid ratio, NI = naringin, NE = naringenin, SH= Sweetheart, M= Marsh, SR = Star Ruby, W= west, E = east, N = north, S=south

Table 5.4 Squared cosines of the variables showing the first three principal components (F1, F2, F3) for the 2015-16 season

Variables	F1	F2	F3
Fruit mass	0.314	**0.445**	0.151
Circumference	0.051	0.320	0.021
Peel mass	**0.601**	0.189	0.011
Segment mass	**0.561**	0.190	0.191
Peel thickness	**0.696**	0.055	0.118
pH	**0.731**	0.177	0.020
Titratable acidity	0.116	**0.455**	0.123
°brix	**0.844**	0.092	0.006
°brix: acid ratio	**0.888**	0.001	0.049
Naringin concentration	0.084	0.059	**0.685**
Naringenin concentration	0.003	0.271	**0.623**

Values in bold correspond to the factor for which the squared cosine is the largest

The PCA biplots in Figures 5.2a and 5.2b depict the distribution of the three varieties sampled in the first season (2015-2016) from the different fruiting quadrants within the tree canopies, based on fruit traits and flavonoid concentration. Figure 5.2a explained 64.95% of the variation in the biplot considering the first two principal components. Figure 5.2b explained 62.60% of the inherent variability in the data of the biplot considering the first and third principal components. In both figures the Sweetheart variety showed a clear cluster opposing the other two varieties along the horizontal axis (F1), explaining approximately 44% of the variation.

In Figure 5.2a, the Sweetheart variety was closely associated with °brix and Ratio. The Star Ruby and Marsh varieties were more spread along the F2 axis. SM and FM had a strong association and in Table 5.1 this was supported with a significant positive correlation ($r=0.816$, $p<0.0001$). SM and °brix plotted opposite each other, thus they were strongly negatively correlated ($r= -0.673$, $p<0.0001$). Marsh fruit collected from the western (M_W) and eastern quadrants (M_E) were more closely associated with FM, and plotted opposite to Sweetheart collected from the southern side (SH_S) of the tree, thus M_W had the highest fruit mass (522.13 ± 12.08 g) and SH_S the lowest (434.53 ± 7.95 g). SH_S had the highest °brix content (11.26 ± 0.06) and plotted opposite Star Ruby east (SR_E) with the lowest °brix content (9.16 ± 0.14).

In Figure 5.2b, the relationship between the flavonoids and other variables was better depicted with the first and third principal components. The reason is that for both naringin and naringenin the third principal component had the largest squared cosine (Table 5.4). The first and third principal components, explained approximately 44% and 18% of the variation, respectively. From this figure it is seen that between naringin and naringenin there was a strong association, and this is supported by Table 5.2 with a significant positive correlation ($r = 0.74$, $p < 0.0001$). Furthermore, neither of the two flavonoids were associated with any of the other variables. As for the grapefruit varieties, all were grouped closely along the F3 axis, except for Marsh south (M_S), which was significantly lower in both naringenin and naringin content (32.49 ± 1.29 mg ml^{-1} and 0.95 ± 0.05 mg ml^{-1}) .

The PCA biplot in Figure 5.3 depicts the distribution of the three varieties sampled in the second season (2016-2017) from the different fruiting quadrants within the tree canopies based on fruit traits and flavonoid concentration. The cumulative variability of F1 and F2 exceeds the minimum explained variation (70%) required, as it explains 80.53% of the inherent variability in the data.

Just as in the previous season, the Sweetheart variety clearly grouped opposite to the other two varieties along the horizontal axis (F1) which explains 49.70% of the variation.

In Figure 5.3 the Sweetheart variety was closely associated with °brix, Ratio and pH, while the Star Ruby and Marsh varieties were more wide spread along the F2 axis. From Table 5.3 it is seen that SM and FM had a strong positive correlation ($r = 0.97$, $p < 0.0001$). In the biplot (Figure 5.3) SM and FM were also closely associated. SM and °brix were plotted in opposite quadrants, indicating a negative interaction, which is supported by Table 5.2 ($r = -0.601$, $p < 0.0001$).

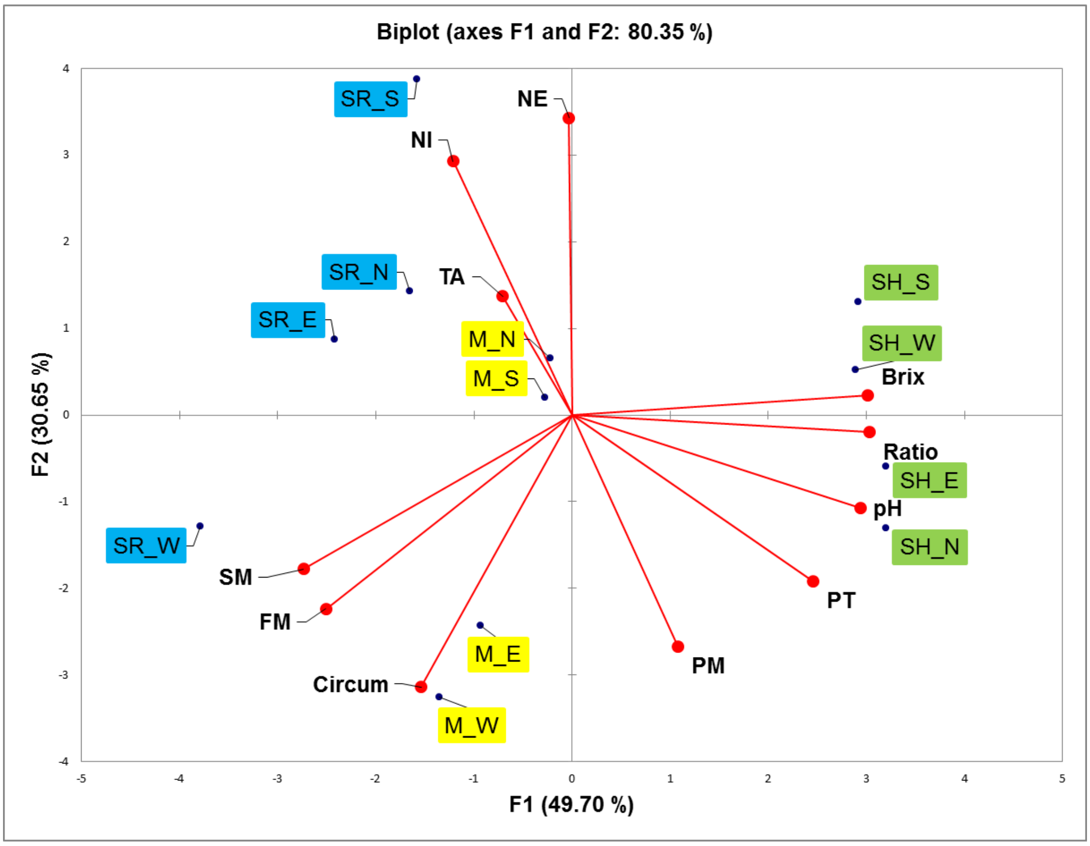

Figure 5.3 PCA biplot of associations between variety and quadrants based on fruit traits and flavonoid concentration for the season 2016-2017
FM = fruit mass, Circum = fruit circumference, PM = peel mass, PT = peel thickness, SM = segment mass, TA = titratable acidity, Ratio = °brix: acid ratio, NI = naringin, NE = naringenin, F= Sweetheart, M= Marsh, SR = Star Ruby, W= west, E = east, N = north, S=south

SR_W, M_W and M_E were more closely associated with FM, SM and Circum, and plotted opposite F_S. Thus, SR_W had the largest fruit mass (436.47 ± 18.09 g), followed by M_W (434.00 ± 21.50 g). SH_S had the smallest fruit mass (309.71 ± 14.34 g). SH_N had the highest °brix content (10.67 ± 0.17) and plotted opposite Star Ruby west (SR_W) with the lowest °brix content (8.03 ± 0.91). Just as in the previous season, naringin and naringenin were strongly positively correlated (r =0.849, p<0.0001). Furthermore neither of the flavonoids were associated with any of the other variables. As for the grapefruit varieties, all three were widely spread along the F2 axis. Star Ruby south (SR_S) had the closest association with both the flavonoids, and the highest content naringin (47.06 ± 0.11 mg ml^{-1}) and naringenin (1.18 ± 0.53 mg ml^{-1}) of all the other varieties and quadrants within. Sweetheart north (F_N) plotted opposite naringin and had the lowest naringin content (26.56 ± 2.15 mg ml^{-1}).

The PCA biplot in Figure 5.4, depicts the distribution of the three varieties sampled over both seasons (2015-2016 and 2016-2017) from the different fruiting quadrants within the tree canopies based on fruit traits and flavonoid concentration and explained 78% of the inherent variability in the data. There was a clear cluster correspondence between the two seasons along the horizontal axis (F1), which explains approximately 49% of the variation. Over both seasons the Sweetheart variety showed a clear cluster corresponding to the other two varieties along the vertical axis (F2), which explained approximately 29% of the variation. The second season was clearly associated with a higher titratable acid (TA) content and a lower fruit mass (FM), segment mass (SM), fruit circumference (FC), peel mass (PM), and °brix:acid ratio (Ratio) , pH, °brix and naringin (NI) and naringenin (NE) content. In Table 5.3 significant positive correlation was seen between FM and SM (r=0.933, p<0.0001), FM and FC (r=0.809, p<0.0001), and °brix and pH (r= 0.855, p<0.0001). This was supported in Figure 5.3 with the associations seen between these variables.

A strong negative correlation was seen between TA and pH in Table 5.3 (r = -0.631, p<0.0001) and between TA and Ratio (r=-0.799, p<0.0001) and is also evident in Figure 5.3 with TA opposite to pH and Ratio. In agreement with both Figures 5.1 and 5.2, naringin and naringenin were positively correlated (r =0.738, p<0.0001), but there were no other significant associations with any of the other variables. Peel thickness (PT) did not show any significant association with any variables. With regard to the grapefruit varieties, for both seasons Sweetheart clustered in the top half of the biplot in association with higher PT, °brix, pH Ratio and PM. The Marsh variety plotted around the F1 axis in the middle of the biplot for both seasons. Star Ruby plotted in the bottom half of the biplot for both seasons, and was strongly associated with higher SM, FM.

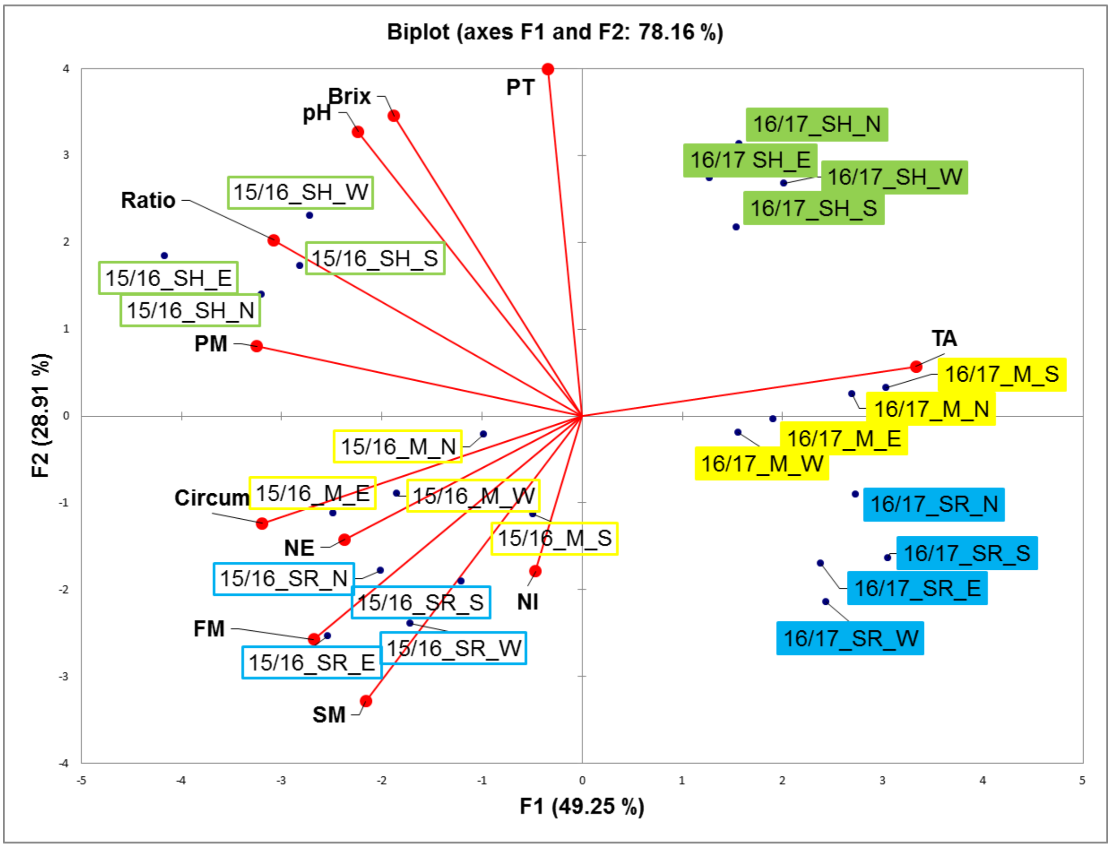

Figure 5.4 PCA biplot of associations between variety and quadrants based on fruit traits and flavonoid concentration for both seasons 2015-2016 and 2016-2017

FM = fruit mass, Circum = fruit circumference, PM = peel mass, PT = peel thickness, SM = segment mass, TA = titratable acidity, Ratio = °brix: acid ratio, NI = naringin, NE = naringenin, SH= Sweetheart, M= Marsh, SR = Star Ruby, W= west, E = east, N = north, S=south, 15/16 = 2015-2016 season, 16/17= 2016-2017 season

5.4 DISCUSSION

5.4.1 Relationship between variables

In seasons one and two positive correlations were seen between fruit mass and segment mass, between pH, °brix and °brix:acid ratio as well as the naringin and naringenin concentration. A negative relationship was seen in both seasons between °brix and segment mass. There was no association between peel thickness and the other variables. Apart from their strong association with each other, there were no associations between naringin and naringenin and the other variables.

The fruit mass recorded is the total mass of an individual fruit. Fruit mass can also be calculated as the sum of segment mass and peel mass (fruit skin and albedo). Thus, as the fruit increases in segment mass, the total fruit mass will increase. This was corroborated by the strong positive correlation seen in both seasons. The positive association seen between naringin and naringenin was also supported by a strong Pearson correlation coefficient calculated for both seasons. The flavonoid naringin is derived from naringenin via the phenylpropanoid pathway (Frydman et al., 2004). As the fruit matures, the anabolism of naringin occurs. Naringin is hydrolysed by the enzyme L-rhamnosidase to produce prunin and L-rhamnose. Prunin is then further hydrolysed to naringenin and D-glucose (Puri et al., 1996). Thus, the amount and availability of naringenin determines the amount of naringin that can be produced, and as the fruit matures, the amount of naringin available also contributes to the total amount of naringenin in the fruit. The negative association seen between segment mass and °brix, can be explained by a dilution effect where the uptake of water increases the fruit size but leads to the dilution of the solids in the juice sacs (Koch, 1984). A study done by Cruse et al. (1982) showed that rain or irrigation during the latter part of the season or during harvest, lowers both the soluble solids and the titratable acid in fruit. The same is true for the opposite where fruit size is reduced under conditions of restricted water which, in turn, also influences the soluble solids and acid contents as soluble solids concentration increases with a decrease in the size of the fruit (Sinclair, 1961; Gardner, 1969).

5.4.2 Relationship between variables and grapefruit varieties

Within each season, the Sweetheart variety was more strongly associated with higher °brix content, pH and °brix:acid ratio, as well as a thicker peel than the other two varieties. In Figures 5.3 and 5.4, Sweetheart was shown to be less associated with naringin content than Star Ruby. These findings of a lower naringin content compared with Star Ruby, and a higher sugar content, is in agreement with the specific Sweetheart traits described by Citrogold (2006).

5.4.3 Relationship between variables and seasons

A difference in fruit quality between seasons was observed (Figure 5.4). Season one was more associated with higher fruit mass, segment mass, peel mass, sugar content, pH, °brix: acid ratio, and flavonoid content. The second season was more associated with a higher titratable acid content. These seasonal differences can be attributed to the environmental effect of water stress, due to a drought and water restrictions that the farmers had to adhere to.

Trees were increasingly under drought stress from the end of the first season right through the second season. During December 2016 to March 2017, the second seasons fruit were in the cell expansion phase, where 40% of the annual water usage is needed (Vahrmeijer, 2016). Water stress was evident with the decline in fruit size as compared to the first season. Studies showed that water stress during the first and second growth period of the fruit causes a decrease in fruit mass, segment mass, peel mass, sugar content, pH, °brix: acid ratio, and flavonoid content (Cruse et al., 1982; Ginestar and Castle, 1996, Vahrmeijer, 2016).

5.4.4 Relationship between variables and canopy quadrant

A classic study done by Reitz and Sites (1950) demonstrated that a higher level of solids in fruit were found in the warmer canopy positions (the south and west quadrants) of Valencia trees. For TSS (°brix) to accumulate, higher levels of carbon fixation is needed which, in turn, is influenced by temperature and light interception (Reitz and Sites, 1950) and light interception of horticultural crops is influenced by spacing, tree height, tree shape and/or row orientation. However, no such clear quadrant association was seen for the °brix and acid content in this study.

Fruit weight, juice content and peel thickness of internal fruit were significantly higher when compared with external fruit (Fallahi and Moon, 1989). Cohen (1988) found in a study with tangelos in the northern hemisphere, that fruit on the southern canopy position were larger than those from the cooler northern side. Verreynne et al. (2004) confirmed this with work done on mandarins in the southern hemisphere where the northern canopy position was warmer and produced the larger fruit. In this study, the orchard row-orientation was east to west, which could explain the higher fruit mass association of Marsh and Star Ruby with the eastern and western quadrants in both seasons.

Flavonoid production in plants is strongly enhanced by oxidative stress (Kumar and Pandey, 2013). Flavonoids protect the plant cells by absorbing the most energetic solar wavelengths (UV-B and UV-A), by inhibiting the generation of ROS, and quenching ROS once they are formed (Kumar et al., 2013). A study was done by Feng et al. (2014) on three apple cultivars to determine how the position of the fruit on the tree might affect the levels of the primary and secondary metabolites in the fruit. One of the study conclusions was that phenolic compound content from both the peel and flesh were significantly higher in the outer-canopy fruit (Feng et al., 2014), however, the sampling of fruit in this study was done randomly in each quadrant from the inner and outer canopy as well as the top and bottom canopy. There were no significant differences in the average levels of naringin or naringenin in the respective quadrants, thus suggesting an equal distribution throughout the canopy. A study done by Jourdan et al. (1985) proved that there is a significant difference in the distribution of naringin in the various tissues of grapefruit fruit. Perhaps, more insight can be gained from investigating the naringin content in respective tissues of fruit sampled from different canopy positions.

5.5 CONCLUSIONS

This study showed that the genetic factors (variety differences) and environmental factors (climate and position of fruit in the canopy) had some degree of interaction. The interactions between the traits, both physical and chemical, were constant over seasons. An environmental effect was observed over seasons and was mainly due to water stress as a result of drought during the second season. This led to a significant decline in fruit mass, segment mass, peel mass, sugar content, pH, °brix: acid ratio, and flavonoid content.

With regard to canopy position, the eastern and western quadrants were associated with larger fruit size while the geographic position of fruit in the canopy had no influence on the flavonoid content nor the °brix and °brix:acid ratio.

As for the interactions between the fruit traits and genetic factors, clear differences were observed over both seasons for the three grapefruit varieties studied. Sweetheart was higher in °brix, pH and °brix:acid ratio and lower in naringin and naringenin than Star Ruby and Marsh, corroborating the variety attributes described by Citrogold (2006). In conclusion, when fruit are screened in a breeding programme for new or improved physical and/or chemical traits, such as higher naringin content, the sampling quadrant does not seem to have an effect, but climatic differences between seasons would affect the fruit naringin content.

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Chapter 6

GENERAL CONCLUSIONS AND RECOMMENDATIONS

A citrus breeding programme starts with the selection of suitable parents and the planning of controlled crosses and ends with a commercialised product welcomed by the consumer (Sippel et al., 2015; Abouzar and Nafiseh, 2016). Information on the breeding value of available parents and the heritability of specific traits is important in a plant breeding programme to aid the breeder in parent selection and the planning of controlled crosses (Abouzar and Nafiseh, 2016).

The major goals of variety breeding in citrus are mostly related to fruit quality, productivity, and harvesting period. Citrus fruit quality includes many physical and chemical attributes like peel and pulp colour, fruit size, ease of peeling, seedlessness, fragrance and pulp taste (Bijzet, 2006; Sippel et al., 2015; Abouzar and Nafiseh, 2016). As a result of their organoleptic properties and health benefits, flavonoids have caused much interest in fruit quality improvement (Puri et al., 1996; Tapas et al., 2008; Owira et al., 2009; Meiyanto et al., 2012). Breeding objectives in the global grapefruit market have shifted from fruit varieties that are seedless and highly pigmented to include high nutritional value (Abouzar and Nafiseh, 2016). However, the specific flavonoid profile of the grapefruit varieties in the current South African gene bank is currently unknown and has therefore not been consciously exploited. Pre-breeding is thus necessary to identify these unexploited traits.

In a citrus breeding programme, many fruit quality traits are only assessed post-harvest and are influenced by several factors, such as genetic factors (variety differences), stages of maturity, environmental factors such as climate, soil conditions, cultivation practices and fruit location within the tree canopy (Sinclair, 1961; Chen, 1990; Hunlun, 2016). Therefore, in a pre-breeding programme, where fruit is sampled for the screening of physical and chemical traits, factors that could have an impact on the selection of sampled fruit need to be taken into account. Hence the reason and importance of this study, in which the physical and chemical traits as well as the naringin and naringenin content of three grapefruit varieties (Star Ruby, Sweetheart, and Marsh) harvested over two seasons (2015-2016 and 2016-2017) were evaluated. For the determination of the naringin and naringenin content of fruit juice, a HPLC-UV/Vis method was optimised. Variety differences, canopy position differences as well as the influence of growing seasons were apparent.

Most targeted breeding objectives such as fruit yield and quality traits are quantitively inherited (Omura and Shimada, 2016). Grapefruit varieties have different genetic traits, which mainly influence the micro constituents and consequently the phenolic and flavonoid content and composition thereof (Hunlun, 2016). In this study, significant varietal differences were observed for fruit size, °brix, pH and naringin content. Overall Marsh and Star Ruby yielded larger fruit. The Sweetheart variety yielded fruit that was higher in °brix, pH and °brix:acid ratio. Literature indicated white grapefruit varieties to be slightly, but not significantly higher in total flavanone content than pink and red varieties (Peterson et al., 2006). In this study Star Ruby (red variety), had a slightly higher naringin content than Marsh (white variety) and Sweetheart (pink variety). The interactions between the genetic factor (variety) and both physical and chemical traits were constant over both seasons.

The canopy position of fruit is related to the amount of radiation the fruit and photosynthetically active leaves are exposed to (Reitz and Sites, 1950). In the southern hemisphere, the north-western canopy quadrant is the warmest and receives the most sunlight. Some studies have shown that this influences the fruit quality directly (Koch, 1984; Cohen, 1988; Fallahi and Moon, 1989).

In this study fruit from different canopy positions varied in physical traits such as fruit mass, circumference, peel mass and segment mass. For the first season, the eastern quadrant yielded the largest fruit. In the second season the western quadrant had the largest fruit, yet not significantly larger than the northern or eastern quadrants. As expected, the eastern quadrant yielded fruit that were larger in size, which is in agreement with the findings of Fallahi and Moon (1989), that fruit from the interior and cooler regions of the tree canopy are characteristically larger in size.

As for the chemical composition of the fruit, those from the eastern quadrant had the highest pH value and °brix:acid ratio for the first season. Fruit from the western and northern quadrants did not show significant differences in pH value or °brix:acid ratio. In the second season, the northern quadrant fruit had the highest °brix content. Overall the northern quadrant fruit were highest in °brix content, and also had a high °brix:acid ratio.

This is in agreement with previous studies, where fruit from warmer tree canopy regions that were more exposed to radiation, had a higher °brix and °brix:acid ratio (Fallahi and Moon, 1989, Verreynne et al. 2004, Freeman and Robbertse, 2003).

As for the flavonoid content, studies have shown that the concentration of plant secondary metabolites such as flavonoids can be influenced by a number of environmental factors such as light intensity, carbon dioxide levels, temperature, fertilization as well as biotic and abiotic stress (Briskin et al., 2001; Jaafar et al., 2008). Feng et al. (2014) concluded in their study, done on three different apple cultivars, that outer and inner canopy fruit had different levels of flavonoid content. Flavonoid levels are also related to specific tissue (Jourdan et al., 1985). A study done by Jourdan et al. (1985) proved that there is a significant difference in the distribution of naringin in the various tissues of grapefruit, with the albedo having the highest level of naringin in mature fruit. Another study done by Sun et al. (2010) supported this, where different amounts of naringin were found in different tissue parts of *Citrus reticulata* 'Chachi' fruit.

In contrast with the other fruit traits investigated in this study, the results indicated no significant differences in the naringin or naringenin content from the different sampling quadrants in both seasons. This equal distribution of naringin and naringenin thoughout the canopy suggest that these traits might not be that sensitive to canopy quadrant differences such as radiation and temperature.

Abiotic stress in the second season affected the results. The second season's fruit suffered drought stress during the critical first and second growth stages. This led to a decline in fruit mass, segment mass, peel mass, sugar content, pH, °brix: acid ratio, and flavonoid content. However, the interactions between the parameters, both physical and chemical, were constant over both seasons.

In season one and two, positive correlations were evident between fruit mass and segment mass, between pH, °brix and °brix:acid ratio as well as the naringin and naringenin concentration. A negative relationship was seen in both seasons between °brix and segment mass. In the literature, an inverse relation between size, soluble solids, acidity and ascorbic acid was documented for oranges (Abouzar and Nafiseh, 2016). This can be explained by a dilution effect where the uptake of water increases the fruit size but leads to the dilution of the solids in the juice sacs (Koch, 1984). No association between peel thickness and the other variables were seen in this study. Apart from their strong association with each other, there were no associations between naringin and naringenin and the other variables, chemical or physical.

In conclusion, when screening the fresh edible part of the grapefruit for naringin and naringenin content, results from this study indicated that the distribution of these flavonoids seems to be equal throughout the canopy quadrants. Further investigation is needed for sampling fruit in a screening programme, as the amount of radiation difference between inner and outer canopy fruit might influence the content of other flavonoids than narignin and naringenin. This study also found that the flavonoid content is affected by genetic differences (varieties). In a review done by Omura and Shimanda (2016), the citrus breeding research done in Japan is discussed with specific focus on Quantitative Trait Loci (QTL) marker development for some flavonoids. The study of Omura and Shimanda (2016), also supports the findings that environmental factors such as water stress, affects fruit quality with regards to size, TSS, pH, °brix:acid ratio and flavonoid content.

It is recommended that additional investigation is done in order to survey the effect of different growing regions within South Africa on the flavonoid content of grapefruit. Furthermore, additional investigation is also required to gain knowledge on the effect of specific agronomic practices used in breeding programmes. Nonetheless, it was demonstrated that the sampling of fruit for determining naringin/naringenin content in grapefruit can be simplified, which is beneficial for screening of a large number of possible parents as well as determining general and specific combining ability as well as genetic gain with regard to naringin/naringenin content in a beeding programme. A simplified screening method is also beneficial for the purpose of genotype by environment interaction investigations with regard to different production areas and different agronomic practices.

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