CHARACTERISING THE FLAVONOID PROFILE OF VARIOUS CITRUS VARIETIES AND INVESTIGATING THE EFFECT OF PROCESSING ON THE FLAVONOID CONTENT

deur

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DECLARATION

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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ABSTRACT

Phenolic compounds in citrus fruit are specific for each species and variety and may be influenced by environmental conditions during the growing season and post-harvest practices. The exact chemical composition of citrus produced in South Africa is currently not known even though 2 million tonnes were produced in 2012. Various citrus varieties are produced for export, local fresh markets as well as processed into value-added products sold on the local market.

In the current study South African citrus fruit (satsuma, clementine, navel and valencia) as well as products such as frozen concentrated orange juice (FCOJ), made-from-concentrate and not-from-concentrate orange juices produced from these varieties were characterised in terms of chemical and phenolic composition as well as total antioxidant capacity (TAC). Samples from two regions and three seasons were evaluated to determine the effect of variety as well as seasonal and regional differences.

Citrus juice characteristics evaluated, included: °Brix, titratable acidity (TA), °Brix:acid ratio, pH as well as ascorbic acid (AA). Furthermore, the phenolic composition of the citrus fruit was quantified using high-performance liquid chromatography coupled with diode-array detection (HPLC-DAD). The TAC was determined using 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, Oxygen Radical Absorbance Capacity (ORAC) assay and Ferric Reducing Antioxidant and Ascorbic Acid (FRASC) assay.

Eight phenolic compounds were quantified and included four flavanone-*O*-glycosides, two flavonols, a flavone and phenolic acid. The phenolic composition of different citrus varieties showed great variation between different seasons. Varietal difference was evident although there was some overlap between citrus varieties within the same season. Hesperidin and narirutin were the predominant flavanone-*O*-glycosides in sweet oranges, which included navel and valencia varieties, with vicenin-2 and ferulic acid-*O*-hexoside also present in high quantities.

Regarding the FCOJ samples the results of the juice characteristics indicated that those from the WC sampling site were more mature compared to those of EC. Varietal differences were evident and variety proved to be the most significant factor that accounted for the variances in juice characteristics and phenolic composition. Seasonal differences were less evident. Variation that could be ascribed to regional differences was found for the

individual phenolic composition. FCOJ from EC were characterised as having higher levels of the individual phenolics, total phenolic composition (TP) and TAC_{DPPH} and TAC_{ORAC}. Of all the FCOJ varieties, navel was found to be the most mature, irrespective of season and region and was the variety with the highest TP.

The predominant flavanone found in the MFC and NFC orange juices were hesperidin (HD) and narirutin (NART) followed by the flavone-*C*-glucoside vicenin-2 (VIC2) and a hydroxycinnamic acid namely ferulic acid-*O*-hexoside. Three other minor phenolic compounds where also quantified. The results indicated that NFC juices had higher levels of the individual phenolics as well as higher TAC_{ORAC}. The results further showed that the phenolic composition of the MFC juices where dependent on the juice formulation, i.e. the quantity of orange juice added and not the treatment type (pasteurisation versus ultra-high temperature pasteurisation). Lastly, the results highlighted the lack of information pertaining to the processing, storage and shelf-life stability of the identified and evaluated phenolic compounds.

UITTREKSEL

Fenoliese verbindings wat in sitrus vrugte voorkom is kenmerkend ten op sigte van spesie en varieteit en word beïnvloed deur omgewingsfaktore gedurende die verbouingseisoen asook as gevolg van na-oes hantering. The presiese chemiese samestelling van sitrus wat in Suid-Afrika verbou word is tans onbekend ongeag die feit dat die land ‘n jaarlikse sitrus produksie van 2 miljoen ton gehad het in 2012. Verskeie sitrus varieteite word vir die uitvoermark geproduseer terwyl ander vrygestel word vir die plaaslike vars mark en/of verwerk word as produkte met toegevoegde waarde wat op die plaaslike kleinhandels mark verkoop word.

In hierdie studie word sitrus vrugte (satsuma, clementine, navel en valencia) eg aan Suid-Afrika asook produkte soos lemoensapkonsentraat (FCOJ) wat as gevries bemark word en ook sap wat gemaak word van lemoensapkonsentraat (MFC) en ook vars uitgedrukte lemoensap (NFC) geevalueer ten opsigte van chemiese-, fenoliese samestelling en antioksidant aktiwiteit (TAC). Lemoensap monsters van twee verbouingstreke en oor drie seisoene is versamel en ontleed sodat die effek wat varieteit, seisoen en verbouingstreke op die chemise en fenoliese samestelling bepaal kon word.

Lemoensap eienskappe is bepaal en sluit in suikergehalte (°Brix), titreerbare suurgehalte (TA), suiker:suur verhouding (°Brix:acid), pH en askorbiensuur (AA). Inwelke die fenoliese samestelling is bepaal deur hoë-druk vloeistof chromatografie met diode-opstelling en deteksie (HPLC-DAD). Die TAC is bepaal deur 2,2’-difeniel-1-picrylhydrazyl (DPPH) radikale blaas toets, Suurstof Radikaal Absorbansie Kapasieit (ORAC) toets en Ferri Vermindering Antioksidant en Askorbiensuur (FRASC) toets.

Agt fenoliese verbindings is bepaal en sluit in vier flavanone-*C*-glikosiede, twee flavonole, ’n flavone en fenoliese suur. Die fenoliese samestelling van die vier sitrus varieteite het groot variasie getoon tussen verskillende seisoene. Varieteitsverskille was duidelik hoewel daar ook oorvleueling was tussen sommige varieteite van dieselfde streek. Hesperidin en narirutin was die hoof flavanoon-*C*-glikosiediese verbindings in soet lemoene wat die navel en valencia varieteite insluit. Vicenin-2 en feroeliese suur-*O*-heksosied was ook teenwoordig in groot hoeveelhede.

Wat die FCOJ aanbetref het die resultate van die sapeienskappe getoon dat die monsters vanaf die Wes-Kaap (WC) streek meer volwasse en ryp was vergeleke met die van die Oos-Kaap (EC) streek. Varieteitsverskille was duidelik en was hoofsaaklik die mees

bestendige faktoor wat aanspreeklik was vir die verskille in die chemise- en fenoliese samestelling. Verskille as gevolg van seisoenale invloed was van mindere belang. Variasie as gevolg van verbouingstreeks verskille was gevind vir die individuele fenoliese verbindings. FCOJ van die EC was gevind om hoër individuele fenole, totale fenoliese vlakke (TP) en TAC te bevat. Dit is bevind dat die FCOJ van die navel varieteit die hoogste TP bevat het en was ook die mees volwasse en/or ryp varieteit ongeag van seisoen of verbouingstreek.

Die hoof fenoliese verbindings in die MFC en NFC lemoensappe was hesperidin en narirutin gevolg deur vicenin-2 en feroeliese suur-*O*-heksosied. Drie ander fenoliese verbindings was bevind om in kleiner hoeveelhede voor te kom. Die resultate het getoon dat NFC sappe hoër individuele fenoliese vlakke en ook hoër TAC gehad het. Verder het die resultate getoon dat die fenoliese samestelling van MFC sappe afhanklik is en beïnvloed word deur die sapformulasie dit wil sê die hoeveelheid lemoensap wat bygevoeg word en nie noodwendig die tipe hitte behandeling (pasteurisasie vs uiterse hoë temperatuur pasteurisasie) wat die sappe aan bloodgestel word nie. Laastens het die resultate van hierdie navorsingstudie die gebrek aan inligting uitgewys wat die stabiliteit van die geïdentifiseerde en bepaalde fenoliese verbindings aanbetref tydens verwerkingsprosesse, opberging en raklewe van lemoensappe.

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CHAPTER 1

INTRODUCTION

The importance of fruit and vegetable consumption has long been known due to its association with a reduced risk of chronic diseases (Williamson, 1996; Müller *et al*., 2010; Khan *et al*., 2014). Recently, identification of new compounds in the chemical composition of fruits and vegetables has received increased attention (Faller & Fialho, 2009). In addition, citrus fruits are receiving much biomedical interest and it has been shown that 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 their antioxidant capacity (Guthrie & Carrol, 1998; Miyagi *et al*., 2000; Poulose *et al*., 2005; Poulose *et al*., 2006; Jayapraksha *et al.*, 2007). Citrus fruit are characterised as having a relatively high phenolic content compared to other edible plants (Hollman & Arts, 2000; Moufida & Marzouk, 2003; Hollman & Arts, 2005; Benavente-García *et al*., 2008). Citrus fruit is the most abundantly grown fruit crop in the world which is produced in 15 countries on different continents and is therefore considered to be one of the most important sources of bioactive compounds (Anon., 2012a; Xi *et al.*, 2014). Juice derived from citrus fruit forms an important dietary source of bioactive compounds which have a direct effect on human health (Roussos, 2011). Citrus bioactive compounds have been reported as being beneficial in cancer prevention, which is one of the many proposed health-promoting properties (Peterson *et al.*, 2006). However, most of the bioactive components responsible for the beneficial effects are currently not known, though it is believed that citrus flavonoids are involved (Peterson *et al*., 2006).

Flavonoids are the major group of phenolic compounds found in citrus fruits. Flavonoids can be classified as flavanones, flavones and flavonols that occur either in the free form and or as glycosides in various parts of citrus fruits (Tripoli *et al.*, 2007). Citrus species or varieties vary with respect to phenolic composition and therefore phenolic compounds are used as chemotaxonomic markers to distinguish between citrus species or varieties (Albach & Redman, 1969; Kanes *et al.*, 1993; Mouly *et al.*, 1994; Marini & Balestrieri, 1995; Ooghe & Detavernier, 1997). The genetic characteristics of citrus varieties, thus mainly influence the microconstituents and consequently the phenolic and flavonoid content and composition (Mouly *et al.*, 1994; Marini & Balestrieri, 1995; Ooghe & Detavernier, 1997; Tripoli *et al.*, 2007). Moreover, citrus flavonoid content is influenced by

the growing season, stages of maturity or ripeness, environmental conditions such as climate and location, and may be influenced by post-harvest processing (Vanamala *et al.*, 2006; Galaverna & Dall'Asta, 2013).

The major flavanones, with a high prevalence in citrus compared to other fruits, have been identified as hesperidin and narirutin (Khan *et al*., 2014). The main flavonols and flavones present in considerable amounts are rutin and vicenin-2 (Gatusso *et al*., 2007). In addition to flavonoid glycosides, citrus juice also contains hydroxycinnamic acids, such as glycoside derivatives of ferulic and sinapic acid (Abad-García et al., 2012b). Identification and characterisation of citrus phenolic composition in various varieties and plant parts of Chinese, Spanish, Mediterranean, Mauritian and North-American origin have been extensively researched over the past 30 years (Mouly *et al.,* 1994; Dhuique-Mayer *et al.*, 2005; Caristi *et al.*, 2006; Cano *et al*., 2008; Ramful *et al*., 2010; Abad-García *et al.*, 2012b). Nevertheless, there are yet unknown phenolic compounds that need to be identified and characterised in citrus. Thus, in order to truly have a global view of the potential health benefits and to fully exploit citrus as a functional food product a database which considers all the relevant information pertaining to phenolic compound levels in different citrus varieties, grown in different continents, countries and regions is required. This includes information relevant to citrus fruit cultivated in South Africa with a unique climate, as well as unique cultivation and post-harvest practices, which is currently not available.

South Africa is the twelfth largest citrus producer in the world and the second largest fresh citrus exporter in the world (Anon., 2012a; Anon., 2012b). Approximately 60 foreign countries receive their citrus fruit requirement from South Africa with an annual citrus production of 2 million tonnes. Altogether 69% of the total amount is exported, 24% is sold on the local market and 7% is processed into commodity and value-added products. The South African citrus industry is divided into four sectors which include the Orange, Soft citrus, Grapefruit and Lemon sectors. Citrus fruits are produced throughout six provinces in South Africa with the Western Cape Province producing 15% of the total citrus production. The Western Cape (WC) Province is responsible for 46% of the total soft citrus production. The Soft Citrus sector includes satsuma and clementine varieties (Anon., 2012a; Anon., 2012b). The Orange sector includes navel and valencia varieties which are also grown and processed in the Western and Eastern Cape (EC). The EC contributes 23% of the total citrus production which makes this province the second largest citrus production area followed by the WC which is the third largest production area in terms of hectares planted (Anon., 2013). The EC production area delivers 14%

valencia, 37% navels, 34% of the total soft citrus fruit production compared to the WC which supplies 8% valencia, 24% navels, 46% soft citrus as previously mentioned (Anon., 2012a; Anon., 2012b). The Limpopo province is by far the largest citrus production area however valencia (56% of total citrus production) is the main variety grown followed by navel (21%) and Soft citrus (10%). Thus the major producers of navels and soft citrus are the EC and WC province with some valencia production in both regions (Anon., 2012a; Anon., 2012b).

The fruit juice industry is the fourth largest under the Beverage agro-economic sector in terms of sales in South Africa (Anon., 2015). Amongst the various fruit juices, pulps and purees produced in South Africa, frozen concentrated orange juice (FCOJ) forms one of the most important export commodities (Anon., 2015). However, the global market has seen a decline in FCOJ consumption between 2007 and 2012 (Anon., 2013). This may be partly due to misperceptions that frozen orange juices possess less health benefits. Furthermore, South African fruit juice producers are currently focusing on fruit juice based drinks because of their functionality and contribution to general health which is an emerging market that is still to be fully developed (Foulds, 2015). The functional properties especially for citrus fruit juice are due to the vitamin C content and phenolic composition. In addition, it has long been the practice to process fruit such as citrus into frozen concentrates in order to supply a stable product and extend the availability throughout the year to various markets such as the fruit juice industry. Moreover, a large portion of South African oranges are further processed into orange juice blended products which are known as made-from-concentrate (MFC) juices undergoing either additional pasteurisation or ultra-high temperature pasteurisation. Alternatively, the juice is extracted and directly pasteurised to produce not-from-concentrate (NFC) orange juice.

In South Africa 80% of the total juice production is sold on the local market and only 20% is exported (Booth, 2015). The fruit juice industry utilises the FCOJ to produce MFC 100% juices, nectars, drinks and squashes, which are all regulated under the Agricultural Product Standards Act (Act 119 of 1990), The Regulation Relating to the Classification, Packing and Marking of Fruit Juice and Drinks intended for sale in the Republic of South Africa (R.286 of 7 November 1980 as amended by Government Notices Nos. R.929 of 1 May 1981, R.1325 of 9 July 1982, R.992 of 13 May 1983, R.602 and R.641 of 30 March 1984, R.1801 of 17 November 1995). Over the years, sophisticated characterisation techniques have been developed for the detection of phenolic compounds in citrus fruit and juices. These techniques rely on high-pressure liquid chromatography (HPLC) in conjunction with diode array detection (DAD) and in some cases liquid chromatography

coupled with electrospray ionisation and mass spectrometry (LC-ESI-MS/MS) for characterisation and identification of citrus specific phenolic compounds (Mouly *et al*., 1999; Caristi *et al*., 2003; Gattuso *et al*., 2006; Abad-Garcia *et al*., 2009; Barreca *et al*., 2011b). However, adulteration of 100% MFC orange juices with cane sugar or other fruit sugars are a concern which misleads the consumer and do not possess the functional attributes of non-adulterated orange juices. This is mainly due to the idling global economy which has placed pressure on fruit juice prices since consumers have a reduced buying power. This resulted in fruit juices being highlighted as one of the top-ten most at-risk foods of being adulterated by the European Union (Richards, 2013). Thus, the economic crisis aggravated food fraud. This is mainly attributable to the potential economic gains and further due to compositional policing which is lacking. This trend does not only affect South Africa but is a growing trend in Europe. The first attempt at overcoming this problem is to promulgate regulations and expand capacity to ensure compliance. In South Africa the SA Fruit Juice Association (SAFJA) played a role in substantially amending the SA regulations pertaining to fruit juices where the sweetened class of fruit juices was removed. This is to the benefit of the consumer and industry (Richards, 2014). In the past dispensation of the regulations the sweetened class of fruit juices allowed the addition of 10% “permitted natural sweeteners” which was typically cane sugar. Thus, in essence adulteration was allowed since it was difficult to police whether only 10% cane sugar is added or more in an attempt to lower the price and increase the profit margin of fruit juices. In addition to the global economic situation, current consumer trends have indicated that traditional MFC orange juice consumption is declining. This is mainly due to consumers’ preference for healthy alternative drinks. This trend is due to general perception that traditional juices such as frozen orange juice is of lesser quality regarding its nutritional properties.

Even though citrus production forms a major part of the South African agronomic and economic sector, knowledge of the exact chemical composition of citrus fruit cultivars and their products (fruit juices, nectars and jams) is necessary, but currently lacking. This information may provide valuable insight regarding the commercial properties of South African citrus fruit varieties and their products. There is currently no information on the variation in phenolic composition of different South African citrus fruit due to varietal and seasonal effects. Furthermore, the effect of growing season on the phenolic composition and total antioxidant capacity (TAC) of South African citrus is unknown. The effect of processing techniques on these phenolic compounds is similarly of interest. There is also a need to establish what the levels of these compounds are in the commodity products

produced from different citrus varieties. Knowledge of factors, such as variety and stages of maturity, which may lead to quantitative differences, is important to better understand their influence on the biological or functional properties of citrus fruit and products derived from these citrus fruit.

Therefore, this study aimed to characterise the phenolic composition and TAC of four citrus varieties grown in the Western Cape of South Africa. Additionally, FCOJ produced in the Western Cape and Eastern Cape region of South Africa was evaluated in terms of individual phenolic composition. Finally, various brands of MFC and NFC orange juice and blends were analysed with a view to establishing their nutritional quality in terms of bioactive phytonutrient level as well as to gauge whether legislative requirements are met.

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CHAPTER 2

LITERATURE REVIEW

BACKGROUND

The importance of fruit and vegetable consumption has long been known due to its association with a reduced risk of chronic diseases (Williamson, 1996; Müller *et al*., 2010; Khan *et al*., 2014). Recently, identification of new compounds in the chemical composition of fruits and vegetables has received increasing attention (Faller & Fialho, 2009). Furthermore, citrus fruits are receiving much biomedical interest and it has been shown that 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 (Guthrie & Carrol, 1998; Miyagi *et al*., 2000; Poulose *et al*., 2005; Poulose *et al*., 2006; Jayapraksha *et al.*, 2007). However, most of the bioactive components responsible for the beneficial effects are currently not known, though it is believed that citrus flavonoids are involved (Peterson *et al*., 2006).

Flavonoids are the major group of phenolic compounds found in citrus fruits. Flavonoids can be classified as flavanones, flavones and flavonols and can occur either in the free form or as glycosides or both in various parts of citrus fruits (Tripoli *et al.*, 2007). Citrus species or varieties vary with respect to phenolic composition and therefore phenolic compounds are used as chemotaxonomic markers to distinguish between citrus species or varieties (Albach & Redman, 1969; Kanes *et al.*, 1993; Mouly *et al.*, 1994; Marini & Balestrieri, 1995; Ooghe & Detavernier, 1997). This approach is based on the fact that the genetic characteristics of citrus varieties are the main influence that determines the microconstituents and consequently the phenolic and flavonoid content. However, citrus flavonoid content is also influenced by the growing season, stages of maturity or ripeness, environmental conditions such as climate and location, and may be influenced by post-harvest processing (Vanamala *et al.*, 2006; Galaverna & Dall'Asta, 2013). Furthermore, sophisticated adulteration detection methods have been developed and these involve investigation of the chemical composition of citrus. Nonetheless, adulteration is still found to be prevalent due to economic reasons such as cost

variation among different raw material varieties and locations which lead to origin adulteration or origin mislabelling (Mouly *et al.*, 1999).

A. CITRUS FRUIT

Classification (Agronomic groups)

Navel oranges are known as sweet oranges and belong to the species *Citrus sinensis* (L.) Osbeck (Dugo & Giacomo, 2002). Navel oranges include cultivars such as Navelina, Washington, Cara-Cara, Thomson, Navelate and Newhall. A distinctive feature of navel orange is the small secondary fruit that is embedded in the apex of the primary fruit. Some desirable characteristics of navels include seedless fruit, larger fruit size than other orange varieties, sweet and pleasant flavour, deep orange flesh colour and a rind that peels easily. Navel oranges are also one of the earliest maturing orange varieties, generally speaking, and are produced in South Africa under the Orange sector (Anon, 2012a).

Valencia is a common orange which also belongs to the species *Citrus sinensis* (L.) Osbeck (Dugo & Giacomo, 2002). Valencia originated from the Azore Islands or Portugal and not Valencia, Spain. Today it is grown in the United States of America, South Africa (under the Orange sector) and many other countries and is a popular variety. Valencia oranges are also called 'white' oranges due to the light flesh colour when compared to others. The common orange is the largest citrus group used for commercial purposes worldwide (Barrett *et al.*, 2005). This is due to the excellent flavour quality in the fruit as well as the juice derived from the fruit. In addition the fruit has a very high juice yield making it desirable to the processor. These factors result in valencia orange juice being the principal citrus product in the world.

Satsuma oranges are one of the main varieties that can be grouped under Mandarins. Satsuma is from the species *Citrus unshiu* (Mak.) Marc. and is mainly grown in Japan, although some are grown in South Africa for commercial purposes because it is a seedless easy peeler fruit and has a good taste and low acid levels (Anon, 2014). This variety is known to have early and late cultivars with different times of ripening and include Owari and Okitsu (Dugo & Giacomo, 2002). This variety is grouped under the Soft Citrus sector of South Africa.

Clementine is a hybrid between sour orange and mandarin (Dugo & Giacomo, 2002). Thus, it is also classified as one of the main groups of Mandarins. Clementine is alternatively known as Tangerine and belongs to the species *Citrus clementina* Hort *ex* Tan. Clementine cultivars include Clemenules, Marisol, Monreal and Fina. Clementine fruit can be distinguished from satsuma since it has a deep rind and flesh colour whilst satsuma has a lighter or paler rind. Since both satsuma and clementine are main groups of mandarins, the parent species is known as *Citrus reticulata* (Dugo & Giacomo, 2002). Clementine fruit is very popular in South Africa (Soft Citrus sector) due to its excellent eating quality (Anon, 2014).

Citrus Fruit Anatomy

Citrus fruit consists of an outer layer known as the flavedo (Fig. 2.1) which houses oil glands containing the essential oil that provides the characteristic odour and flavour (Kimball, 1984). Colour pigments are also found in the exterior flavedo. The albedo is situated beneath the flavedo and can be described as a spongy white layer. Fruit sections follow the albedo and are divided by a membrane. Each fruit section contains elongated vesicles that are attached to the centre of the fruit. These vesicles contain juice cells which is primarily engorged vacuoles and it has been shown that the juice in the juice cells are completely absent of cloud material. The cell nuclei and other organelles are located in the membrane (Barrett *et al.*, 2005). During fruit maturation, water and carbohydrates accumulate in the juice cell while the active mitochondria in the membrane of the juice cell produce citric acid via the Krebs cycle. This results in citric acid accumulation in the juice cell, which is responsible for the varying acidity during fruit maturation. Thus, in early season the acidity is high due to citric acid accumulation and as the fruit matures the acidity decreases due to dilution (growth) or citric acid depletion through increased metabolic activity during the warmer climate (Kimball, 1984).

B. CULTIVATION: GEOGRAPHICAL & ENVIRONMENTAL CONDITIONS

Citrus fruit cultivation is dependent on a number of factors that include soil type (depth and structure), cultural or production practices (fertilization and plant nutrition), irrigation and climatic conditions(Dugo & Giacomo, 2002; Anon, 2014).

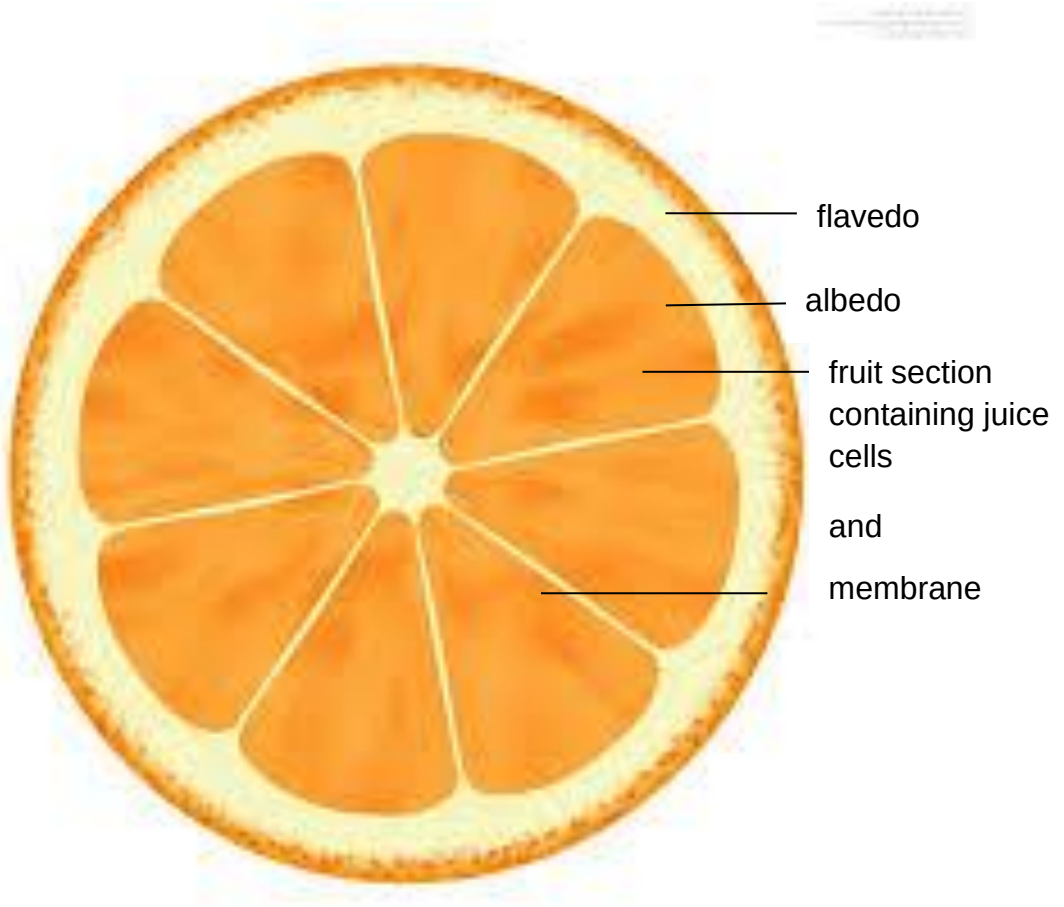


Figure 2.1 A typical cross section of citrus fruit depicting the various parts of the fruit anatomy.

The latter, which include temperature, relative humidity, rainfall, wind velocity, and sunshine, are uncontrollable factors that willgreatly impact on the growth, quality and size of the citrus fruit obtained during the harvest season (Dugo & Giacomo, 2002; Anon, 2014). Citrus fruit is inherently characterised as a tropical fruit but trees also yield good quality fruit in sub-tropical climates. Temperature is the most important climatic condition that affects the growing citrus fruit. Temperatures that are considered optimum for fruit growth range from 20° - 28°C. However, temperatures vary for sub-tropical regions and differ as altitudes (above sea level) change from region to region. Typically, sub-tropical temperatures range from 15° - 20°C, with lower temperatures during harsh winter months when temperatures can decrease to approximately 5° - 10°C. Sub-tropical regions situated inland and away from the seashore, can on the other hand, experience extremely high temperatures during summer months with temperatures reaching 35° - 40°C. Temperatures below 12.5°C adversely affect the

vegetative growth of citrus (Ladaniya, 2008). Therefore, a heat summation unit (HU) based on this temperature (12.5°C) can be used in order to determine the overall effect temperature of a region will have during the growing season. For example, if a growing region has an average temperature of 15°C during a 30 day period the HU can be calculated by subtracting the minimum temperature (12.5°C) as follows: 15 - 12.5 = 2.5 x 30 = 75 HU over a 30 day period / month (Ladaniya, 2008). Thus, when this HU is compared to a growing region with an average temperature of 20°C (for a 30 day period) a much higher HU of 225 will be obtained. Consequently, the HU can be used to indicate whether citrus from one region will grow faster or slower compared to another region. Higher HU values indicate faster growth rates which are regarded as undesirable since the fruit will be of poor quality due to faster respiration which will lead to low carbohydrate content. Hence, citrus fruit that grows slower will accumulate carbohydrates more slowly which will lead to fruit with higher acid compared to sugar content which is general for sub-tropical climates with frost, fog and cloud cover. In this context, valencia citrus fruit grown in Columbia in tropical conditions matures within 6 - 7 months in comparison with valencia fruit that matures within 14 months grown in California in an arid sub-tropical coastal climate (Ladaniya, 2008). The significant effect of climate on citrus fruit quality is regarded as having a higher importance compared to that of soil quality, cultural practices and even genetic factors. This is evident in navel oranges grown in different regions of the USA. The navel fruit from Florida (lowland tropics) has higher respiration rates and subsequently faster maturation and acid depletion. The °Brix-to-acid ratio increases rapidly with the decrease in acidity resulting in fruit that has less intense colour and has a bland taste (Dugo & Giacomo, 2002; Ladaniya, 2008). The navel fruit grown in the coastal region of California accumulates carbohydrates (sugars) slowly and therefore matures more gradually taking longer and having a higher acidity, which is desirable. In addition, tropical growing conditions lead to continuous vegetative growth that competes with fruit growth and contributes to a lower total soluble solids level. In contrast, the lower temperature of sub-tropical growing regions, at the time of fruit maturity, results in a decrease in vegetative growth and hence the total soluble solids are higher. Moreover, it is believed that the best citrus fruit quality is achieved in regions with relative dryness (low rainfall), hot summers and cool wet winter months when fruit matures. These conditions are typically obtained in Mediterranean climates and the Western Cape region of Southern Africa (Ladaniya, 2008).

Other climatic factors that play a role in fruit development are humidity and rainfall. In humid and heavy rainfall regions fruit growth tend to be excellent yielding citrus fruit with a high percentage of sugar and a low acid level (Dugo & Giacomo, 2002). Hence, seasonal differences will affect fruit growth and size.

C. SOUTH AFRICAN CITRUS INDUSTRY

South Africa has been a citrus producing and exporting country for more than 100 years (Anon, 2012b). Currently South Africa is the twelfth largest citrus producer in the world, however the second largest fresh citrus exporter in the world. Approximately 60 foreign countries receive their citrus fruit requirement from South Africa with an annual citrus production of 2 million tonnes (Anon, 2012b). Although only 69% of the total amount is exported, 24% is sold on the local market and 7% is processed into commodity and value-added products. Citrus fruit are exported practically throughout the year due to South Africa's eighteen regions with different climatic conditions that is divided into six provinces (Fig. 2.2).

The South African citrus industry consists of citrus sectors as per agronomic group or type. The four sectors are Oranges, Soft Citrus, Grape Fruit and Lemons (Fig. 2.3). The major sector in South Africa is the Orange sector (60 000 ha) and constitute two thirds of the total citrus production. The Orange sector consists mainly of navel and valencia sub-varieties with approximately 14 000 ha and 24 000 ha planted, respectively. Navel orchards are planted in the Western Cape, Eastern Cape and some in the Limpopo and Mpumalanga provinces, while valencia is mainly produced in Limpopo and Mpumalanga with some in the Western and Eastern Cape (Fig 2.4.). In general, on an annual basis most of the orange sector is exported which is about 750 000 tonnes followed by processing of about 300 000 and 120 000 tonnes sold on the local market. Table 2.1 indicates actual tonnage for the past few years as recorded by the Perishable Product Exports Control Board (PPECB) and the South African Department of Agriculture, Forestry and Fisheries (DAFF). The Orange sector has a gross value of over 4 billion Rand and fetching R 4030 per ton of export fruit, R 529 per ton of processed fruit and on average R1 762 per tonnes of fruit sold on the local market during 2011 (sales on the 20 major fresh produce markets) (Anon, 2012b). This sector has also showed an increase in growth since 2006.

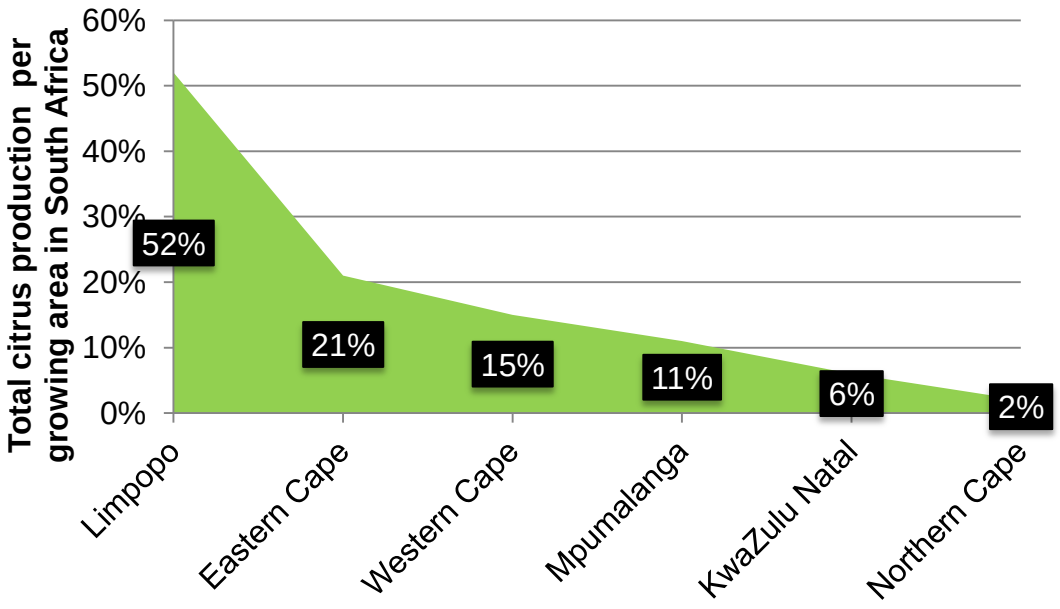


Figure 2.2 Citrus production areas in South Africa (Anon, 2012b).

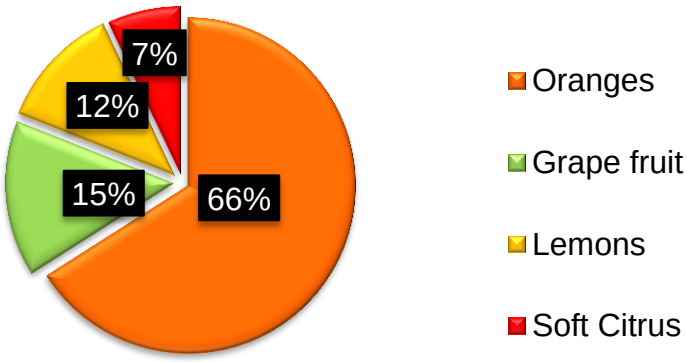


Figure 2.3 Citrus industry sectors and percentage of total exports in 2011 (Anon, 2012b).

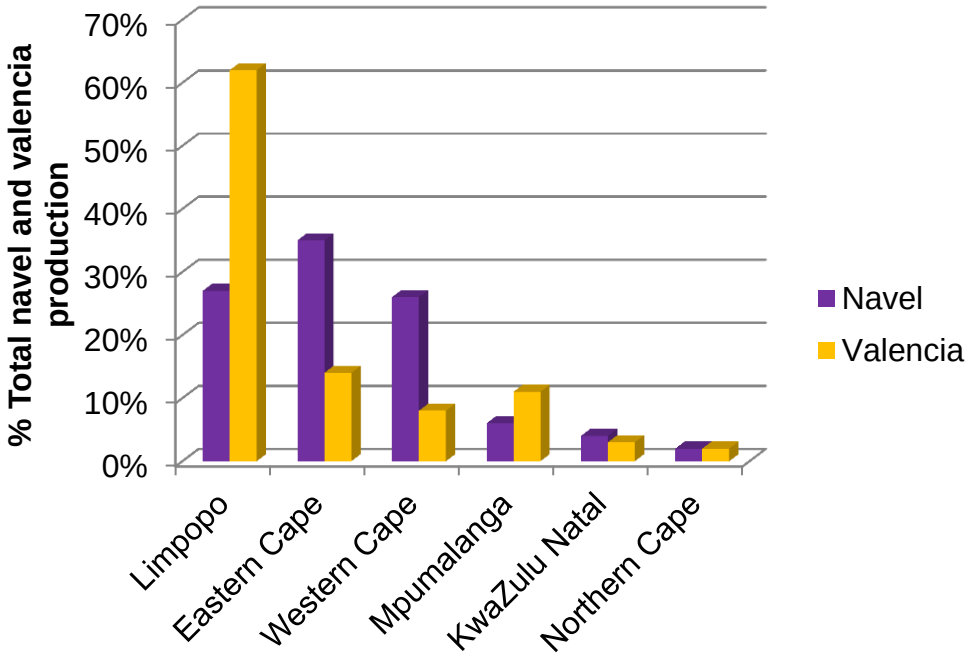


Figure 2.4 Production area distributions of navel and valencia fruit (Orange Sector) (Anon, 2012b).

During 2011 the Soft citrus sector comprised 5 200 ha of the total citrus production (Anon, 2012b). Soft citrus fruit are mainly produced in the Western Cape (46%), Eastern Cape (31%) and some in Limpopo (11%) and Mpumalanga (9%) provinces. In total, 103 618 tonne were exported in 2011 of which 22 620 tonnes were clementine and 20 983 tonnes were Satsuma. Soft citrus that were processed accounted for 16 212 tonnes while 10 283 tonnes were sold on the local market. The Soft citrus sector was responsible for a gross value of approximately 7.5 million Rand which was derived mostly from foreign currency. The export prices was approximately R 5 618 per tonne, whilst the lower local price was R 4 082 per tonne and the processed fruit fetching a price of R 214 per tonne in 2011. A price comparison between the Orange and Soft citrus sector is illustrated in figure 2.9. It is interesting to note that this sector is mainly for the fresh market and thus the demand is higher and supply is lower resulting in higher market prices (Anon, 2012b).

Table 2.1 Orange sector production figures from 2009 – 2013

Year	Total production (tonnes)	*Exports (tonnes)	Processed (tonnes)	**Local Market (tonnes)
2009	1 369 474	868 521	254 138	133 552
2010	1 459 417	1 045 254	266 508	134 456
2011	1 428 027	941 695	270 607	137 841
2012	1 428 059	1 036 955	300 468	128 636

*Export values as per PPECB passed for export.

**Local Market as per sales of 20 major fresh produce groups

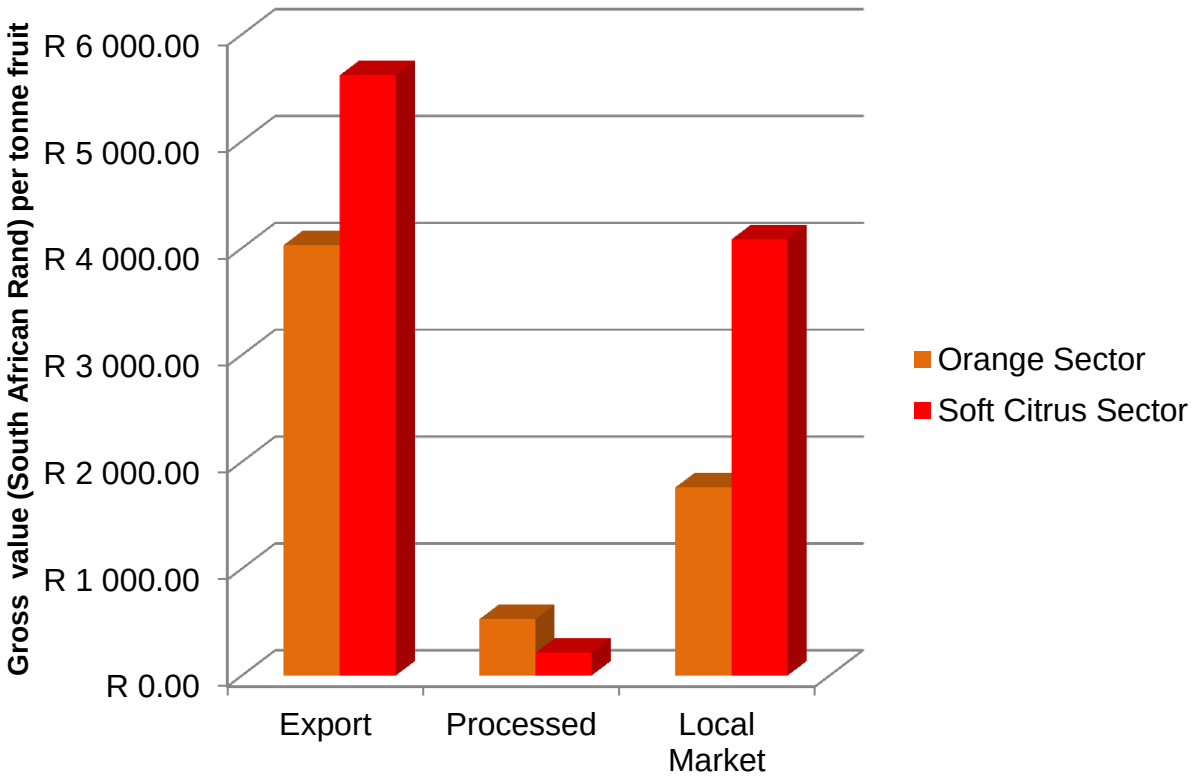


Figure 2.5 Price comparisons between the Orange Sector and Soft Citrus sector during 2012.

D. POST-HARVEST TREATMENTS: JUICE PRODUCTION

Processing of citrus fruit into juice is one of the major post-harvest treatments applied to citrus fruit. In South Africa around 270 000 tonnes of oranges are processed annually into juice for the established juice market (Anon, 2012a; Anon, 2014). Citrus fruit is mainly used to produce frozen orange juice concentrate (FOJC) which is then used in value added citrus juices that is sold on the retail market. A typical flow diagram of juice production is depicted in Figure 2.6. Juice can be extracted from the citrus fruit using one of the two major extraction methods known as the FMC Citrus juice extractor and Brown extractors (Kimball, 1999; Dugo & Giacomo, 2002; Barrett *et al.*, 2005). There is a difference in the extraction method, with the former a hole is cut in the fruit and the juice and pulp is squeezed out of the fruit, while with the latter the fruit is cut in half and the juice and pulp is reamed from the fruit. A filtration step follows the extraction process to remove large solid particles including seeds, peel pieces and heavy pulp by using a Finisher. The juice is subsequently deaerated, deoiled and pasteurised in order to reduce the microbial load and inactivate enzymes. The juice can then be further processed into frozen concentrated orange juice commodity or other products.

The concentration of orange juice involves controlled evaporation of water which is a major constituent of orange juice. It offers many advantages to processors such as reducing the bulk volume which has an influence on storage and transportation costs and additionally offers a prolonged shelf life at refrigerated or frozen temperatures since yeast growth is less likely at such high sugar levels. The sugar level is standardised to approximately 60 - 65 °Brix and can be used to prepare ready-to-drink juices (11.5 - 12 °Brix). The concentrated juices are normally obtained using Thermally Accelerated Short-Time Evaporators (TASTE) which heats the juice under vacuum using hot steam in order to avoid excessive heat damage (Galaverna & Dall'Asta, 2014).

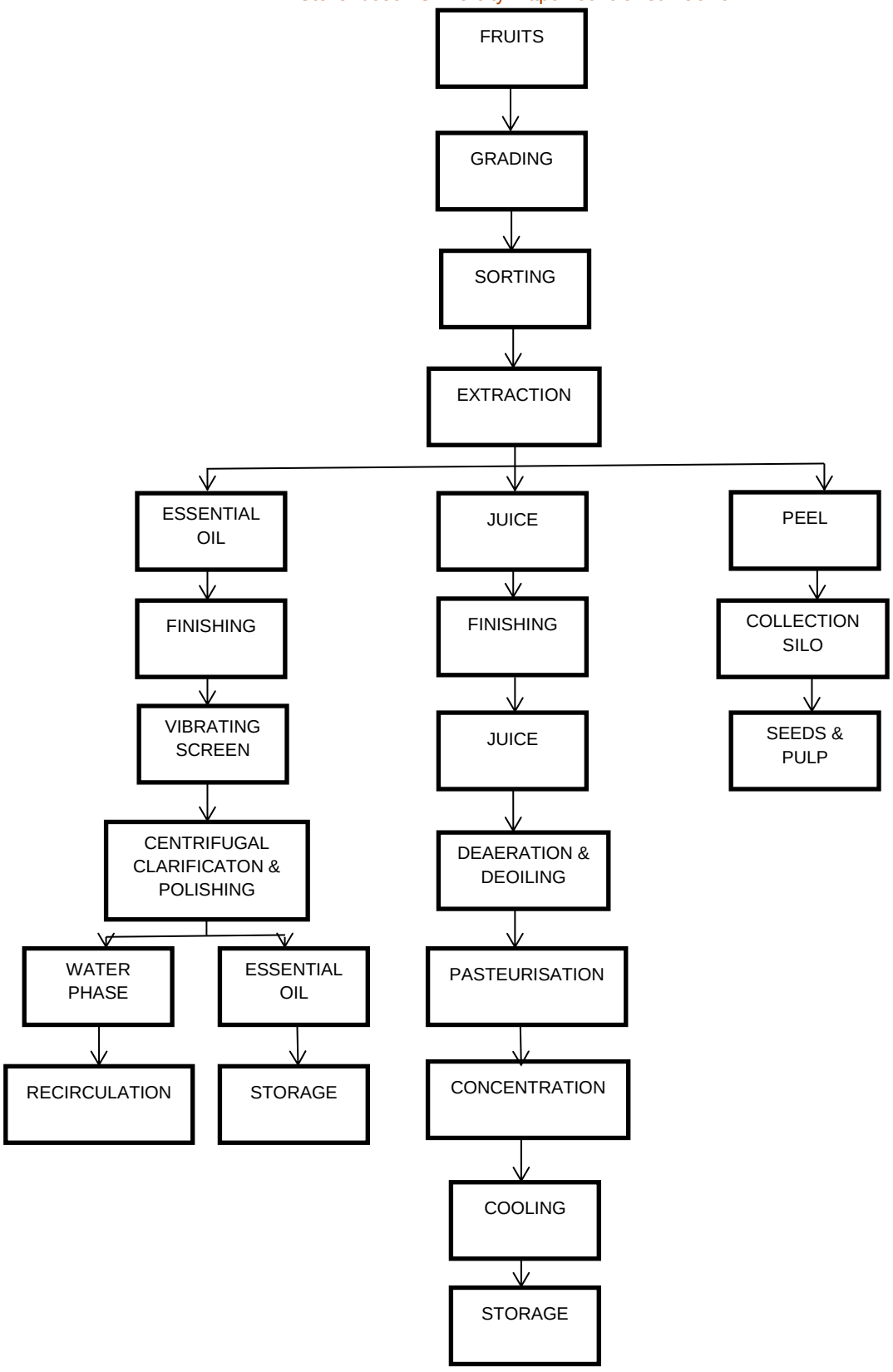


Figure 2.6 A flow diagram of citrus fruit processing into concentrated orange juice and essential oil (Dugo & Giacomo, 2002).

E. CHEMICAL AND PHENOLIC COMPOSITION

Citrus Flavonoids

Flavonoids are widely distributed in nature and can be found in many fruits and vegetables and are mainly yellow plant pigments (Harborne *et al.*, 1975; Gattuso *et al*., 2007). These natural plant pigments are derivatives of benzopyrane and have a typical chemical structure (Fig. 2.7a), consisting of two benzene rings that enclose a heterocyclic six membered ring that contains an oxygen atom (pyrone ring) (Harborne *et al.*, 1975; Gattuso *et al*., 2007). They are differentiated and classified by the presence of some phenolic hydroxyl groups (which can be free, methylated or bound to a sugar moiety) and differences in the heterocyclic ring (Park *et al.*, 1983).

Citrus flavonoids can be divided into six classes according to their molecular structures. These classes are flavones, flavanones, flavonols, iso-flavones, flavan-3-ols (catechins) and anthocyanidins. The latter only occurs in blood oranges (Tripoli *et al.*, 2007). The predominant citrus flavonoid is flavanones.

Flavanones can occur in the aglycone form of which naringenin and hesperetin are the most important, or glycoside form (Harborne *et al.*, 1975, Tripoli *et al.*, 2007). Generally, *O*-glycosides are the most common in citrus fruit and juices and contain a sugar moiety at the C-7 or C-3 position, for example rutin which is a 3-*O*-rutinoside. The two disaccharides commonly linked as *O*-glycosides in citrus are the neohesperidosides and rutinosides (Fig. 2.7 b & c). Flavanone *O*-glycosides with neohesperidose moiety (rhamnosyl-α-1,2-glucose) are naringin, neohesperidin and neo-eriocitrin which have a bitter taste (Horowitz, 1961; Tripoli *et al.*, 2007). Flavanone *O*-glycosides with a rutinose (rhamnosyl-α-1,6-glucose) moiety are hesperidin, narirutin, eriocitrin and didymin (neoponcirin) (Fig 2.7 d). These compounds are without taste. Although *C*-glycosides are also present in large numbers in citrus juices, they are mostly found in the di-*C*-glycoside form with smaller quantities of mono-*C*-glycosides.

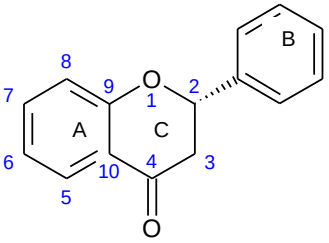


Figure 2.7a Flavanone skeleton.

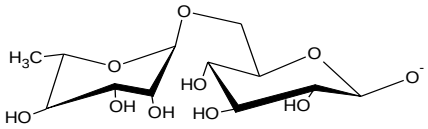


Figure 2.7b Rutinose sugar moiety presenting an α-1,6-interglycosidic linkage.

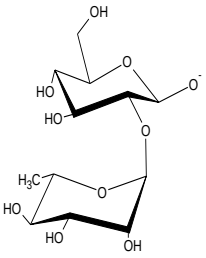
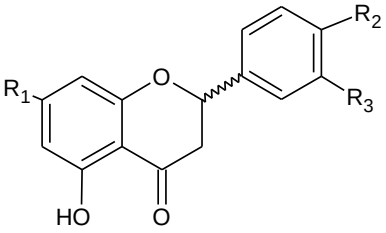


Figure 2.7c Nehesperidose sugar moiety with a α-1,2-interglycosidic bond.



	Hesperidin	Narirutin	Didymin
R1	*O*-Rut	*O*-Rut	*O*-Rut
R2	OCH_3	OH	OCH_3
R3	OH	H	H

Figure 2.7d Flavanone-*O*-glycosides.

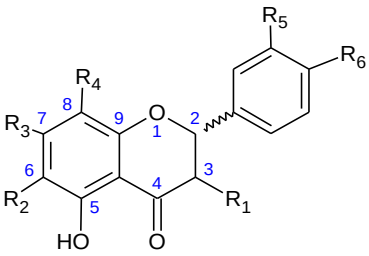


Figure 2.7e Flavone *O*-glycosides

	Rutin	Scoparin	Vicenin-2
R1	*O*-Rut	H	H
R2	H	H	*C*-Glu
R3	OH	OH	OH
R4	H	*C*-Glu	*C*-Glu
R5	OH	OCH_3	H
R6	OH	OH	OH

Figure 2.7 Typical chemical structures of the different types of flavonoids based on the differences in the heterocyclic ring (Ooghe *et al.*, 1994a). Rutinose sugar moiety (*O*-Rut); Glucose monosaccaharide (*C*-Glu).

Generally, diglycosides are found in citrus fruit which provides the characteristic taste. Typically, sugar moieties are found on the C-6 and or C-8 position and currently only D-glucosyl derivatives have been reported (Gattusso *et al*., 2007). An example of a mono-*C*-glycoside with substitution at the C-6 position is isovitexin (apigenin-6-*C*-glucoside) compared to for example, vitexin (apigenin-8-*C*-glucoside), where substitution is at the C-8 position. Nevertheless, substitution can occur at both C-6 and C-8 positions to form a di-*C*-glycoside for example vicenin-2 (apigenin-6,8-di-*C*-glucoside) (Gattusso *et al*., 2007).

Flavanones that occur naturally display the (*S*) configuration at the C-2 position, this is due to enantioselectivity of the chalcone isomerase catalysed intramolecular Michael addition of the chalcone precursor (Rouseff *et al.*, 1987; Rouseff, 1988; Gattusso *et al*., 2007). It has been documented that true citrus species may only contain one of the structural isomers as described above. However, Si-Ahmend *et al*. (2010) found that (*2R*) and (*2S*)-hesperidin epimers exist in a ratio of 1:6 in orange juice. This was ascribed to possible epimerization of flavanone glycosides at the C-2 position. Generally, the citrus cultivar will determine the kind, amount and distribution in number of the flavanone glycoside (Rouseff *et al.*, 1987; Rouseff, 1988). When both isomers are found in a cultivar it can be considered to be hybrid species.

Additionally, it is known that citrus flavonoid content is not only determined by genetic influences but is also dependent on the growing season, location and may well be influenced by post-harvest practices or treatment as is the case with grapefruit (*C.paradisi*) (Albach *et al.*, 1981; Vanamala *et al.*, 2005).

Biosynthesis of phenols and flavonoids

Phenols are plant secondary metabolic products characterised by hydroxyl groups attached to a benzene ring (Shahidi & Naczk, 2004; Davies & Schwinn, 2006). These organic aromatic compounds are widely distributed in plants and are produced for their important role in plant defences, fruit and flower colour, flavour and hormone balance. Thus, these compounds do not form part of the metabolic processes responsible for the growth and development of plants such as for proteins, carbohydrates, lipids and nucleic acids. Nevertheless, they have an equally important and essential role (Shahidi & Naczk, 2004; Davies & Schwinn, 2006). Phenols are formed during the production of aromatic amino acids such as tryptophan, tyrosine and phenylalanine via the shikimic acid pathway. During the formation of phenols,

other aromatic compounds, cinnamic acids and derivatives such as caffeic, sinapic and ferulic acid are formed. Cinnamic acids are formed from phenylalanine and the reaction is catalysed by phenyl ammonia lyase enzyme (PAL). From tyrosine, *p*-coumaric acid is formed, and the reaction is catalysed by tyrosine ammonia lyase (TAL), which is important in the formation of flavonoids. Furthermore, cinnamic acids are bound to sugars in ester or glycoside forms and when bound to the latter, flavonoids are formed (Shahidi & Naczk, 2004; Davies & Schwinn, 2006).

Phenols occur either as monocyclic (catechol, hydroquinone, *p*-hydroxycinnamic acid), dicyclic- (flavanones) and polycyclic (cyanins - pigments) forms (Shahidi & Naczk, 2004; Davies & Schwinn, 2006). Phenols are mostly found conjugated with other molecules including mono- and disaccharides which form glycosides, as is the case with flavonoids. Phenols are mostly tasteless, phenolic acids are sour in taste, condensed flavan-3-ols are astringent and in the case of glycosylated phenols they are bitter in taste. Cinnamic acids and flavan-3-ols decrease with fruit maturity, whilst the level of other phenolic compounds such as anthocyanidins and anthocyanins increases. The solubility of phenols depends on the presence of polar or hydrophilic groups (hydroxyl groups). Typically glycosylation increases the solubility of flavonoids. Flavonoids also have the ability to form complexes with metals due to the hydroxyl and carbonyl groups in their structure (Potter & Hotckiss, 1999; Shahidi & Naczk, 2004; Davies & Schwinn, 2006). In conjunction with polyphenols' ability to terminate oxidation reactions affords flavonoids antioxidant properties.

The polyphenolic content of citrus fruit vary and increases in flavanone concentration (Ladaniya, 2008). For example, neohesperidose-derivatives in grapefruit and rutinose-derivatives in lemon and sweet oranges have been found during the early growth stages. However, increased phenol levels have also been documented after infection by pathogens. Polyphenols are oxidised by a polyphenol oxidase enzyme or enzymes in order to form potent antifungal agents which inhibit the pectolytic enzymes from invasive fungal pathogens. Citrus fruit are known to protect themselves from intrusive fungal pathogens by lignification of wounds, which is derived from lignin and in turn originates from hydroxylated cinnamic acid (Ladaniya, 2008). Ferulic and *p*-coumaric acid have also been implicated in the formation of lignin-like molecules which helps citrus fruit protect themselves. In valencia citrus fruit, injured cells showed an increase in the lignin layer and free phenolics as well as a decrease in conjugated phenolics when grown under humid conditions. This increased lignin layer

provided a physical barrier that impeded and inhibited the infiltration of *Penicillium digitatum* (Ismail & Brown, 1975). Therefore, the variability in polyphenolic content of citrus fruit is dependent on various factors such as growth stage as well as periods of cell stress and repair.

The flavonoid biosynthetic pathway has been well-documented and completely characterised. Flavonoids are produced from carbohydrate metabolic and phenylpropanoid pathway products (Shahidi & Naczk, 2004; Davies & Schwinn, 2006; Khan *et al.*, 2014). The two precursors responsible for the initiation of flavonoid formation are malonyl-coenzyme A (CoA) (derived from carbohydrate metabolism) and *p*-coumaryl-CoA (originating from the phenylpropanoid pathway). Three malonyl-CoA molecules condenses with *p*-coumaryl-CoA to form 2',4,4',6'-tetrahydroxychalcone (yellow compound), while chalcone synthase (CHS) catalyses the reaction. The product is unstable and soon forms 4',5,7-trihydroxyflavanone by chalcone isomerase (CHI) (Fig. 2.8). The latter is believed to be the foundation for the synthesis of all other flavonoid classes (flavones, flavonols, iso-flavone, flavan-3-ols). Consequently, flavonoids are characterised by a common structure, the flavan nucleus containing two aromatic rings (A and B) and a pyran ring (C) (Fig 2.7a). In addition, variances in the location of the B-ring and C-ring lead to classification of flavonoids (2-phenylbenzopyrans) and isoflavonoids (3-phenylbenzopyrans) as well as neoflavonoids (4-phenylbenzopyrans). The 2-phenylbenzopyrans can be further classified into 3-hydroxyflavonoids, which include the flavonols, flavan-3-ols, anthocyanidins and dihydroflavonols, as well as flavanones and flavones which are without substitution at the C-3 position. The latter classes are also the most abundantly distributed in plants. Furthermore, flavanones are distinguished from flavones by a double bond at the C-2-C-3 position (Marais *et al.*, 2006).

Biosynthetic enzymes are responsible for catalysing flavanone formation and consequently will influence the distribution of flavanones in different fruit and plant parts. This is mainly due to flavonoid functions in the various parts of plants and may include defence against pathogens, functions in plant reproduction, disease resistance and protection against UV radiation. For example, flavanones are found in highest concentration in the citrus peel compared to the fleshy part of citrus fruit (Nogata *et al.*, 2006). Additionally, enzymes from *Citrus* species, such as UDP-glucose flavanone-7-*O*-glucosyltransferase (UFGT) and UDP-

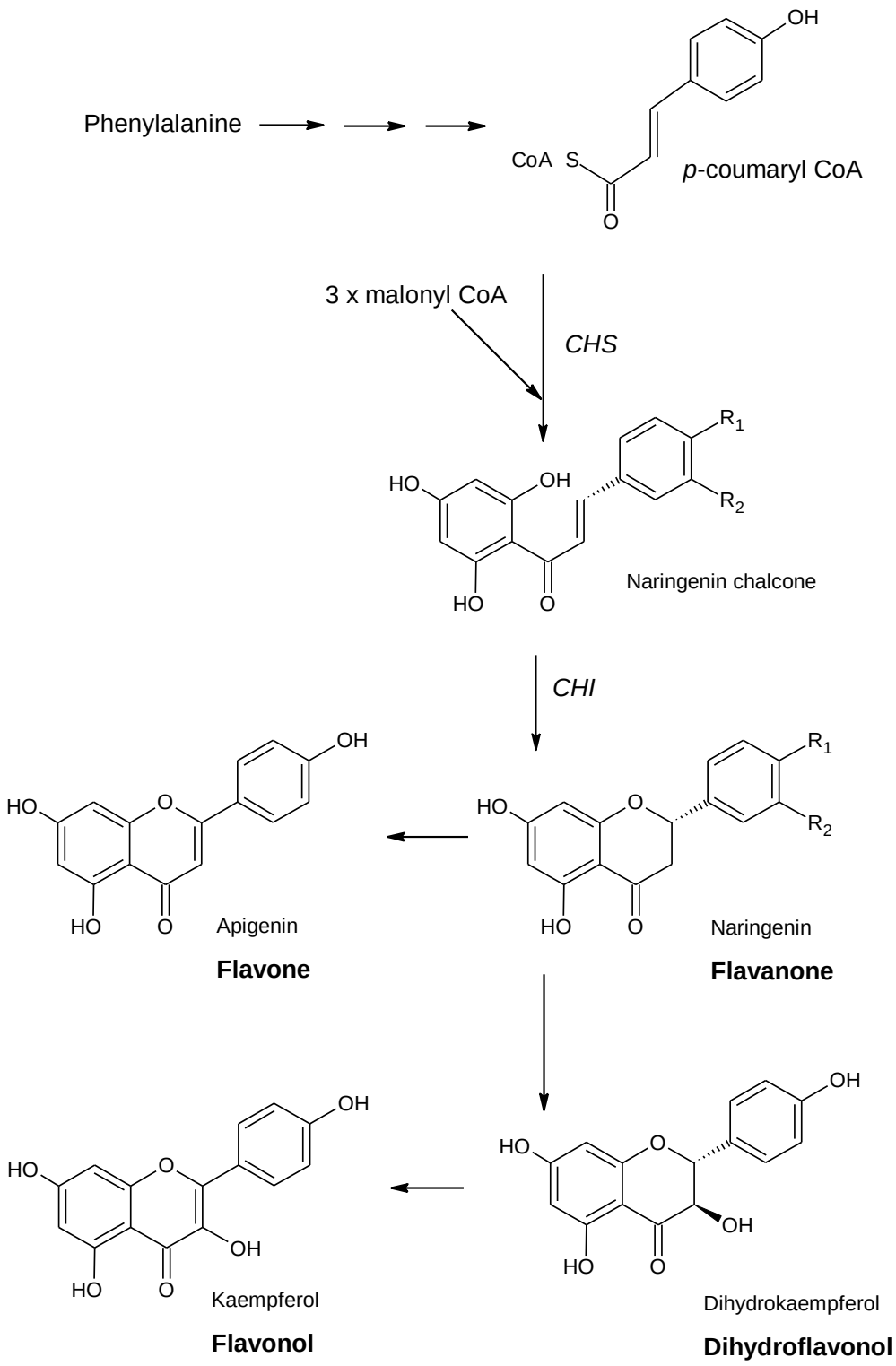


Figure 2.8 Basic synthesis of citrus flavonoids initiated by chalcone synthase (CHS) and chalcone isomerase (CHI).

rhamnose flavanone glucoside rhamnosyltransferase (UFGRT) are also responsible for the conversion of flavanone aglycones into their 7-*O*-β-D-glucosides and rhamnoglucosides (Lewinsohn *et al.*, 1989).

Distribution of flavonoids amongst citrus varieties

Every citrus species has specific flavanone composition and therefore specie identification is possible in commercial citrus juices (Marini & Balestrieri, 1995; Mouly *et al.*, 1994; Ooghe & Detavernier, 1997). In grapefruit (*Citrus paradise* Macf.) and sour oranges (*Citrus aurantium* L.) such as bergamot (*C. bergamia*), neohesperidoside flavanones like naringin, neohesperidin and neo-eriocitrin are mostly present (Horowitz, 1986; Mouly *et al.*, 1994). Although rutinoside flavanones like hesperidin, narirutin and didymin are also present in bergamot (*C. bergamia*) in low concentrations, they are mostly found in mandarin (*C. unshiu* and *C. clementine*) as well as in sweet oranges (*C. sinensis*) (Table 2.2).The neohesperidoside flavanone naringin is never found in sweet oranges (*C. sinensis*) and its presence indicates adulteration (Mouly *et al.*, 1994; Ooghe *et al.*, 1994a).

Flavanones have previously been used as markers to identify various citrus varieties (Mouly *et al.*, 1994; Ooghe *et al.*, 1994a). However, it is sometimes difficult to rely on the flavonoid glycosides (FG) only in order to differentiate between mandarin as well as sweet orange species. Therefore, it is more useful to use the differences in FG as well as polymethoxylated flavones (PMF) as a tool to discriminate between species (Mouly *et al*., 1998). PMF (Fig. 2.9) are mainly found in the flavedo of citrus fruit. During citrus fruit processing into juice, the PMF enters the juice with the essential oil that remains in the juice after deoiling the juice. Since these two compound classes belong to different families of compounds due to structural differences, different separation methods are normally employed to quantify it in orange juice. Thus, Mouly *et al*. (1998) proposed a method that will separate and quantify FG and PMF simultaneously using liquid chromatography. Furthermore, Luezzi *et al*. (2000) studied the FG and PMF of pigmented orange (*Citrus sinensis*) in single-strength juice, concentrated juice and second pressure extracts (SPE) and detected both groups of compounds simultaneously at wavelengths 278 nm and 325 nm, respectively. Flavanone glycosides included narirutin, hesperidin and didymin whilst the PMF included sinensetin, qeurcetogetin, nobiletin, isoscutellarein, heptamethoxyflavone and tangeretin. Similar results

were obtained when the FG for the single-strength juice and concentrate was compared. During the industrial juice production of single-strength juice and concentrate it was found that there is an initial reduction of hesperidin levels which is ascribed to its low solubility. Hesperidin values obtained for the juice ranged from 555 - 761 $mg.L^{-1}$ compared to 470 - 614 $mg.L^{-1}$ in the concentrate. No significant changes in the other FG levels were observed between the single-strength juice and concentrate. In addition, sinensetin and nobiletin were the major PMF found in the juice and concentrate, though in trace concentrations. Considerably higher concentrations of PMF were found in SPE. The authors concluded that higher amounts of PMF found in juices can indicate adulteration with SPE. Moreover the results indicated that sampling at different stages in the juice production line at different temperatures also resulted in no changes in the PMF levels detected. Thus, indicating that FG and PMF remained relatively stable during juice production after initial juice extraction.

Dhuique-Mayer *et al.* (2005) found that the major flavanone glycoside in orange species cultivated in the Mediterranean area were hesperidin and narirutin. It was reported that clementine and mandarin varieties contained the highest amount of hesperidin from all the species and varieties analysed. Hesperidin in clementine juice was found to be 754 $mg.L^{-1}$, whilst the lowest hesperidin content was reported for valencia orange juice (257 $mg.L^{-1}$). In a similar study, Cano *et al.* (2008) substantiated that hesperidin and narirutin was the most abundant flavanone glycoside found in oranges. In the study the hesperidin content for juice from the various orange species and varieties (obtained in Valencia, Spain) was reported to range from 132 - 606 $mg.L^{-1}$ for clementine, 374 - 585 $mg.L^{-1}$ for satsuma, 609 - 1046 $mg.L^{-1}$ for navel and 577 $mg.L^{-1}$ for valencia. The satsuma group displayed the highest narirutin concentration (276 $mg.L^{-1}$) and clementine the lowest concentration (71 $mg.L^{-1}$). Furthermore, the study found that there were no significant differences in the hesperidin concentrations of navel and valencia orange juice. However, a significant difference was found between the clementine and satsuma oranges. There was no significant difference in the narirutin concentrations of the navel and satsuma orange juices. Likewise, there was no significant difference in the narirutin concentrations of navel and valencia juices.

Vanamala *et al.,* (2006) investigated the difference in total bioactive flavonoid content in made-from-concentrate (MFC) and not-from-concentrate (NFC) juices. These juices were not species or variety specific and the difference in bioactive flavonoid content was based on post-harvest treatment. The study indicated that the major flavonoids present in MFC orange

Table 2.2 Flavanone-*O*-glycosides content (mg.L^{-1}) in Citrus fruit juice documented in literature

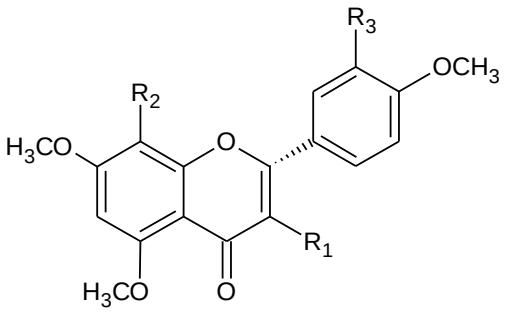
	Hesperidin	Narirutin	Naringin	Neohesperidin	Eriocitrin	Neo-eriocitrin	Didymin (Neoponcirin)	References
Sour orange (*C.aurantium*)	traces	traces	136-362	97-209	-	-	-	Rouseff *et al.*, 1987;
	-	0.55-0.66	21.89-24.85	16.91-19.28	0.72-0.87	8.19-9.87		Barreca *et al*., 2011;
	-	0.8	188.3	110.9	53	140.1	28.9	Peterson *et a*l., 2006
	-	-	19.7	8.7	-	7.7	-	Gattuso *et al*., 2007
Sweet orange (*C.sinensis* Valencia)	151	27	nd	nd	-	-	-	Goulas & Manganaris., 2012
	6024 ug/g DW*	787 ug/g DW*	13 ug/g DW*	-	-	-	-	Peterson *et al*., 2006
	152.5	23.3	1.7	-	2.8	0.4	4.5	Dhuique-Mayer *et al*., 2005
	257 577	51.4	-	-	-	-	18.9	;Gattuso *et al*., 2007
Sweet orange (*C.sinensis* Navel)	135-232 609 – 1046	31-56	nd	nd	-	-	-	Wu et al., 2004
Mandarin (*C.reticulata*)	192.6	27	nd	nd	0.2	-	11.1	Peterson *et al*., 2006
	243	39.2	-	-	3.1	0.2	14.4	Dhuique-Mayer *et al*., 2005;
								Gattuso *et al*., 2007
	2610 ug/g DW* 374 - 585	439 ug/g DW* 276	-	-	-	-	-	Ye *et al*., 2011 Cano *et al*., 2008
Clementine (*C.clementina*)	399 754 132 - 606	46.4 71	0.8	-	-	-	-	Dhuique-Mayer et al., 2005; Gattusoet al., 2007; Cano et al., 2008

*results expressed as µg.g-1 dry weight

Table 2.2 Flavone-*C*-glycosides, Flavone-*O*-glycosides content (mg.L^{-1}) and antioxidant activity of Citrus fruit

	Flavone-*C*-glycosides				Flavone-*O*-glycosides				Antioxidant activity (µM TE/ ml)				
	Lucenin-2	**Vicenin-2**	**Lucenin-2,4'-methyl ether**	**Rhoifolin 4'-glucoside**	**Narirutin 4'-*O*-glucoside**	**Diosmin**	**Rhoifolin**	**Total flavonoid content mg/L**	**DPPH**	**ABTS given as TEAC**	**FRAP**	**ORAC**	**References**
Sour orange (*C.aurantium*)	0.07-0.16	1.33-1.54	0.4-0.51	0.33-0.38	0.12-0.18		0.61-0.96	65.6	10.8	21.3			Barreca *et al*., 2011;
	-	-	-	-	-	-	-		188.67 – 303.33				Moulehi *et al*., 2012 Peterson *et al*., 2006
Sweet orange (*C.sinensis* Navel)	-	-	-	-	-	-	-	0.78 – 1.21			10.2-12	18.1	Abeysinghe *et al*., 2007 Wu *et al*., 2004
Mandarin (C.reticulata)	-	-	-	-	-	-	-	*0.65 -2.78*	*210 – 577*		*10 – 13*		Abeysinghe *et al*., 2007 Moulehi *et al.,* 2012 Peterson *et al.,* 2006

Lucenin-2 (Luteolin-6,8-di-*C*-glucoside)
Vicenin-2 (Apigenin-6,8-di-*C*-glucoside)
Lucenin-2,4'-methyl ether (Diosmetin-6,8-di-*C*-glucoside)
Rhoifolin-4'-glucoside (apigenin-7-*O*-neohesperidoside-4'-glucoside)
Diosmin (Diosmetin-7-*O*-rutinoside)
Rhoifolin (apigenin-7-*O*-neohesperidoside)



Polymethoxyflavones	R1	R2	R3
Hexamethoxyflavone	OCH_3	H	OCH_3
3,3',4',5.6,7,8-Heptamethoxyflavone	OCH_3	OCH_3	OCH_3
Natsudaidain	OH	OCH_3	OCH_3
Nobiletin	H	OCH_3	OCH_3
Sinensetin	H	H	OCH_3
Tangeretin	H	OCH_3	H
5-hydoxy-6,7,4'-trimethoxyflavone	H	H	H

Figure 2.9 Compounds classified as polymethoxyflavones generally found in the essential oil fraction of citrus peels.

juices were hesperidin, narirutin and didymin in concentrations ranging from 329 - 548, 44 - 80 and 117 - 257 $mg.L^{-1}$ respectively. Moreover, the NFC orange juices were found to have hesperidin, narirutin and didynim in concentrations ranging from 180 - 428, 295 - 541 and 116 - 314 $mg.L^{-1}$. An interesting finding was that MFC orange juices had a significantly higher total flavonoid content (532 $mg.L^{-1}$) compared to NFC orange juices with a total flavonoid content of 365 $mg.L^{-1}$.

Bioactive properties of citrus flavonoids

Citrus fruit have been associated with relatively high concentrations of vitamin C, carotenoids, flavonoids and other polyphenolic compounds which all contribute to its health-promoting properties. High consumption of citrus fruit and juices have been linked with reducing the risk of contracting different types of cancer, cardiovascular and neurological diseases, mainly due to antioxidants naturally present in the fruit and juice (Diplock, 1994; Kaur & Kappoor, 2001;

Landbo & Meyer, 2001; Heim *et al*., 2002; Vinson *et al*., 2002). Furthermore, flavonoids occur as glycosides and other polymers and are generally digested to a variable extent in the human digestive tract where absorption occurs which reduces plasma indices of oxidative status (Hollman *et al.,* 1996). Nevertheless, it is the chemical structure of flavonoids which affords it the ability to inhibit free-radical mediated events. The radical scavenging and chelating activity of flavonoids are dependent upon the number, types and position or spatial arrangement of phenolic hydroxyl (OH) groups (Khan *et al.,* 2014). Although literature offers vast discrepancies in documented antioxidant activities of flavonoids, it can be ascribed to the diversity, multiple mechanisms of action together with various methods of initiation, detection and measurement *in vivo* and *in vitro*. Despite the latter, some flavonoid structure-activity relationships do exist and have been well established *in vitro*. The presence of multiple OH groups typically increases the chelating, antioxidant and pro-oxidant potential (Heim *et al.,* 2002; Khan *et al.*, 2014). Methoxy groups (*O*-methylation) increases the lipophilicity of flavanones which shows a decrease in antioxidant activity (such as neohesperidin, hesperetin, isosakuranetin) and others even become pro-oxidant (such as naringin, narirutin, naringenin, neo-eriocitrin, eriodictyol). Flavanones therefore exhibit higher antioxidant activity in hydrophilic environments. Furthermore, flavanones with a double bond, carbonyl group in the heterocyclic structure or polymerization of the nuclear structure increase the antioxidant activity. This is due to the flavonoid radical being more stable through conjugation and electron delocalisation (Heim *et al*., 2002).

Reactive oxygen species (ROS) and Reactive Nitrogen species (RNS) are responsible for the oxidation of cellular proteins, nucleic acids and lipids. They are necessary and produced in low concentrations for physiological signalling pathways. However, larger amounts are produced in order to destroy bacteria, viruses and leucocytes during infection due to inflammatory responses in the human body. This is known as oxidative stress. The initiation of degenerative diseases is due to chronic exposure to oxidative stress (Khan *et al.,* 2014). Phenolic compounds are comprehensively studied for their capability to reduce ROS/RNS which includes superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), hypochlorite ion (ClO^-), nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), lipid oxyl ($RO^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$) which are produced during autoxidation of polyunsaturated fatty acids. The defensive capacity of flavonoids is due to the following functions in biological

systems: transferring electron radicals, chelating metal catalysts, activation of antioxidant enzymes, reducing α-tocopherol radicals and inhibition of oxydases (Heim *et al*., 2002).

As mentioned previously vast discrepancies in total antioxidant capacity has been documented in literature due to many reasons. However, some findings will be discussed and compared. Abeysinghe *et al.*, (2007) evaluated and compared the bioactive compounds (flavonoids, vitamin C and total antioxidant capacity) in different edible tissues of four citrus varieties. The contribution of flavonoids (naringin and hesperidin) and vitamin C to overall total antioxidant capacity (TAC) was investigated based on ferric reducing antioxidant power (FRAP) assays. The four citrus varieties included species of *Citrus unshiu*, *Citrus reticulata*, *Citrus sinensis* and *Citrus changshanensis*. Various parts of the fruits were analysed and included juice sacs (JS), segment membrane (SM) and segment (Seg). Hesperidin was found to contribute to 18.5 - 38.5% of the total phenolics in three of the citrus species. The segment membrane was also found to have the highest TAC (*C*. *changshanensis).* Furthermore, it was found that vitamin C was the major contributor to TAC for all species analysed and ranged from 26.9 - 45.9%. However, this was contrary to what was described in other studies for other fruits (Sun *et al*., 2002). Nonetheless, Gardner *et al*. (2000) and Yoo *et al*. (2004) likewise reported that vitamin C as contributing 65 - 100% of TAC in citrus juice. Hesperidin was found to contribute 5.9% to 54% to the TAC for all tissues and was highest in the segment membrane. Moreover, naringin was found to contribute the lowest percentage to the TAC in all the species and fruit parts. The marked difference in the contribution of the two flavanones hesperidin and naringin was ascribed to the possible differences in flavanone structure and more specifically the different number and position of the hydroxyl groups.

F. IMPACT OF GEOGRAPHICAL AND ENVIRONMENTAL CONDITIONS ON FLAVONOID COMPOSITION AND ANTIOXIDANT CAPACITY OF CITRUS

In the late 1970's the first research pertaining to origin discrimination of frozen concentrated orange juice (FCOJ) was conducted by McHard *et al.* (1976). The authors' aim was to use pattern recognition of the trace elemental composition in order to distinguish whether the FCOJ originated from Florida, Brazil, Mexico and or California. Interestingly, origin discrimination is still a hot topic after decades of research and over the years flavonoid profiling of various citrus varieties from various growing regions over different continents have

been conducted with the view to identify and quantify species markers and, in addition, possibly result in specie and also origin discrimination. Mouly *et al.* (1994) were able to successfully distinguish between sweet orange species using the FG profile of valencia, navel, blood, Thompson and Malta citrus fruit. Dhuique-Mayer *et al*. (2005) used the FG, carotenoids and vitamin C content to differentiate between 8 orange varieties and 2 mandarin species grown in the Mediterranean area. Furthermore, Green *et al*. (2007) isolated and quantified the major PMF present in the peel of 20 citrus varieties grown in Jamaica and Mexico with a view to ascertain information regarding geographical origin and chemotaxonomic markers.

It has been well documented that geographical, environmental and climatic conditions affect the growth and development of citrus fruit and will therefore result in distinguishable phytonutrient contents, which includes flavonoids (Hagen *et al*., 1966; Albach *et al*., 1981; Girennavar *et al*., 2008; Hilal *et al*., 2008). However, since climatic conditions can vary from season to season, vast variability in phytonutrient composition of citrus fruit cultivated in the same region over different seasons can be expected. This adds to the degree of difficulty for the establishment of a database containing information about the phytonutrients of different citrus species cultivated worldwide. Hagen *et al*. (1966) reported that FG of Texas Ruby Red Grapefruit varied during the growing season and concluded that it was not only due to changes in maturity resulting in dilution of FG but that other factors are also involved. Furthermore, Albach *et al*. (1981) studied the annual and seasonal changes in naringen of Texas Ruby Red Grapefruit juice over 5 consecutive seasons. The study showed that naringin concentrations were subject to seasonal variation (during the growing season as well as crop years). 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. Girennanvar *et al*. (2008) studied the influence of growing location, processing and storage on the levels of furocoumarins (dihydroxybergamottin [DHB], paradisin A and bergamottin) in Rio Red and White Marsh grapefruit cultivars. The study found that DHB decreased throughout the season for both cultivars. In addition, the cultivars differed with Marsh White having higher DHB concentrations than Rio Red grapefruit. Similarly, paradisin A and bergamottin was found to decrease as the season progressed in Rio Red grapefruit. On the other hand, bergamottin levels showed an increase during the middle of the season for the Marsh White cultivar. Geographical differences were also

observed for the individual furocoumarin levels of the Rio Red and Marsh White cultivars. Besides, processing effects and storage further influenced the individual furocoumarin concentrations with clear variation due to different processing and storage conditions.

Furthermore, extensive research done on other fruits such as pomegranates showed that agro-climate and seasonal variation in bioactive compounds do occur (Mphahlele *et al*., 2014). Different total phenolic contents were observed for pomegranates grown in different regions (elevation and temperature). It was concluded that altitude may affect the biosynthesis of phenols (Mditshwa *et al*., 2013). Moreover, seasonal variation in the total polyphenol content and gallotannins were observed in pomegranates harvested during different seasons, whilst no difference was observed in the total anthocyanin and total flavonoid content. It was concluded that the latter was not affected by the growing season (Fawole & Opara, 2013).

Moulehi *et al*. (2012) studied the effect of variety and ripening on the phenolic composition and antioxidant capacity of mandarin (*C. reticulata*) and bitter orange (*C.aurantium*) seed extracts. A total of 22 phenolic compounds were identified in both species. Naringin, hesperidin and gallic acid were the most abundant in mandarin in comparison with naringin and neohesperidin in bitter orange. Antioxidant activity was measured using DPPH free radical scavenging and β-carotene/linoleic acid systems. In addition, three stages of maturity were included (immature - IM, semimature - SM, commercial mature - CM). The results for the major flavanones are given in Table 2.2. Variety and fruit ripening was associated with variation in total polyphenols and flavonoids. Highest values was seen during the SM ripening stage for both mandarin and bitter orange, with flavonoids ranging from 1.31 - 2.52 mg and catechin equivalents.g^{-1} DW and total polyphenols ranging from 0.68 - 2.11 mg gallic acid equivalents. g^{-1} DW. Overall, mandarin was found to contain higher amounts of flavonoids. The antioxidant activity, measured using DPPH, of both varieties followed a similar trend, with increases in antioxidant activity during ripening. Therefore, at the CM stage the highest antioxidant activity was obtained with IC_{50} values of 210 µg.mL^{-1} for mandarin and 188.70 µg.mL^{-1} for bitter orange. On the other hand, bitter orange was found to have twice as high antiradical activity (1.91-2.93 mg.mL^{-1}), as measured by β-carotene bleaching capacity, compared to mandarin (3.65 – 4.90 mg.mL^{-1}) which was significant. Thus, it was concluded that variety and stages of ripening had a significant effect on total flavonoids, total polyphenols and antioxidant activity.

G. IMPACT OF POST-HARVEST PROCESSING ON FLAVONOID AND ANTIOXIDANT CAPACITY

The effect of thermal processing on vitamin C has been studied extensively in order to understand the degradation that occurs during and after processing. It is due to the instability of vitamin C which causes a decline in antioxidant activity after processing and during storage. The decomposition of vitamin C follows a first order kinetic reaction and the rate at which it is depleted is temperature dependent and is described by the Arrhenius equation (Vervoort *et al*., 2011; Juhász *et al*., 2012; Galaverna & Dall'Asta; 2014).

Vervoort *et al*. (2012) investigated the effect of thermal and non-thermal (high pressure - HP and pulsed electric field - PEF) processing on the chemical and biochemical characteristics of orange juice and found that there were no significant differences in the vitamin C content after processing and storage. However, the author made use of "mild" thermal pasteurisation (72°C for 20 s) which did not render the juice "shelf-stable" since the orange juice showed some pectin methylesterase (PME) activity. It was fair to compare the thermal and non-thermal processes (even though some heat is generated during HP and PEF) since the degree of treatment for all the processes were similar in terms of PME inactivation and product shelf life. Similarly, Bull *et al*. (2004) and Plaza *et al*. (2006) found that ascorbic acid degraded at a slower rate after thermal processing (65°C for 60 s; 70°C for 30 s and 85°C for 25 s) when compared to HP and or PEF processing. On the other hand, other authors found that heat processed orange juices showed a greater loss in ascorbic acid when compared to an enhanced retention of ascorbic acid in PEF processing (Polydera *et al*., 2003; Polydera *et al*., 2005; Elez-Martínez & Martín-Belloso, 2007). This result can be attributed to the fact that the authors made use of harsher thermal treatments (90 - 95°C for 20 - 90 s and 80°C for 30 - 60 s) in order to produce shelf-stable juices and comparing it to non-shelf stable juices such as the case with PEF processed juices. Furthermore, the variation in FG and PMF in industrially squeezed, pasteurised, concentrated, refrigerated or storage at 20°C valencia orange juice was studied (Sentandreu *et al.*, 2007). The total antioxidant activity (TAA) measured by DPPH assay was ascribed to the contribution of three different compounds; those characterised by fast-kinetics (FAA) such as vitamin C, fast and slow-kinetics such as hydroxycinnamic acids and slow-kinetics antiradical activity (SAA) such

as flavanones and flavones. After comparing the results obtained at each step (industrial squeezing, pasteurisation and concentration) to that of hand-squeezed juice, no significant changes were observed between the FG and PMF. This indicated that industrial extraction and thermal stabilisation methods does not affect the flavonoid content. Although, a slight decrease in the PMF was found as well as a significant decrease in vitamin C (FAA) and soluble hesperidin during storage. These changes were deemed to be temperature dependent, since a slower decrease was observed at lower temperatures. In general, heat treatment will affect vitamin C the most, while storage conditions have a greater impact on the overall degradation of bioactive compounds in orange juice.

In summary, thermal stabilising treatments negatively affect orange juice antioxidant components which include carotenoids, anthocyanins and most importantly decreases vitamin C. It has been shown that HP processing can increase carotenoid and flavanone content due to improved extraction of these compounds and have limited to no thermal damage occurs. However, no increase in antioxidant capacity was noted. PEF treatment has a marginal effect on vitamin C and protects other components from degradation. Finally, thermally accelerated short-time evaporation (TASTE) which is used in the production of FCOJ is very effective in maintaining antioxidant components due to its low operating pressure and therefore temperature; this better preserves the vitamin C content which is the major contributor to TAA.

H. METHODS OF FLAVONOID, ANTIOXIDANT AND VITAMIN C ANALYSIS

Various methods for the quantification of total phenols and specific groups of phenolic compounds have been developed and employed. Chromatographic techniques, specifically high-pressure liquid chromatography (HPLC), are mostly employed for the identification and quantification of specific phenolic compounds. This is mainly due to the specificity and accuracy of HPLC methods. In addition, it allows for the simultaneous determination of various phenolic compounds within a group, in this case flavonoid glycosides. This results in the quantification of flavanone-*O*-glycosides, flavone-*O*-glycosides, flavone-*C*-glucosides and polymethoxyflavones using one method. Therefore, amongst the various analytical techniques, HPLC methods will be discussed together with LC-MS as well as various antioxidant assays.

High performance liquid chromatography (HPLC)

This is the most widely applied method documented in literature for the quantification of phenolics and specifically for citrus flavonoid classes. Flavonoid profiles can be obtained for fruit juice samples using HPLC without prior sample preparation or extraction. This method is extensively used and typically involves reversed-phase chromatography 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. Chromatographic conditions used and documented in literature is summarised in Table 2.3. Generally, diglycosides (more polar) elute before monoglycosides, followed by aglycones. This is due to the polarity of the groups of compounds. 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 280 - 290 nm, flavones at 304 - 350 nm and flavonols at 352 - 385 nm (Gattuso *et al*., 2007).

Structural characterization of phenolics is often done using HPLC coupled with tandem mass spectroscopy (HPLC-MS). Ionisation techniques employed includes fast bombardment mass spectrometry (FAB-MS), electrospray ionization mass spectrometry (ESI-MS) and atmospheric pressure chemical ionization (APCI-MS), although ESI-MS is most common. ESI and APCI ionizes compounds to obtain pseudomolecular ions $[M+H]^+$ and fragments may be formed using collision-induced dissociation (CID). Acetic acid or formic acid added to the mobile phase generally assists with ionization. The molecular mass of a compound can be determined from the pseudomolecular ion, while the fragments give information on the nature and/or position of substituents of the A and B rings. However, it is not always possible to obtain enough data from MS detection for a complete flavonoid structure analysis. Flavonoids are normally detected in negative ionisation mode due to a lower sensitivity obtained for many flavonoids in positive ionisation mode (Gattuso *et al*., 2007). Recent developments have shown the use of HPLC-DAD-ESI-MS-MS for qualitative and quantitative analysis of citrus juices. This allows for simultaneous monitoring of chromatograms at different wavelengths giving UV spectrum and full MS-MS spectra for each peak.

Table 2.3 Various HPLC methodologies employed for the characterisation of citrus products documented in literature

	Variety	Plant part	Sample preparation / Extraction	Column	Solvents	Compounds	Reference
1	*C. sinensis* L., var. Valencia late	RTD and freshly squeezed juice	UAE and acid hydrolysis	RP C18 Hypersil ODS (250 x 4.6 mm, 5 µm)	Milli-Q water pH 2.5 + 50 mM ortho-phosphoric acid (85%) and MeCN	naringenin hesperetin	Plaza *et al*., 2011
2	*C. sinensis*	peel	Instant controlled pressure drop (ICPD) technology and UAE	RP C18 (250 x 4 mm, 5 µm)	0.5% acetic acid and 100% MeCN	naringin hesperidin	Allaf *et al.*, 2013
3	*C. unshiu* Marc. Satsuma mandarin	peel	UAE	RP C18 (250 x 4.6 mm, 5 µm)	4% acetic acid/100% MeOH (80:20, v/v)	narirutin hesperidin	Ma *et al.*, 2008
4	*C. clementina*	fruit	solvent extraction of γ-irradiated fruits	RP C18 (150 x 4.5 mm, 5 µm)	4% acetic acid/100% MeCN	naringin hesperidin didymin eriocitrin poncirin neoeriocitrine	Oufedjikh *et al*., 2000
5	*C. reticulata* Blanco, *C. tankan* Hayata, *C. reticulate x C. sinensis*, *C.sinensis* (L.) Osbeck	edible and inedible fruit portions	soxhlet extraction	RP C18 (250 x 4 mm, 5 µm)	2% acetic acid/ 0.5% acetic acid-MeCN (50:50, v/v)	naringin hesperidin neohesperidin diosmin luteolin sinensetin rutin quercetin kaempferol caffeic acid chlorogenic acid ferulic acid sinapic acid *p*-coumaric acid	Wang *et al*., 2007
6	Unspecified	MFC and NFC orange juices	none	RP C18 (250 x 4.6 mm, 5 µm)	4% acetic acid and MeCN/water	hesperidin narirutin didymin	Vanamala *et al.*, 2006
7	*C.aurantium* , *C. reticulata* Blanco	juice and seeds	solvent extraction of seeds	RP C18 (250 x 4.6 mm, 4 µm)	MeCN/water and 0.2% sulphuric acid	epigallocatechin catechin rutin naringin hesperidin quercetin amentoflavone gallic acid cafeic acid chlorogenic acid vanillic acid syringic acid ferulic acid rosmarinic acid trans-2-hydroxycinammic acid	Moulehi *et al.*, 2012

Table 2.3 continues

	Variety	Plant part	Sample preparation / Extraction	Column	Solvents	Compounds	Reference
8	*C. reticulata*	fruit	solvent extraction of freeze dried sample	RP C18 (150 x 4.6 mm, 3 µm)	acetic acid-water (0.5:99.5 v/v) and MeOH	quercetin glycoside apigenin derivate kaempherol glycoside eriocitrin narirutin rutin hesperidin kaempherol-3-O-rut isorhamnetin-3-O-rut	Abad-García *et al.*, 2007
9	*C. clementina* Hort. Ex Tan. X *C. tangerina* Hort. Ex Tan, *C. unshiu* Marc. X *C. nobilis* Lour, *C. reticulata* Blanco x *C. sinensis* L. Osb.	juice	solvent extraction	RP C18 (250 x 4 mm, 5 µm)	4% acetic acid and MeOH/water	eriocitrin neoeriocitrin narirutin naringin hesperidin didymin naringenin apigenin diosmin sinensetin nobiletin tangeretin quercetin gallic acid chlorogenic acid	Sdiri *et al.*, 2012
10	*C. reticulata x C. paradisi*	juice	solvent extraction	RP C18 (250 x 4.6 mm, 5 µm)	MeOH/water	lucenin-2 vicenin-2 rutin narirutin-4'-O-glucoside neoeriocitrin narirutin neohesperidin didymin sinensetin nobiletin tangeretin	Barreca *et al*., 2013

Ready-to-drink (RTD)

Ultrasound assisted solvent extraction (UAE)

Instant controlled pressure drop technology (ICPD)

Made-from-concentrate (MFC)

Not-from-concentrate (NFC)

This method was used for trace analysis of citrus flavanones and is expected to improve future development of LC-MS applications (Abad-García *et al*., 2012).

Total antioxidant capacity

Total antioxidant capacity (TAC) assays can be divided into those that are based on hydrogen atom transfer (HAT) or electron transfer (ET) (Huang *et al.*, 2005). The HAT-based assays involve a competitive reaction scheme where the antioxidant and substrate compete for thermally generated peroxyl radicals through decomposition of azo compounds. Amongst these assays is oxygen radical absorbance capacity (ORAC), total radical trapping parameter (TRAP), inhibition of low-density lipoprotein autoxidation and crocin bleaching assays. ET-based assays include the total phenols assay by Folin- Ciocalteu reagent (FCR), Trolox equivalent antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). These assays measure the capacity of an antioxidant to reduce an oxidant which will change colour when reduced. The degree of colour change is correlated with the TAC of the samples (Huang *et al.*, 2005). Some of the aforementioned assays have been applied to functional food as well as botanical samples. The major antioxidant capacity assays which are applied to fruit and vegetable juices will be discussed further.

TRAP, ORAC and crocin bleaching assays involve the use of molecular probes. These colorimetric and fluorometric antioxidant capacity assays apply a radical reaction but without a chain propagation step which is an essential step during lipid autoxidation (Huang *et al.*, 2005). The relevance of this approach to radical chain-breaking antioxidant capacity is thus debatable. The mechanisms in general apply a thermal radical generator to give a steady flux of peroxyl radicals in an air-saturated solution. Consequently, the added antioxidant competes with the molecular probes for the radicals and retards the molecular probe oxidation. The components of the assay involve: i) an azo radical initiator usually AAPH, ii) a molecular probe (UV or fluorescence) in order to monitor the progress of reaction, iii) antioxidant; and iv) reaction kinetic parameters collected for antioxidant capacity quantification. The kinetic curves derived from the reaction are similar for ORAC, TRAP and crocin bleaching assays, but different end-points are used to quantify the TAC, namely the

area under the kinetic curve (AUC) for ORAC, the lag time for TRAP and the reaction rate for crocin bleaching assay (Huang *et al.*, 2005).

ORAC was first developed by Cao *et al*. (1993). Their method was however flawed due to the use of β-phycoerythrin (β-PE) a fluorescent protein as probe. β-PE was found to have large variability between batches since it was produced from *Porphyridium cruentum,* was bleached under plate-reader conditions, interacts with polyphenols and loses fluorescence without the addition of the radical generator. Hence, β-PE was consequently replaced by fluorescein (FL) (3',6'-dihydroxyspiro{isobenzofuran-1[3*H*], 9'[9*H*]-xanthen]-3-one). This improved method allows for direct measure of hydrophilic and lipophilic chain-breaking antioxidant capacity versus peroxyl radicals (Huang *et al.*, 2005). This method may for some antioxidant samples result in a different kinetic curve to that of the Trolox standard curve (AUC). This is normally caused by sample matrix interference when the sample have a low antioxidant activity and thus large amounts of sample is required in order to give a measurable AUC. Advantages of this method are that it applies equally well for antioxidants that exhibit specific lag phases and those that have no lag phases. This is useful for food samples with complex reaction kinetics since it unifies the lag time and initial rate method. A broad range of samples; raw fruit and vegetable extracts, plasma, and pure phytochemicals have shown a direct linear correlation of AUC. ORAC has many advantages and has been proven to be reliable. This is also the reason why ORAC is so broadly applied by researchers and the food and supplement industry. Therefore, it is no surprise that an antioxidant database using ORAC in combination with the total phenols assay have been established (Prior *et al.*, 2003; Wu *et al*., 2004).

DPPH which is an ET-based assay is convenient since the organic nitrogen radical is stable and commercially available. The reaction can also be monitored using a simple spectrophotometer (Huang *et al.*, 2005). The organic nitrogen radical has an absorption maximum at 515 nm and upon reduction loses colour. Even though this method is simple by nature it does offer some limitations in its application. DPPH is a long-lived nitrogen radical with no similarity to that of the highly reactive transient peroxyl radicals involved in lipid peroxidation. There are many antioxidants that may react slowly or not at all with DPPH. As a result, the antioxidant capacity is not properly determined. Moreover, the reaction kinetics between the antioxidants and DPPH is not linear to DPPH concentrations. In addition the presence of adventitious acids and bases present in the solvent can cause a reduction or

amplification in the measured rate constants due to the influence on the ionization equilibrium of phenols. This renders the DPPH assay less chemically sound as an antiradical activity assay (Foti & Ruberto, 2001).

The FRAP assay is based on the reduction of the pale yellow triazine-complexed ferric ($Fe^{3+)}$ to its ferrous (Fe^{2+}) form as the signal or indicator. This is a measurement of the total reductive antioxidant action within a sample. The 2,4,6-tripyridyl-*s*-triazine (TPTZ)- Fe^{2+} complex results in a colorimetric product which is deep blue with an absorption maximum at 593 nm (Huang *et al.*, 2005; Benzie & Choi, 2014). This method can be used with relatively simple instruments and the reagents are stable and of low toxicity. It is also regarded as a sensitive and precise method since the stoichiometric factors of reacting antioxidants are constant and small differences in reaction conditions will not affect the results. A modification of this method aiming to quantify ascorbic acid (vitamin C) is known as the FRASC, ferric reducing and ascorbic acid assay (Benzie & Choi, 2014). This method has been validated against HPLC methods and is a one-step modification of the FRAP method. This allows for simultaneous quantification of reductive antioxidants and ascorbic acid. In brief, the FRASC assay involves the preparation of two aliquot samples. One is treated with ascorbate oxidase which depletes the ascorbic acid present in the sample. The other is treated with the same volume of water. The paired samples are run similarly to the FRAP method. The difference between the absorbance of the paired samples is used to calculate the ascorbic acid concentration. Thus, FRAP offers a speedy, inexpensive and flexible alternative for the determination of the combined activity of redox-active antioxidants in food and can offer the added benefit of ascorbic acid determination (Benzie & Choi, 2014).

Vitamin C determination

Vitamin C or total ascorbic acid (TAA) determination in orange juices is common. Though, the method employed by various researchers is not as common. In general, HPLC is mostly used for academic research, whilst industry utilises indophenol (official AOAC method) or iodine (I_2) titration methods. These conventional volumetric titration methods involve I_2 as reagent and require lengthy standardisation processes and excessive amounts of reagents. In addition, interferences from other antioxidants such as flavonoids can lead to incorrect estimations. The instability of AA during storage and even analysis hampers the

determination process and affect the repeatability and reliability of these methods. Moreover, methods that rely on the determination of AA using ascorbate oxidase enzymes have been developed, such as FRASC and is said to be more specific in the quantification of TAA. The above three methods will be discussed further.

It is widely accepted to use HPLC for the determination of AA. However, it is not only the development of the method that is of importance for reliable results, but the sample storage, sample preparation and extraction conditions (Spínola *et al*., 2014). Sample preparation, HPLC analysis conditions and standardisation is the three critical components of HPLC determination. Sample preparation normally includes a stabilisation and extraction phase. This is generally achieved using an acid solution. Typically metaphosphoric acid (MPA) (HPO_3) is used and may be combined with perchloric ($HClO_4$), acetic-, citric- or oxalic acid. The former has been reported to be more efficient (Hernández *et al*., 2006). Subsequently, the available oxidised vitamin C forms is reduced using Tris-[2-carboxyethyl] phosphine hydrochloride (TCEP_HCl). Reverse phase HPLC analysis follow coupled with DAD or UV-Vis detection of AA at 246 nm. Mobile phases may consist of ammonium dihydrogenphosphate (NH_4-H_2PO_4) of which the pH is adjusted to 3.5 which is below the pK_a (4.17) of AA and prevents degradation (Spínola *et al*., 2014; Valente *et al*., 2014). It is important to consider that stabilisation and extraction solutions such as MPA may interact with C18 Silica based columns which can cause a drift in the baseline and retention time of AA (Valente *et al.*, 2014). This can be overcome by using a combination of MPA and perchloric acid instead as described by Valente *et al*. (2014). The column temperature is typically kept at 25°C to avoid thermal degradation of AA and the analysis has a short run time of approximately 5 -15 min. Furthermore, validation of the optimized method is required to ensure its suitability. Key elements of validation studies include: selectivity, linearity, stability, accuracy, precision and lower limit-of-detection (LOD) or limit-of-quantification (LOQ). Other parameters that may be evaluated include: recovery, matrix effect and robustness (Spínola *et al*., 2014).

Another widely accepted method is volumetric titration using sodium 2,6-dichlorophenolindophenol (DCIP) solution. In brief the AOAC method involves stabilisation and extraction of AA using 3% MPA and 8% acetic acid solution which is then titrated against the indophenol solution (25% DCIP and 21% sodium bicarbonate, $NaHCO_3$, in water). Once a rose pink colour appears and persists for 5 seconds the end-point has been reached

(AOAC, 1999). The alternative is to use iodine titration methods. It involves titration of the sample mixed with a starch-acid solution against Iodine solution. The drawback of titrimetric methods is the need for lengthy standardisation processes and the use of excessive amounts of reagents as well as the generation of large amounts of waste.

Vitamin C determination using ascorbate oxidase (AO) enzymes has gained popularity due to the reaction specific oxidation of vitamin C. This coupled with spectrophotometric techniques or a plate reader results in rapid and inexpensive assays (Vislisel *et al*., 2007). Vislisel *et al*., 2007 developed a rapid method using AO and a plate reader. After centrifugation, orange juice samples were dissolved in methanol-water solution ($MeOH/H_2O$, 75:25 v/v) containing diethylene-triaminepentaacetic acid (DETAPAC). After incubation (10 min) followed by centrifugation the supernatant was transferred to a 96-well micro-plate. Tempol stock solution (2.32 mM Tempol in acetate buffer) was added to each well and allowed to incubate for 10 min after which *o*-phenylenediamine (OPDA) (5.5mM OPDA in acetate buffer) was added to each well. The fluorescence was measured at 345 nm excitation filter and 425 nm emission filter. The fluorescence was recorded every 26 sec for 9.5 min. This kinetic assay is based on the formation of dehydroascorbic acid(DHA)-OPDA over time. The initial rate of the reaction is proportional to the initial DHA present in the sample and or standard. The kinetic considerations of the assay are fully explained by Vislisel *et al*. (2007). This assay can detect DHA concentrations between 6.2 - 150 µM. This method is sensitive, rapid and affordable when compared to alternative methods and find application in a wide variety of sample matrices.

CONCLUSION

Over the past 30 years research pertaining to functional properties of citrus has been conducted as the growing awareness of diet linked to health continues. Nevertheless, comprehensive insight into the exact composition of citrus and citrus derived products has yet to be accomplished even though analytical methodologies have improved over the years with increased sensitivity and efficiency. Moreover, it is not only the exact composition of bioactive compounds that are lacking but the mechanisms involved in active metabolism which affords flavanone metabolites its health promoting activities which are unknown. Furthermore, additional information pertaining to the influence of climate, fruit maturity and processing on these beneficial compounds are also required. Interestingly, the Florida Citrus Industry Research Co-ordinating Council ranked the “Health Benefits” of citrus as sixth on the list of research priorities and deemed it as “essential” whilst the “health/nutrition” of fresh juice was ranked in 18th place and deemed as “important” during 2010 (Anon, 2015). In South Africa, there is no evidence of research pertaining to bioactive compounds of South African citrus species and cultivars or their derived products and therefore valuable information is currently missing. This knowledge is required to add to the body of knowledge that already exists, internationally, and can possibly increase the economic value of this already substantial agronomic sector of South Africa and by finding ways to fully exploit citrus fruit, juices and even waste products.

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CHAPTER 3

EVALUATION OF THE TOTAL POLYPHENOL CONTENT AND FLAVONOID CONTENT OF FOUR CITRUS VARIETIES GROWN IN THE WESTERN CAPE

ABSTRACT

Phenolic compounds in citrus fruit are specific for each species and variety and may be influenced by environmental conditions during the growing season and post-harvest practices. The exact chemical composition of citrus produced in South Africa is currently not known even though the country produced 2 million tonne in 2012. Various citrus varieties are produced for export, local fresh markets as well as processed into value-added products sold on the local market.

In this initial screening study, four citrus varieties; clementine; satsuma; navel and valencia, were evaluated to determine whether differences exist in the total polyphenol content (TPC) and total flavonoid content (FLAV) of the four citrus varieties grown in the Western Cape. All citrus fruit were sampled during 2012. One variety, namely navel was sampled during 2011 as well and compared to those sampled in 2012 in order to provide information on the influence of seasonal variation. Juice analysis consisted of °Brix, °Brix:acid ratio, titratable acidity (TA), pH, TPC using a modified Folin-Ciocalteu method and FLAV using the Davis Test.

The results indicated that the TPC and FLAV of navel varieties grown and harvested during 2011 and 2012 did not differ significantly ($p>0.05$) from each other. However, significant differences ($p<0.05$) in the TPC and FLAV were found between the different varieties harvested during 2012. Satsuma had the highest FLAV level (747.61 $mg.L^{-1}$ NE) and differed significantly ($p<0.05$) from the rest. Valencia samples had the lowest TPC of 114.51 $mg.L^{-1}$ GAE which differed significantly ($p<0.05$) and similarly the TA (1.53%) differed significantly and was the highest. The high TA of valencia resulted in a clear grouping on the PCA biplot and was thus negatively associated with TPC and FLAV. The results of this study indicated that varieties grown in the same geographical region differ in terms of TPC and FLAV content and that seasonal influence was not significant. On this basis specific phenolic compounds responsible for the distinction should be further investigated.

INTRODUCTION

South Africa is the twelfth largest citrus producer in the world and the second largest fresh citrus exporter in the world (Anon., 2012a; Anon., 2012b). Approximately 60 foreign countries receive their citrus fruit requirement from South Africa with an annual citrus production of 2 million ton. Although only 69% of the total amount is exported, 24% is sold on the local market and 7% is processed into commodity and value-added products. Citrus fruits are produced throughout six provinces in South Africa with the Western Cape Province producing 15% of the total citrus production. The Western Cape Province is responsible for 46% of the total soft citrus production. The Soft Citrus sector includes satsuma and clementine varieties (Anon., 2012a; Anon., 2012b). The Orange sector includes navel and valencia varieties which are also grown and processed in the Western Cape. Even though citrus production forms a major part of the South African agronomic and economic sector, knowledge of the exact chemical composition of Citrus fruit cultivars and their products (fruit juices, nectars and jams) is necessary, but lacking currently. This information may provide valuable insight regarding the commercial properties of South African citrus fruit varieties and their products.

Citrus fruit has received increasing attention internationally, due to its phenolic composition (Faller & Fialho, 2009). Citrus phenolic compounds are characteristic of species and variety and therefore suitable as chemotaxonomic markers (Mouly *et al.*, 1994; Marini & Balestrieri, 1995; Ooghe & Detavernier, 1997; Tripoli *et al.*, 2007). Nevertheless, quantitative differences do occur since the following may have an impact on phenolic content: climatic or environmental conditions; storage conditions; post-harvest treatments as well as stages of maturity and especially fruit variety (Spanos *et al*., 1990a; Spanos & Wrolstad, 1990; Bengoechea *et al*., 1997; Djoukeng *et al*., 2008).

Flavonoids are the major group of phenolic compounds found in citrus fruits. Flavonoids can be classified as flavanones, flavones and flavonols and can occur either in the free form and/or as glycosides in various parts of citrus fruits (Vanamala *et al.*, 2006). Very little information exists on the variation in flavonoid content of different South African varieties (which are grown in different regions) and the effect of processing techniques on these levels. There is also a need to establish what the levels of these compounds are in the commodity products produced from different citrus varieties. Knowledge of factors, such as variety and stages of maturity, which may lead to quantitative differences, is important to better understand their influence on the biological or functional properties of citrus fruit and products derived from these citrus fruit.

Over the years, sophisticated characterisation techniques have been developed for the detection of phenolic compounds in citrus fruit. These techniques rely on high-pressure liquid chromatography (HPLC) in conjunction with diode array detection (DAD) and in some cases liquid chromatography coupled with electrospray ionisation and mass spectrometry (LC-ESI-MS/MS) for characterisation and identification of citrus specific phenolic compounds (Mouly *et al*., 1999; Caristi *et al*., 2003; Gattuso *et al*., 2006; Abad-Garcia *et al*., 2009; Barreca *et al*., 2011b). However, in this initial screening study the aim was to evaluate whether quantitative differences in the total polyphenol content and flavonoid content occur between four citrus varieties grown in the Western Cape of South Africa using less sophisticated spectrophotometric methods.

MATERIALS AND METHODS

Chemicals

Deionised water prepared using a Milli-Q ultrapure water purification system (Millipore, Microsep, Bellville, SA) was used during all experiments. All reagents used were of analytical grade and included sodium carbonate ($NaCO_3$), sodium hydroxide (NaOH) and diethylene glycol which were sourced from Merck (Darmstadt, Germany). In addition Folin-Ciocalteu reagent and the pure reference standards, gallic acid and naringin were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

Navel fruit samples were collected during the June and July 2011 harvesting season for analysis (n=5). Furthermore, clementine (n=5), satsuma (n=8), navel (n=7) and valencia (n=5) samples were collected during the March – September 2012 harvesting season for analysis. All samples were collected from a local fruit processor in the Western Cape. Sampling consisted of collecting approximately 2 kg composite samples from bins containing the citrus fruit that was located on-site on the day of sampling. Information on variety and harvest date was recorded (Table 3.1). Composite juice samples were obtained for each variety, containing 16 – 45 fruit, depending on variety.

The composite juice samples were prepared by manually hand squeezing the citrus fruit samples. The juice was stored at -18°C prior to analysis. The samples were thawed

Table 3.1 Harvest date, sample weight and number of fruit of various citrus varieties harvested in 2012

Variety	Sample number	Number of fruit	Composite weight (Kg)	Harvest date
Satsuma	1	38	1.83	19 April 2012
	2	50	2.10	18 April 2012
	3	43	2.02	19 April 2012
	4	33	1.69	18 April 2012
	5	39	1.61	18 April 2012
	6	21	2.18	19 April 2012
	7	34	1.99	03 April 2012
	8	30	2.22	20 March 2012
	9	26	1.63	07 March 2012
			17.26	
Clementine	1	31	2.31	11 April 2012
	2	31	2.29	22 May 2012
	3	32	2.48	25 May 2012
	4	34	2.24	29 May 2012
	5	45	2.37	31 May 2012
	6	35	1.71	04 June 2012
	7	38	2.09	08 June 2012
			15.50	
Navel	1	31	2.31	17 May 2012
	2	31	2.29	22 May 2012
	3	32	2.48	24 May 2012
	4	34	2.24	28 May 2012
	5	45	2.37	29 May 2012
	6	35	1.71	30 May 2012
	7	38	2.09	06 June 2012
			15.50	
Valencia	1	16	2.28	22 August 2012
	2	20	2.06	05 September 2012
	3	16	2.34	18 September 2012
	4	16	2.13	19 September 2012
	5	19	2.15	21 September 2012
			10.96	

and centrifuged at 10 000 x *g* (Thermo Electron Industries centrifuge MR 18 – 12 Model#: 11174584, France) for 5 minutes at room temperature. The supernatant was used for analysis.

Determination of total polyphenol content

Total phenolic content of samples were determined by the modified Folin-Ciocalteu method of Cai *et al.* (2004) as reported by Abeysinghe *et al.*, (2007). Aliquots of 0.5 mL supernatant of the juice samples were each diluted with 4.0 mL distilled water. This solution was mixed with 0.5 mL Folin-Ciocalteu reagent and allowed to react for 3 min before the addition of 1.0 mL saturated sodium carbonate. After incubation at room temperature for two hours, the absorbance of the reaction mixture was measured at 760 nm against a deionised water blank on a spectrophotometer (Perkin Elmer Lambda 25 UV/VIS Spectrometer, Singapore). The total polyphenol concentration (TPC $mg.L^{-1}$) was expressed as gallic acid equivalents (GAE).

Determination of total flavonoid content

The total flavonoid content was determined according to the Davis Test (Davis, 1947). The spectrophotometer (Perkin Elmer, Lambda 25 UV/VIS Spectormeter) was zeroed at 420 nm with the reagent blank and then the background absorbance of samples were measured against the reagent blank. Aliquots of 0.5 mL juice supernatant were mixed with 25.0 mL diethylene glycol by repeated inversion of test tubes after which 0.5 mL of 4 M sodium hydroxide (NaOH) was added to samples. After incubation at room temperature for 10 min, the absorbance of the reaction mixture was measured at 420 nm. The total flavonoid concentration (FLAV $mg.L^{-1}$) was expressed as naringin equivalents (NE).

Juice analysis (Total soluble solids, titratable acidity and pH)

Samples (50 mL) were titrated againts 0.1 M NaOH until the end-point of pH 8.1 was reached. The results were expressed as % $w.w^{-1}$ citric acid. The remainder of the samples were used for the determination of pH (pH meter), and total soluble solids (TSS, expressed as °Brix) (hand-held refractometer). In addition, a °Brix:acid ratio was calculated and used as maturity index when comparing fruit maturity (Izquierdo & Sendra, 1993; Wedding & Horspool, 1995).

Statistical analysis

The experimental layout consisted of four fruit types (satsuma, clementine, navel and valencia) of which composite samples were obtained on different harvest dates during the 2012 season. The TPC and FLAV of the 4 fruit types were compared. Only one fruit type (navel) of which composite samples were obtained on different harvest dates during the 2011 and 2012 season, was selected to evaluate whether seasonal differences occur. The TPC and FLAV content of the navel variety was compared for 2011 and 2012 seasons.

Data were expressed as means ± standard deviations (SD). All analyses were performed on two replicate samples. One-way analysis of variance (ANOVA) was performed and the Least Significant Difference t-test were used as a post-hoc test. Differences were considered statistically significant at the 95% confidence level ($p< 0.05$). In addition, the results were analysed using multivariate analysis (Principal Component Analysis). The data for the PCA's were pretreated using correlation matrix.

RESULTS AND DISCUSSION

Seasonal differences

No significant differences ($p>0.05$) were observed between the °Brix (TSS), titratable acidity (TA) and consequently the °Brix:acid ratio of the 2011 and 2012 navel samples (Fig 3.1). Although a significant difference ($p<0.05$) was observed for pH, with 2011 navel samples having a higher pH of 3.57 than the 2012 navel samples (pH of 3.17). This is interesting since TA and pH have an inverse relationship, namely when TA increases, pH decreases. Nevertheless, the TA for the 2011 and 2012 season differed marginally and therefore cannot explain the significant difference in pH observed. The TPC and FLAV of the 2011 and 2012 navel samples (Fig. 3.2) did not differ significantly ($p>0.05$). Both the TPC and FLAV of the 2011 navel samples were slightly higher 534.93 $mg.L^{-1}$ GAE and 276.62 $mg.L^{-1}$ NE compared to 480.37 $mg.L^{-1}$ GAE and 219.10 $mg.L^{-1}$ NE of the 2012 navel samples, with latter accompanied by a slightly higher (although not significant) °Brix:acid ratio. The lower °Brix:acid ratio of the 2011 navel samples may be related since increased maturity in citrus fruits were previously shown to decrease the phenolic content and antioxidant activity (Barreca *et al*., 2011b; Rekha *et al*., 2012).

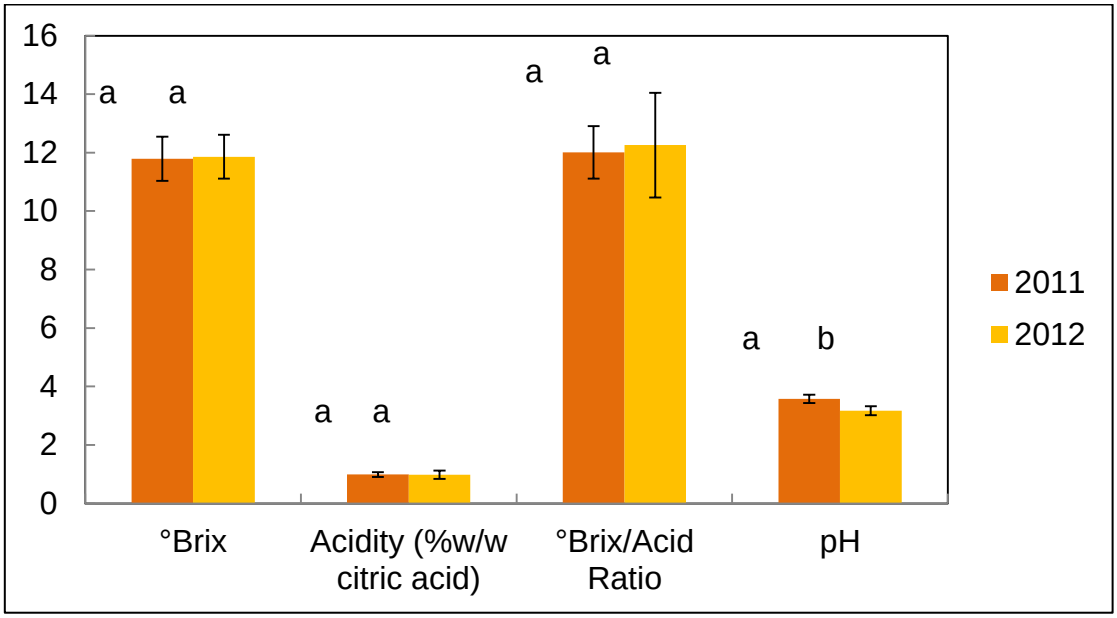

Figure 3.1 Juice parameters (°Brix, titratable acidity, °Brix:acid ratio and pH) of navel citrus fruit harvested during 2011 and 2012 growing seasons. Different letters indicate a significant difference at $p<0.05$.

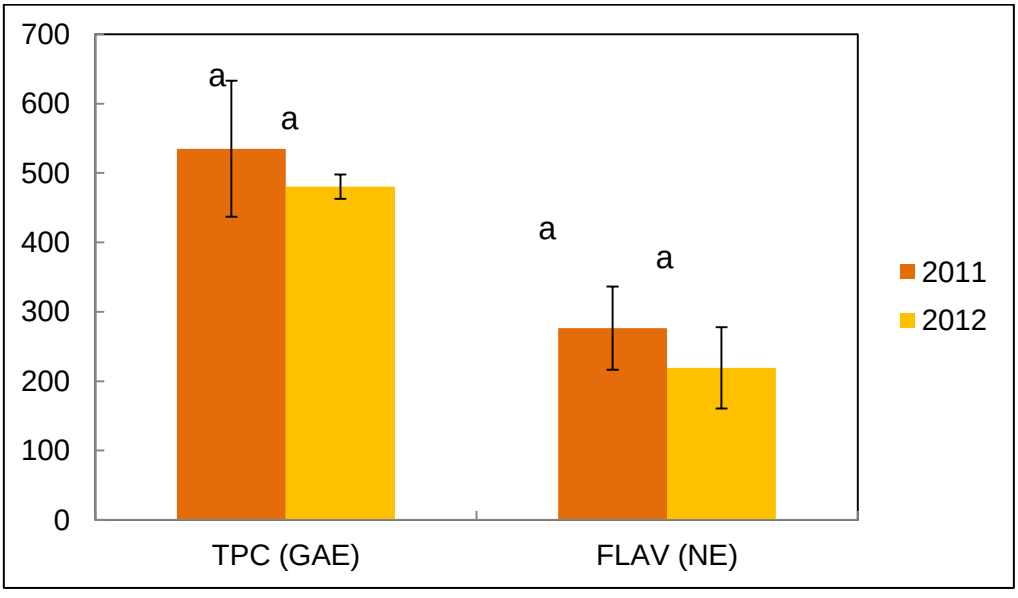

Figure 3.2 Total polyphenol (TPC) and flavonoid content (FLAV) of navel citrus fruit harvested during the 2011 and 2012 growing season. Different letters indicate a significant difference at $p<0.05$.

Seasonal differences (Fig 3.3) were evident from the Principal Component Analysis (PCA). The 2011 season was associated with higher TPC, FLAV, pH and a higher TA. This could possibly further indicate that fruit maturity influences the TPC and FLAV content. °Brix (TSS) and °Brix:acid ratio were found to be significant ($p<0.05$) in its overall contribution to the variability in the data and can be associated with 68.2% of the inherent variability in the data. The 2012 season was associated with a higher °Brix (TSS) and °Brix:acid ratio again proving that the 2012 navel samples were slightly more mature and the lower TPC and FLAV levels may be attributed to this.

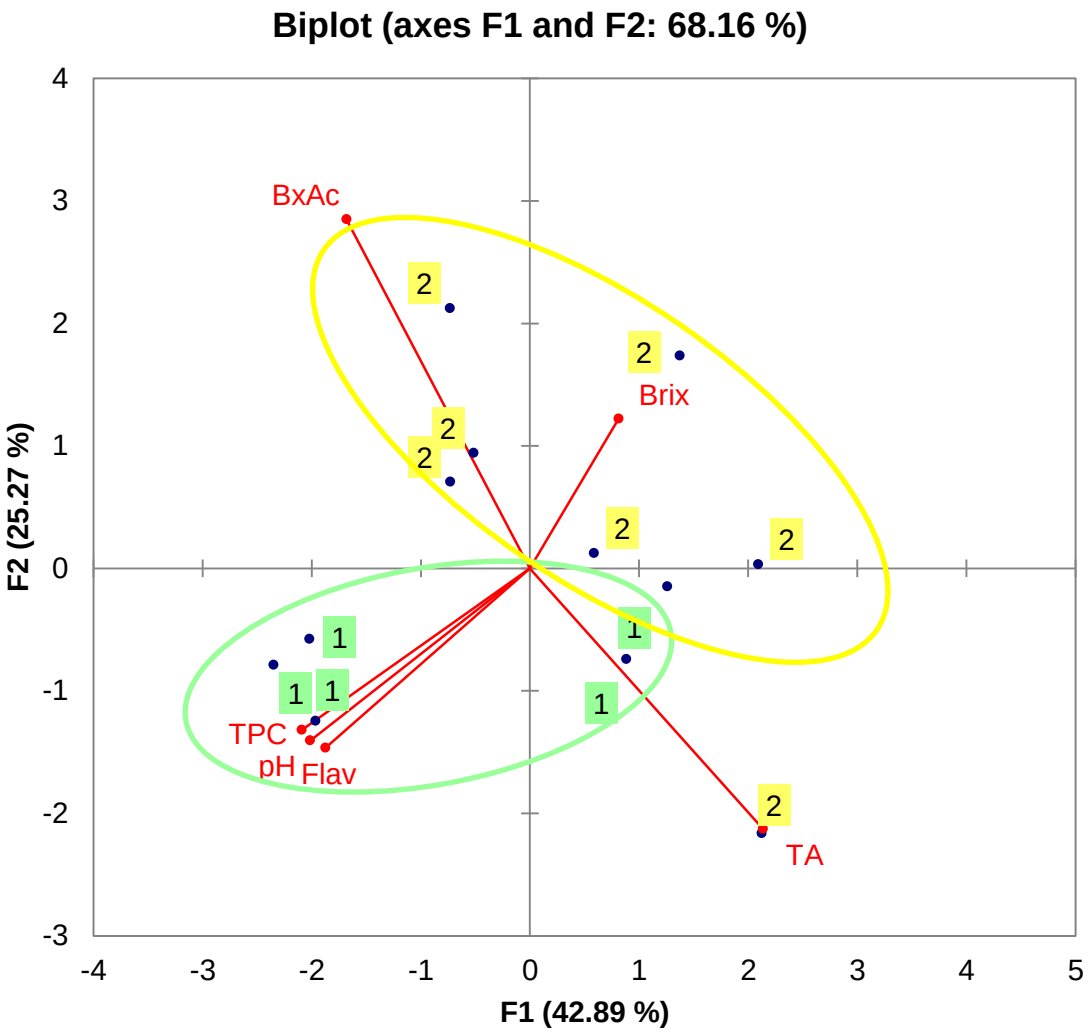

Figure 3.3 PCA biplot of juice parameters, total polyphenol and flavonoid content of navel citrus fruit samples collected during 2011 and 2012 harvesting seasons. TPC = total polyphenol content, Flav = total flavonoid content, Brix = °Brix, TA = titratable acidity, BxAc = °Brix:acid ratio, 1 = samples from 2011 season, 2 = samples from 2012 season.

Varietal differences

Significant differences ($p<0.05$) were observed between the chemical composition of the four citrus varieties analysed. The °Brix (TSS) ranged from 10.95 – 12.50 °Brix and was significantly different ($p<0.05$) with clementine having the highest °Brix (TSS) and valencia having the lowest (Fig 3.4). The results of the post hoc test indicated that the °Brix (TSS) of clementine and navel did not differ significantly (Fig 3.4). The TA differed significantly ($p<0.05$) with valencia having the highest TA of 1.53 % $w.w^{-1}$ citric acid and satsuma having the lowest of 0.84 % $w.w^{-1}$ citric acid. This resulted in valencia having the lowest °Brix:acid ratio and significantly differing ($p<0.05$) from the rest. Clementine and satsuma had the highest °Brix:acid ratio of 14.6 and 13.7, respectively and did not differ significantly ($p>0.05$) indicating these fruit samples were of similar maturity and most mature of all four varieties. In agreement with the TA results, the pH of clementine and satsuma did not differ significantly from each other whilst valencia had the lowest pH of 3.01 which was significantly different ($p<0.05$) to the rest.

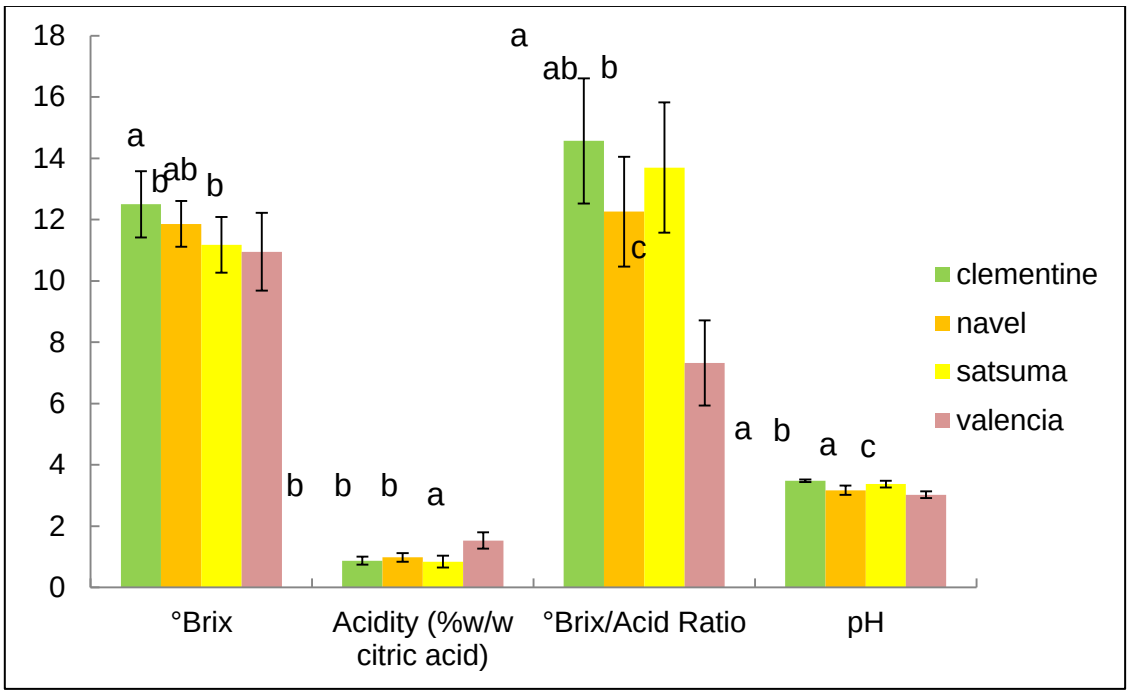

Figure 3.4 Juice parameters (°Brix, titratable acidity, °Brix:acid ratio and pH) of four citrus varieties harvested in the 2012 growing season. Different letters indicate a significant difference at $p<0.05$.

The TPC of the four varieties also differed significantly ($p<0.05$) with valencia having the lowest TPC of 114.51 $mg.L^{-1}$ GAE and clementine having the highest of 481.97 $mg.L^{-1}$ GAE, but the latter did not differ significantly ($p>0.05$) from the TPC of navel and satsuma (Fig. 3.5). The TPC for clementine and satsuma was in line with values obtained for mandarin varieties grown in Spain which ranged from 541.2 – 738.8 $mg.L^{-1}$ GAE (Sdiri *et al*., 2012). However, mandarin hybrids grown in China were reported to have higher TPC levels which ranged from 774.54 – 1555.49 $mg.L^{-1}$ GAE (Xu *et al*., 2008). Roussos (2011) also reported higher TPC levels (964 – 1215 $mg.L^{-1}$ Tannic Acid Equivalents) for sweet orange varieties grown in Greece than what was found for the navel and valencia 2012 samples in this study. Interestingly, the FLAV content of all three varieties (clementine, navel and valencia) were found not to differ significantly from each other and ranged from 124.76 – 338.78 $mg.L^{-1}$ NE. The satsuma variety was the only variety that differed significantly with the highest FLAV content of 747.61 $mg.L^{-1}$ NE. Remarkably, clementine and satsuma varieties were found to be most mature (having the highest °Brix:ratio) and in conjunction also having the highest TPC and FLAV content, thus indicating that fruit maturity is not the only factor impacting on the total polyphenol and flavonoid content but that genetic and species type have a major effect. Thus, when comparing varieties, fruit maturity becomes a less important factor. In addition, the fact that clementine and satsuma had similar results was expected since they are sub-species of the *Citrus reticulata* family.

Furthermore, from the PC Biplot (Fig. 3.6) it is clear that valencia varieties are associated with higher TA levels and thus are separated from the other varieties on that basis. Clementine varieties are positively associated with °Brix (TSS), °Brix:acid ratio and TPC levels while satsuma varieties are positively associated with FLAV. In Figure 3.6 it can also be seen that there is no clear groupings between the navel and satsuma varieties. However, it is notable that the navel variety was positively associated with TPC compared to a negative association of valencia which had a significantly lower TPC level according to the post hoc test. Moreover, the variation (84.4%) in the data can be ascribed to TA, °Brix:acid ratio, TPC and FLAV levels which were associated with a considerable contribution to varietal differences.

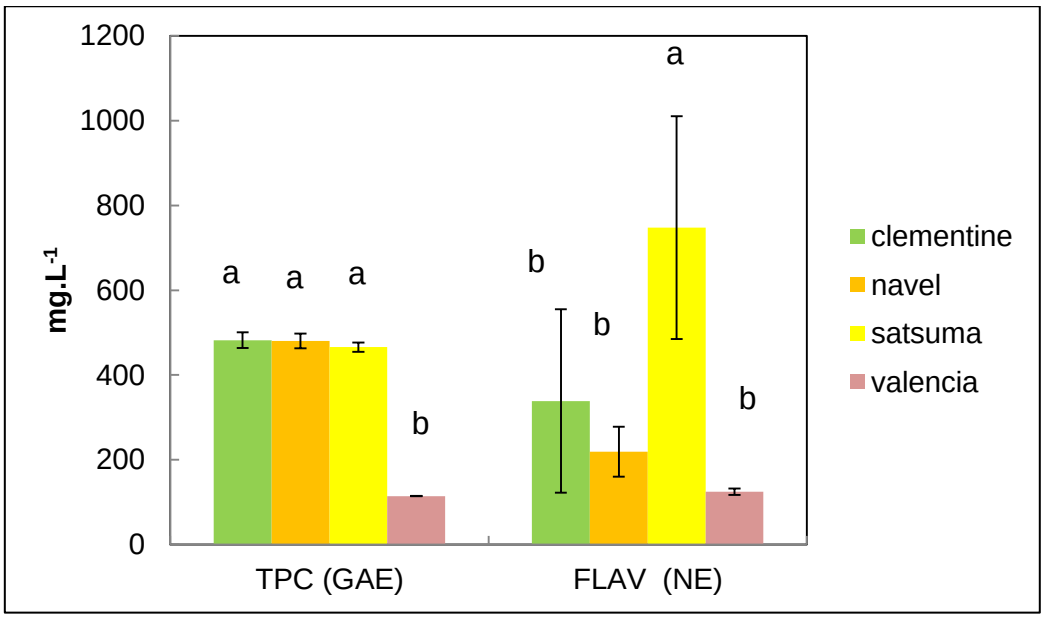

Figure 3.5 Total pholyphenol (TPC) and flavonoid content (FLAV) of four citrus varieties harvested in the 2012 harvesting season.

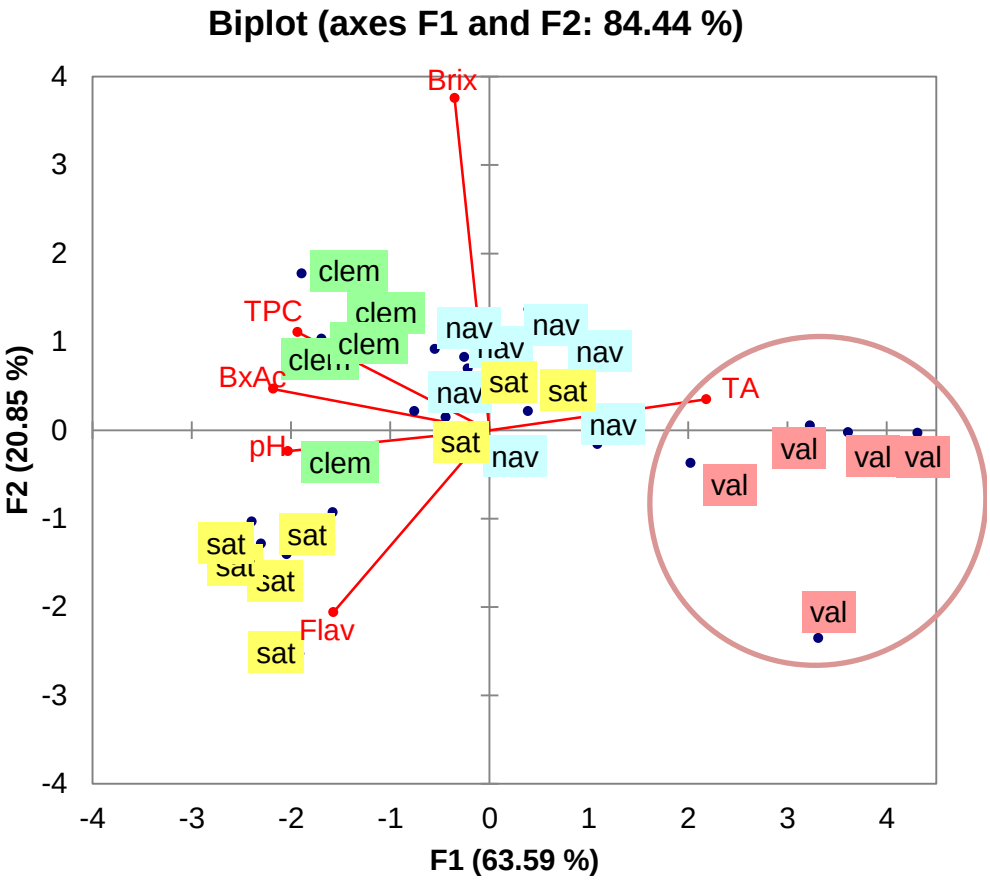

Figure 3.6 PCA biplot of juice parameters, total polyphenol and flavonoid content for four citrus varieties harvested in 2012. TPC = total polyphenol content, Flav = total flavonoid content, Brix = °Brix, TA = titratable acidity, BxAc = °Brix:acid ratio, clem = clementine, sat = satsuma, nav = navel, val = valencia.

CONCLUSION

South Africa produces large volumes of citrus fruits and derived products throughout the year due to the various growing regions throughout the country. The four major varieties found on the fresh and commodity market includes satsuma and clementine (known as soft citrus) and navel and valencia (known as sweet oranges). The polyphenolic content of citrus fruits are greatly influenced by specie genetics, environmental conditions and post-harvest handling.

In this study the TPC and flavonoid content of navel varieties harvested over two seasons from the Western Cape region were evaluated and compared. It was found that the TPC and flavonoid content of navel varieties did not differ significantly from season to season (2011 vs 2012). Moreover, the TPC and flavonoid content of four varieties (satsuma, clementine, navel and valencia) harvested in 2012 were evaluated and compared. It was found that significant differences exist and that valencia varieties had the lowest TPC. Similarly, valencia varieties exhibited the lowest flavonoid content while satsuma varieties showed the highest flavonoid concentration.

Finally, it was found that seasonal conditions were less important regarding differences in phenolic content and that the variety was the determining factor. In addition, there was no clear evidence that maturity of the fruit affected the TPC and flavonoid content of the various varieties investigated. It is however recommended to investigate the individual phenolic profile of each variety to better determine how these varieties differ on species and inter-species level in South Africa in order for it to be compared to varieties grown internationally.

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CHAPTER 4

CHARACTERISATION OF THE FLAVONOID COMPOSITION AND TOTAL ANTIOXIDANT CAPACITY OF JUICE FROM DIFFERENT CITRUS VARIETIES FROM THE WESTERN CAPE REGION

ABSTRACT

Citrus species are known for their nutritional quality and have been described as sources of vitamin C, dietary fibre, folate and other minerals as well as phytonutrients which include flavonoids and phenolic acids. In addition many have reported that citrus species can be distinguished based on their flavonoid composition. Moreover, it has been documented that pre-harvest factors such as climatic conditions and post-harvest treatments such as processing can have an effect on the phenolic composition and antioxidant capacity of citrus fruit.

The phenolic composition and antioxidant capacity of juice from four citrus varieties grown in the Western Cape of South Africa and collected during different seasons (2012 & 2014) were evaluated and compared. Citrus juice characteristics evaluated, included °Brix, titratable acidity (TA), °Brix:acid ratio, pH as well as ascorbic acid (AA). Furthermore, the phenolic composition of the citrus fruit was quantified using high-performance liquid chromatography coupled with diode-array detection (HPLC-DAD). The total antioxidant capacity (TAC) was determined using 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, Oxygen Radical Absorbance Capacity (ORAC) assay and Ferric Reducing Antioxidant and Ascorbic Acid (FRASC) assay.

The phenolic composition of different citrus varieties showed great variation between different seasons. Varietal difference was evident although there was some overlap between citrus varieties within the same season. Hesperidin and narirutin were the predominant flavanone-*O*-glycosides in sweet oranges, which included navel and valencia varieties, with vicenin-2 and ferulic acid-*O*-hexoside also present in high quantities. The satsuma and clementine varieties (soft citrus fruit) differed greatly in terms of phenolic composition, which was unexpected since the two varieties are generally grouped as mandarin fruit. The satsuma citrus fruits had the highest quercetin-3-*O*-rutinoside-7-*O*-glucoside, narirutin and rutin concentrations, while clementine had the lowest in terms of total phenolic compound content. The satsuma citrus variety had the

highest TAC_{ORAC} values and was among the lowest TAC_{DPPH} and TAC_{FRAP} values. The valencia variety was found to have the highest AA content, which resulted in the highest TAC_{FRAP} value. The navel variety had the highest total phenolic compound content; however this was not directly reflected in its TAC values. Overall the citrus fruit samples, collected in 2014, irrespective of variety, had higher AA and as a result also the highest TAC_{FRAP} and TAC_{DPPH} values compared to the samples collected in 2012. Finally, the TAC_{ORAC} proved to be the best assay for measuring TAC of citrus juice due to phenolic compounds since it was the least affected by the AA content.

INTRODUCTION

Citrus fruit are characterised as having a relatively high phenolic content compared to other edible plants (Hollman & Arts, 2000; Moufida & Marzouk, 2003; Hollman & Arts, 2005; Benavente-García *et al*., 2008). Citrus fruit is the most abundantly grown fruit crop in the world, is produced in 15 countries on different continents and is therefore considered to be one of the most important sources of bioactive compounds (Anonymous, 2012; Xi *et al.*, 2014). Juice derived from citrus fruit forms an important dietary source of bioactive compounds which have a direct effect on human health (Roussos, 2011). Citrus bioactive compounds have been reported as being beneficial in cancer prevention, being one amongst the many proposed health-promoting properties (Peterson *et al.*, 2006). The major phenolic compounds found in citrus are flavonoids. More specifically, citrus fruits have a restricted distribution of flavonoid glycosides which are characteristic to the genotype and can be used along with polymethoxylated flavones to differentiate between cultivars (Mouly *et al.*, 1994; Tripoli *et al.*, 2007). The most common flavonoid glycoside classes found in citrus juices are flavanones, flavones and flavonols (Horowitz & Gentilli, 1977). The major flavanones, with a high prevalence in citrus compared to other fruits, have been identified as hesperidin and narirutin (Khan *et al*., 2014). The main flavonols and flavones present in considerable amounts are rutin and vicenin-2 (Gatusso *et al*., 2007). Moreover, citrus species or varieties can be distinguished on the basis of their flavonoid composition (Abad-García *et al*., 2012a; Mouly *et al.*, 1994; Marini & Balestrieri, 1995; Ooghe & Detavernier, 1997). In addition to flavonoid glycosides, citrus juice also contains hydroxycinnamic acids, such as glycoside derivatives of ferulic and sinapic acid (Abad-García *et al*., 2012b). Identification and characterisation of citrus phenolic composition in various varieties and plant parts of Chinese, Spanish, Mediterranean, Mauritian and North-American origin have been extensively researched over the past 30

years (Abad-García *et al.*, 2012b; Mouly *et al.,* 1994; Dhuique-Mayer *et al.*, 2005; Caristi *et al.*, 2006; Cano *et al*., 2008; Ramful *et al*., 2010). Nevertheless, there are yet unknown phenolic compounds that need to be identified and characterised in citrus. Moreover, the natural variability in phenolic composition of citrus fruits and products not only can be ascribed to genetic characteristics, but also to factors such as stages of maturity, climatic conditions or post-harvest processing (Vanamala *et al.*, 2006; Galaverna & Dall'Asta, 2013). Thus in order to truly have a global view of the potential health benefits and to fully exploit citrus as a functional food product, a database which considers all the relevant information pertaining to phenolic compound levels in different citrus varieties, grown on different continents, in different countries and regions are required. This includes information relevant to citrus fruit cultivated in South Africa with a unique climate, as well as unique cultivation and post-harvest practices.

The South African citrus industry is divided into four sectors which include the Orange, Soft citrus, Grapefruit and Lemon sectors. The bulk of citrus fruit is exported, but some are sold on the local retail market or processed into value-added products. There is currently no information on the variation in phenolic composition of different South African citrus varieties. Furthermore, the effect of growing season on the phenolic composition and total antioxidant capacity (TAC) of South African citrus is unknown. Thus, the aim of this study was to characterise the phenolic composition, as well as the TAC of the juice from four citrus varieties (satsuma, clementine, navel and valencia) grown in the Western Cape and to evaluate whether varietal and/or seasonal differences (2012 vs 2014) occur.

MATERIALS AND METHODS

Chemicals

Deionised water used during experiments were purified using an Elix Millipore system (Merck Millipore, Darmstadt, Germany) which was subsequently treated with a Milli-Q Reference A+ system (Millipore) water purification system in order to obtain HPLC-grade water. HPLC-grade acetonitrile was purchased from Sigma-Aldrich (St. Louis, MO, USA), while analytical grade chemicals and reagents including 2,2'-diphenyl-1-picrylhydrazyl (DPPH), (±)-6-hydroxy-2,5,7,8-tertramethylchromane-2-carboxylic acid (Trolox), potassium phosphate (KH_2PO_4), sodium fluorescein, 2,2'-azobis-(2-methylpropionamidine) dihydrochloride (AAPH), sodium hydroxide (NaOH), methanol (MeOH), dimethylsufoxide (DMSO), ascorbic acid and glacial acetic acid were obtained from either Merck Millipore or

Sigma-Aldrich. Pure reference standards (purity ≥ 95%) included rutin from TransMIT (Gießen, Germany), narirutin from Extrasynthese (Genay, France), hesperidin and ferulic acid from Sigma-Aldrich, vicenin-2 and neoponcirin from Phytolab (Vestenbergsgreuth, Germany). An Ascorbic Acid Assay Kit II (MAK075) was purchased from Sigma-Aldrich for Ferric Reducing/Antioxidant and Ascorbic Acid (FRASC) assays.

Sample preparation

Citrus fruit samples were collected during the 2012, 2013 and 2014 harvesting seasons for analysis. Unfortunately, the 2013 samples were destroyed due to freezer failure. All samples were collected from a local fruit processor in Citrusdal in the Western Cape, South Africa. Sampling consisted of collecting *ca* 2 kg composite samples (containing 16 - 50 fruits, depending on variety, which were pooled for one observation) from bins containing the citrus fruit that was located on-site on the day of sampling. Information on variety and harvest date was recorded (Table 4.1a & b). Composite juice samples were obtained for each observation by manual-squeezing the citrus fruit samples. The juice was stored at -18°C prior to analysis. The samples were thawed and centrifuged at 10 000 x g (centrifuge model M-24A, BOECO, Germany) for 5 min at room temperature to remove solids from the juice. The juice supernatant was used for TAC, vitamin C and HPLC-DAD analyses. Prior to HPLC analysis all samples were filtered using 0.45 µm Acrodisc syringe filters with a GHP membrane (Pall Life Sciences, Separations, USA).

Determination of citrus juice characteristics

Titratable acidity (TA) of juice samples was determined using an automatic titrator (Metrohm Titrino 702 autotitrator, Johannesburg, South Africa). Duplicate samples (10 mL) were diluted with 50 mL deionised water and titrated against 0.1 M sodium hydroxide solution (NaOH) until the end-point of pH 8.1 was reached. The results were expressed as citric acid (% $w.w^{-1}$).

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

The pH of juice samples were measured in duplicate with a standard pH meter after calibration with pH 4.0 and 7.0 buffers.

Table 4.1a Citrus samples collected during the 2012 harvesting season

Variety	Sample number	Number of fruit	Composite weight (Kg)	Harvest date
Satsuma	1	38	1.83	19 April 2012
	2	50	2.10	18 April 2012
	3	43	2.02	19 April 2012
	4	33	1.69	18 April 2012
	5	39	1.61	18 April 2012
	6	21	2.18	19 April 2012
	7	34	1.99	03 April 2012
	8	30	2.22	20 March 2012
	9	26	1.63	07 March 2012
			17.26	
Clementine	1	31	2.31	11 April 2012
	2	31	2.29	22 May 2012
	3	32	2.48	25 May 2012
	4	34	2.24	29 May 2012
	5	45	2.37	31 May 2012
	6	35	1.71	04 June 2012
	7	38	2.09	08 June 2012
			15.50	
Navel	1	31	2.31	17 May 2012
	2	31	2.29	22 May 2012
	3	32	2.48	24 May 2012
	4	34	2.24	28 May 2012
	5	45	2.37	29 May 2012
	6	35	1.71	30 May 2012
	7	38	2.09	06 June 2012
			15.50	

Table 4.1b Citrus samples collected during the 2014 harvesting season

Variety	Sample number	Number of fruit	Composite weight (Kg)	Harvest date
Mandarin	1	26	1.62	27 May 2014
	2	20	1.67	10 June 2014
	3	28	2.30	26 June 2014
	4	39	1.50	02 July 2014
	5	18	1.53	03 September 2014
			8.62	
Navel	1	14	1.68	03 June 2014
	2	14	1.70	05 June 2014
	3	13	1.60	05 June 2014
	4	5	1.42	06 June 2014
	5	7	1.31	07 June 2014
	6	8	1.54	07 June 2014
	7	12	1.62	10 June 2014
	8	14	2.34	23 June 2014
	9	12	2.40	24 June 2014
	10	16	2.40	25 June 2014
	11	11	2.33	25 June 2014
	12	15	2.11	26 June 2014
	13	16	2.10	26 June 2014
	14	18	2.25	27 June 2014
	15	12	2.13	27 June 2014
	16	16	2.40	30 June 2014
	17	13	2.10	30 June 2014
	18	13	1.47	30 June 2014
			34.90	
Valencia	1	11	1.64	27 August 2014
	2	8	1.75	02 September 2014
	3	9	1.73	03 September 2014
	4	9	1.40	19 September 2014
	5	7	1.25	21 September 2014
	6	8	1.45	22 September 2014
	7	9	1.44	22 September 2014
	8	7	1.58	22 September 2014
	9	7	1.28	24 September 2014
	10	7	1.21	24 September 2014
	11	9	1.62	29 September 2014
	12	8	1.37	29 September 2014
	13	11	1.37	29 September 2014
	14	14	1.48	01 October 2014
	15	9	1.42	02 October 2014
	16	8	1.53	02 October 2014
	17	8	1.38	03 October 2014
			24.90	

Determination of phenolic composition using high-performance liquid chromatograpy coupled with diode-array detection (HPLC-DAD)

The individual phenolic compounds were quantified using an Agilent 1200 series HPLC (Agilent, Santa Clara, CA, USA). The instrument was equipped with an in-line degasser, quaternary pump (maximum pressure 400 bar), autosampler, column thermostat and diode-array detector (standard 13 µL flow cell and 10 mm path length). Chemstation software controlled the system and was used for the acquisition and analysis of data. A Gemini-NX C18 (3 µm particle size, 110 Å pore size,150 × 4.6 mm ID, Phenomenex, Santa Clara, CA, USA) column was used and fitted with a guard column (4.0 x 3.0 mm ID, Phenomenex).

The gradient described by Leuzzi *et al*. (2000) was adapted to a 150 mm column length and evaluated using a navel and mandarin juice sample. The gradient was subsequently adapted to provide optimal separation for the major phenolic compounds present. Separation was performed at 31°C with a flow rate of 1.0 $mL.min^{-1}$ using 0.2% acetic acid (A) and acetonitrile (B) in a gradient described in Table 4.2. The injection volume was varied depending on the concentration of phenolic compounds in the sample to obtain peak areas within the calibration curves.

Table 4.2 Final solvent gradient program for analysis of citrus fruit samples

Time (min)	% Acetonitrile
0	5
3	5
40	27
41	100
43	100
45	5
55	5

Phenolic compounds were identified based on co-elution with authentic reference standards and/or comparison of UV-Vis and mass spectrometric (MS) spectra with those of authentic reference standards and literature reports. LC-DAD-MS analysis were performed on an Acquity UPLC system equipped with a binary solvent manager, sample manager, column heating compartment and diode-array detector coupled to a Synapt G2 Q-TOF system with an electrospray ionisation source (Waters, Milford, USA). Data were

acquired in resolution mode (scanning from 150–1000 amu) and MS^E mode and processed using MassLynx v.4.1 software (Waters). The negative ionisation mode was used and the instrument was calibrated using a sodium formate solution. Leucine enkephalin was used for lockspray. The MS parameters were as follows: capillary voltage 2.5 kV, sampling cone voltage 15.0 V, source temperature 120 °C, desolvation temperature 275 °C, desolvation gas flow (N_2) 650 $L.h^{-1}$, cone gas flow (N_2) 50 $L.h^{-1}$. For MS/MS experiments, the trap collision energy was ramped from 20 to 60 V. The eluent was split 1:1 prior to introduction into the ionisation source. UV-Vis spectra were recorded from 220 to 400 nm.

The flavone-*O*-glycosides, namely rutin and quercetin-3-*O*-rutinoside-7-*O*-glucoside were quantified at 255 nm, flavanone-*O*-glycosides, namely narirutin, naringenin-7-*O*-rutinoside-4′-*O*-glucoside, hesperidin and neoponcirin, were quantified at 288 nm, ferulic acid-*O*-hexoside a hydroxycinnamic acid was quantified at 320 nm and lastly vicenin-2 a flavone-*C*-glucoside was quantified at 350 nm.

The method was validated by evaluating the linearity of the calibration curves, the stability of compounds in the calibration mixture and two juice samples (navel and mandarin), as well as the intra- and inter-day repeatability for the calibration mixture and two juice samples as described below.

Stock solutions of the phenolic standards were prepared at approximately 1 $mg.mL^{-1}$ concentrations in dimethylsulfoxide (DMSO). The individual stock solutions were combined to form calibration mixtures. Ten-point calibration curves were set up for all authentic reference standards. The calibration mixtures consisted of rutin, narirutin, hesperidin, neoponcirin, vicenin-2 and ferulic acid. Injection volumes were at 1, 5, 10, 20, 30, 40, 50, 60, 80 and 100 µL in order to obtain on-column calibration ranges as follows: 0.02 - 2.00 µg for rutin, narirutin, hesperidin and neoponcirin, 0.01 - 1.00 µg for vicenin-2 and 0.005 - 0.5 µg for ferulic acid. The various calibration ranges were selected in order to accommodate the wide variability in the quantities that occur naturally in citrus. Furthermore, linear regression (Microsoft Excel 2003, Microsoft Corporation, Redmond, WA) was performed using the least squares method on the data to test for linearity. The slope, *y* intercept and correlation coefficients (R^2 and R) were determined and used to quantify the phenolic compound content in citrus juice. Since no authentic reference standards were available for quercetin-3-*O*-rutinoside-7-*O*-glucoside, naringenin-7-*O*-rutinoside-4′-*O*-glucoside and ferulic acid-*O*-hexoside, they were quantified as rutin, narirutin and ferulic acid equivalents, respectively.

Repeated injections (n=7) at 40 µL over 24 hours of two citrus juice samples (mandarin and navel) and the calibration mixture, with and without the addition of 10% ascorbic acid, were tested in order to evaluate the stability of the phenolic compounds and samples in the autosampler. The sample aliquots were thawed at room temperature. The sample (1.5 mL) was combined with 150 µL 10% ascorbic acid and mixed before it was filtered using an 0.45 µm Acrodisc syringe filter with GHP membrane (Pall Life Sciences, Separations, USA) into an amber autosample vial or filtered without addition of ascorbic acid. The percentage change in peak area for each compound over the 7 time points during the 24 h period was calculated and the percentage relative standard deviation (% RSD) determined.

Moreover, inter- and intra-day repeatability was performed by injecting the standard mixture and citrus samples containing 10% ascorbic acid 6 times per day over 3 consecutive days. Once again the % RSD was determined for replicate injections on the same day to obtain the intra-day repeatability while the % RSD of the mean values for each day was determined to evaluate the inter-day repeatability.

The samples were analysed in duplicate at 40 µL and 10 µL injection volumes with the addition of ascorbic acid. All samples were analysed within 24 h of preparation to ensure compound stability. Calibration curves were created weekly.

Total antioxidant capacity

2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The juice samples were analysed using the DPPH radical scavenging assay which was adapted from Rangkadilok *et al*. (2007). The DPPH reagent was prepared by dissolving 5 mg in 50 mL methanol and sonicating the solution for 5 min in a covered waterbath to protect it from light. After preparation, the solution was stored in a dark cupboard until analysis and prepared freshly every day prior to use. Trolox was used as standard to prepare a calibration curve. Trolox (0.01250 g) was prepared in 50 mL methanol and sonicated as well to ensure a fully dissolved homogeneous solution. The standard series consisted of concentrations ranging from 50 – 400 µM.

Deep-well plates with 96 wells (300 µL capacity) were used and 30 µL each of deionised water blanks, standard solutions and diluted samples were pipetted into assigned wells. The DPPH solution (270 µL) was added to each well using a multi-channel pipette before the deep-well plate was sealed with a silicone mat and mixed for 30

s at 1650 rpm, using an Eppendorf MixMate (Merck Millipore). The plate was incubated at room temperature in a dark cupboard for 2 h. After incubation the mixtures (200 µL) were transferred to a 96-well flat-bottom clear microplate into corresponding wells using a mutli-channel pipette. The absorbance was read at 515 nm, percentage (%) inhibition was calculated based on blank wells and the results were expressed as µmol Trolox equivalents (TE).mL^{-1}.

Oxygen Radical Absorbance Capacity (ORAC) assay

In contrast to the DPPH radical scavenging assay, the ORAC assay is based on fluorescence measurements in order to obtain the response. In this case a method was adapted from Huang *et al*. (2002) to suit the specific microplate reader that was used. Trolox was used to prepare a standard series for the calibration curve. The Trolox (12.5 mg) was dissolved in 10 mL methanol before the addition of deionized water to obtain a 500 µM stock solution. After further dilution, a standard series was obtained that ranged from 4 - 25 µM. The respective standards, reagent blanks (75 mM K-phosphate buffer, pH 7.4) and samples (25 µL) were pipetted into allocated wells of a 96-well flat-bottom clear microplate. The standards, reagent blanks and samples were protected by a thermal barrier by pippeting 300 µL of deionised water into the surrounding outside wells. Fluorescein solution ($8.16x10^{-2}$ mM) was prepared by dissolving 0.0031 g of fluorescein disodium in 100 mL of 75 mM K-phosphate buffer (pH 7.4). Subsequently, the fluorescein solution was further diluted with K-phosphate buffer (73 µL in 100 mL K-phosphate buffer) to obtain the working solution. A multi-channel pipette was used to add 150 µL of fluorescein working solution ($8.16x10^{-5}$ mM fluorescein disodium) into each well (standards, blanks and samples). The plate was incubated at 37°C for 10 min after which the 153 mM AAPH in K-phosphate buffer was added automatically by the dispenser of the microplate reader. The content was mixed before fluorescence measurements commenced. Measurements were recorded at one minute intervals for a duration of 35 min. The area under the curve (AUC) was determined and the net AUC was used for the final antioxidant capacity calculations. The results were expressed as µmol $TE.mL^{-1}$

Ferric Reducing/Antioxidant and Ascorbic Acid (FRASC) assays

This assay was used to determine the ascorbic acid (AA) concentration of the juice samples. In this assay Fe^{3+} is reduced to Fe^{2+} by antioxidants present in the sample which

results in a colorimetric (593 nm) product. When ascorbate oxidase enzyme is added any AA, which is also a powerful antioxidant, will be oxidised. Thus, when parallel samples contain ascorbate oxidase, the net measurement will result in the measurement of the AA concentration. The reagents were supplied in kit form and contained FRASC buffer, AA probe, iron chloride ($FeCl_3$) solution, ascorbate oxidase enzyme and 20 µM AA standard. The AA standard solution was reconstituted in 200 µL deionised water to generate a 100 mM stock solution. This stock solution was further diluted (10 µL in 990 µL deionised water) to obtain a 1 mM AA solution. The 1 mM AA solution was pipetted into a clear flat bottom 96-well microplate to obtain a 0, 2, 4, 6, 8 and 10 nmole per well standard series. Deionised water was added to bring the final volume of the standard series to 100 µL. Appropriately diluted samples were pipetted into the assigned wells and brought to a final volume of 100 µL. Deionised water was added (10 µL) to the standards and sample wells. For the sample blanks, 10 µL of ascorbate oxidase was added. The plate was mixed by pipetting and incubated for 15 min at room temperature. A master reaction mix was prepared containing FRASC buffer, AA probe, iron chloride solution in a ratio of 8:1:1. A 100 µL volume of the master reaction mix was added to each well. The plate was mixed by pipetting and then incubated for 2 min. The absorbance was measured at 593 nm for maximum sensitivity. The net absorbance was used to calculate the AA concentration.

Statistical analysis

The experimental layout consisted of four fruit types (satsuma, clementine, navel and valencia) of which replicate composite samples were obtained on different harvest dates during the 2012 season. During the 2014 season, satsuma and clementine varieties were grouped under mandarin and consequently only three varieties were collected, namely mandarin, navel and valencia.

Data were expressed as means ± standard deviations (SD). All analyses were performed in duplicate, except for DPPH and ORAC which was performed in triplicate. Univariate analysis of variance (ANOVA) was performed and the Least Significant Difference (LSD) Student's t-test was used as a post-hoc test. The Shapiro-Wilk test was used to test for normality. Differences were considered statistically significant at a 95% confidence level ($P < 0.05$). SAS statistical software (SAS®, Version 9.2, SAS Institute Inc., Cary, NC, USA) was used for univariate statistical analysis. In addition, multivariate statistical analysis was performed using XLSTAT software (Version 7.5.2, Addinsoft, New York, USA). Principal Component Analysis (PCA) was used to evaluate relationships

between fruit characteristics and varieties/seasons. The data for the PCA's were pretreated using Correlation matrix.

RESULTS AND DISCUSSION

HPLC method development, identification of phenolic compounds and method validation

The method described by Leuzzi *et al*. (2000) used to quantify flavonoid glycosides and polymethoxyflavonoids in orange juices, was modified to improve the separation of a number of phenolic compounds present at lower concentrations. The method (Leuzzi *et al*., 2000) proved successful for the separation and identification of nine phenolic compounds. The method employed 0.2% acetic acid (Solvent A) and acetonitrile (Solvent B) with a Discovery C18 250 x 4.6 mm column (Supelco, Sigma-Aldrich) column at 30°C. These parameters were used as the starting point, except that a Gemini-NX C18 150 x 4.6 mm column was used after adapting the gradient to the shorter column length. Subsequently, the concentration of the acidified aqueous phase, column temperature and gradient program was systematically adjusted until the method gave the best separation for the major phenolic compounds in the test samples (navel and mandarin juice).

The Gemini-NX C18 column used during initial testing of the juice samples provided satisfactory peak shapes and thus it was decided that no other column would be investigated for performance. Throughout the entire method development process acetonitrile was used as organic modifier. The separation of some phenolic compounds was problematic and therefore different concentrations of acetic acid (0.2% versus 2.0%) were compared. The best separation was obtained using 0.2% acetic acid which was used for further optimisation. The gradient program was adjusted to include a 3 min isocratic holding period in order to avoid co-elution during the first part of the chromatogram. In addition, different column temperatures (28°C, 29°C, 30°C, 31°C and 38°C) were evaluated. A column temperature of 31°C gave the best separation. Chromatograms for a typical sample of each citrus variety using the optimised method are provided in Figure 4.1.

Identification of the phenolic compounds in various juices (navel, mandarin, satsuma and other retail juices distinguished as pasteurised and ultra-high temperature treated juices) was performed using LC-DAD-MS analysis data. The comparison of the retention times, UV-Vis spectral properties, LC-MS spectra and LC-MS/MS fragmentation patterns with those of commercial authentic reference standards and/or those cited in

literature made identification of 8 phenolic compounds possible (Table 4.3). Five of the compounds, namely vicenin-2 (VI2), rutin (RUT), narirutin (NART), hesperidin (HD) and neoponcirin (NEOP), could be confirmed with authentic reference standards whilst another 3 were compared to previously identified compounds found in literature (Abad-García *et al*., 2012b). Quercetin-3-*O*-rutinose-7-*O*-glucoside (QRG), ferulic acid-*O*-hexoside (FHX) and naringenin-7-*O*-rutinose-4'-*O*-glucoside (NRGLC) were previously identified in orange and tangerine juice by Abad-García *et al*. (2012b).

The reliability of the method was evaluated during method validation, before routine analysis was performed on the juice samples. Two juice samples (navel and mandarin) were selected and included in the validation study to confirm the compatibility of the method. The results of the calibration curves are given in Table 4.4. The linearity of the calibration curves was excellent since the *y*-intercepts were relatively low with the Pearson correlation coefficient (r) equal to 1.00 for all standard reference compounds. The stability of all of the compounds in the calibration mixtures was very good over a 26 h period since the percentage relative standard deviation (%RSD) for all the compounds ranged from 0.4% – 0.9%. In addition the percentage change ranged from -1.3 to 2.2% which is considered excellent for stability testing since it is within -5 and 5% specification. However, some of the compounds in the juice samples proved to be less stable and resulted in a %RSD greater than 5%. This was seen for RUT (7.2 and 5.3%) as well as NRGLC (5.3 and 9.4%) in both juice samples. Nevertheless, the stability for the other compounds in fruit juice was excellent and ranged from 0.3 to 4.8% (Table 4.5). The addition of AA ensured that most of the compounds remained stable during the entire 26 h period. Moreover, the intra and inter-day precision was evaluated and had a %RSD of less than 5% for all standard compounds except for the ferulic acid standard compound (Table 4.6). The %RSD for compounds identified in navel juice for inter-day precision was all below 5% while RUT was the only compound in mandarin juice which had a %RSD greater than 5% (11.46%).

Comparison of 2012 and 2014 harvest season

The citrus juice characteristics which include °Brix, titratable acidity (TA) expressed as % w.w^{-1} citric acid, °Brix:acid ratio and pH of various citrus varieties harvested in 2012 and 2014 respectively, are given Figure 4.2. During the 2012 season clementine and satsuma fruit were received and processed separately by the fruit processor which allowed for the separate evaluation of these two mandarin species. Unfortunately, in 2014 clementine

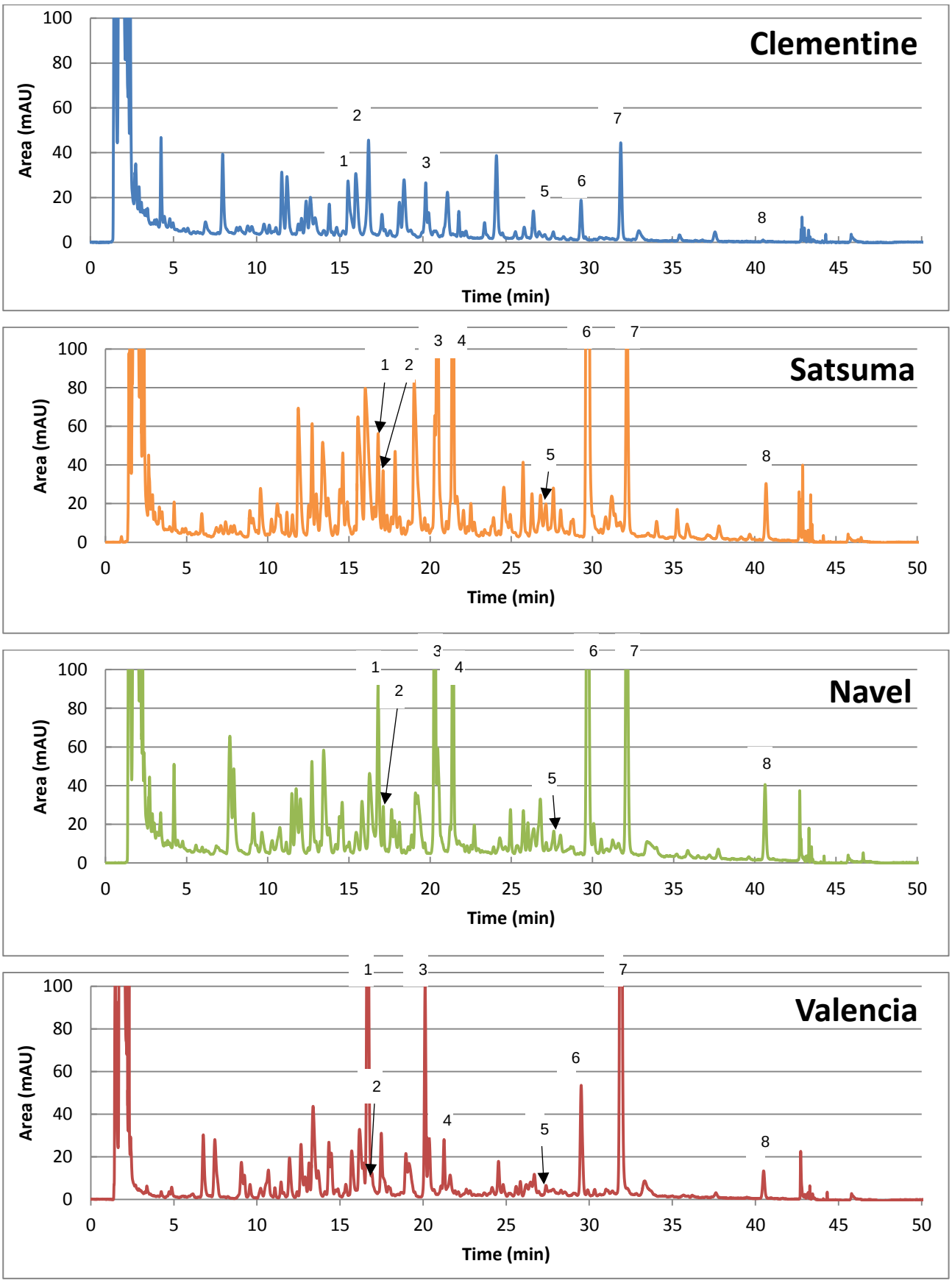

Figure 4.1 Chromatograms (recorded at 288 nm) of clementine, satsuma, navel and valencia juice showing the major phenolic compounds identified (numbers correspond to those in Table 4.3).

Table 4.3 UV-Vis, LC-MS and LC-MS/MS characteristics of the major phenolic compounds identified in various citrus juices

Peak	t_R (min)	UV-Vis λ_{max} (nm)	[M-H]-	Accurate mass[a]	Molecular formula	Error (ppm)	Fragment ions (MS/MS)	Compound	Nav	Man	Past	UHT	Sat
1	16.8	255, 352	771*	771.1984	$C_{33}H_{39}O_{21}$	0.1	609*, 462, 299	Quercetin-3-*O*-rutinose-7-*O*-glucoside	x	nd	x	nd	x
2	16.8	236, 329	355*	355.1029	$C_{16}H_{19}O_9$	-0.3	175*, 160, 132	Ferulic acid-*O*-hexoside	x	x	x	x	x
3	20.2	270, 334	593*	593.1506	$C_{27}H_{29}O_{15}$	-1.3	503, 473, 383, 353*	Apigenin-6,8-di-*C*-glucoside (vicenin-2)	x	x	x	x	x
4	21.2	284, 332sh	741*	741.2242	$C_{33}H_{41}O_{19}$	-3.4	271	Naringenin-7-*O*-rutinose-4'-*O*-glucoside	x	nd	x	x	x
5	27.02	255, 351	609*	609.1456	$C_{27}H_{29}O_{16}$	2.8	300*, 153	Quercetin-3-*O*-rutinoside (rutin)	x	x	x	x	x
6	29.6	283, 322sh	579*	579.1714	$C_{27}H_{31}O_{14}$	0.5	271*, 151	Naringenin-7-*O*-rutinoside (narirutin)	x	x	x	x	x
7	31.9	283, 328sh	609*	609.1819	$C_{28}H_{33}O_{15}$	0.2	301	Hesperetin-7-*O*-rutinoside (hesperidin)	x	x	x	x	x
8	40.5	282, 338sh	593*	593.187	$C_{28}H_{33}O_{14}$	2.0	285	Isosakuranetin-7-*O*-rutinoside (neoponcirin)	x	x	x	x	x

[a] accurate mass determined experimentally; * ion with highest relative intensity; nd, not detected; Abbreviations: tR , retention time; Nav, Navel juice; Man, Mandarin juice; Past, pasteurised orange juice blend; UHT, Ultra-high temperature treated orange juice blend.

Table 4.4 Linear regression data for calibration curves

Compound	Regression Equation	r
Rutin	y = 1413x – 2.64	1.00
Narirutin	y = 1532x – 3.31	1.00
Hesperidin	y = 1442x – 2.52	1.00
Ferulic acid	y = 3362x – 2.89	1.00
Vicenin-2	y = 1916x – 1.43	1.00

Table 4.5 Compound stability in standard calibration mixtures and two juices over a 26h period.

Compound	%RSD (% change)		
	Samples with ascorbic acid addition		
	Calibration mixture (30 µL)	Navel juice (40 µL)	Mandarin juice (40 µL)
Rutin	0.86 (2.2)	7.23 (20.1)	5.31 (16.1)
Narirutin	0.51 (1.7)	0.45 (1.3)	0.97 (0.1)
Hesperidin	0.60 (1.6)	0.56 (1.7)	1.53 (-0.9)
Ferulic acid	0.46 (-1.3)	nd	nd
Vicenin-2	0.41 (1.2)	0.40 (1.1)	1.32 (0.8)
Quercetin-3-*O*-rutinoside-7-*O*-glucoside	nd	1.01 (2.8)	0.57 (-1.2)
Neoponcirin	nd	2.75 (-0.8)	4.82 (-3.4)
Naringenin-7-*O*-rutinoside-4'-*O*-glucoside	nd	5.33 (-12.0)	9.42 (-19.6)
Ferulic acid-*O*-hexoside	nd	0.33 (-0.6)	1.94 (4.1)
	Samples without ascorbic acid addition		
		Navel juice (40 µL)	Mandarin juice (40 µL)
Rutin		44.97 (1.9)	6.12 (-1.1)
Narirutin		0.11 (0.1)	0.94 (1.6)
Hesperidin		0.39 (-1.0)	0.56 (1.6)
Ferulic acid		nd	nd
Vicenin-2		0.16 (-0.16)	1.38 (2.4)
Quercetin-3-*O*-rutinoside-7-*O*-glucoside		16.91 (1.32)	0.49 (-1.0)
Neoponcirin		2.23 (-5.2)	10.0 (3.0)
Naringenin-7-*O*-rutinoside-4'-*O*-glucoside		8.1 (-14.7)	8.3 (-17.2)
Ferulic acid-*O*-hexoside		0.76 (-2.0)	2.9 (-5.3)

Abbreviations: %RSD, % relative standard deviation; nd, not detected.

Table 4.6 Intra- and inter-day precision (% relative standard deviation) for individual phenolic compounds as determined using standard calibration mixtures as well as two juice samples

Compound	Intra-day (n = 6/day)			Inter-day (n = 3)
	Day 1	Day 2	Day 3	
Calibration mixture (30 µL)				
Rutin	0.31	0.41	0.38	0.51
Narirutin	0.34	0.19	0.53	2.41
Hesperidin	0.25	0.53	2.40	1.59
Ferulic acid	0.26	0.36	2.20	6.14
Vicenin-2	0.18	0.39	2.20	4.48
Navel juice (40 µL)				
Rutin	0.85	2.26	3.05	4.50
Narirutin	0.18	0.12	0.19	1.60
Hesperidin	0.19	0.40	0.45	1.40
Vicenin-2	0.25	0.42	0.62	0.40
Quercetin-3-*O*-rutinose-7-*O*-glucoside	0.42	0.26	0.16	0.80
Neoponcirin	0.22	0.78	0.91	1.00
Naringenin-7-O-rutinose-4'-*O*-glucoside	0.30	0.75	1.10	0.90
Ferulic acid-*O*-hexoside	1.67	4.23	1.67	2.20
Mandarin juice (40 µL)				
Rutin	2.48	1.28	1.95	11.46
Narirutin	1.14	0.95	0.61	1.66
Hesperidin	1.47	0.41	0.29	0.75
Vicenin-2	1.08	1.72	1.10	0.43
Quercetin-3-*O*-rutinose-7-*O*-glucoside	2.75	1.73	2.41	2.67
Neoponcirin	3.69	0.56	1.06	1.90
Naringenin-7-*O*-rutinose-4'-*O*-glucoside	7.43	6.34	2.23	2.71
Ferulic acid-*O*-hexoside	3.20	7.54	5.84	2.90

and satsuma fruit was combined as mandarin fruit by the fruit processor due to consumer demands and was sampled as such to form the mandarin 2014 sample. Thus, clementine 2012 and satsuma 2012 will be directly compared to mandarin 2014 samples to determine whether seasonal variation occurs. In addition, in 2012 the fruit processor did not receive any valencia fruit due to unfavourable fruit prices. Thus, no valencia fruit was sampled during the 2012 harvesting season.

The °Brix of the various citrus samples harvested in 2012 and 2014 did not differ significantly with the exception of clementine 2012 which had a significantly ($p<0.05$) higher °Brix (Fig 4.2) than other samples. Regarding TA, valencia fruit sampled in 2014 had the highest TA and differed significantly ($p<0.05$) from the rest. This is expected since valencia, grown in the Western Cape region of South Africa, is characterised by its acidic nature (K Smuts, 2014, Cape Fruit Processors, Citrusdal, South Africa, personal communication). There was no seasonal variation in terms of TA for the 2012 and 2014 navel for clementine, satsuma and mandarin samples. However, varietal differences were evident since the TA of the satsuma 2012 and mandarin 2014 were found to be significantly lower ($p<0.05$) compared to the other varieties, except for navel 2014. In terms of °Brix:acid ratio, varietal differences were once again more evident than seasonal differences. Clementine 2012 and mandarin 2014 had the highest °Brix:acid ratio, followed by satsuma 2012, as well as the 2012 and 2014 navel samples which did not differ from each other ($p>0.05$). Valencia had the lowest °Brix:acid ratio which is directly linked to the high TA. The pH was found to differ significantly ($p<0.05$) between seasons (2012 & 2014) as well as between varieties. The fruit harvested in 2014 had a higher pH compared to those sampled in 2012. This may indicate that seasonal differences have an effect on parameters such as fruit pH. Mouly *et al*. (1994) suggested that pH can have an influence on the quantification of phenolic compounds such as hesperidin and narirutin. Nonetheless, the pH of all the samples was in the optimum range described by Mouly *et al*. (1994). Furthermore, it is evident that varietal differences play a bigger role than seasonal differences in the variation for the results obtained for the citrus juice characteristics.

Large variation was observed within varieties of a specific harvest season and between varieties when the individual phenolic compound content was evaluated. Figure 4.3 displays the distribution of the data with the aid of boxplots. Large variability within the data for satsuma 2012 samples was especially noted for QRG and RUT , while VIC2 content had a notably high variation for navel 2014 and mandarin 2014 samples (Fig. 4.3). The relatively low variation obtained for phenolic compound content of mandarin 2014

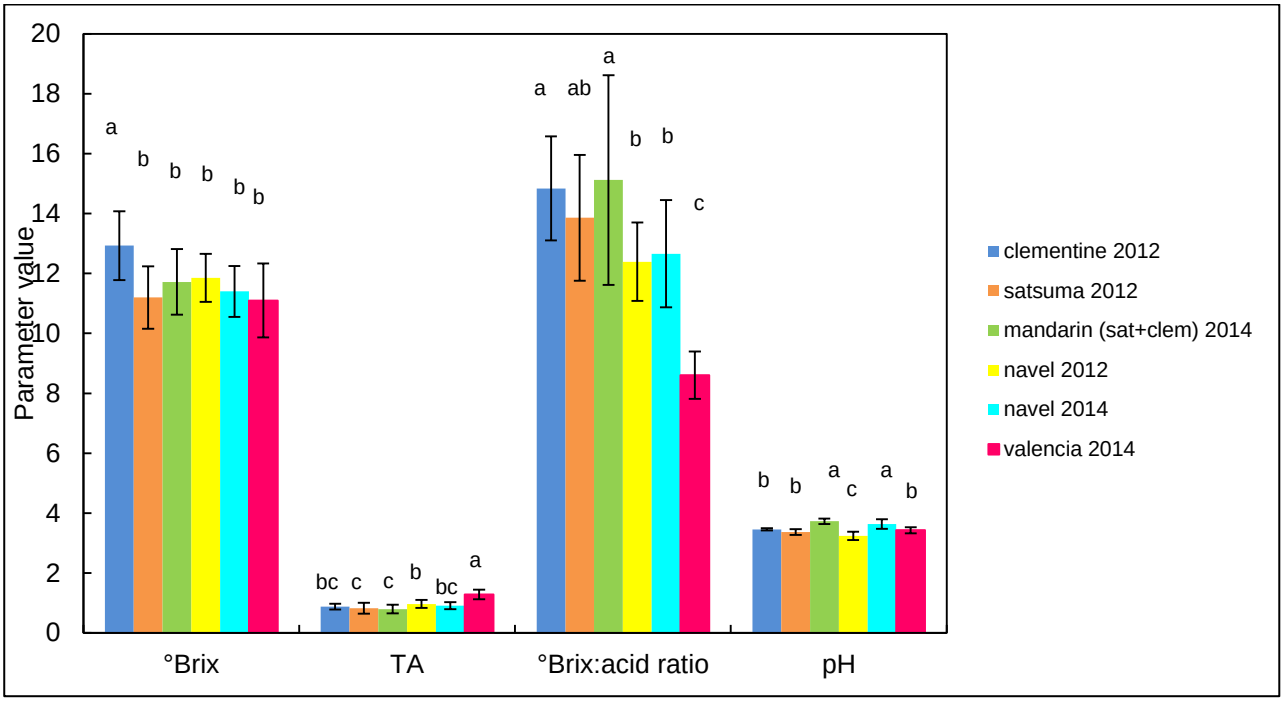

Figure 4.2 °Brix, titratable acidity (TA expressed as %w.w^{-1} citric acid), °Brix:acid ratio and pH of different citrus varieties harvested in 2012 and 2014.*Different letters over columns for a specific paramater represent significant differences at p≤0.05.

samples was unexpected, since these were samples from two different varieties showing differences in phenolic composition for the 2012 harvest season.

The AA content of fruit samples was determined using the FRASC assay, however, the results obtained indicated very low or undetectable AA levels for all samples from the 2012 harvest season (Fig. 4.3). This clearly indicates that most of the AA present in these samples degraded during the storage period of the samples and the values are therefore reported as not determined (ND). In addition, the TAC_{DPPH} and TAC_{FRAP} values determined for the 2012 samples were substantially lower than that of 2014 samples (data not shown), indicating that most of the TAC measured for citrus fruit in these assays is due to AA. The data are not reported nor discussed further, since it can not be considered in any conclusions. The low AA content of the 2012 samples did not seem to affect the TAC_{ORAC} values since they were in the same range for both harvest seasons, although a higher level of variation was observed for clementine and satsuma 2012 samples compared to other samples. Variation in the AA content was similar for the different varieties sampled during 2014. The TAC_{FRAP} values of valencia 2014 samples were higher than those for other varieties from the 2014 harvest season.

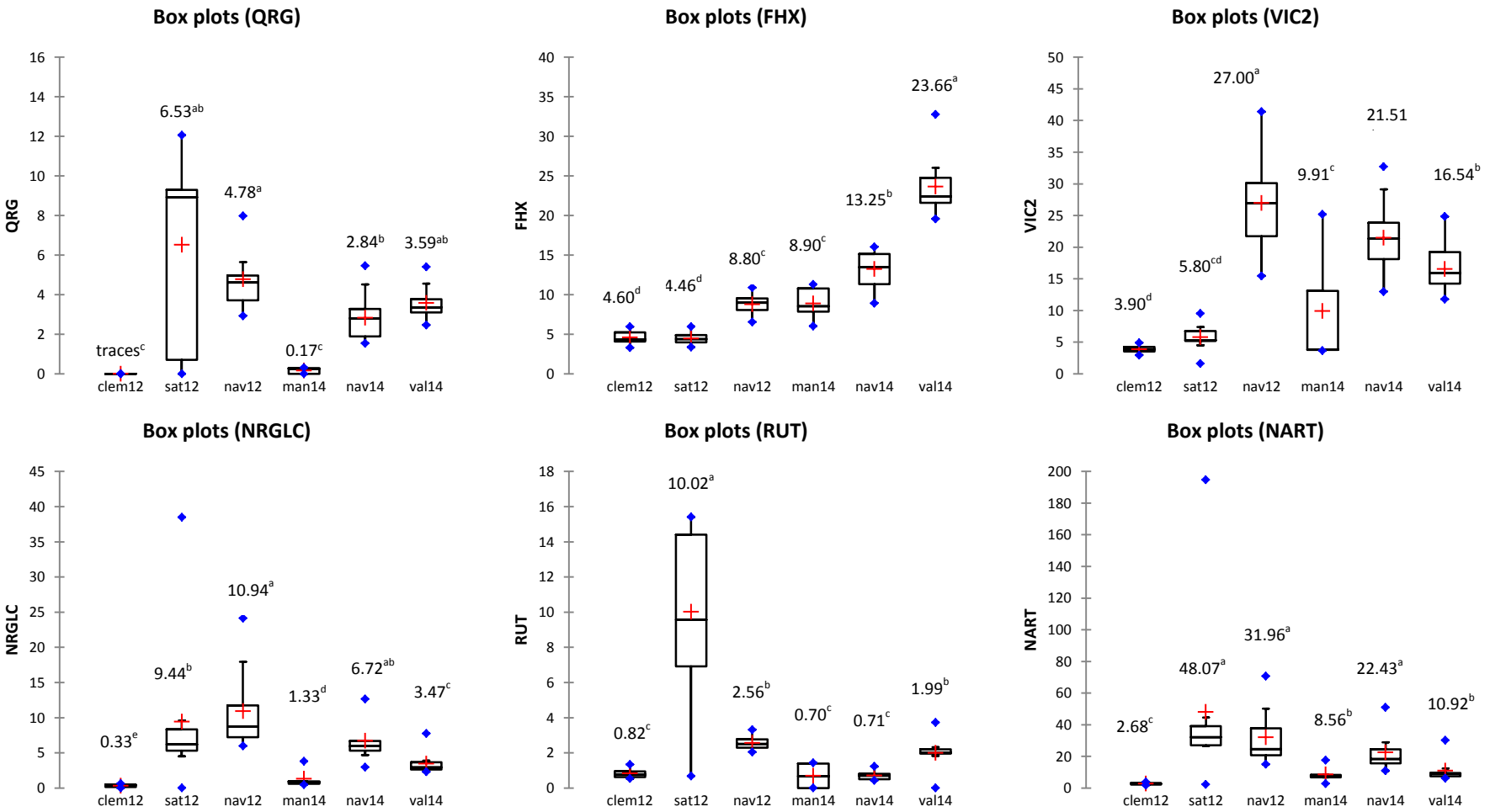

Figure 4.3 Boxplots displaying the distribution of phenolic compound and ascorbic acid content (mg.L^{-1}) as a function of citrus variety and harvest season. (QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds, AA = ascorbic acid; clem = clementine; sat = satsuma; nav = navel; man = mandarin; val = valencia; 12 = 2012; 14 = 2014). Significant differences ($p<0.05$) are indicated by different alphabetical letters.

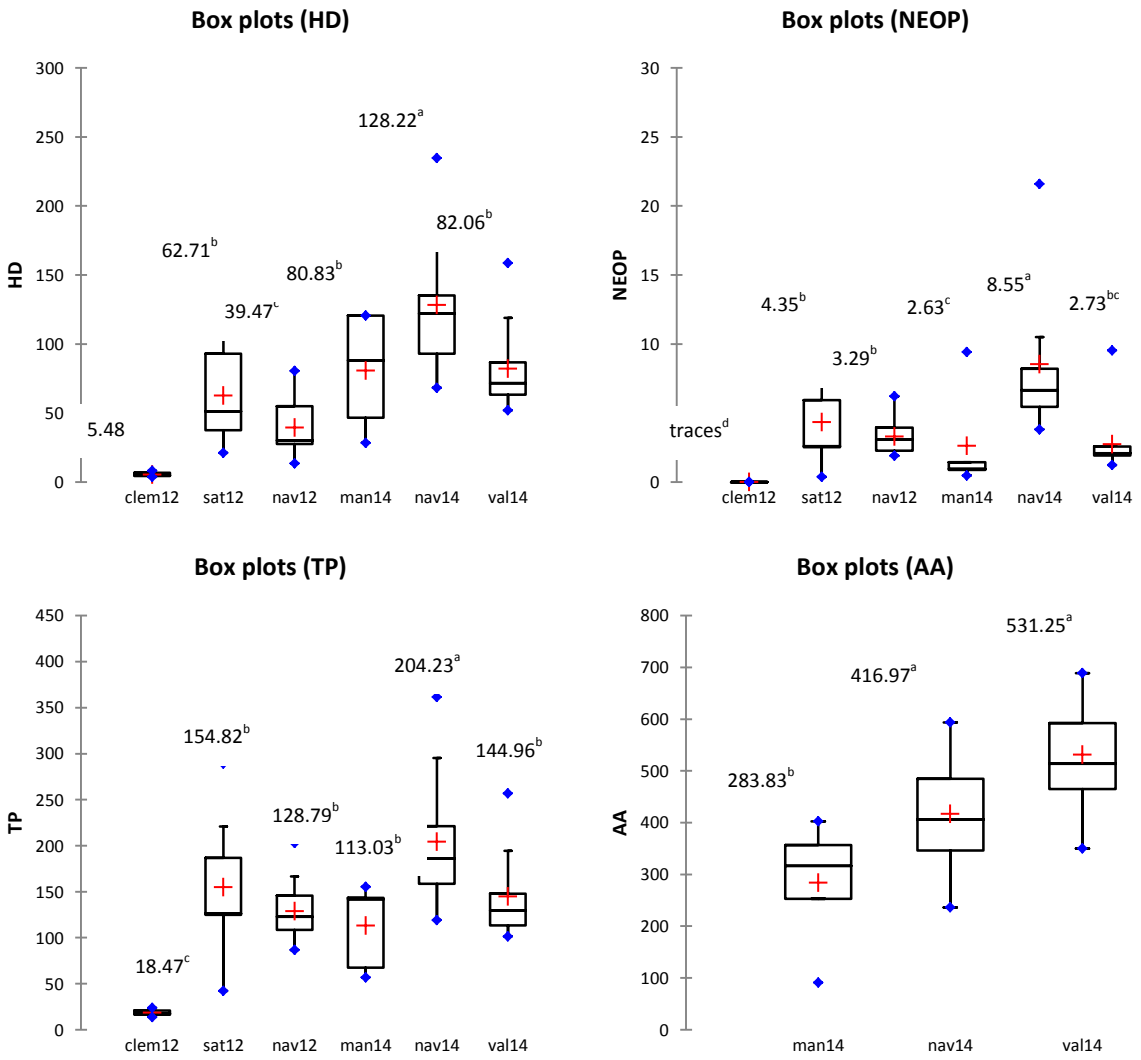

Figure 4.3 (continued)

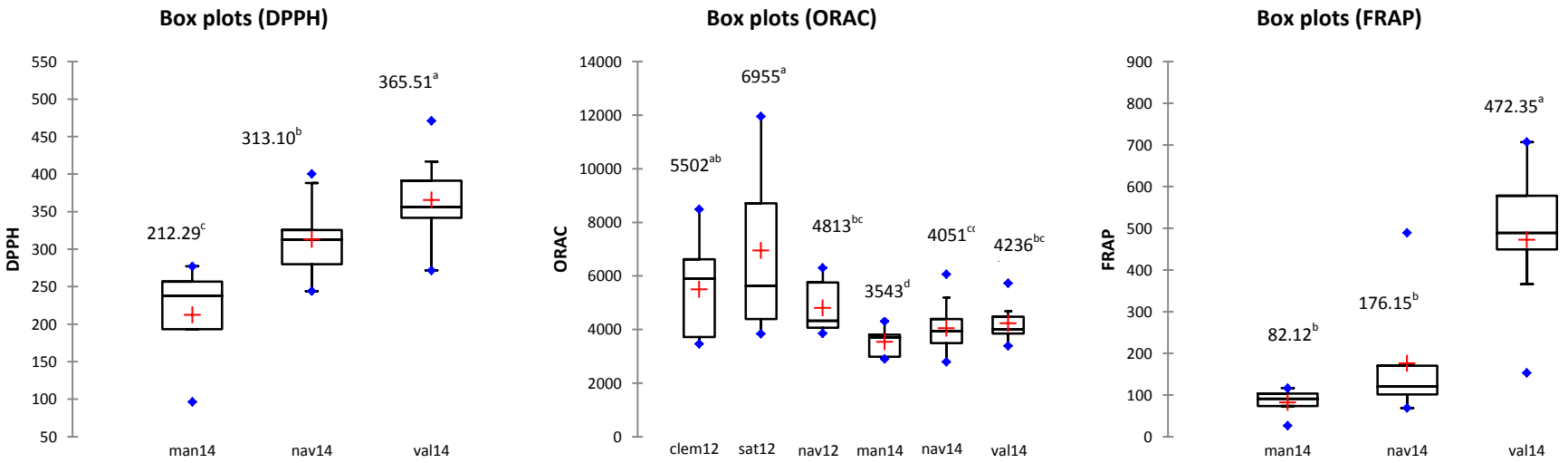

Figure 4.4 Boxplots showing the distribution of total antioxidant capacity (TAC) as measured by DPPH, ORAC and FRAP expressed as µmol Trolox equivalents (TE).mL^{-1} as a function of citrus variety and harvest season (clem = clementine; sat = satsuma; nav = navel; man = mandarin; val = valencia; 12 = 2012; 14 = 2014). Significant differences ($p<0.05$) are indicated by different alphabetical letters.

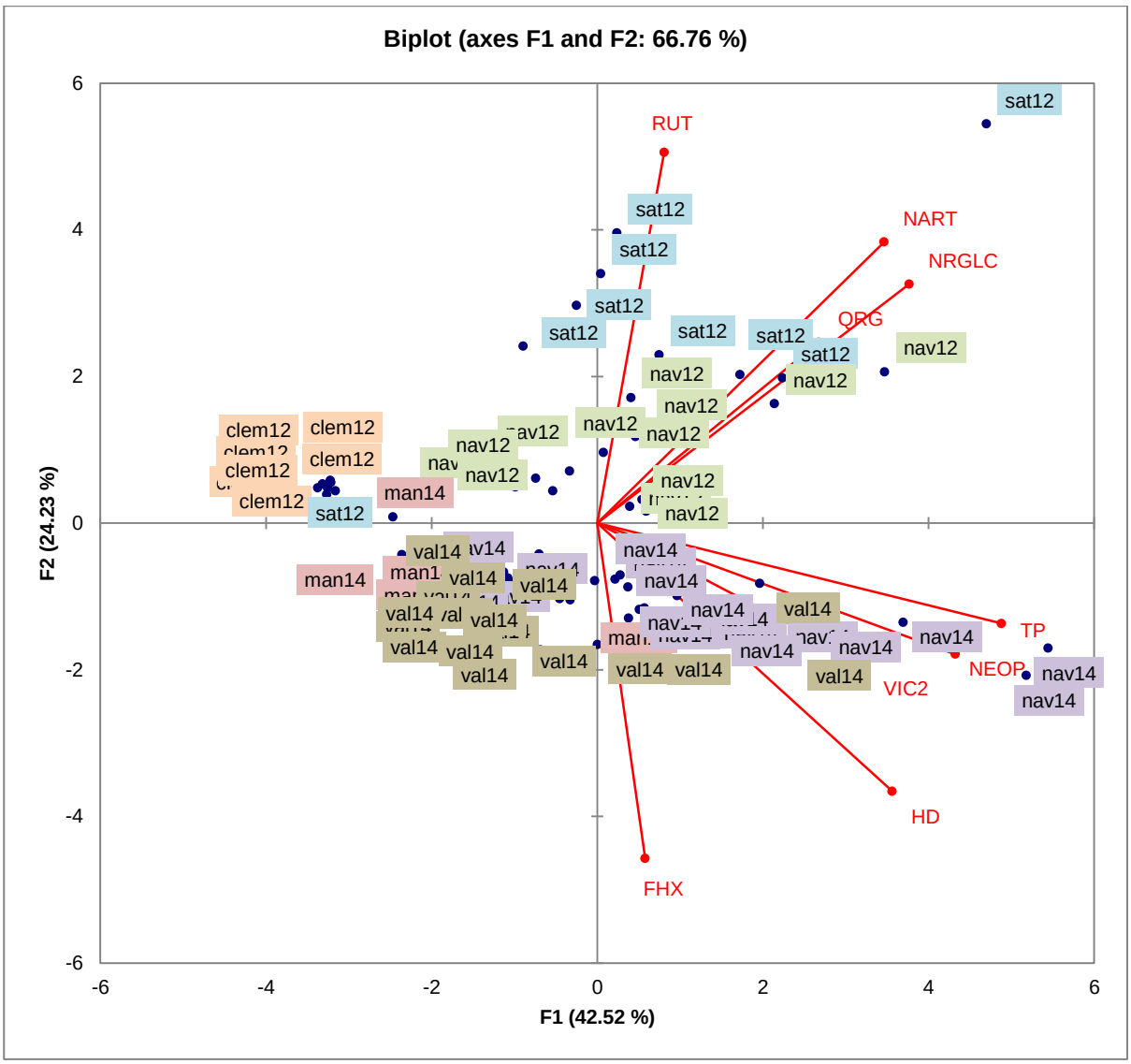

Figure 4.5 PCA biplot displaying associations between phenolic composition and citrus varieties harvested in 2012 and 2014. (QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = naririutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds, clem = clementine; sat = satsuma; nav = navel; man = mandarin; val = valencia; 12 = 2012; 14 = 2014).

Principal component biplots were evaluated for any associations between the individual phenolic compound content of the different citrus varieties and harvesting seasons. The biplot explains 66.8 % of the variation in phenolic composition for citrus varieties and harvesting season. Harvesting seasons form clear clusters with 2012 samples in the upper quadrants and 2014 samples in the lower quadrants. Citrus varieties, however, are mostly overlapped for 2014 samples, while 2012 samples show clearer grouping for citrus varieties. The 2012 samples, irrespective of variety, were associated with higher RUT,

NART, QRG and NRGLC contents. A close relationship between clementine and satsuma fruit was expected since they are sub-species of the *Citrus reticulata* family, however, these samples were clearly separated from each other on the PCA biplot. Furthermore, navel and valencia harvested in 2014 showed a close relationship and this was associated with higher FHX, VIC2, HD, NEOP and total phenolic compound (TP) content. The mandarin samples harvested in 2014 were more closely associated with the 2012 clementine samples compared to the 2012 satsuma samples. This may suggest that the 2014 mandarin samples contained mostly clementine fruit as opposed to satsuma fruit.

In all samples, the flavanone glycoside HD was the predominant compound with navel 2014 samples having a significantly ($p<0.05$) higher mean content (128.22 $mg.L^{-1}$) than other samples. The mean HD content of valencia 2014 (82.06 $mg.L^{-1}$), mandarin 2014 (80.83 $mg.L^{-1}$), and satsuma 2012 (62.71 $mg.L^{-1}$) samples were lower but similar, followed by that of navel 2012 (39.47 $mg.L^{-1}$) and clementine 2012 (5.48 $mg.L^{-1}$). The significantly ($p<0.05$) lower hesperidin content in navel 2012 samples compared to navel 2014 samples indicates that season had a significant effect on the hesperidin content. It was also evident that all the varieties collected in 2014 had a higher hesperidin level compared to the 2012 samples. Substantially higher hesperidin concentrations have been reported previously for valencia (257 - 577 $mg.L^{-1}$), navel (135 – 232 $mg.L^{-1}$), mandarin (192.6 – 585 $mg.L^{-1}$) and clementine (64 – 606 $mg.L^{-1}$)(Dhuique-Mayer *et al*., 2005; Peterson *et al*., 2006; Cano *et al*., 2008; Milella *et al*., 2011). Gattuso *et al*. (2007) combined data from a large number of publications showing ranges of hesperidin content in *C. sinensis* juices (35 – 550 $mg.L^{-1}$), including valencia, navel and others and *C. reticulata* juices (8 – 458 $mg.L^{-1}$), including clementine and others. Differences in values obtained between studies may be due to a number of factors, including growing region, cultivar and differences in sample preparation. In some studies (including the present study) juice was filtered and directly injected, while in other studies the unfiltered juice samples were extracted using organic solvents such as methanol prior to filtering and analysis. With regards to NART content, no seasonal variation was observed for the navel 2012 and 2014 samples. The highest NART levels were observed for the satsuma 2012 (48.1 $mg.L^{-1}$), navel 2012 (31.96 $mg.L^{-1}$) and navel 2014 (22.43 $mg.L^{-1}$) samples with significantly ($p<0.05$) lower values for valencia 2014 (10.92 $mg.L^{-1}$) and mandarin 2014 (8.56 $mg.L^{-1}$). Clementine 2012 samples had the lowest NART content (2.68 $mg.L^{-1}$). The values obtained in the current study for navel samples were slightly lower than that reported by Wu *et al*. (2004) for navel oranges grown in China (31 - 56 $mg.L^{-1}$). NART content observed for satsuma 2012 samples were in line with the figures reported by Wu

et al. (2004). The low NART levels for mandarin 2014 and clementine 2012 was in contrast to various studies reporting levels for mandarin between 27 - 276 mg.L^{-1} and clementine varieties between 6 - 27 mg.L^{-1} (Dhuique-Mayer *et al*., 2005; Peterson *et al*., 2006; Cano *et al*., 2008; Millela et al., 2011). Gattuso *et al.* (2007) reported NART levels of 6 - 142 mg.L^{-1} for *C. sinensis* and 1 - 90 mg.L^{-1} for *C. reticulata*. Similar to results for HD, navel 2014 was characterised with the highest NEOP concentration (8.55 mg.L^{-1}), which significantly ($p<0.05$) differed from all other variety x season combinations. The NEOP levels decreased in the order: satsuma 2012 (4.35 mg.L^{-1}), navel 2012 (3.29 mg.L^{-1}), valencia 2014 (2.73 mg.L^{-1}) and mandarin 2014 (2.63 mg.L^{-1}). The NEOP concentrations found in this study were also lower than those described in literature for sweet orange varieties like valencia as well as mandarin, which ranged from 11.1 - 14.4 mg.L^{-1} (Dhuique-Mayer *et al*., 2005; Peterson *et al*., 2006; Cano *et al*., 2008). Gattuso *et al*. (2007) reported NEOP levels of 8 - 31 mg.L^{-1} for *C. sinensis* (valencia*)* juice and 0.5 - 31 mg.L^{-1} for *C. reticulata* (mandarin) juice. While the upper limit of the range was the highest reported yet, the range for mandarin juice included the levels reported in the present study.

Another minor flavanone glycoside, namely NRGLC, was highest in navel samples with 2012 samples having 10.94 mg.L^{-1} and 2014 samples having 6.72 mg.L^{-1}, with the difference not significant ($p>0.05$). Satsuma 2012 significantly differed ($p<0.05$) from clementine 2012 as well as mandarin 2014 with regards to NRGLC concentrations. Satsuma 2012 had considerably higher NRGLC levels (9.44 mg.L^{-1}) compared to clementine 2012 (0.33 mg.L^{-1}) and mandarin 2014 (1.33 mg.L^{-1}). This once again strengthens the argument that the mandarin 2014 samples could possibly have consisted mainly of clementine fruit. Furthermore, valencia 2014 had significantly lower NRGLC levels (3.47 mg.L^{-1}) compared to the navel variety. Previously, levels of 19 – 74 mg.L^{-1} have been reported for NRGLC in sweet oranges (Abad-García *et al*., 2012a; Abad-García *et al*., 2014), which are considerably higher than levels observed in the current study.

Only one phenolic acid, namely FHX, was quantified in citrus juices, since it occurred in high quantities in all varieties evaluated. Valencia 2014 samples were characterised with the highest FHX levels (23.66 mg.L^{-1}) which significantly differed from the rest, followed by navel 2014 (13.25 mg.L^{-1}) and mandarin 2014 (8.89 mg.L^{-1}) which also differed significantly from the 2012 samples. Navel 2012 (8.79 mg.L^{-1}) samples did not differ from mandarin 2014 with respect to FHX levels. Clementine and satsuma 2012 had the lowest FHX levels, namely 4.60 mg.L^{-1} and 4.50 mg.L^{-1}, respectively. FHX was previously reported at similar levels from 6 to 33 mg.L^{-1} in sweet oranges (Abad-García *et al*., 2012a; Abad-García *et al*., 2014).

The major flavone in citrus varieties, namely VIC-2, was also determined with the navel 2012 and 2014 samples having the highest levels. However, 2012 navel samples had the highest level (26.99 $mg.L^{-1}$) which differed significantly from 2014 (21.51 $mg.L^{-1}$). Valencia 2014 samples had a relatively high VIC-2 content of 16.54 $mg.L^{-1}$ and differed significantly from mandarin 2014 (9.91 $mg.L^{-1}$) as well as satsuma (5.80 $mg.L^{-1}$) and clementine 2012 (3.90 $mg.L^{-1}$) which had the lowest vicenin-2 level. The VIC-2 concentrations reported in literature for sweet orange varieties are considerably higher (39 - 80 $mg.L^{-1}$) (Caristi *et al*., 2006; Gattuso *et al*., 2007; Abad-García *et al*., 2012a) than those obtained for the valencia and navel samples in the current study. This may be ascribed to the same factors as described for HD. The VIC-2 levels reported by Caristi *et al*. (2006) for clementine and mandarin hybrids ranged from 4 - 27 $mg.L^{-1}$ which was in line with the aforementioned values recorded in this study. Two flavonols were quantified in citrus juices, namely RUT and QRG. For both these compounds, satsuma 2012 had the highest content values, namely 10.02 and 6.53 $mg.L^{-1}$ for RUT and QRG, respectively. In the case of RUT, clementine 2012, mandarin 2014 and navel 2014 had the lowest values, while clementine 2012 and mandarin 2014 had the lowest QRG values. Seasonal differences were found for the navel variety, with 2014 samples having significantly ($p<0.05$) lower RUT and QRG concentrations. The clementine 2012 samples did not differ from the mandarin 2014 samples for both these compounds. Millela *et al*. (2011) previously reported rutin content of 30 - 75 $mg.L^{-1}$ for a range of clementine cultivars, while QRG levels of 3 - 6 $mg.L^{-1}$ have been reported in sweet oranges (Abad-García *et al.,* 2012a; Abad-García *et al,* 2014). In terms of total phenolic compound content, navel 2014 had the highest 204.23 $mg.L^{-1}$ and differed significantly from satsuma 2012 (154.82 $mg.L^{-1}$), valencia 2014 (144.96 $mg.L^{-1}$), navel 2012 (128.79 $mg.L^{-1}$) and mandarin 2014 (113.03 $mg.L^{-1}$). Clementine 2012 showed the lowest total phenolic compound content 18.47 $mg.L^{-1}$ which differed significantly ($p<0.05$) from the rest. The varieties collected in 2014 were all associated with higher AA and consequently higher TAC_{DPPH} and TAC_{FRAP}, while the varieties harvested in 2012 displayed higher TAC_{ORAC} and considerably lower AA values (Fig. 4.3 & 4.4).

The results obtained in the study showed that seasonal differences played a larger role in the variation in the data compared to varietal differences. On some occasions varietal differences were distinctive, but for the most part varieties showed overlap within a season suggesting that seasonal rather than genotypic differences differentiated the citrus fruit samples.

CONCLUSION

The phenolic compound profile and antioxidant capacity of four South African citrus varieties were characterised. Varietal differences were evident and the influence of growing season was apparent from the results. Some of the varieties showed similarity in terms of juice characteristics and individual phenolic compound content. Narirutin and hesperidin were the major flavanone *C*-glycosides, while the major flavone *C*-glycoside and phenolic acid were vicenin-2 and ferulic acid-*O*-hexoside, respectively. Samples collected during the 2014 harvest season were found to have higher TAC_{DPPH} and TAC_{FRAP} which was as a direct result of the higher AA content. The length of storage for 2012 samples negatively affected the AA content of these samples which also dramatically impacted on the TAC_{DPPH} and TAC_{FRAP} values. The TAC_{ORAC} gave a better indication of the overall TAC of the different varieties which can be attributed to the phenolic composition and not only linked to the AA content, as values for 2012 and 2014 samples were in similar ranges. The samples were also clearly separated based on growing season, demonstrating the impact of growing season on juice characteristics, phenolic composition and TAC.

The results obtained from this research can be used as a baseline, since it is the first of its kind in terms of the establishment of a phenolic profile specific to South African citrus fruit varieties. Further investigation is required in order to survey other important agronomic citrus varieties and growing regions. It is also important to further characterise South African citrus varieties in terms of polymethoxylated flavone and carotenoid composition, as well as trace elemental analysis. This will generate specific knowledge on the quality of South African citrus in terms of their nutritional and antioxidant potential.

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ADDENDA

Table 4.7 Citrus juice characteristics (°Brix, titratable acidity, °Brix:acid ratio and pH; mean ± standard deviation) of three citrus varieties harvested over two seasons

Variety and Year	°Brix	Titratable Acidity (% w.w^{-1} citric acid)	°Brix:acid ratio	pH
clementine 2012	12.93 ± 1.15^{a}	0.88 ± 0.10^{bc}	14.84 ± 1.74^{a}	3.46 ± 0.04^{b}
mandarin 2014	11.72 ± 1.10^{b}	0.80 ± 0.14^{c}	15.12 ± 3.50^{a}	3.73 ± 0.09^{a}
navel 2012	11.85 ± 0.80^{b}	0.97 ± 0.13^{b}	12.39 ± 1.31^{b}	3.24 ± 0.14^{c}
navel 2014	11.40 ± 0.85^{b}	0.91 ± 0.12^{bc}	12.66 ± 1.79^{b}	3.64 ± 0.16^{a}
satuma 2012	11.20 ± 1.04^{b}	0.83 ± 0.18^{c}	13.86 ± 2.10^{ab}	3.37 ± 0.10^{b}
valencia 2014	11.10 ± 1.23^{b}	1.29 ± 0.16^{a}	8.61 ± 0.79^{c}	3.43 ± 0.10^{b}

*Different letters represent significant differences at p≤0.05.

Table 4.8 Phenolic compound content (mg.L^{-1}; mean ± standard deviation) of three citrus varieties harvested over two seasons

Variety and Year	QRG	FHX	VIC2	NRGLC	RUT	NART	HD	NEOP	TP	AA
clementine 2012	Traces ^{c}	4.60 ± 0.97^{d}	3.90 ± 0.66^{d}	0.33 ± 0.25^{e}	0.82 ± 0.28^{c}	2.68 ± 0.73^{c}	5.48 ± 1.64^{d}	Traces ^{d}	18.47 ± 3.5^{c}	ND
mandarin 2014	0.17 ± 0.16^{c}	8.90 ± 2.17^{c}	9.90 ± 9.45^{c}	1.33 ± 1.39^{d}	0.70 ± 0.71^{c}	8.56 ± 5.45^{b}	80.83 ± 42.28^{b}	2.63 ± 3.81^{c}	113.03 ± 46.77^{b}	283.83± 121.08^{b}
navel 2012	4.78 ± 1.5^{a}	8.80 ± 1.46^{c}	26.99 ± 7.24^{a}	10.94 ± 5.23^{a}	2.56 ± 0.42^{b}	31.96 ± 16.1^{a}	39.47 ± 19.89^{c}	3.29 ± 1.25^{b}	128.79 ± 31.96^{b}	ND
navel 2014	2.84 ± 1.1^{b}	13.25 ± 2.33^{b}	21.51 ± 4.84^{ab}	6.72 ± 2.52^{ab}	0.71 ± 0.21^{c}	22.43 ± 11.3^{a}	128.22 ± 47.55^{a}	8.55 ± 5.35^{a}	204.23 ± 70.54^{a}	416.97± 104.91^{a}
satuma 2012	6.53 ± 4.82^{ab}	4.46 ± 0.78^{d}	5.80 ± 2.17^{cd}	9.44 ± 11.22^{b}	10.02 ± 4.82^{a}	48.10 ± 56.3^{a}	62.71 ± 38.58^{b}	4.35 ± 3.01^{b}	154.82 ± 70.96^{b}	ND
valencia 2014	3.59 ± 0.83^{ab}	23.66 ± 3.90^{a}	15.54 ± 3.89^{b}	3.47 ± 1.43^{c}	1.99 ± 0.75^{b}	10.92 ± 6.8^{b}	82.06 ± 31.12^{b}	2.73 ± 1.95^{bc}	144.96 ± 45.79^{b}	531.3± 95.02^{a}

QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds; AA = ascorbic acid; ND = not determined.

Different letters represent significant differences at p≤0.05.

Table 4.9 Total antioxidant capacity (TAC; μM.L^{-1}; mean ± standard deviation) as determined by the DPPH, ORAC and FRAP assays of three citrus varieties harvested over two seasons

Variety and Year	TAC_{DPPH}	TAC_{ORAC}	TAC_{FRAP}
clementine 2012	ND	5502.04 ± 1940.97^{ab}	ND
mandarin 2014	212.29 ± 71.79^{c}	3542.56 ± 592.6^{d}	82.12 ± 34.86^{c}
navel 2012	ND	4812.73 ± 878.58^{bc}	ND
navel 2014	313.10 ± 41.83^{b}	4051.38 ± 808.22^{cd}	176.15 ± 133.60^{b}
satuma 2012	ND	6954.67 ± 3221.86^{a}	ND
valencia 2014	365.51 ± 47.7^{a}	4235.80 ± 631.79^{bcd}	472.35 ± 167.92^{a}

Different letters represent significant differences at p≤0.05.

ND = not determined

CHAPTER 5

CHARACTERISATION AND COMPARISON OF THE PHENOLIC COMPOSITION AND TOTAL ANTIOXIDANT CAPACITY OF FROZEN CONCENTRATED ORANGE JUICE: EVALUATION OF VARIETAL, SEASONAL AND REGIONAL DIFFERENCES

ABSTRACT

South Africa relies on its agro-economic sector for economic growth and sustainability. One of the major sectors that earn foreign currency is the Beverage sector. Fruit juices are the fourth largest under the beverage sector and current trends that promote national market growth are the use of fruit juice bases as functional food vehicles. Orange juices have been shown to possess phytonutrients such as bioactive phenols and vitamin C that promotes general health. However, in recent years a decline in frozen orange juice consumption has been recorded and may be ascribed to consumer perception that "processed" orange juices possess less health benefits.

Three commercially important citrus varieties (mandarin, navel and valencia), which are used for frozen concentrated orange juice (FCOJ) production in two regions of South Africa namely Western Cape (WC) and Eastern Cape (EC), were evaluated. The juice characteristics of the FCOJ samples were evaluated and compared which included °Brix, titratable acidity (TA), °Brix:acid ratio (BX/AC) and pH. In addition the individual phenolic composition using high-performance liquid chromatography coupled with diode-array detection (HPLC-DAD) and total antioxidant capacity (TAC) using 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and Oxygen Radical Absorbance Capacity (ORAC) assays were determined.

Climatic data (average monthly minimum and maximum temperatures, average monthly rainfall, average monthly radiation and evapotranspiration - ET_0) was evaluated and compared for the WC and EC region. Differences were evident based on average rainfall, maximum monthly temperatures and ET_0. The EC had the highest average rainfall (37 mm vs 25 mm) and thus lowest ET_0. The WC region was characterised with lower summer rainfall, higher monthly maximum temperatures (35°C vs 31°C) and higher ET_0 which can indicate water stress experienced by the citrus orchards and may influence the fruit quality.

The results of the juice characteristics indicated that the FCOJ from the WC were more mature compared to those of EC, having a significantly ($p<0.05$) lower TA and higher BX/AC. Varietal differences were evident and variety proved to be the most significant factor that accounted for the variances in juice characteristics and phenolic composition. Seasonal differences were less evident. Variation that could be ascribed to regional differences were found for the individual phenolic composition. FCOJ from EC were characterised having significantly ($p<0.05$) higher levels of the individual phenolics, total phenolic composition (TP) and TAC_{DPPH} and TAC_{ORAC}. Of all the FCOJ varieties, navel was found to be the most mature, irrespective of season and region and was the variety with the highest ($p<0.05$) TP. FCOJ of navel and valencia varieties did not differ significantly in terms of TAC_{DPPH} and TAC_{ORAC}. Interestingly, the EC mandarin FCOJ had the highest TP (192 $mg.L^{-1}$) compared to the WC mandarin which were found to have the lowest (122 $mg.L^{-1}$).

INTRODUCTION

The fruit juice industry is the fourth largest under the Beverage agro-economic sector in terms of sales in South Africa (Anon., 2015). Amongst the various fruit juices, pulps and purees produced in South Africa, frozen concentrated orange juice (FCOJ) forms one of the most important export commodities (Anon., 2015). However, the global market has seen a decline in FCOJ consumption between 2007 and 2012 (Anon., 2013). This may be partly due to misperceptions that frozen orange juices possess less health benefits. Furthermore, South African fruit juice producers are currently focusing on fruit juice based drinks because of its functionality and contribution to general health, which is an emerging market that is still to be fully developed (Foulds, 2015). The functional properties especially for citrus fruit juice are due to the vitamin C content and phenolic composition. In addition, it has long been the practice to process fruit, such as citrus, into frozen concentrates in order to supply a stable product and extend the availability throughout the year to various markets such as the fruit juice industry. Therefore, it is important to illustrate that FCOJ possibly have similar health benefits compared to fresh juice in the form of its bioactive phenolic composition and not only the vitamin C content.

The fruit juice industry utilises the FCOJ to produce made-from-concentrate (MFC) 100% juices, nectars, drinks and squashes which are all regulated under the Agricultural Product Standards Act (Act 119 of 1990), The Regulation Relating to the Classification, Packing and Marking of Fruit Juice and Drinks intended for sale in the Republic of South

Africa (R.286 of 7 November 1980 as amended by Government Notices Nos. R.929 of 1 May 1981, R.1325 of 9 July 1982, R.992 of 13 May 1983, R.602 and R.641 of 30 March 1984, R.1801 of 17 November 1995). However, adulteration of 100% MFC orange juices with cane sugar or other fruit sugars are a concern which misleads the consumer and does not possess the functional attributes of non-adulterated orange juices.

The phenolic composition of citrus has been shown to be specific and markers can be used to detect adulteration of orange juices with grapefruit for example (Rouseff, 1988; Mouly *et al*., 1994). It is also important to consider the effect of orange juice extraction and processing (heat treatment) on these phenolic chemical markers during the production of FCOJ. Moreover, it is necessary to consider that natural variation in the phenolic composition due to climatic growing conditions and seasonal changes may occur. Therefore, this study proposes to characterise the phenolic composition and total antioxidant capacity of FCOJ produced in the Western Cape and Eastern Cape region of South Africa. The phenolic composition of three varieties (mandarin, navel and valencia) will be compared with a view to evaluate the effect of varietal, seasonal and regional differences.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were purchased and prepared as described in Chapter 4 of this dissertation.

Sample preparation

Frozen concentrated orange juice (FCOJ) samples, from three varieties were collected from two citrus fruit processors over a three year period (2012, 2013 and 2014). The FCOJ types included mandarin (containing satsuma and clementine varieties which are grouped and processed together), navel and valencia. The fruit processors were situated in different regions of South Africa, one in Citrusdal in the Western Cape province and the other in Kirkwood in the Eastern Cape. The samples consisted of retention samples taken by the Quality Control department of each company. Samples where distinguished on the basis of processing or batch date and variety. All samples were kept frozen at -18°C. Prior to analysis the samples were diluted with ultrapure water (Milli-Q Reference A+

system, Merck Millipore, Darmstadt, Germany) in order to standardise the total soluble solids (°Brix) to approximately 11.5 °Brix.

The samples were centrifuged at 10 000 x g (centrifuge model M-24A, BOECO, Germany) for 5 min at room temperature. The supernatant was used for total antioxidant capacity determinations (DPPH and ORAC). Prior to HPLC analysis all samples were filtered using 0.45 µm Acrodisc syringe filters with GHP membrane (Pall Life Sciences, Separations, USA).

Determination of citrus juice characteristics and phenolic composition

The FCOJ samples were reconstituded to single-strenght (11.5°Brix) before all analyses. Total soluble solids (TSS, expressed as °Brix), titratable acidity (TA), pH and phenolic composistion were deteremined as described in Chapter 4.

Additionally, information on the climatic growing conditions pertaining to the sampling sites (Citrusdal and Kirkwood) from the two regions (WC & EC) which was used during this study was obtained from the Agricultural Research Council of South Africa (I Joubert, 2015, Agricultural Research Council, Institute of Soil, Weather and Water, Agromet Division, South Africa). In brief the monthly average maximum and minimum temperatures of the two regions were compared from 2011 – 2014 since the climatic conditions in 2011 could have impacted on the fruit quality which was harvested in 2012 and subsequent harvesting seasons (Fig 5.1).

Statistical analysis

The experimental layout consisted of three FCOJ types: mandarin (n=62), navel (n=63) and valencia (n=32). The chemical, flavonoid composition and antioxidant capacity of the three FCOJ types were compared, per variety, season (year: 2012; n=53, 2013; n=63, 2014; n=41) and region (Western Cape; n=117 and Eastern Cape; n=40 respectively).

Data were expressed as means ± standard deviations (SD). All analyses were performed in duplicate except those done for DPPH and ORAC which was in triplicate. Univariate analysis of variance (ANOVA) and the Least Significant Difference Student's t-test were used as a post-hoc test. SAS statistical software (SAS®, Version 9.2, SAS Institute Inc., Cary, NC, USA) was used for data processing. The Shapiro-Wilk test was used to test for normality. In addition, the results were analysed using multivariate statistical analysis in XLSTAT software (Version 7.5.2, Addinsoft, New York, USA). Principal Component

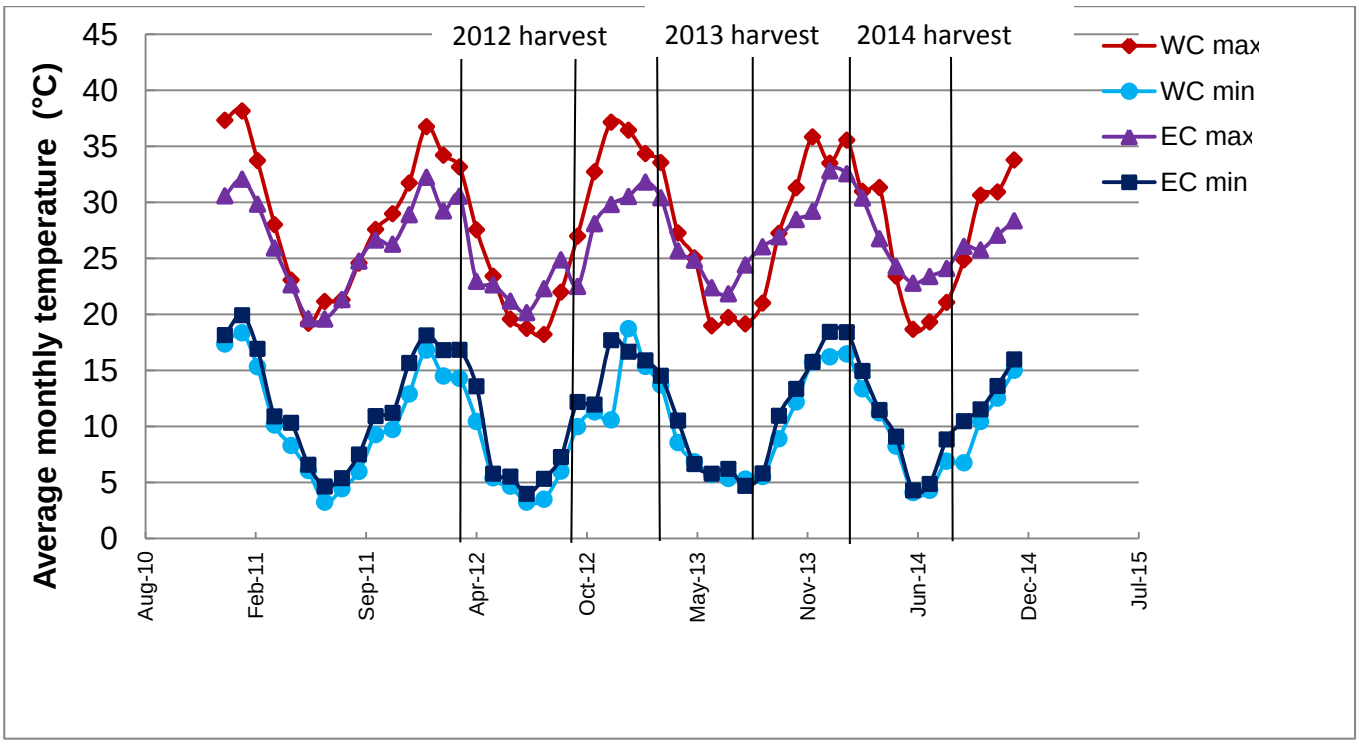
Analysis (PCA) was used to evaluate relationships between the characteristics, FCOJ type, seasons and region. The data for the PCA's were pretreated using Correlation matrix. Differences were considered statistically significant at 95% confidence level (P values < 0.05).

RESULTS AND DISCUSSION

Climatic differences based on growing region

The minimum and maximum monthly average temperatures follow a similar trend and correspond to the change in seasons (Fig 5.1a). There is a clear increase in the average maximum and minimum temperatures during spring which is the flowering months for citrus fruit produced in South Africa. There are 3 normal growth flushes for citrus in South Africa; during spring (August/September) followed by a second in November/December and lastly during the summer months February/March (Anon., 2009). Citrusdal (CD) in the WC region, in general reached a higher maximum monthly temperature during all the harvesting seasons (2011 - 2014). The WC sampling site had a maximum monthly average of 35°C, 34°C and 36°C for 2011, 2012 and 2014. In comparison to Kirkwood (KW) in the EC which had a maximum monthly average of 31°C, 30°C and 31°C for 2011, 2012 and 2014. In addition, the WC sampling site attained lower maximum temperatures during the winter months (Jun – Aug) which proves to be a colder climate overall during the harvesting months. Interestingly, the minimum monthly temperatures were similar for both regions. The EC region (KW) was characterised with considerably higher rainfall (average 37 mm) than the WC (25 mm) during 2011 - 2014 (Fig 5.1b). It is also clear that the WC experienced most of its rainfall during the winter months (Jun – Aug) while the EC received most of its rainfall during the summer months (Dec – Feb), which is expected. The data in figure 5.2 depicts the reference evapotranspiration (ET_0) of the two regions. Evapotranspiration can be used to point out possible periods of water stress experienced by the growing citrus crop and can be consequently linked to lower fruit quality such as an increase in °Brix and TA (Mougheith *et al*., 1977). Once again WC experienced a higher ET_0 even though the radiation for the sites in the WC and EC was similar. This may be due to the lower rainfall in the WC, which can possibly contribute to water stress in the citrus orchards. In Fig. 5.3 it can be seen that the ET_0 values are higher during those periods of little rainfall in the WC, which was expected. This implies that during those

a.)



b.)

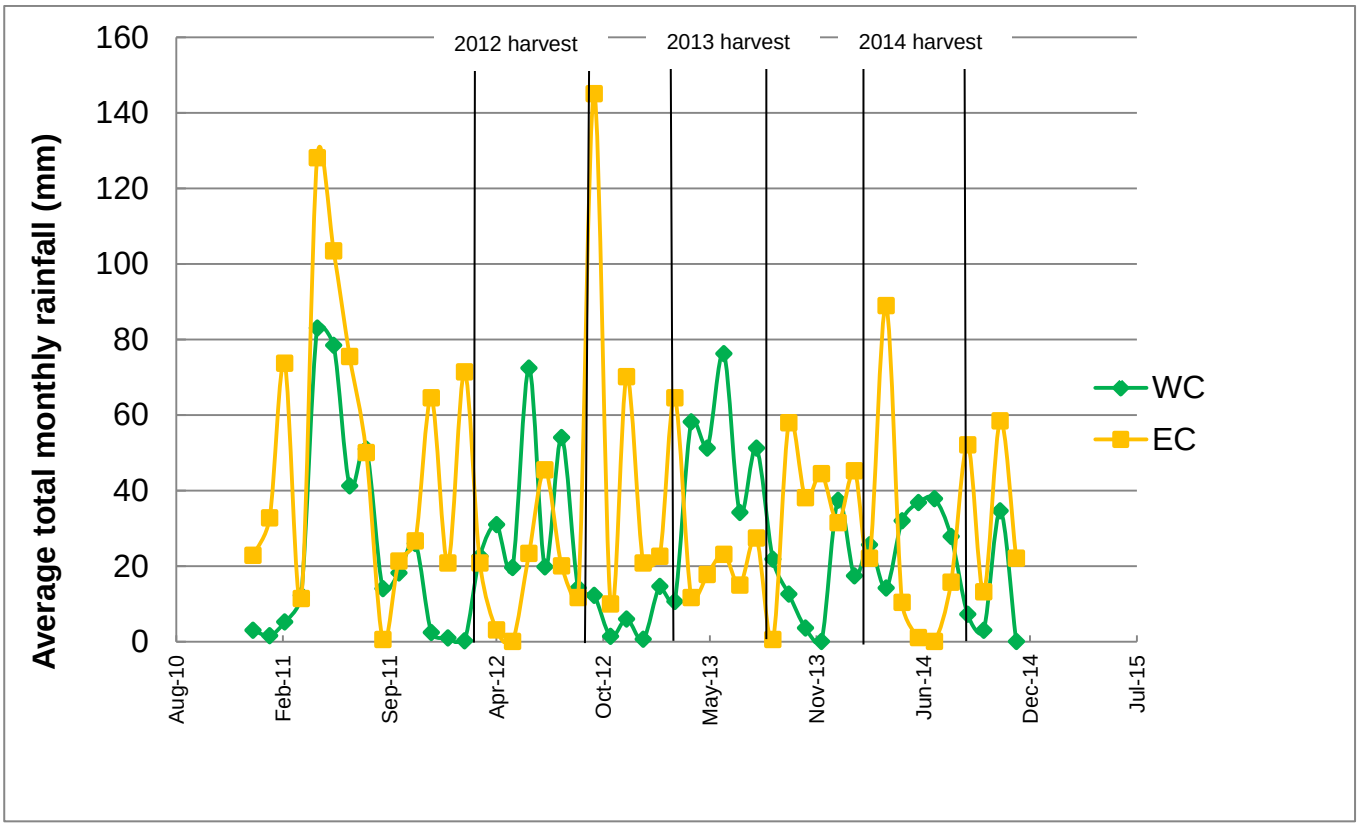
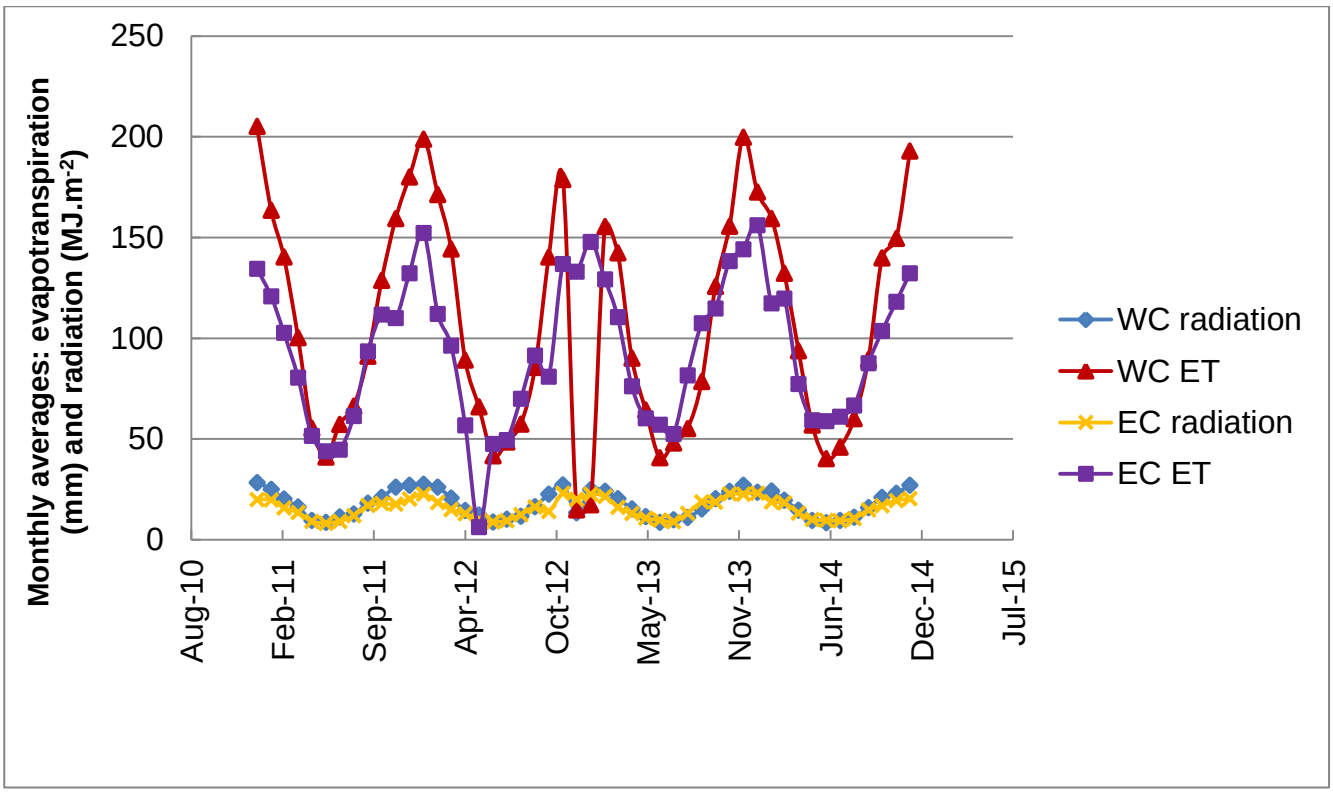
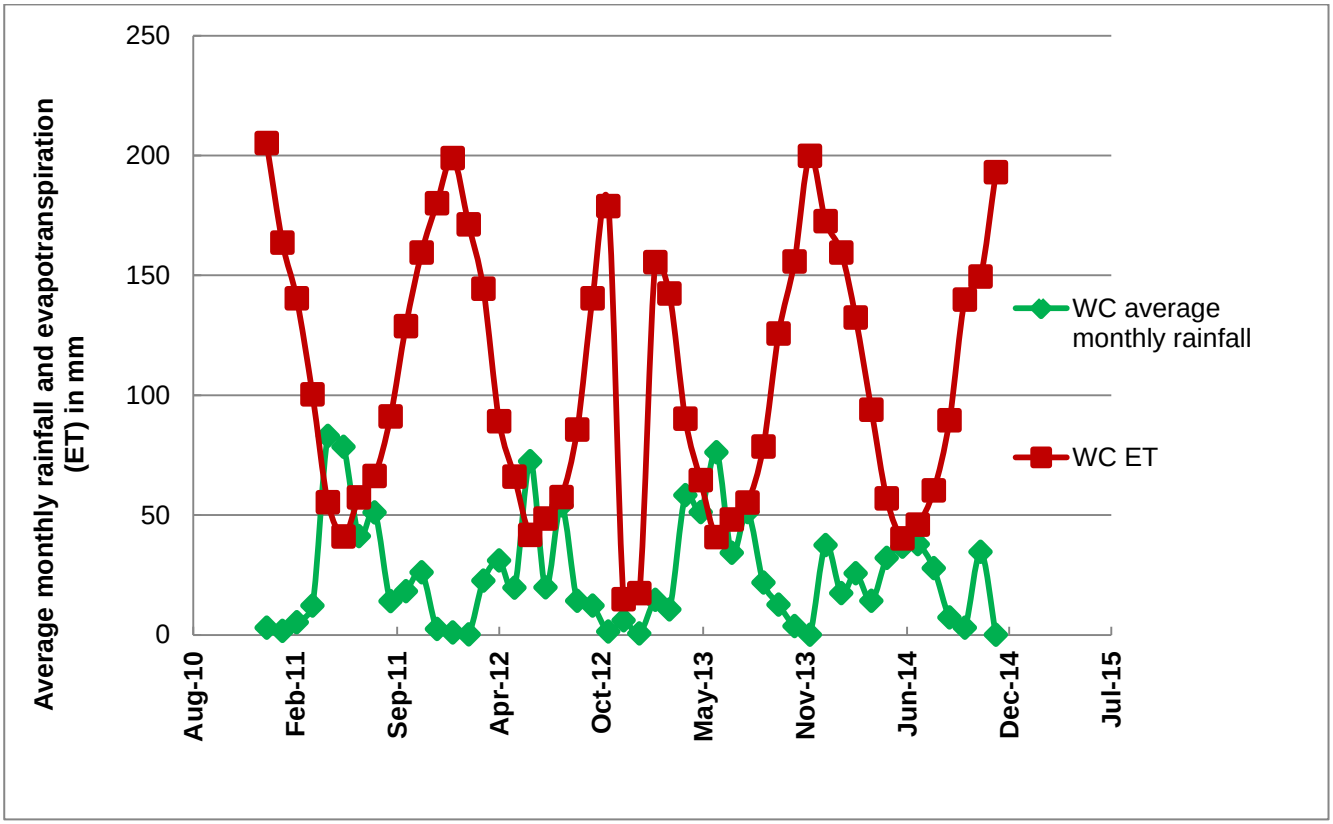


Figure 5.1 Climatic growing conditions, minimum and maximum monthly temperatures (a) and average total monthly rainfall (precipitation) (b) for the two regions evaluated in South Africa for 2011 - 2014. WC = Western Cape; EC = Eastern Cape.



Figures 5.2 Radiation and evapotranspiration recorded for 2011 – 2014 growing seasons in the Western Cape (WC) and Eastern Cape (EC) region of South Africa.



Figures 5.3 Relationship between average monthly rainfall and evapotranspiration (ET) recorded for 2011 – 2014 growing seasons in the Western Cape (WC) region of South Africa.

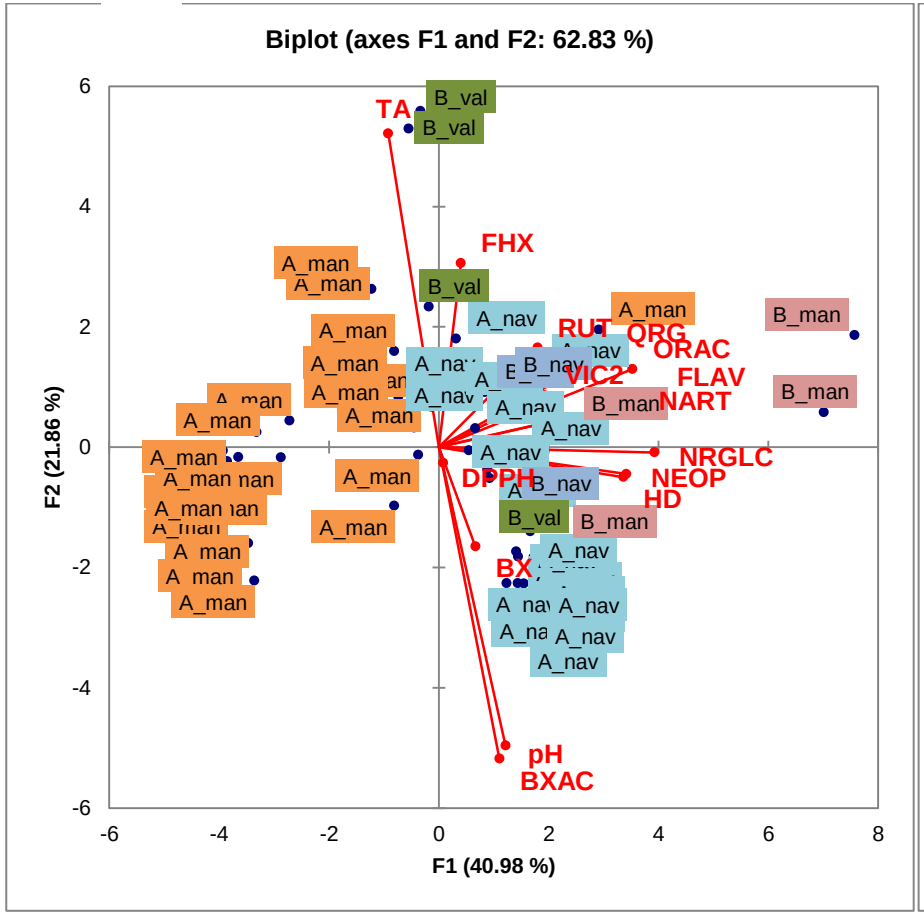
times irrigation of the growing crop would have been necessary. If the orchards experienced a lack of moisture during October – January it could result in acid fruit or fruit with a higher TA (Anon., 2009). This was the case for the WC sampling site in terms of average monthly rainfall. This resulted in all the varieties from this region having a significantly ($p<0.05$) lower TA compared to those from the EC sampling site, which is in contradiction to literature. However, this may be attributed to orchard irrigation during the lower rainfall periods at the WC sampling site resulting in lower TA. Unfortunately, information on irrigation practices or orchard management of the various farms who contributed to the final samples from both regions were unavailable. Therefore, attempts will be made to derive and link information regarding climatic growing conditions to possible differences in the chemical and phenolic composition of the samples based on regional differences only.

Principal Component Analysis (PCA)

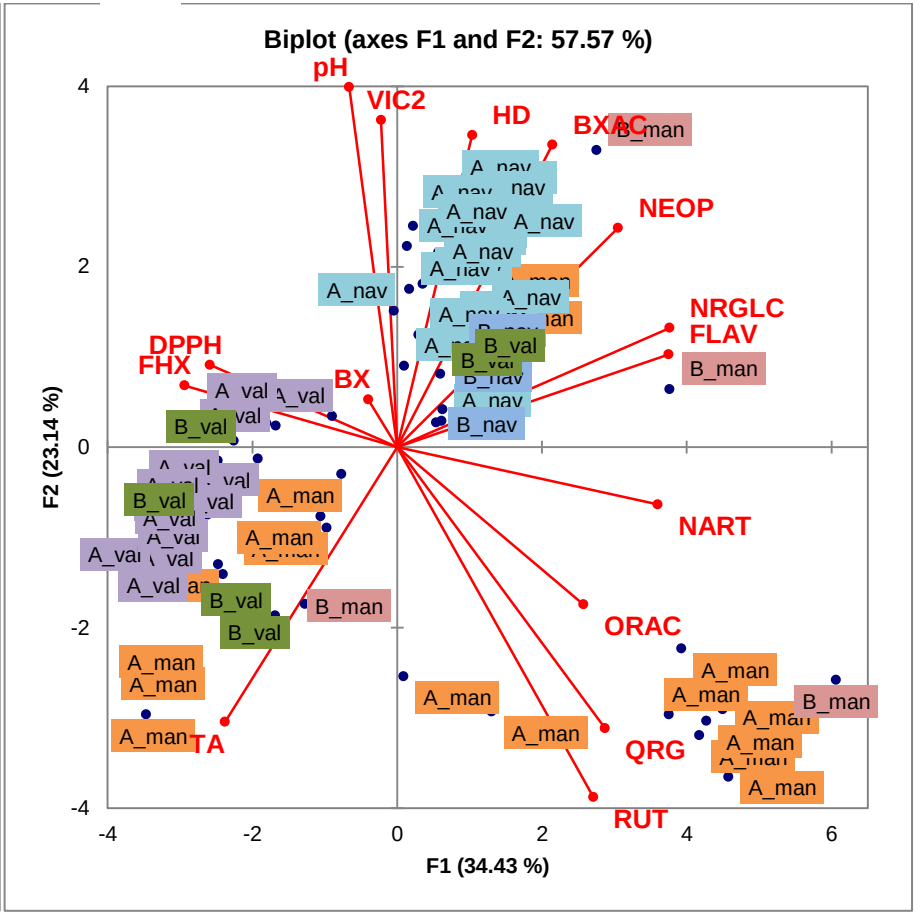
Associations between the FCOJ varieties, juice characteristics and region per season were evaluated using PCA. The biplots compiled from the PCA are given in Figure 5.4. ANOVA was used to evaluate and compare the individual characteristics of the FCOJ variety, season (year) and region. The main observations and comparisons will be further discussed. The PCA biplot (Fig. 5.4a) depicts the distribution of the three varieties sampled in 2012 from the WC and EC sampling sites based on chemical and phenolic composition and explains 63% of the inherent variability in the data. The mandarin variety shows clear clusters corresponding to the two regions. Mandarin from the EC sampling site (B_man) was positively associated with higher TP and TAC_{ORAC} and negatively associated with lower TA. Mandarin from the WC sampling site (A_man) was negatively associated and had lower phenolic composition and higher TA. The navel samples from both sampling sites (A_nav and B_nav) was closely associated in terms of phenolic composition with some overlap with the valencia (B_val) from the EC sampling site. Although B_val was associated with higher TA and FHX.

The PCA biplot (Fig. 5.4b) depicts the distribution of the three varieties sampled in 2013 from the WC and EC sampling sites based on chemical and phenolic composition and explains 58% of the inherent variability in the data. The distribution of the different varieties from the different sampling sites based on chemical and phenolic composition is widespread for the 2013 season and differs from the 2012 season. However, some varietal and regional differences in terms of associations may be noted. Most of the

a.)



b.)



c.)

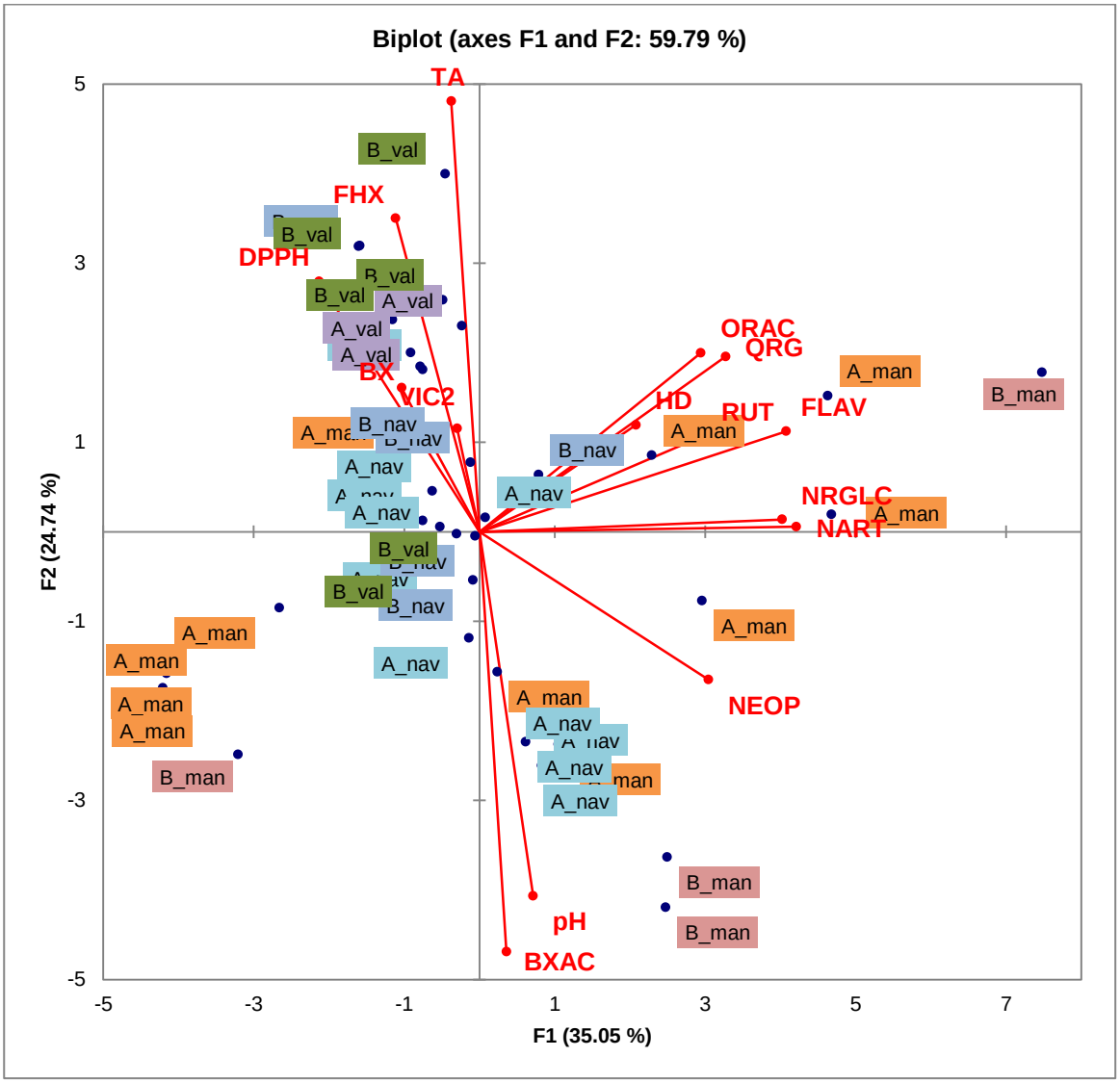


Figure 5.4 PCA biplot of associations between variety and region based on chemical and phenolic composition for the a.) 2012, b.) 2013 and c.) 2014 processing seasons. (TA = titratable acidity, BX/AC = °Brix:acid ratio, QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds, DPPH and ORAC = total anitioxidant capacity, nav = navel; man = mandarin; val = Valencia, A = Western Cape sampling site, B = Eastern Cape sampling site).

A_man samples were positively associated with higher TA, RUT, QRG and TAC_{ORAC}. The B_man samples were scattered with no distinct associations. This is also seen in figure 5.6 showing the large variation in the results for the mandarin varieties. Valencia varieties from both regions were associated with higher FHX and TAC_{DPPH}. The navel varieties formed a cluster and were associated with higher BX/AC, NRGLC, HD, NEOP, and TP.

The PCA biplot (Fig. 5.4c) depicts the distribution of the three varieties sampled in 2014 from the WC and EC sampling sites based on chemical and phenolic composition and explains 60% of the inherent variability in the data. It is clear that the 2014 season showed even more natural variation in chemical and phenolic attributes within the specific varieties based on the past seasons (2012 and 2013). Once again the mandarin variety had varying associations which could not be related to specific chemical or phenolic attributes. Although most of the B_man samples were positively associated with higher NRGLC, RUT, NART, HD, NEOP and TP others were negatively associated with lower concentrations for these compounds. Once again the valencia varieties from both regions were associated with higher TA, FHX and TAC_{DPPH}. Similarly, the navel samples, irrespective of region were mostly associated with higher Bx, BX/AC, pH and VIC2. Differences between valencia and navel based on the distribution of the phenolic compounds were unclear with overlap between the two varieties. Although valencia from the WC region, irrespective of season, were positively associated with higher FHX and DPPH values. Navel was positively associated with higher VIC2, NEOP, HD, NRGLC, FLAV and ORAC. Furthermore in Fig. 5.6 b&c it is clear that the mandarin variety showed large variation for all the individual phenolic compounds as well as TAC_{ORAC}.

Effect of variety, season and region on chemical and phenolic composition of FCOJ

The statistical results have been tabulated in Table 5.1 displaying the ANOVA results and significance ($p<0.05$) for the main effects and interactions. According to the results the main effects variety and region was the most significant ($p<0.05$) with season (year) having a significant ($p<0.05$) effect on some parameters such as BX/AC, pH, HD and NEOP only. In addition there were various significant ($p<0.05$) interactions between region x variety and region x year.

The results obtained indicated that the effect of variety was not significant ($p>0.05$) for the BX, QRG and TAC_{ORAC}. Moreover, the effect of region was not significant ($p>0.05$) for the BX, pH, RUT and TAC_{DPPH}. Finally, the effect of season (year) was not significant ($p>0.05$) for most parameters and included BX, TA, QRG, FHX, VIC2, NRGLC, RUT,

NART, HD, NEOP, TP, TAC_{DPPH} and TAC_{ORAC}. Thus, seasonal differences did not have a significant ($p>0.05$) effect on phenolic composition apart from two flavanones (HD and NART). In terms of chemical composition, the BX/AC and pH differed significantly ($p<0.05$) between the different seasons. The significant ($p<0.05$) effects for individual parameters will be discussed further.

Varietal differences (FCOJ type)

Three varieties of FCOJ produced over three seasons in different regions of South Africa were evaluated in order to obtain information on the chemical and phenolic profile. There were no significant differences ($p>0.05$) in the °Brix since all FCOJ samples were prepared by reconstitution at a standardised single strength °Brix level, with a tolerance range of 11.3 - 11.7 °Brix, to ensure that the samples were at the same concentration for direct comparison (Fig 5.5). Therefore, corroborating that the effect of variety was not significant ($p<0.05$). However, on the other hand significant differences ($p<0.05$) were found between the titratable acidity (TA) of the three varieties. As expected the valencia variety was characterised with the highest TA of 1.22 % $w.w^{-1}$ citric acid, followed by mandarin which was not expected since mandarin is described as having low acid levels (Rodrigo & Zacarías, 2006; Anon, 2012). The navel variety was found to have the lowest acidity level (0.93% $w.w^{-1}$ citric acid) and can be ascribed to the fact that in general this variety is the earliest maturing variety of all Orange sector varieties. The lower TA can be due to the accumulation of carbohydrates and water in the fruit which results in a decrease in the tartness or characteristic acidity because of a dilution effect as the fruit matures (Kimball, 1999; Rodrigo and Zacarías, 2006). In addition, when citrus fruit, especially navels are grown in warmer climates where higher temperatures are observed the respiration rate will be higher and the citric acid reserves will decrease as a result (Kimball, 1999; Rodrigo and Zacarías, 2006). This makes navels less suited for warm environments. The growing conditions in terms of average temperature experienced by navels grown in South Africa may have contributed to the lower TA as previously shown in Fig 5.1a. Consequently, navel varieties had the highest °Brix:acid ratio (BX/AC) (12.70) which differed significantly ($p<0.05$) from mandarin (11.5) and valencia (9.5). The pH was also found to differ significantly ($p<0.05$) between navel and the other varieties. Navel fruit were found to have a mean pH of 3.6 compared to mandarin and valencia having a pH of 3.5 and 3.4 respectively. The higher pH of navel can be ascribed to the lower TA levels.

Table 5.1 P values for ANOVA of individual chemical parameters, phenolic composition and total antioxidant capacity for different citrus varieties, growing regions and processing seasons

Source	BX	TA	BX/AC	pH	QRG	FHX	VIC2	NRGLC	RUT	NART	HD	NEOP	TP	DPPH	ORAC
Region (R)	0.3942	0.0002	0.0018	0.7194	0.0407	0.0094	<0.0001	0.0062	0.4164	<0.0001	<0.0001	0.0028	<0.0001	0.3886	<0.0001
Variety (V)	0.3742	<0.0001	<0.0001	<0.0001	0.2583	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	<0.0001	0.3980
R x V	0.3584	0.0003	<0.0001	0.0024	0.876	0.0002	0.7729	0.0002	0.9852	<0.0001	<0.0001	<0.0001	<0.0001	0.0008	0.0045
Year (Y)	0.1601	0.1086	0.0103	<0.0001	0.1856	0.2185	0.5360	0.0790	0.2120	0.2440	<0.0001	0.0012	0.0926	0.4342	0.1556
R x Y	0.4769	0.7109	0.7000	0.0589	0.0971	0.1254	0.4092	0.0102	0.0934	0.0407	0.0925	0.0720	0.0565	0.8111	<0.0001
V x Y	0.6377	0.4880	0.5058	0.0024	0.1070	0.3101	0.7993	0.2093	0.1683	0.2287	0.0779	0.5789	0.0967	0.2371	<0.0001
R x V x Y	0.1971	0.4211	0.1651	0.6476	0.2546	0.5480	0.9410	0.0419	0.2390	0.0540	0.0377	0.2372	0.1034	0.4222	0.0014

BX = °Brix, TA = titratable acidity (expressed as %w.w^{-1} citric acid), BX/AC = °Brix:acid ratio, QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds, $TAC_{DPPH/ORAC}$ = total antioxidant capacity as measured by DPPH and ORAC expressed as μM TE.

Table 5.2 The effect of variety on the individual chemical parameters and phenolic composition including the total antioxidant activity of FCOJ

variety	TA	BX/AC	pH	FHX	VIC2	NRGLC	RUT	NART	HD	NEOP	TP	DPPH
mandarin	1.0 ± 0.10 ^{b}	11.49 ± 1.35 ^{b}	3.45 ± 0.20 ^{b}	6.97 ± 2.82 ^{c}	19.1 ± 15.47 ^{b}	12.62 ± 9.44 ^{b}	7.13 ± 7.31 ^{a}	54.14 ± 44.59 ^{a}	24.25 ± 10.75 ^{b}	5.77 ± 4.88 ^{a}	136.77 ± 78.61 ^{b}	211.8 ± 80.24 ^{b}
navel	0.93 ± 0.10 ^{c}	12.67 ± 1.78 ^{a}	3.57 ± 0.20 ^{a}	9.08 ± 1.42 ^{b}	34.28 ± 4.74 ^{a}	18.58 ± 1.88 ^{a}	1.9 ± 0.61 ^{b}	45.7 ± 4.71 ^{a}	40.66 ± 4.8 ^{a}	6.46 ± 1.05 ^{a}	161.94 ± 11.15 ^{a}	331.21 ± 27.85 ^{a}
valencia	1.23 ± 0.20 ^{a}	9.55 ± 1.33 ^{c}	3.44 ± 0.10 ^{b}	16.35 ± 3.38 ^{a}	31.28 ± 7.1 ^{a}	11.73 ± 3.8 ^{b}	2.4 ± 0.7 ^{b}	30.9 ± 10.86 ^{b}	38.65 ± 7.92 ^{a}	4.4 ± 1.6 ^{b}	141.58 ± 24.57 ^{b}	346.37 ± 42.3 ^{a}

QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds, TAC_{DPPH} = total antioxidant capacity as measured by DPPH expressed as μM TE WC = Western Cape, EC = Eastern Cape. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

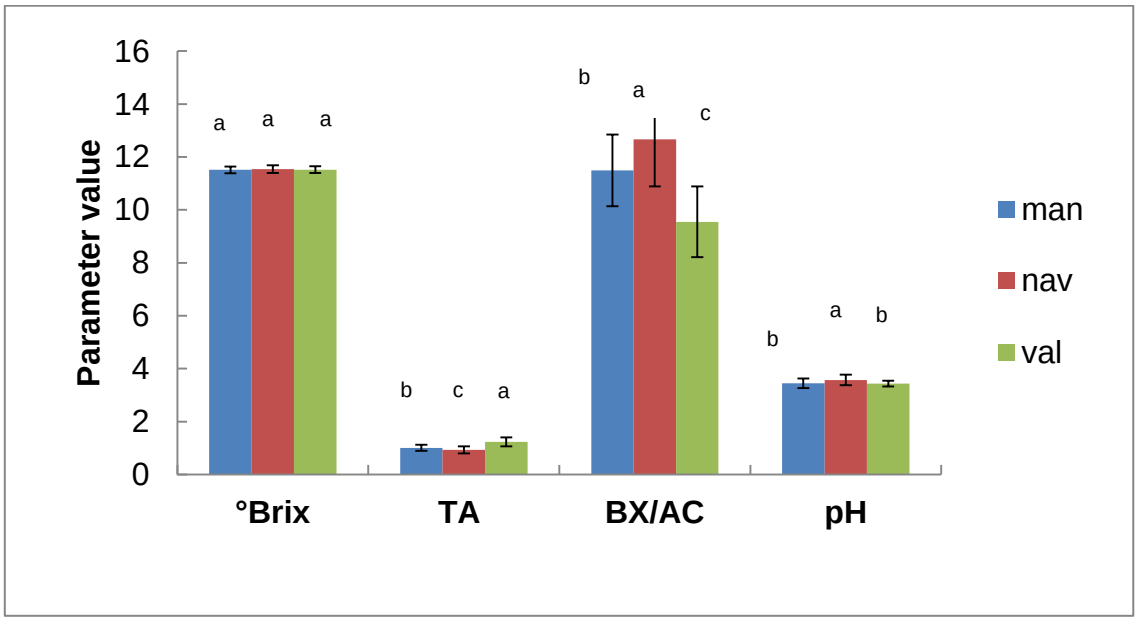


Figure 5.5 Chemical composition of three varieties of FCOJ samples collected from 2012 – 2014 processed in South Africa. TA = titratable acidity (expressed as % $w.w^{-1}$ citric acid); BX/AC = °Brix:acid ratio. Significant differences ($p<0.05$) are indicated with the use of different alphabetical letters.

Seven phenolic compounds were identified and quantified for the three varieties (Fig 5.6). The flavone-*O*-glycosides specifically 3-*O*-rutinosides were quantified at 255 nm and included quercetin-3-*O*-rutinose-7-*O*-glucoside (QRG) as well as rutin (RUT). Flavanone-*O*-glycosides specifically 7-*O*-rutinosides were quantified at 288 nm and included naringenin-7-*O*-rutinose-4'-*O*-glucoside (NRGLC) as well as narirutin (NART), hesperidin (HD) and neoponcirin (NEOP). One hydroxycinnamic acid was quantified at 320 nm namely ferulic acid-*O*-hexoside (FHX) and lastly a flavone-*C*-glucoside was quantified at 350 nm namely vicenin-2 (VIC2). No significant difference ($p>0.05$) was found in the QRG of the three varieties (Fig 5.6). There was however a significant difference ($p<0.05$) in the RUT levels with mandarin having the highest concentration (7.13 $mg.L^{-1}$) (Table 5.2). The RUT concentration of navel and valencia did not differ significantly ($p>0.05$). These two varieties are closely associated since they are sub-species of *Citrus sinensis* family which may explain this similarity. Navels showed a significant difference ($p<0.05$) for all the quantified flavanone-*O*-glycosides and had the highest concentration of NRGLC (18.58 $mg.L^{-1}$), HD (40.66 $mg.L^{-1}$) and NEOP (6.46 $mg.L^{-1}$). The NART and NEOP concentration of navels (45.70 $mg.L^{-1}$ and (6.46 $mg.L^{-1}$) were similar to that of mandarin (54.14 $mg.L^{-1}$ and 5.77 $mg.L^{-1}$) ($p>0.05$), whilst the HD concentration were similar for navels and valencia. In addition the NRGLC of mandarin and valencia were found not to differ ($p>0.05$). The FHX concentration varied significantly ($p<0.05$) between the varieties with valencia having the highest (16.35 $mg.L^{-1}$) followed by navel (9.10 $mg.L^{-1}$) and mandarin (6.97 $mg.L^{-1}$). Mandarin was found to contain significantly lower ($p<0.05$) VIC2 levels, 19.06 $mg.L^{-1}$, compared to navel and valencia, 34.28 $mg.L^{-1}$ and 31.28 $mg.L^{-1}$ respectively. Ultimately, the navel variety was characterised with the highest total phenolic concentration (TP) of 161.94 $mg.L^{-1}$ which was significantly ($p<0.05$) higher than that of valencia (141.58 $mg.L^{-1}$) and mandarin (136.77 $mg.L^{-1}$). Interestingly, this observation did not result in the navel variety exhibiting the highest TAC. The valencia variety showed the highest TAC_{DPPH} of 346.37 µM $TE.mL^{-1}$, which did not differ significantly ($p>0.05$) from navel having a TAC_{DPPH} of 331.21 µM $TE.mL^{-1}$. Mandarin exhibited a significantly ($p<0.05$) lower TAC_{DPPH} of 211.8 µM $TE.mL^{-1}$. The TAC_{ORAC} were found not to differ significantly ($p>0.05$) between the varieties and ranged from 5538.2 - 5575.0 µM $TE.mL^{-1}$. Furthermore, increased maturity (higher BX/AC) in citrus fruits was previously shown to decrease the phenolic content and antioxidant activity (Barreca *et al*., 2011; Rekha *et al*., 2012). However, in contradiction to what was found in Chapter 3 and in literature the results for mandarin in terms of TP and TAC_{DPPH}

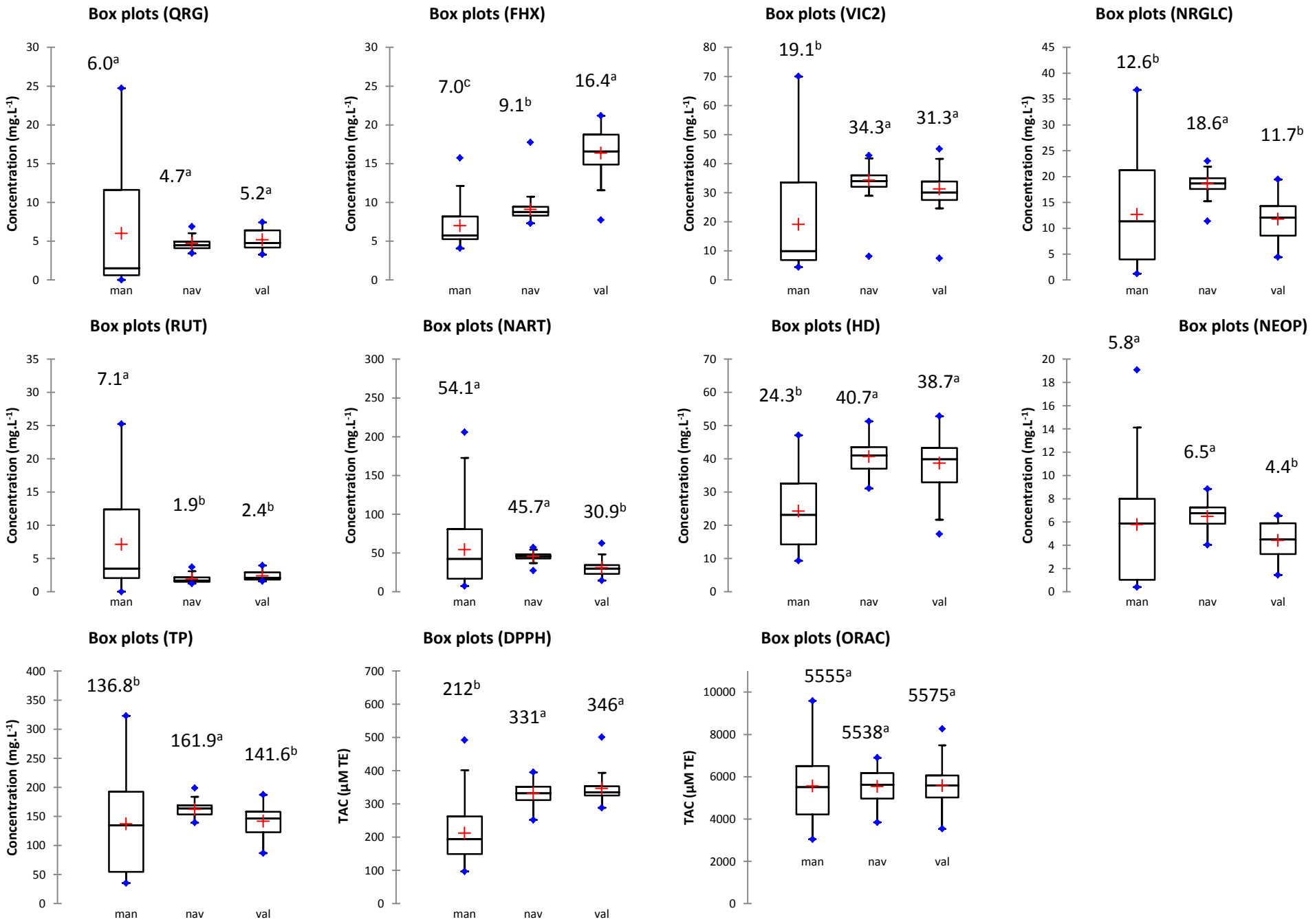


Figure 5.6 Distribution of phenolic compounds (with means in mg.L^{-1}) and total antioxidant capacity (TAC expressed as µM TE and measured by DPPH and ORAC) of three varieties of FCOJ samples collected from 2012 – 2014 and processed in South Africa (QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds, nav = navel; man = mandarin; val = valencia).

were significantly ($p<0.05$) lower than navel which had the highest (Bx/AC) and considered more mature. In previous studies done by Leuzzi *et al.* (2000) on blood orange cultivars from *Citrus sinensis* the HD, NART and NEOP levels was reported to be much higher in the FCOJ samples compared to what was found in this study. The mean levels were found to be 513 $mg.L^{-1}$ for HD, 73 $mg.L^{-1}$ for NART and 23.3 $mg.L^{-1}$ for NEOP. The authors also found that the HD and NEOP levels in the FCOJ samples were slightly lower in the concentrate compared to the single-strength juice. In contrast the NART was found to be slightly higher in the concentrate.

Significant ($p<0.05$) effects due to region x variety interaction were observed for the TA, BX/AC and pH (Table 5.3) as well as for five phenolic compounds and both TAC assays. The TA of navel was the highest for the varieties sampled at the WC site and corresponded to the TA of the samples from the EC sampling site. Two varieties, namely mandarin and valencia from the WC site therefore had significantly ($p<0.05$) lower TA and correspondingly higher BX/AC and pH. The mandarin from EC had significantly ($p<0.05$) higher FHX which was found to be the highest level of 11.20 $mg.L^{-1}$. This significantly differed ($p<0.05$) from the mandarin and valencia varieties in the WC site. The navel samples from the WC site had similar FHX levels to those from the EC site. The NRGLC level of the EC mandarin once again was found to be the highest (19.39 $mg.L^{-1}$) and significantly ($p<0.05$) differed from the mandarin and navel varieties sampled in the WC. However, the valencia from both regions had similar levels with WC valencia having levels of 16.1 $mg.L^{-1}$ and EC valencia having 16.92 $mg.L^{-1}$. All the varieties from the EC site had higher NART levels with mandarin once again having significantly ($p<0.05$) higher levels of 71.42 $mg.L^{-1}$, followed by navel with 58.42 $mg.L^{-1}$. The valencia variety irrespective of region had similar NART levels, while the WC mandarin had the lowest NART level of 36.33 $mg.L^{-1}$. Concerning the HD levels, EC Valencia had the highest (42.57 $mg.L^{-1}$) and WC mandarin the lowest (27.64 $mg.L^{-1}$). The navel variety from both regions had similar concentrations. The varieties from the EC in general had higher TP and consequently TAC_{DPPH} and TAC_{ORAC}. The EC mandarin had significantly ($p<0.05$) higher TP concentrations of 192.25 $mg.L^{-1}$. On the other hand, the WC mandarin had significantly ($p<0.05$) lower TP levels (122.35 $mg.L^{-1}$) compared to other varieties from the same or different region. Similarly, the TAC_{ORAC} showed that the interaction between variety and regions was significant ($p<0.05$) with the varieties from the EC having higher activity which is linked to the phenolic composition. The TAC_{DPPH} results were found to differ from the TAC_{ORAC} and did not relate the TAC to the TP content, since the higher TP of EC mandarin resulted in the lowest TAC_{DPPH} which cannot be explained.

Table 5.3 Means and standard deviation for chemical parameters and phenolic compounds ($mg.L^{-1}$) with a significant ($p<0.05$) region x variety interaction effect

Region	Variety	TA	BX/AC	pH	FHX	NRGLC	NART	HD	NEOP	TP	DPPH	ORAC
Western Cape	mandarin	0.99 ± 0.10 ^{b}	11.67 ±1 .42 ^{ab}	3.53 ± 0.10 ^{ab}	8.16 ± 2.28 ^{c}	12.34 ± 7.15 ^{c}	36.33 ± 16.70 ^{d}	27.64 ± 12.63 ^{c}	4.45 ± 2.54 ^{c}	122.35 ± 51.39 ^{d}	270.65 ± 80.25 ^{ab}	4952.66 ± 1115.83 ^{e}
	navel	1.02 ± 0.20 ^{ab}	11.52 ± 1.80 ^{b}	3.41 ± 0.20 ^{c}	10.6 ± 5.01 ^{ab}	14.65 ± 6.29 ^{bc}	42.37 ± 20.60 ^{cd}	33.92 ± 10.18 ^{b}	5.76 ± 2.49 ^{bc}	143.55 ± 38.02 ^{cd}	299.18 ± 75.52 ^{ab}	5327.63 ± 1062.23 ^{de}
	valencia	0.96 ± 0.20 ^{b}	12.46 ± 2.27 ^{ab}	3.59 ± 0.20 ^{a}	9.27 ± 4.21 ^{bc}	16.10 ± 6.65 ^{ab}	47.95 ± 25.8 ^{bcd}	36.41 ± 12.48 ^{b}	6.28 ± 2.79 ^{ab}	151.52 ± 50.89 ^{bc}	280.47 ± 67.53 ^{ab}	5942.4 ± 1017.04 ^{bc}
Eastern Cape	mandarin	1.10 ± 0.20 ^{a}	10.83 ± 1.90 ^{b}	3.47 ± 0.10 ^{bc}	11.2 ± 4.70 ^{a}	19.39 ± 8.90 ^{a}	71.42 ± 51.92 ^{a}	37.01 ± 3.43 ^{b}	6.84 ± 4.39 ^{ab}	192.25 ± 64.99 ^{a}	267.02 ± 107.25 ^{b}	6810.51 ± 1318.49 ^{a}
	navel	1.10 ± 0.20 ^{a}	10.78 ± 1.54 ^{b}	3.48 ± 0.10 ^{bc}	9.49 ± 4.10 ^{bc}	14.8 ± 6.61 ^{bc}	58.42 ± 52.80 ^{ab}	34.28 ± 5.99 ^{b}	5.60 ± 4.67 ^{bc}	170.53 ± 57.53 ^{abc}	304.25 ± 119.27 ^{a}	6483.28 ± 952.18 ^{ab}
	valencia	1.10 ± 0.30 ^{a}	11.18 ± 2.62 ^{b}	3.54 ± 0.20 ^{ab}	10.99 ± 5.04 ^{a}	16.92 ± 6.28 ^{ab}	52.27 ± 31.31 ^{bc}	42.57 ± 7.22 ^{a}	7.84 ± 4.63 ^{a}	173.55 ± 47.61 ^{ab}	303.18 ± 86.41 ^{a}	5598.05 ± 1181.82 ^{cd}

QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds, TAC_{ORAC} = total antioxidant capacity as measured by DPPH and ORAC expressed as μM TE, WC = Western Cape, EC = Eastern Cape. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

Seasonal differences (processing year 2012, 2013 and 2014)

The FCOJ samples processed in 2012, 2013 and 2014 were compared to evaluate the effect of processing season on juice characteristics (Addendum Table 5.9a). No significant differences were found in the °Brix and TA for the three production seasons. The BX/AC was found to be higher for samples produced in 2014 but did not differ significantly ($p>0.05$) from those produced in 2012, only from those produced in 2013. A similar trend was seen for the pH recorded. The pH of the samples produced in 2012 was similar to those produced in 2014 but differed significantly ($p<0.05$) from the 2013 samples. Thus, the 2013 FCOJ samples had a significantly ($p<0.05$) lower BX/AC and resulting pH (Table 5.4). Therefore, it can be concluded that variability due to seasonal variation in terms of climate and growing conditions had a minimal to no effect on the juice characteristics of the different FCOJ varieties evaluated.

The phenolic composition (QRG, VIC2, RUT and NART) did not differ significantly ($p>0.05$) between the production seasons (Addendum Table 5.9b). FHX and NRGLC did not differ significantly ($p>0.05$) for the 2013 and 2014 production season. However, 2012 had significantly ($p<0.05$) lower concentrations. HD and NEOP were the only compounds to show significant ($p<0.05$) differences in concentration for the three production seasons (Table 5.4). The TP once again resembled a similar trend with 2013 and 2014 seasons having comparable levels differing from the 2012 season. This is also seen for the TAC_{ORAC} and TAC_{DPPH} which is associated with the TP concentration (Velioglu *et al*., 1998).

When the juice characteristics are compared between varieties and processing seasons it is clear that the TA and BX/AC for each variety was not significantly ($p>0.05$) different for each season (Addendum Table 5.10a). Furthermore, for the individual phenolic composition it is clear the mandarin 2012 had the lowest QRG, NRGLC, RUT, NART, NEOP, TP and TAC_{ORAC} (Addendum Table 5.10b). Similarly, valencia 2014 had the highest HD and NEOP which differed significantly ($p<0.05$) from valencia 2012 and 2013. Overall the effect of season (year) on HD indicated that 2014 had significantly ($p<0.05$) higher concentrations (38.82 $mg.L^{-1}$), followed by 2013 (33.99 $mg.L^{-1}$) and 2012

Table 5.4 Means ($mg.L^{-1}$) and standard deviation for parameters with a significant ($p<0.05$) year main effect

year	BX/AC	pH	HD	NEOP
2012	11.50 ± 1.55 [ab]	3.52 ± 0.10 [a]	29.59 ± 11.95 [c]	4.95 ± 3.12 [b]
2013	11.37 ± 1.76 [b]	3.42 ± 0.20 [b]	33.99 ± 9.43 [b]	5.73 ± 3.02 [b]
2014	11.96 ± 2.47 [a]	3.57 ± 0.20 [a]	38.82 ± 11.06 [a]	6.89 ± 3.65 [a]

BX/AC = °Brix:acid ratio, HD = hesperidin, NEOP = neoponcerin. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

Table 5.5 Means and standard deviation for parameters with a significant ($p<0.05$) variety x year interaction effect

Variety	Year	pH	ORAC
Mandarin	2012	3.49 ± 0.05 [bcd]	4841 ± 1723 [d]
	2013	3.28 ± 0.19 [e]	6257 ± 1226 [a]
	2014	3.61 ± 0.12 [a]	5810 ± 1404 [abc]
Navel	2012	3.58 ± 0.11 [ab]	5749 ± 684 [abc]
	2013	3.54 ± 0.22 [abc]	5201 ± 948 [cd]
	2014	3.58 ± 0.25 [ab]	5709 ± 713 [abc]
Valencia	2012	3.40 ± 0.17 [de]	6204 ± 491 [a]
	2013	3.43 ± 0.09 [cd]	5245± 914 [bcd]
	2014	3.48 ± 0.12 [bcd]	5992 ± 1158 [ab]

TAC_{ORAC} = total antioxidant capacity as measured by ORAC expressed as µM TE. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

(29.59 mg.L^{-1}). This was also seen for NEOP with 2014 having significantly ($p<0.05$) higher levels of 6.89 mg.L^{-1}.

Furthermore, a significant ($p<0.05$) variety x year interaction for pH and TAC_{ORAC} was observed (Table 5.5). Regarding all varieties, pH was found to be the highest for the 2014 season. The mandarin variety had the highest pH of 3.61 followed by navel (pH = 3.58) and valencia (pH = 3.48). The 2013 season, irrespective of variety, had the lowest pH for all sampling years. Both navel and Valencia sampled in 2012 had the highest TAC_{ORAC}, 5749 and 6204 μM TE respectively, and was similar to the TAC in 2014. Whereas, for mandarin the 2013 season had the highest TAC_{ORAC} of 6257 μM TE followed by 2014 (5810 μM TE).

Regional differences

Overall the FCOJ samples, irrespective of variety, collected from the Eastern Cape (EC) had a significantly ($p<0.05$) higher phenolic composition for all the individual compounds characterised. The samples from the WC site were found to be more mature, having a BX/AC of 11.78 compared to those from the EC site (10.96) which is as a result of a significantly ($p<0.05$) higher TA of 1.09% w.w^{-1} citric acid (Table 5.6). This is very interesting and may be attributed to the different rainfall patterns experienced by the two regions. However, the results of this study indicated that fruit with a significantly ($p<0.05$) higher TA is obtained irrespective of good rainfall during October – January prior to harvesting as opposed to the lack of moisture during those months which result in more acidic fruit (Anon., 2009). BX/AC is commonly regarded as the best available indicator of maturity in oranges since it can be readily obtained through simple equipment (Wedding & Horspool, 1995). Moreover, the EC FCOJ samples were found to have the highest individual phenolic composition and was significant ($p<0.05$) for all compounds apart from RUT (Table 5.6). Thus, it can be concluded that the lower average temperature, higher average rainfall, higher TA and thus lower BX/AC (less mature) of the EC had a positive effect on the bioactive phenolic composition and thus better quality FCOJ may be produced. This is also in line with previous research that showed an increase in maturity (higher BX/AC) results in a decrease in phenolic composition and TAC (Barreca *et al*., 2011; Rekha *et al*., 2012).

When the effect of production season is evaluated within a specific region significant differences ($p<0.05$) are observed between the phenolic composition (NRGLC, NART and TAC_{ORAC}) (Table 5.7). Concerning significant ($p<0.05$) region x year

interactions, the Eastern Cape (EC) region was characterised with an overall higher TA for 2012, 2013 and 2014 (Addendum Table 5.11a). This differed significantly ($p<0.05$) between the EC & WC as previously mentioned. The WC region had significantly ($p<0.05$) lower TA with 2014 having the lowest followed by 2012 and 2013 (Addendum Table 5.11a). This resulted in an identical development in the BX/AC observed. In addition for the individual phenolic compounds QRG, and RUT did not differ significantly ($p>0.05$) irrespective of region or season (Addendum Table 5.11b). Likewise, the HD levels of the WC 2013, WC 2014, EC 2012 and EC 2013 did not differ significantly ($p>0.05$). Although the WC 2012 had the lowest HD of 27.64 $mg.L^{-1}$ and EC had the highest HD of 42.57 $mg.L^{-1}$ which differed significantly ($p<0.05$) from the rest. However, regional and seasonal interactions were significant ($p<0.05$) for NRGLC and NART differences were clear for the TAC_{ORAC} (Table 5.7). The NRGLC of the WC 2014 samples were found to be significantly ($p<0.05$) higher, 16.10 $mg.L^{-1}$ compared to 2013 and 2014. The NRGLC concentration of the WC and EC 2014 samples corresponded with the EC site having 16.92 $mg.L^{-1}$. On the other the EC 2012 samples had the highest NRGLC concentration of 19.39 $mg.L^{-1}$. The exact observation was made for NART and TAC_{ORAC} as well. This indicates that regional and seasonal interactions may influence the natural levels and variation of these specific phenolic compounds. However, the results indicated that variation due to seasonal differences within a region was marginal since only some parameters where significantly ($p<0.05$) affected.

Significant ($p<0.05$) effects due to region x variety x season interaction was observed for NRGLC, HD and TAC_{ORAC} (Table 5.8). EC mandarin 2012 and 2014 had significantly ($p<0.05$) higher NRGLC levels of 26.14 $mg.L^{-1}$ and 19.96 $mg.L^{-1}$ respectively. Compared to WC mandarin 2012 had the lowest NRGLC of 6.79 $mg.L^{-1}$. Regarding the HD levels EC mandarin had significantly ($p<0.05$) higher levels compared to WC mandarin for 2012, 2013 and 2014. Although, navel and valencia varieties had significantly ($p<0.05$) higher HD levels in 2014. Again, EC mandarin 2012 had the highest TAC_{ORAC} (8071 µM TE) followed by 2013 (7030 µM TE), while WC mandarin 2012 had the lowest (4254 µM TE). It is evident that the effect growing region was significant ($p<0.05$) for the juice characteristics in terms of TA as previously mentioned (Addendum Table 5.12a). More importantly, it is interesting to note that the growing region had a significant ($p<0.05$) effect on the phenolic composition, especially for the mandarin varieties (Addendum Table 5.12b). From the results it is clear that distinctions based on regional differences can be made using the VIC2 concentrations since the FCOJ varieties from the WC region had significantly ($p<0.05$) lower VIC2 levels.

Table 5.6 Means and standard deviation for parameters with a significant ($p<0.05$) region main effect

region	TA	BX/AC	QRG	FHX	VIC2	NRGLC	NART	HD	NEOP	TP	ORAC
Western Cape	1.00 ± 0.20 [b]	11.78 ± 1.81 [a]	4.82 ± 4.92 [b]	9.44 ± 4.16 [b]	25.55 ± 12.52 [b]	14.12 ± 6.78 [b]	41.39 ± 20.84 [b]	32.20 ± 12.04 [b]	5.40 ± 2.66 [b]	137.64 ± 47.13 [b]	5324 ± 1123 [b]
Eastern Cape	1.09 ± 0.20 [a]	10.96 ± 2.08 [b]	6.70 ± 5.56 [a]	10.56 ± 4.59 [a]	33.83 ± 11.43 [a]	16.91 ± 7.22 [a]	59.54 ± 44.58 [a]	38.35 ± 6.89 [a]	6.83 ± 4.56 [a]	177.71 ± 55.3 [a]	6219 ± 1241 [a]

TA = titratable acidity (expressed as %w.w^{-1} citric acid), BX/AC = °Brix:acid ratio ,QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds, TAC_{ORAC} = total antioxidant capacity as measured by ORAC expressed as μM TE. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

Table 5.7 Means and standard deviation for phenolic compounds ($mg.L^{-1}$) and total antioxidant capacity (μM TE) with a significant ($p<0.05$) region x year interaction effect

Region	Year	NRGLC	NART	ORAC
Western Cape	2012	12.34 ± 7.15 ^{c}	36.33 ± 16.70 ^{d}	4952 ± 1115 ^{e}
	2013	14.65 ± 6.29 ^{bc}	42.37 ± 20.60 ^{cd}	5327 ± 1062 ^{de}
	2014	16.10 ± 6.65 ^{ab}	47.95 ± 25.8 ^{bcd}	5942 ± 1017 ^{bc}
Eastern Cape	2012	19.39 ± 8.9 ^{a}	71.42 ± 51.92 ^{a}	6810 ± 1318 ^{a}
	2013	14.79 ± 6.61 ^{bc}	58.42 ± 52.79 ^{ab}	6483 ± 952 ^{ab}
	2014	16.92 ± 6.28 ^{ab}	52.3 ± 31.31 ^{bc}	5598 ± 1181 ^{cd}

NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, TAC_{ORAC} = total antioxidant capacity as measured by ORAC expressed as μM TE. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

Table 5.8 Means and standard deviation for phenolic compounds (mg.L^{-1}) and total antioxidant capacity (μM TE) with a significant ($p<0.05$) region x variety x year interaction effect

Region	Variety	Year	NRGLC	HD	ORAC
Western Cape	mandarin	2012	6.79 ± 5.47 ^{f}	17.13 ± 6.87 ^{h}	4254.19 ± 935.43 ^{g}
		2013	13.77 ± 8.23 ^{bcde}	24.15 ± 4.49 ^{gh}	6074.76 ± 1191.15 ^{bcde}
		2014	13.71 ± 9.26 ^{bcde}	25.76 ± 10.45 ^{g}	5792.67 ± 1240.89 ^{cdef}
	navel	2012	18.45 ± 1.52 ^{bcd}	39.21 ± 4.81 ^{bcde}	5720.98 ± 730.31 ^{cdef}
		2013	18.76 ± 1.63 ^{bc}	41.3 ± 3.88 ^{bcd}	4982.27 ± 809.74 ^{efg}
		2014	18.96 ± 1.98 ^{bc}	43.16 ± 5.23 ^{ab}	5891.14 ± 613.28 ^{cdef}
	valencia	2013	9.46 ± 2.91 ^{ef}	35.36 ± 8.67 ^{cdef}	4881.95 ± 687.49 ^{fg}
		2014	14.07 ± 0.37 ^{bcde}	50.75 ± 1.82 ^{a}	6679.36 ± 1371.24 ^{bc}
Eastern Cape	mandarin	2012	26.14 ± 11.97 ^{a}	39.21 ± 0.99 ^{bcde}	8071.2 ± 1474.47 ^{a}
		2013	15.99 ± 8.99 ^{bcd}	29.12 ± 7.38 ^{fg}	7030.01 ± 1212.4 ^{ab}
		2014	19.96 ± 11.81 ^{ab}	39.81 ± 13.56 ^{bcde}	5855.84 ± 2016.23 ^{cdef}
	navel	2012	18.24 ± 2.91 ^{bcd}	38.70 ± 3.75 ^{bcde}	5938.32 ± 116.04 ^{bcdef}
		2013	18.52 ± 1.69 ^{bcd}	34.15 ± 1.1 ^{def}	6656.65 ± 96.62 ^{bc}
		2014	17.93 ± 3.41 ^{bcd}	43.01 ± 4.59 ^{abc}	5376.21 ± 817.35 ^{def}
	valencia	2012	13.49 ± 2.55 ^{cde}	33.53 ± 1.81 ^{ef}	6203.97 ± 491.21 ^{bcd}
		2013	12.13 ± 6.10 ^{def}	37.80 ± 4.13 ^{bcde}	6032.11 ± 888.16 ^{bcde}
		2014	13.88 ± 1.82 ^{bcde}	43.97 ± 4.01 ^{ab}	5648.03 ± 983.74 ^{cdef}

NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, HD = hesperidin, TAC_{ORAC} = total antioxidant capacity as measured by ORAC expressed as μM TE. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

Regional differences were not significant ($p>0.05$) when it came to QRG concentrations. Regarding the other phenolic compounds FHX, NRGLC, RUT, NART, NEOP, TP, TAC_{DPPH} and TAC_{ORAC} was found to be similar for navel and valencia FCOJ samples from both regions. Therefore, regional and varietal distinction was not possible. Moreover, it is remarkable that the mandarin FCOJ variety can be distinguished based on regional differences when comparing the QRG, FHX, NRGLC, RUT, NART, HD, NEOP, TP and TAC_{ORAC} which differed significantly ($p<0.05$) between the WC and EC. The mandarin FCOJ samples from the WC had the lowest concentrations of the above mentioned phenolic compounds and TAC_{ORAC} in comparison with EC mandarin FCOJ. In general, it can thus be concluded that in terms of phenolic composition the mandarin FCOJ samples from EC was of better quality.

CONCLUSION

The distribution and concentration of phenolic compounds and total antioxidant activity of three FCOJ varieties grown and processed during three seasons (2012, 2013 and 2014) in two regions (WC and EC) of South Africa was evaluated. The phenolic profile of each variety evaluated indicated that varietal and regional differences proved to be the most important factors affecting the individual phenolic concentrations. Varietal and regional similarities were mainly found for QRG and HD levels. The navel FCOJ variety was shown to be the most mature, irrespective of season and region, and had the highest TP levels. In contrast, mandarin in the WC had the lowest TP and it was concluded that the mandarin from EC was of better quality in terms of bioactive phenolic compounds. Futhermore, when the results for individual phenolic composition and TAC are compared to those reported in Chapter 4 for citrus fruit from the WC, it is clear that during the processing of the citrus fruit into FCOJ it is not only the sugars that are concentrated, but that there is also an increase in the individual phenolic composition, thus overall TP concentration and TAC. Therefore it can be concluded that FCOJ have at least similar and possibly improved health benefits (in terms of phenolic composition only and not vitamin C content) compared to fresh citrus fruit or juice.

The results from this study are important as it highlights the importance of varietal and regional effects on phenolic composition of citrus products. In addition, seasonal variation is a limiting factor and the contribution to differences in phenolic composition is marginal. It is thus important to use this data and evaluate whether juices sold on the retail market as made-from-concentrate (MFC) orange juice have similar TP

concentrations and TAC. This will provide evidence on whether the minimum amount of orange juice is used in the formulation of the MFC orange juices, which is legislated, but more importantly whether consumers do receive the expected health benefits associated with the phenolic content after consuming MFC orange juices.

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ADDENDUM TABLES

Table 5.9a Differences in juice characteristics based on processing season

	°Brix	Titratable Acidity (%w.w^{-1} citric acid)	°Brix:acid ratio	pH
year				
2012	11.50 ± 0.1 ^{a}	1.02 ± 0.2 ^{a}	11.50 ± 1.55 ^{ab}	3.52 ± 0.1 ^{a}
2013	11.52 ± 0.1 ^{a}	1.04 ± 0.2 ^{a}	11.37 ± 1.76 ^{b}	3.42 ± 0.2 ^{b}
2014	113.55 ± 0.1 ^{a}	10.10 ± 0.2 ^{a}	11.96 ± 2.47 ^{a}	3.57 ± 0.2 ^{a}

Table 5.9b Phenolic composition of FCOJ sampled in 2012, 2013 and 2014

year	QRG	FHX	VIC2	NRGLC	RUT	NART	HD	NEOP	TP	DPPH	ORAC
2012	4.4 ± 4.56 ^{a}	8.79 ± 3.14 ^{b}	27.54 ± 12.96 ^{a}	13.8 ± 7.99 ^{b}	3.64 ± 4.44 ^{a}	43.61 ± 30.73 ^{a}	29.59 ± 11.95 ^{c}	4.95 ± 3.12 ^{b}	136.86 ± 60.94 ^{b}	269.90 ± 85.39 ^{b}	5338.25 ± 1376.45 ^{b}
2013	5.73 ± 5.01 ^{a}	10.37 ± 4.82 ^{a}	27.81 ± 13.51 ^{a}	14.68 ± 6.3 ^{ab}	4.22 ± 5.41 ^{a}	45.68 ± 30.29 ^{a}	33.99 ± 9.43 ^{b}	5.73 ± 3.02 ^{b}	149.12 ± 43.64 ^{ab}	300.23 ± 85.24 ^{a}	5566.1 ± 1135.55 ^{ab}
2014	5.81 ± 5.96 ^{a}	9.95 ± 4.57 ^{a}	27.58 ± 11.48 ^{a}	16.4 ± 6.44 ^{a}	3.37 ± 5.94 ^{a}	49.63 ± 27.78 ^{a}	38.82 ± 11.06 ^{a}	6.89 ± 3.65 ^{a}	160.12 ± 50.22 ^{a}	289.33 ± 75.25 ^{ab}	5808.02 ± 1083.19 ^{a}

QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

Table 5.10a Influence of variety and year on juice characteristics of FCOJ

	°Brix	Titratable Acidity (%w.w^{-1} citric acid)	°Brix:acid ratio	pH
Variety+year				
mandarin 2012	11.50 ± 0.20 ^{a}	1.04 ± 0.10 ^{b}	11.18 ± 0.96 ^{b}	3.49 ± 0.10 ^{bcd}
mandarin 2013	11.50 ± 0.10 ^{a}	1.04 ± 0.10 ^{b}	11.21 ± 1.02 ^{b}	3.28 ± 0.20 ^{e}
mandarin 2014	11.51 ± 0.10 ^{a}	0.95 ± 0.10 ^{bc}	12.44 ± 1.88 ^{a}	3.61 ± 0.10 ^{a}
navel 2012	11.51 ± 0.20 ^{a}	0.95 ± 0.10 ^{bc}	12.24 ± 1.53 ^{ab}	3.58 ± 0.10 ^{ab}
navel 2013	11.54 ± 0.10 ^{a}	0.89 ± 0.10 ^{c}	13.02 ± 1.17 ^{a}	3.54 ± 0.20 ^{abc}
navel 2014	11.57 ± 0.10 ^{a}	0.94 ± 0.20 ^{bc}	12.78 ± 2.60 ^{a}	3.58 ± 0.30 ^{ab}
valencia 2012	11.48 ± 0.10 ^{a}	1.28 ± 0.30 ^{a}	9.35 ± 2.38 ^{c}	3.40 ± 0.20 ^{de}
valencia 2013	11.5 ± 0.1 ^{a}	1.22 ± 0.1 ^{a}	9.55 ± 0.93 ^{c}	3.43 ± 0.1 ^{cd}
valencia 2014	11.57 ± 0.1 ^{a}	1.23 ± 0.2 ^{a}	9.63 ± 1.66 ^{c}	3.48 ± 0.1 ^{bcd}

WC = Western Cape, EC = Eastern Cape. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

Table 5.10b Effect of varietal and seasonal differences on the individual phenolic composition and total antioxidant capacity of FCOJ

Variety+year	QRG	FHX	VIC2	NRGLC	RUT	NART	HD	NEOP	TP	DPPH	ORAC
mandarin 2012	3.53 ± 6.38 ^{b}	7.68 ± 2.92 ^{cd}	19.49 ± 14.31 ^{b}	9.77 ± 9.65 ^{e}	4.99 ± 6.10 ^{bc}	42.66 ± 43.91 ^{b}	20.53 ± 10.29 ^{g}	4.31 ± 4.2 ^{bc}	113.63±80.46 ^{c}	201.01 ±66.01 ^{b}	4841.42 ± 1722.9 ^{d}
mandarin 2013	8.06 ± 8.19 ^{a}	6.44 ± 2.43 ^{d}	20.21 ± 19.05 ^{b}	14.19 ± 8.20 ^{bcd}	8.92 ± 7.41 ^{a}	62.68 ± 45.62 ^{a}	25.10 ± 8.36 ^{fg}	6.39 ± 4.49 ^{a}	153.10 ± 68.68 ^{ab}	220.13 ± 97.54 ^{b}	6256.71 ± 1226.1 ^{a}
mandarin 2014	7.35 ± 9.79 ^{ab}	6.49 ± 3.10 ^{d}	16.69 ± 12.23 ^{b}	15.38 ± 9.96 ^{abc}	8.32 ± 8.61 ^{ab}	62.10 ± 42.73 ^{a}	29.51 ± 12.6 ^{ef}	7.41 ± 6.01 ^{a}	154.10 ± 83.13 ^{ab}	218.83 ± 79.42 ^{b}	5809.51 ± 1404.21 ^{abc}
navel 2012	5.02 ± 0.79 ^{ab}	8.73 ± 0.85 ^{bc}	35.75 ± 3.27 ^{a}	18.42 ± 1.66 ^{ab}	2.11 ± 0.51 ^{c}	45.99 ± 3.81 ^{ab}	39.15 ± 4.61 ^{bc}	5.92 ± 1.03 ^{ab}	161.55 ± 10.45 ^{ab}	334.37 ± 30.37 ^{a}	5749.33 ± 683.7 ^{abc}
navel 2013	4.41 ± 0.77 ^{ab}	9.10 ± 0.85 ^{bc}	33.19 ± 6.47 ^{a}	18.73 ± 1.6 ^{a}	1.69 ± 0.69 ^{c}	45.02 ± 3.46 ^{ab}	40.36 ± 4.37 ^{bc}	6.73 ± 0.96 ^{a}	159.96 ± 10.29 ^{ab}	328.17 ± 21.68 ^{a}	5200.67 ± 948.45 ^{cd}
navel 2014	4.63 ± 0.92 ^{ab}	9.52 ± 2.33 ^{bc}	33.77 ± 3.14 ^{a}	18.6 ± 2.52 ^{a}	1.88 ± 0.53 ^{c}	46.21 ± 6.95 ^{ab}	43.11 ± 4.87 ^{ab}	6.85 ± 0.96 ^{a}	165.16 ± 12.99 ^{a}	331.05 ± 32.61 ^{a}	5709.4 ± 712.86 ^{abc}
valencia 2012	6.46 ± 0.98 ^{ab}	16.36 ± 2.79 ^{a}	32.7 ± 3.56 ^{a}	13.49 ± 2.55 ^{cde}	3.6 ± 0.38 ^{c}	36.04 ± 8.7 ^{b}	33.53 ± 1.81 ^{de}	3.45 ± 0.7 ^{c}	145.91 ± 12.82 ^{abc}	347 ± 48.86 ^{a}	6203.97 ± 491.21 ^{a}
valencia 2013	4.75 ± 1.21 ^{ab}	16.27 ± 3.97 ^{a}	29.68 ± 8.13 ^{a}	10.31 ± 4.2 ^{de}	2.1 ± 0.54 ^{c}	27.68 ± 12.37 ^{b}	36.13 ± 7.49 ^{cd}	3.79 ± 1.47 ^{c}	131.62 ± 26.39 ^{bc}	354.92 ± 47.32 ^{a}	5245.16 ± 914.29 ^{bcd}
valencia 2014	5.48 ± 0.97 ^{ab}	16.51 ± 2.42 ^{a}	34.03 ± 5.1 ^{a}	13.95 ± 1.45 ^{cde}	2.51 ± 0.44 ^{c}	35.39 ± 4.84 ^{b}	46.23 ± 4.73 ^{a}	6.12 ± 0.48 ^{ab}	160.68 ± 7.39 ^{ab}	328.04 ± 21.05 ^{a}	5991.8 ± 1157.94 ^{ab}

QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds, WC = Western Cape, EC = Eastern Cape. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

Table 5.11a Differences in juice characteristics of FCOJ based on region and season

	°Brix	Titratable Acidity (%w.w^{-1} citric acid)	°Brix:acid ratio	pH
region + year				
WC 2012	11.51 ± 0.20 ^{ab}	0.99 ± 0.10 ^{b}	11.67 ± 1.42 ^{ab}	3.53 ± 0.10 ^{ab}
WC 2013	11.51 ± 0.10 ^{ab}	1.02 ± 0.20 ^{ab}	11.52 ± 1.80 ^{b}	3.41 ± 0.20 ^{c}
WC 2014	11.58 ± 0.10 ^{a}	0.96 ± 0.20 ^{b}	12.46 ± 2.27 ^{a}	3.6 ± 0.20 ^{a}
EC 2012	11.48 ± 0.10 ^{b}	1.10 ± 0.20 ^{a}	10.83 ± 1.90 ^{b}	3.47 ± 0.10 ^{bc}
EC 2013	11.52 ± 0.10 ^{ab}	1.10 ± 0.20 ^{a}	10.78 ± 1.54 ^{b}	3.5 ± 0.10 ^{bc}
EC 2014	11.51 ± 0.10 ^{ab}	1.10 ± 0.30 ^{a}	11.18 ± 2.62 ^{b}	3.54 ± 0.2 ^{ab}

WC = Western Cape, EC = Eastern Cape. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

Table 5.11b The influence of region and season on phenolic composition of FCOJ

region + year	QRG	FHX	VIC2	NRGLC	RUT	NART	HD	NEOP	TP	DPPH	ORAC
WC 2012	3.42 ± 3.39 ^{b}	8.16 ± 2.28 ^{c}	26.43 ± 13.53 ^{bc}	12.34 ± 7.15 ^{c}	2.98 ± 3.24 ^{b}	36.33 ± 16.70 ^{d}	27.64 ± 12.63 ^{c}	4.45 ± 2.54 ^{c}	122.35 ± 51.39 ^{d}	270.65 ± 80.25 ^{ab}	4952.66 ± 1115.83 ^{e}
WC 2013	5.58 ± 5.02 ^{ab}	10.60 ± 5.01 ^{ab}	25.58 ± 12.23 ^{bc}	14.65 ± 6.29 ^{bc}	4.22 ± 5.46 ^{ab}	42.37 ± 20.60 ^{cd}	33.92 ± 10.18 ^{b}	5.76 ± 2.49 ^{bc}	143.55 ± 38.02 ^{cd}	299.18 ± 75.52 ^{ab}	5327.63 ± 1062.23 ^{de}
WC 2014	5.66 ± 6.38 ^{ab}	9.27 ± 4.21 ^{bc}	24.00 ± 11.63 ^{c}	16.10 ± 6.65 ^{ab}	4.77 ± 6.14 ^{ab}	47.95 ± 25.80 ^{bcd}	36.41 ± 12.48 ^{b}	6.28 ± 2.79 ^{ab}	151.52 ± 50.89 ^{bc}	280.47 ± 67.53 ^{ab}	5942.39 ± 1017.04 ^{bc}
EC 2012	8.12 ± 6.45 ^{a}	11.20 ± 4.7 ^{a}	31.77 ± 9.9 ^{ab}	19.39 ± 8.90 ^{a}	6.14 ± 7.13 ^{a}	71.42 ± 51.92 ^{a}	37.01 ± 3.43 ^{b}	6.84 ± 4.39 ^{ab}	192.25 ± 64.99 ^{a}	267.02 ± 107.25 ^{b}	6810.51 ± 1318.49 ^{a}
EC 2013	6.30 ± 5.11 ^{ab}	9.49 ± 4.1 ^{bc}	36.38 ± 15.22 ^{a}	14.79 ± 6.61 ^{bc}	4.23 ± 5.42 ^{ab}	58.42 ± 52.79 ^{ab}	34.28 ± 5.99 ^{b}	5.60 ± 4.67 ^{bc}	170.53 ± 57.53 ^{abc}	304.25 ± 119.27 ^{a}	6483.28 ± 952.18 ^{ab}
EC 2014	6.04 ± 5.43 ^{ab}	10.99 ± 5.04 ^{a}	33.17 ± 8.94 ^{a}	16.92 ± 6.28 ^{ab}	3.76 ± 5.77 ^{ab}	52.3 ± 31.31 ^{bc}	42.57 ± 7.22 ^{a}	7.83 ± 4.63 ^{ab}	173.55 ± 47.61 ^{ab}	303.18 ± 86.41 ^{a}	5598.05 ± 1181.82 ^{cd}

QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds, WC = Western Cape, EC = Eastern Cape. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

Table 5.12a The influence of variety and growing region on the juice characteristics of FCOJ

	°Brix	Titratable Acidity (%w.w^{-1} citric acid)	°Brix:acid ratio	pH
region + variety				
WC mandarin	11.51 ± 0.20 ^{ab}	0.99 ± 0.10 ^{b}	11.67 ± 1.42 ^{ab}	3.53 ± 0.10 ^{ab}
WC navel	11.51 ± 0.10 ^{ab}	1.02 ± 0.20 ^{ab}	11.52 ± 1.80 ^{b}	3.41 ± 0.20 ^{c}
WC valencia	11.58 ± 0.10 ^{a}	0.96 ± 0.20 ^{b}	12.46 ± 2.27 ^{ab}	3.59 ± 0.20 ^{a}
EC mandarin	11.48 ± 0.10 ^{b}	1.10 ± 0.20 ^{a}	10.83 ± 1.90 ^{b}	3.47 ± 0.10 ^{bc}
EC navel	11.52 ± 0.10 ^{ab}	1.10 ± 0.20 ^{a}	10.78 ± 1.54 ^{b}	3.48 ± 0.10 ^{bc}
EC valencia	11.51 ± 0.10 ^{ab}	1.10 ± 0.30 ^{a}	11.18 ± 2.62 ^{b}	3.54 ± 0.20 ^{ab}

WC = Western Cape, EC = Eastern Cape. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

Table 5.12b Differences in individual phenolic composition and total antioxidant capacity based on regional and varietal influence

region + variety	QRG	FHX	VIC2	NRGLC	RUT	NART	HD	NEOP	TP	DPPH	ORAC
WC mandarin	3.42 ± 3.39 ^{b}	8.16 ± 2.28 ^{c}	26.43 ± 13.53 ^{bc}	12.34 ± 7.15 ^{c}	2.98 ± 3.24 ^{b}	36.33 ± 16.70 ^{d}	27.64 ± 12.63 ^{c}	4.45 ± 2.54 ^{c}	122.35 ± 51.39 ^{d}	270.65 ± 80.25 ^{ab}	4952.66 ± 1115.83 ^{e}
WC navel	5.58 ± 5.02 ^{ab}	10.60 ± 5.01 ^{ab}	25.58 ± 12.23 ^{bc}	14.65 ± 6.29 ^{bc}	4.22 ± 4.46 ^{ab}	42.37 ± 20.60 ^{cd}	33.92 ± 10.18 ^{b}	5.76 ± 2.49 ^{bc}	143.55 ± 38.02 ^{cd}	299.18 ± 75.52 ^{ab}	5327.63 ± 1062.23 ^{de}
WC valencia	5.66 ± 6.38 ^{ab}	9.27 ± 4.21 ^{bc}	24.00 ± 11.63 ^{c}	16.10 ± 6.65 ^{ab}	4.77 ± 6.14 ^{ab}	47.95 ± 25.80 ^{bcd}	36.41 ± 12.48 ^{b}	6.28 ± 2.79 ^{ab}	151.52 ± 50.89 ^{bc}	280.47 ± 67.53 ^{ab}	5942.4 ± 1017.04 ^{bc}
EC mandarin	8.12 ± 6.45 ^{a}	11.20 ± 4.70 ^{a}	31.77 ± 9.90 ^{ab}	19.39 ± 8.90 ^{a}	6.14 ± 7.13 ^{a}	71.42 ± 51.92 ^{a}	37.01 ± 3.43 ^{b}	6.84 ± 4.39 ^{ab}	192.25 ± 64.99 ^{a}	267.02 ± 107.25 ^{b}	6810.51 ± 1318.49 ^{a}
EC navel	6.30 ± 5.11 ^{ab}	9.49 ± 4.10 ^{bc}	36.38 ± 15.22 ^{a}	14.80 ± 6.61 ^{bc}	4.23 ± 5.42 ^{ab}	58.42 ± 52.80 ^{ab}	34.28 ± 5.99 ^{b}	5.6 ± 4.67 ^{bc}	170.53 ± 57.53 ^{abc}	304.25 ± 119.27 ^{a}	6483.28 ± 952.18 ^{ab}
EC valencia	6.04 ± 5.43 ^{ab}	10.99 ± 5.04 ^{a}	33.17 ± 8.94 ^{a}	16.92 ± 6.28 ^{ab}	3.76 ± 5.77 ^{ab}	52.27 ± 31.31 ^{bc}	42.57 ± 7.22 ^{a}	7.84 ± 4.63 ^{a}	173.55 ± 47.61 ^{ab}	303.18 ± 86.41 ^{a}	5598.05 ± 1181.82 ^{cd}

QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds, WC = Western Cape, EC = Eastern Cape. Significant ($p<0.05$) differences are indicated by different alphabetical letters.

CHAPTER 6

COMPARISON OF ORANGE JUICE ATTRIBUTES, PHENOLIC COMPOSITION AND ANTIOXIDANT CAPACITY OF THREE TYPES OF READY-TO-DRINK ORANGE JUICES

ABSTRACT

Health promoting bioactive compounds has been identified in citrus fruit and juices. The levels of these compounds vary in terms of citrus variety and may be influenced during citrus juice production in the form of frozen concentrated orange juice (FCOJ), which is used for the manufacturing of made-from-concentrate (MFC) juices. The MFC juices are typically pasteurised (PAST) or treated using ultra-high temperature pasteurisation (UHT). Certain citrus varieties are freshly squeezed (FSQ) to produce not-from-concentrate (NFC) juices. These orange juices form part of the South African juice industry which sells mostly to the local market with 20% exports. In the past few years there has been a decline in MFC orange juices due to consumers desiring “healthier” juices and drinks options. Furthermore, traditional MFC juices consist of blending 50% orange juice (the named fruit) with deflavoured apple, pear and/or grape juice to form a 100% Orange juice blend which is allowed by current legislation.

Therefore, the phenolic composition and total anti-oxidant capacity (TAC) of three brands of MFC and two brands of NFC juices were evaluated. The predominant flavanones were hesperidin (HD) (FSQ = 112.73 $mg.L^{-1}$, PAST = 42.23 $mg.L^{-1}$ and UHT = 50.10 $mg.L^{-1}$) and narirutin (NART) (FSQ = 33.33 $mg.L^{-1}$, PAST = 28.51 $mg.L^{-1}$ and UHT = 26.23 $mg.L^{-1}$) followed by the flavone-*C*-glucoside vicenin-2 (VIC2) (FSQ = 23.89 $mg.L^{-1}$, PAST = 22.26 $mg.L^{-1}$ and UHT = 18.54 $mg.L^{-1}$) and a hydroxycinnamic acid namely ferulic acid-*O*-hexoside (FHX) (FSQ = 9.55 $mg.L^{-1}$, PAST = 5.25 $mg.L^{-1}$ and UHT = 5.33 $mg.L^{-1}$). Three other minor phenolic compounds where also quantified. The TAC was determined using the 2,2’-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and Oxygen Radical Absorbance Capacity (ORAC) assay. The results indicated that NFC juices had significantly ($p<0.05$) higher levels of the individual phenolics as well as higher TAC_{ORAC} (5621.1 μM TE). MFC

juices on the other hand showed lower TAC levels (4152.27 – 4688.75 μM TE) apart from two brands, PAST_WW (6471.96 μM TE) and UHT_WLD (5061.44 μM TE). The results further showed that the phenolic composition of the MFC juices were dependent on the juice formulation, i.e. the quantity of orange juice added and not the treatment type (PAST versus UHT). Lastly, the results highlighted the lack of information pertaining to the processing, storage and shelf-life stability of the identified and evaluated phenolic compounds.

INTRODUCTION

Citrus have been reported as important health promoting dietary sources of phenolic compounds. Amongst the potential beneficial effects of citrus flavonoids are the protection they offer against cancer and cardiovascular diseases (Guthrie & Carrol, 1998; Miyagi *et al*., 2000; Poulose *et al*., 2005; Poulose *et al*., 2006; Jayapraksha *et al.*, 2007). These important possible health promoting nutrients are consumed as fresh citrus fruit or citrus containing fruit juice. Citrus phenolic compounds together with vitamin C are the major nutrients receiving active research since they are related to quality and quality changes during processing and storage (Vanamala *et al.*, 2006; Galaverna & Dall'Asta, 2014). The varietal, seasonal and regional variabilities of phenolic compounds included in South African oranges and frozen concentrated orange juice (FCOJ) was investigated in previous chapters. However, a large portion of South African oranges are further processed into orange juice blended products which are known as made-from-concentrate (MFC) juices undergoing either additional pasteurisation or ultra-high temperature pasteurisation. Alternatively, the juice is extracted and directly pasteurised to produce not-from-concentrate (NFC) orange juice.

In South Africa 80% of the total juice production is sold on the local market and only 20% is exported (Booth, 2015). In recent times the industry was affected by the idling global economy which has placed pressure on fruit juice prices since consumers have a reduced buying power. This resulted in fruit juices being high-lighted by the European Union as one of the top-ten most at-risk foods of being adulterated (Richards, 2013). Thus, the economic crisis aggravated food fraud. This is mainly due to the potential economic gains and further due to compositional policing which is lacking. This trend does not only affect South Africa but is a growing trend in Europe. The first attempt at overcoming this problem is to promulgate regulations and expand capacity to ensure compliance. In South Africa the SA

Fruit Juice Association (SAFJA) played a role in substantially amending the SA regulations pertaining to fruit juices where the sweetened class of fruit juices was removed. This is to the benefit of the consumer and industry (Richards, 2014). In the past dispensation of the regulations the sweetened class of fruit juices allowed the addition of 10% “permitted natural sweeteners” which was typically cane sugar. Thus, in essence adulteration was allowed since it was difficult to police whether only 10% cane sugar was added or more in an attempt to lower the price and increase the profit margin of fruit juices.

In addition to the global economic situation, current consumer trends have indicated that traditional MFC orange juice consumption is declining. This is mainly due to consumers’ preference for healthy alternative drinks. This trend is due to general perception that traditional juices such as frozen orange juice, are of lesser quality regarding its nutritional properties. Therefore, the aim of this study was to evaluate the TP and TAC of various brands of MFC and NFC orange juice and blends with a view to establish its nutritional quality in terms of bioactive phytonutrient level as well as to gauge whether legislative requirements are met.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents purchased and prepared as described in Chapter 4 of this dissertation.

Sample preparation

Made-from-concentrate orange juice (MFC) samples as well as not-from-concentrate (NFC) orange juice samples were purchased from the local market. The MFC juices were divided according to brand and processing type. The processing types included freshly-squeezed (FSQ, 2 brands) which is classified as NFC, pasteurised (PAST, 3 brands) and ultra-high temperature treated (UHT, 3 brands) orange juice which is classified as MFC. All samples were kept frozen at -18°C.

The samples were defrosted and centrifuged at 10 000 x g (centrifuge model M-24A, BOECO, Germany) for 5 min at room temperature. The supernatant was used for total antioxidant capacity determinations (DPPH and ORAC). Prior to HPLC analysis all samples were filtered using 0.45 µm Acrodisc syringe filters with GHP membrane (Pall Life Sciences, Separations, USA).

Determination of citrus juice characteristics and phenolic composition

Total soluble solids (TSS, expressed as °Brix), titratable acidity (TA), pH and phenolic composition were deteremined as described in Chapter 4 of this dissertation.

Statistical analysis

The experimental layout consisted of two MFC types namely PAST (n=18) and UHT (n=18); and one NFC type; FSQ (n=11). Each brand was designated using abbreviations and included PAST_CLV, PAST_RL, PAST_WW, UHT_WLD, UHT_LF, UHT_PJ, FSQ_WW and FSQ_CHKRS. Replicate samples were distinguished based on different batch and "best before" dates. The chemical juice characteristics, flavonoid composition and antioxidant capacity of the juices were compared.

Data were expressed as means ± standard deviations (SD). All analyses were performed in duplicate except for DPPH and ORAC analysis which was in triplicate. Univariate analysis of variance (ANOVA) and the Least Significant Difference Student's t-test were used as a post-hoc test. SAS statistical software (SAS®, Version 9.2, SAS Institute Inc., Cary, NC, USA) was used for data processing. The Shapiro-Wilk test was used to test for normality. In addition, the results were analysed using multivariate statistical analysis using XLSTAT software (Version 7.5.2, Addinsoft, New York, USA). Principal Component Analysis (PCA) was used to evaluate relationships between the characteristics and juice type. The data for the PCA's were pretreated using Correlation matrix. Differences were considered statistically significant at 95% confidence level (P values < 0.05).

RESULTS AND DISCUSSION

The results (means ± standard deviations) obtained for the juice characteristics are summarised in Fig. 6.1. The °Brix of the MFC and NFC juices differed significantly ($p<0.05$). The FSQ_WW samples had the highest °Brix of 13.4 while the UHT_WLD samples had the lowest (10.8) which was still above the required minimum of 8.6°Brix as legislated (Anon., 2013). The prescribed minimum Bx/Ac is 8.5 and TA is 0.65% $w.w^{-1}$ citric acid. The FSQ_WW samples had the highest TA of 1.1% $w.w^{-1}$ citric acid and differed significantly ($p<0.05$) from the rest. The TA of the PAST and UHT did differ significantly ($p<0.05$), however the reason for this is most likely based on formulation due to brand difference as the same brands could not be collected for the different juice types (UHT vs PAST). Consequently, the °Brix:acid ratio was also found to differ significantly ($p<0.05$) and again was likely due to brand difference and not ascribed to juice type (FSQ vs UHT vs PAST). The pH of the juices did not differ significantly ($p>0.05$) between all of the samples apart from FSQ_CHKR which had the highest 3.9.

The distribution of the individual phenolic compounds and TAC is given in Fig.6.2. The QRG level differed significantly ($p<0.05$) and PAST_WW was found to have the highest level of 3.96 $mg.L^{-1}$. Interestingly the FSQ_WW samples had the lowest of 1.10 $mg.L^{-1}$ QRG concentration but the highest FHX of 10.6 $mg.L^{-1}$ ($p<0.05$). It is also clear from the boxplots (Fig. 6.2) that the variation in QRG for the FSQ samples was quite large. The VIC2 concentrations of PAST_WW, 31.6 $mg.L^{-1}$, FSQ_WW, 28.3 $mg.L^{-1}$ and UHT_WLD, 32.6 $mg.L^{-1}$ were significantly higher ($p<0.05$) than the other samples and did not differ from each other. Thus, in this specific case juice brand and not juice type was the determining factor, which may indicate that the processing of the orange juices, NFC orange juice compared to MFC orange juice (PAST vs UHT), had no effect on the VIC2 levels. This was also the case for NART. Likewise, the NRGLC of PAST_WW (21.5 $mg.L^{-1}$) and UHT_WLD (21.2 $mg.L^{-1}$) were significantly ($p<0.05$) higher than the rest. Regarding RUT PAST_CLV and PAST_WW had the highest concentration of 1.94 $mg.L^{-1}$ and 1.89 $mg.L^{-1}$ respectively. This differed significantly ($p<0.05$) from UHT_LF which had the lowest concentration of 0.90 $mg.L^{-1}$. In general it seems that the RUT levels are higher for PAST type MFC juices followed by FSQ and with UHT types having the lowest levels. PAST_WW (47.21 $mg.L^{-1}$) had

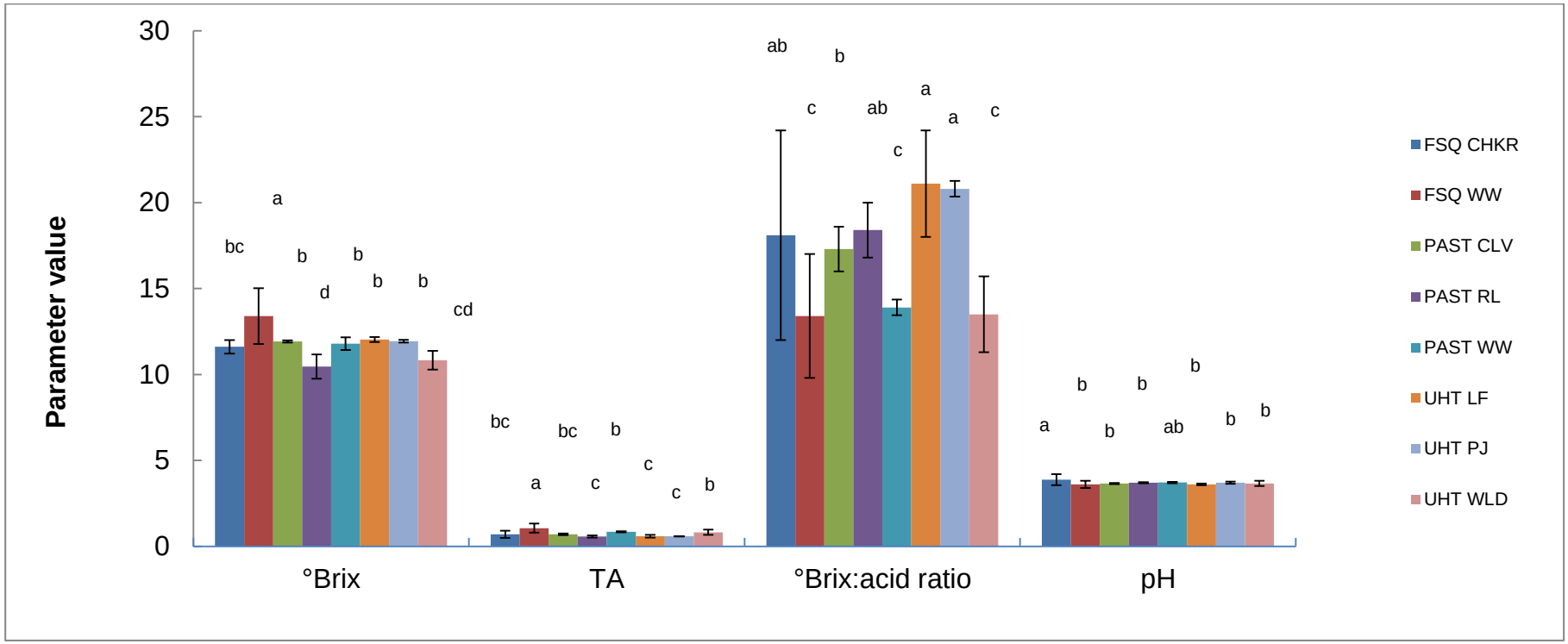

Figure 6.1 Juice characteristics (°Brix, titratable acidity, °Brix:acid ratio and pH) of different orange juices obtained from the local retail market. TA = titratable acidity expressed as %w.w^{-1} citric acid. Significant differences ($p<0.05$) are indicated using different alphabetical letters.

comparable NART concentrations to that of UHT_WLD (47.02 $mg.L^{-1}$) and FSQ_WW (46.04 $mg.L^{-1}$) which did not differ significantly ($p>0.05$). These NART levels were close to those reported for orange juices in literature, found to be 51.4 $mg.L^{-1}$ (Dhuique-Mayer *et al.*, 2005; Gattuso *et a*l., 2007). When compared to the study done by Vanamala *et al.* (2006), slightly lower NART levels were found in the MFC orange juices compared to the 44 - 80 $mg.L^{-1}$ reported. UHT_LF had the lowest NART level of 14.79 $mg.L^{-1}$. This may indicate that PAST_WW, UHT_WLD and FSQ_WW are of similar variety. The HD levels differed significantly ($p<0.05$) with the FSQ types having the highest concentrations (FSQ_WW 107.21 $mg.L^{-1}$ and FSQ_CHKR 119.35 $mg.L^{-1}$). In general the PAST types had the lowest HD apart from PAST_WW but did not differ significantly from the UHT type. The HD levels for all the juice types were found to be lower than those reported in literature for sweet orange juices (Dhuique-Mayer *et al.*, 2005; Gattuso *et a*l., 2007). This may be ascribed to the fact that the phenolic composition is affected during processing due to skin and/or peel contact which increases the individual flavanone concentration. Likewise, the levels reported for MFC orange juices were found to be lower than 329 - 548 $mg.L^{-1}$ reported by Vanamala *et al.* (2006). Again for the NEOP levels it was found that FSQ_WW and PAST_WW had significantly ($p<0.05$) higher levels (9.91 $mg.L^{-1}$ and 9.55 $mg.L^{-1}$, respectively). Similarly, the results obtained for NEOP was lower than that for juice derived from sweet oranges as described by Dhuique-Mayer *et al.* (2005) and Gattuso *et a*l. (2007) and the range (117 – 257 $mg.L^{-1}$) for MFC orange juices analysed by Vanamala *et al.* (2006). The TP followed a similar trend and FSQ_WW and PAST_WW consequently had the highest overall phenolic content (221.25 $mg.L^{-1}$ and 201.79 $mg.L^{-1}$, respectively) which differed significantly from the rest ($p<0.05$). Moreover, FSQ_WW was found to possess a significantly ($p<0.05$) lower TAC_{DPPH} compared to PAST_WW which had the highest of 362.14 μM TE. In contrast, FSQ_WW and PAST_WW were found to have similar TAC_{ORAC} which did not differ significantly ($p>0.05$) and were found to be the highest together with FSQ_CHKR. This differed significantly from the rest.

The results indicated, for the most part, that large variation was seen for QRG, FHX VIC2, NARGLC, RUT, NART, HD and NEOP for the FSQ_WW, PAST_WW and UHT_WLD juice types. This variation is ascribed to raw material variation, in other words different raw material varieties that are available and used in the production of the above juices. However, differences in the levels of individual phenolic compounds could not be established on the

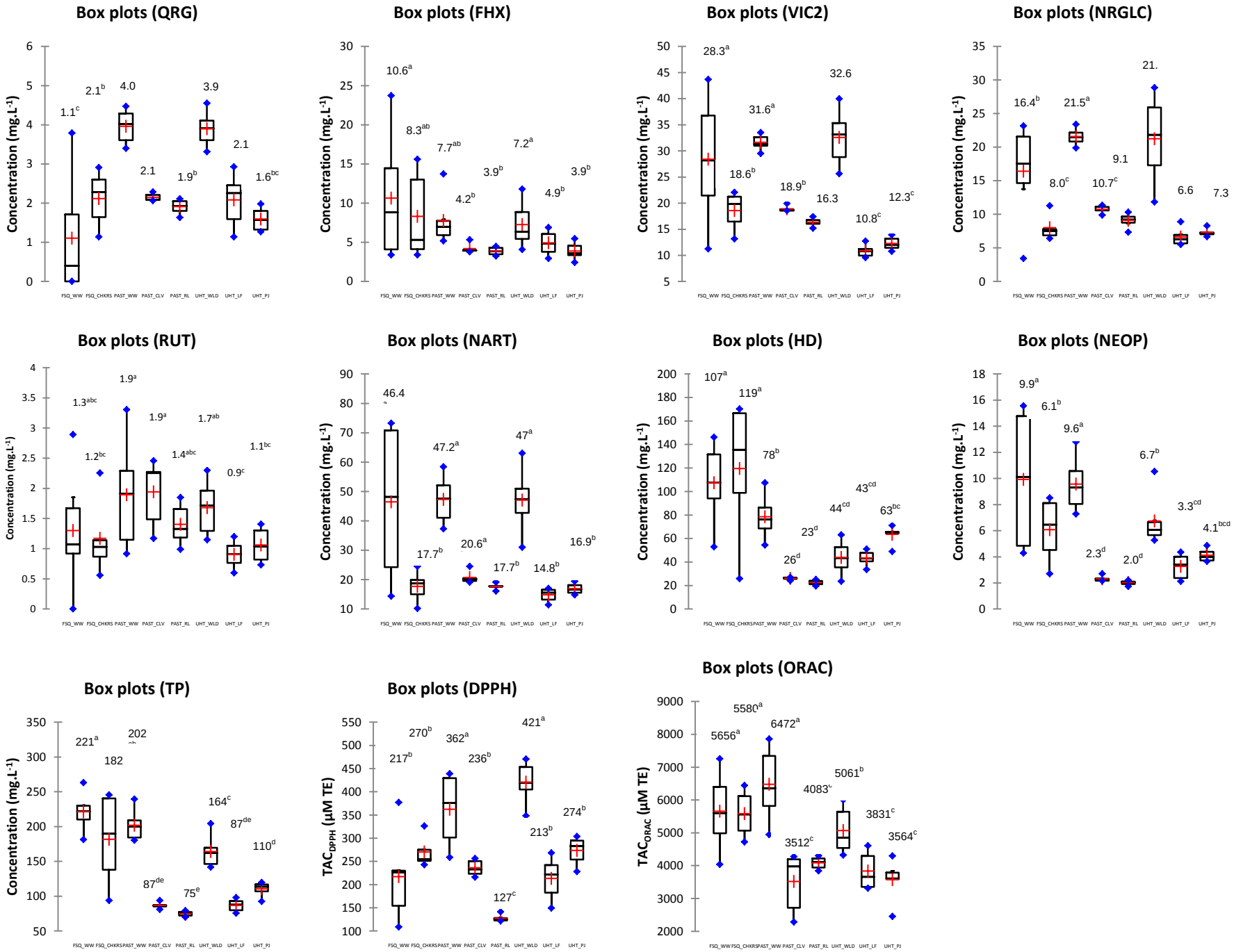

Figure 6.2 Distribution of phenolic compounds (with standard deviation) in various orange juices obtained from the local market. (QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds). Different alphabetical letters indicate significant differences at p<0.05.

basis of MFC juice type i.e. PAST versus UHT processing. There are no clear results indicating that the process reduced the antioxidant components and that the selected packaging may have played a role in preserving these compounds.

The NFC FSQ juices were characterised with significantly ($p<0.05$) higher TP concentration. The HD levels were found to have a larger variation in the FSQ juices (Fig. 6.2) and this is in-line with what was reported by Vanamala *et al*. (2006). Similarly, for NEOP it was found that one brand of NFC juices had higher levels compared to MFC juices which correspond to literature (Mouly *et al.*, 1998; Vanamala *et al*., 2006). NEOP was also shown to be the most labile flavanone which can be lost through concentration techniques during FCOJ production. It has been documented that NEOP can decrease by 52% during concentration, while NART and HD only showed slight decreases (Gil-Izquierdo *et al*., 2002). This may explain the lower NEOP concentrations in most of the MFC juices. Moreover, the difference between MFC and NFC juices were unexpected. The TP for MFC juices have been reported to be significantly higher compared to NFC juices since the juice concentration process can double the TP concentration in FCOJ (Gil-Izquierdo *et al*., 2002; Vanamala *et al*., 2006). In this study the opposite was observed.

The PCA biplot describes 66% of the inherent variability in the data (Fig. 6.3). The orange juices are mostly separated based on the juice type, except for PAST_WW and UHT_WLD brands. This may point to formulation similarities since the composition in terms of phenolic compounds are comparable. It is also clear that there are differences in phenolic composition of NFC and MFC juices. Furthermore, only one brand of PAST and UHT (PAST_WW and UHT_WLD) MFC juices were associated with higher NART, VIC2, NRGLC, QRG, RUT and TAC_{DPPH}. This may indicate that the composition, based on formulation and phenolic concentrations, are similar and consists mainly of orange juice. Whereas, PAST_RL and PAST_CLV were of similar formulation (containing other fruit juices such as apple, pear and/or grape juice as indicated by the label) and possibly containing similar quantities of orange juice concentrate, which is legislated. This explains the negative association of PAST_RL and PAST-CLV and with NART, VIC2, NRGLC, QRG, RUT and TAC_{DPPH} indicating lower values. Moreover, only one brand of FSQ NFC orange juice (FSQ_WW) was positively associated with higher HD, NEOP, FHX, TP and TAC_{ORAC}. Similarly, UHT_PJ and UHT_LF was negatively associated with all of the individual phenolic compounds which can again be ascribed to the juice formulations containing lower percentages of orange juice concentrate

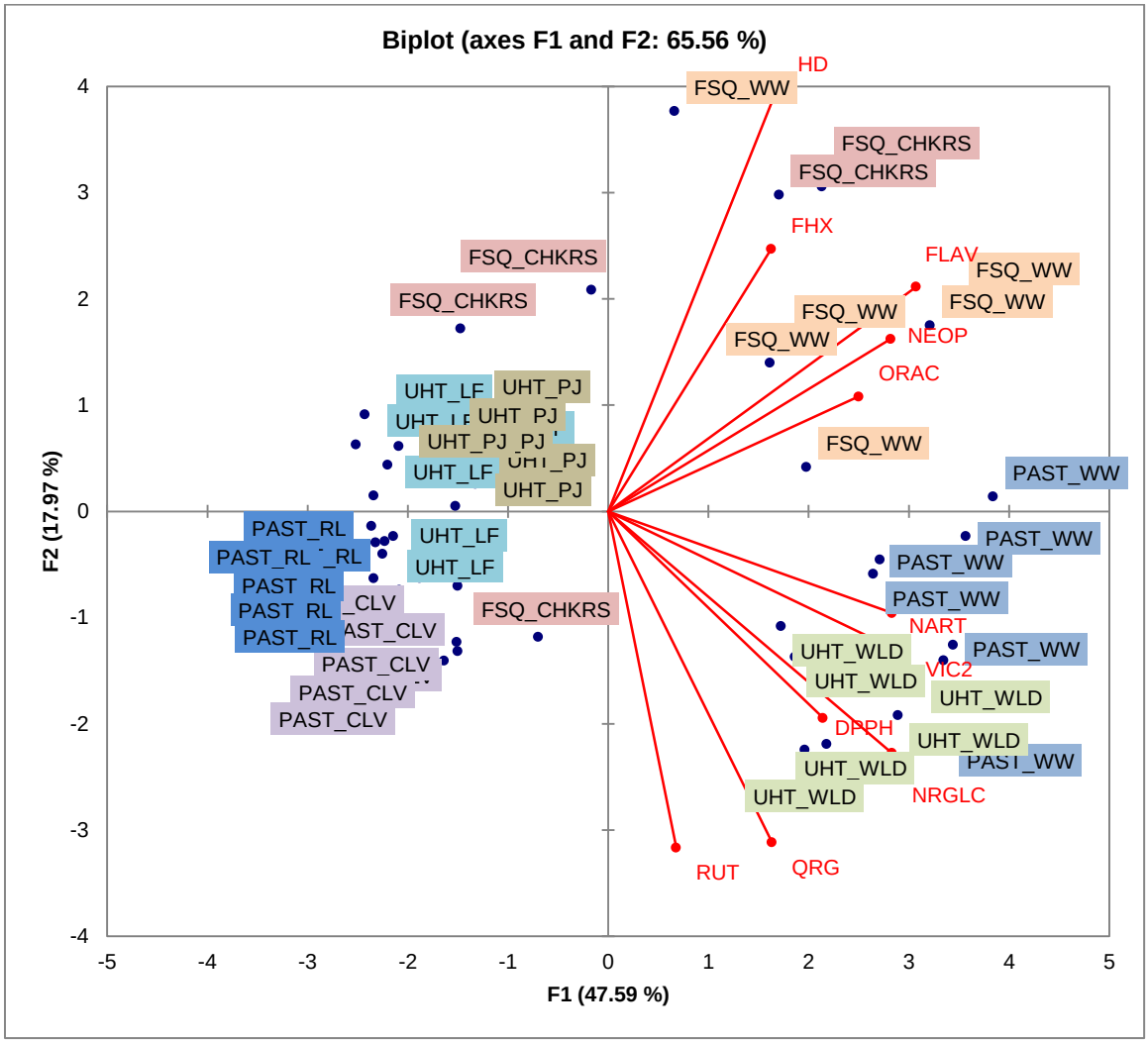

Figure 6.3 PCA biplot of individual phenolic composition and total anti-oxidant capacity and associations between different orange juices obtained from the local retail market.

(QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds).

compared to UHT_WLD and PAST_WW. Another possibility is the effect of the heat treatment which may influence these levels which is well documented for vitamin C (Trifirò *et al.*, 1995; Del Caro *et al*., 2004; Galverna & Dall'Asta, 2014). Processing (production of FCOJ) was shown not to decrease the individual phenolic concentrations in Chapter 5 and could be ascribed to the thermally accelerated short-time evaporation process which is effective in maintaining antioxidant components (Gil-Izquierdo *et al*., 2002; Galverna & Dall'Asta, 2014). It may however be affected during subsequent heat treatments such as further pasteurisation or ultra-high temperature treatment which are thermal processes that will affect bioactive components such as vitamin C (Gil-Izquierdo *et al*., 2001) and citrus flavonoids (Galverna & Dall'Asta, 2014). Furthermore, it was also expected that the MFC juices that have undergone UHT processing and packaged in laminate packaging material, with light transmission barriers, would protect the phenolic compounds against light oxidation and would therefore have higher TP concentrations compared to PAST samples which is filled into clear polyethylene terephthalate (PET) plastic packaging which allows light exposure leading to oxidation.

Finally, when comparing the phenolic composition of NFC to MFC juices it is clear that NFC juices had higher concentrations of the individual phenolic compounds (Fig. 6.4). This was surprising since it has been well documented that MFC juices produced from FCOJ have higher phenolic levels (Mouly *et al.*, 1998; Vanamala *et al*., 2006). This result can be ascribed to factors such as juice formulation and processing technique. The juice formulation for 100% Orange juice blends is legislated. According to the regulations a juice blend of a specific fruit for example "100% Orange juice blend" needs to be made up of at least 50% $v.v^{-1}$ standard juice strength of the total juice content of the blend and must have the typical appearance and taste of that fruit (Anon., 2013). The remainder of the juice content may be comprised of deflavoured apple, pear and/or grape juice. Thus, the phenolic composition of MFC juices found on the South African market may be well diluted since the 100% juice blend consist of only 50% orange juice. Although the phenolic content of MFC juices were not 50% lower than NFC juices, consumers, however, still do not receive the full benefit of citrus phenolic compounds in MFC juices since it contains significantly ($p<0.05$) lower levels as previously reported. However, other fruit juices added may also contribute flavonoids, such as flavonols, dihydrochalcones and flavan-3-ols (Schieber *et al*., 2001; Makris *et al*., 2006; Kalinowska *et al*., 2014). Furthermore, the different processing methods (pasteurisation

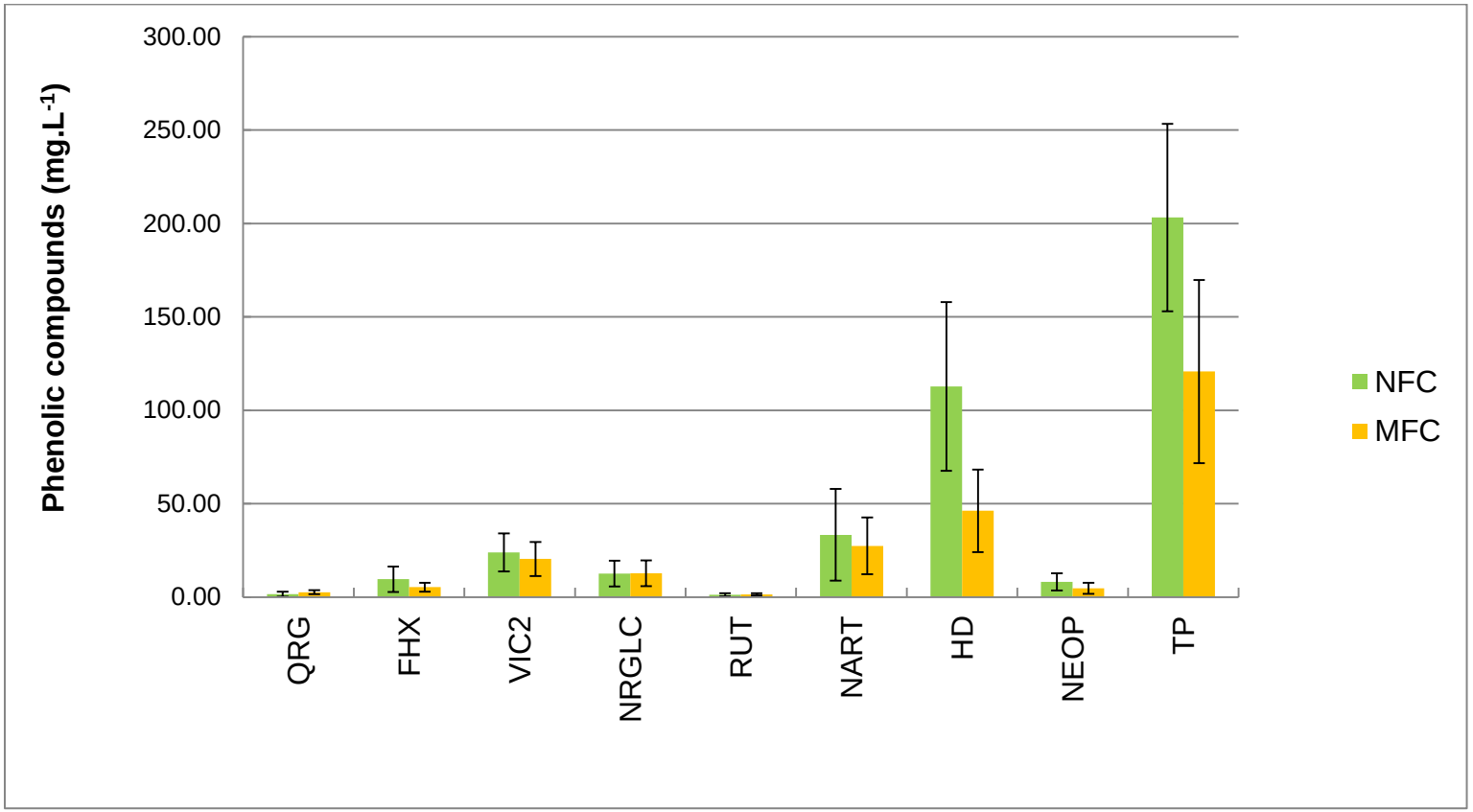

Figure 6.4 Differences in phenolic composition of made-from-concentrate (MFC) and not-from-concentrate (NFC) juices. (QRG = quercetin-3-*O*-rutinose-7-*O*-glucoside, FHX = ferulic acid-*O*-hexoside, VIC2 = vicenin-2, NRGLC = naringenin-7-*O*-rutinose-4'-*O*-glucoside, NART = narirutin, HD = hesperidin, NEOP = neoponcerin, TP = total phenolic compounds).

versus ultra-high temperature treatment) and packaging materials (clear plastic versus light protected laminate materials) may further influence the phenolic compounds in MFC orange juices and unfortunately no conclusions could be made regarding these factors from this study. Concerning the nutritional value of South African MFC juices, a standard serving of 240 mL (Food and Drug Administration's standard serving size for fruit juices) would contain on average, 11 mg of HD and 6.5 mg of NART compared to NFC juices which will contain 27 mg of HD and 8 mg of NART. In total, a standard serving (240 mL) of MFC and NFC orange juice will provide 29 mg and 49 mg of phenolic compounds, respectively. According to Petrick *et al*. (2015) the average dietary intake of flavonoids commonly consumed by Americans is between 123.55 and 125.03 $mg.d^{-1}$. Therefore, based on the total phenolic content a single serving of MFC orange juices will provide approximately 23% of the daily intake compared to NFC juices which will provide approximately 39%. Therefore, NFC and MFC juices contribute considerably to average daily flavonoids intake. However, there is definitely a need for preserving or increasing the TP concentration. A possible means of increasing the TP concentration of MFC orange juices is to further change legislation for orange juice and blends in order to eliminate blending the orange juice with other fruits. Moreover, information on the effect of processing techniques, storage conditions and packaging materials influencing the TP levels and protection of the individual phenolic is still required.

CONCLUSION

The phenolic content of three brands and three orange juice types were evaluated. The orange juice samples consisted of two MFC types namely PAST (n=18) and UHT (n=18) and one NFC type namely FSQ (n=11). The distribution and concentration of individual phenolic compounds indicated that NFC juices had higher levels and had a varied distribution. The MFC juices had significantly ($p<0.05$) lower TP levels which was unexpected since MFC juices produced from FCOJ has been shown to possess increased TP levels. The major flavanones were HD and NART in both juice types followed by VIC2 which was the major flavone-*C*-glucoside and FHX the major hydroxycinnamic acid. The TAC_{DPPH} for two brands of MFC juices namely PAST_WW and UHT_WLD were found to be the highest and significantly ($p<0.05$) differing from NFC juices. The TAC_{ORAC}, on the other hand, showed that NFC juices had significantly ($p<0.05$) higher anti-oxidant capacity compared to MFC

juices. From a nutritional stand point and considering the results obtained from this study, a standard serving of MFC and NFC orange juices will deliver 23% and 39% of the average daily intake of 124.29 $mg.d^{-1}$ respectively. Furthermore, the two MFC types selected, PAST versus UHT, showed no significant difference in the TP levels of these two types based on the heat treatment. This statement can be justified since the results showed no significant ($p>0.05$) difference between PAST_RL, PAST_CLV, UHT_LF and UHT_PJ. The only two MFC brands that were similar to NFC juices in terms of TP levels were PAST_WW and UHT_WLD. Thus it was concluded that the lower levels in the PAST_RL, PAST_CLV, UHT_LF and UHT_PJ are due to formulation and that these juices contained only approximately 50% orange juice $v.v^{-1}$ of the total juice content. This is due to current juice manufacturing practices and legislation that allows this practice.

The results from this study have shown that the phenolic composition of orange juices found in the South African market is much lower than that reported elsewhere in the world. This may be related to the lower TP levels found for South African citrus fruit varieties as previously discussed. However, the lower TP levels found in MFC juices is unexpected and not acceptable. The results can be used to motivate in conjunction with discussions with the fruit juice industry to move towards inclusion of higher percentages of the named fruit in 100% fruit juice blends in current legislation. This will have a cost implication and may be resisted by industry, however the preceived health benefits of the juice will improve and thus benefit the consumer, which may be more likely to pay the higher price if there are increased health benefits. In addition, the results from this study neccesitates further investigation of how bioactive compounds change during different processing techniques, storage (temperature abuse) and the entire shelf-life of ready-to-drink juices.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

The chemical and phenolic composition of various citrus varieties have been characterised over the past 30 years in fruit from Chinese, Spanish, Mediterranean, Mauritian and North-American origin (Mouly *et al.,* 1994; Dhuique-Mayer *et al.*, 2005; Caristi *et al.*, 2006; Cano *et al*., 2008; Ramful *et al*., 2010; Abad-García *et al.*, 2012). This is mainly due to the importance attached to the array of health promoting properties exhibited by citrus bioactive compounds. Furthermore, over the years sophisticated characterisation techniques have been developed for the detection of phenolic compounds in citrus fruit. These techniques rely on high-pressure liquid chromatography (HPLC) in conjunction with diode array detection (DAD) and in some cases liquid chromatography coupled with electrospray ionisation and mass spectrometry (LC-ESI-MS/MS) for characterisation and identification of citrus specific phenolic compounds (Mouly *et al*., 1999; Caristi *et al*., 2003; Gattuso *et al*., 2006; Abad-Garcia *et al*., 2009; Barreca *et al*., 2011). Nevertheless, there are yet unknown phenolic compounds that need to be identified and characterised in citrus. However, very little information exists on the variation in phenolic composition of different South African varieties (which are grown in different regions) and the effect of processing techniques on these levels. Hence the reason and importance of this research study in which the phenolic profile of four citrus fruit varieties (satsuma, clementine, navel and valencia) grown and harvested over three seasons (2012, 2013 and 2014) in the Western Cape (WC) were evaluated using an optimised HPLC-DAD method. Varietal differences were evident and the influence of growing season was apparent. Some of the varieties showed overlap in terms of juice characteristics and individual phenolic compound content. Narirutin and hesperidin were the major flavanone-*O*-glycosides, while the major flavone-*O*-glycoside and phenolic acid were vicenin-2 and ferulic acid-*O*-hexoside. Samples collected during the 2014 harvest season were found to have higher total antioxidant capacity (TAC) as determined using TAC_{DPPH} and TAC_{FRAP} which was as a direct result of the higher ascorbic acid (AA) content.

Similarly, three citrus varieties (mandarin, navel and valencia) of frozen concentrated orange juice (FCOJ) from the Western and Eastern Cape (EC) produced

over three seasons (2012, 2013 and 2014) were characterised in terms of 8 phenolic compounds and antioxidant capacity. The results indicated that varietal and regional differences proved to be the most important factors affecting the individual phenolic concentrations. Varietal and regional similarities were found for mainly quercetin-3-*O*-rutinoside-7-*O*-glucoside and hesperidin levels. The navel FCOJ variety was shown to be most mature, irrespective of season and region, and had the highest total polyphenol concentration. In contrast, mandarin in the WC had the lowest TP and it was concluded that the mandarin from EC was of better quality in terms of bioactive phenolic compounds. Furthermore, when the results for individual phenolic composition and TAC were compared to the data obtained for the citrus fruit varieties from the WC, it was clear that during the processing of the citrus fruit into FCOJ it is not only the sugars that are concentrated but that there is an increase in the individual phenolic composition, which can be attributed to the extraction process (pressing of the peel), thus increasing the overall TP concentration and TAC. Therefore it can be concluded that FCOJ have at least similar and possibly improved health benefits (in terms of phenolic composition only and not AA content) compared to fresh citrus fruit or juice. The results from this study are important as it highlights the importance of varietal and regional effects on phenolic composition of citrus products. In addition, seasonal variation is a limiting factor and the contribution to differences in phenolic composition is marginal.

Furthermore various ready-to-drink orange juices were also evaluated. The phenolic content of three brands and three orange juice types were evaluated. The orange juice samples consisted of two made-from-concentrate (MFC) types, namely pasteurised (PAST) and ultra-high temperature pasteurisation (UHT) and one not-from-concentrate (NFC) type namely freshly squeezed (FSQ). The distribution and concentration of individual phenolic compounds indicated that NFC juices had higher levels and had a varied distribution. The MFC juices had lower TP levels which was unexpected since MFC juices produced from FCOJ has been shown to possess increased TP levels. The major flavanones were hesperidin and narirutin in both juice types followed by vicenin-2 which was the major flavone-*C*-glucoside and ferulic acid-*O*-hexoside the major hydroxycinnamic acid. These were also the major phenolic compounds characterised in the citrus fruits. The TAC_{DPPH} for two brands of MFC juices were found to be the highest and differing from NFC juices. The TAC_{ORAC} on the other hand showed that NFC juices had higher antioxidant capacity compared to MFC juices. From a nutritional stand point and considering the results obtained from this study a standard serving of MFC and NFC orange juices will deliver 23% and 39% of the average daily intake of 124.29

mg.d^{-1} respectively. Furthermore, the two MFC types selected, PAST versus UHT, showed no difference in the TP levels of these two types based on the heat treatment. Thus it was concluded that the lower TP levels and TAC are due to formulation and that these juices contained only approximately 50% orange juice v.v^{-1} of the total juice content. This is due to current juice manufacturing practices and legislation that allows this practice.

The results obtained from this research can be used as a baseline, since it is first of its kind in terms of the establishment of a phenolic profile specific to South African citrus fruit varieties. The results are important as it highlights the importance of varietal and regional effects on phenolic composition of citrus products. In addition, seasonal variation is a limiting factor and the contribution to differences in phenolic composition is marginal when citrus varieties are processed into products such as FCOJ. The results showed that the phenolic composition of orange juices found in the South African market is much lower than that reported for elsewhere in the world. The reasons behind this occurrence may be related to the lower TP levels found for the South African citrus fruit varieties. However, the lower TP levels found in MFC juices is unexpected and not acceptable. The results can be used to motivate in conjunction with discussions with the fruit juice industry to move towards inclusion of higher percentages of the named fruit in 100% fruit juice blends in current legislation. This will have a cost implication and may be resisted by industry, however the preceived health benefits of the juice will improve and thus benefit the consumer, who may be more likely to pay the higher price if there are increased health benefits.

Further it is recommended that additional investigation is required in order to survey other important agronomic citrus varieties and growing regions. It is also important to further characterise South African citrus varieties in terms of polymethoxylated flavone and carotenoid composition as well as trace elemental analysis. This will generate specific knowledge on the quality of South African citrus in terms of their nutritional and antioxidant potential. In addition, the results from this study neccesitates further investigation of how bioactive compounds change during different processing techniques, storage (temperature abuse etc) and the entire shelf-life of ready-to-drink juices.

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