Non-destructive prediction of ‘Marsh’ grapefruit (*Citrus* x *paradisi* MacFad) postharvest quality and physiological rind disorders using visible to near infrared spectroscopy

By

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Declaration

I, Khayelihle Ncama (Student No.: 211552523), declare that this is my original work and it is referenced where some information is taken from other sources. I did all the analyses involved and this work has never been submitted in any intention for any other degree or at other institution. I will be held responsible for plagiarism should this work or any part of it be found to belong to someone else.

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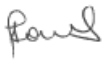
Co-Supervisor signature:  Date: …06/03/2017…

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General overview

In this study, visible to near infrared spectroscopy (Vis/NIRS) was investigated as a tool for non-destructive prediction of 'Marsh' grapefruit susceptibility to postharvest rind physiological disorders, specifically chilling injury and rind pitting. However, before such objective techniques were developed, specific pre-symptomatic biochemical markers related to chilling injury and rind pitting disorders were identified. After which, the potential to predict these biomarkers and susceptibility of fruit to these disorders was investigated.

The first chapter is a general introduction outlining aims and objectives of this research. The second chapter reviews previous literature in an attempt to gain an understanding of the biological mechanisms for citrus rind physiological disorders. This chapter also reviews the applications of Vis/NIRS for non-destructive prediction of citrus fruit quality. The third chapter is evaluating the conditions and potential of using Vis/NIRS to estimate internal citrus fruit parameters. The study in chapter 3 was out of 'Marsh' grapefruit harvest season and therefore, 'Star Ruby' grapefruit and 'Valencia' orange were used instead of 'Marsh' because they possess similar characteristics. The fourth chapter is identifying physiological attributes that can be used as pre-symptomatic markers during prediction of 'Marsh' grapefruit postharvest rind disorders. The fifth chapter is the prediction of 'Marsh' grapefruit physiological rind disorders and their pre-symptomatic biochemical markers. Overall discussions and conclusions are made in chapter six, where future research prospects are also recommended.

Publications

All chapters of this work, except chapter 1 and 6, were intended for publication and as a result, are written in the form of manuscripts. Due to the time limit, only chapter 3 is currently published but all chapters are or will be submitted to relevant journals.

Chapter 3 was published in the Journal of Food Engineering and is referenced as:

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Chapter 1: General Introduction

1.1 Introduction

South African citrus fruit production is mainly intended for exports to international markets and is one of primary exported fresh fruit from the country. Some of the most exported citrus species include oranges, grapefruit, lemons, and mandarins. Grapefruit contributes a significant amount of the country's citrus exports with over 375 000 mega tons during the 2013/2014 season (FAO, 2014). Since the South African citrus industry is export oriented, cold storage is the common practice used to prolong the postharvest life of fruit. Cold temperatures are also used for quarantine treatment of phytosanitary pests such as Mediterranean fruit flies (*Ceratitis capitata* and *Ceratitis rosa*) (Bassal and El-Hamahmy, 2011). During cold quarantine treatments, citrus fruit are stored at -0.6 °C for at least 14 days and then stored at 5 °C for the subsequent weeks of shipping. Cold storage treatments have been shown to cause chilling damage on susceptible cultivars such as 'Marsh' grapefruit (*Citrus* x *paradisi* MacFad) (Ezz and Awad, 2009). The fruit suffer from chilling injury (CI) and other chilling related disorders (Alferez *et al*., 2010; Chaudhary *et al.*, 2014).

Chilling injury is a physiological disorder occurring in tropical and subtropical fruits after a period of exposure to temperatures below 10 °C but above the freezing point (0 °C) (Bassal and El-Hamahmy, 2011). The period of exposure and threshold temperature resulting to the disorder development varies across citrus types and cultivars (Magwaza *et al*., 2013). Chilling injury is often characterized by water-soaked patches on the flavedo (the outer-most pigmented part of the rind) during cold storage or rind pitting (RP) at shelf life, which is manifested as small, irregular and slightly sunken patches of 3-6 mm in diameter that are randomly distributed about the flavedo of the fruit (Fig. 1) (Alferez *et al*., 2010; Cronje *et al*., 2011a; Magwaza *et al*., 2013). Rind staining, red blotches, internal breakdown, off-flavour, decay and necrosis on the fruit's rind can also appear (Chaudhary *et al.*, 2014).

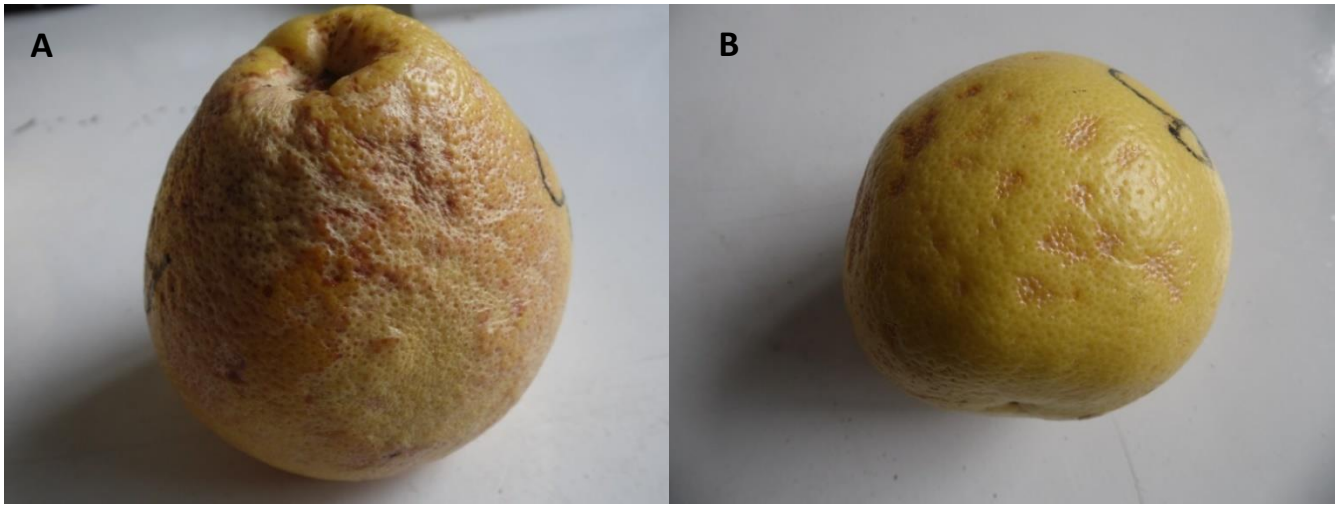

Fig. 1: The ‘Marsh’ grapefruit with chilling injury (A) and rind pitting (B) disorders that developed during and after cold storage assimilating commercial cold chain.

Several causal factors such as difference in water potential and nutrition during fruit growth and development, fruit position within the canopy and postharvest handling procedures have been found to contribute to fruit susceptibility to rind physiological disorders (Magwaza *et al*., 2013). However, the physiological and biochemical mechanism underlying the occurrence of these disorders is still not well understood.

Rind physiological disorders that do not manifest during harvesting, grading or sorting, such as RP and CI, but only show after 3 to 5 weeks during postharvest handling coincides with the time that South African citrus fruit reach international markets and point of sale (Cronje *et al*., 2011b; Magwaza *et al.*, 2013). Although rind disorders do not affect the edible internal part of the fruit, consumers use the external appearance as the primary indicator of the fruit quality (Alquezar *et al.*, 2010). The symptoms of postharvest rind disorders reduce the market value of fresh citrus fruits and results to drastic economic loss considering the investments made from the orchard practices through to the point of sale (Alferez and burns, 2004). Previous studies reported ‘Nules Clementine’ fruit harvested from the inside canopy position of a tree to be highly susceptibility to rind physiological disorders (Cronje *et al.*, 2011a). In ‘Marsh’ grapefruit, Ezz and Awad (2009) observed a negative correlation between rind ascorbic acid content and susceptibility to CI. In that study, a relationship between

the internal parameters composition and RP was also investigated and the authors noticed a positive correlation of total soluble sugars (TSS) to RP.

Citrus rind disorders stand high chances of being aligned with the results from the activity of free radicals formed during the breakdown of larger molecules such as carbohydrates, proteins and lipids during respiration (Purvis and Shewfelt, 1993). Radicals are reactive oxygen species (ROS) responsible for damaging cells and cause loss of cell compartmentalization which results in loss of cell respiration and causes cell death. The death of cells expands and reaches a point when it can be detected visually as pitting patches. ROS activity is known to be antagonistically inhibited by the antioxidant activity of fruit (Ramful *et al*., 2010). In previous studies of antioxidant capacity of white 'Marsh' grapefruit, ascorbic acid was found to make little contribution to the total antioxidant capacity of a fruit (Bahorun *et al.*, 2007), while flavonoid derivatives were found to have a significant contribution to antioxidant capacity of the flavedo (Di Majo *et al*., 2005). However, in another study by Del Caro *et al.* (2004), antioxidant capacity was correlated with ascorbic acid content rather than the presence of flavanone glycoside. Since the antioxidant capacity of fruit flavedo reduces the activity of ROS substance, it can be related to reduced flavedo cell death and therefore render fruit more susceptible to RP.

Current packing lines grade fruit with a potential of developing defects in postharvest storage together with sound fruit, which reduces the quality of the batch as symptoms manifest during the postharvest storage. Currently, not much is understood about the relationship of rind biochemical profile, physiological attributes and 'Marsh' grapefruit sensitivity to CI and RP disorders. Since symptoms of postharvest rind physiological disorders manifest late during the postharvest life of grapefruit, development of methods for predicting fruit susceptibility to these disorders during packing operations is of great importance. Development of non-invasive fruit quality assessing techniques such as visible to near infrared spectroscopy (Vis/NIRS) has made it possible to develop objective, fast and non-destructive assessment of internal biochemical characteristics of fruits. Vis/NIRS studies internal characteristics of biological samples by illuminating the product with radiation and

measuring the reflected, absorbed or transmitted radiation. The radiation changes its spectral characteristics as it penetrates through the product depending on its chemical composition and microstructures. The spectral change is dependent on wavelength and causes scattering or absorption at certain spectral regions, which can be used to study important internal characteristics of a sample (Nicolaï *et al*., 2007).

Vis/NIRS, together with chemometric softwares, has been successfully applied for prediction of different parameters on various citrus fruit in previous studies (Peiris *et al*., 1998; Tsuchikawa *et al*., 2003; Lee *et al*., 2004; Kim *et al*., 2004; Gomez *et al.,* 2006; Cayuela and Weiland, 2010; Zheng *et al.,* 2010; Liao *et al.,* 2013; Sànchez *et al.,* 2013; Magwaza *et al*., 2014; Liu *et al*., 2015). Physio-chemical properties such as rind dry matter (DM), non-structural carbohydrates and antioxidants content, that might be linked with susceptibility of 'Nules Clementine' mandarin fruit to rind breakdown disorder, can be predicted using Vis/NIRS (Magwaza *et al*., 2012; Magwaza *et al.,* 2014a, b). However, the physiological and biochemical mechanism underlying susceptibility of 'Marsh' grapefruit to rind physiological disorders such as rind pitting and chilling injury are different from that of 'Nules Clementine' mandarins. Furthermore, the rind thickness of the fruit plays an important role on its optical properties when it is illuminated with a Vis/NIR spectrophotometer. Therefore, models for predicting the quality of 'Nules Clementine' mandarins with thin rinds cannot be used to predict the quality of fruit with thick rinds such as grapefruit.

Therefore, in this study, Vis/NIR spectroscopy will be investigated as a tool for non-destructive prediction of 'Marsh' grapefruit susceptibility to postharvest rind physiological disorders, specifically chilling injury and rind pitting. However, before the ability of Vis/NIRS to predict grapefruit parameters is investigated, specific pre-symptomatic biochemical markers related to these rind physiological disorders will be identified.

1.2 Aims and objectives

This master's research aims to develop Vis/NIRS based models that could be used by the citrus industry to predict the susceptibility of 'Marsh' grapefruit to postharvest chilling injury and rind pitting disorders. The models will allow discrimination of fruit with high chances of surviving cold storage, usually used during shipping, and export only fruit guaranteed to reach a consumer whilst in its high quality status. The objectives are:

- To evaluate optimum conditions for Vis/NIRS to predicting physiological attributes of 'Marsh' grapefruit.
- To identify pre-symptomatic biochemical markers that can be used to predict the susceptibility of 'Marsh' grapefruit to CI and RP.
- To develop Vis/NIRS models to predict the disorders and rind biochemical markers associated with fruit susceptibility to CI and RP disorders.

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Chapter 2: Literature review: Application of Vis/NIR spectroscopy for non-destructive prediction of citrus fruit quality and postharvest rind physiological disorders

2.1 Abstract

Rind physiological disorders are known to affect different types of citrus fruit, but the conditions leading to their development are still unclear. Intensive research has focused on finding the mechanisms governing them and means to alleviate their occurrence, which does not completely solve the problem considering the fruit to fruit differences. Methods for predicting rind physiological disorders before their development are required as an approach for discriminating fruit with different susceptibility during packaging and therefore, targeting a relevant market for each category. The purpose of this review was to outline the physiological, molecular and biochemical basis of most important citrus postharvest rind physiological disorders; to evaluate the capability of different techniques used for their alleviation; and to evaluate the potential of Visible to near infrared spectroscopy (Vis/NIRS) to non-destructively predict citrus fruit quality and postharvest rind disorders. Previous studies have shown that there is a strong relationship between physiological attributes and rind physiological disorders of citrus fruit. The high capability of Vis/NIRS to non-destructively predict citrus internal and external parameters combined with the correlation of certain physiological parameters to rind disorders motivated the idea for predicting physiological disorders using Vis/NIRS prior to shipping of fruit to international markets.

Keywords: Citrus, ‘Marsh’ Grapefruit, Rind Disorders, Chilling Injury, Rind Pitting, Near Infrared Spectroscopy (NIRS)

2.2 Introduction

South African citrus fruit industry is one of the major contributors to the country's economy. The country produces large amounts of different citrus cultivars including oranges, lemons, soft citrus and grapefruit. During 2013/2014 season, a total of 375 000 MT of grapefruit was produced, but the local market consumes very little, not even 1 %, of the fruit production (FAO, 2014). Citrus production in South Africa is mainly intended for fresh fruit exports to international markets. However, international markets have strict requirements for imported fruit. These requirements include the consistent and reliable supply of required volumes of high-quality fruit. It is vital to secure a trusting marketing relationship by ensuring that fruit exported meet or exceeds market quality standards, satisfies international consumers' needs and is profitable to the industry.

The South African citrus industry has a successful history in international trade. Cold storage is the common practice used when shipping fresh fruit to international markets. It is also used as a quarantine treatment for the sterilization of the Mediterranean fruit flies (*Ceratitis capitata* and *Ceratitis rosa*) (Bassal and El-Hamahmy, 2011). During quarantine treatment, citrus fruit are stored at -0.6 °C for about 14 to 22 days, depending on the specific requirements of the importing country. However, if stored at such low temperatures, grapefruit suffer from rind disorders such as chilling injury (CI) and rind pitting (RP), reducing external quality and general acceptance of fruit in the competitive market.

Chilling injury is a physiological disorder occurring on tropical and subtropical fruit that is caused by a period of exposure to temperatures below 10 °C but above the freezing point (0 °C) (Siboza *et al*., 2014). It is usually characterized by rind pitting, rind staining, red blotches, internal breakdown, off-flavour, decay and necrosis on the flavedo (the outer-most pigmented part of the rind) of the fruit (Chaudhary *et al.*, 2014). RP is manifested as small, irregular and slightly sunken patches of 3 to 6 mm in diameter that are randomly distributed about

the flavedo of the fruit (Alferez *et al*., 2010; Cronje *et al*., 2011a; Magwaza *et al*., 2013a). The symptoms of postharvest rind disorders reduce the market value of fruit and drastically increase the economic loss considering the investment from orchard practices to postharvest handling and shipping costs (Magwaza *et al*., 2013a). Susceptibility to the rind physiological disorder differs among the fruit of the same cultivar, spurring the need for sorting fruit suitable for the export market. However, the susceptibility of grapefruit to these postharvest rind physiological disorders is difficult to identify during harvest and packing because the symptoms only manifest 3 to 5 weeks postharvest. In the exporting citrus industry such as the one in South Africa, this usually coincides with the shipping period and the point of sale (Magwaza *et al.,* 2012; Magwaza and Opara, 2015).

The development of fruit postharvest disorders is affected by a number of factors experienced during pre-harvest, harvest and postharvest handling procedures (Lee *at al*., 2015). Fruit physico-chemical properties such as rind dry matter, carbohydrates and antioxidants content at harvest are among vital factors determining fruit susceptibility to different postharvest physiological disorders (Di Majo *et al*., 2005). Previous studies on 'Nules clementine' mandarins showed that rind dry matter content, carbohydrate concentration and antioxidant capacity differed with fruit susceptibility to rind breakdown disorder (Magwaza *et al*., 2012, 2013a; 2014a, b, c).

Previous studies of CI on grapefruit have indicated high lycopene concentration found on red peel areas to increase the fruit tolerance to the disorder (Lado *et al.,* 2015; Lado *et al.,* 2016). A positive correlation of total soluble sugars (TSS) to rind pitting was found in the study by Ezz and Awad (2009) on 'Marsh' grapefruit. The authors noticed a negative correlation between CI and ascorbic acid content. There is, therefore, a potential of using these biochemical properties as biochemical markers for predicting the susceptibility of grapefruit to rind physiological disorders. However, analytical methods used to determine these biochemical parameters are destructive, time consuming and require specialized sample preparation. Also, these analyses can only be

performed on a limited number of representative samples. Considering significant fruit-to-fruit variability due to a number of pre-harvest factors such as position within the canopy and flowering time, the results from representative samples may not be enough. The recent trend in the agricultural industry is moving towards developing non-destructive analytical methods for predicting fruit quality parameters. Therefore, there is a need to develop non-destructive methods for determining physico-chemical properties related to fruit susceptibility to rind physiological disorders.

Citrus biochemical compounds are characterized by bonds such as O-H, N-H, C-O and C-H that can be studied by illuminating the fruit with radiation and investigating light transmission, reflection and refraction pattern (Liu *et al*., 2015). The introduction of near infrared radiation application to study intact biological samples has necessitated the investigation of internal and external quality of fruit without causing any damage to the fruit (Magwaza *et al*., 2012; 2013b). Near infrared spectroscopy (NIRS) is arguably the latest and most advanced technology used for such an objective. Previous studies of using NIRS on citrus have shown the technique to be appropriate and successful with relevant chemometrics to analyze spectra (Liao *et al*., 2013; Sànchez *et al*., 2013; Magwaza *et al*., 2013b; 2014b, c; Liu *et al*., 2015). This paper aims to discuss current knowledge about CI, RP, and application of NIRS to predict quality parameters and physiological disorders of citrus fruit.

2.3 Chilling injury

Low temperature storage is a postharvest technology widely used to extend the postharvest life of fresh fruit and vegetables. It allows quality preservation after harvest because low temperatures decrease the rate of cellular metabolism and delay plant senescence and fruit ripening (Sevillano *et al*., 2009). However, some tropical and subtropical fruits such as certain citrus cultivars cannot tolerate exposure to very low temperatures and suffer from chilling injury.

Physiological basis of chilling injury

The primary sites for CI development are cell membranes (Rui *et al*., 2010). Chilled tissues change their membrane flexibility from flexible to solid gel structure. The existence of temperature induced phase transition is mainly determined by the fatty acid composition of membrane lipids (Aghdam and Bodbodak, 2013). A higher proportion of unsaturated to saturated fatty acids provided higher tolerance to low temperature in banana (Mirdehghan *et al*., 2007), pomegranate (Promyou *et al.,* 2008) and loquat fruit (Cao *et al*., 2009). Membrane integrity maintenance at low temperatures has been reported to be important in resistance to chilling injury (Wonsheree *et al.,* 2009), and can be measured indirectly by measuring electrolyte leakage and malondialdehyde (MDA) content (Sharom *et al*., 1994; Siboza *et al*. 2014). MDA is the end product of the peroxidation of membrane fatty acids and the level of this compound is used as a marker of oxidative stress since its rise is indicating damage on the cell membrane (Hodges *et al*., 1999). Therefore, if the tissue, organ or whole plant is exposed to damaging temperatures for a long period of time, the cell membranes rupture causes leakage of intracellular liquid, ions, and metabolites, which can be monitored by determination of electrolyte leakage (Sharom *et al*., 1994).

The rupturing of the membrane and therefore, intense of electrolyte leakage, depends on the degree of membrane saturation that is correlated to fatty acid desaturases (FAD), the enzymes responsible for increasing the degree of membrane unsaturation (Hernández *et al.,* 2011). This group of enzymes includes phospholipase D (PLD) and phospholipase C (PLC), which are responsible for the degradation of unsaturated fatty acids and reduction of cell membrane integrity and therefore increase the impact of CI (Aghdam *et al*., 2012). In cucumbers and tomatoes, PLD and PLC have been shown to be reduced by subjecting fruit to heat and salicylic acid treatments, respectively, leading to an increase in CI resistance as a result of improved cell membrane integrity and diminishing lipid peroxidation (Mao *et al*., 2007; Aghdam *et al*., 2012).

Other two metabolic energy parameters involved in lipid peroxidation of unsaturated fatty acids are adenosine triphosphate (ATP) and adenylate energy charge (AEC) according to their associated role in the biosynthesis of fatty acids and their unsaturation. The decrease of adenosine diphosphate (ADP), ATP and AEC, while there is an increase of adenosine monophosphate (AMP), and results in the reduction of metabolic energy levels which negatively affect the integrity of cell membranes (Crawford and Braendle, 1996). Apart from the direct effect of low temperatures on the molecular arrangement of lipids constituting cell membranes, the loss of membrane integrity is itself boosted by oxidative processes. This is because cold stress increases the levels of reactive oxygen species (ROS) that stimulates lipid peroxidation in cell membranes (Fig. 1; Sevillano *et al*., 2009).

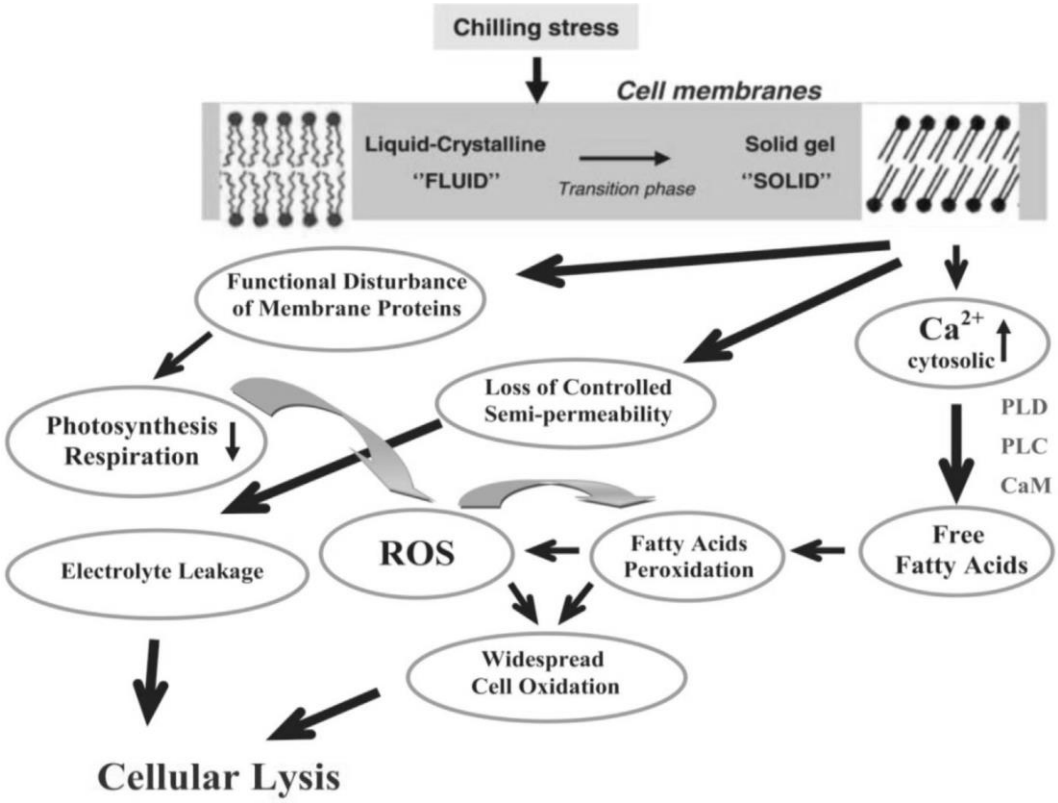

Fig. 1: Alterations in the cell membrane integrity and de-organization due to cold stress led to solute leakage and oxidative stress, which could trigger widespread cell decompartmentalisation and oxidation, which causes cell death in prolonged stressful conditions (Aghdam and Bodbodak, 2013).

Moller (2001) suggested two mechanisms used by plant defense system against oxidative stress. The first one is by activation of enzymes responsible for the expression of genes encoding proteins involved in activating ROS avoidance such as alternative oxidase (AOX). The second one is by inducing the activity or the gene expression for ROS scavengers such as antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), glutathione-S-transferase (GST), mono-dehydro-ascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) (Moller, 2001). The AOX pathway is a branch of the respiratory electron transport chain in mitochondria that emerges from the cytochrome (Mittler, 2002). When over reduction occurs in this electron transport chain, AOX intervenes by inhibiting the excessive reduction of ubiquinol and thus assists in avoiding ROS accumulation (Moller, 2001). Higher activity of the enzymatic antioxidant system leads to a reduction of ROS, therefore maintaining membrane integrity and inducing tolerance towards abiotic stresses such as CI (Fig. 2; Mittler, 2002).

Aghdam and Bodbodak (2013) suggested that a high ratio of unsaturated to saturated FA, high levels of cell metabolic energy (ATP and AEC), and a lower level of PLD and Lipoxygenase (LOX) pro-oxidant enzymes activities accompanied by boosted activities of enzymes from the antioxidant system (SOD, CAT, GPX, GST, APX, DHAR and MDHAR) result in reduction of cell membrane damage because of membrane lipid peroxidation and the avoidance of ROS accumulation, all of which have a positive effect on CI tolerance, which is reflected as an improved cell membrane integrity.

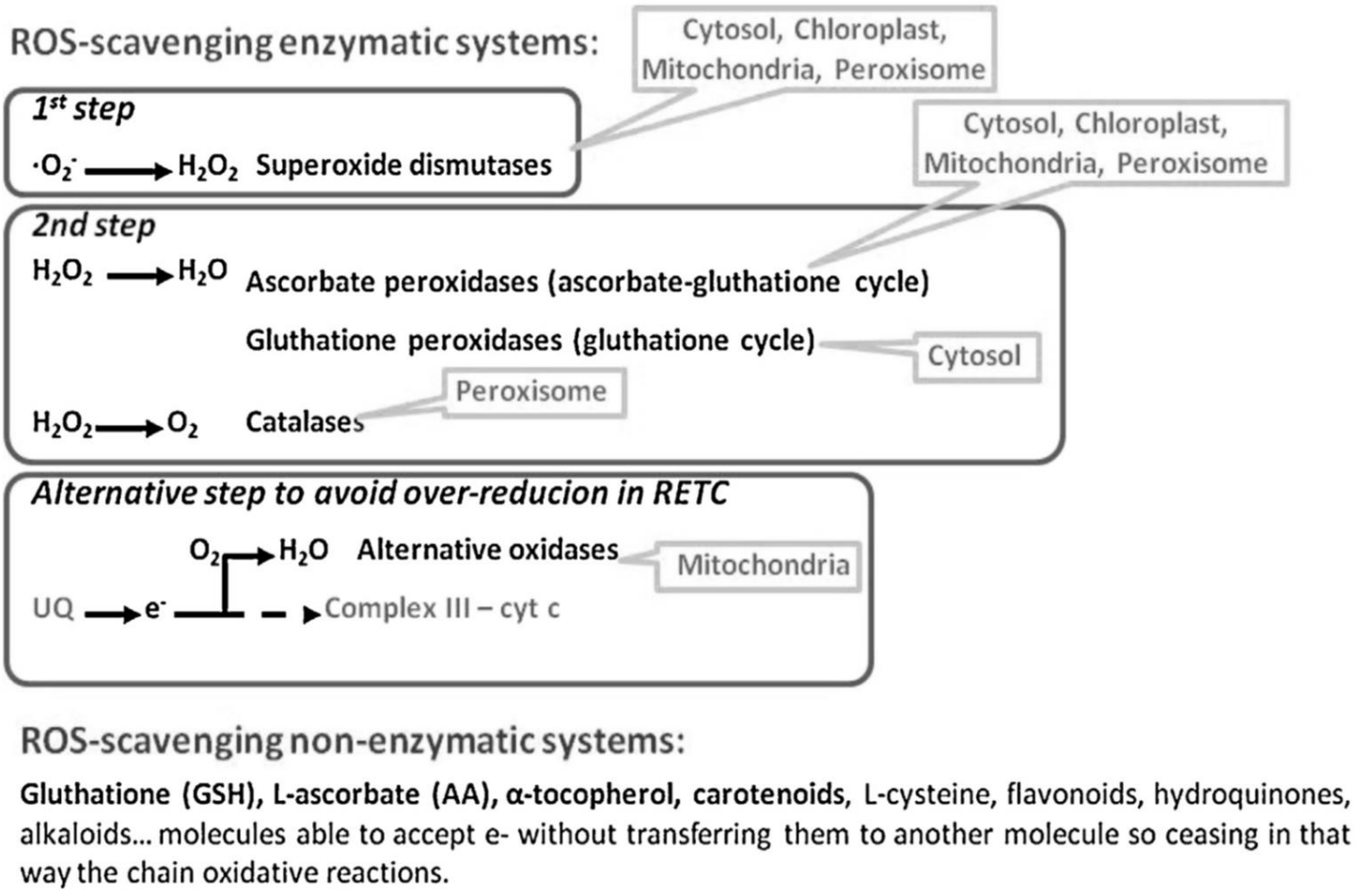

Fig. 2: The antioxidant systems that plant cells use to counteract oxidative stress, based on Mittler (2002).

Chilling injury alleviation techniques

Chilling injury has been studied for a long time and a number of its alleviation techniques have been suggested. Bassal and El-Hamahmy (2011) studied water dip and preconditioning treatment effect on reducing chilling injury and maintaining the postharvest quality of 'Navel' and 'Valencia' oranges during cold storage. They reported that hot water dipping (HWD) at 41± 1 °C for 20 minutes or 50±1 °C for 5 minutes reduced the CI incidence in both cultivars, especially HWD at 41°C for 20 minutes. However, the treatments they used were complicated to be practiced by the commercial citrus industry since it consisted of 20 minutes water dipping and temperature and humidity alterations that cannot be applied to a large number of fruit usually shipped commercially.

Siboza *et al.* (2014) hypothesized that treating lemon fruit with methyl jasmonate (MJ) and salicylic acid (SA) may enhance chilling tolerance by increasing the synthesis of total phenolic compounds and phenylalanine ammonia-lyse (PAL) which activate the key enzyme regulating the shikimic acid pathway whilst inhibiting the activity of peroxidase (POD) and polyphenol oxidase (PPO). These authors discovered that 10 µM MJ plus 2 mM SA treatment was highly effective in enhancing chilling tolerance, significantly reducing chilling-induced membrane permeability and membrane lipid peroxidation of flavedo tissue. After coating 'Nules clementine' mandarins (*Citrus reticulate* Blanco) with shellac-based wax (SBW) and polyethylene-based wax (PBW) and storing at 2, 5 or 8 °C for 90 days, Bajwa and Anjum (2006) found that storing fruit at either very low (2 °C) or high cold storage (8 °C) temperatures had significant difference. The fruit stored at 8 °C had higher disorder compared to 5 °C and had higher rind staining and physiological loss in fruit weight. It could then be deducted from their findings that fruit have their optimal cold storage temperatures that satisfy both prolonging the fruit postharvest lifespan while avoiding chilling injury. They also demonstrated that temperature increase in cold storage is not always a solution to avoid chilling injury.

2.4 Rind pitting

Rind pitting (also called rind breakdown) manifests itself on the flavedo of the rind as randomly distributed dark/brown spots, usually appearing as a "leopard spot" pattern (Cronje, 2005). It is associated with the collapse of oil glands as is the case with CI. Its occurrence is erratic and unpredictable (Alquezar *et al*., 2010), showing high variability from year to year, among orchards and even among the fruit of the same tree.

Rind pitting (RP) disorder can develop as a symptom of CI or at non-chilling temperatures in pre- and postharvest. On oranges, tree water stress at fruit maturity (Albrigo, 1972) and fruit water loss while still attached to the tree (Tamim *et al*., 2001) have been reported to cause RP at pre-harvest. Exposure of mandarin

fruit to direct sunlight during development and delaying harvest until high maturity level was also reported as the cause of pre- and postharvest RP at non-chilling temperatures (Duarte and Guardiola, 1995). Rind Pitting has been used by many authors as a symptom of CI. Its index has been used as an appropriate CI level expression on lemons (Siboza *et al*., 2014) and grapefruit (Chaudary *et al*., 2014). In the study by Cronje *et al.* (2011a) on 'Nules Clementine' mandarin, fruit stored at 7.5 °C showed higher rind breakdown disorder (RBD) when compared to fruit stored at -0.5 °C. The resulted RBD could be interpreted as CI symptoms because it did not occur on fruit stored at temperatures below freezing point (0 °C).

Alferez and Burns (2004) conducted a study investigating the effect of relative humidity, its alterations and the effect of waxing during postharvest storage of 'Marsh' grapefruit at non-chilling temperatures (20 °C). After storing fruit at either 30% RH or 90% RH, fruit developed low RP even if waxed immediately after harvest. When fruit stored at 30% RH were transferred to 90% RH, the incidence of peel pitting markedly increased, while transferring fruit from 90% to 30% RH did not lead to increased pitting. Basically, increasing relative humidity increases the vapour pressure in the atmosphere around the fruit, which decreases the fruit respiration potential and leads to cells collapsing due to abnormal water potential between rind cells and the atmosphere.

Increasing relative humidity may reduce the respiration of the rind cells (Alférez *et al*., 2010). That effect can reduce the fruit internal oxygen which is needed by the fruit to maintain its rind quality and appearance. Petracek *et al*., (1998a, b) found that fruit with RP have low internal O_2 and higher CO_2 compared with fruit without the disorder. Table 1 shows an overview of previous studies on grapefruit RP.

Table 1: An overview of symptomology, time of occurrence, and causal factors of different physiological rind pitting disorders on grapefruit

Cultivar	Disorder name	Time of occurrence	Pre/postharvest	Causal factors	Symptoms	Reference
Marsh	Peel pitting	2-6 days	Postharvest	Low relative humidity during harvest before storage at high RH	Depression in the flavedo that ultimately affects oil gland	Alférez *et al.* (2005)
Marsh	Rind Pitting	.....	Postharvest	Waxing	Clusters of collapsed oil glands over the surface of the fruit	Sun and Petracek (1999)
Marsh	Peel pitting	2-20 days	Postharvest	Waxing, warm temperature storage, and sudden changes from low to high relative humidity	Sunken areas on the flavedo followed by browning and dryness	Alférez and Burns (2004)
Marsh	Peel pitting	2-20 days	Postharvest	Variation in water, osmotic, and turgor potentials	Sunken areas on the flavedo followed by browning and dryness	Alférez *et al.* (2010)
Marsh	Rind Pitting	10 days	Postharvest	Waxing coupled with warm temperature storage	Clusters of collapsed oil glands over the surface of the fruit	Petracek *et al.* (1998a)
Star ruby	Stem End Rind Breakdown	9 days	Pre- and postharvest	Rootstock and storage temperature	Collapse and browning of the peel at the stem end of the fruit	Ritenour *et al.* (2008)
Star Ruby	Chilling Injury	12 weeks	Postharvest	Cold storage at 2 °C	Rind pitting	Chaudhary *et al.* (2014)

In ‘Navelina’ and ‘Navelate’ oranges, a sharp increase in RH after episodes of fruit dehydration was responsible for peel pitting in the field (Agusti *et al*., 2001) or during postharvest handling and storage (Alférez *et al*., 2003). Such variation in RH changes the peel water status in fruit, alters turgor pressure of the flavedo and albedo cells, and ultimately leads to peel damage (Alferez and Burns, 2004; Alquezar *et al*., 2010). It is noticeable that fruit moisture content plays an important role in the development of rind pitting whether in fruit’s pre- or postharvest stage. However, it is vital to note that there are various grapefruit rind disorders caused by different attributes.

2.5 Causes of physiological citrus rind disorders

Pre-harvest factors

Precursory conditions leading to the development of physiological disorders on fresh fruit can be manifested prior to harvesting. Scion and rootstock cultivar, fruit canopy position, fruit maturity status at harvest and fruit developmental nutrition are among pre-harvest factors determining the fruit susceptibility to the disorders.

Scion cultivar and rootstock

Plant improvement techniques such as grafting and breeding enable the ability to combine the required characteristics of two plants of the same species into one tree. Fruit of different cultivars of a species differ in sensitivity to develop physiological rind disorders. There are cultivars highly sensitive to CI such as ‘Navelate’ orange (Bower *et al*., 2013) and ‘Eureka’ lemons (Siboza *et al*., 2014), and cultivars highly sensitive to RP such as ‘Marsh’ grapefruit (Petracek *et al*., 1998a; Alferez and Burns, 2004), ‘Navelina’ oranges (Lafuente and Sala, 2002) and ‘Fallglo’ tangerine (Petracek *et al*., 1998b). A good example of fruit cultivar difference is the ‘Pinalate’ orange fruit that is tolerant to CI while they are very prone to non-chilling RP. The other one is

'Navelate' orange fruit which is prone to CI while tolerant to non-chilling RP (Magwaza *et al*., 2013a). Therefore, combining the scion of a cultivar resistant to RP can reduce or alleviate the incidence of RP on the fruit of that tree even if the rootstock had traits for higher RP incidence. In such cases, the scion would be grafted on the rootstock with traits for surviving harsh soil conditions, although its natural scion cannot resist RP, to produce a tree with both the rootstock and scion advantageous to high production capability.

Canopy position

Fruit found in different tree positions differ in biochemical composition as a result of shading and direct exposure to sunlight (Cronje *et al*., 2011b). Shading affects biological processes that occur during fruit growth and development. Canopy position affects the light and temperature the fruit is exposed to and, therefore, the photosynthesis and respiration processes. 'Nova' mandarin fruit directly exposed to sunlight developed pre- and postharvest rind pitting in 14 to 28 days, while shaded fruit did not develop the disorder in the study by Duarte and Guardiola (1995). Moreover, shaded 'Nules Clementine' mandarin developed a higher rind breakdown, a similar disorder, at postharvest after 3 to 5 weeks (Cronje *et al*., 2011a) and had higher disorder incidence earlier than fruit from outside canopy. Fruit directly exposed to sunlight loses water more than fruit in the inner canopy, and possibly have different biochemical compounds content in their flavedo which gives them their characteristic difference.

Postharvest fruit water loss was proven to induce peel pitting on 'Navelate' orange (Alquezar *et al*., 2010). Therefore, fruit at the outer canopy may lose water to certain extent under stressed conditions. Cronje *et al*. (2011b) demonstrated a possible link between fruit position, rind sugar, and development of rind breakdown disorder of 'Nules Clementine' mandarin. The flavedo of fruit borne on the outer canopy had higher sucrose; glucose and fructose content than fruit from the inner canopy and had a significant lower sensitivity to the disorder.

Fruit maturity

Mature fruit have a higher susceptibility to rind pitting (Cronje, 2009). Their susceptibility has been correlated with earlier rind senescence when they are compared with immature fruit. Green fruit are not susceptible to RP (Daurte and Guardiola, 1995). Fruit susceptibility to RP is mainly determined at the time when fruit change colour during pigmentation until harvest (Assimakopoulou *et al*., 2009). The occurrence of RP on hanging fruit was reported to extend from the beginning of fruit colour change and continue depending on climatic conditions thereafter (Agusti *et al*., 2001). Daurte and Guardiola (1995) conducted a study on the incidence of peteca spot on lemons, a disorder similar to RP, and found that fruit harvested green did not develop the disorder while those harvested yellow had the highest incidence. The authors further investigated the application of exogenous plant regulators using gibberellic acid (GA_3). Its application at colour break was reported to reduce the incidence of peteca spot, but the result was correlated to the chlorophyll retaining ability. The primary benefit of applying GA_3 could be delaying carotenoids accumulation and senescence, and therefore, retarding RP development.

Fruit mineral nutrition and on-tree water potential

Although the citrus tree requires all the 17 essential mineral elements for normal growth and reproduction, calcium (Ca), nitrogen (N) and potassium (K) are the most important with regard to fruit rind pitting. Ca is known to have a positive effect on fruit rind physiology as the element is correlated to fruit membrane quality. Storey *et al.* (2002) reported the negative correlation to exist between creasing and Ca concentration on flavedo and albedo of oranges. Ca sprays were also reported to reduce the incidence of RBD of oranges (Treeby and Storey, 2002) and peteca spot of lemons (Storey and Treeby, 2002). On the other hand, nitrogen increases the

size of the fruit and the thickness of the peel and therefore, the fruit softness which also increases its susceptibility to rind disorders.

Fruit found in the inner canopy, which are aligned with a higher incidence of RBD, have thicker and softer rind tissues when compared with fruit found on the outer canopy (Cronje *et al*., 2011b; Magwaza *et al.,* 2013a). Cronje *et al.* (2011b) also demonstrated fruit from the inner canopy to have lower Ca and Mg and higher K concentrations. The authors suggested that higher concentration of K may be related to stress response due to reduced respiration conditions, as the element is responsible for stomatal regulation (Storey and Treeby, 2002). However, Assimakopoulou *et al.* (2009) reported no significant differences in mineral elements of 'Clementine' mandarins affected by RP and those not affected. The damage was reported to be on the cuticle since strata with oil glands remained intact. The results observed by Assimakopoulou *et al.* (2009) correlated the disorder to microclimatic conditions rather than nutritional imbalance.

Fruit get water from the tree when they are still attached to it. When the tree experiences stress, either by low rainfall or reduced irrigation, it will reduce the amount of water it provides to fruit and stress the fruit as well. Pre-harvest water stress induced by blocking irrigation and rainfall for 49 days before harvest was reported to increase the incidence of peel breakdown on grapefruit (Ritenour *et al*., 2008).

Postharvest factors

Fruit susceptibility to disorders is mainly affected by pre-harvest conditions while its extent is affected by postharvest factors. Important factors that determine the susceptibility of fruit to physiological disorders are highly about the water status of fruit and its surroundings. Most studies of citrus rind have shown RH to be the main inducing factor. Some studies also confirm the fruit water status to play a significant role regarding fruit susceptibility.

Fruit water loss

Fresh fruit lose water during postharvest storage through processes called transpiration and respiration. Those processes are reduced during cold storage but proceed at a normal rate when fruit are transferred back to shelf life. Water loss accounts for 90% postharvest fresh fruit weight loss (Ben-Yehoshua *et al*., 2001). Alférez and burns (2004) reported 1% water loss rate per day for the first 6 days after harvest on 'Marsh' grapefruit stored at 21 °C and 30% RH. The rate decreased to 0.6% thereafter. Alférez *et al.* (2010) also reported water loss between 9% and 15% when 'Marsh' grapefruit was stored at 21 °C and 45% RH. Fruit water losses can reach a point when it significantly reduces the quality of a fruit. Excessive water loss of fruit results to shriveling which appears as loss of shine, softening, and senescence (Magwaza *et al*., 2012).

Change of flavedo and albedo water status was reported as the primary factor determining the susceptibility of 'Navelate' orange to rind pitting at non-chilling temperatures (Alférez *et al*., 2010). Alquezar *et al.* (2010) investigated the morphological and structural alterations in flavedo and albedo of 'Navelate' fruit exposed to rind breakdown inducing conditions. Fruit stored at high RH (95%) had no signs of flavedo cells disruption or collapse as was the condition of the albedo cells of fruit stored for the same period at low RH (45%). The albedo cells dehydrated and compacted, forming clusters of flattened cells that were typical symptoms of rind pitting. However, the authors suggested the flavedo to be more sensitive to water loss than albedo after investigating the water potentials of the two parts. Water potentials of the albedo and flavedo were investigated before and after storage. A 30% decrease in water potential of flavedo was observed while the albedo water potential was unaltered.

'Navelate' oranges (Lafuente and Sala, 2002) and 'Marsh' grapefruit (Alférez and Burns, 2004) stored at high RH have been reported to develop a higher incidence of rind pitting when compared with fruit stored at low

RH. The contrasting results presented by the authors, and the low RH by Alquezar *et al.* (2010) and RP development, suggests that there could also be another factor responsible for the disorder incidence. There is, therefore, a need to investigate whether RH is the most important factor, or whether there are other factors responsible for RP of citrus. Previous studies have confirmed the change in RH from low to high as a typical cause of RP on 'Marsh' grapefruit (Alférez and Burns, 2004; Alférez *et al*., 2010) and 'Navelate' orange (Alférez *et al*., 2003; Alquezar *et al.,* 2010). Changing the RH from high to low did not induce RP in both fruits. The above studies increased the RH after a week of storage at low RH. Alférez *et al*. (2004) investigated the time it takes for the fruit to sense the RH change. The authors demonstrated that the period as short as 3 hours is sufficient for inducing RP on 'Marsh' grapefruit when RH was increased, which increases the confidence on the previous results obtained by Alférez *et al*. (2003) and Alquezar *et al.* (2010).

Postharvest wax application

Water loss of citrus fruit (except organic fruit) is usually reduced by application of waxes. Waxes are also applied to improve fruit appearance and to serve as a medium to apply fungicides for protection against diseases and thus increase shelf life (Alférez *et al*., 2010). Fruit are washed before they are waxed on commercial packing lines. The natural wax is brushed down and reorganized but not removed by normal commercial washing and brushing (Albrigo, 1972). Removal or re-organization of natural waxes on the fruit surface was hypothesized to alter water and osmotic potentials, which gradually reduce turgor pressure potential of fruit (Alférez *et al*., 2010). In the study conducted by Alférez *et al* (2010) on 'Marsh' grapefruit, fruit stored at 45% RH for 30 days had higher (15%) water loss when they were washed by a packing line system compared with those hand-washed and waxed with shellac based wax which had 9% water loss. In a previous study by Alférez and Burns (2004), fruit exposed to prolonged periods of dehydration at lower RH had higher water loss. The authors suggested that waxing enhances the severity of the damage already caused by the dehydration period.

In a study conducted by Petracek *et al.* (1998), results showed that shellac-based waxes caused higher RP incidence when compared with carnauba- or polyethylene-based waxes. Non-waxed fruit did not develop the disorder. Internal O_2 levels were lower (1.8 to 3.5%) on fruit coated with shellac-based waxes, higher (10%) for carnauba and polyethylene-based waxes, and highest on non-waxed fruit (19%). Internal CO_2 levels were high on shellac- based wax (7.8 to 8.3%), lower on carnauba based wax (4.9%) and polyethylene- based wax (5.8%), and lowest for non-waxed fruit (1.5%). The study conducted by Alférez and Zacarias (2001) showed that waxing the fruit on its own had no damage with regard to RP development, but alteration in RH of waxed fruit increased the RP incidence.

2.6 Methods used to estimate rind physiological disorders

Researchers working on citrus have used visual inspection for estimating the intensity of both CI and RP damage. They take fruit samples, expose them to similar conditions as the condition they want to use for massive fruit storage and investigate the disorders after they have developed sample fruit. The procedure is carried out by scoring on a scale from 0 (not injured) to 3 (severe injury) based on the percentage surface area of a fruit covered by the injury (Maul *et al.,* 2011; Chaudhary *et al*., 2014; Lado *et al.,* 2015). Chilling injury index is calculated by multiplying the number of fruit in each category by their corresponding score, summing the products and dividing the sum by the total number of fruits per treatment (Eq. 1) (Lado *et al.*, 2015).

$$\text{CI index} = \frac{\text{Rind pitting } (0-3) \text{ x No.of affected fruit}}{\text{Total No.of fruit}} \quad (1)$$

Chilling injury is usually evaluated according to the necrotic surface area and browning intensity, which is not accurate considering that CI symptoms appearance differs from one fruit to another. For example, some fruit may show RP (Chaudhary *et al.*, 2014), red blotches (Martinez-Tellez *et al.*, 1997) or decay (Porat *et al.*, 2002) which are all symptoms of CI. Comparison under such condition may be biased based on the physical

appearances of the symptoms. Furthermore, the symptoms development and scoring is not reliable when the fruit of the same batch show different CI symptoms.

Chilling injury can be indirectly estimated by the tolerance of fruit to the disorders. In such cases, it is estimated by investigating enzymes responsible for its alleviation. It is possible to estimate CI if the trend or relationship between CI and a certain enzyme is known. Bassal and El-Hamahmy (2011) assessed enzymes associated with oxidative stress and metabolic changes occurring during induction of CI in the 'Navalate' orange fruit. The authors used hot water dips as a treatment and found that peroxidase (POX) and catalase (CAT) increased as CI was reduced. Therefore, the higher amount of these compounds correlates with lower incidence of CI. Other researchers have used the membrane integrity as an estimate of the CI (Sharom *et al*., 1994; Wonsheree *et al*., 2009; Mirdehgham *et al*., 2007), which is the closest estimate considering that cell membranes are primary sites of CI development (Rui *et al.*, 2010).

Although those methods are still believed to be some of the best for researchers on citrus rind disorders, they cannot be applied to a large number of fruit normally aimed for fresh fruit market because of two reasons. Firstly, they involve destructive analysis, which means analyzed fruit cannot be returned to the fruit going to market. Secondly, the estimate of fruit going to the market will be based on the representative samples. Yet, characteristics of each fruit differ from fruit to fruit in every batch. The only useful way of estimating disorders would be predicting the potential of fruit to develop the disorders while they are still fresh, of which, non-destructive techniques stands the highest potential based on its previous applications on citrus.

2.7 Visible to near infrared spectroscopy application

Basic concepts

The electromagnetic spectrum is the range of all possible frequencies of electromagnetic radiation. Visible radiation covers 380 to 760 nm whilst near infrared (NIR) radiation is the range between 750 and 2500 nm. Visible to NIR spectroscopy studies internal characteristics of biological samples by illuminating a product with radiation and measuring its reflection, absorption or transmission (Nicolaï *et al*., 2007). Near infrared spectroscopy is of overtone vibrational spectroscopy occurring in the near infrared region, where overtones or combinations of fundamental stretching bands respond, which is often plotted as log (1/R) versus wavelength (Lin and Ying, 2009). The radiation changes its spectral characteristics while it penetrates the product depending on chemical composition and microstructures of the examined product. Reflection is caused by the external characteristics of a sample. Specular reflection is caused by gloss, whereas external diffuse reflection is induced by rough surfaces. Reflection only provides information about the surface of a sample. The cell wall interfaces of fruit and vegetables are the main elements of scattering since they induce abrupt changes in refractive index. Other suspended particles such as starch granules, chloroplasts and mitochondria may also cause diffraction when their refractive index differs to that of surroundings (McGlone *et al.*, 1997; Mehinagic *et al*., 2004; Nicolaï *et al*., 2007).

The size, shape and microstructure of the particle have an effect on the light scattering properties. Scattering may also be caused by interferences such as pores, openings and capillaries randomly distributed in the sample. Most absorption bands in the NIR region are overtone or combination bands of fundamental absorption due to vibrational and rotational transitions (Nicolai *et al.*, 2007). In Figure 3, typical NIR reflectance spectra of an apple; pear; orange and nectarine are shown. The spectra are very similar with overtones at the point of responsible bonds such as O-H, N-O, C-O, C-H and H-N. The spectra in figure 3 are all dominated by water

spectrum with overtone bands of O-H bonds at 760, 970 and 1450 nm and a combination band at 1940 nm (Polessello and Giangiacomo, 1981). This is a reason why sophisticated multivariate statistical packages are applied to analyze and extract useful information from NIR spectrum (Nicolaï *et al*., 2006b).

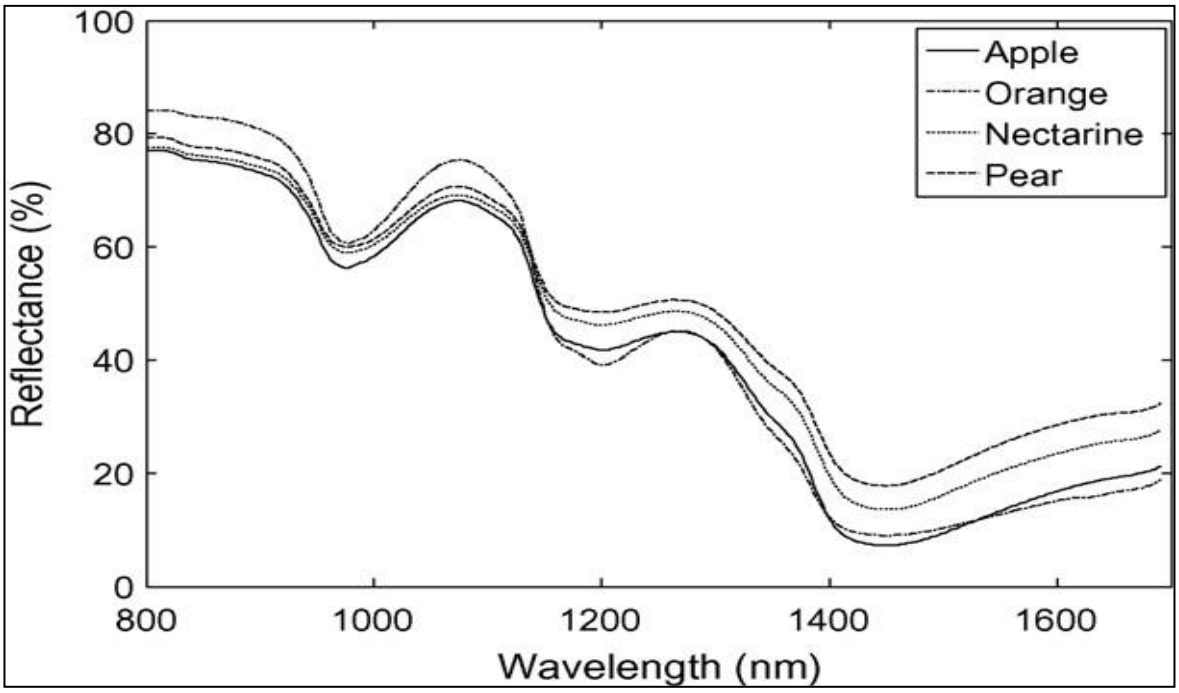

Fig. 3: Typical NIR reflectance spectra of an apple, orange, nectarine and pear (Nicolaï *et al*., 2007).

Spectrophotometers

An NIR spectrophotometer consists of a light source (usually a tungsten light bulb), sample presentation accessory, monochromator, detector and optical components such as lenses, collimators, beam splitters, integrating spheres and optical fibers. Spectrophotometers are classified based on the kind of monochromator. In *filter* instrument, the monochrometer is a wheel holding a number of absorption or interference filters with limited spectral resolution. In a *scanning monochromator* instrument, a grating or a prism is used to separate the individual frequencies of the radiation either entering or leaving the sample. It has a wavelength separator

that rotates so that the radiation of the individual wavelengths subsequently reaches the detector (Nicolaï *et al*., 2007).

In *Fourier transform* spectrophotometers, an interferometer is used to generate modulated light. The time domain signal of the light reflected or transmitted by the sample onto the detector can be converted into a spectrum via a fast Fourier transform. In *photodiode array* (PDA) spectrophotometers, a fixed grating focuses the dispersed radiation onto an array of silicon (350–1100 nm) or InGaAs (Indium Gallium Arsenide, 1100–2500 nm) photodiode detectors. *Laser* based systems do not contain a monochromator but have different laser light sources or a tunable laser. *Acoustic optic tunable filter* (AOTF) instruments use a diffraction based optical-band-pass filter that can be rapidly tuned to pass various wavelengths of light by varying the frequency of an acoustic wave propagating through an anisotropic crystal medium. In *liquid crystal tunable filter* (LCTF) instruments, a birefringent filter is used to create constructive and destructive interference based on the retardation, in phase between the ordinary and extraordinary light rays passing through a liquid crystal. This way enables them to act as an interference filter to pass a single wavelength of light. High spectral resolution can be achieved by combining several electronically tunable stages in series (Stratis *et al.*, 2001; Nicolaï *et al*., 2007).

High acquisition speed and absence of moving parts characteristics of PDA systems have caused a significant shift towards them. Their integration time is ±50 ms. Moreover, the absence of moving parts enables them to be mounted on online fruit grading lines. Miniaturised versions are available from companies, such as Ocean Optics (Dunedin, FL, USA), Zeiss (Jena, Germany), Oriel (Stratford, CT, USA), and Integrated Spectronics (Baulkham Hills, Australia). Guidelines for selecting an appropriate spectrophotometer are given by Walsh *et al.* (2000).

Equipment setup during measurement

Depending on the sample and the Vis/NIRS instrument design, five modes of measurement exist. These modes are transmittance, interactance, transflectance, diffuse transmittance and reflectance (Huang *et al*., 2008). However, three modes normally used are reflectance, transmittance and interactance (Fig. 4). In *reflectance* mode, light source and the detector are placed on the same side of the sample in the position of certain specific angle. In *transmittance* mode, light source and the detector are positioned directly on the opposite sides of the sample. In *interactance* mode, light source and the detector are positioned on the same side of the sample parallel to each other using bifurcated cable that channels the light from the light source and to the detector by means of a light barrier (McGlone *et al.*, 2003; Cayuela and Weiland, 2010).

Choosing an appropriate measurement setup mode to apply depends on the sample and the light penetrating properties. Lammertyn *et al.* (2000) found a penetration depth of up to 4 mm in the 700–900 nm range and between 2 and 3 mm in the 900–1900 nm range for apple. Fraser *et al*. (2000) showed that the penetration depth in apple in the 700–900 nm range was at least 25 mm, while it was less than 1 mm in the 1400–1600 nm range. The limited penetration depth reduces the accuracy of using reflectance or interactance measurements for detecting internal characteristics of the thick-skinned fruit, such as citrus. Reflectance spectra can only provide information about the rind with maximum accuracy on fruit such as citrus.

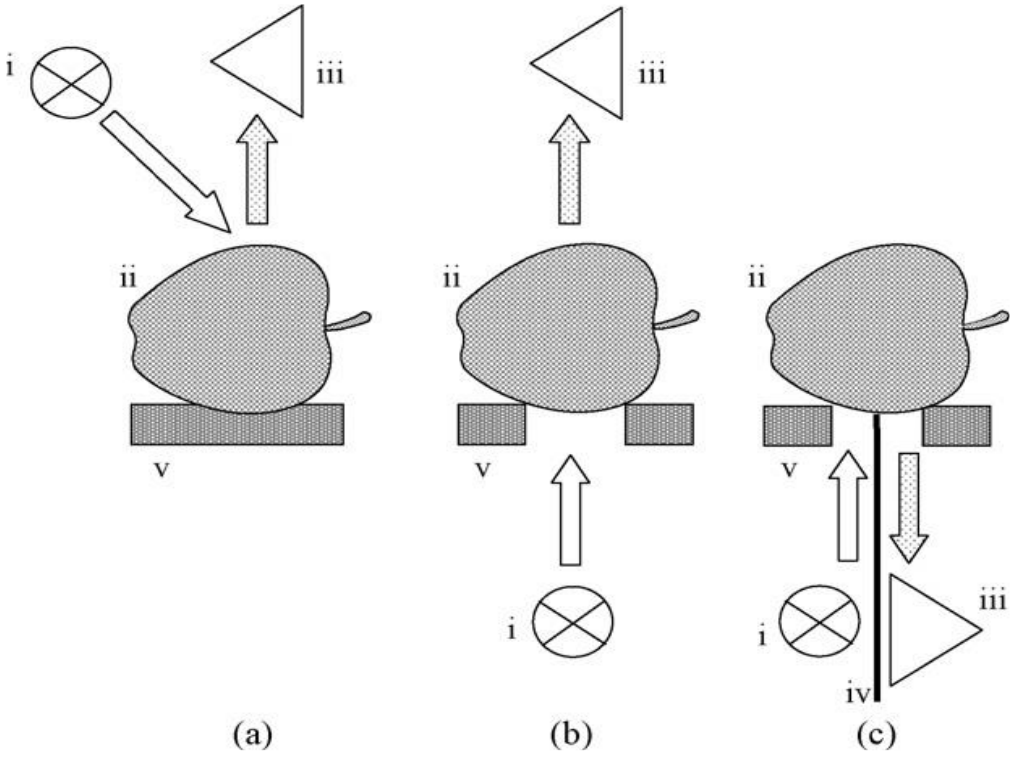

Fig. 4: The three common measurement setup for the acquisition of (a) reflectance, (b) transmittance, and (c) interactance spectra, with (i) the light source, (ii) fruit, (iii) monochromator/detector, (iv) light barrier, and (v) sample support (Nicolaï *et al*., 2007).

Rind characteristics are usually used as accepted criteria for citrus external quality evaluation (Magwaza *et al*., 2013a). Transmission mode is frequently used and considered as an appropriate technique to assess the internal quality of thick-skinned fruit such as mandarin and oranges (Kawano *et al*., 1993; Cayuela, 2008). However, the mode does not separate spectra of fruit with different sizes, peel thickness and fruit shape since those factors may result in the different optical path and optical density (Krivoshiev *et al*., 2000). On the other hand, transmittance mode needs very high light intensities which can easily burn the fruit surface and alter its spectral properties. Transmitted light may also provide information about fruit skin that may be useful or useless depending on the application (Nicolaï *et al*., 2006a; 2007). However, Dull *et al.* (1989) and Krivoshiev *et al.* (2000) argued that with transmittance mode, internal fruit quality can be predicted with sufficient accuracy neglecting the peel spectrum.

McGlone *et al*. (2003) compared the accuracy of three different modes of Vis/NIRS measurement on 'Satsuma' mandarin fruit. Transmittance mode was more accurate regarding R^2 and RMSEP with 0.96 and 0.32 °Brix values, respectively. Interactance mode did better (R^2 = 0.85, RMSP = 0.47 °Brix) compared with reflectance mode (R^2 = 0.75, RMSP = 0.63 °Brix). Furthermore, in the study comparing transmittance and reflectance modes on predicting brown heart disorder of pears, Fu *et al.* (2007) obtained results indicating transmission to be better than reflectance for internal disorder prediction. Transmittance mode excellence, when compared with other modes in determining internal characteristics or disorders, can be aligned with the light passing through the fruit from side to side and not missing any information from any part of a fruit. The results obtained by the previous authors somehow contradict but they show no damage on the fruit caused by the reflectance mode. In another previous study, reflectance mode was applied to assess internal parameters and succeeded obtaining R^2-values of 0.98 (Magwaza and Opara 2015). It is, therefore, the choice of a researcher whether to use a transmittance mode for internal fruit characteristics with a caution about the risk of burning fruit as mentioned by NicolaÏ *et al.* (2007) or use the safe but not always accurate reflectance mode.

Data acquisition position

Another important factor regarding spectra collection on Vis/NIRS system is the part of a fruit at which radiation is illuminated by the source and collected by the detector. Apart from the difference brought by maturation status, growing conditions and size of a fruit, different parts of the single fruit have a different amount of internal attributes. For example in a single grapefruit distal ends, a significant difference can be found in attributes such as TSS if compared with the regions around the fruit circumference (Peiris *et al*., 1999). The previous authors demonstrated variation in grapefruit regions by measuring TSS content from proximal to distal ends, around the fruit circumference and radial orientation, and found 10.2, 1.8 and 5.6%, respectively. The chemical composition of bigger fruit such as pineapple and melons has been reported to vary from sun exposed to shaded sides of a single fruit. For example, in the study conducted by Guthrie and Walsh (1997) on

pineapples, SSC was consistently 1 °Brix higher on the sun exposed side compared with the shaded side. It was further suggested that citrus fruit acquisition should be around the fruit circumference in order to best represent the fruit characteristics (Magwaza *et al*., 2012). However, the chemical composition of the region around the circumference of the fruit may not be applicable when investigating certain disorders such as stem end pitting of citrus. In such cases, it is involuntary that certain spectra are obtained from the stylar end of the fruit.

2.8 Chemometrics

Spectral preprocessing

Preprocessing techniques are normally applied to spectra for removing useless or extracting useful information. Spectral pre-processing methods are used to sample set and spectral data, for removal of system error and data smoothing. Savitzky-Golay polynomial smoothing and moving smoothing are the main smoothing types usually applied for de-noising of spectra. Spectral resolution is increased by using derivatives (usually first and second). Fourier Transform compresses the principal information from dataset and enhances the ratio of signal to noise in order to improve the signal quality. Another transformer employed is the wavelet, which works similar to Fourier except that it has window width which varies with frequency resulting in faster data decomposition represented by wavelet coefficients. Multiple scatter correction (MSC), standard normal variate (SNV); orthogonal signal correction (OSC), and net analyte are other methods of data pre-processing available and employed by certain researchers. Lu *et al.* (2006) conducted a study using OSC, SNV and MSC to evaluate whether different techniques have different results. The authors obtained similar statistical parameters using all three methods and concluded that the preprocessing techniques have no effect on quality of prediction models.

A number of studies have used pre-processing before calibration and model development. However, some authors obtained better results without preprocessing. Preprocessing includes removal of certain information such as outliers and selection of wavelength range to use during predictions. The useful information may be removed together with useless information and result in models performance lower than when the preprocessing was not done. Xudong *et al*. (2009) and Wang *et al*. (2014) conducted a study investigating the importance of preprocessing and found that the results without it were better. In contrary, other studies conducted for the same investigation found preprocessing useful (Gomez *et al*., 2006; Liu *et al*., 2010a; Sànchez *et al.,* 2013). The inconsistency of performance among studies may lead to confusion in determining biological sample parameters using NIRS systems (Liu *et al*., 2015). It is, therefore, the choice of a researcher on what pre-processing techniques to use and whether to use them or not.

Model accuracy

The accuracy of Vis/NIRS models is measured by the value of the coefficient of determination (R) (Eq. 2), the root mean square error of calibration (RMSEC) (Eq. 3) and the root mean square error of prediction (RMSEP) (Eq. 4) (Liu *et al*., 2010b; Magwaza *et al*., 2012). An R-value is the measure of correlation between the calibrated and laboratory-based actual data. A good model should have a high R-value approaching 100% accuracy. An RMSEC is the error of calibration during the development of prediction formulae, while RMSEP is the error of the developed formulae to predict the validation data values. A good model should have low RMSEC and RMSEP with a small difference between the two errors. Another statistical parameter measuring the model accuracy is the bias (Eq. 5) (the average difference between predicted and measured values), which should be low for the higher precision of the model.

$$R = 1 - \sqrt{\frac{\sum(y_{cal} - y_{act})^2}{\sum(y_{cal} - y_{mean})^2}} \qquad (2)$$

$$\text{RMSEC} = \sqrt{\sum(y_{cal} - y_{act})^2/n} \quad (3)$$

$$\text{RMSEP} = \sqrt{\sum(y_{pred} - y_{act})^2/n} \quad (4)$$

$$\text{Bias} = \frac{1}{n}\sqrt{\sum(y_{pred} - y_{act})^2} \quad (5)$$

Where n = the number of spectra; y_{act} = the actual value; y_{mean} = the mean value; y_{cal} = the calculated value; y_{pred} = the predicted value of fruit attribute

Another important parameter commonly used to determine the models reliability is the residual predictive deviation (RPD), which is the ratio of standard deviation of the reference data for validation to RMSEP (Magwaza *et al*., 2013a). The RPD measures the model robustness based on three model reliability categories:

(1) excellent models, with RPD >2

(2) fair models, with 1.4 < RPD < 2

(3) non-reliable models, with RPD <1.4

However, statistical bases were not used in determining those thresholds and some researchers use different values (Bellon-Maurel *et al*., 2010).

Model robustness

Model robustness is the stability of the model to accurately predict across different external conditions. It can be lost when: (i) calibration models from another instrument are transferred to another instrument that responds

differently compared with the first instrument; (ii) When instrument response changes because of temperature fluctuations, electronic drift, and changes in wavelength or detector over time; and (iii) the samples belong to different batches (Nicolaï *et al.*, 2007). Fruit from different trees, locations, seasons, and different cultivars differ in their physico-chemical attributes and thereby, imposing difficulties for models to accurately measure the required parameters. Kawano (1998) conducted a study to test model robustness on kiwifruit of different sizes, different origins and different maturity stage. The author obtained higher validation errors when fruit were not categorized based on differences. Similar results were also obtained on the soluble solids content of apples (Peiris *et al.*, 2002) and melon (Guthrie *et al*., 2006). In order to increase model robustness, increasing the number of samples and involving samples with all possible difference during prediction model development is recommended. For example, using fruit from different orchards, different canopy, and different seasons may significantly reduce errors.

2.9 Vis/NIRS applications on citrus

Citrus chemical parameters such as soluble sugar content (SSC) and total acidity (TA) are dominated by C-H-O bonds that can be traced or quantified using Vis/NIRS. It is, therefore, possible to estimate their amount upon accurate calibration techniques and use of appropriate chemometrics. Vis/NIRS has been used in a variety of fruit and vegetables (For further reviews of NIRS application on fruit please refer to Nicolaï *et al*., 2007; Huang *et al*., 2008; Lin and Ying, 2009; Magwaza *et al*., 2012). In table 2, only Vis/NIRS applications on citrus will be highlighted. Vis/NIRS is used for measuring external and internal quality attributes, for discrimination and for defect determination. The system may be based in the laboratory, portal or in/on-line with grading and sorting lines.

Table 2: An overview of Vis/NIRS applications on citrus fruit

Variety	Acquisition mode	Application/attribute (s)	Reference
Mandarins	Transmittance	Sugar content	Kawano *et al.* (1993)
Tangerine	Reflectance	Drying internal disorder	Peiris *et al*. (1998)
Mandarins	Transmittance	Sugar content, acid content	Tsuchikawa *et al*. (2003)
Lemon	Transmittance	Defects, maturity	Kim *et al*. (2004)
Lemon	Transmittance	Sugar content	Lee *et al*. (2004)
Mandarins	Reflectance	Soluble sugar content, acidity, firmness	Gomez *et al.* (2006)
Orange	reflectance	Soluble solids	Cayuela (2008)
Orange	Reflectance	Soluble sugar content, total acidity	Cayuela and Weiland (2010)
Different citrus varieties	Reflectance	Oleocellosis	Zheng *et al*. (2010)
Different citrus varieties	Reflectance	Cluster analysis of citrus genotypes	Liao *et al.* (2013)
Orange	Reflectance	Weight, size, colour, texture, yield, chemical parameters	Sànchez *et al.* (2013)
Orange	Reflectance	Titratable acidity, Total soluble solutes, citrus colour index, mass and vitamin C	Magwaza *et al*. (2013b)
Mandarin	Reflectance	Rind breakdown disorder, Rind hue angle ($h°$), rind dry matter, non-structural carbohydrates	Magwaza *et al*. (2014)
Orange	Reflectance	Juice vitamin C content	Liu *et al*. (2015)
Orange and Grapefruit	Reflectance	Titratable acidity, Total soluble solutes, BrimA, Maturity index	Ncama *et al.* (2017)

2.10 Conclusion

The Vis/NIRS system can be used to predict internal/external characteristics of many fruits in time as short as a second. However, the challenge to researchers is to develop a standard application procedure and similar analytical chemometrics software. Different studies have used different Vis/NIRS settings such as pre-treatment techniques (others did not use any) for improving prediction, which makes the comparison unfair and causes loss of communication with other researchers. Vis/NIRS application has necessitated the idea of discriminating fruit based on pre-symptomatic indicators to develop rind disorders prior to shipping, which can save a lot of money for the fruit industry. Although Vis/NIRS has been used for decades in citrus studies, information of its application on predicting physiological rind disorders is limited. However, it shows a high capability of predicting citrus fruit attributes correlating to rind disorders, and the correlation of those attributes to disorders may be used to estimate the possibility of disorders. Future studies would greatly benefit the citrus industry by focusing on developing models that could be applied with grading and sorting on commercial lines to select fruit with high ability to survive cold storage and lower chances of developing disorders on shelves in the market. Fruit with lower chances of surviving cold shipping would then be processed and exported as processed products since those products will have extended shelf life.

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Chapter 3: Application of Vis/NIR spectroscopy for predicting sweetness and flavour parameters of 'Valencia' orange (*Citrus sinensis*) and 'Star Ruby' grapefruit (*Citrus* x *paradisi* Macfad)

3.1 Abstract

Sweetness and flavour are desirable attributes used for quality control and assurance of citrus fruit, which are largely determined by total soluble solids (TSS), titratable acidity (TA) and TSS: TA ratio. However, the accuracies of TSS, TA and TSS: TA as flavour indices have been recently criticized. BrimA (Brix minus acids), on the other hand, is an accurate organoleptic parameter that has been shown to be highly related to sweetness and flavour of citrus fruit. In this study, the ability of visible to near infrared spectroscopy (Vis/NIRS), in reflectance mode, to non-destructively quantify BrimA, TSS, TA and TSS: TA ratio of 'Valencia' orange and 'Star Ruby' grapefruit was evaluated. Vis/NIR spectral data was acquired using a laboratory bench-top monochromator NIR Systems. Reference measurements and spectral datasets were subjected to partial least square (PLS) regression analysis. The best prediction models were observed for BrimA of 'Valencia' oranges with the coefficient of determination (R^2) = 0.958; root mean square error of prediction (RMSEP) = 0.006 and residual predictive deviation (RPD) = 3.96, followed by TSS: TA ratio (R^2 = 0.958; RMSEP = 0.605; RPD = 4.92). Good models for predicting flavor of grapefruit were also attained, with TSS having the best model (R^2 = 0.896, RMSEP = 0.308 and RPD = 2.94), followed by BrimA (R^2 = 0.858; RMSEP = 0.429; RPD = 2.45). These results demonstrated the ability of Vis/NIRS to non-destructively predict sweetness and flavour attributes of oranges and grapefruit. Vis/NIRS was recommended as a possible fast and accurate technique to be used for fruit discrimination based on flavour parameters during packing and for pricing of fruit in the market.

Keywords: BrimA, Total soluble solids, Maturity index, Citrus flavour, near infrared spectroscopy

3.2 Introduction

Citrus fruit quality assessment by consumers is highly based on external appearance, which influences the decision to purchase. The decision for subsequent purchases is governed by the certainty that the visual appearance of purchased citrus fruit will be matched by a rewarding sensory experience at the time of consumption. However, visual appearance is not always related to biochemical properties responsible for sweetness and flavour. Therefore, quantification of internal sensory and organoleptic quality is of great importance. Commercially, organoleptic quality of citrus fruit is currently evaluated by measuring total soluble solids (TSS), titratable acidity (TA), and TSS: TA ratio which contributes to sweetness and flavour (Chen and Opara, 2013; Magwaza and Opara, 2015).

Total soluble solids content, usually measured from extracted juice using a refractometer, is currently the most important quality parameter to indicate sweetness of citrus fruit used by the industry to determine marketing standards (Kader, 2002). However, in citrus fruit, sugars contribute between 75 and 85% of TSS. The rest of TSS is contributed by acids, together with small amounts of dissolved vitamins, fructans, proteins, pigments, phenolic compounds, and minerals (Magwaza and Opara, 2015). Therefore, it is not surprising that TSS is not always aligned with consumer perception of sensory sweetness because TSS alone does not have practical importance regarding consumer perception of citrus fruit sweetness.

Titratable acidity is another factor affecting consumer perception of taste in citrus fruit. TSS, TA and their ratios are not static, but vary considerably during fruit maturation and ripening. The combination of these attributes is commonly used as laboratory and commercial indicators of maturity and flavour of citrus fruit (Genizi and Cohen, 1988; Kader, 1999, 2008a, b). Furthermore, TSS: TA ratio is currently used as a maturity and flavour index for citrus fruit. However, it has been recognized that this measurement does not always

correlate well with the perception of sweetness or tartness of citrus fruit (Baldwin *et al*., 1998; Jordan *et al*., 2001; Obenland *et al*., 2009). One difficulty with this index is that the same ratio may be derived from different concentrations of TSS and TA, leading to different flavour perceptions for the same ratio.

The necessity for correlating TSS, TA, and TSS: TA ratio to sensory flavour and to include alternatives, such as subtracting acidity has recently been recognized. As a result, Jordan *et al*. (2001) developed and introduced a sweetness (or maturity) index called BrimA (pronounced bree-mah) as an alternative to TSS:TA ratio. BrimA (an abbreviation for Brix minus Acids) measures the balance between Brix (sweetness) and acidity (sourness). Studies by Jordan *et al*. (2001) and Obenland *et al*. (2009) demonstrated that the hedonic flavour and sweetness score of citrus fruit was more closely related to BrimA than TSS: TA ratio. Since it compensates for acids, BrimA would be a better indicator of sweetness and quality and may better align with a prediction of human perception of good taste.

The sweetness and flavour of citrus fruit are commonly quantified by instrumental assessment and sensory evaluation including taste panels (Genizi and Cohen, 1988; Shewfelt, 2009). The procedures currently used to measure the sensory parameters are considered to be time-consuming, not cost effective and labour intensive due to sample pre-treatment and the need for expensive chemicals. In addition, consumers nowadays are capable of distinguishing sensory attributes with a high degree of sensitivity and hence, demanding better quality and consistent supply of quality produce with appropriate taste (Jamshidi *et al*., 2012). Therefore, there is much incentive linked to using non-destructive methods to sort and grade fruit based on their internal quality for the fresh market, which leads to an increase in profit margins for the industry, through price differentiation for different grades (Mendoza *et al*., 2014).

Among non-destructive techniques for measuring fruit quality, visible to near infrared spectroscopy (Vis/NIRS) has been used successfully to measure internal quality of different varieties of citrus fruit (Gómez

et al., 2006; Liu *et al*., 2010 a, b; Cayuela and Weiland, 2010; Magwaza *et al*., 2013). Although work on non-destructive methods to measure quality using Vis/NIR-based systems has led to commercial use in a packing line to select fruit with acceptable flavour quality, there is a need for continued development of non-destructive sensing of flavor quality. This should include the use of Vis/NIRS to estimate concentrations of sweetness and flavour related parameters. A recent review of literature by Magwaza and Opara (2015) also suggested that further research is needed to investigate the use of Vis/NIR spectroscopy for determination of taste attributes (TSS and TA) and to assess the feasibility of using the technique for non-destructive prediction of taste indices such as TSS:TA ratio and BrimA. Therefore, in this study, the capability and conditions of visible to near infrared spectroscopy to non-destructively quantify BrimA, TSS, TA and TSS: TA ratio of intact 'Valencia' orange and 'Star Ruby' grapefruit was evaluated.

3.3 Materials and methods

Fruit samples

The research was carried out during 2014/15 season using 'Valencia' orange (*Citrus sinensis*) and 'Star Ruby' grapefruit (*Citrus x paradisi* Macfad). A total of 120 grapefruit and 120 oranges were randomly picked, mixing all possible sizes of mature fruit, from two orchards at Olifant's River Estates, a commercial citrus farm, located at Hoedspruit in Limpopo Province, South Africa (24°23'39.02"S; 30°49'20.65"E). Harvested fruit were transported in ventilated vehicle to the Postharvest Technology Laboratory of the University of KwaZulu-Natal where experiments for Vis/NIRS and destructive analyses were performed. Upon arrival at the laboratory, fruit were equilibrated at room temperature (21±1 °C; 65±1% RH) for 24 h before Vis/NIR spectra was acquired.

Vis/NIR spectroscopy collection

Vis/NIR spectral data was acquired in reflectance mode using a laboratory bench-top monochromator NIR Systems Model XDS spectrometer (FOSS NIR Systems, Inc.; Maryland, USA) equipped with a quartz halogen lamp and lead sulfide (PbS) detector. To reduce baseline shift of spectral data, the system was calibrated by scanning a 100% white reference tile prior fruit scanning and consistently after every 30 min interval. The visible to near infrared reflectance spectrum ranging from 450 to 2500 nm was acquired from two opposite sides along the fruit equator and recorded as log 1/reflectance (log 1/R). The scanned spots were marked A or B. Each spectrum was the average of 32 scans recorded using Vision software (Vision TM, version 3.5.0.0, Tidestone Technologies Inc., KS, USA).

Destructive TSS and TA analysis

The same set of fruit was used for destructive measurements after acquiring Vis/NIR spectra. Each fruit was peeled and the juice collected from the marked spots where spectra were collected to avoid variation of internal parameters within the fruit (Peiris *et al.*, 1999). The collected juice was homogenized using a stirrer (ULTRA-TURRAX, IKA® T25 digital, Staufen, Germany) and tested for TSS using a digital refractometer with a thermodynamic control system (RFM340+ refractometer, Bellingham and Stanley Ltd, Basingstoke, Hants, UK). TSS was recorded as °Brix which is equivalent to TSS %. The remaining juice was snap frozen in 120 mL plastic specimen jars and stored in a freezer at -20 °C for further analysis of titratable acids.

TA analysis was carried out by mixing 10 mL juice with 50 mL distilled water and titration with 0.1 M sodium hydroxide (NaOH) to the end point (pH of 8.1). The titre was recorded and the acid formula for citrus fruit samples (Eq. 1) was applied to calculate TA, expressed as % citric acid.

$$TA\ (\%\text{citric acid}) = \frac{0.0064 \times \text{titre (NaOH) mL}}{10\ \text{mL (juice)}} \times 100 \quad (1)$$

The ratio of TSS to TA, also known as maturity index was calculated using Eq. 2.

$$\textit{TSS to TA ratio (maturity index)} = \frac{\text{TSS}}{\text{TA}} \quad (2)$$

BrimA, an index that measures the balance between sweetness and acidity was calculated using Eq. 3 described by Jordan et al. (2001).

$$\text{BrimA} = \text{TSS} - k\ (\text{TA}) \quad (3)$$

Where, *k* is a constant that reflects the tongue's higher sensitivity to TA compared to TSS. The k constant allows TA amounts smaller than TSS to make the same numerical change to BrimA. The BrimA equation (Eq. 3) was modified as suggested by Obenland *et al*. (2009) who replaced the value of the constant (*k*) of 5 by Jordan *et al*. (2001) with 3 and 4 in order to eliminate the generation of negative BrimA values for oranges. In the current study, all three constant (*k*) values of 3, 4, and 5 were tested and are referred to as BrimA3, BrimA4 and BrimA5, respectively.

Statistical analysis

Statistical analyses of reference and predicted fruit properties were carried out using GenStat statistical software (GenStat®, 14th edition, VSN International, UK). The coefficient of variation (CV), defined as the

ratio of the standard deviation to the mean of the reference values was calculated, multiplied by 100 and reported as a percentage (Magwaza *et al*., 2014a).

Chemometric analysis

The chemometric analysis was performed using Vision software (Vision® Single and Multi-User ("S/M") Version 3.5.0.0, FOSS NIRSystems, Inc.; Maryland, USA). Two individual spectra from each sample were averaged prior to calibration and validation, hence, results reported herein are based on average spectra. The spectral data were first subjected to principal component analysis (PCA) to compare spectral characteristics, using leave-out-one cross validation, to determinate effective wavelengths and detect spectral outliers (Magwaza *et al*., 2014b).

Furthermore, partial least square (PLS) regression models were developed from the collected spectra using test set validation with 50 samples used for calibration, 40 for validation and 30 used for external validation. The 30 samples used for external validation and to test validation were harvested from another orchard on the same farm. The spectra collected were converted to absorbance values (log 1/R) to obtain a linear correlation between the spectrum and the sample molecular concentration (Miller and Zude-Sasse, 2004). Several pre-processing methods were applied including no preprocessing, Standard Normal Variate (SNV) and Smoothing using Savitzky-Golay with segment size of seven for averaging at 1^{st} and 2^{nd} polynomial order of derivative were tested with different spectrum ranges and evaluated for model performance. These spectral pre-processing methods were applied to correct baseline shift, correct for light scatter, reduce changes in light path length and smooth spectral data prior to regression. Calibration models were selected based the spectral pre-processing which provided the lowest root mean square error of prediction (RMSEP).

Spectral outliers were identified using sample residual variance and leverage on PCA spectral data exploration and PLS regression exercises (Shiroma and Rodriguez-Saona, 2009; Magwaza *et al*., 2014a, b). Samples in the dataset with large residual and/ or high leverage and located far from the zero line of the residual variance plot were identified and recorded as potential outliers. A more stringent Hotelling T^2 ellipse statistics was used as a diagnostic tool for confirming outlier values of samples (Magwaza *et al*., 2014a). Only one and two spectral outliers were recognised and removed from orange and grapefruit datasets, meaning that samples for analysis were reduced to 119 and 118, respectively. In the current study, the number of factors to be included in the models was determined as the minimum number of factors corresponding to the first lowest value of residual *X*-variance from the plot of the residual *X*-variance for increasing a number of factors (Davey *et al*., 2009).

The performances of calibration models were assessed based on getting the lowest root mean square error of calibration (RMSEC) (Eq. 4), highest coefficient of determination for calibration (R^2_c) (Eq. 5). The ideal prediction model was determined by having the lowest RMSEP (Eq. 6) and the measure of favour in samples evaluation (bias) (Eq. 7) while having higher coefficient of determination for validation (R^2_v) and residual predictive deviation (RPD) (Sun *et al*., 2009; Liu *et al*., 2010a, b; Bobelyn *et al*., 2010). RPD values of the prediction models were calculated using Eq. 8 and rated according to Chang *et al.* (2001) in which three quality categories were defined to account for the model's reliability, with excellent models having RPD > 2, fair models having 1.4 < RPD < 2 and non-reliable models having RPD < 1.4.

$$RMSEC = \sqrt{\sum (y_{cal} - y_{act})^2 / n} \quad (4)$$

$$R^2 = 1 - \frac{\sum (y_{cal} - y_{act})^2}{\sum (y_{cal} - y_{mean})^2} \quad (5)$$

$$RMSEP = \sqrt{\sum (y_{pred} - y_{act})^2 / n} \quad (6)$$

$$Bias = \frac{1}{n}\sqrt{\sum (y_{pred} - y_{act})^2} \quad (7)$$

$$RPD = \frac{\mathrm{SD}}{\mathrm{RMSEP}} \quad (8)$$

Where, n is the number of fruit samples used in calculation; y_{act} is the actual value measured by a destructive method; y_{mean} is the average value of predicted data; y_{pred} is the Vis/NIRS predicted the value of fruit parameter and SD is the standard deviation of reference data values.

3.4 Results and discussion

Optimum chemometric conditions for calibration models

As a common practice in Vis/NIRS, different pre-treatment techniques were evaluated before calibration, to determine the one that will lead to better model accuracy. Table 1 summarizes results obtained using different spectral pre-treatments and wavelength region combinations, as well as the statistics used for selecting the most accurate model during calibration for predicting quality parameters of 'Valencia' oranges. For example, models for predicting TSS in 'Valencia' oranges using spectra without pre-processing had poor prediction accuracy (R^2 = 0.747, RMSEP = 0.527 and RPD = 1.73) compared to that of model calibrated using Savitzky-Golay second derivative spectral pre-processing with a second order polynomial which had the lowest RMSEP (0.283) and highest R^2–value (0.927) and RPD (3.57). Therefore, Savitzky-Golay second derivative was chosen for all calibration models in this study (Table 1). Consistently, Savitzky-Golay second derivative with second order polynomial showed superior results for both cultivars, possibly because this pre-processing corrected light scattering properties of the fruit (Magwaza *et al*., 2012). These results confirm the importance of spectral

pre-processing when analysing quality parameters of intact fruit samples which are characterised by scattering properties. Wavebands at the beginning and end of the spectra were characterised by noise, hence, were removed from the original spectra.

The full spectral range, including both visible and NIR region of the electromagnetic spectrum (400-2500 nm) was compared with the NIR region (850-2500 nm) to select the optimal range for predicting each of the quality parameters. Models developed using the NIR spectral region from 850-2500 nm performed better than those based on the region that included visible range (Table 1). For example, 'Valencia' orange TSS prediction using the model developed with the 450-2500 nm range without preprocessing was poor (R^2 = 0.659; RMSEP = 0.668; RPD = 1.22) when compared with prediction using the model developed using the 850-2500 nm range without preprocessing (R^2 = 0.747; RMSEP = 0.527; RPD = 1.73). The results were consistent across all predicted parameters. Since intact fruit were scanned, the deduction made was that there was high noise effect in the 450-800 nm wavelength range. This is probably due to thick citrus peel interfering with spectral information for internal quality parameters and attributing to noise in the visible range (Magwaza *et al*., 2013; Wang *et al*., 2014; Liu *et al*., 2015). The low predictability of models developed using the visible range may also suggest limited penetration depth in this region of the spectrum (Lammertyn *et al*., 2000). The results are in contradiction with Fraser *et al.* (2001) who got that the penetration was greatly reduced at higher wavelengths (1400–1600 nm) due to water absorption, and concluded that this range is not capable of investigating parameters in the flesh greater than 1 mm into the fruit.

Table 1: The PLS models developed for comparing the combinations of pre-processing methods and wavelength region on 'Valencia' orange parameters during calibration.

Quality attribute	Pre-proc.	R^2	RMSEP	Bias	Factor	Mean	SD pred	SD ref	CV% pred	CV% ref	RPD	Wavelength region (nm)
TSS	None	0.66	0.668	0.063	10	10.24	1.05	0.81	10.29	7.94	1.22	450 – 2500
	None	0.75	0.527	0.051	9	10.24	1.05	0.91	1.29	8.91	1.73	856 – 2292
	SNV	0.70	0.577	0.055	8	10.24	0.88	1.05	8.61	8.60	1.82	450 – 2500
	SNV	0.69	0.581	0.055	10	10.24	1.05	0.88	10.29	8.57	1.51	856 – 2292
	SG-1st Der	0.81	0.459	0.044	9	10.24	0.95	1.05	10.29	9.25	2.30	456 – 2492
	SG-1st Der	0.90	0.331	0.031	9	10.24	1.00	1.05	9.76	8.68	3.18	856 – 2292
	SG-2nd Der	0.91	0.314	0.031	9	10.24	1.01	1.05	9.20	9.64	3.36	456 – 2492
	SG-2nd Der	**0.93**	**0.283**	**0.027**	**9**	**10.24**	**1.05**	**1.01**	**10.29**	**9.91**	**3.57**	**856 – 2292**
TA	None	0.54	0.045	0.004	10	0.66	0.05	0.07	7.28	9.95	1.47	450 – 2500
	None	0.47	0.045	0.004	10	0.66	0.05	0.07	7.28	9.95	1.47	850 – 2500
	SNV	0.53	0.045	0.004	10	0.66	0.05	0.07	7.26	9.95	1.47	450 – 2500
	SNV	0.56	0.897	0.085	9	0.66	1.05	0.07	15.29	9.95	0.07	850 – 2500
	SG-1st Der	0.65	0.039	0.004	9	0.66	0.05	0.07	8.01	9.95	1.69	456 – 2492
	SG- 1st Der	0.80	0.029	0.003	8	0.66	0.06	0.07	8.91	9.95	2.28	856 – 2292
	SG-2nd Der	0.83	0.024	0.002	9	0.66	0.06	0.07	9.24	9.95	2.75	456 – 2492
	SG- 2nd Der	**0.93**	**0.017**	**0.002**	**9**	**0.66**	**0.06**	**0.07**	**9.58**	**9.95**	**3.88**	**856 – 2292**
BrimA3	None	0.50	0.953	0.091	12	8.18	0.96	1.36	11.75	16.58	1.42	450 – 2500
	None	0.55	0.996	0.095	12	8.18	0.92	1.36	11.21	16.58	1.36	850 – 2500
	SNV	0.46	0.996	0.095	**9**	8.18	0.92	1.36	11.21	16.58	1.36	450 – 2500
	SNV	0.63	1.467	0.141	8	8.18	1.07	1.36	13.12	16.58	0.93	850 – 2500
	SG- 1st Der	0.68	0.762	0.073	10	8.18	1.12	1.36	13.69	16.58	1.78	456 – 2492
	SG-1st Der	0.85	0.592	0.051	10	8.18	1.25	1.36	15.25	16.58	2.29	856 – 2292
	SG- 2nd Der	0.82	0.568	0.054	9	8.18	1.23	1.36	15.04	16.58	2.39	456 – 2492
	SG- 2nd Der	**0.96**	**0.281**	**0.027**	**9**	**8.18**	**1.33**	**1.36**	**16.22**	**16.58**	**4.83**	**856 – 2292**
BrimA4	None	0.43	1.027	0.098	9	7.52	0.90	1.37	11.95	18.21	1.33	450 – 2500
	None	0.55	0.909	0.087	9	7.52	1.02	1.37	13.55	18.21	1.5	850 – 2500
	SNV	0.46	1.001	0.096	9	7.52	0.93	1.37	12.32	18.21	1.37	450 – 2500
	SNV	0.56	0.908	0.087	9	7.52	1.01	1.37	13.56	18.21	1.51	850 – 2500
	SG-1st Der	0.68	0.765	0.073	9	7.52	1.13	1.37	15.05	18.21	1.79	456 – 2492
	SG-1st Der	0.45	1.014	0.097	8	7.52	0.91	1.37	12.15	18.21	1.35	856 – 2292
	SG- 2nd Der	0.82	0.511	0.049	8	7.52	1.27	1.37	16.87	18.21	2.68	456 – 2492
	SG- 2nd Der	**0.96**	**0.276**	**0.026**	**8**	**7.52**	**1.09**	**1.08**	**14.34**	**14.23**	**3.92**	**856 – 2292**
BrimA5	None	0.46	1.015	0.097	9	6.85	0.93	1.38	13.62	13.62	1.36	450 – 2500
	None	0.56	0.916	0.088	9	6.85	1.03	1.38	15.05	20.17	1.51	850 – 2500
	SNV	0.51	0.961	0.092	9	6.85	0.99	1.38	14.44	20.17	1.44	450 – 2500
	SNV	0.56	0.917	0.088	9	6.85	1.03	1.38	15.03	20.17	1.51	850 – 2500
	SG-1st Der	0.38	1.351	0.129	9	6.85	0.85	1.38	12.41	20.17	1.02	456 – 2492
	SG-1st Der	0.85	0.535	0.051	8	6.85	1.27	1.38	18.58	20.17	2.58	856 – 2292
	SG- 2nd Der	0.83	0.575	0.055	8	6.85	1.26	1.38	18.32	20.17	2.4	456 – 2492
	SG- 2nd Der	**0.96**	**0.277**	**0.027**	**8**	**6.93**	**1.10**	**1.10**	**15.93**	**15.81**	**3.96**	**856 – 2292**
TSS: TA	None	0.21	2.239	0.214	14	15.48	1.17	2.53	7.52	16.37	1.13	450 – 2500
	None	0.54	1.716	0.164	15	15.48	1.86	2.53	12.01	16.37	1.48	850 – 2500
	SNV	0.51	1.765	0.169	10	15.48	1.81	2.53	11.71	16.37	1.44	456 – 2500
	SNV	0.54	1.708	0.164	10	15.48	1.87	2.53	12.05	16.37	1.48	850 – 2500
	SG- 1st Der	0.69	1.402	0.134	10	15.48	2.11	2.53	13.61	16.37	1.81	456 – 2492
	SG- 1st Der	0.84	0.999	0.096	9	15.48	2.33	2.53	15.03	16.37	2.54	856 – 2292
	SG- 2nd Der	0.83	1.031	0.099	9	15.48	2.31	2.53	14.95	16.37	2.46	456 – 2492
	SG- 2nd Der	**0.96**	**0.515**	**0.049**	**9**	**15.48**	**2.48**	**2.53**	**16.02**	**16.37**	**4.92**	**856 – 2292**

Pre-proc, preprocessing method, TSS, total soluble solids; TA, titratable acidity; BrimA3, 4, 5, BrimA with k = 3, 4 and 5 respectively; None, predicted without pre-processing; R^2, correlation coefficient between Vis/NIRS predicted and measured values; SNV, standard normal variate; SG-1st Der, Savitzky-Golay with 1st order of derivative; SG-2nd Der, Savitzky-Golay with 2nd order of derivative; SD pred, Standard deviation of predicted data values; SD ref, standard deviation of reference data values; Mean, mean of predicted values; CV% pred, the coefficient of variation of predicted data; CV% ref, the coefficient of variation of reference data; RPD, residual predictive deviation.

The number of factors used during calibration and to evaluate model prediction was determined by the log of Prediction Residual Error minus Sum of Squares (PRESS). A typical example of factor selection method used is presented in Fig. 1 where a log (PRESS) for oranges BrimA3, using factor 1 resulted in lower correlation co-efficient (R^2), lower loadings intensity and higher log (PRESS) compared with using factor 10. The log (PRESS) is determined by the loadings intensity of a factor or the wideness of the factor curve in Fig. 2. Factor 10 is normally used under a similar condition since it had the lowest log (PRESS) compared with any other factors and higher R^2 -value when compared with factor 1 to factor 9. Increasing the loadings intensity increases the model accuracy since the wide curve will be able to accommodate most important amplitude it will be validating during prediction. This satisfied the common rule that states that samples used to develop a Vis/NIR model should be similar to those the model will be used to predict (Cozzolino *et al*., 2011, Magwaza *et al*., 2014a). Using factor 1 during calibration for predicting grapefruit BrimA3 with Savitzky-Golay second order of derivative in 856-2292 nm range of spectrum gave an R^2–value of 0.048, RMSEP = 6.245 and Bias = 0.373, while using factor 10 gave an R^2 – value of 0.957, RMSEP = 0.281 and Bias = 0.027. The results consistently agreed on the fact that choosing the factor with the lowest log (PRESS), when there is significant fluctuation of log (PRESS) along the factors, increases predicting accuracy of the model.

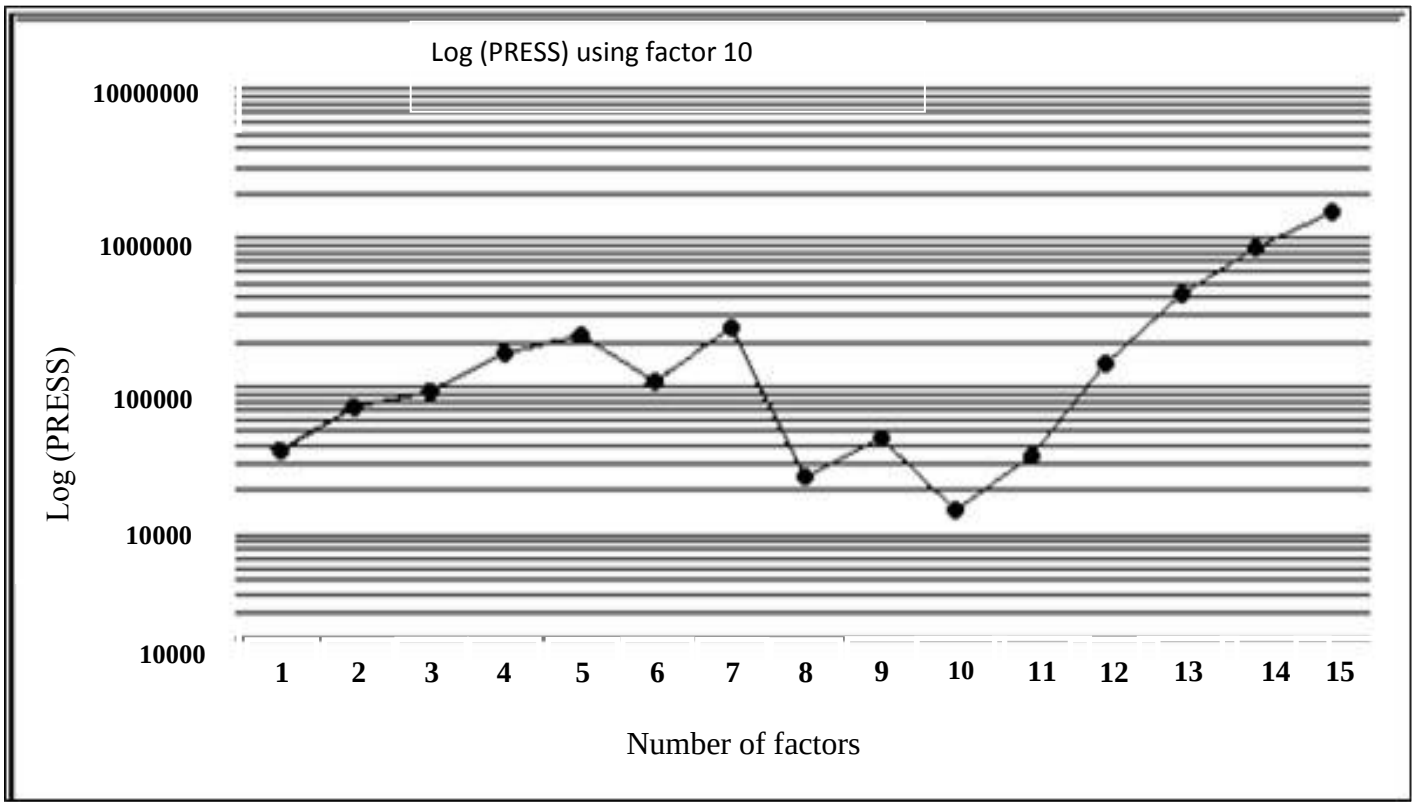

Fig. 1: The log (PRESS) that determined the factor to use on predicting BrimA3 of 'Valencia' oranges. Factor 10 was used since it had the lowest log (PRESS).

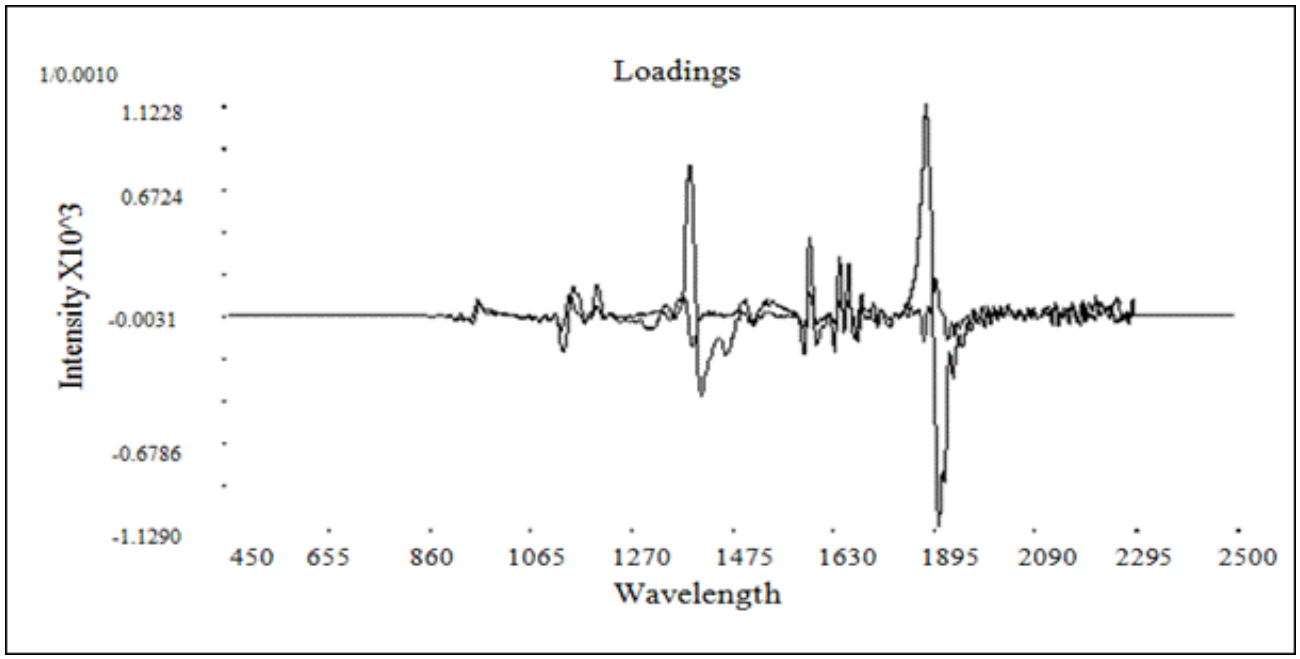

Fig. 2: The factor-depended loadings intensity that determined the factor to use when predicting with a model. Factor 1 had narrow loadings intensity compared with factor 10.

Ideal models for predicting sweetness and flavour parameters of oranges and grapefruit

The prediction statistics obtained from best models developed for all sweetness and flavour quality parameters of both 'Valencia' oranges and 'Star Ruby' grapefruit are presented in Tables 2 and 3, respectively. For 'Valencia' oranges, TSS: TA ratio model was the most accurate (R^2 = 0.958; RMSEP = 0.605; RPD = 4.92) followed by BrimA5 (R^2 = 0.958; RMSEP = 0.006; RPD = 3.96), BrimA3 (R^2 = 0.957; RMSEP = 0.011; RPD = 4.83), BrimA4 (R^2 = 0.957; RMSEP = 0.006; RPD = 3.92), TA (R^2 = 0.929; RMSEP = 0.017; RPD = 3.88) and TSS (R^2 = 0.927; RMSEP = 0.283; RPD = 3.57) (Table 2). Scatter plots of the relationships between conventionally measured quality parameters and those predicted using NIR models developed for 'Valencia' oranges are presented in Fig. 3. High regression co-efficient between NIR predicted and measured sweetness and flavour parameters were observed.

The mean of all tested quality parameters for oranges was predicted accurately (Table 2). The ease of the mean prediction was correlated to a slight difference between the samples within the batch. The standard deviation of predicted data was slightly lower than that of the actual parameters values for TA, TSS: TA ratio and BrimA3, which meant that the predicted values were closer to the mean when compared with the reference data. It was a good trend because predicted samples deviated normally about the mean, which meant there were very low chances of predicting a sample as an outlier if its actual value was close to the mean. Moreover, the change in SD after prediction may affect the skewness of the data and result in bias statistical parameters such as RPD (Bellon-Maurel *et al*., 2010). All parameters for the orange had RPD values above 3.5 and were considered excellent when rated according to the scale by Chang *et al*. (2001). The predicted CV% was smaller than the actual CV% for TA, TSS: TA ratio and BrimA3. The ability of the prediction models to maintain the coefficient of variation was vital and indicated that the prediction was closer to the actual values. The Bias values of all calibration models were below 1, showing a higher degree of prediction models robustness.

Table 2: The statistics of the values predicted by models chosen based on higher prediction of 'Valencia' orange parameters.

Parameter	Mean Pred	Mean ref	R^2	SD $_{pred}$	SD $_{ref}$	CV% $_{pred}$	CV% $_{ref}$	RPD	RMSEC	RMSEP	Bias
TSS	10.24	10.24	0.93	1.05	1.01	10.29	9.91	3.57	0.28	0.28	0.03
TA	0.66	0.66	0.93	0.06	0.07	9.58	9.95	3.88	0.02	0.02	0.00
TSS:TA	15.48	15.48	0.96	2.48	2.53	16.02	16.37	4.92	0.52	0.61	0.05
BrimA3	8.18	8.18	0.96	1.33	1.36	16.22	16.58	4.83	0.28	0.01	0.03
BrimA4	7.52	7.52	0.96	1.09	1.08	14.34	14.23	3.92	0.28	0.01	0.03
BrimA5	6.93	6.93	0.96	1.10	1.10	15.93	15.81	3.96	0.28	0.01	0.03

TSS, total soluble solutes; TA, titratable acidity; Mean $_{pred}$, mean of predicted data; Mean $_{ref}$, mean of reference data values; R^2, correlation coefficient between Vis/NIRS predicted and measured values; SD $_{pred}$, Standard deviation of predicted data values; SD $_{ref}$, standard deviation of the reference data; RPD, residual predictive deviation; CV% $_{ref}$, the coefficient of variation for the reference data; CV% $_{pred,}$ the coefficient of variation for the predicted data; BrimA3,4,5, BrimA with k = 3,4 and 5 respectively; RPD, residual predictive deviation; RMSEC, root mean square error of calibration; RMSEP, root mean square error of prediction.

Furthermore, other authors such as Lu *et al*. (2006) and Liu *et al*. (2010b) successfully predicted SSC from intact 'Gannan' citrus fruit (R = 0.99; RMSEP = 0.79 °Brix) using the range of 350-1800 nm and predicted TA (R^2 = 0.92; RMSEP = 0.65) on 'Nanfeg' mandarin fruit using the range of 350-1040 nm, respectively. The previous results and results of this study were hypothesized to demonstrate that internal quality parameters of citrus fruit are less likely to be directly detected in the visible range. Their prediction in the spectral region including the visible range is likely to represent the secondary correlation of rind quality attributes related to internal quality.

For 'Star Ruby' grapefruit, a different trend was observed where TSS model showed best prediction (R^2 =0.896; RMSEP = 0.308; RPD = 2.94) followed by BrimA3 (R^2 = 0.858; RMSEP = 0.429; RPD = 2.68); BRIMA4 (R^2 = 0.846; RMSEP = 0.411; RPD = 2.45); TA (R^2 = 0.835; RMSEP = 0.066; RPD = 2.46); TSS:

TA ratio (R^2 = 0.812; RMSEP = 0.451; RPD = 2.32) and BrimA5 (R^2 = 0.810; RMSEP = 0.498; RPD = 2.19) (Table 3). In Fig. 4, the values for TSS, TA, TSS: TA ratio, and BrimA values of grapefruit predicted using NIR are plotted against the destructively analyzed values. The prediction of BrimA was hypothesized to be increased by increasing the accuracy of predicting TSS and TA, of which that accuracy was not very high on the grapefruit in this study. BrimA3 and BrimA5 showed better predictability when compared with BrimA4 on grapefruit and orange respectively, which was of high interest because other than developing the models, the study also aimed at evaluating the citrus BrimA constant to use during calculations for development of Vis/NIRS models.

The mean of all grapefruit parameters was also predicted with significant accuracy (Table 3). The standard deviation of predicted data was lower than SD of reference data for all parameters. A similar trend also occurred on CV% except for TSS, which showed that there was a wider deviation after predictions for TSS compared to the deviation of the reference data. That was bad as the predicted data should fall within the actual variation to be considered valuable. On the RPD perspective, the order of the grapefruit models performance could be ranked as TSS > BrimA3 > TA > BrimA4 > TSS: TA ratio > BrimA5. The overall results demonstrated higher chances of predicting BrimA than predicting TSS: TA ratio, which is good considering the critics of using the ratio for estimating the citrus flavor. The Bias of predictability was very low for all grapefruit parameters (< 0.03), indicating the high robustness of the models.

The reason for the lack of excellence in the predictability of the grapefruit parameters was correlated to TSS, TSS: TA ratio and BrimA mainly referring to the fruits' sweetness, which is not a characteristic flavour of grapefruit. Moreover, the thick rind of grapefruit was also related to complications of collecting ideal spectra reflecting internal parameters. Citrus peel is known to reduce the light penetration, which complicates the assessment of internal parameters. Working on 'Satsuma' mandarin, Fraser *et al*. (2003) discovered that there is a rapid reduction in light level across the thick illuminated skin and a less rapid but still high reduction occurs

as the light continues to diffuse into the flesh. In this study, the path taken by radiation into the grapefruit flesh and reflected back to the detector may have been too long and hence, decreased the accuracy of the obtained spectra as the radiation would have lost important information due to other particles such as seeds, pith and voids within the fruit causing perturbation (Wang *et al*., 2014). Reflectance mode on fruits with thick rind such as grapefruit is highly suitable for prediction of rind characteristics rather than internal parameters since the path taken by radiation would be significantly reduced and can, therefore, be reflected back to the detector without losing important information (Nicolaï *et al*., 2007).

Table 3: Statistics of the values predicted by models chosen based on higher prediction accuracy of 'Star Ruby' grapefruit parameters.

Parameter	Mean $_{Pred}$	Mean $_{Ref}$	R^2	SD $_{pred}$	SD $_{ref}$	CV% $_{pred}$	CV% $_{ref}$	RPD	RMSEC	RMSEP	Bias
TSS	10.32	10.32	0.90	0.91	0.96	9.28	8.79	2.94	0.31	0.31	0.02
TA	1.43	1.43	0.84	0.15	0.16	10.40	13.66	2.46	0.07	0.07	0.00
TSS:TA	7.31	7.31	0.81	0.94	1.04	12.84	14.25	2.32	0.45	0.45	0.03
BrimA3	6.04	6.04	0.86	0.90	0.97	14.93	16.13	2.68	0.36	0.43	0.02
BrimA4	4.61	4.61	0.85	0.97	1.05	20.94	22.77	2.45	0.41	0.41	0.03
BrimA5	3.22	3.22	0.81	0.97	1.08	30.24	33.40	2.19	0.50	0.50	0.03

TSS, total soluble solutes; TA, titratable acidity; BrimA3, 4, 5, BrimA with k = 3, 4 and 5 respectively; Mean $_{pred}$, mean of predicted data; Mean $_{ref}$, mean of reference data values; R^2, correlation coefficient between Vis/NIRS predicted and measured values; SD $_{pred}$, Standard deviation of predicted data values; SD $_{ref}$, standard deviation of the reference data; RPD, residual predictive deviation; CV% $_{ref}$, the coefficient of variation for the reference data; CV% $_{pred,}$ the coefficient of variation for the predicted data; RPD, residual predictive deviation; RMSEC, root mean square error of calibration; RMSEP, root mean square error of prediction.

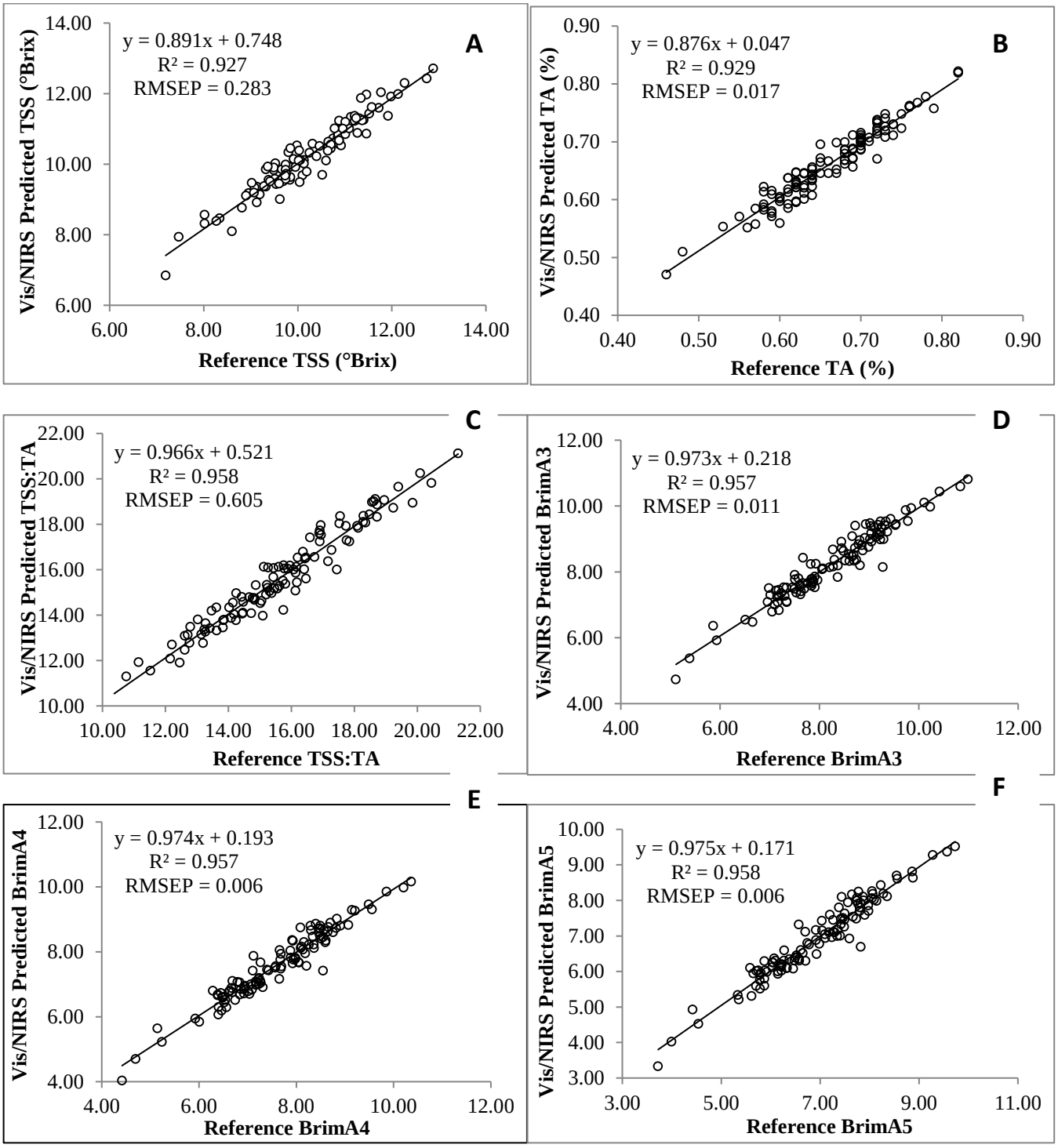

Fig. 3: Scatter plot of Vis/NIRS predicted versus measured or calculated values of 'Valencia' oranges Total Soluble Solutes (TSS) (A), Titratable Acidity (TA) (B), TSS: TA ratio (C), BrimA with k = 3(BrimA3) (D), BrimA4 (E) and BrimA5 (F). RMSEP, root mean square error of prediction; R^2, the regression coefficient of Vis/NIRS predicted data to reference data; Vis/NIRS, visible to near infrared spectroscopy.

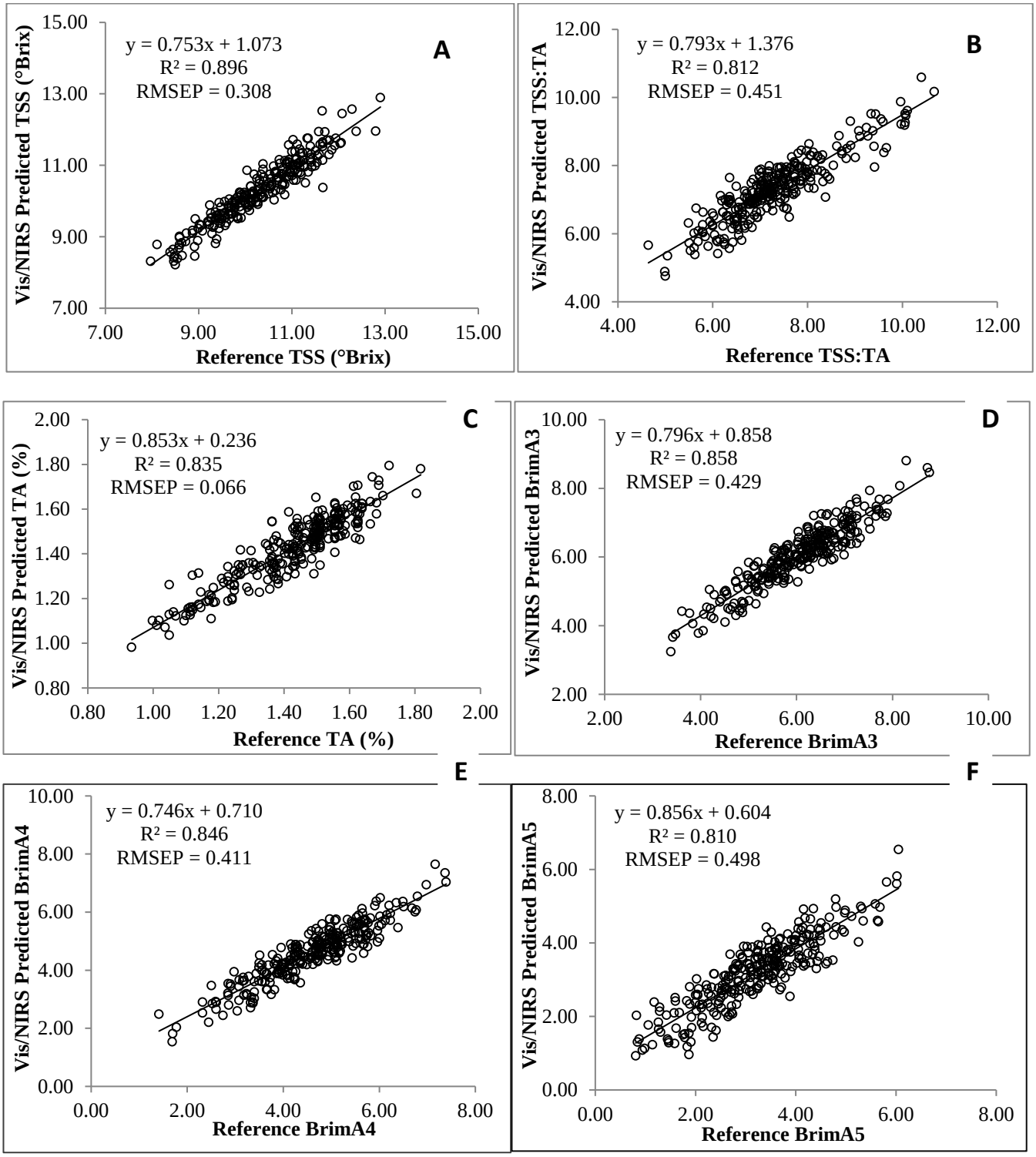

Fig. 4: Scatter plot of Vis/NIRS predicted versus measured or calculated values of 'Star Ruby' grapefruit's TSS (A), TSS: TA ratio (B), TA (C), BrimA3 (D), BrimA4 (E) and BrimA5 (F). RMSEP, root mean square error of prediction; R^2, regression coefficient of Vis/NIRS predicted data to reference data; Vis/NIRS, visible to near infrared spectroscopy.

The spectra of both cultivars showed similar absorbance characteristics except for the higher peak for oranges compared with grapefruit at 650-700 nm (Fig. 5). This was hypothesized to be because of the orange colour of oranges which is found at 590- 680 nm range of the visible spectrum (Chittka and Waser, 1997). The continous decrease in absorbance with the minimum at 720 nm was observed. In both cultivars, the penetration of radiation into the fruit (absorbance) was low in the visible region of the spectrum compared with the NIR region. This was correlated with bright coloured flavedo of fruit rind causing reflection and therefore, the poor performance of predicting models developed using wavelength region below 850 nm. An increase in absorbance was observed from 850 nm until the maximum was reached at 1980 nm, absorbance dropped again and reached a maximum at 2500 nm. The absorbance along the wavelengths showed better absorbance in the NIR region compared with a visible region which correlated with the superior performance of models developed using NIR region. These results were consistent with previous publications that recognised poor performance of long wavelengths to penetrate thick-skinned fruit such as citrus (Gómez *et al*., 2006; Magwaza *et al*., 2011).

The ability of Vis/NIRS to accurately predict BrimA was a success considering that the parameter is believed to be the closest estimate of consumer perception for flavour and sweetness of citrus fruit. Furthermore, current pricing of fruit in the market is based on fruit weight, which is not accurate because some fruit are just big but lack preferable flavour for consumers. Prediction of BrimA will not only enable the discrimination of fruit for export purpose but could also be used commercially for determining the price of fruit based on flavour rather than fruit size.

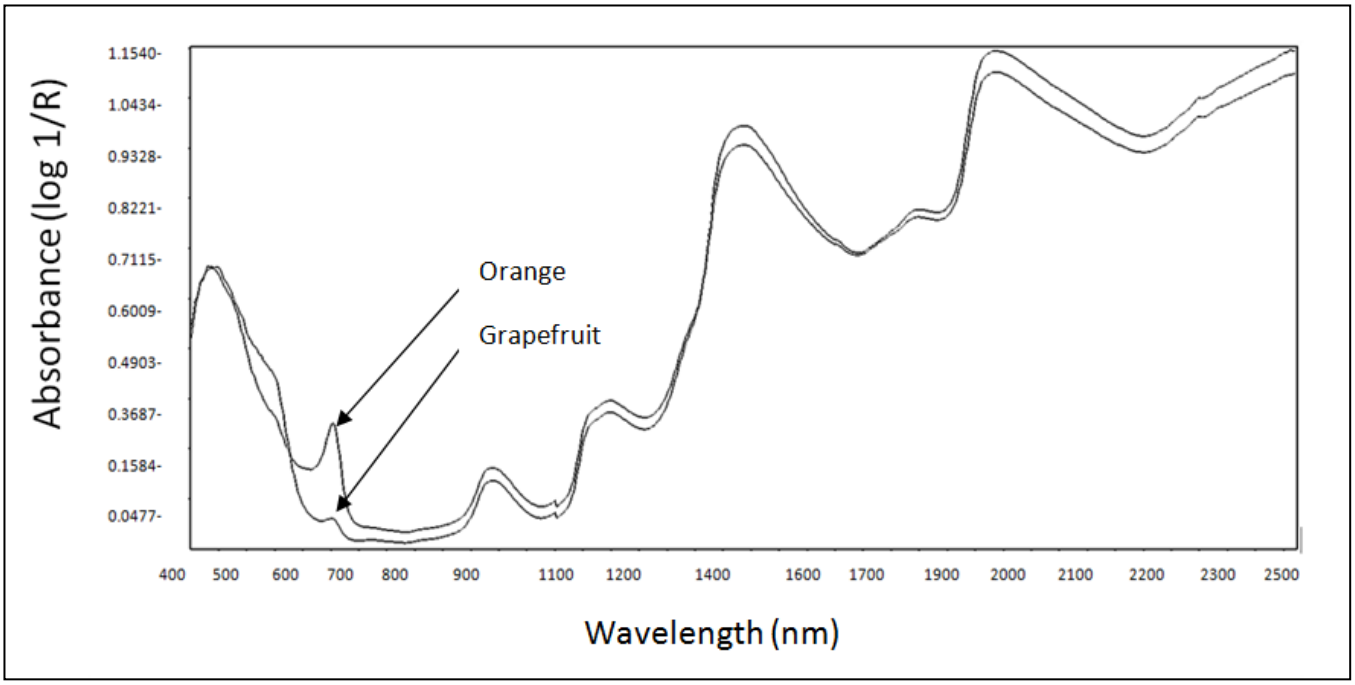

Fig. 5: The typical average visible to near infrared (400-2500 nm) absorbance intensity spectra of 'Valencia' orange and 'Star Ruby' grapefruit.

3.5 Conclusion

The 850-2300 nm range of spectrum showed to be the optimum range reflecting internal parameters of 'Valencia' orange and 'Star Ruby' grapefruit. Application of Vis/NIRS showed that sweetness and flavour parameters, namely, BrimA and TSS: TA ratio, were predicted with the same level of accuracy. Although the models of this study did predict BrimA with high accuracy on both cultivars, this is the first study to report the application of Vis/NIR spectroscopy to predict this sweetness and flavour parameter. Therefore, more studies with similar objectives were recommended for these and other citrus cultivars as well. It was further suggested that improving the accuracy of juice extracting technique or increasing the number of samples may increase the models performance. The results from this study confirmed the ability of Vis/ NIR spectroscopy, combined with chemometrics, to nondestructively predict sweetness and flavour attributes of oranges and grapefruit.

Vis/NIRS was recommended as a possible fast and accurate technique that could be used for fruit discrimination based on flavour parameters during packing and for pricing of fruit in the market.

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Chapter 4: Determination of pre-symptomatic biochemical markers that can be used to predict susceptibility of 'Marsh' grapefruit to chilling injury and rind pitting

4.1 Abstract

This study was conducted to examine the relationships between fruit position within the tree canopy, fruit physico-chemical composition and susceptibility of 'Marsh' grapefruit to rind physiological disorders during postharvest storage. Fruit position within the canopy had a significant effect ($p < 0.01$) on fruit susceptibility to rind physiological disorders. The difference was aligned with parameters determining physiological status of fruit, which differed with fruit canopy position on the tree. Fruit from inside canopy positions of the two orchards, in Limpopo and KwaZulu-Natal provinces, had higher chilling injury incidence of 27% and 24% compared to outside canopy 12% and 0%, respectively. In both sites, rind pitting was lower on inside canopy fruit (13 *vs*. 19% and 2 *vs*. 9%) compared to outside canopy fruit. Fruit from inside canopy had higher concentrations of sucrose (3.25 and 2.78 *vs*. 3.12 and 2.46 mg/g dry mass), antioxidant activity (72.52 and 69.71 *vs*. 70.08 and 67.47 µmol Trolox/g dry mass) and fructose (2.71 and 2.46 *vs*. 2.61 and 2.53 mg/g dry mass) and lower dry matter (28.67 and 29.94% vs. 26.93 and 29.2%), total antioxidant compounds (3.15 and 4.80 *vs*. 2.68 and 4.76 mg ascorbic acid/g dry mass), total phenolic compounds (337.31 and 356.36 *vs*. 288.87 and 303.22 µg gallic acid equivalent/g dry mass) compared with outside canopy fruit. The relationships (correlations) of parameters to rind disorders were also examined for possibility to develop techniques for pre-symptomatic prediction of the disorders. Parameters with positive correlations to rind pitting did not show a positive correlation to chilling injury, which excited the idea of using fruit parameters to predict the possibility of the disorders.

Keywords: Citrus, chilling injury, rind pitting, principal component analysis (PCA)

4.2 Introduction

The South African citrus industry is ranked the second largest exporter of fresh citrus fruit after Spain (Potelwa, 2015). The position of the industry in the international market is strengthened by the production of a wide range of cultivars over an extended period of time - from March through to November (Department of Agriculture, Forestry and Fisheries, 2015). Markets in the European Union, Japan, China and the United States of America are of particular importance to the citrus fruit industry (Pryke and Pringle, 2008). However, the risk of fruit consignment getting rejected due to the presence of phytosanitary pests such as fruit fly (*Ceratitis capitata* and *Ceratitis rosa*) and false codling moth (*Thaumatotibia leucotreta*) are the major threats to fruit export (Pryke and Pringle, 2008).

Many countries in which South Africa send its citrus fruit have stringent cold sterilization requirements. For example, Japan demands that citrus fruit be kept at -0.6 °C for at least 12 days during transit while the requirements of the American and Chinese markets are even more extreme, extending the period to 24 days (Hofmeyr et al., 2016; Cronje, 2016). However, for fruit such as grapefruit which are sensitive to chilling temperatures, there is a very narrow window between combating the insects and damaging the fruit. Grapefruit rinds, typically develop pits or scalds at temperatures below 4.5 °C, a phenomenon known as chilling injury, which reduce fruit quality and increase postharvest losses (Ezz and Awad, 2009; Magwaza *et al.*, 2013a; Chaudhary *et al.*, 2014; Siboza *et al.*, 2014).

Chilling injury is often characterized by rind pitting (RP), rind staining, red blotches, internal breakdown, off-flavour, decay and necrosis on the flavedo (the outer-most pigmented part of the rind) of the fruit (Chaudhary *et al.*, 2014). During early stages, RP is manifested as small, irregular and slightly sunken patches of 3-6 mm in diameter that are randomly distributed about the flavedo of the fruit (Alférez *et al.*, 2010; Cronje *et al.*, 2011; Magwaza *et al.*, 2013a). Susceptibility of citrus fruit to rind physiological disorders differs from one cultivar

to another. Grapefruit cultivars with red rind, such as 'Ruby Red' are known to have lower susceptibility, which has been related to higher antioxidant capacity, phenolic compounds and lycopene content (Lado *et al*., 2015), higher ascorbic acid content (Del Caro *et al*., 2004) and higher dry matter as well as non-structural carbohydrates in 'Nules Clementine' mandarin fruit (Cronje *et al*., 2011; Magwaza *et al*., 2014a).

'Marsh' is a white grapefruit cultivar, which is related to lower antioxidant capacity, because the red colour in red cultivars is believed to be caused mainly by phenolic compounds and carotenoids (Jayaprakasha *et al*., 2008). Phenolic compounds are one of the important groups of compounds increasing antioxidant capacity and the level of fruit tolerance to rind physiological disorders (Lee *et al*., 2003; Magwaza *et al*., 2013). Rind physiological disorders have a significant impact on exported 'Marsh' grapefruit because symptoms manifest about 4 to 6 weeks post-harvest. This is the main problem that faces the industry and importers alike, because if fruit are exported this usually coincide with the shipping period and point of sale (Magwaza *et al*., 2013; 2014a). Although these physiological disorders affect the rind and in general do not compromise the edible internal portion of the fruit, they are, however extremely problematic as they can lead to tremendous financial losses and customer complaints.

Furthermore, consumers estimate fruit quality based on external appearance (Chen and Opara, 2013). The appealing outside appearance is normally aligned with a satisfying flavor during consumption. While in fact, the important flavor attributes of citrus are internal parameters (Ncama *et al*., 2017). Understanding the basis of physiological rind disorders prior exports can reduce fruit quality loss during the postharvest chain, as appropriate management techniques may be applied. Fruit from different orchards or different canopy positions differ in their biochemical compounds content and therefore, susceptibility to non-chilling rind physiological disorders (Cronje *et al*., 2011; Magwaza *et al*., 2014a, b, c). Another area that still needs further research is the relationship between the biochemical profile of the rind, antioxidant system and development of citrus chilling physiological disorders. Understanding this cause-response relationship will help identify potential pre-

symptomatic physico-chemical markers for these disorders. Cronje *et al*. (2011a) showed a negative relationship between rind carotenoids concentration measured postharvest and rind breakdown of 'Nules Clementine' mandarin. Previous studies have also suggested the participation of the antioxidant system in chilling and non-chilling conditions causing rind pitting. These investigations have showing that phenolic metabolism may be required for building protective barriers that helped 'Navelate' fruit to reduce non-chilling peel pitting (Lafuente *et al*., 2003; Sala *et al*., 2005). However, further investigations are needed to understand the participation of the antioxidant system in protecting other citrus fruit such as 'Marsh' grapefruit against other rind physiological disorders, such as chilling injury and rind pitting.

A recent review of the literature by Magwaza *et al.* (2013b) shows that, to date, knowledge of the biochemical changes occurring in the rind of the citrus fruit that could be used to predict fruit rind condition, and therefore susceptibility to rind disorders, is limited. It is, therefore, crucial to identify potential biochemical markers of rind condition that are related to fruit susceptibility to chilling injury and rind pitting. The assessment of such biochemical markers and correlation with the disorder constitute the principal framework of this research towards understanding the mechanism(s) which influence chilling rind physiological disorders, which in turn, may lead to a pre-symptomatic detection and/or prediction of the disorder. Therefore, this study was conducted to identify pre-symptomatic bio-markers associated with 'Marsh' grapefruit susceptibility to rind physiological disorders. Susceptibility difference brought by fruit position with the tree canopy was also investigated.

4.3 Materials and methods

Fruit sampling

This research was conducted during 2015/2016 season using 'Marsh' grapefruit (*Citrus* x *paradisi* Macfad). A total of 150 fruit were harvested from inside or outside canopy position of randomly selected trees in the

following two orchards: one located at Hoedspruit in Limpopo Province (24°23'39.02"S; 30°49'20.65"E) and at Enkwalini in KwaZulu-Natal Province (32°75'28.S; 35°89'31.E), South Africa. The fruit were transported in a ventilated vehicle to postharvest technology laboratory of the University of KwaZulu-Natal, where postharvest storage and biochemical analysis were performed. Upon arrival at the laboratory, fruit treatments simulating commercial chain were applied.

Postharvest treatments and storage

Fruit were washed with Imazalil® fungicide (Farmalinx Pty. Ltd.; Bondi Jucntion; Australia) and coated with Citrashine® wax (Citrashine Pty. Ltd.; Decco; Johannesburg; South Africa) with concentrations prepared based on recommendations on container labels. They were left overnight in open space at room temperature to allow the coating to dry. In the next morning, 20 fruit per treatment were taken and analyzed for sampling just after harvest; fruit were labeled and transferred into the cold room, simulating normal export conditions. Cold storage started as a quarantine treatment for 2 weeks at -0.6 ºC; 95 ± 1% relative humidity (RH), and the storage container temperature was raised to 5 ºC for the subsequent 4 weeks (Department of Agriculture Forestry and Fisheries, 2014). Thereafter, fruit were taken back to room temperature to simulate shelf life, which would happen after reaching designated market, and sampled after a week.

Data collection

Since predictions for fruit susceptibility to chilling injury disorders should be done before shipping, rind parameters sampling was only done immediately after harvesting. Sampling of other quality parameters such as colour, mass loss and internal parameters was done just after harvesting (week 0) and at two weeks intervals until the end of the six weeks of cold storage. During each sampling date, fruit were kept at room temperature (21 ±1 ºC) for seven days to simulate shelf life at a retail supermarket. After seven days, disorders were rated

from 1 to 3 based on the fruit surface covered by the disorder. The CI and RP incidences were calculated and expressed as an index according to Eq. 1 (Magwaza *et al*., 2012).

$$\text{Disorder index} = \frac{\Sigma(disorder\ (1-3)\ x\ no.of\ fruit\ in\ each\ class)}{total\ No.of fruit} \quad (1)$$

Mass and colour measurements

Fruit mass was measured using a calibrated weighing scale (RADWAG Wagi Electronic Inc., Poland). The fruit colour was measured from three random spots on the equatorial position of a fruit using portable colorimeter (Chroma Meter, Konica Minolta Sensing, INC., Japan), which was calibrated by scanning a 100% white reference brick with Y= 87.0, X = 0.3146 and y = 0.3215 prior fruit scanning and periodically at 30 min intervals. The fruit colour was expressed as a citrus colour index (CCI) according to Eq. 2 (Pathare *et al.*, 2013; Vidal *et al*., 2013).

$$CCI = \frac{1000 * a}{L*b} \quad (2)$$

Total Soluble Solutes, Titratable Acidity, BrimA and maturity index

During sampling, each fruit was cut in half and squeezed to collect juice for TSS and TA analysis. The collected Juice was homogenized using a stirrer (ULTRA-TURRAX; IKA® T25 digital; Germany) and tested for TSS using a digital refractometer with a dynamic control system (RFM340+ BS®, Bellingham and Stanley Ltd, Basingstoke, Hants, UK). TSS was recorded as ºBrix which is equivalent to TSS %. The remaining juice was snap frozen in 120 mL plastic specimen jars and stored in a freezer set at -20 °C for further analysis of TA.

Titratable acids were analyzed by mixing 10 mL juice with 50 mL distilled water and titrating with 0.1 M sodium hydroxide (NaOH) to the end point (pH of 8.1). The titre was recorded and the citrus acids formula (Eq. 3) was applied to calculate TA, expressed as % citric acid.

$$TA\ (\%\ citric\ acid) = \frac{0.0064\ X\ titre\ (NaOH)\ ml\ X\ 100}{10\ mL\ juice} \quad (3)$$

BrimA was calculated based on the formula by Obenland *et al.* (2009), Eq. 4.

$$BrimA = TSS - k\ (TA) \quad (4)$$

Where, *k* is a constant that reflects the tongue's higher sensitivity to TA compared to TSS. In this study *k*-values used were presented as BrimA*k*. For example, BrimA4 is BrimA with factor 4.

The ratio of TSS to TA, also known as citrus maturity index, was calculated using Eq. 5.

$$TSS\ to\ TA\ ratio\ (maturity\ index) = \frac{\mathrm{TSS}}{\mathrm{TA}} \quad (5)$$

Rind dry matter determination

To measure rind dry matter, the mass of fresh rind sample was measured to obtain fresh mass; freeze-dried; and measured again to obtain dry mass. The dried mass was divided by the fresh mass to calculate a percentage dry matter (DM %) (Eq.6):

$$Dry\ matter\ (DM\%) = \frac{dried\ mass}{initial\ fresh\ mass} \quad (6)$$

Free radical scavenging assay and total antioxidants capacity

Antioxidant activity was based on Trolox equivalent antioxidant capacity (TEAC), which was studied using 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) scavenging assay. DPPH free radical scavenging assay and total antioxidant capacity of 'Marsh' grapefruit rind extraction and analysis were executed according to a method by Aliyu *et al*. (2013). Briefly, freeze dried rind samples were ground into powder using a pestle and mortar. The sample (1 g) was extracted using 25 mL of distilled water. The mixture was allowed to stand at room temperature for 1 h in the dark, with agitations at every 15 min intervals. The aqueous extract was obtained by filtering the liquid sample mixture through 0.25 µm syringe filter before analysis.

The DPPH free radical scavenging activity of each sample was determined using the Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, USA) according to the method described by Wong *et al.* (2006). Briefly, a 0.1 mM solution of DPPH in methanol was prepared. The initial absorbance of the DPPH in methanol at different concentrations (0.05 to 2.5 mg/mL; $R^2 = 0.994$) was measured at 515 nm to create a standard curve. An aliquot (40 µL) of an extract was added to 3 mL methanolic DPPH solution. The samples were allowed to react for 30 min. The change in absorbance for samples was measured and antioxidant activity determined based on Trolox standard curve. The antioxidant capacity based on the DPPH free radical scavenging ability of the extract was expressed as µmol Trolox equivalents per gram of plant material on dry matter basis.

The total antioxidant capacity of the extracts was evaluated by the phosphor-molybdenum method (Aliyu *et al*., 2013). Briefly, 80% v/v methanol (5 ml) was used for extracting the rind powder sample (0.5 g). The aqueous extract was obtained by filtering the liquid mixture through 0.25 µm syringe filter before analysis. The methanolic extracts (0.3 mL) were combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 85

ºC for 90 min. The solution was allowed to cool to room temperature before the absorbance was measured at 695 nm using a UV-1800 Spectrophotometer (Shimadzu Scientific Instruments INC., Columbia, USA), against blank. Methanol (0.3 mL) instead of the extract was used as the blank. The total antioxidant activity was expressed as milligram equivalent of ascorbic acid per gram of plant in dry matter based on known standard curve.

Rind non-structural carbohydrates extraction and determination

The extraction and determination of non-structural carbohydrates were carried out according to Magwaza *et al*. (2014a) with slight modification. Briefly, non-structural carbohydrates were extracted from 0.5 g of dried rind powder using 80% (v/v) aqueous methanol (5 mL). The samples were left to stand for 1 hour with occasional agitation at room temperature, filtered through a 0.25 μm syringe nylon filter and put in vials for high performance liquid chromatography (HPLC) analysis.

Concentrations of glucose, sucrose and fructose were determined using a HPLC binary pump system (Agilent Technologies, UK). Sample extracts were injected into a Rezex RCM monosaccharide Ca+ (8%) column of 7.8 mm diameter x 300 mm (Phenomenex, Torrance, CA, USA). The column temperature was set at 86 °C using a thermo-stated column compartment (G1316A, Agilent). The mobile phase used was HPLC-grade water at a flow rate of 0.6 mL/min. The presence and concentration of the selected non-structural carbohydrates were calculated by comparing peak area of samples against peak area of known standard concentrations (0.05-1.25 mg/mL; $R^2 = 0.991$).

Phenolic compounds extraction and determination

Total phenolic compound extraction and quantification were carried out using a method described by Lamien-Meda *et al.* (2008) with slight modification. Briefly, a ground rind sample powder (0.5 g) was extracted with 80% acetone (5 mL). The solution was kept for 30 min at room temperature with constant stirring. The samples were filtered, pooled and centrifuged at 10000 rpm for 10 min in a 5 ºC pre-cooled centrifuge (Centrifuge 5810R, Eppendorf AG, Germany) to obtain clear extracts.

Total phenolic compounds contents were determined by Folin-Ciocalteau method (Lamien-Meda *et al*., 2008). Each extract (0.1 mL) was mixed with 2N Folin-Ciocalteau reagent (2.5 mL) and allowed to stand for 5 min. Thereafter, 75 g/L sodium carbonate (2 mL) was added and samples were incubated for 2 h at 65 ºC. Samples were allowed to cool and the absorbance was measured at 750 nm using a UV-1800 Spectrophotometer (Shimadzu Scientific Instruments INC., Columbia, USA) against acetone as blank. A standard calibration curve was plotted using gallic acid (0-200 µg/mL; $R^2 = 0.959$) in the reagent and the total phenolic compounds were expressed as µg gallic acid equivalents (GAE)/g of plant material on a dry matter basis.

Data analysis

Statistical analyses of fruit properties were carried out using statistical software (GenStat®, 14th edition, VSN International, UK). Data was subjected to analysis of variance (ANOVA) and means were separated by least significant difference ($p = 0.01$). The coefficient of variation (CV), defined as the ratio of the standard deviation of the mean was multiplied by 100 and reported as a percentage (Magwaza *et al*., 2014b). Data was also subjected to multivariate statistical analyses, including principal component analysis (PCA). PCA was performed on Unscrambler® Software (Version 10.3, CamoSoftware, AS, Norway).

4.4 Results and discussion

Canopy position effect on susceptibility to rind physiological disorders

The incidence of chilling injury was significantly affected by both the fruit position within the tree canopy ($p < 0.01$) as well as the growing region ($p < 0.01$), and canopy position by growing region interaction ($p < 0.01$). In both orchards, fruit from inside canopy position had a higher CI incidence (Fig. 1) compared to those harvested from outside canopy position. The highest incidence of CI (27%) was observed on fruit harvested from Limpopo's inside canopy (LP IN) followed by Kwazulu-Natal's inside canopy (KZN IN) (24%). Outside canopy fruit had a lower incidence of CI with 12% and 0% on fruit from LP and KZN, respectively. Since the position of a fruit within the canopy affects its physiological attributes (Cronje *et al*., 2011), citrus fruit borne on different canopy positions differ in their susceptibility to physiological disorders (Ezz and Awad, 2009; Cronje *et al*., 2011; Magwaza *et al.,* 2013a, b).

Fruit from KZN had lower CI incidence compared to fruit from LP. The difference in fruit susceptibility was further aligned with the difference in climatic conditions of the two orchards. The orchard in KZN is situated in the area that reaches a minimum temperature of 5 ºC during the month of May, which is the month of early season fruit maturity, while the LP orchard could only reach 8 °C as minimum temperature. Exposure of plant tissues to cold temperatures gives plants an important signal to produce defensive proteins to induce tolerance to chilling disorders (Gill and Tuteja, 2010). The ability of KZN OUT fruit to adapt cold storage was hypothesized to be because of lower minimum temperatures KZN fruit experienced during development compared with the fruit from LP.

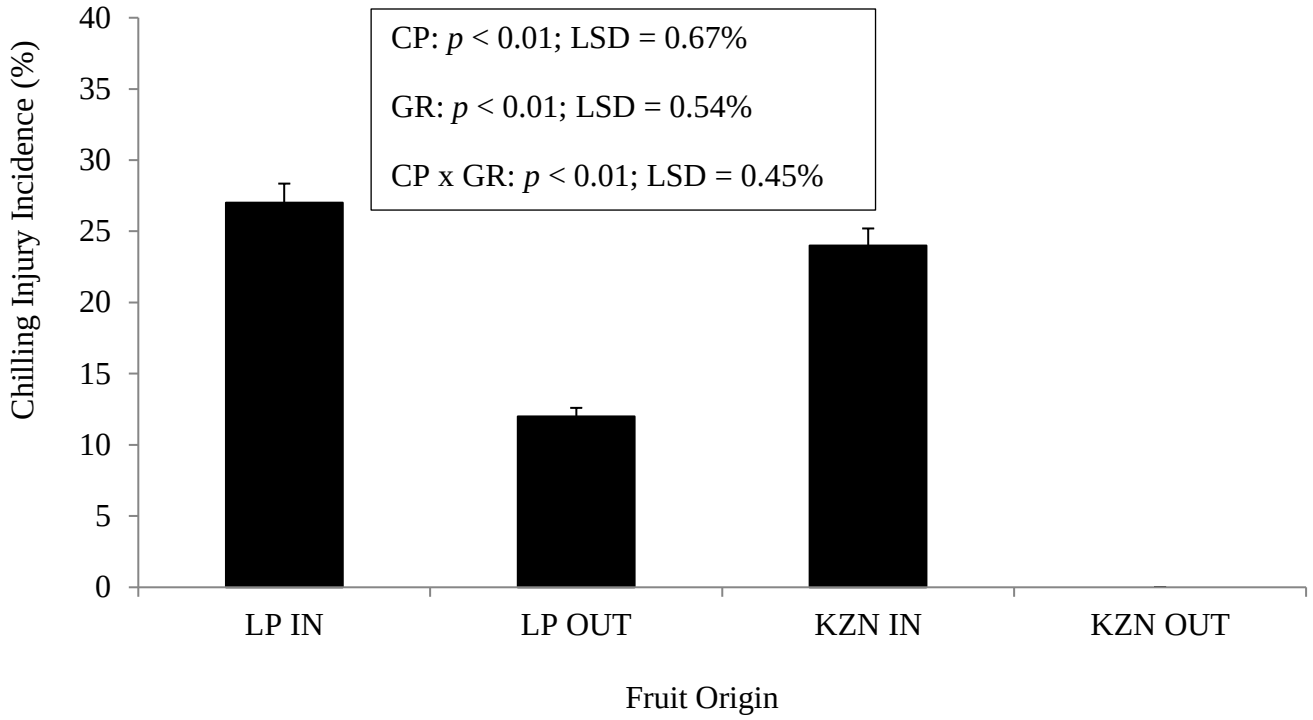

Fig. 1: The chilling injury incidence difference based on fruit origin and canopy position. Data presented as mean ±standard deviation (SD); LP, Limpopo; LP IN, fruit from inside canopy of the orchard in Limpopo; LP OUT, fruit from outside canopy position of the orchard in Limpopo; KZN, KwaZulu-Natal; CP, canopy position; GR, growing region.

The incidence of RP was significantly affected by both canopy position of fruit ($p < 0.01$) as well as growing region ($p < 0.01$), and canopy position by growing region interaction ($p = 0.003$). The trend of RP occurrence can be presented as LP OUT (19%) > LP IN (13%) > KZN OUT (9%) > KZN IN (2%). In both orchards, inside canopy fruit had significantly lower RP. Fruit from KZN had a lower incidence than fruit from LP (Fig. 2).

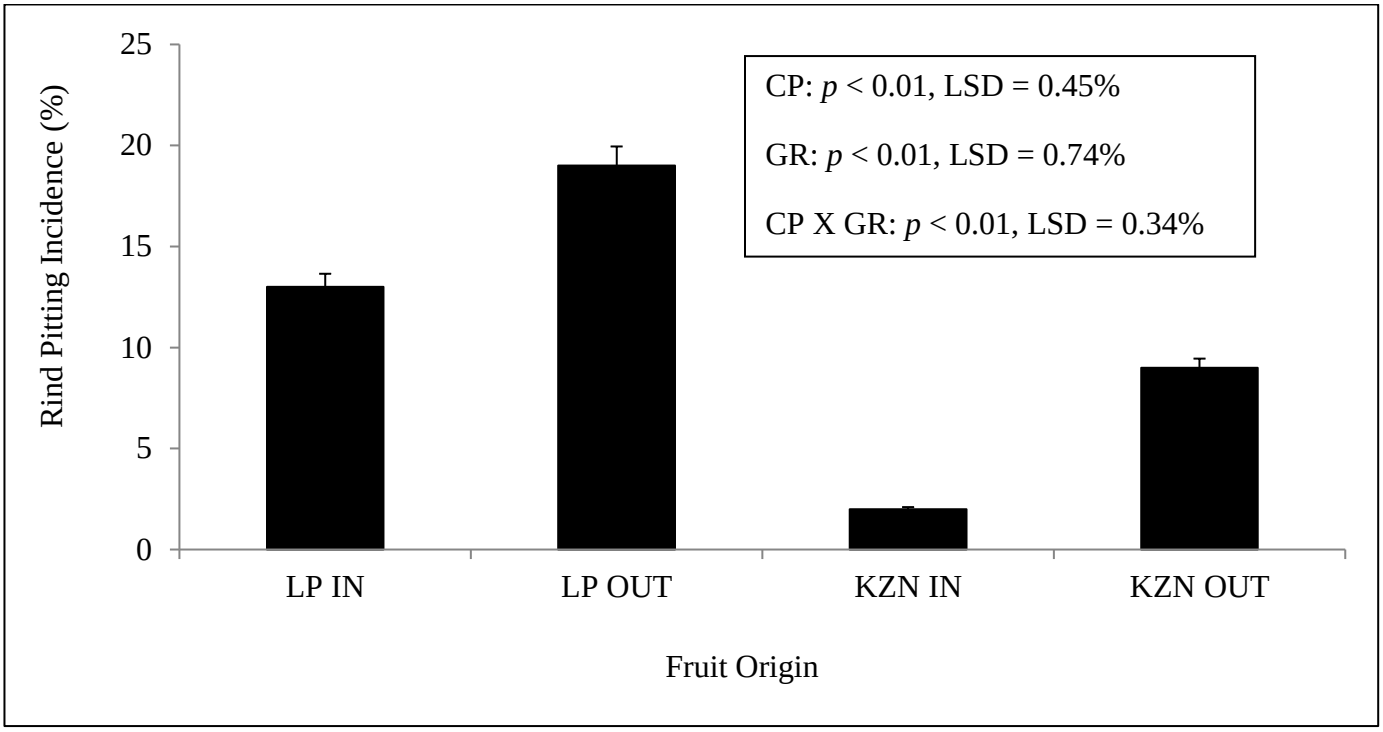

Fig. 2: The Rind pitting incidence difference based on fruit origin and canopy position. Data presented as mean ± 5% standard error; LP, Limpopo; LP IN, fruit from inside canopy of the orchard in Limpopo; LP OUT, fruit from outside canopy position of the orchard in Limpopo; KZN, KwaZulu-Natal; CP, canopy position; GR, growing region.

The results of incidences of rind pitting and chilling injury disorders from this study were related to fruit from inside canopy position having thicker rinds (Cronje *et al*., 2011; Magwaza *et al.,* 2013b), constituted by high moisture content, which was hypothesized to cause the less biological transformation to adapt to cold storage, and that reduced the ability of the inside canopy fruit to tolerate CI. Fruit from outside canopy developed more RP compared to fruit from inside canopy, which was related to fruit from outside canopy having lower rind moisture content, which did not allow their rind to shrink after chilling damage, but their cells collapsed and dehydrated when taken back to shelf life at increased temperature and lower relative humidity (RH).

Antioxidant activity, total antioxidant capacity and phenolic compounds

Fruit from inside canopy in both orchards had significantly higher ($p < 0.01$) antioxidant activity compared to outside canopy fruit. There was a statistically nonsignificant difference between the growing regions. The trend can be presented as LP IN (72.52) > KZN IN (70.08) > LP OUT (69.75) > KZN OUT (67.47) in units of µmol Trolox equivalent per gram on the dry mass basis (Fig. 3). Fruit from outside canopy had significantly higher ($p < 0.01$) total antioxidant capacity compared with inside canopy fruit (Fig. 4). The nonsignificant differences existed from the growing regions. The trend of the total antioxidant capacity can be presented as LP OUT (4.80) > KZN OUT (4.76) > LP IN (3.15) > KZN IN (2.68) in units of mg ascorbic acid per gram of dry mass sample. It was hypothesized that the inside canopy fruit were utilizing more antioxidants compared to fruit from outside canopy due to the relatively higher need for defense mechanism against reactive oxygen species (ROS). Under cold stress, plant tissues can experience an increased level of ROS, which are species that stimulate lipid peroxidation and result to cell membrane damage (Rui *et al*., 2010; Hernández *et al*., 2011). The antioxidant compounds deactivate the ROS reactions, resulting in reduced membrane rupturing (Sevillano *et al*., 2009). Fruit from inside canopy experienced higher CI incidence, which required them to utilize more antioxidants than outside canopy fruit.

However, it is also possible that the method of using ascorbic acid as equivalent to total antioxidants, used in this study, did not determine the main compound contributing to total antioxidant capacity. In previous studies of white grapefruit antioxidant capacity, ascorbic acid was found to make little contribution to the total antioxidant capacity of the fruit (Bahorun *et al.*, 2007). The higher total antioxidant capacity of outside canopy fruit was related to other possible attributes, other than ascorbic acid, that enhanced the resistance of outside canopy fruit to CI and resulted in utilization of less ascorbic acid as an antioxidant compound in deactivation of ROS during maintenance of membrane integrity in adaptation to cold storage. These results were similar to Ezz and Awad (2009), who noticed a negative correlation of ascorbic acid content to CI. Ascorbic acid was

used as total antioxidants equivalent in this study, and fruit with higher total antioxidant capacity showed lower susceptibility to CI.

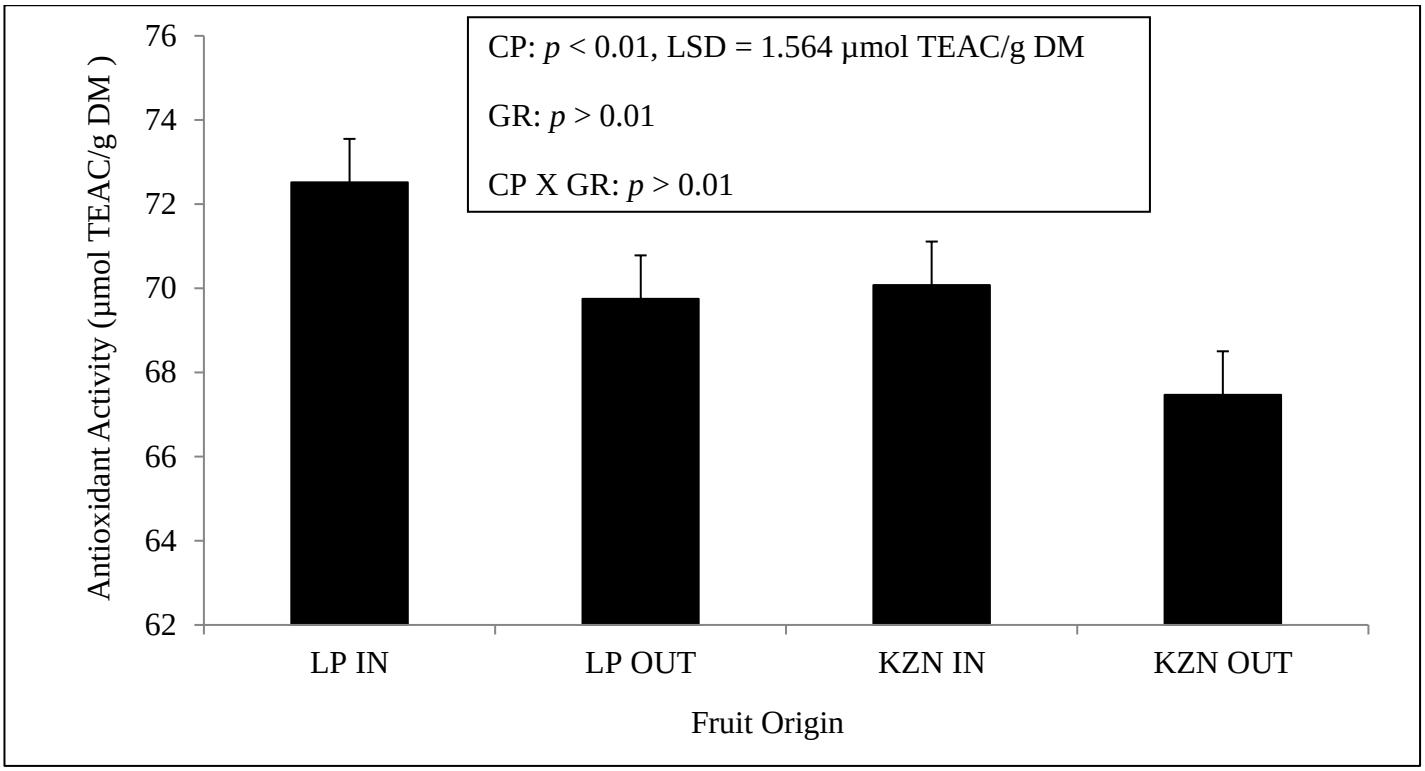

Fig. 3: Trolox equivalent antioxidant capacity (TEAC) based on DPPH scavenging activity of 'Marsh' grapefruit from different origins and canopy position. Data presented as mean ± standard error; LP, Limpopo; LP IN, Limpopo inside canopy; LP OUT, Limpopo outside canopy; KZN, KwaZulu-Natal; DM, dry mass basis; CP, canopy position; GR, growing region.

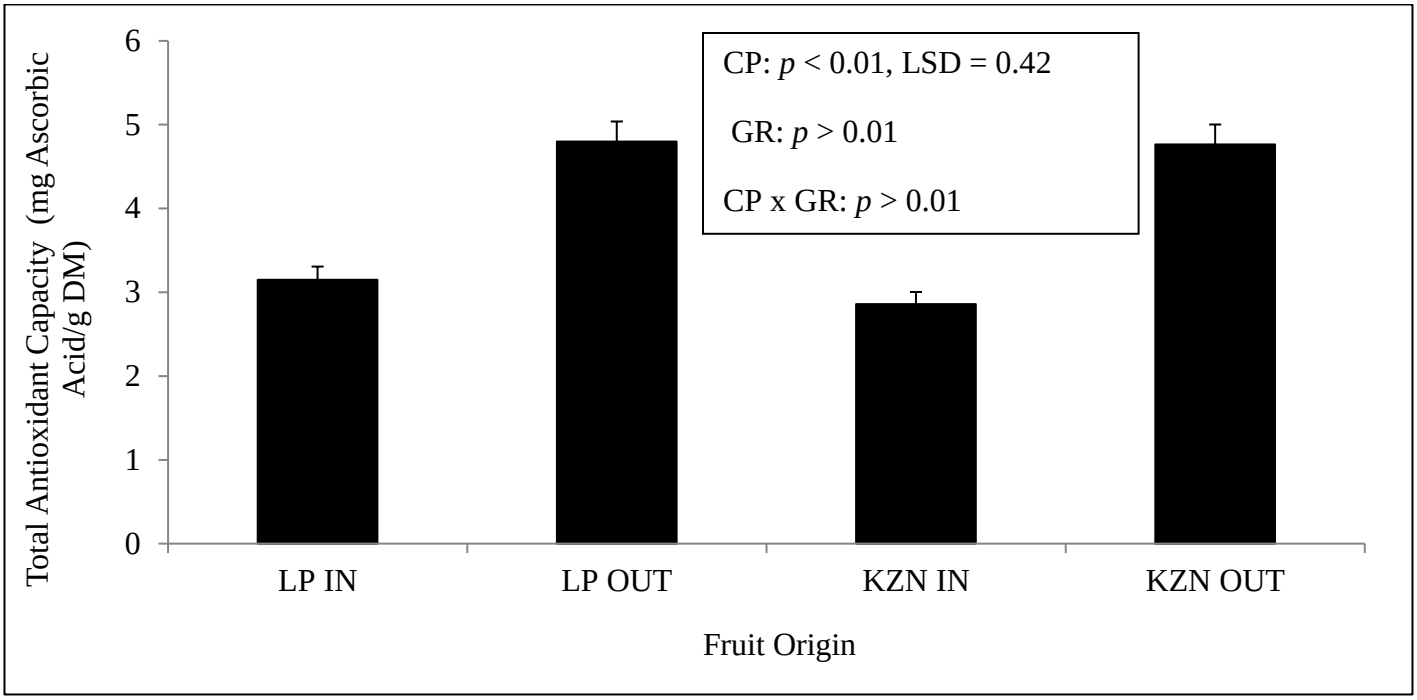

Fig. 4: Total antioxidant capacity of 'Marsh' grapefruit from different origins based on canopy position. Data presented as mean ± standard error; LP, Limpopo; LP IN, Limpopo inside canopy; LP OUT, Limpopo outside canopy; KZN, KwaZulu-Natal; DM, dry mass basis; CP, canopy position; GR, growing region.

In both orchards, fruit from outside canopy had significantly higher ($p < 0.01$) total phenolic compounds compared to fruit from inside canopy (356.36 vs. 337.31 and 303.22 vs. 288.87 µg gallic acid/g DM for KZN and LP, respectively) (Fig. 5). There was a significant difference between growing regions ($p < 0.01$), and significant difference existed at an interaction between canopy position and growing region ($p < 0.01$). Fruit from KZN had higher phenolic compounds compared to fruit from LP, irrespective of the canopy position. Phenolic compounds were believed to have had a higher contribution to the antioxidant activity of the outside canopy fruit and thereby, increasing their resistance to CI incidence. Phenolic compounds are one of the important groups of compounds increasing antioxidant capacity and the level of fruit tolerance to physiological disorders (Lee *et al*., 2003). From the antioxidant compounds perspective, the results may be summarized to say antioxidants have a low relationship to the resistance of 'Marsh' grapefruit to RP. This is also emphasised by the results of correlations in table 4. The possible explanation of the RP occurrence may be that it is mainly

caused by the rind moisture content alteration. Unlike CI that is affected by certain biochemical compounds such as ROS reaction.

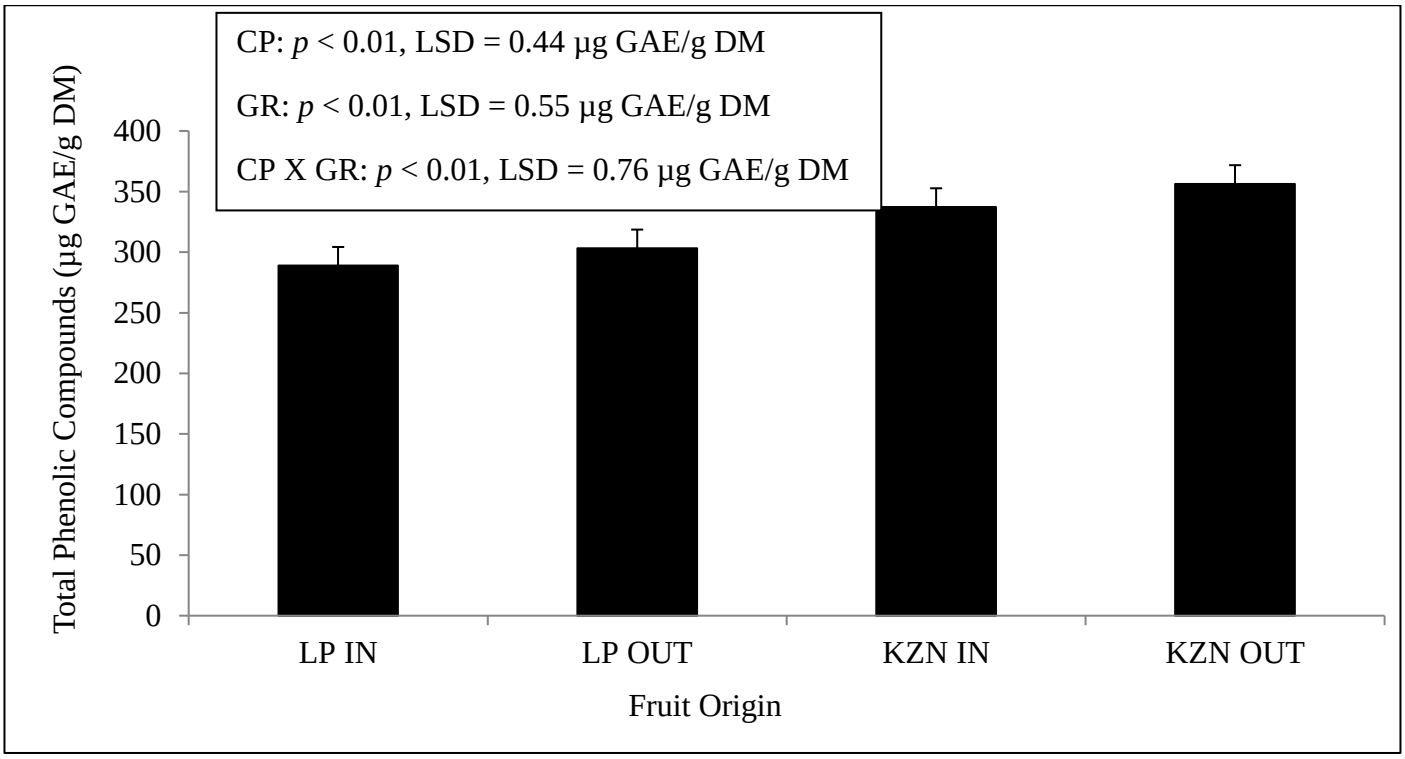

Fig. 5: Total phenolic compounds of 'Marsh' grapefruit. Data presented as mean ± SD; LP, Limpopo; LP IN, fruit from inside canopy of the orchard in Limpopo; LP OUT, fruit from outside canopy position of the orchard in Limpopo; KZN, KwaZulu-Natal; db, dry basis; CP, canopy position; GR, growing region.

Canopy position effects on 'Marsh' grapefruit's rind non-structural carbohydrates and dry matter

The rind of fruit from inside canopy had significantly ($p < 0.01$; LSD = 1.97 mg/g DM) higher sucrose (3.25 vs. 2.78 and 3.12 vs. 2.46 mg/g db for LP and KZN, respectively); insignificantly higher fructose (2.71 vs. 2.46 and 2.61 vs. 2.53 mg/g db for LP and KZN, respectively), and significantly ($p < 0.01$) lower glucose (1.79 vs. 3.21 and 1.98 vs. 2.64 mg/g db for LP and KZN, respectively) concentration compared to fruit from outside canopy in both orchards (Fig. 6). There was significant difference between the two growing origins in glucose

content ($p < 0.01$; LSD = 1.56 mg/g DM) and significant difference existed in the interaction of canopy position and growing regions ($p < 0.01$; LSD = 1.16 mg/g DM) and insignificant difference existed on sucrose and fructose content ($p > 0.01$) on growing regions and canopy positions, and growing region by canopy position interaction. The higher sucrose and fructose content from inside canopy results are in contradiction with the results obtained in studies by Magwaza *et al.* (2012; 2013b), who found higher concentrations of all total non-structural carbohydrates in rinds of 'Nules Clementine' mandarin fruit from outside canopy compared to inside canopy fruit. From these results, it could be deduced that even if there is a positive contribution of rind carbohydrates to 'Marsh' grapefruit CI, it is not all non-structural carbohydrates that contribute to higher susceptibility. For example, glucose had a negative correlation ($R^2 = -0.23$) to CI (Table 4), and fruit from outside canopy had higher glucose concentration but had lower CI incidence.

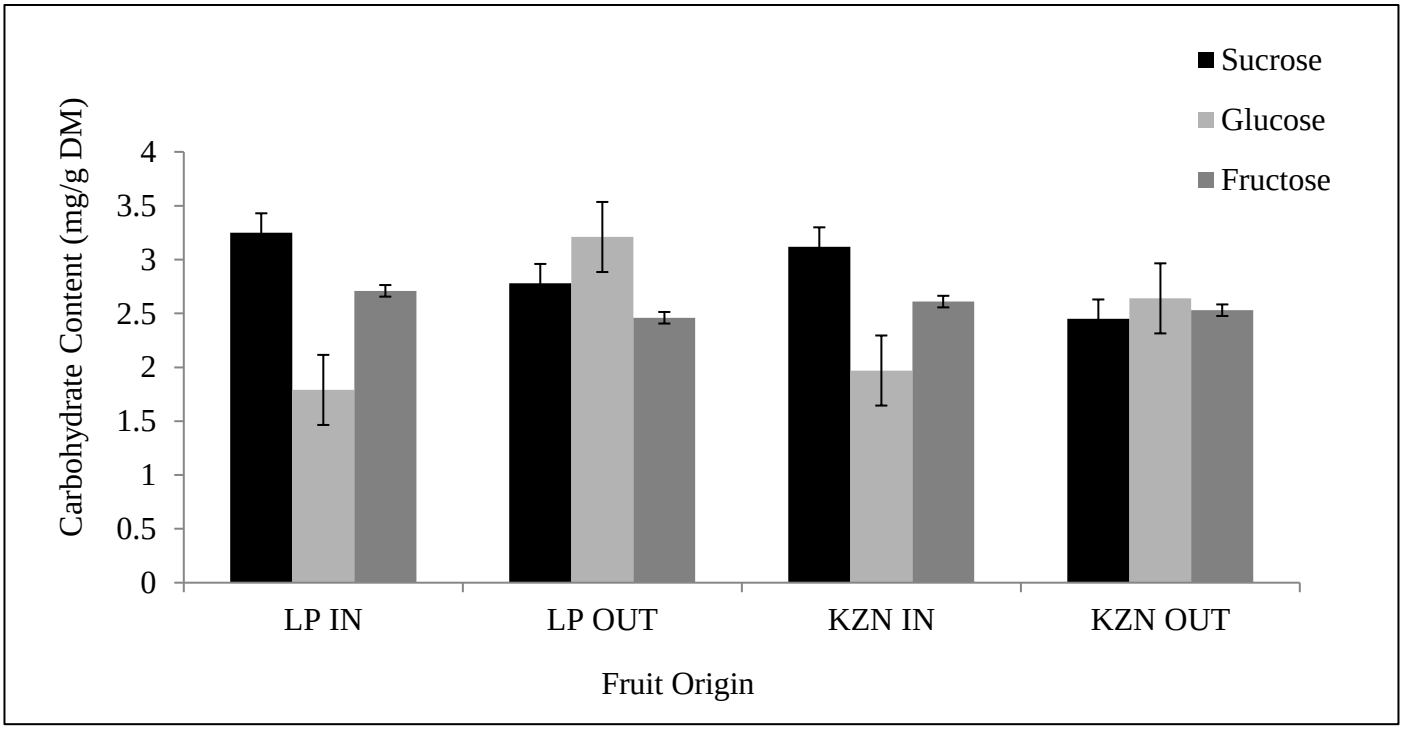

Fig. 6: The amount of rind non-structural carbohydrates of fruit from different origins. Data presented as mean ± SD; LP, Limpopo; LP IN, fruit from inside canopy of the orchard in Limpopo; LP OUT, fruit from outside canopy position of the orchard in Limpopo; KZN, KwaZulu-Natal; DM, dry matter basis; CP, canopy position; GR, growing region.

Although there were nonsignificant differences brought by fruit growing regions on dry matter, inside canopy fruit had lower rind dry matter compared with outside canopy fruit for both orchards (28.67 vs. 29.94% and 26.93 vs. 29.2% for LP and KZN, respectively) (Fig. 7). It was hypothesized that the lower temperature and humid environment inside the tree canopy kept the shaded fruit away from sunlight so much that they did not lose significant moisture through evapotranspiration to toughen their rinds during fruit development. Therefore, the fruit from such conditions had thicker moistened soft rinds, with weak structural compounds that easily got damaged under cold stress conditions.

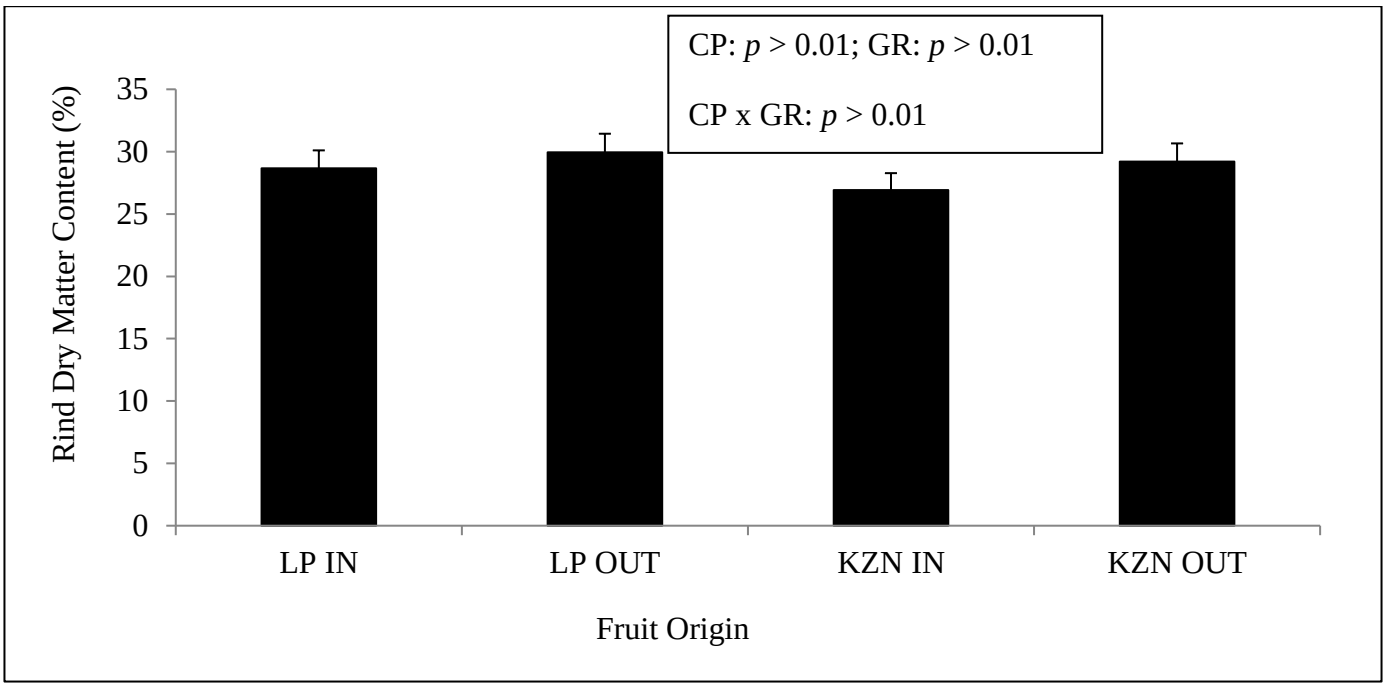

Fig. 7: The rind dry matter contents of fruit from different origins. Data presented as mean ± SD; LP, Limpopo; LP IN, fruit from inside canopy of the orchard in Limpopo; LP OUT, fruit from outside canopy position of the orchard in Limpopo; KZN, KwaZulu-Natal; CP, canopy position; GR, growing region.

Fruit borne on the inside canopy experience shading from other fruit and leaves on outside canopy, which is related to lower photosynthesis, lower respiration, greener colour, higher structural carbohydrates and lower carotenoids content when compared with fruit from outside canopy (Cronje *et al*., 2011). Shaded leaves found

in the inner canopy may have very low photosynthesis because of reduced sunlight interception (Fitter and Hay, 2012), which can lead them to compete with inside canopy fruit for assimilates and results to reduced fruit quality in general.

Canopy position effect on 'Marsh' grapefruit internal parameters

Fruit from outside canopy had higher TSS (10.7 ±0.21 vs. 9.54 ±0.53 and 9.82 ±0.1 vs. 9.75 ±0.12) and lower TA (1.34 ±0.04 vs. 1.41 ±0.35 & 1.24 ±0.15 vs. 1.45 ±0.66) compared with fruit from inside canopy in LP and KZN, respectively (Table 1). The results meant that fruit from outside canopy were at higher maturity level. That was related to lower CI and higher RP incidence observed when they were compared to fruit from inside canopy. Similar results were also observed by Ezz and Awad (2009). In addition, the maturity index (TSS: TA) (6.97 ±0.27); citrus colour index (1.38 ±0.1); BrimA3 (5.52 ±0.18); BrimA4 (4.11 ±0.22) and BrimA5 (2.70 ±0.26) of LP IN was lower than that of LP OUT (8.19 ±0.27; 6.70 ±0.18; 4.11 ±0.22; 4.03 ±0.27 and 1.47 ±0.1, respectively). The higher maturity of fruit from outside canopy was related to higher fruit ability to resist storage conditions causing CI compared to less mature inside canopy fruit. However, the weakness of that discussion was shown by the complete opposite results observed in KZN.

The maturity index (TSS: TA) (8.00 ±0.27), citrus colour index (1.69 ±0.1); BrimA3 (5.81 ±0.18); BrimA4 (4.57 ±0.22) and BrimA5 (3.33 ±0.28) of KZN IN were higher, meaning higher maturity, than that of KZN OUT (6.98 ±0.27; 1.51 ±0.1; 5.48 ±0.18; 4.03 ±0.22 and 2.58 ±0.29, respectively). However, KZN IN had higher CI incidence compared to KZN OUT. The mass of fruit from KZN IN (401.5 ±10.5) was higher than that of KZN OUT (381.5 ±10.5), LP IN (378.0 ±10.5) and LP OUT (344.5 ±10.5). Fruit rind can become wrinkled after prolonged postharvest storage. Generally, citrus fruit with such wrinkled rinds develop sour taste because of accumulation of acidity and/or reduction of sugars (Ladanyia and Ladaniya, 2010). Therefore,

citrus internal parameters direct or indirectly affect the rind quality of fruit, probably because they can serve as storage compounds a fruit can utilize during postharvest.

Table 1: The fruit parameters of 'Marsh' grapefruit from different regions and canopy positions

Parameter	Fruit Origin	Mean ± SE	SD	CV%	LSD	Overall *p*-value
TSS (%)	LP IN	9.75 ±0.12a	0.58	5.90	-	-
	LP OUT	10.7 ±0.21b	0.48	4.44	-	-
	KZN IN	9.54 ±0.53c	0.59	6.22	-	-
	KZN OUT	9.82 ±0.10ab	0.45	4.55	-	-
				*5.50	*0.36	*<0.01
TA (%)	LP IN	1.41 ±0.35a	0.14	9.90	-	-
	LP OUT	1.34 ±0.04ab	0.20	14.83	-	-
	KZN IN	1.24 ±0.15b	0.26	20.82	-	-
	KZN OUT	1.45 ±0.66a	0.26	18.18	-	-
				*15.5	*0.14	*<0.01
Maturity index	LP IN	6.97 ±0.37a	0.71	10.18	-	-
	LP OUT	8.19 ±0.25b	1.30	15.86	-	-
	KZN IN	8.00 ±0.67b	1.77	22.10	-	-
	KZN OUT	6.98 ±0.26a	1.22	17.47	-	-
				*0.79	*0.79	*<0.01
BrimA3	LP IN	5.52 ±0.48a	0.79	29.13	-	-
	LP OUT	6.70 ±0.43c	1.04	25.76	-	-
	KZN IN	5.81 ±0.55b	1.30	39.06	-	-
	KZN OUT	5.48 ±0.18a	1.36	52.56	-	-
				*14.10	*0.54	*<0.05
BrimA4	LP IN	4.11 ±0.25a	0.70	16.92	-	-
	LP OUT	5.36 ±0.12c	0.87	16.13	-	-
	KZN IN	4.57 ±0.26d	1.07	23.50	-	-
	KZN OUT	4.03 ±0.52ab	1.11	27.60	-	-
				*22.60	*0.65	*<0.05
BrimA5	LP IN	2.70 ±0.26a	0.62	11.28	-	-
	LP OUT	4.03 ±0.27b	0.71	10.56	-	-
	KZN IN	3.33 ±0.28c	0.87	14.91	-	-
	KZN OUT	2.58 ±0.29d	0.88	16.02	-	-
				*39.70	*0.77	*<0.05
Mass	LP IN	378.0 ±11.55a	50.69	13.41	-	-
	LP OUT	344.5 ±13.48b	58.69	17.04	-	-
	KZN IN	401.5 ±9.53c	45.02	11.21	-	-
	KZN OUT	381.5 ±15.49d	32.367	8.48	-	-
				*13.60	*30.70	*<0.01
Citrus Colour Index	LP IN	-1.38 ±0.12a	0.22	16.19	-	-
	LP OUT	-1.47 ±0.09ab	0.38	25.48	-	-
	KZN IN	-1.69 ±0.10c	0.37	21.78	-	-
	KZN OUT	-1.51 ±0.15b	0.29	19.18	-	-
				*29.10	*0.29	*<0.05

TSS, total soluble solutes; TA, titratable acidity; BrimA3, Brima4 and BrimA5, TSS minus TA with factor 3, 4 and 5, respectively; LP, Limpopo; LP IN, fruit from inside canopy of the orchard in Limpopo; LP OUT, fruit from outside canopy position of the orchard in Limpopo; KZN, KwaZulu-Natal; SE, standard error; SD, standard deviation; CV%, coefficient of variation; LSD, least significant difference; values with * presents the overall statistics between all treatments; data reported with letters show the significance of difference if different from each other based on Duncan analysis

Grapefruit parameters changing pattern during postharvest storage

To assess the average changing pattern of fruit's parameters during storage, all samples were analyzed as one batch without considering the difference in growing regions and canopy positions.

Total soluble solutes, titratable acidity, maturity index and BrimA

The sugars were analyzed as TSS while acidity analysis was based on titratable acids. TSS increased with storage while TA gradually decreased (Table 2). Citrus fruit ripening process is characterized by the increasing juice sugar level while its acidity depreciates with time, which results in their characteristic sweetness and flavour (Magwaza *et al*., 2013). The difference (BrimA) and the ratio (maturity index) between the two parameters increased as the TSS was increasing.

Table 2: The changing pattern of 'Marsh' grapefruit parameters during storage

Time	TSS (%)	TA (%)	TSS: TA	BrimA5	BrimA4	BrimA3
Week 0	10.04 ±0.7a	1.42 ±0.3a	7.28 ±1.3c	2.95 ±1.1a	4.37 ±0.9a	5.78 ±1.3a
Week 2	10.04 ±0.8a	1.45 ±0.2b	7.10 ±1.1b	2.76 ±1.2b	4.21 ±1.1b	5.67 ±1.0a
Week 4	10.25 ±0.8b	1.52 ±0.2c	6.82 ±0.9a	3.35 ±1.4c	4.73 ±1.2c	6.11 ±1.1b
Week 6	10.12 ±0.7ab	1.46 ±0.1b	6.95 ±0.9b	2.82 ±1.0a	4.3 ±0.9ab	5.74 ±0.9a
Week 7	10.35 ±0.9c	1.34 ±0.2a	7.36 ±0.9c	3.2 ±1.1bc	4.59 ±1.0c	6.0 ±0.9ab

Data presented as mean ±standard deviation (p = 0.01). TSS, total soluble solids (%); TA, titratable acidity (%); BrimA= TSS- k. TA; Week 0, at harvest; Week 4, four weeks in cold storage.

Mass loss

Fruit mass loss was also investigated (Fig. 8). The average mass depreciated with time, and the depreciation rate was temperature and RH dependent. The mass loss of fruit during cold storage showed an increasing trend until week 4 and decreased thereafter. The decrease in mass loss rate was hypothesized to be caused by the fruit ability to develop tolerance to cold storage. Plant tissues produce heat shock proteins to reduce sensitivity to uncommon conditions such as after extreme temperatures (Siboza *et al.*, 2014). Those proteins reduce respiration rate of fruit and therefore, reduce mass loss. A change in respiration rate after 4 weeks in cold storage was also observed on 'Nules Clementine' mandarins stored at 7.5 ºC (Cronje *et al*., 2011). During respiration, fruit degrade storage compounds such as proteins, carbohydrates and fats (Kruger, 1990). Therefore, returning fruit to room temperature was hypothesized to increase rates of physiological reactions such as respiration and evaporation which led to an increase in mass loss.

The rate of mass loss during shelf life (week 6 to week 7) at room temperature was higher than when fruit were in cold storage (week 2 to week 6) at 5 °C and 95% RH. The increase in mass loss rate during shelf life compared with cold storage conditions was related to increased temperature and decrease in RH causing higher respiration rate. Fruit mass is the one parameter determining the selling price of fruit in supermarkets. Fruit are weighed and the price rated based on the mass the customer buys. The harvest fruit size and rate of mass loss are vital parameters during packing, packaging and export, considering that fruit shrink with prolonged postharvest period and lose their fresh appearance, which gradually depreciates their quality (Ladanyia and Ladaniya, 2010).

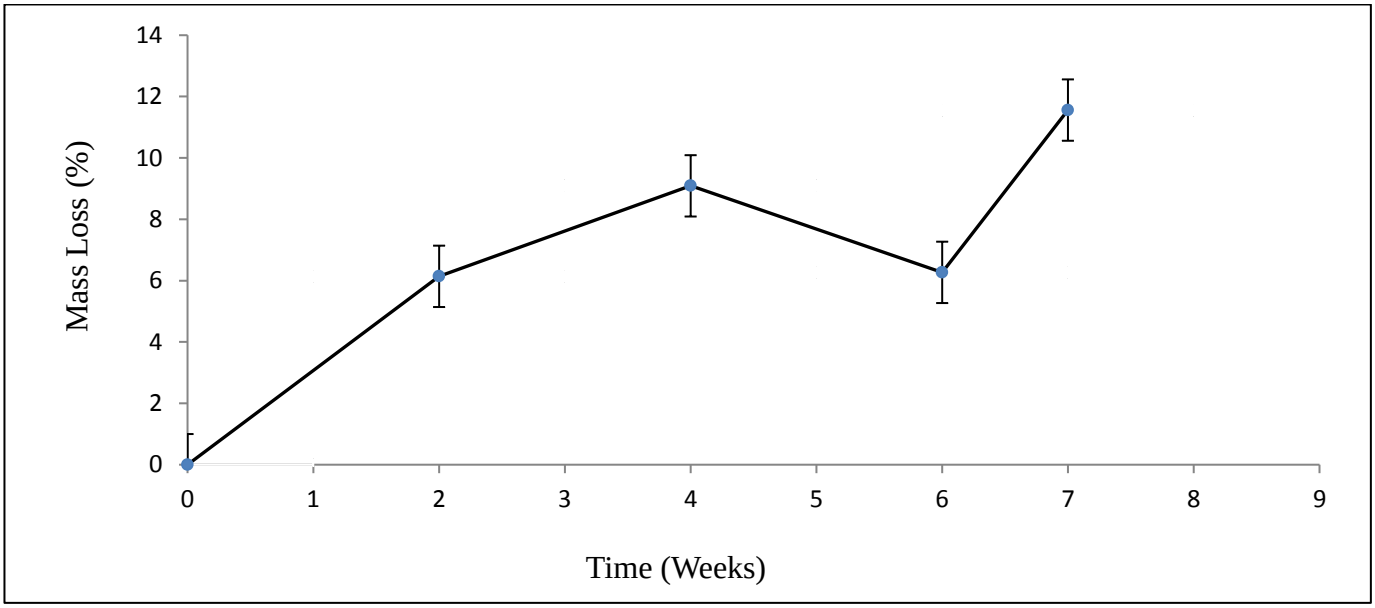

Fig. 8: The overall mass loss trend during postharvest storage of fruit. Data presented as means ± 5% standard error.

Colour

Colour is one of the most important characteristics for attracting consumers to edible produce. In this study, the average 'Marsh' grapefruit citrus colour index (CCI) was -1.56 just after harvesting and decreased to -2.32 after six weeks of cold storage (Fig. 9). The average CCI of fruit increased rapidly during shelf life to the value of -1.75. The sudden increase was related to increased temperatures when fruit were taken out of cold storage, which accelerated the physiological processes occurring in the fruit (Kader, 1997). Citrus is harvested when fully matured, as it is a non-climatic fruit, while over-maturity is avoided to ensure a longer postharvest quality maintenance by the fruit (Kader, 2002). In white grapefruit such as 'Marsh', the colour of fruit is difficult to be used as a harvesting parameter and the fruit does not significantly change its colour upon maturity and ripening. Therefore, the slight changes observed could not be clearly aligned with the maturity or ripening level of the fruit.

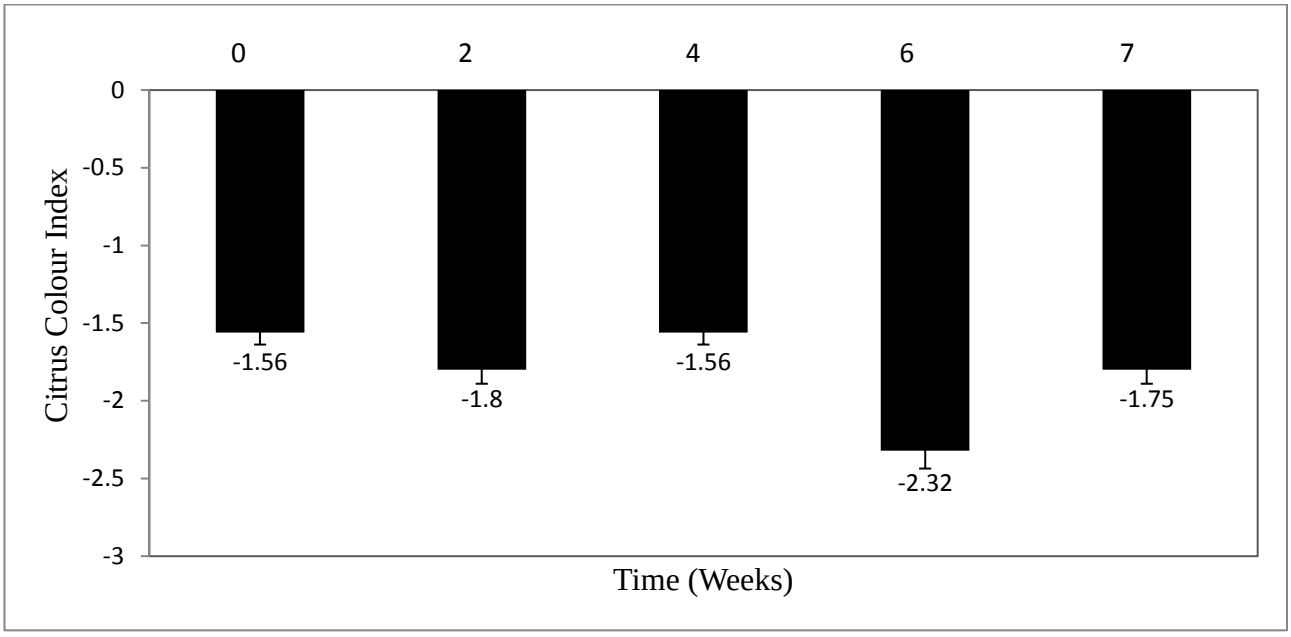

Fig. 9: The citrus colour index changing pattern during 'Marsh' grapefruit cold storage and one week on shelf. Data presented as mean ± 5% standard error.

Disorders occurrence trend during the storage period

There was an increasing incidence of disorders over time (Table 3). Chilling injury occurrence appeared earlier than RP in as early as sampling at 2 weeks of cold storage. Limpopo inside canopy was the only fruit origin that developed CI after 2 weeks and had a chilling injury index (CI_i) of 0.02. The rind pitting development began to show after four weeks of cold storage, which was after 2 weeks of quarantine treatment (-0, 5 ºC) and 2 weeks of cold storage (5 ºC). This phenomenon was thought to be caused by a sudden increase in temperature of the atmosphere surrounding the fruit rind from -0, 5 ºC to 5 ºC, and further increased to room temperature during the week of shelf life assimilation. There was lower CI_i on fruit from inside canopy in all sampling intervals. Fruit from LP developed higher CI than fruit from KZN. Fruit from KZN OUT did not develop CI and had consistently lower RP when compared with LP OUT fruit.

It was deduced from the results that exporting fruit with a faster transport or shipping to closest international markets can save fruit from injuries and ensure a trustworthy exporting warranty. However, most South African citrus exports are aimed at countries situated in climacteric regions such as Netherlands and Japan that rely on imports of citrus as they cannot grow their own because of unsatisfactory climatic conditions. These countries are far from South Africa, which reduces the possibility of using other means of faster transport such as vehicles or railway transport. The only option is shipping, which takes a long time (6 weeks) that allows development of disorders. This excites the requirement for alternative means to reduce the disorders or strict selection of fruit capable for exports.

Table 3: The incidence of disorders during storage period of fruit (expressed as indexes)

Sampling time	LP IN		LP OUT		KZN IN		KZN OUT	
	CI_i	RP_i	CI_i	RP_i	CI_i	RP_i	CI_i	RP_i
Week 0	-	-	-	-	-	-	-	-
Week 2	0.02	-	-	-	-	-	-	-
Week 4	0.11	-	-	0.01	0.01	-	-	0.01
Week 6	0.22	0.01	0.02	0.03	0.14	0.01	-	0.01
Week 7	0.46	0.22	0.2	0.17	0.42	0.02	-	0.15

LP IN, Limpopo inside canopy fruit; LP OUT, Limpopo outside canopy fruit; KZN, KwaZulu-Natal; Week 2, two weeks after harvest; RP_i, rind pitting index; CI_i, chilling injury index

The correlation of grapefruit parameters to rind disorders and to one another

The Pearson correlations of internal parameters, rind parameters and rind disorders were tested after normalization of data to equalize every parameter or disorder values contributions (Table 4). The correlation of parameters to disorders was very weak. This was interpreted to express the difficulty of spotting the exact

factors contributing to the disorders. However, the nature of the relationship between each parameter and the disorders was observed. Fruit luminosity, titratable acidity, rind dry matter and fructose showed a negative correlation to RP with R^2- values of -0.07; -0.01; -0.02 and -0.02, respectively. This meant that fruit with dimmer colour, sweeter in taste and had lower moisture in the rind had higher RP incidence. Low rind moisture content and postharvest rind moisture loss have been correlated to rind disorders previously (Cohen *et al*., 1994; Alférez and Burns, 2004; Magwaza *et al*., 2014c). Although all parameters had weak correlation to RP, BrimA3 had highest positive correlation ($R^2 = 0.36$) followed by TSS ($R^2 = 0.33$), glucose ($R^2 = 0.33$) and total antioxidants capacity ($R^2 = 0.30$), respectively.

Weak correlation of parameters to CI also existed. Citrus colour index, total antioxidant capacity, phenolic compounds, and rind glucose had a negative correlation to CI incidence with R^2-values of -0.43; -0.25; -0.85 and -0.47, respectively. This means fruit with greener colour; higher antioxidant capacity, higher phenolic compounds and higher rind glucose content had lower CI occurrence. The fruit surface hue, luminosity, antioxidant activity and fructose had a positive correlation to CI with R^2-values of 0.39; 0.35; 0.29 and 0.28, respectively. Although the correlations of parameters to disorders were weak, it was important that the relationship of each parameter to disorders was explored. An alternative aim of this study was to find the relationship between grapefruit physiological parameters and the disorders, which was slightly satisfied by finding whether the relationship was positive or negative.

The noticeable trend on the correlation between grapefruit parameters was that there was a higher correlation of parameters from the same tissue. The rind parameter with the highest correlation to internal parameter was an antioxidant activity which had a correlation value (R^2) of 0.67 and 0.63 to TSS and TA, respectively. Rind total antioxidant capacity had the lowest correlation to internal parameters with an R^2-value of -0.03 and -0.05 to TSS: TA ratio and TA, respectively. Colour indices (Luminosity; Hue; CCI) correlated with rind parameters more than they correlated with internal parameters, which was aligned with the proximity of those parameters.

RP, rind pitting; CI, chilling injury; L, luminosity; H, HUE CCI, citrus colour index; DM, dry matter;TSS, total soluble solids; TA, titratable acidity; BrimA3, BrimA with factor 3; Total Ant, total antioxidant capacity; Ant Act, antioxidant activity; Phenols, total phenolic compounds

Table 4: The correlation between parameters and their correlation to rind disorders

	RP	CI	L	H	CCI	TSS%	TA%	BrimA3	TSS:TA	DM%	Mass Loss	Total Ant	Ant Act	Phenols	Glucose	Sucrose	Fructose
RP	1																
CI	0.13	1															
L	-0.07	0.35	1														
H	0.03	0.39	**0.94**	1													
CCI	0.10	-0.43	**-0.77**	**-0.83**	1												
TSS%	0.33	0.19	0.50	0.69	-0.35	1											
TA%	-0.01	0.05	0.44	0.50	-0.26	0.40	1										
BrimA3	0.36	0.17	0.29	0.46	-0.23	**0.85**	-0.13	1									
TSS:TA	0.23	0.25	0.41	0.49	-0.35	0.64	-0.37	**0.90**	1								
DM%	-0.02	0.22	**0.74**	0.67	-0.51	0.39	0.08	0.38	0.51	1							
Mass Loss	0.06	0.23	**0.80**	**0.81**	**-0.71**	0.48	0.50	0.23	0.25	0.62	1						
Total Ant	0.30	-0.25	-0.52	-0.42	**0.70**	0.20	-0.05	0.25	-0.03	-0.34	-0.45	1					
Ant Act	0.09	0.29	**0.82**	**0.91**	**-0.75**	0.67	0.63	0.37	0.32	0.52	**0.77**	-0.45	1				
Phenols	0.11	-0.48	**-0.85**	**-0.92**	**0.99**	-0.39	-0.29	-0.26	-0.40	-0.56	**-0.79**	**0.78**	**-0.83**	1			
Glucose	0.33	-0.23	-0.47	-0.34	0.59	0.36	-0.13	0.47	0.20	-0.30	-0.44	**0.89**	-0.34	0.65	1		
Sucrose	0.20	0.20	**0.71**	**0.66**	-0.48	0.32	0.48	0.07	0.08	0.49	**0.74**	-0.28	0.52	-0.53	-0.35	1	
Fructose	-0.02	0.28	**0.76**	**0.70**	**-0.75**	0.09	0.37	-0.11	0.05	0.58	**0.74**	**-0.74**	0.64	**-0.83**	**-0.79**	**0.77**	1

The highest correlation of colour indices to rind parameters was found on the correlation of rind phenolic compounds content to citrus colour index ($R^2 = 0.99$), and hue correlation to phenolic compounds ($R^2 = 0.92$) and antioxidant activity ($R^2 = 91$). These results were interpreted to mean that fruit with greener colour had higher phenolic compounds and higher antioxidant activity. It was found interesting that fruit mass loss had a high positive correlation to Luminosity and Hue with R^2-values of 0.80 and 0.81, respectively. This was aligned to a fruit changing colour while it continually loose mass after it was harvested.

The use of PCA models to separate fruit based on canopy position and PCA-based correlation of fruit parameters to disorders

Two separate clusters of data points, one on the right and another on the left side of the y-axis, revealed distinct grouping of fruit spectra from inside and outside canopy, respectively (Fig. 10; Fig. 11). The first two principal components (PC) accounted for 71% of total variability of fruit from Limpopo (Fig. 9). PC-1 contributed 60% of variation and PC-2 contributed 11% of the variation during mapping. The clustering results were also observed in a study by Magwaza *et al*. (2014a). In that study, the combination of colour, moisture content, and carbohydrates of 'Nules Clementine' mandarin rind was reportred to play an important role in discriminating fruit from different canopy positions. Those parameters were also hypothesized as the major characteristics enabling distinction of grapefruit from different canopy position in this study.

For a clear view of how canopy position and rind disorders incidence correlated with each other as well as other fruit quality parameters, data sets for both LP and KZN were subjected to principal component analysis (PCA) loadings and bi-plots in Fig. 10 and Fig. 11. PCA bi-plot was used to recognise the main contributions or correlations of fruit parameters to fruit susceptibility. A positive correlation was based on parameters proximity to fruit origin. Parameters on the same quadrant with a disorder had a positive correlation to the disorders and contributed significantly to the difference in fruit susceptibility, and fruit distinction according to canopy during mapping. In Fig. 10, a positive correlation of rind pitting to rind dry matter, rind total antioxidants, fruit citrus colour index, juice BrimA, juice TSS and rind phenolic compounds content was observed. The positive correlation of RP to these parameters was also presented in previous figures showing higher RP incidence and higher amount of the parameters on LP OUT fruit compared to LP IN fruit. The fruit from LP IN had a higher CI incidence which was aligned with a positive contribution of fruit mass loss, rind sucrose, fruit hue angle, fruit lightness, rind antioxidant capacity, juice titratable acidity, rind fructose and rind sucrose (Fig. 10).

In the PCA loadings and bi-plot represented in Fig. 11, the first two PCs contributed 67% of the distinction of KZN fruit from inside or outside canopy position. PC-1 contributed 49% and PC-2 contributed 18% of variation and mapping. The KZN OUT fruit had higher RP incidence compared to KZN IN, which was aligned to the positive contribution of TSS, glucose, TA, total antioxidants compounds, citrus colour index and phenolic compounds. A positive correlation between TSS and RP was also found in the study by Ezz and Awad (2009) on 'Marsh' grapefruit. Fruit from KZN IN had higher CI incidence when compared to fruit from KZN OUT, which was aligned with a positive contribution of fruit mass loss, rind fructose, rind dry matter, juice TSS: TA ratio, juice BrimA, rind antioxidant capacity, fruit lightness and hue angle.

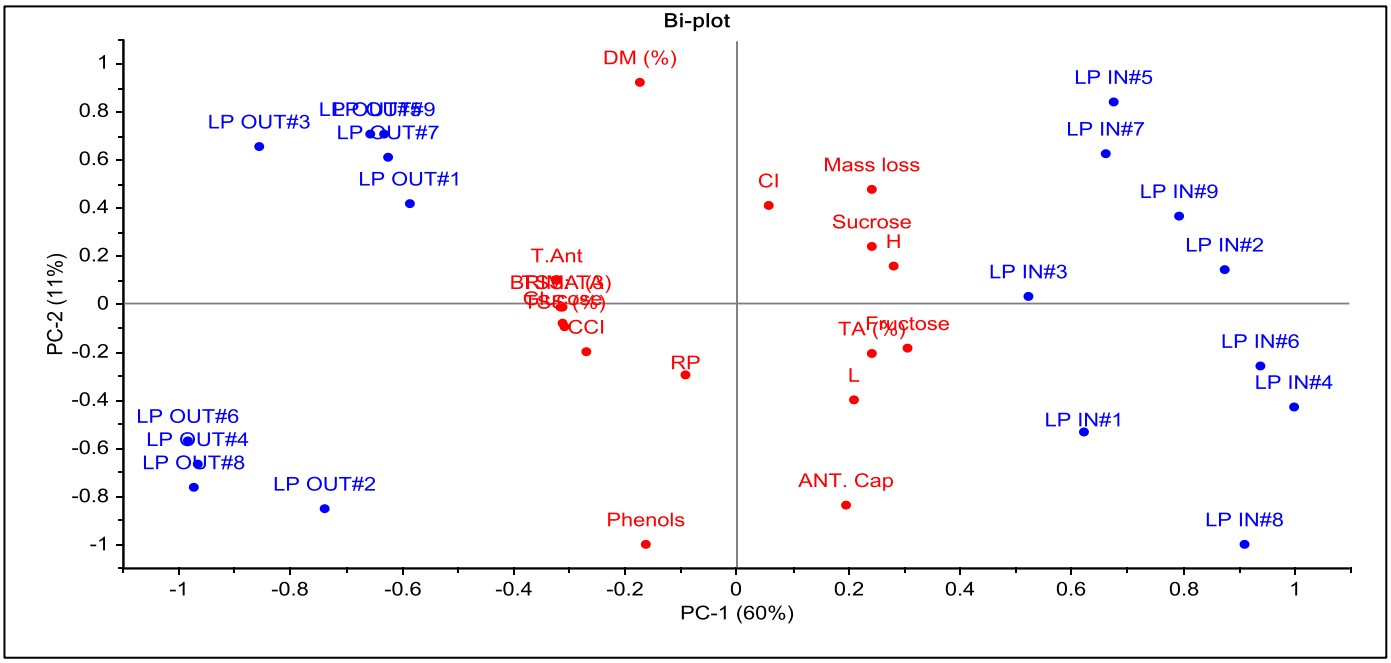

Fig. 10: The principal component analysis-based correlation of parameters to fruit origin. LP, Limpopo; LP IN, Limpopo inside canopy fruit, LP OUT, Limpopo outside canopy fruit; DM (%), percentage rind dry matter; CI, chilling injury; H, hue angle; L, Lightness; TA (%), titratable acidity percentage; ANT. Cap, antioxidant capacity; RP, rind pitting; CCI, citrus colour index; T. Anti, total antioxidant capacity; TSS (%), total soluble solutes

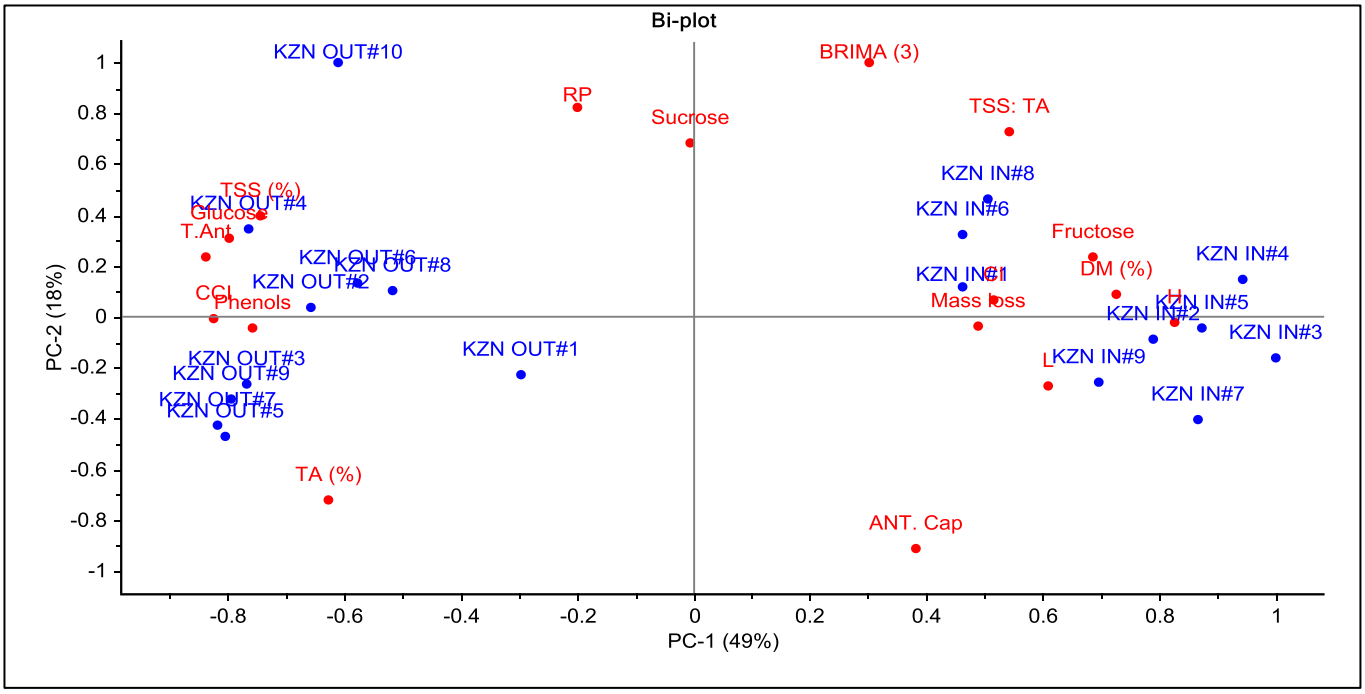

Fig. 11: The principal component analysis-based correlation of parameters to fruit origin. KZN, KwaZulu-Natal; KZN IN, KwaZulu-Natal inside canopy fruit, KZN OUT, KwaZulu-Natal outside canopy fruit; DM (%), percentage rind dry matter; CI, chilling injury; H, hue angle; L, Lightness; TA (%), titratable acidity percentage; ANT. Cap, antioxidant capacity; RP, rind pitting; CCI, citrus colour index; T. Anti, total antioxidant capacity; TSS (%), total soluble solutes

4.5 Conclusion

In this study, BrimA, TSS, rind glucose and rind total antioxidants capacity were found to have a positive correlation to RP. Citrus colour index, rind total antioxidant capacity, rind phenolic compounds and rind glucose had a negative correlation to CI incidence. The fruit surface hue, luminosity, rind antioxidant activity and rind fructose had a positive correlation to CI. Although the correlations of parameters to disorders were weak, it was important that the relationship, whether negative or positive, of each parameter and the disorders, was explored. Fruit from different canopy position had different susceptibility to disorders and the content of physico-chemical parameters differed significantly. PCA-based discrimination of fruit from different canopy

positions was also successful. This can enable the prediction of parameters related to disorders for estimation of disorder developing chances. Further studies with similar objectives on determining the parameters correlated to rind disorders, and their predictions non-destructively are recommended.

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Chapter 5: Non-destructive prediction of rind pitting and quality of 'Marsh' grapefruit (*Citrus x paradisi* MacFad) using Vis/NIR spectroscopy

5.1 Abstract

Postharvest rind pitting (RP) of 'Marsh' grapefruit (*Citrus* x *paradis* MacFad) reduces the guarantee of shipping fresh fruit to international markets. However, the disorder develops 3-5 weeks after harvest, which makes it difficult to detect during sorting and packing. In this study, visible to near infrared (Vis/NIR) spectral data was acquired from fruit, just after harvest. Spectral data was acquired in reflectance mode using a laboratory bench-top monochromator NIR Systems Model XDS spectrometer equipped with a quartz halogen lamp and lead sulfide (PbS) detector. The data was correlated to the visual scores of RP disorder development after six weeks in cold storage and a week in shelf life. Partial least square (PLS) regression models were developed using Unscrumbler® chemometric software. Good prediction of RP was obtained (R^2_p = 0.78; RPD = 2.03; RMSEP = 1.41). Prediction models of rind quality parameters successfully developed included total antioxidant capacity (RMSECV = 0.04; R^2_{cv} = 0.95), β carotene (RMSECV = 0.002; R^2_{cv} = 0.99), total carotenoids (RMSECV = 2.51; R^2_{cv} = 0.92), chlorophyll a (RMSECV = 0.008; R^2_{cv} = 0.89), chlorophyll b (RMSECV = 0.008; R^2_{cv} = 0.93), dry matter (RMSECV = 0.297; R^2_{cv} = 0.88), sucrose (RMSECV = 0.021; R^2_{cv} = 0.91), glucose (RMSECV = 0.013; R^2_{cv} = 0.93) and fructose (RMSECV = 0.023; R^2_{cv} = 0.94). PLS models for internal quality parameters were also developed successfully. The ability of Vis/NIR spectroscopy coupled with chemometric analysis to cluster fruit based on original canopy position during sorting and packaging was recognised and recommended as a secondary approach to discriminate fruit with high chances of developing RP, since its occurrence was high on fruit from outside canopy.

Keywords: Citrus, Rind Disorders, Prediction, NIRS, Grapefruit

5.2 Introduction

Citrus production is a very expensive business considering the amount of capital involved in orchard establishment, management, irrigation and fertilization, wages, postharvest fruit treatment and lastly, exports. Management of all these practices, especially exporting, is crucial since it's in the last part of the business chain. Failure to manage exports may result in loss of all the investments previously made. A good appearance of edible produce is critical for attracting consumers in the market. Customers use the outside appearance to estimate the internal quality of citrus fruit, and the appealing rind is usually preferred for purchases because it is aligned with satisfaction in flavour at a time of consumption (Magwaza *et al.*, 2013a).

During exports, South African fresh citrus fruit are shipped under cold storage at temperatures below 10 °C to international markets (Department of Agriculture Fisheries and Forestry, 2015). However, horticultural fruit crops originating from subtropical and tropical regions may develop chilling physiological rind disorders when stored for extended periods of cold, but above freezing temperatures (Bassal and El-Hamahmy, 2011; Chaudhary *et al.*, 2014). In citrus, particularly 'Marsh' grapefruit, these disorders are of great concern as grapefruits are exposed to ± -0.6 °C for at least 14 days for quarantine purpose against the Mediterranean fruit flies (*Ceratitis capitate* and *Ceratitis rosa*) and stored at 5°C during shipping (Sevillano *et al.*, 2009; Bassal and El-Hamahmy, 2011). White grapefruit ('Marsh') was noticed to have lower resistance to chilling injury (CI) and rind pitting (RP) disorders compared to red cultivars in previous studies (Alferez and Burns, 2004; Lado *et al.*, 2015; Lado *et al.*, 2016).

The 'Marsh' grapefruit higher susceptibility to physiological rind disorders was hypothesized to be caused by various internal biochemical attributes, including lower antioxidant and phenolic compounds, compared to other cultivars with red rinds (Sevillano *et al.*, 2009; Lado *et al.*, 2016). Rind dry matter, non-structural carbohydrates, chlorophyll and carotenoid contents, and harvest maturity are among physiological attributes

related to a difference in susceptibility to physiological disorders of citrus fruit within a single cultivar (Assimakopoulou *et al*., 2009; Cronje, 2009; Magwaza *et al.,* 2013a). Postharvest handling factors such as waxing, fungicides application, storage temperature, and relative humidity have been explored as other factors determining the extent of the disorders' incidence (Alférez *et al*., 2003; Alferez and Burns, 2004; Cronje *et al.* 2011a; Magwaza *et al.,* 2013a).

Rind physiological disorders of citrus fruit develop symptoms after 3-5 weeks in postharvest, which coincides with the time they reach the designated international market (Magwaza *et al*., 2013b). Although the disorder does not affect the internal quality, fruit with rind disorders are considered as waste in the fresh fruit market. Therefore, means to predict the possibility of each fruit developing chilling disorders at postharvest prior to shipping is needed. The factors or combination of factors leading to fruit susceptibility may be used as pre-symptomatic markers. In literature, previous studies have shown a high potential of visible to near infrared radiation spectroscopy (Vis/NIRS), together with capable chemometric software, to accurately predict internal and external physiological attributes of citrus (Gómez *et al*., 2006; Nicolaï *et al*., 2007; Huang *et al*., 2008; Lin and Ying, 2009; Magwaza *et al*., 2012; Liu *et al*., 2010 a, b; Cayuela and Weiland, 2010; Magwaza *et al*., 2013b; 2014a, b, c; 2015). Vis/NIRS studies biological sample characteristics by illuminating a sample with radiation and measuring radiation reflection or transmission. The radiation changes its spectral characteristics while it penetrates the product depending on the chemical composition and microstructures of the product. The spectral change is wavelength dependent and causes scattering or absorption at certain spectral regions which can be used to extract important information from the interior of the sample (Nicolaï *et al*., 2007).

It was previously proposed that the application of Vis/NIRS for predicting rind physiological disorders of citrus is possible (Magwaza *et al.,* 2012a). Studies predicting rind disorders on grapefruit are limited. However, near infrared spectroscopy was successfully used for predicting surface defects on peach fruit (Miller and Delwiche, 1991), surface bruising on apples (Geeola *et al*., 1994), internal drying disorder in tangerine citrus (Peiris *et*

al., 1998), storage disorders of kiwifruit (Clark *et al*., 2004) and pericarp hardening of mangosteen fruit (Teerachaichayut *et al*., 2011). Moreover, the alignment of fruit susceptibility with physio-chemical attributes and the ability of Vis/NIRS to predict rind internal attributes of fruit excite the possibility of predicting rind physiological disorders. Magwaza *et al*. (2012b) attempted to predict rind breakdown disorder of 'Nules Clementine' mandarins harvested from different canopy positions within the tree. Although their study did not successfully predict the disorder, it was deduced that a good correlation of spectra to rind carbohydrates content, rind dry matter, colour index and mass loss, and ability of NIRS to segregate samples into canopy position-based clusters demonstrated a high potential of Vis/NIRS to non-destructively predict mandarin fruit susceptibility to rind breakdown disorder. In the previous chapter, biochemical compounds with positive or negative correlation to 'Marsh' grapefruit CI and RP were explored. In this study, Vis/NIRS is evaluated as a tool to predict postharvest physiological rind pitting of 'Marsh' grapefruit and its pre-symptomatic markers.

5.3 Material and methods

Fruit sampling

The 'Marsh' grapefruit (*Citrus* x *paradisi* Macfad) for this study was harvested during 2015/2016 season. A total of 240 mature fruit, mixing all possible sizes, were harvested from inside or outside canopy position of randomly selected trees from the following two orchards: Hoedspruit in Limpopo Province (24°23'39.02"S; 30°49'20.65"E) and at Enkwalini in KwaZulu-Natal province (32°75'28.S; 35°89'31.E), South Africa. The fruit were transported with a ventilated vehicle to postharvest physiology laboratory of the University of KwaZulu-Natal where analysis was done. Upon arrival at the laboratory, fruit treatments assimilating commercial chain were applied.

Postharvest treatments and storage

Fruit were washed with Imazalil® fungicide (Farmalinx Pty. Ltd.; Bondi Jucntion; Australia) and coated with Citrishine® wax (Citrashine Pty. Ltd.; Decco; Johannesburg; South Africa) with concentrations prepared based on recommendations on container labels. They were left overnight in open space at room temperature to allow the coating to dry. In the next morning, 20 fruit from each treatment (growing region by canopy position interactions) were taken and analyzed for after-harvest sampling. The remaining fruit were labeled and transferred into the cold room. Cold storage started as a quarantine treatment for 2 weeks at ± -0.6 ºC; 95 ±1% relative humidity (RH), and the storage container temperature was later raised to 5 ºC for the subsequent 6 weeks (NDA, 2014). Thereafter, fruit were taken back to room temperature (21°C) to assimilate shelf life that would happen after reaching designated market.

Vis/NIRS spectra collection

Vis/NIR spectral data was acquired in reflectance mode using a laboratory bench-top monochromator NIR Systems Model XDS spectrometer (FOSS NIR Systems, Inc.; Maryland, USA) equipped with a quartz halogen lamp and lead sulfide (PbS) detector. The system was calibrated by scanning a 100% white reference tile to provide background reference prior fruit scanning, and periodically at 30 min intervals of scanning fruit, to reduce baseline shift of spectral data (Magwaza *et al*., 2014b, c). The full visible to near infrared reflectance spectrum (450 to 2500 nm) was acquired from two opposite sides along equatorial region of the fruit and recorded as log 1/reflectance (log 1/R). Each spectrum was the average of 32 scans recorded using Vision software (Vision TM, version 3.5.0.0, Tidestone Technologies Inc., KS, USA).

TSS, TA, BrimA and Maturity index

Each fruit was cut in half and squeezed to collect juice for total soluble solutes (TSS) and titratable acidity (TA) analysis. The collected juice was homogenized using a solution stirrer (ULTRA-TURRAX; IKA® T25 digital; Germany) and tested for TSS using a digital refractometer with a dynamic control system (RFM340+ BS®, Bellingham and Stanley Ltd, Basingstoke, Hants, UK). TSS was recorded as ºBrix which is equivalent to TSS %. The remaining juice was snap frozen in 120 mL plastic specimen jars and stored in a freezer set at -20 °C for analysis of TA in less than two days after extraction.

Titratable acidity was analyzed by mixing 10 mL juice with 50 mL distilled water and titrating with 0.1 M sodium hydroxide (NaOH) to the end point (pH of 8.1). The volume of NaOH titrated to endpoint was recorded and the citrus acid formula (Eq. 1) was applied to calculate TA, expressed as % citric acid.

$$\text{TA (\% citric acid)} = \frac{0.0064\ X\ titre\ (NaOH)\ ml\ X\ 100}{10\ ml\ juice} \quad (1)$$

BrimA was calculated based on the formula by Obenland et al. (2009), Eq. 2.

$$BrimA = TSS - k\ (TA) \quad (2)$$

Where, *k* is a constant that reflects the tongue's higher sensitivity to TA compared to TSS. In this study the *k*-value used is 4 and presented as BrimA*k*. For example, BrimA4 is BrimA with factor 4.

The ratio of TSS to TA, also known as citrus maturity index was calculated using Eq. 3.

$$TSS\ to\ TA\ ratio\ (maturity\ index) = \frac{\mathrm{TSS}}{\mathrm{TA}} \quad (3)$$

Rind dry matter determination

To measure rind dry matter, a fresh rind sample was weighed to obtain fresh mass, freeze-dried and weighed again to obtain dried mass. The dried mass was divided by fresh mass to calculate a percentage dry matter (DM %) (Eq.5):

$$Dry\ matter\ (DM\%) = \frac{dried\ mass}{initial\ fresh\ mass} \quad (5)$$

Determination of carotenoids and chlorophyll

The extraction and quantification of chlorophyll and carotenoids were executed following a procedure by Lichtenthaler (1997) with slight modification. Briefly, rind powder (1g) was extracted using 80% v/v acetone (8ml). The samples were left in glass tubes to stand for 10 min on ice covered with aluminium foil. Thereafter, they were homogenized in Ultra-Turrax using 1-minute bursts twice and centrifuged for 10 min at 4 °C in pre-cool centrifuge. The absorbance of samples was read using a UV-1800 Spectrophotometer (Shimadzu Scientific Instruments INC., Columbia, USA) at the wavelengths required for calculations of the pigments. The concentrations of chlorophyll a (Chl_a), chlorophyll b (Chl_b), total chlorophylls (Chl_{a+b}), and total carotenoids (C_{x+c}) were calculated using the equations below (Lichtenthaler, 1987). The β-carotene concentration was calculated using formula after Svjetlana and Kristina (2010). Results were expressed as µg pigment/ mL extracting solution, which was later converted to µg /g on dry mass basis.

$$Chl_a = 12.25\ A_{663.2} - 2.79\ A_{646.8} \quad (6)$$

$$Chl_b = 21.50\ A_{646.8} - 5.10\ A_{663.2} \quad (7)$$

$$Chl_{a+b} = 7.15\ A_{663.2} + 18.71\ A_{646.8} \quad (8)$$

$$C_{x+c} = (1000\ A470 - 1.82\ Chl_a - 85.02\ Chl_b) / 198 \quad (9)$$

$$\beta\ \text{carotene} = 0.216\ A_{663.2} - 1.22\ A_{645.0} - 0.304\ A_{505.0} + 0.452\ A_{453.0} \quad (10)$$

Where, A- absorbance of a sample at subscript wavelength, e.g. $A_{453.0}$ is sample absorbance at 453.0 nm.

Total antioxidants capacity

The total antioxidant capacity of the extracts was evaluated by the phosphor-molybdenum method adopted from Aliyu *et al*. (2003), with slight modification. Briefly, 80% v/v aqueous acetone was used for extraction. The rind powder sample (0.2 g) was extracted with 5 mL of acetone. The glass tubes containing extracts were immersed in hot water bath (85.5 °C) for 90 min to evaporate acetone. Thereafter, 5 ml of distilled water was added to the samples, which were then allowed to cool to room temperature. Clean extracts were obtained by filtering the mixture through 0.25 µm syringe nylon filter before analysis. The extract (0.3 mL) was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 ºC for 90 min. The solution was allowed to cool to room temperature before the absorbance was measured at 695 nm using a UV-1800 Spectrophotometer (Shimadzu Scientific Instruments INC., Columbia, USA), against blank. The blank was prepared similar to samples but acetone (0.3 mL) instead of an extract was used. The total antioxidant activity

was calculated using a formula from known ascorbic acid standard curve (0-200 µg/ml, R^2 = 0.9595) and expressed as the number of milligrams ascorbic acid per gram of plant on dry matter basis.

Rind non-structural carbohydrates extraction and determination

The extraction and determination of non-structural carbohydrates were carried out according to a method previously used by Magwaza *et al*. (2014a) with slight modification. Briefly, non-structural carbohydrates were extracted from 0.5 g of dried rind powder using 80% v/v methanol (10 mL). The samples were left to stand for 1 h with occasional agitation at room temperature, filtered through Whatman™ filter paper to obtain liquid extract without particles and evaporated in Genvac evaporator (Genevac® EZ 2.3; IPSWICH; England) to remove alcohol. The alcohol was replaced with 10 mL distilled water before samples were filtered through 0.25 µm syringe nylon filter and put in glass vials for high performance liquid chromatography (HPLC) analysis.

Concentrations of glucose, sucrose and fructose were determined using a HPLC binary pump system (Agilent Technologies, UK). Sample extracts were injected into a Rezex RCM monosaccharide Ca+ (8%) column of 7.8 mm diameter x 300 mm (Phenomenex, Torrance, CA, USA). The column temperature was set at 86 °C using a thermostated column compartment (G1316A, Agilent). The mobile phase used was HPLC-grade water at a flow rate of 0.6 mL/min. The presence and concentration of the selected non-structural carbohydrates were calculated by comparing peak area of samples against peak area of known standard concentrations using formulae from known standard curves (0.05-1.25 mg/mL).

Disorder evaluation

Rind pitting was evaluated after cold storage and a week in shelf life. It was recorded as a scale from 0 (no pitting) to 3 (severe pitting) based on the fruit surface covered by the pitting (Fig. 1).

Fig 1: The levels used to categorize rind pitting occurrence

Statistical analysis

Statistical analyses of fruit properties were carried out using Genstat statistical software (GenStat®, 14th edition, VSN International, UK). Data was subjected to analysis of variance (ANOVA) and means were separated by least significant difference (LSD; $p = 0.01$). The coefficient of variation (CV), defined as the ratio of the standard deviation to the mean was multiplied by 100 and reported as a percentage.

Spectra analysis

Spectra collected were analyzed using Unscrambler® chemometric software (Version 10.3, CamoSoftware, AS, Norway). Visible (450 -850 nm), Near infrared (850-2500 nm), infrared (1100-2500 nm) and full spectral range (400-2500 nm) were tested separately, and the range that showed best results on reflecting fruit parameters was selected to create the final model. PLS regression models were developed using the measured parameter and spectral data. As a general practice during calibration and validation in chemometric analyses of spectral data, various preprocessing techniques were applied to the spectra to convert spectral data and fruit parameters data to a standardized investigable state. Preprocessing techniques that resulted in good prediction during cross-validation were chosen to develop a final model and are presented in Table 1 (normalization and smoothing using standard normal variate). Hoteling T^2 outlier detection technique was used to discriminate outliers and improve model's performance in cross-validation before external validation (Magwaza *et al*., 2014a). Removal of outliers was unnecessary during rind pitting and parameters prediction because all parameters predictions were considered excellent with R^2-values over 0.9. Useful detection and removal of outliers were only observed during principal component cluster analysis of fruit spectra from inside KZN canopy (3) and outside KZN canopy (5). External validation of the developed PLS model was done by samples from an orchard that was not used during model creation.

Models' performance evaluation

The ideal performance of prediction models were evaluated based on the following statistical terms: lowest root mean square error of calibration (RMSEC; Eq. 11), lowest root mean square error of cross validation (RMSECV), lowest root mean square error of prediction (RMSEP; Eq. 12), lowest measure of favour in samples evaluation (bias; Eq. 13), highest correlation coefficient of calibration (R^2_c; Eq. 14), highest correlation

coefficient of cross-validation (R^2_{cv}), highest correlation coefficient of prediction (R^2_p) and highest residual predictive deviation (RPD; Eq. 15).

$$RMSEC = \sqrt{\sum (y_{cal} - y_{act})^2 / n} \quad (11)$$

$$RMSEP = \sqrt{\sum (y_{pred} - y_{act})^2 / n} \quad (12)$$

$$Bias = \frac{1}{n}\sqrt{\sum (y_{pred} - y_{act})^2} \quad (13)$$

$$R^2 = 1 - \frac{\sum (y_{cal} - y_{act})^2}{\sum (y_{cal} - y_{mean})^2} \quad (14)$$

$$RPD = \frac{\text{SD}}{\text{RMSEP}} \quad (15)$$

Where, n is the number of fruit samples used in calculation; y_{act} is the actual value measured by destructive methods; y_{mean} is the average value of predicted data; y_{pred} is the Vis/NIRS predicted value of fruit parameter and SD is the standard deviation of reference data values.

5.4 Results and discussion

Ability of Vis/NIRS-based models to predict rind pitting and grapefruit parameters

Good prediction of rind pitting on 'Marsh' grapefruit was obtained (RMSEC =5.3x10^{-4}; R^2_c = 0.88; RMSECV = 5.2x10^{-4}; R^2_{cv} = 0.89; RMSEP = 1.41; R^2_p = 0.78; RPD = 2.03). Rind pitting prediction model was developed using fruit from LP and externally validated using fruit from KZN. The model's RPD was regarded suitable

for quantitative prediction according to a system by Davey *et al.* (2009), since its value was higher than 2 (Chang *et al.*, 2001; Saeys *et al.*, 2005). Davey *et al.* (2009) categorized RPD accuracy level as unusable (RPD < 1.5), suitable for rough prediction (1.5 < RPD < 2.0), suitable for quantitative predictions (2.0 < RPD < 2.5), good (RPD > 2.5) and excellent (RPD > 3.0). The RP prediction model had a very low bias (-0.17) indicating model robustness on predicting RP of 'Marsh' grapefruit from KZN and probably other regions (Nicolai *et al.*, 2007; Magwaza *et al.*, 2012a). Figure 2 depicts the correlation of predicted to reference RP index values recorded from fruit after six weeks in cold storage and a week in shelf life.

The prediction of parameters was excellent during calibration using fruit from LP. However, there were difficulties when their respective prediction models were externally validated using fruit from KZN. All models developed using KZN fruit also did not do well, which indicated that the difficulty in lower performance (R^2_p = 0.78 vs R^2_{cv} = 0.89) of RP model developed using LP fruit in KZN fruit was due to certain unknown factors in KZN fruit analysis. The possible error that may result in a condition as such is if there was little control during spectra collection or analysis in the laboratory. However, it is not clear on when or how the error may have happened since all data collection and data analysis was done similarly.

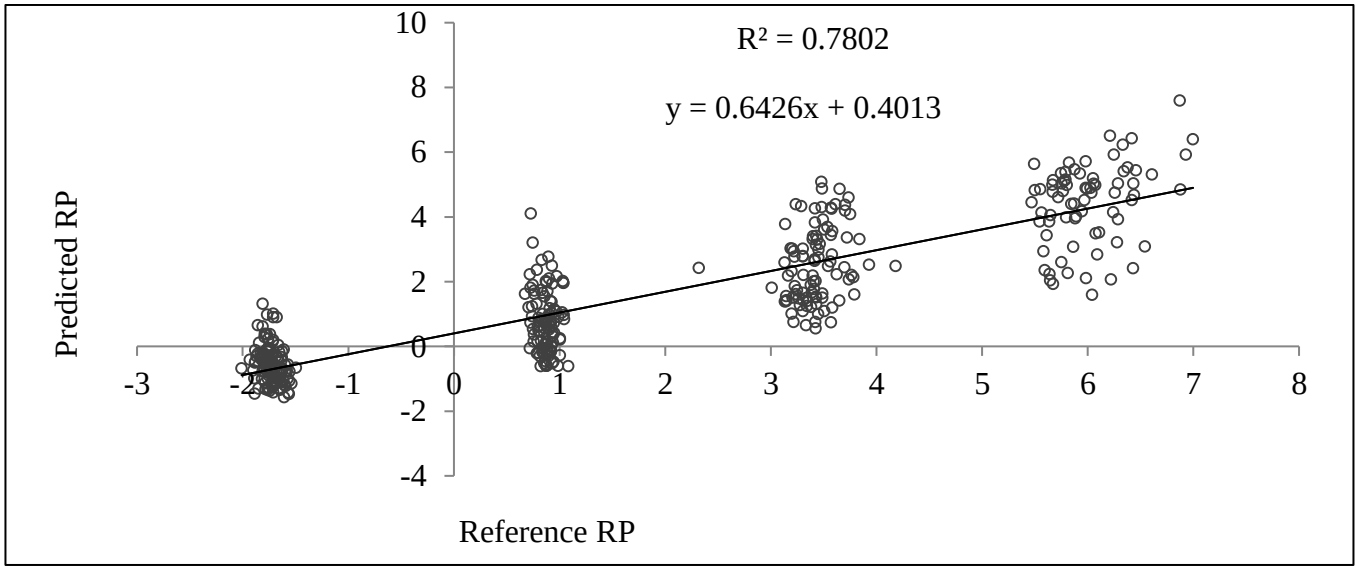

Fig. 2: The correlation of rind pitting values predicted using Vis/NIRS compared to subjective visual scoring.

Although prediction of grapefruit parameters was excellent during models calibration (Table 1), the developed models struggled to predict KZN fruit parameters. The models of internal parameters successfully developed were TSS (R^2_{cv} = 0.97; RMSECV = 3.47x10^{-4}); TA (R^2_{cv} = 0.96; RMSECV = 3.18x10^{-5}); maturity index (R^2_{cv} = 0.95; RMSECV = 5.42x10^{-4}); BrimA (R^2_{cv} = 0.95; RMSECV = 3.02x10^{-4}) and Juice pH (R^2_{cv} = 0.99; RMSECV = 0.04). Internal quality parameters were previously predicted successfully in the study of sweetness and flavour parameters of 'Valencia' orange and 'Star Ruby' grapefruit using the same Vis/NIR system by Ncama *et al.* (2017). In this study, the internal parameters' prediction was used to check for the possibility of noticing certain errors before prediction of the actual RP-related 'Marsh' grapefruit rind parameters. They all showed accurate predictability during cross validation from LP fruit, which strengthened the reliability of all prediction models although they were not validated externally.

Rind parameters prediction models developed successfully were total antioxidant capacity (R^2_{cv} = 0.95; RMSECV = 0.04); β carotene (R^2_{cv} = 0.99; RMSECV = 0.002); total carotenoids (R^2_{cv} = 0.92; RMSECV = 2.51); chlorophyll a (R^2_{cv} = 0.89; RMSECV = 0.008); chlorophyll b (R^2_{cv} = 0.93; RMSECV = 0.08); glucose (R^2_{cv} = 0.93; RMSECV = 0.013); fructose (R^2_{cv} = 0.94; RMSECV = 0.023); sucrose (R^2_{cv} = 0.91; RMSECV = 0.021) and dry matter (R^2_{cv} = 0.88; RMSECV = 0.297).

Wavelength region that best reflects each parameter was evaluated. Full wavelength (400 to 2500 nm) was the most efficient for predicting rind pitting. This study slightly contradicts previous studies on non-destructive assessment of citrus canker of grapefruit using hyperspectral imaging which showed the range from 400 to 900 to be optimum wavelength for predictions (Qin *et al*., 2008; Balasundaram *et al*., 2009). Maturity index was the only parameter predicted best using the full wavelength. Total antioxidant capacity and β carotene were predicted best in the near infrared region (850 to 2500 nm) while all other parameters were best predicted in

the visible range (400 to 850 nm) of the spectrum. Obviously, the colour-related parameters such as total carotenoids and chlorophylls were best predicted in the visible range of spectrum except for β carotene.

The phenomenon was aligned with the fact that β-carotene is the most abundant carotenoid in rinds of fruits (Lichtenthaler, 1987), and therefore, that enables its prediction even under less accurate spectral regions. The ability of visible range to accurately predict rind parameters can be aligned with their proximity to the bright-coloured, and colour changing flavedo of fruit. Each fruit in every batch differs in colour and any rind physiological parameter (Magwaza *et al*., 2013a), which makes their composition and alteration easily picked by radiation reflectance approaches such as spectral absorbance.

Table 1: The statistics obtained during calibration (n = 40) and validation (n = 40) of models for fruit parameters and rind pitting model with n = 120 for calibration and validation, and n = 120 for external validation.

Pre-proc, pre-processing technique; LV, latent variables; R^2, regression value; RMSEC, root mean square error

Parameter	Pre-proc	LV	Calibration			Validation			Region (nm)
			R^2_c	RMSEC	Slope	R^2_{cv}	RMSECV	Slope	
Rind pitting	Norm	10	0.88	$5.3x10^{-4}$	0.89	0.89	$5.2x10^{-4}$	0.89	400-2500
TSS	Norm	4	0.96	$3.81x10^{-4}$	0.96	0.97	$3.47x10^{-4}$	0.97	400-850
TA	Norm	4	0.96	$3.46x10^{-5}$	0.96	0.96	$3.18x10^{-5}$	0.96	400-850
MI	Norm	2	0.95	$5.62x10^{-4}$	0.95	0.95	$5.42x10^{-4}$	0.95	400-2500
BrimA	Norm	4	0.94	$3.21x10^{-4}$	0.95	0.95	$3.02x10^{-4}$	0.95	400-850
Juice pH	SNV	6	0.99	0.05	0.97	0.99	0.04	0.99	400-850
TAO	Norm	2	0.95	0.05	0.95	0.95	0.04	0.95	850-2500
β car	SNV	4	0.99	0.002	0.99	0.99	0.002	0.99	850-2500
T car	SNV	2	0.91	2.69	0.92	0.92	2.51	0.92	400-850
Chl a	SNV	4	0.86	0.009	0.86	0.89	0.008	0.89	400-850
Chl b	SNV	4	0.91	0.008	0.92	0.93	0.008	0.92	400-850
Glucose	SNV	8	0.88	0.017	0.87	0.93	0.013	0.93	400-850
Fructose	SNV	8	0.92	0.028	0.99	0.94	0.023	0.94	400-2500
Sucrose	SNV	6	0.79	0.031	0.77	0.91	0.021	0.91	400-2500
DM	SNV	8	0.88	0.303	0.88	0.88	0.297	0.88	400-850

calibration; RPD, residual predictive deviation; TSS, juice total soluble solutes; TA, titratable acidity; MI, maturity index; BrimA, TSS-4*TA; TAO, total antioxidant capacity; β car, β carotene; T car, total carotenoids; Chl a, chlorophyll a; Chl b, chlorophyll b; DM, dry matter

Features of Vis/NIR spectra

The ability to use spectra collected from the fruit of different canopy positions for indirect prediction of fruit with a potential of higher RP incidence was then tested. The average spectra of fruit from inside or outside canopy position did not show a significant difference (Fig. 3). Both spectra are characterised by a gradual decrease in absorbance in the 400-700 nm range; having peaks at 676, 982; 1200; 1450; 1926 and rise in absorption from 1100 until 2500 nm. The absorbance peak observed at 676 was related to a red colour absorbing pigments such as chlorophylls that give fruits their green colour (Goméz *et al*., 2006). The peaks observed at 982 and 1200 nm can be related to water and carbohydrates absorption that occurs at 935 and 958 nm (Kawano *et al.*, 1993; McGlone and Kawano, 1998). The absorption peaks occurring in 1450 and 1926 were believed to be water absorption bands normally found at 970, 1200, 1450, 1950 and 2250 nm in biological samples (Williams and Norris, 2001). Strong water absorption peaks are usually observed at 1184 and 1457 nm (Magwaza *et al*., 2012a).

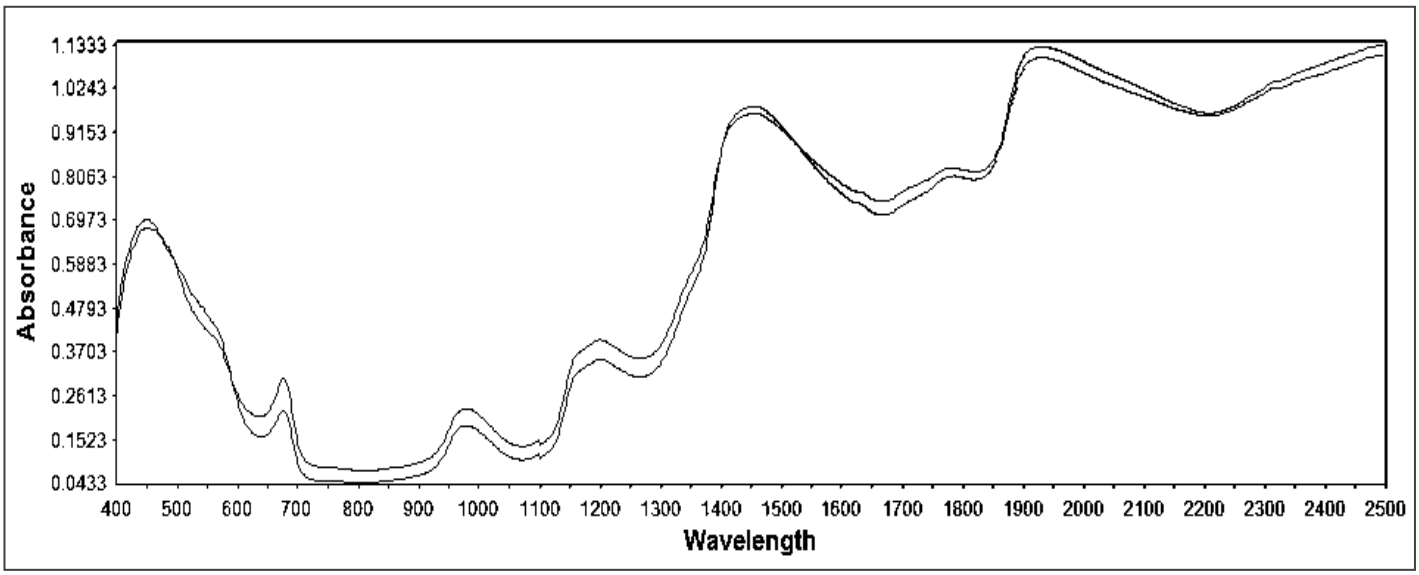

Fig. 3: The average spectra of fruit from inside and outside canopy position

Fruit susceptibility to rind pitting as affected by canopy position

Attempts to indirectly estimate the probability of fruit developing disorders were taken after noticing poor prediction model from KZN. The RP incidence difference of grapefruit from inside or outside canopy was significant ($p < 0.01$). Fruit from inside canopy had a higher number of fruit developing low (level 1) RP compared with outside canopy fruit. Fruit from outside canopy developed the highest incidences severe of RP, which proceeded the deductions made by previous studies about the difference in fruit susceptibility to physiological rind disorders brought by canopy position on citrus (Ezz and Awad, 2009; Cronje *et al*., 2011; Magwaza *et al*., 2014c). Citrus fruit from different canopy positions differs in susceptibility to rind disorders, which enables the valuable but less accurate discrimination based on canopy position.

These results are consistent with those reported by Duarte and Guardiola (1995), who found that 'Nova' mandarin fruit directly exposed to sunlight developed higher pre- and postharvest rind pitting compared to shaded fruit that had no disorder. Basically, shading reduces the sunlight and average temperature the fruit is exposed to and, therefore, the rate of its photosynthesis and evapotranspiration processes. If taking rind moisture content of fruit into consideration, fruit at the outer canopy loses more water than shaded fruit due to higher evapotranspiration and photosynthesis which results to higher susceptibility to RP disorder. The water loss of fruit at postharvest was proven to induce peel pitting on 'Navelate' orange (Alquezar *et al*., 2010). In the study of rind breakdown of 'Nules Clementine' mandarin, Cronje *et al*. (2011) and Magwaza *et al*. (2014c) found that fruit exposed to sunlight had lower disorder incidence than shaded fruit. Magwaza *et al*., (2014c) argued that the higher susceptibility of shaded fruit to rind breakdown disorder was due to the observed higher postharvest mass loss, compared to exposed fruit, which was correlated to moisture loss through transpiration which accounts for 90% water loss.

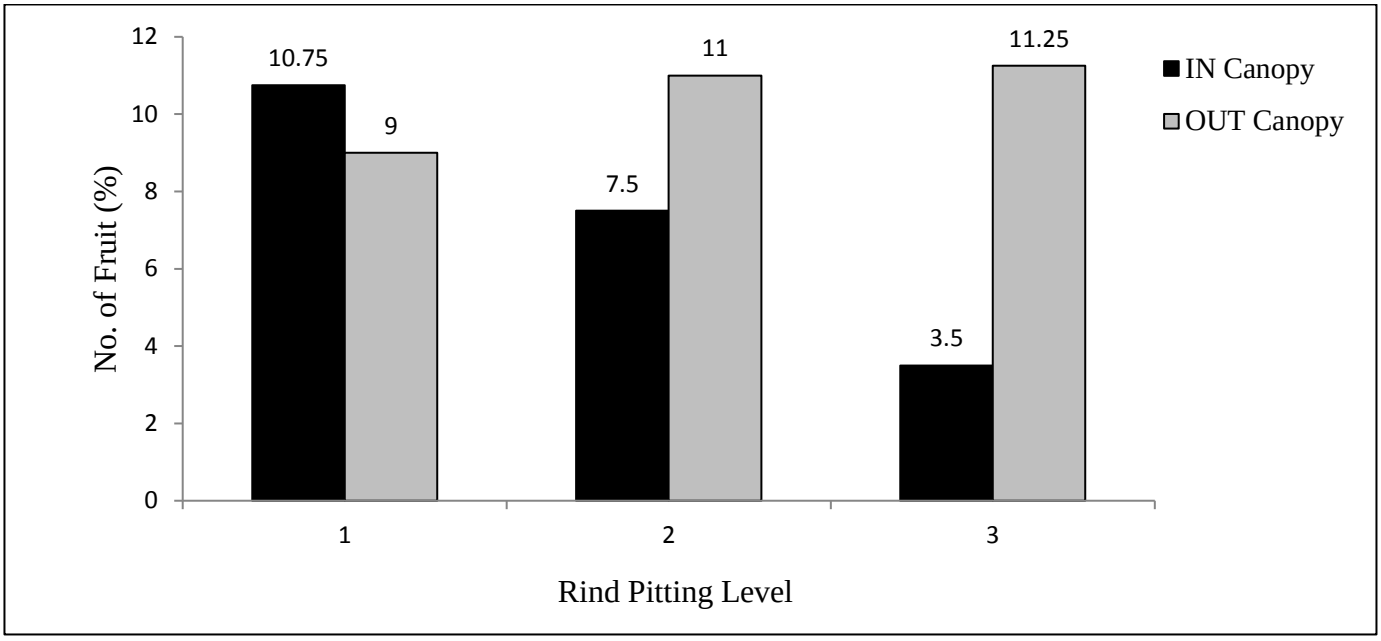

Fig. 4: The levels-based classification of fruit affected by rind pitting. IN Canopy, inside canopy; OUT Canopy, outside canopy

The use of Unscrambler® principal component cluster analysis enabled grouping of spectra collected from fruit of different canopy positions. Fruit from KZN were separated successfully (Fig. 5). The grouping of fruit spectra based on canopy position may be applied during sorting and packaging to discriminate fruit from outside or inside the canopy. Since 'Marsh' grapefruit from outside canopy proved to have a higher potential of developing disorders, canopy discrimination would enable postharvest managers to apply relevant treatments and/or to aim for appropriate markets. Fruit from inside canopy may be used for fresh fruit market while outside canopy fruit may be processed into products with longer shelf life before they are sent to markets. Moreover, fruit from outside canopy can be sent to local market to reduce their chances of developing chilling disorders by eliminating exposure to cold storage usually practiced during shipping.

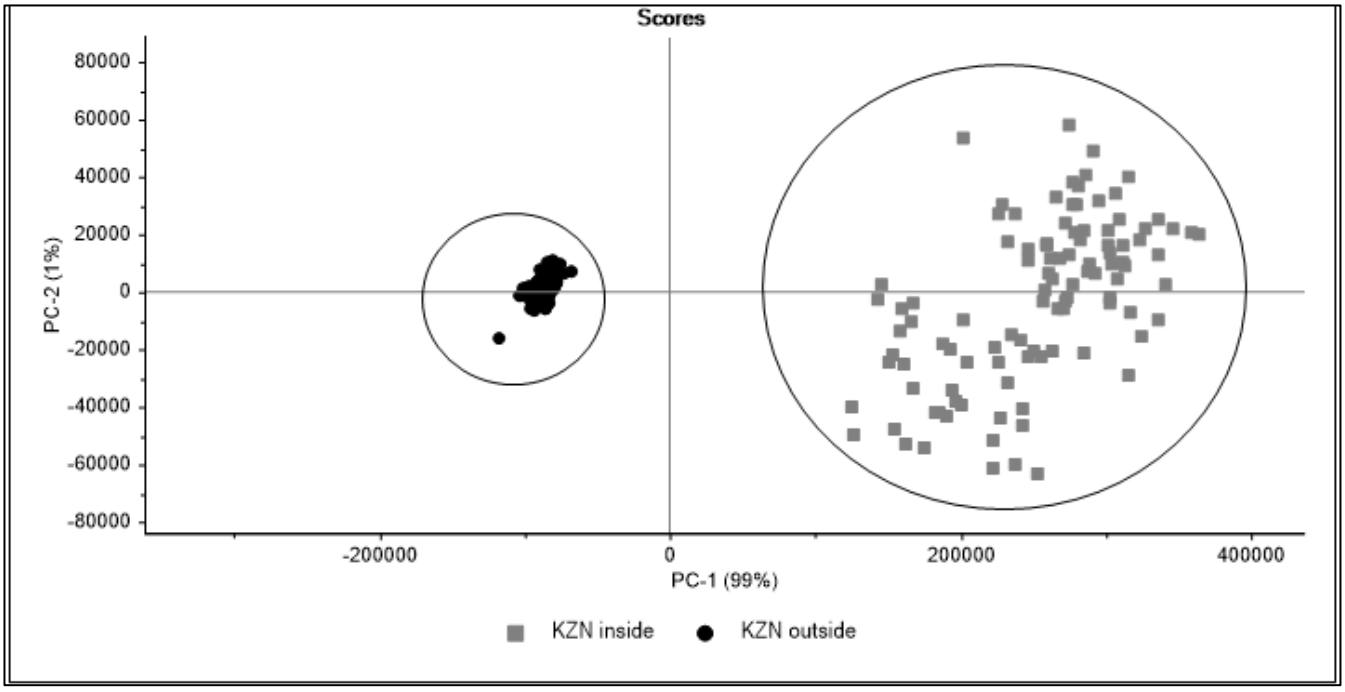

Fig 5: The principal components analysis based clustering of fruit spectra from different canopy position. KZN inside, inside canopy fruit from KwaZulu-Natal; KZN outside, outside canopy position fruit from KwaZulu-Natal

Statistics of the analysed parameters

The reference data of all fruit used to develop prediction models of parameters was analysed and presented in Table 2. The TSS, TA; juice pH; maturity index and BrimA were normally distributed about the mean values (11.37%; 1.12%; 2.98; 11.09 and 6.90%, respectively) with respective standard deviations of 1.33; 0.40; 0.52; 3.84 and 1.24. The ranges of the parameters were 8.61-13.37%; 0.24-2.49%; 1.18-4.14; 5.20-49.96 and 2.67-10.87%, respectively. The wide distribution of samples during calibration is vital for enhancement of model robustness which improves its performance during external validation. It is vital that the range of samples used during calibration and validation must cover a wide range so that it will cover most samples used for external validation and increase chances of being used on samples from various places (Lu *et al*., 2006; Clément *et al*.,

2008). A wide variation of internal parameters of samples was also indicated by CV% of TSS (11.70%), TA (35.71%), juice pH (17.45%), maturity index (34.63%) and BrimA (17.97%).

There was also range on rind parameters analysed. The means of dry matter (23.14 %); chlorophyll a (0.54 µg/g); chlorophyll b (0.58 µg/g); β carotene (0.04 µg/g); total carotenoids (358.07 mg/g); total antioxidant capacity (884. 10 µg/g); fructose (2.22 mg/g); sucrose (2.52 mg/g) and glucose (2.64 mg/g) were captured between values with standard deviations of 2.22; 0.54; 0.81; 0.05; 77.47; 632.08; 0.93; 1.10 and 1.49, respectively.

Table 2: The statistics obtained from the amounts of 'Marsh' grapefruit's parameters

Parameter	TSS (%)	TA (%)	Juice pH	MI	BrimA	DM (%)	Chl a (µg/g)	Chl b (µg/g)	β car (µg/g)	T car (mg/g)	TAO (µg/g)	Fructose (mg/g)	Sucrose (mg/g)	Glucose (mg/g)
Min	8,61	0,24	1,18	5,20	2,67	18.74	-1,77	-2,46	-0,20	263,44	141,99	0,25	0,68	0,77
Max	13,83	2,49	4,14	57.63	10,87	38,43	2,74	4,73	0,11	538,12	3280,32	5,06	10,57	11,14
Mean	11,37	1,12	2,98	11,09	6,90	23,14	0,54	0,58	0,04	358,07	884,10	2,22	2,52	2,64
SD	1,33	0,40	0,52	3,84	1,24	2,22	0,54	0,81	0,05	77,47	632,08	0,93	1,10	1,49
% CV	11,70	35,71	17,45	34,63	17,97	9.59	100	139,66	125	21,64	71,49	41,89	43,65	56,44

SD, standard deviation; TA, titratable acidity; BrimA, TSS-4*TA; DM, rind dry matter; Chl a, chlorophyll a; Chl b, chlorophyll b; β carotene; T car, total carotenoids; MI, maturity index; TAO, total antioxidant capacity

The correlations of analysed parameters

The correlation between parameters was analysed and presented in Table 3. Negative and positive correlations were observed. Correlations higher than 0.70 are highlighted in bold. Most significant was a correlation of juice pH to TA (0.99) followed by chlorophyll a to chlorophyll b (0.96). The correlations observed were not surprising. Titratable acidity is measured by titration of acidic juice to the final pH of 8.1. Therefore the amount

of TA should correspond to the pH initially obtained in the sample, hence, the high positive correlation. Citrus is characterised by their orange or yellow colour at full maturity (Vidal *et al*., 2013). The green chlorophylls gradually vanish as orange carotenoids expression enhances during colour-break maturity stage of citrus. This results in negative relationship observed by the correlation of chlorophyll b to β carotene (-0.92) and chlorophyll a to β carotene (-0.87). Sucrose is a macromolecule made up of glucose molecules (Bewley and Black, 1994). Increase in glucose increases the amount of sucrose and hence, the high positive relationship observed in sucrose to glucose correlation (0.81).

Other parameters also showed important information as to whether they possess positive or negative relationships with one another. Also noticed was a negative correlation value of maturity index to TA (-0.69). The reason for the negative relationship was aligned with the fact that TA depreciates with maturity as fruit approach their characteristic sweet flavour (Jashmidi *et al*., 2012).

Table 3: The correlation (Pearson R^2-value) between the investigated fruit parameters

	TA	BrimA	DM	TSS	Chl a	Chl b	β car	T car	Juice pH	MI	TAO	Fructose	Sucrose	Glucose
TA	1,00													
BrimA	-0,58	1,00												
DM	-0,58	0,04	1,00											
TSS	0,65	0,24	-0,65	1,00										
Chl a	0,60	-0,13	-0,64	0,59	1,00									
Chl b	0,59	-0,17	-0,59	0,55	**0,96**	1,00								
β car	-0,63	0,18	0,61	-0,58	**-0,87**	**-0,92**	1,00							
T car	0,15	-0,08	-0,13	0,10	0,54	0,55	-0,20	1,00						
Juice pH	**0.99**	-0,58	-0,58	0,65	0,60	0,59	-0,63	0,15	1,00					
MI	-0,69	0,60	0,28	-0,26	-0,28	-0,28	0,29	-0,10	-0,69	1,00				
TAO	-0,50	0,06	0,56	-0,53	-0,56	-0,51	0,55	-0,12	-0,50	0,25	1,00			
Fructose	-0,54	-0,05	0,53	-0,69	-0,59	-0,56	0,60	-0,15	-0,54	0,21	0,50	1,00		
Sucrose	0,42	-0,08	-0,46	0,43	0,50	0,45	-0,47	0,07	0,42	-0,19	-0,42	-0,12	1,00	
Glucose	0,52	0,02	-0,52	0,64	0,51	0,45	-0,47	0,07	0,52	-0,24	-0,49	-0,34	**0,81**	1,00

TA, titratable acidity; BrimA, TSS-4*TA; DM, rind dry matter; Chl a, chlorophyll a; Chl b, chlorophyll b; β car, β carotene; T car, total carotenoids; MI, maturity index; TAO, total antioxidant capacity

5.5 Conclusion

Rind pitting of 'Marsh' grapefruit was predicted successfully. PCA-based fruit spectra grouping technique was recommended as a secondary tool for canopy based fruit discrimination during sorting and packaging. The technique may be applied in/online with the fact that 'Marsh' grapefruit from outside canopy showed higher susceptibility to rind pitting. The models for predicting internal and rind parameters were also developed, and due to time limit will be externally validated in the next season. It will be more effective to do external validation in the next season since the models robustness will be tested in two factors (between seasons and place of origin). The lack chilling injury incidence on the fruit of this study led to the recommendation of further studies focusing on developing disorders so as to guarantee the success of studies predicting those disorders.

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Chapter 6: Overall discussions and conclusions

6.1 Literature review

The literature clearly indicated that Vis/NIRS system can be used to predict internal/external characteristics of many fruits in time as short as a second. However, its application on prediction of fruits disorders is limited. Previously, it was successfully used for predicting surface defects on peach fruit (Miller and Delwiche, 1991), surface bruising on apples (Geeola *et al*., 1994), internal drying disorder in tangerine citrus (Peiris *et al.,* 1998), storage disorders of kiwifruit (Clark *et al*., 2004) and pericarp hardening of mangosteen fruit (Teerachaichayut *et al*., 2011), but has never succeeded on citrus. It was stated in the literature that future studies would greatly benefit the citrus industry, which is a major exporter in fruit industry of South Africa, by focusing on developing non-destructive techniques that could be utilized during grading and sorting on commercial lines to discriminate fruit with high chances of surviving cold storage and maintain its quality and lower chances of developing disorders in international markets. This study was based on that gap.

6.2 Evaluating optimum conditions for using Vis/NIRS to predict physiological attributes of 'Marsh' grapefruit

The use of visible to near infrared spectroscopy (Vis/NIRS) based models for predicting internal quality parameters of grapefruit and orange was proven. Various factors contributing to successful prediction and application were noticed including:

(i) The number of samples used. Using as many samples as possible during model calibration results to higher chances of obtaining a normal distribution of reference data. That results to model stability across various

conditions (robustness), which is very important as the developed model will not only be used to predict the samples from that specific area or growing season but also on samples from other areas and the following seasons. This robustness is vital as fruit from each season will vary due to factors such as alternate bearing, climate change, and management inconsistency that usually occur in two consecutive harvest seasons.

(ii) Wavelength region that best reflects sample parameters. This is important as most studies begin by pretreatment techniques that determine what range of spectra the authors will use to predict sample parameters. The difference in wavelength regions used in the literature to predict any one parameter emphasizes how authors need to clearly indicate which region they use, or it may complicate the communication and application of NIR between scholars and technicians. For example, the visible to near infrared range can be referred to 350-1800 nm (Liu *et al*., 2010) or 500-1900 nm (Lammertyn *et al*., 2000). Therefore, it won't be enough to mention the range using only words and not specifying it as some authors did (Blasco *et al.*, 2007; Lopez-Glarcia *et al*., 2010).

(iii) Pretreatment techniques. Repeatability of the models developed from each study conducted is crucial so that they can be adopted by anyone from anywhere in the absence of the author. Currently, there is a wide variation on pretreatments utilized. However, that does not reduce the model's performance ability since the authors would specify whether they used pretreatment and what techniques they used. The complications begin when communicating to a non-scholarly person is considered. For example, some authors call a number of factors used during calibration as Factors (Cayuela, 2008; Sánchez *et al*., 2013) while other authors call them Latent Variables (Gomez *et al.*, 2006; Magwaza *et al*., 2012), which complicates the terminology. The communication would be much clearer if there was a standard terminology across all researchers working on the NIR systems, which is now difficult to switch.

6.3 Identifying pre-symptomatic biochemical markers that can be used to predict susceptibility of 'Marsh' grapefruit to chilling injury and rind pitting

The contributions of investigated quality parameters to chilling injury and rind pitting were explored. The principal components analysis (PCA) based correlation and clustering was applied. There were weak correlations of quality attributes to rind disorders, which were interpreted to mean that there is a wide range of various factors contributing to the development of the disorders. Rind total antioxidants capacity was found to have a weak positive correlation to rind pitting (RP) and negative correlation to chilling injury (CI), which means the higher the antioxidants, the lower the CI incidence. This was interpreted to say RP is mostly affected by the rind moisture status rather than oxidizing attributes such as reactive oxygen species. There was also a positive correlation of rind dry matter to CI, while it had a negative correlation to RP. The deduction was further supported by the change in colour, yellowish to reddish-brown, of the CI affected fruit rind while the RP affected rind changed from yellow to brown which is a normal colour changing the pattern of plant tissues when they become dehydrated. Positive correlation of total soluble solutes (TSS) to both CI and RP was observed. However, rind disorders were merely believed to be affected by internal parameters in this study. Apart from the poor correlation of internal parameters to disorders, it was also considered more useful to correlate rind parameters with rind disorders because of their proximity to the actual site of the disorders' action.

This study showed a significant difference on fruit susceptibility based on canopy position. Fruit from inside canopy suffered higher CI and fruit from outside canopy suffered higher RP. The disorder occurrence was aligned with the difference in microclimate that the fruit developed in. Fruit from inside canopy had higher rind moisture content probably because of lower exposure to sunlight and reduced aeration inside the tree. Therefore, when those fruit got damaged it resulted to CI which was characterized by affected parts of rind

becoming water soaked patches. When fruit from outside canopy got damage, they suffered from RP which was characterized by dry patches of dead cells on the flavedo.

6.4 Development of Vis/NIRS models to predict chilling injury, rind pitting and rind biochemical markers associated with the disorders

Vis/NIR spectroscopy was explored as a tool to predict rind pitting of 'Marsh' grapefruit. The tool did not only predict the pitting but also successfully predicted rind quality parameters. The combination of Vis/NIRS, to non-destructively collect accurate and fruit-specific spectra, and chemometric softwares to specify and cluster spectra into groups based on fruit origin was also noticed. The grouping technique was highly recommended as it allows canopy based discrimination during sorting and packaging. Canopy-based sorting is good news to farmers and postharvest managers as it assures that most of their harvest reaches a consumer. Fruit with higher chances of developing disorders may be sent to local markets for eliminating cold storage exposure that normally takes place during shipping or they can be processed to other products such as juices, dried fruits and sweets which have a lifespan longer than the optimum shelf life of fresh fruit.

Only RP developed during the study of predicting the disorders. The inability of fruit to develop CI was aligned with seasonal alternate bearing since fruit from the previous season developed significant CI, with occurrence even higher than RP. This was hypothesized to emphasize the pre-harvest conditions as important factors determining physiological disorders' incidences on 'Marsh' grapefruit. The result of this study exposed the need for studies focusing on techniques to manually develop fruits disorders. The prediction of the disorders requires their presence, which if there are adequate scholarly articles about developing physiological disorders would ensure the success of prediction studies.

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