Non-destructive determination of pre-symptomatic biochemical markers for Peteca spot and evaluation of edible coatings for reducing the incidence of the disorder on 'Eureka' lemons

By

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Declaration

I, Muriel Mbhoni Rikhotso, hereby declare that all work in this thesis is my original work and a result of my own investigations. Information taken from other sources is correctly referenced and acknowledged. This work has never been submitted to any other institution for any degree.

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“They tell you: Follow your dreams. Listen to your spirit. Change the world. Make your mark. Find your inner voice and make it sing. Embrace failure. Dream. Dream and dream big. As a matter of fact, dream and don’t stop dreaming until your dream comes true.

I think that’s nonsense.

I think a lot of people dream. And while they are busy dreaming, the really happy people, the really successful people, the really interesting, powerful, engaged people? Are busy doing.”

Rhimes, S., 2015. Year of yes: How to dance it out, stand in the sun and be your own person.

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Preface

This Dissertation is presented as a compilation of manuscripts where each chapter is an independent entity introduced separately. Some repetition between chapters has, therefore been unavoidable. The chapters in this dissertation are written in accordance with the requirement of Elsevier BV Publishers of Scientia Horticulturae. Chapter 3 and 4 have been submitted as follows:

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Abstract

International markets that import citrus fruit from South Africa have imposed regulations that involve cold sterilization at low temperatures, which cause physiological disorders such as peteca spot in lemon. The aim of this study was to, non-destructively determine pre-symptomatic biochemical markers for Peteca spot and the evaluation of edible coatings for reducing the incidence of the disorder on 'Eureka' lemons. The first chapter is general background which introduces the key words and clearly outlines the aim and objectives of the study. The second chapter is review of literature, which motivated the three research chapters due to the gaps found. Presymptomatic biochemical markers that are related to peteca spot were evaluated in the third chapter. The Principal Component Analysis (PCA) was able to separate fruit harvested from the inside and outside canopy positions based on their susceptibility to the disorder. Fruit harvested in the inside canopy were more susceptible to peteca spot and these were correlated with physic-chemical properties, which were typically low in the inside canopy. The efficacy of carboxymethyl cellulose (CMC) and chitosan (CH) incorporated with moringa leaf extracts (M) edible coatings on reducing the incidence of peteca spot was also evaluated in the fourth chapter. Fruit harvested from inside and outside canopy positions were assigned to five coating treatments: control, M+CMC, CMC, CH and M+CH. The most effective coating treatment in reducing the susceptibility of 'Eureka' lemon to peteca spot was M+CMC followed by CMC and CH. The fifth chapter focused on, non-destructively predicting peteca spot using visible to near infrared spectroscopy (vis/NIRS). Presymptomatic biochemical markers that have been related to peteca spot were successfully predicted. Lastly, general discussions and conclusions were made in chapter six as well as recommendations.

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Chapter 1: General Introduction

1.1. Introduction

South Africa is among the top three global exporters of fresh citrus fruit, with an estimate of over a million tons exported per season (Philp, 2006). Being the third largest horticultural industry in South Africa after deciduous fruit and vegetables, the citrus industry has a significant contribution to the total gross value of the country's agricultural production. During 2016/17 growing seasons, the industry contributed R17.7 billion, which represents 24 % of the total horticultural gross value (R74.2 billion) (Citrus Growers' Association of Southern Africa, 2018; DAFF, 2018). The strength of the citrus industry in the export market is due to the wide range production of different cultivars over an extended period, from March until November (Magwaza, 2013). The leading export cultivars are 'Valencia' oranges (37 %), 'Navel' oranges (21 %), soft citrus (17 %), lemons (15 %) and grapefruit (10 %) (Citrus Growers' Association of Southern Africa, 2018).

Citrus fruit have long been considered a valuable part of a healthy and tasty diet. As a result, it is widely grown and consumed, because of its thirst quenching ability as well as nutritional and functional properties. They provide an array of essential key nutrients such as vitamin A & C, folate, dietary fibre and minerals, which provide significant health benefits (Turner and Burri, 2013). Citrus fruit contain several phytochemicals, which play a role of nutraceuticals, including flavanones, carotenoids, limonoids as well as vitamin B complex (Ladanyia, 2010). As stated by Filatova and Kolesnova (1999), citrus fruit contain flavonoids in the juice which are effective in improving blood circulation and also possess anti-carcinogenic, antiviral and anti-allergic properties.

The South African citrus industry is mainly export orientated. More than 72 731 ha is currently used for citrus production with the main production regions located in Limpopo – 42%, Eastern Cape – 26%, Western Cape – 17%, Mpumalanga – 7%, KwaZulu-Natal and Northern Cape – 8%. Lemons, which play an important role in the export market are mainly grown in the Western and Eastern Cape provinces and over 270 000 tons of lemons were exported in 2017 (Citrus Growers' Association of Southern Africa, 2018). Amongst other cultivars, 'Eureka' is one of the important cultivated lemon variety in South Africa, Australia, California, Argentina and Israel (DAFF, 2018). Lemon has been used for both flavouring and therapeutic purposes

for many years (FAO, 2013). It contains citric acid, which is an excellent fat burner, hence it is used for weight loss. Obesity is a major health problem in the world today (Ahima and Lazar, 2013). Flegal et al. (2016) reported an estimated 300 million people around the world who are known to be obese. Therefore, replacing high calories foods with lemons is arguably one of the effective strategies for weight management.

The South African export market is often faced with a big threat of the Mediterranean fruit fly, Natal fly and Oriental fruit fly, which has led to many countries requiring specialized quarantine inspections before fruits enter their markets (Grout and Moore, 2015). Although there are preharvest control measures that are mostly used for the prevention of citrus infestation by fruit flies, most markets require assurance of fly-free fruits, which is achieved through cold disinfestation (Manrakhan et al., 2018). During cold disinfestation, fruits are stored at -0.6 °C for 14 days (Mathaba, 2012), followed by 5 °C during shipping (Ncama, 2016). However, fruit that are shipped to China, Korea, Thailand and the United States of America require even longer periods of 24 days or more (Schirra et al., 2004).

The use of cold storage in citrus is mainly to extend postharvest shelf life by reducing respiration, mass loss and decay incidence (Maul et al., 2011) but lemon originates from tropical and subtropical regions, and they tend to develop chilling injury and physiological disorders when stored under low temperatures. Chilling injury was reported in 'Eureka' lemons that were cold-stored at 1 °C for 2 weeks followed by 5 °C for 3 weeks (which was to meet the cold disinfestation requirement for Australian lemon for quarantine against fruit flies) (Underhill et al., 1995). Wild (1991) reported a high incidence of peteca spot in 'Meyer' lemon stored at 3 °C. Peteca spot is one of the commercially important postharvest physiological disorders affecting the citrus industry (Cronje, 2005).

The disorder is associated with the collapse of oil glands of the flavedo, which ultimately reduces the quality and shelf life of the fruit (Undurraga et al., 2002; Cronje, 2015). There is limited information with regard to the actual cause of the disorder (Undurraga et, al., 2009), but research has shown that certain factors (preharvest and postharvest) could be responsible for enhancing the incidence of its occurrence (Wild, 1991; Fichet et al., 2012). For example, low rainfall accompanied with cold/wet conditions 3 months before harvest have been strongly linked to high incidence of peteca spot in South African 'Eureka' lemons (Cronje, 2015)

Peteca spot disorder resembles rind pitting, but it is are different in that it has rounded depressions at the edges and the oil glands tend to darken before the surrounding epidermal cells (Pyle, 2015). It can be seen as early as 3-5 days after harvest, but it may take time to appear and be visible later during storage, which then becomes a problem to growers (Cronje, 2015). The symptoms include light brown lesions developing on the fruit surface (Fig. 1).

Fig. 1: Peteca spot symptoms on 'Eureka' lemons

Postharvest conditions that can aggravate peteca in lemon fruit include the use of wax. Wild (1991) found an increase in the incidence of peteca spot after citrus wax application (commercial polyethylene based citrus wax with 16% solids) and also with fruit brushing. Similarly, waxing of 'Marsh' grapefruit with polyethylene waxes was reported to cause chilling injury (Dou, 2004). This, and many other complaints regarding waxes has risen the interest of switching to edible coatings (Baldwin et al., 2011). The use of edible coatings was discovered many years ago in food products. Edible coatings are environmentally friendly substances that are used in the replacement of waxes to extend the shelflife of fruits and vegetables. Edbile coatings have many adavantages, which includes forming a semi-permeable layer, which reduces oxidation reaction rates (Perez-Gago et al., 2005). The most important characteristics of edible coatings are, they are biodegradable and they provide protection against physical and mechanical damages (Palou et al., 2015)

Coatings that have been found to dominate the food industry include, chitosan, moringa and carboxymethyl cellulose (CMC) (Tesfay and Magwaza, 2017). Chitosan is a natural biopolymer that has been associated with high antimicrobial activity, which gives it the ability to reduce postharvest diseases (Palou et al., 2015). Campos et al. (2011) reported that the antimicrobial activity of chitosan depends on the presence of other ingredients in the coating. When incorporated with moringa extracts, chitosan was found to extend shelflife of avocado (Tesfay and Magwaza, 2017). Similar results were found in citrus fruit when chitosan was combined with CMC (Arnon et al., 2014).

There is limited information regarding the occurrence of biochemical changes in the citrus rind that could be used for predicting fruit rind susceptibility to physiological disorders (Magwaza, 2013), therefore, there is a need for the identification of potential biochemical markers of rind condition that are related to lemon susceptibility to peteca spot. Since the actual cause of peteca spot is not well known, identifying these biochemical markers may lead to a better understanding of the factors influencing its occurrence and the prediction of the disorder before the symptoms become visible. Previous studies have shown that the biochemical profile of the rind is affected by tree canopy position and different microclimatic conditions during the growing season (Cronje et al., 2011a; Cronje et al., 2011b; Magwaza et al., 2013). Shading affects the biochemical processes occurring during fruit growth and development. When the fruit is shaded, the amount of light it is exposed to is limited hence affecting respiration and photosynthesis processes (Ncama, 2016).

Fruit from the outside canopy are directly exposed to sunlight, which makes them lose more water, but they are characterized with higher sucrose, glucose and fructose than fruit in the inside canopy. This is the reason why the sensitivity of ‘Nules Clementine’ mandarin to rind breakdown was found to be lower in the outside canopy (Magwaza et al., 2013). There is limited research relating peteca spot development to biochemical attributes, but it has been reported in other citrus fruits. The incidence of rind spot in ‘Fortune’ mandarins was found higher in fruit from the outside canopy than shaded fruit in the inside canopy (Almela et al., 1992), whilst a high susceptibility of ‘Nules Climantine’ mandarin to rind breakdown was found in fruit harvested from the inside canopy (Cronje et al., 2011a). Interestingly, Ezz and Awad (2009) reported a negative correlation between ascorbic acid concentration and the susceptibility of ‘Marsh’ grapefruit to rind breakdown.

These studies and many more suggest that the concentration of rind carbohydrates, antioxidants, including phenolics and vitamin C, may be used as potential biochemical markers of fruit susceptibility to rind disorders. However, the current methods used for the detection of carbohydrates are time-consuming and require a laborious sample preparation (Magwaza, 2013). Destructive analyses are only done in a few samples and the results found only reflect properties of that specific fruit. There is a high demand in the industry for innovative tools for detecting biochemical attributes and this has risen an interest among researchers to develop rapid, cost-effective and non-destructive tools, which can be used for the detection, prediction, segmentation and monitoring of physiological disorders (Zheng et al., 2010).

There is, therefore, a need to develop non-destructive methods that can be used to monitor and predict citrus fruit susceptibility to physiological rind disorders such as peteca spot on lemons. To achieve this, non-destructive, instrument-based methods are used and this is because they allow for the analysis of individual fruit and repeated measures on the same fruit over time, while reducing waste and increasing sample size (Nicolai et al., 2007). Among other instruments, research has found visible to near infrared (Vis/NIR) spectroscopy to be more advanced and effective, hence it is widely used for non-destructive determination of fruit physiological disorders. This this is largely attributed to its instrumentation, applications, accessories and chemometric software packages (Magwaza, 2013).

Most of the NIR spectroscopy research on citrus fruit has focused on assessing internal quality attributes by means of measuring the amount of reflected, absorbed or transmitted radiation that is illuminated through scanning the fruit (Liu et al., 2010; Ncama, 2016). There is limited information on the use of NIR spectroscopy in predicting the internal and rind physiological disorders (Cronje, 2009). The NIR exists between the visible and infrared region which is defined as stretching from 780 to 2500 nm (Nicolai et al., 2007). This electromagnetic radiation with the kind of energy range interacts with molecular overtones and combination bands of the fundamental molecular vibrations which are found in the mid-infrared region (Engelsen, 2016).

The NIR is characterized by absorption bands, which are caused by stretching vibrations of molecular groups such as C-H, O-H and N-H (Nicolai et al., 2007). It has many advantages, which include the ability to non-destructively determine the biochemical properties of the fruit.

Samples with high moisture content can be measured, the method is fairly simple and rapid since there is no need for sample preparation and can monitor fast process dynamics. Engelsen (2016) highlighted that NIR Spectroscopy has the ability to simultaneously measure several quality parameters, it does not use any chemicals, which makes it environmentally friendly.

1.2. Research aims and objectives

The aim of this research project is to non-destructively determine pre-symptomatic biochemical markers for Peteca spot and the evaluation of edible coatings on reducing the incidence of the disorder on Eureka lemons.

The objectives of this study are to:

1. Determine pre-symptomatic biochemical markers related to Peteca spot
2. Evaluate the efficacy of Moringa extracts, Chitosan and Carboxylmethyl Cellulose (CMC) and their combinations on reducing Peteca spot
3. Develop non-destructive methods for predicting Peteca spot using Near-infrared spectroscopy (NIRS)

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Chapter 2: Literature Review

An overview of factors affecting citrus rind physiological disorders and non-destructive prediction and control using Vis/NIR spectroscopy and edible coatings, respectively

Abstract

Citrus is one of the most important and commonly consumed fruit around the world. Major citrus producing countries such as Spain and South Africa export their fresh fruit to distant and lucrative international markets such as Western Europe and the United Kingdom. However, citrus fruit are prone to physiological disorders which are becoming a major constraint in the export market. There has been an ongoing research aimed at finding safe and effective treatments such as edible coatings for reducing the incidence of the disorders as well as maintaining the postharvest quality of citrus fruit. The growing interest in the potential use of edible coatings is also due to environmental and health concerns linked to the use of synthetic waxes. Currently, there is limited information on the pre-symptomatic biochemical markers that are associated with the rind physiological disorders of citrus fruit. The objective of this chapter was to review published and unpublished information on rind physiological disorders of citrus fruits as well as highlighting the factors aggravating the incidence of these disorders. The efficacy of edible coatings to retard the development of physiological disorders of citrus fruit is also reviewed. It concludes by highlighting the use of visible to near infrared spectroscopy (vis/NIRS) for predicting physiological disorders; the fundamental theory, spectral pre-processing methods, robustness and the previous applications of this technology in citrus fruit are reviewed.

Keywords: Citrus, Physiological disorders, Coatings, Biochemical markers, Near Infrared Spectroscopy

2.1. Introduction

Citrus is one of the most important fruit consumed and grown all over the world throughout the tropics and subtropics (FAO, 2013). Major citrus producing countries include the United States, Japan, Spain, South Africa, Cuba, Israel, Brazil and Turkey (Manner et al., 2006). Citrus fruit can be consumed as fresh, juice and can be derived for medicinal and herbal uses. The

increased consumption of citrus around the world is due to therapeutic properties that are linked to phytovitamins, antioxidants and nutrients possessed by the fruit (Ladaniya, 2008; Huang and Ho, 2010). Amongst other tapes of citrus fruit, lemons and oranges are the top two cultivars which by virtue of their richness in vitamins and minerals have been found to have considerable health benefits. Epidemiological studies have shown that lemon consumption can help in reducing chronic diseases such as arthritis, obesity and coronary heart diseases (Silalahi, 2002).

In South Africa, the production of citrus fruit is aimed at exports to international markets and during shipping the fruit are stored under low temperatures in order to meet the quarantine requirements imposed by international markets for prevention against Mediterranean fruit fly (*Ceratitis capitata*), Natal fly (*Ceratitis rosa*) and Oriental fruit fly (*Bactrocera dorsalis*) (Manrakhan et al., 2018). In order to provide assurance for fly-free consignments, the fruit are disinfected by exposing them to a low storage temperature of -0.6 °C before shipping for about 14-22 days depending on the specific requirements of the importing country (Van Wyk et al., 2009). The problem related to this however, is that not all citrus cultivars are tolerant to low temperatures due to chilling sensitivity and as a result, they tend to develop rind physiological disorders (Magwaza et al., 2013a). Temperatures below 10 °C have been reported to cause chilling injury in 'Marsh' grapefruit (Maul et al., 2011), lemon (Siboza et al., 2014) and other citrus fruit (Rehman et al., 2018). Undurraga et al. (2006) found a high incidence of PS in lemon under low storage temperature of 3 °C.

Previously, synthetic waxes were used in packhouses to coat the fruit in order to extend shelf life and reduce the occurrence of some physiological disorders (Palou et al., 2015). However, health and environmental concerns have been raised regarding the use of waxes in fresh horticultural produce, moreover, some waxes have been found to aggravate the occurrence of rind disorders in citrus fruit. Carnauba wax coating was reported to cause chilling injury in grapefruit cultivars (Dou, 2004) and Wild (1991) found a high incidence of peteca spot in 'Meyer' lemon coated with polyethylene-based waxes. This could be due to physiological stress produced by increased CO_2 concentrations associated with wax application. As a result, there are concerted efforts aimed at finding safe and alternative ways to reduce the incidence of disorders as well as maintaining the postharvest quality of fruit which includes the use of edible coatings (Tesfay and Magwaza, 2017).

Amongst a wide variety of edible coatings that have been developed in the fruit industries, moringa, carboxymethyl cellulose (CMC) and chitosan coatings are the most dominant (Tesfay and Magwaza, 2017). In citrus fruit, CMC incorporated with moringa extracts has been found to reduce firmness loss and maintain the quality of oranges by reducing moisture loss and conserving water content of the fruit (Adetunji et al., 2013). Shao et al. (2015) reported that chitosan effectively controlled green mould and inhibited mycelial growth in 'Satsuma' mandarin. This is because chitosan coatings have been reported to display antifungal effects when applied to citrus fruit. The use of edible coatings in extending shelflife and reducing the incidence of some rind physiological disorders in citrus fruit has been summarized in Table 1.

Rind physiological disorders in citrus fruit have been associated with a change in biochemical attributes such as non-structural carbohydrates, ascorbic acid, phenolics, flavonoids and total carotenoids in the fruit. These biochemical properties have shown the ability to be used as pre-symptomatic markers that can be used to predict disorders, however, the current methods used for their determination is time consuming, labor intensive and costly (Ncama et al., 2018). As a result, many industries are advancing to innovative tools that will rapidly detect and monitor physiological disorders. This has led to the recent increasing use of visible to Near Infrared Spectroscopy (NIR) for quality monitoring and evaluation of citrus rind physiological disorders. Near Infrared Spectroscopy has been successfully used to predict rind pitting in 'Marsh' grapefruit (Ncama et al., 2018), rind breakdown in mandarins (Magwaza et al., 2014a) and oleocellosis in sweet oranges (Zheng et al., 2010).

The objective of this review was to highlight postharvest rind physiological disorders of citrus fruit and factors that affect their development. Attempts have also been made to discuss recent advances on the potential of edible coatings to control rind disorders and their non-destructive prediction using vis/NIR Spectroscopy.

Table 1: Effect of edible coatings on various citrus fruit.

Coating treatment	fruit	Effect of coating	Reference
CMC+ Moringa; Corn starch+Moringa	Oranges	. Reduced firmness loss more than 50% . Reduced ascorbic acid loss and conserved water content	Adetunji et al., 2013
CMC + Chitosan	Mandarin, 'Star Ruby' grapefruit, 'Navel' oranges	. Enhanced fruit gloss, increased fruit firmness . Decreased flavor of mandarins and was not effective in preventing postharvest weight loss	Arnon et al., 2014
HPMC + Moringa	Oranges	. Decreased weight loss, increased firmness and reduced ascorbic acid loss . Maintained fruit quality	Adetunji et al., 2012
MC, HPMC, CMC & CH	Mandarins	. CMC- best firmness, lowest weight loss and good gloss . CMC+CH had best results in maintaining fruit quality	Arnon et al., 2015
Paraffin oil, C_aCl_2, Salicylic acid and Arabic gum	Grapefruit	. Significantly improved storage life, reduced weight loss and ascorbic acid loss	Abdel-Salam, 2016
Corn starch	Assam lemon	. Maintained higher TSS, acidity, ascorbic acid and retained colour	Ghosh et al., 2015
Chitosan + Clove oil	'Satsuma' mandarin	. Pure chitosan effectively controlled green mould than the combination	Shao et al., 2015

CMC= Carboxymethyl cellulose, HPMC= Hydroxypropylmethyl-cellulose, CH= Chitosan, MC= Methyl-cellulose

2.2. Rind physiological disorders associated with citrus fruit

Citrus fruit are usually produced in areas that are far from the market which results in delay between harvest and consumption (Eckert and Eaks, 1989). This delay results in physiological disorders if the fruit is not well stored and handled. South African fruit are exported to countries

that are very far such as Japan and the most suitable means of exporting the fruit is through shipping. However, shipping fruit for over long distances takes about 6 weeks which gives more time for the occurrence of rind disorders by the time the fruit reach the market (Ncama, 2016). Many researchers have identified and reviewed disorders that are associated with citrus fruit as well as factors that lead to their susceptibility (Magwaza et al., 2013a). Sometimes differentiating these disorders is difficult since there are a lot of factors that can lead to the development of similar symptoms and the relationship between these disorders is not fully understood.

2.2.1. Peteca spot

Peteca spot (PS) is a disorder that occurs from harvest until cold storage in citrus fruit, which is associated with the collapse of oil glands of the flavedo, ultimately reducing the quality and shelf life of the fruit (Cronje, 2005). The disorder was probably first observed in Italy since the name peteca is derived from *petecchia* as it was known in Italy back in 1924 (Fawcett, 1936). The incidence of PS of lemons in South Africa was first reported in 2004 in all citrus producing areas. Limpopo, KwaZulu-Natal, and Mpumalanga provinces of South Africa are faced with greater challenges with this disorder (Citrus Growers Association of South Africa, 2018). Lemon fruit are more susceptible to PS and this disorder causes a great economic loss for citrus growers since lemons make a significant contribution to the export market (Cronje, 2015). The incidence of the disorder is usually higher during the initial weeks of the year. The susceptibility of PS was found to be higher during the first 4 months of the year which is followed a sudden reduction in the successive weeks/months (Fig. 1) (Cronje, 2015).

The disorder is also known as ‘rumple’ (Knorr, 1963). The symptoms tend to appear 3 to 4 days after fruit packing and sorting (Khalidy et al., 1969; Offers, 1987). They include deep depressions with round edges on the peel as a result of collapse of oil glands found in the flavedo, leaking the oil into the lower flavedo and albedo cells (Undurraga et al., 2009). This results in the browning of the flavedo of which Cronje (2005) explained that it could be resulting from a change in gaseous exchange from the albedo due to oxygen depletion rather than enzymatic browning. The disorder causes browning of the albedo which can been seen when the flavedo is removed. The development of sunken lesions which are visible in yellow fruit occur without physical or mechanical damage of the epidermal rind tissue (which is the opposite for oleocellosis) (Cronje, 2005).

There is limited information with regard to the actual cause of PS (Undurraga et al., 2009) but research has shown that pre-harvest factors (low temperature, high relative humidity, direct rainfall, mineral nutrition, fruit maturity) and post-harvest factors (storage temperature, wax application) could be responsible in enhancing its occurrence (Wild, 1991; Fichet et al., 2012; Cronje, 2015). Temperature has been reported to affect PS where sudden changes in day and night temperature close to harvest was reported to result in the occurrence of the disorder (Undurraga et al., 2006). It was also observed that fruit from the outside canopy (exposed to sunlight) are directly affected by rainfall and are subject to abrupt changes in temperature and relative humidity, so if the fruit are harvested in winter or any time after heavy rainfall, a high susceptibility to PS development may occur which renders nearly 30% of the fruit unacceptable for exports (Undurraga et al., 2009).

Cronje (2015) also reported that harvesting fruit during cold, wet weather conditions coupled with low rainfall three months prior harvest could result in high susceptibility of 'Eureka' lemons to PS. Undurraga et al. (2006) reported that maturity stage and fruit size can influence the development of the disorder. The authors found fruit with yellow rind to be more susceptible than those in silver stage which means finding ways to delay colour change in lemon after harvest could reduce the susceptibility of fruit to PS. After fruit harvesting, it is important for fruit to be stored in a suitable environment in order keep the quality of the fruit as well as reduce the risk of fruit developing rind physiological disorders. This is because postharvest storage temperature has been found to play a significant role in inducing the incidence of peteca spot.

Undurraga et al. (2014) conducted a study in 'Eureka' lemon fruit using 3 different storage temperatures (3, 7 and 11 °C). The results showed 3 °C to result in more peteca development whereas Undurraga (1998) reported a temperature of 8 °C to cause no peteca in a study conducted in Chile. Cronje (2015) explained the relationship between temperature and peteca development to be linked with stress which is associated with the formation of different compounds under these temperatures. Low temperatures are known to lower the activity of enzymes which causes denaturing/breakdown of the structure (Sala, 1998). As the fruit defends itself against stress conditions caused by low temperatures, compounds such as oxalates, calcium and peroxides increase their concentrations (Hu et al., 2003), which are conciliated by

enzymatic activities (glutathione peroxidase, ascorbate peroxidase, polyphenol oxidase and peroxidases) (Hodges et al., 2004).

The role of peroxide as a secondary messenger in plants is the transduction of reactive oxygen species signals that are involved in the adaptation and induction of defense genes (Undurraga et al., 2009). This could explain the development of PS in the reported cold storage of 3 °C. Peteca spot has been correlated with an imbalance of calcium and potassium in the flavedo of lemons (Gomes et al., 2017). This happens because during development, cells present a strong concentration of calcium oxalate from the calcium that is found in the cell wall and vacuoles which then results in the collapse in the cell followed by dehydration that collapses the flavedo of the fruit (Fichet et al., 2012). This makes mineral nutrition one of the important factors that influence the susceptibility of PS, therefore, it is important to ensure a suitable balance between calcium and potassium during growth and development of the fruit.

Other postharvest factors that can aggravate the incidence of peteca include wax application and fruit brushing. Wild (1991) found commercial polyethylene based waxes containing 16% solids to have a high significant effect on the development of the disorder. The author also reported that the incidence was high when fruit were waxed immediately upon arrival to the laboratory after harvest. Although these factors (preharvest and postharvest) have been reported to be involved in peteca spot development, up to date, there is still no single factor that has been identified to be directly responsible for its occurrence, therefore, more knowledge on the pre-symptomatic biochemical markers that could lead to its development is needed.

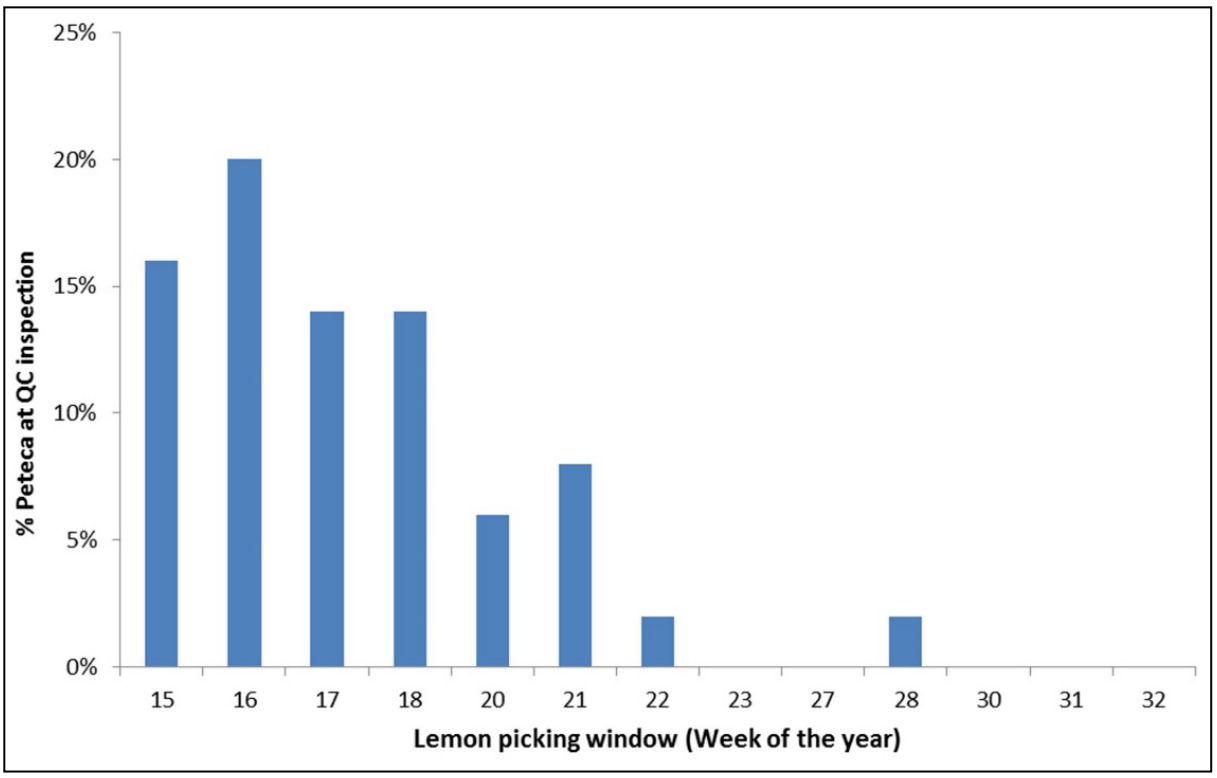

Fig. 1: Peteca spot incidence in citrus packhouse in South Africa, Limpopo in 2012 (Cronje, 2015).

2.2.2. Rind oil spot

Oil spotting, also known as oleocellosis, is a common peel injury of citrus fruit (Shomer and Erner, 1989). This physiological disorder is caused by the phytotoxic action of oil released from oil glands upon surface cells of the rind (Xie et al., 2018). It has been identified in lemon fruit (Cahoon et al., 1964) and in oranges (Erner, 1982). The severity of the disorder is mostly seen in cold wet periods during harvesting and fruit handling particularly in humid areas or arid areas with heavy dew (Shomer and Erner, 1989).

The susceptibility of citrus fruit to oleocellosis development has been associated with ethylene degreening. Citrus fruit are non-climacteric, the fruit usually take time to change colour after harvest hence the use of ethylene degreening in order to accelerate colour change and make the fruit more marketable. The problem associated with the use of ethylene is that is can also accelerate the incidence of the disorder. A study done in Washington 'Navel' oranges showed that the application of ethephon (380 ppm) increased the susceptibility of the fruit to oleocellosis (Levy et al., 1979). However, Cronje (2015) applied ethephon (200 and 400 ppm) in lemon and found that both concentrations significantly reduced the incidence of peteca spot.

Levy et al. (1979) reported that the high susceptibility of the fruit to oleocellosis could be a result of stomatal closure that might have been caused by the application of the treatment.

Apart from ethylene degreening, mechanical harvesting has also been reported to influence the development of the disorder. This is because mechanical harvesting may cause bruising which results in a rupture of oil glands that spreads over the surface of the rind, causing a collapse and death of the flavedo cells that are surrounding the oil glands. The oil kills nearby parenchyma, epidermal and sub epidermal cells of the flavedo preventing the usual differentiation of chloroplasts into chromoplasts hence causing the disorder development (Wardowski et al., 1998; Xie et al., 2018). Both green fruit and fruit that have already developed colour are susceptible to rind oil spot. Research has also found that small fruit are more susceptible than larger fruit and this can be related to slow metabolic processes in smaller fruit which affect colour development (Xie et al., 2018). Once a single fruit is affected, it can spread to the adjacent fruit which is how the disorder spreads (Soule and Grierson, 1986).

Oleocellosis can occur at any time during harvesting, handling, and marketing, but it usually occurs at or near harvesting time (Wardowski et al., 1998). The symptoms are irregularly shaped spots around the surface of the fruit (Fig. 2), which may be seen as directly after harvest until the fruit reach the markets. The affected areas are initially yellow and change to dark brown sunken spot as oil spreads and oxidizes (Wardowski et al., 1998). Although the spots do not affect the storage and internal quality of the fruit, it lowers the fruit grade which causes extensive financial loss to growers, shippers, and handlers everywhere in the world. The disorder may occur in all citrus varieties around the world but commercial lemons, sweet oranges and limes are more susceptible (Montero et al., 2012).

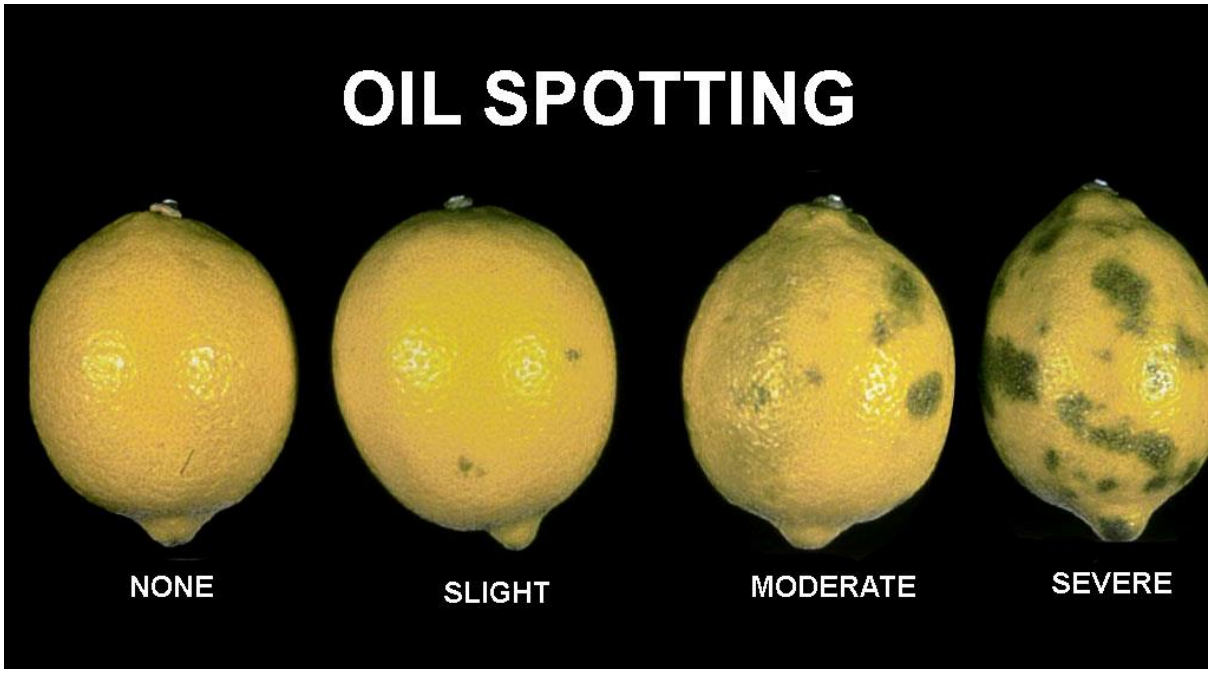

Fig. 2: Rind oil spot symptoms in lemon fruit https://ucanr.edu/repository/view.cfm?article=83419.

2.2.3. Stem-end rind breakdown

Stem end rind breakdown (SERB) is a physiological disorder found on citrus fruit from humid and high rainfall areas. It affects the pericarp tissue that surrounds the button of the fruit (Ritenour and Dou, 2003). Different terms are usually used interchangeably with SERB and these include brown stem, burnt stem, rind breakdown and aging. The symptoms are seen as sunken brown depressions on the rind which also becomes hard and leathery to the touch (Fig. 3) (Ritenour et al., 2004). Grierson (1986) also reported that this disorder is characterized by dark and sunken irregular shape around the stem end of the fruit which develop 2-7 days after packing. The actual cause of this is not known but it is associated with a collapse in the rind tissue (Ritenour and Dou, 2003).

Rind breakdown is a similar disorder caused by prolonged cold storage which may have similar symptoms as SERB. Just like peteca spot on lemons, SERB results in the collapse of oil glands which results in the discolouration on the collapsed area affecting both the flavedo (exocarp) and the albedo (mesocarp) of the fruit. But the difference is that in PS, the oil glands remain intact and the sunken areas are rounded shaped which appear on all parts of the fruit whereas in SERB the brown depressions on the peel appear at the stem end of the fruit. The citrus type

and variety, growing conditions, fruit maturity and growing seasons influence the severity of the disorder (Grierson, 1965).

The most susceptible citrus varieties to this disorder are orange and grapefruit. Stem end rind breakdown has a major economic loss in the citrus industry and this is because the symptoms usually develop after the fruit have been processed and packed for shipment which results in the rejection of the fruit in the export market (Ritenour and Dou, 2003). Hopkins and McCornack (1958) and Grierson (1965) reported that SERB was highly influenced by degreening where fruit showed a high incidence of the disorder after they had been exposed to ethylene treatment. Wax application (Carnauba, polyethylene, and shellac based) has also been found to increase the susceptibility of fruit to SERB (Mohamed, 2001). Stem end rind breakdown can be a result of preharvest factors, but it is difficult to pinpoint specific ones (How, 2003).

Tree nutrition is one of the main factors that has been investigated and found to influence the development of SERB (Grierson 1986; Agustí, 1999). Different researchers have associated it with nutritional imbalances such as nitrogen and potassium (Agustí, 1999). Ritenour et al. (2004) found that rootstock plays a role in the development of the disorder due to the fact that it has a direct influence on fruit and tree characteristics. Harvesting the fruit from water stressed trees was also found to aggravate the severity of the disorder compared to non-stressed fruit. A recent study by Torres et al. (2018) has reported SERB as a new postharvest physiological disorder in lemon. An overview of rind physiological disorders that occur in citrus fruit are summarized in (Table 2).

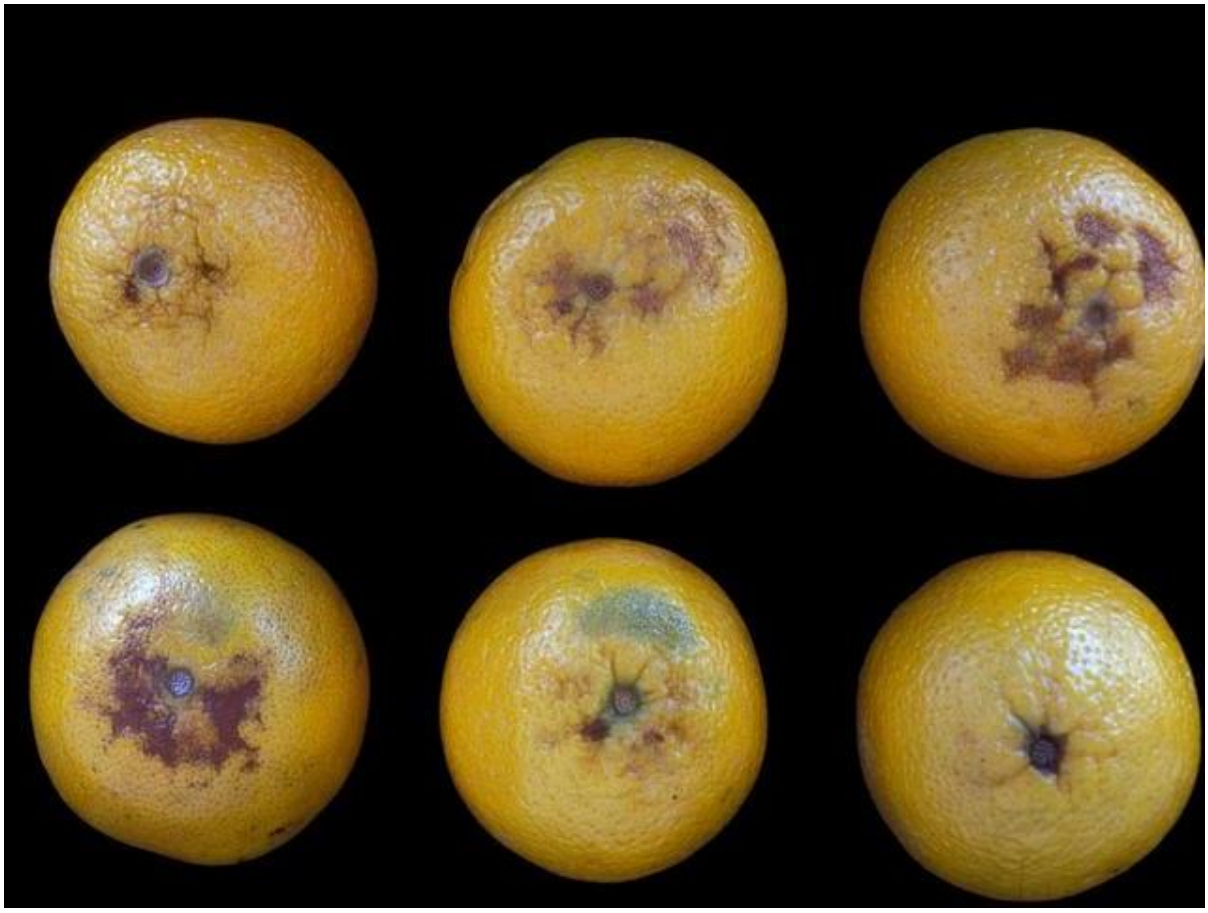

Fig. 3: Symptoms of stem end rind breakdown in citrus (Gerald Holmes, California Polytechnic State University at San Luis Obispo, Bugwood.org, 1992).

Table 2: An overview of symptomology, causes, and affected cultivars of different physiological rind disorders in various citrus fruit quality.

Disorder name	Affected cultivar/s	Causes	Symptoms	Reference
Chilling injury	Lemon	Temperatures below 9 °C	Unusual fruit respiration, surface lesions, pitting	Lafuente et al. (2001)
	Star Ruby	2 °C temperature	Large sunken areas in the rind, fruit decay, pitting	Chaudhary et al. (2014)
	Navel orange	Temp below 10 °C but above freezing point	Collapse of the individual oil glands, dark brown lesions	Eaks (1960)
Non-chilling rind pitting	'Navelate' orange	Dehydration, lack of epicuticular wax	Irregular depressions on the flavedo that may turn brown with time	Cajuste et al. (2010)
Rind pitting	All citrus cultivars are susceptible	Low storage temp, waxing, relative humidity	'Leopard' spot pattern on the flavedo	Alférez et al. (2003)
Peteca spot	'Eureka'and 'Meyer' lemon	Ethylene degreening, waxes, 3°C temperature	Dark brown lesions on the rind of the fruit	Wild (1991); Cronje (2015)
Stem end rind breakdown	Oranges	Collapse of rind tissue	Hard and leathery sunken brown depressions	Ritenour and Dou (2003)
Oleocellosis	Lemon and Orange	Ethylene degreening, mechanical harvesting	Irregularly shaped spots on the surface of the fruit	Wardowski et al. (1998)

2.3. Preharvest factors affecting the incidence of postharvest rind physiological disorders

Most of the postharvest rind physiological disorders do not show symptoms immediately after harvest which has made it difficult to identify specific conditions that could lead to their development. Certain postharvest factors such as ethylene, storage temperature and coatings have been identified to cause physiological disorders and researchers have been recently focusing on preharvest factors due to a number of disorders being related to climatic conditions. Fruit maturity, mineral nutrition and canopy position are some of the factors that have been reported to determine fruit susceptibility to physiological disorders.

2.3.1. Mineral nutrition

Essential mineral nutrients of which plants cannot complete life cycle without include (in no order) nitrogen, phosphorus, potassium, copper, boron and magnesium (Fageria, 2001). Mineral nutrition in plants has long been studied and was reviewed since about 50 years ago by Chapman (1968) and was further elaborated by Mattos Junior et al. (2010), however, the reviews did not highlight the interactions of nutrients with fruit quality and physiological disorders. A recent review by Aular et al. (2017) focused on the effect of mineral nutrition on citrus fruit. Mineral nutrition plays an important role in fruit quality which makes nutritional balance a key factor for vigorous plant growth and better tolerance to biotic and abiotic stresses. A change in mineral nutrition in the fruit not only affects the quality of the fruit, but may also result in the development of physiological disorders (Aular et al., 2017).

Concentrations of nitrogen and potassium in the leaf contents have been reported to determine the production and quality of ‘Clementine’ tangerine (Hammami et al., 2010) and ‘Kinnow’ tangerine (Khan et al., 2011). A high supply of essential nutrients in plants results in toxicity, while an undersupply results in deficiency (Fageria, 2001). High potassium concentration in the fruit was found to cause high water loss and fruit wilting. This has been previously related to the development of physiological disorders in citrus fruit by delaying magnesium, calcium and manganese absorption (Aular et al., 2017). Water loss which leads to fruit wilting was found to cause rind pitting in ‘Navelina’ and ‘Navelate’ oranges (Agustí et al., 2001). The authors explained the mechanism to be due to alterations in turgor pressure of the flavedo and

albedo cells caused by reduced water status in the fruit which ultimately leads to a damage in the peel.

Previous studies showed a positive relationship between mineral nutrition and the development of physiological disorders in citrus fruit. Cronje et al. (2011b) reported that mineral nutrition is one of the important factors that play a role in the development of postharvest rind pitting. For example, nitrogen plays a role in the size, thickness and texture of the peel which may increase fruit susceptibility to rind disorders. Storey and Treeby (2000) also found mineral nutrition to cause severe outbreaks of albedo breakdown (Creasing) rind disorder in 'Bellamy Navel' orange caused by a higher ratio of k/ca and mg/ca in the albedo of the fruit. Magwaza et al. (2013a) reported that shading and light effects caused by the position of fruit in the tree has an influence on the nutrients and the development of rind physiological disoders in citrus fruit.

Lower concentrations of calcium and magnesium and higher potassium are found in the inside canopy of the tree which is associated with thicker and softer rind tissues hence high incidence of rind breakdown (Cronje et al., 2011b). Gomes et al. (2017) reported that an imbalance of calcium and potassium in the flavedo of lemon fruit causes PS. Khalidy et al. (1969) also identified calcium and boron to play a role in the development of the disorder. This might be attributed to higher concentration of potassium inside the flavedo of fruit which is strongly linked to stress response. The imbalance, therefore, compromises the rind integrity resulting to the development of the disorder.

As reported in most studies assessing the effect of mineral nutrition on the quality and physiological disorders in citrus fruit, the effect is mainly due to excess or little availability as well as an imbalance of essential nutrients. This means the application of these nutrients could help in reducing the incidence of the disorders. Li et al. (2008) found that the development of peel pitting in 'Navel' orange was effectively decreased by almost 50% following the application of 1% of $CaCl_2$. Calcium plays an important role in the cell wall of citrus fruit, resulting in the overall healthy growth and development of the fruit. Its ability to reduce physiological disorders is due to its relation to electrical conductivity of the cells and the ability to reduce enzymes activities that could lead to the development of the disorder (Cajuste and Lafuente, 2007).

2.3.2. Canopy position

Fruits are produced throughout the canopy around the tree and are exposed to varying irradiation and temperature which may result in difference in colour of fruit from the same tree (Cronje et al., 2011a). The major influence caused by this difference is the effect it has on consumer preference with regard to eating quality and physical appearance (Magwaza et al., 2013a; Cronje, 2014). Canopy position has an influence on the biochemical composition of the fruit caused by the difference in shading and direct exposure to sunlight (Cronje et al., 2011b). Shading affects the biochemical processes occurring during fruit growth and development and this is because when the fruit is shaded, the amount light it is exposed to is limited hence affecting respiration and photosynthesis processes (Ncama, 2016).

Fruits outside the canopy are directly exposed to sunlight, which makes them lose more water, but they are characterized with higher sucrose, glucose and fructose than fruit in the inside canopy, this is one of the reasons for lower susceptibility of the outside canopy fruit to rind disorders (Cronje et al., 2011a). The susceptibility of fruit to certain physiological disorders differs according to seasonal climatic and orchard environmental conditions (Wild, 1991). The incidence of rind spot in 'Fortune' mandarins was found to be higher in fruit from the outside canopy than shaded fruit (Almela et al., 1992). Several studies conducted in mandarins showed a higher incidence of rind pitting in the fruit exposed to direct sunlight (Duarte and Guardiola, 1995; Chikaizumi, 2000; Victor et al., 2000). The difference in fruit susceptibility of different canopy positions to physiological disorders is related to temperature, water potential and irradiation.

Fouche et al. (2010) investigated the irradiance levels within 'Granny smith' apple trees. The results showed that fruit from the outside canopy which were on the Northern side of the tree had 53% exposure to full sunlight while fruit from the inside canopy only received 2% full sunlight. Outside canopy fruit are associated with high TSS concentration, high antioxidants and low total acidity and this could be attributed to their easy access to photoassimilates, which are produced by outside canopy leaves (Cronje, 2014). Antioxidants in plant cells protect the plant against harmful effects of ROS, including hydrogen peroxide, ozone and singlet oxygen, which are known to be partially reduced forms of atmospheric oxygen (Mittler, 2002; Purvis, 2004).

2.3.3. Fruit maturity

Fruit maturity was found as one of the determinants for the development of PS in 'Meyer' lemon (Wild, 1991). It has an influence on morphology and composition of cells and cell walls in the flavedo and albedo of citrus fruit. Fruit maturity influences the susceptibility of fruit to rind pitting development by affecting water flow through rind layers (Alférez and Zacarías, 2014). When evaluating the influence of fruit maturity on the development of rind pitting on 'Navel' oranges, Alférez and Zacarías (2014) found that rind pitting increased with maturation except for mature green fruit. As the fruit reached maturity, there was an increase in water potential differences of fruit that were maintained at 45% or 95% RH due to the rind tissue reducing the ability of water adjustment during maturation.

Grierson (1981) reported that large sized mature fruit are more susceptible to rind physiological disorders than small and immature fruit. This is probably because immature fruit have a high probability of failing to develop the yellow/orange acceptable colour in citrus fruit with only shades of green/yellow colour development on the flavedo. This has been reported to cause oleocellosis in citrus fruit (Wardowski et al., 1998). Most rind physiological disorders like peteca spot, stem end rind breakdown and oleocellosis have been associated with the collapse of oil glands and epidermal rupture above glands, mature fruit are characterized with high oil content in the rind compared to immature fruit and if the oil is directly released into the rind tissue, it causes the occurrence of the above mentioned rind disorders (Knight et al., 2002).

In mandarin development, the occurrence of rind breakdown was found higher during fruit maturity (Almela et al., 1992). The susceptibility of 'Clementine' mandarin to rind spotting was found to be at the beginning of colour break and develops until harvest (Assimakopoulou et al., 2009). When the fruit is still attached to the tree, its susceptibility to rind pitting starts from colour change and will continue until senescence depending on climatic conditions (Agustí et al., 2001). However, during postharvest storage, the disorder will occur irrespective of harvest time although mature fruit have high incidence of development probably because the synthesis of biochemical attributes in the fruit increases as the fruit matures (Alférez and Zacarìas, 2001).

Undurraga et al. (2009) reported that fruit maturity has an influence on the development of PS. The authors concluded that there is a significant interaction between maturity and temperature

on the concentrations of peroxide and calcium in the albedo of the fruit that lead to the development of the disorder. This was also confirmed by Duarte and Guardiola (1995) who reported a positive correlation between rind pitting and fruit maturity. These authors reported that fruit harvested at yellow stage had more disorder than those that were harvested green. This could be because during ripening, there is a loss of calcium from the cell walls which causes solubilization of pectin and accelerates senescence. The authors suggested that the development of the disorder can be controlled by the application of gibberellic acid (GA3). This is because the application of this hormone at colour break acts as a barrier to delay chlorophyll loss as well as the accumulation of carotenoids pigment in rind tissue. GA3 is a hormone that is responsible for delaying rind senescence hence it had positive results when used to control rind pitting.

2.4. Postharvest factors affecting the incidence of the disorders

The susceptibility of fruit to physiological disorders is mainly due to preharvest factors, but the severity is caused by postharvest factors. Citrus fruit tend to develop physiological disorders after harvesting which is mainly influenced by factors that follow after storage. Fruit water loss, ethylene, storage temperature and coatings are some of the key factors influencing the development of rind disorders in citrus fruit (Magwaza, 2013).

2.4.1. Coatings and waxes

In citrus packhouses, there are postharvest treatments as well as conventional synthetic waxes and chemical fungicides (including Imazalil (IMZ), Thiabendazole (TBZ) and sodium orthophenylphenate (SOPP)) that are used in order to extend shelf life, improve quality and control postharvest losses (Palou et al., 2015). The cause of postharvest losses in citrus fruit could be due to mechanical damages causing rind wounds or bruises during harvest. The wound act as an infection site for postharvest fungal pathogens, causing deterioration of the fruit. Losses are also caused by poor postharvest handling mainly storage conditions which lead to the development of most serious physiological disorders (Ismail and Zhang, 2004).

Although these fungicides have been used and were previously found successful for many years, there's a recent rising concern with regard to health and environmental issues that are associated with synthetic waxes, chemical residues and the proliferation of pathogen strains.

This issue has led to the EU market banning the use of synthetic waxes (Kruger, 2013). Many other countries are also increasingly restricting the use of agrochemicals, and there's a huge demand from export markets to reduce the residue levels of fruit lower than those established by official regulations (Palou et al, 2015). These changes are causing significant losses in the citrus export industry, which has led to the need to develop edible coatings that will be used as an alternative to synthetic waxes but providing the same advantages of preserving the quality of the fruit and extending shelf life (Palou et al., 2015, Tesfay and Magwaza, 2017).

Postharvest application of coatings in fruit is largely practiced in order to reduce moisture loss, lower respiration rate and to extend shelf life and quality of fruit (Palou et al., 2015). Hagenmaier and Baker (1993) applied wax coatings to citrus fruit and found that it restricted gaseous exchange in the fruit resulting in high concentration of internal carbon dioxide hence reducing weight loss. The problem associated with the use of waxes is that some of them can aggravate the incidence of physiological disorders in fruits. Wild (1991) reported that commercial polyethylene based citrus wax and carnauba based wax formulations had a highly significant effect on PS development in 'Meyer' lemon. The author concluded that the application of these waxes greatly enhances the incidence and the severity of the disorder.

Young and Biale (1968) explained that the reason behind this could be because some of the waxes restrict gaseous exchange resulting in increased carbon dioxide concentration that increases organic content. The increase results in an overproduction of volatiles which are associated with anaerobic conditions. This causes calcium imbalances that may lead to the development of PS (Khalidy et al., 1969). Petracek et al. (1995) reported that waxing 'Marsh' grapefruit with shellac-based waxes enhances rind pitting. Polyethylene and shellac based coatings cause poor water distribution in the rind and show more disorder than unwaxed fruit (Bajwa et al., 2006). There has been an increased interest in many research groups to develop new natural, biodegradable, edible coatings that will replace the currently used synthetic waxes.

Edible coatings are described as ecologically friendly substitutes that are applied on fresh produce to reduce water loss, gaseous exchange and respiration (Dhall, 2013). The most important characteristics of edible coatings are the edibility, biodegradability, migration, permeation as well as physical and mechanical protection, which improves the shelf life of the fruit and preserve quality (Han, 2014). Some edible coatings also have antimicrobial activity (Krochta, 2002) hence they can be applied on fruit to enhance safety. The benefits of using

edible coatings is that they are environmentally friendly and do not pose any threat to human health. Another different functional property of edible coatings is that they have a high ability to be used as carriers of postharvest active ingredients. For example they can be used as anti-browning agents, and antimicrobial activity, food colourants, and they can improve shelf life by reducing the risk of pathogens on food surfaces (Pranoto et al., 2005).

Among other edible coatings, chitosan and carboxymethyl cellulose (CMC) and moringa extracts have made a significant contribution to the food industry (Tesfay and Magwaza, 2017). Moringa (*Moringa oleifera*) is a perennial tree that is grown in most parts of the world including South Africa, India, Florida, Ethiopia and Tropical Asia. It is well known for its medicinal uses and it can also be used for industrial purposes. All parts of the Moringa tree (The leaf, root, bark, flower, sap) are edible and mostly consumed by humans. These parts are considered healthy as they possess antimicrobial and antioxidant activity (Tesfay et al., 2016). Edible coatings are also known to have high concentration of phenolics, vitamins and carotenoids (Yang et al., 2006).

These characteristics of moringa have made it possible for the plant to be used as edible coating which has shown positive results in improving postharvest quality and extend shelf-life of various fruit. A research done by Adetunji et al. (2012) showed that the addition of moringa to edible coatings (corn starch and CMC) reduced mass loss and extended shelf life. Chitosan is biopolymer which is also used as an edible coating due to its antimicrobial and non-toxic nature (Silva et al., 2017). Chitosan has also been used in many studies as an edible coating to prolong shelf life of fruits and has been found effective. Chitosan coating forms a semi-transparent layer on smooth surface which reduces respiration and transpiration rates (Pagno et al., 2018).

Chitosan application was evaluated on mango where it showed positive results in extending shelf life (Silva et al., 2017). Coatings containing chitosan have also been used to extend the shelf life of strawberries (Vargas et al., 2006; Badawy et al., 2017) and avocadoes (Tesfay and Magwaza, 2017). Plácido et al. (2016) reported the effectiveness of chitosan-based coating on postharvest quality of tangerines. Edible coatings that are based on cellulose gums are known to significantly delay ripening of some climacteric fruits such as banana, mangoes and papayas. They can also reduce enzymatic browning on sliced mushrooms (Nisperos-Carriedo et al., 1991).

2.4.2. Ethylene degreening

The internal edible part of citrus fruit usually reaches maturity while the flavedo is still green. This has led to the application of ethylene for degreening the fruit in order to accelerate colour change and makes it marketable and attractive to consumers (Porat, 2008). Ethylene is an unsaturated hydrocarbon that has physiological impacts on living plant material. When applied to a fruit, it destroys the chlorophyll in the flavedo that gives the fruit its green colour which then allows for the existing yellow and orange pigments that are associated with edible mature fruit to be more visible (Jacob-Wilk et al., 1999). The initial practice of degreening process was to extend marketing season for early varieties and for fruit that take long to change colour due to environmental and growing conditions (Porat, 2008).

Ethylene is a hormone that plays a role in senescence of fruits and is also involved in the development of cellular degradation that is associated with stress conditions (Porat et al., 1999). The hormone is also known to protect fruit against stress conditions that lead to damaged tissues. It has been found to control citrus fruit from stress that cause injury and non-chilling peel pitting (Lafuente et al., 2001). Notably, ethylene can speed up senescence, button abscission and it can also result in the development of physiological disorders. Mayuoni et al. (2011) reported that the application of ethylene for degreening in packhouses may have a negative influence on the quality of the fruit. Degreening has been associated with inducing the incidence of rind breakdown in 'Nules Clementine' mandarin (Cronje et al., 2011a).

In the early published studies on the effect of ethylene in citrus fruit, Abeles et al. (2012) reported that it could increase the susceptibility of the fruit to superficial infection by the *colletotrichum* fungus which is due to ethylene injury in instances where fruit is not handled properly. According to Poole and Gray (2002), this hormone is able to reduce the occurrence of physiological disorders in mature fruit that are harvested after colour break, however, in citrus industries it is only applied for degreening purposes and the concentration of ethylene used, time exposure and external conditions should be well managed (Cohen, 1978). The application of ethylene combined with temperature can cause some serious physiological disorders in citrus fruit (Eaks, 1970).

McCollum and Naul (2007) reported that 3-5 days of ethylene exposure (5 ppm) to fruit at 20-29 °C results in increased respiration which enhances physiological aging of the peel tissue and

causes the appearance of rind disorder symptoms. Some of citrus varieties are more sensitive to ethylene degreening and show more disorder development and examples include 'Villa Franka' lemon (Cohen et al., 1983) and 'Fallglo' tangerines (Petracek et al., 1998). Another effect of degreening in citrus is a defect known as green islands which is seen by the appearance of green spots around the fruit. This disorder has been reported in 'Navel' oranges (Porat, 2008). It has also been reported to enhance calyx senescence which results in calyx abscission and browning (Carvalho et al., 2008).

2.4.3. Storage temperature

Citrus fruit are characterized by a long shelflife, the fruit have the ability to be stored for as long as 6-8 weeks in cold storage without losing quality. The fruit are non-climacteric, they produce very little ethylene and have low respiration rate which makes them undergo little compositional changes after harvesting (Kader, 2002). Low temperatures have been used effectively for many years to prolong shelf life of fresh citrus and this is because when used correctly, it can retard respiration, slows the production of ethylene, senescence and undesirable metabolic changes (Hardenburg et al., 1986). Fruit are shipped over long distances during export which means they have to be stored at low temperatures in order to preserve their biochemical properties. Some countries have requirements for quarantine treatments for the purpose of sterilization against Mediterranean fruit fly (*Ceratitis capitate*), Natal fly (*Ceratitis rosa*) and oriental fruit fly (*Bactrocera dorsalis*) (Grout & Moore, 2015) where fruit are stored at -0.6 °C for 14 to 22 days depending on the required time frame of the importing country (Bassal and El-Hamahmy, 2011).

However, most citrus fruit are sensitive to low temperatures, which leads to the development of physiological disorders (Magwaza et al., 2013a). Grapefruit and lemons are treated at 0 – 2.2 °C for the fruit fly quarantine and this has been reported to lead to a development of chilling injury (Chalutz et al., 1985). Temperatures below 10 °C but above 0 °C are known to cause physiological disorders in tropical and subtropical fruits and storage organs of plants (Siboza et al., 2014). A high incidence of PS in lemon was found at 3 °C (Undurraga et al., 2009). Most fungi related disorders in citrus fruit are negatively correlated to temperature because the decay producing fungi is retarded under low temperatures. After about 4 weeks of storage at 10 °C, there could be a development of blue mold, stem-end decay or alternaria core rot but the rate of decay is not usually commercially significant. Soon after the fruit are removed from cold

storage, the decay producing fungi continues its activity, so the rate of decay depends on the period of storage (Miller, 1946).

2.5. Pre-symptomatic biochemical markers related to physiological disorders

2.5.1. Carbohydrates

Carbohydrates play an important role in fruit development and maintaining postharvest life (Ladaniya, 2008). They make up a structure of all living cells and the compound is made up of carbon, hydrogen and oxygen which is defined by the formula $(CH_2O)_n$ (Holland et al., 2005). Carbohydrates are the main determinants of flavor, colour and texture and they are classified into three sugars which are monosaccharide, oligosaccharide and polysaccharide (Ladaniya, 2008). Monosaccharide sugars include D-glucose, D-fructose and galactose of which the first 2 are the most dominant in citrus fruit.

Carbohydrates are related to plant stress responses in the flavedo of citrus fruit. They are also involved in cold stress resistance in fruits (Holland et al., 2005). Holland et al. (1999) studied the changes in carbohydrate content and metabolism in the flavedo of 'Fortune' mandarin to determine the involvement of carbohydrates in the fruit's ability to tolerate chilling injury. There was an increase in fructose and glucose during the seasons with sucrose showing little change. The author concluded that sucrose is involved in chilling resistance of the fruit. Soluble sugars play a role in the structure and functioning of all living cells (Couée et al., 2006). They are sources of carbon and energy and sucrose is mostly involved in photosynthesis, transport and heterotrophic utilization.

2.5.2. Antioxidants

Fruit colour is one of the important attributes used by consumers to assess fruit quality (Lado et al., 2016). The change of colour in fruit is highly influenced by carotenoids and chlorophyll concentration and distribution. In citrus fruit, there are certain carotenoids that determine the colour in the rind and pulp of different varieties (Kato, 2012; Zhang et al., 2011). Carotenoids are a precursor of vitamin A which makes them beneficial in human nutrition (Rao and Rao, 2007). Britton (2008) defined carotenoids as C40 isoprenoid molecules that play different roles in plants. They are pigments that are responsible for giving citrus fruit colour and they range

from yellow coloured lemon to white and red grapefruit and the orange colour of sweet orange and mandarin. The pink/ red colour in grapefruit is a result of lycopene accumulation.

Carotenoid concentration is affected in the inside canopy because light is an important (but not only) factor for carotenoid synthesis (Steyn, 2012). An earlier research by Sites and Reltz (1949) on oranges and mandarins showed that fruit from the inside canopy developed low peel colour than those in the outside canopy and this is because of the high light intensity in the outside canopy. Carotenoids transfer energy to chlorophyll and act as a photoprecusor, which is responsible for dispersing excess light energy. They are also known as substances that are produced in small quantities, which are able to prevent the oxidation of easily oxidisable materials (Frankel and Meyer, 2000). One of the most important carotenoids in citrus is lycopene, a red carotene that is characterized by a high antioxidant capacity because of the presence of double bonds (Aizawa et al., 2011). Carotenoids play an important role in chloroplast and chromoplast stabilization and they also act as powerful scavengers of reactive oxidation species (ROS). Antioxidants in plant cells protect the plant against harmful effects of ROS including hydrogen peroxide, ozone and singlet oxygen which are known to be partially reduced forms of atmospheric oxygen (Mittler, 2002; Purvis, 2004).

These ROS are generated by photosynthesis and respiration processes and can also be produced when the cell undergoes stress. Their sources are NADPH and amine oxidases as well as cell wall bound peroxidases (Purvis, 2004). The main sites for ROS generation are in the chloroplast, mitochondria, cytoplasm and the apoplastic region (Mittler, 2002). The interest that arose in studying these ROS is because is the threat they cause to cells when there is excess production, but at the same time they are signals that activate stress and defense mechanisms by the cell. In cases where the generation of ROS exceeds the capacity of the cell to remove them, there's an occurrence of oxidative stress (Hodges et al., 2004).

An increase in carotenoid content in citrus fruit is positively correlated with high stress tolerance (Zhang et al., 2011). Kim et al. (2012) reported that stress conditions were favorable for the growth of sweet potato transgenic cells accumulating high amount of β carotene. Lycopene increment was studied in tomato and was found to result in less incidence of chilling damage (Whitaker, 1944). Rugkong et al. (2011) confirmed that fruit which are damaged have four times lower lycopene content compared to undamaged fruit. Zhang et al. (2013) showed that other antioxidants also play a role in reducing disease development on fruit. The author

reported that anthocyanin accumulation during cold storage of transgenic tomatoes led to a reduction in postharvest deterioration and disease incidence.

Lado et al. (2016) assessed the association between carotenoids and chilling injury of grapefruit, their findings showed that the fruit susceptibility to chilling injury was strongly linked with the presence of lycopene in the peel. Yellow 'Marsh' and red 'Star Ruby' grapefruit were subjected to a low temperature of 2 °C and the results showed a high susceptibility to chilling injury in the yellow fruit, while red fruit showed no symptoms. This could be due to a high carotenoid content in the red grapefruit which was found to be 14 times higher than in the yellow fruit. Interestingly, the authors concluded that lycopene plays a role in protecting citrus fruit from the development of chilling injury (Lado et al., 2016). The authors have also established the relationship between fruit colour (highly contributing to the external quality) and the composition of pigments in the tissue.

Ascorbic acid, phenolics and glutathione are some of the other non-enzymatic antioxidants found in citrus fruit that can influence the development of physiological rind disorders. Ascorbic acid is the chemical name for vitamin C, which is located in the apoplast and chloroplast of plant tissues (Foyer, 1993). This antioxidant helps in protecting cells from substances that damage DNA (Blokhina et al., 2003). It is also known as the generic term for all compounds exhibiting the biological activity of L. ascorbic acid. In citrus fruit, ascorbic acid was found to retard browning (Liao, 1988). It is a hexose metabolism product that can be detected in most plant cell types (Foyer, 1993). Ascorbic acid is able to reduce hydrogen peroxide to water through the metabolism of ascorbate peroxidase and this makes it an important antioxidant that can prevent all metabolic disruption as well as cellular damage (Foyer, 1993; Khumalo, 2006).

2.5.3. Phenolic compounds and flavonoids

Phytochemicals such as phenolics are major bioactive compounds, which are well known for health benefits in fruit and vegetables. All parts of the plant, whether edible or not, show a presence of phenolics with multiple biological effects (Jacob et al., 2008). As reported by Shahidi (1997), phytophenolics play a role in health promotion and they have an ability to prevent diseases. They are well known for their ability to defend the plant from stress and to adapt from a change in environmental conditions (Ćetković et al., 2007). The mechanism

behind this is that they are directly related to cell differentiation, deactivation of pro-carcinogens, DNA repair maintenance and the ability to suppress the formation of N-nitrosamine. The antioxidant mechanisms of phenolics in functional foods are known as free radical scavenging and metal chelatin activities (Rafiq et al., 2018). Most importantly, a lot of antioxidants found in plants show a wide range of characteristics such as antibacterial properties, antiviral, and antimicrobial (Cook and Samman, 1996).

Flavonoids are known as polyphenolic compounds with a phenyl benzopyrone structure. They have a 2-benzene ring (C_6) structure joined by a linear C_3 carbon chain that has a carbonyl group at the C position (Bar et al., 1990). Flavonoids are regarded as non-nutritive agents but play an important role in preventing diseases in human beings. Citrus fruit contain flavonoids such as glycosides (e.g. hesperidin and narigin) as well as other types of flavones. The accumulation of these flavonoids is in the peel where there's high concentration compared to the rest of the fruit. Some flavonoids are responsible for giving citrus fruit its sweetness (Bar et al., 1990). Assini et al. (2013) studied the biochemical functions of flavonoids in the peel of oranges and they were found to increase serum antioxidant capacity against lipid peroxidation.

2.6. The basis of Vis/NIRS application on fruit

Physiological disorders occur unpredictably and mostly manifest after postharvest storage, this poses a serious challenge for exporters (Magwaza et al., 2014a). This has led to an interest amongst researchers to develop models that will be able to predict fruit susceptibility to the disorders during packing and sorting which has been achieved through visible to near infrared spectroscopy (Vis/NIRS) (Nicolai et al., 2007). The use of vis/NIRS is to non-destructively analyze the internal quality of the fruit by means of measuring the amount of reflected, absorbed or transmitted radiation that is illuminated through scanning the fruit (Magwaza et al., 2013b; Ncama et al., 2017).

Vis/NIRS was first used in agricultural applications to measure moisture in grain by Norris (1964), thereafter, its application became more common for rapid analyses of protein, fat and moisture content for different agricultural food products (Davies and Grant, 1987). It has become a key focus in postharvest technology, as demonstrated by the increasing number of publications reviewing and applying in various fruits and vegetables (Table 3). Also, many

manufacturers of on-line grading lines have implemented NIR systems to be used for the measurement of several quality attributes of fruits and vegetables (Nicolai et al., 2007).

Near infrared radiation exists between the visible and infrared region which is defined as stretching from 780nm to 2500nm. This electromagnetic radiation with the kind of energy range interacts with molecular overtones and combination bands of the fundamental molecular vibrations which are found in the mid-infrared region (Engelsen, 2016). The NIR is characterized by absorption bands which are caused by stretching vibrations of molecular groups such as C-H, O-H and N-H (Magwaza et al., 2012). It has many advantages which include the ability to determine biochemical properties of a fruit nondestructively (Magwaza, 2013, 2014a, 2014b).

Samples with high moisture content can be measured, the method is fairly simple and rapid since there is no need for sample preparation and can monitor fast process dynamics. Engelsen (2016) highlighted that NIRS has the ability to simultaneously measure several quality parameters, it doesn't use any chemicals which makes it environmentally friendly and it makes it possible to contrast or fingerprint incoming raw materials and ingredients, process streams as well as the end-products. The long wavelength NIR region stretching from 1900 to about 2500 nm is used for complex fingerprinting purposes due to its hypersensitivity to matrix effects and intermolecular interactions and because it contains the weakest photon energies (Nicolai et al., 2007).

The next region is called the first overtone and it's from 1300 nm to 1900 nm. The first overtone region possesses the same spectral pattern as the fundamental stretching vibrations in mid-infrared. The last region from 850 to 1050 nm is called the shortwave NIR region (Engelsen, 2016). The molar extinction coefficient for each overtone is reduced by a factor of 10. This means that more sample materials can be measured at high overtones and this makes sampling easier and provides a better mass overview (Lebotsa, 2017). NIRS has a lower absorptiveness which makes diffuse reflectance an efficient sampling method which is not biased by the state (solid, semi-solid or liquid) of the components in the sample (Ncama, 2016).

The change in spectrum of the radiation is highly dependent on the fruit being analyzed. When the sample is radiated, the incident radiation may be absorbed, transmitted or reflected depending on the chemical and physical composition of the sample (Walsh, 2005). Previous

studies have focused on using NIRS for predicting internal quality of the fruit and there's been limited research using it for the prediction of physiological disorders. This has led to researchers now shifting focus and evaluating vis/NIRS for predicting physiological disorders. NIRS could be used to predict the susceptibility of lemon to PS by evaluating the physiochemical properties that are linked with the disorder, which include non-structural carbohydrates, dry matter and antioxidants (Magwaza et al., 2014b)

The typical NIR reflectance spectra for various fruit shown in (Fig. 4) is very similar to that of other plant materials such as apples (Liu and Ying, 2005), mandarins (Gomez et al., 2006) and lemons (Reddy et al., 2016).

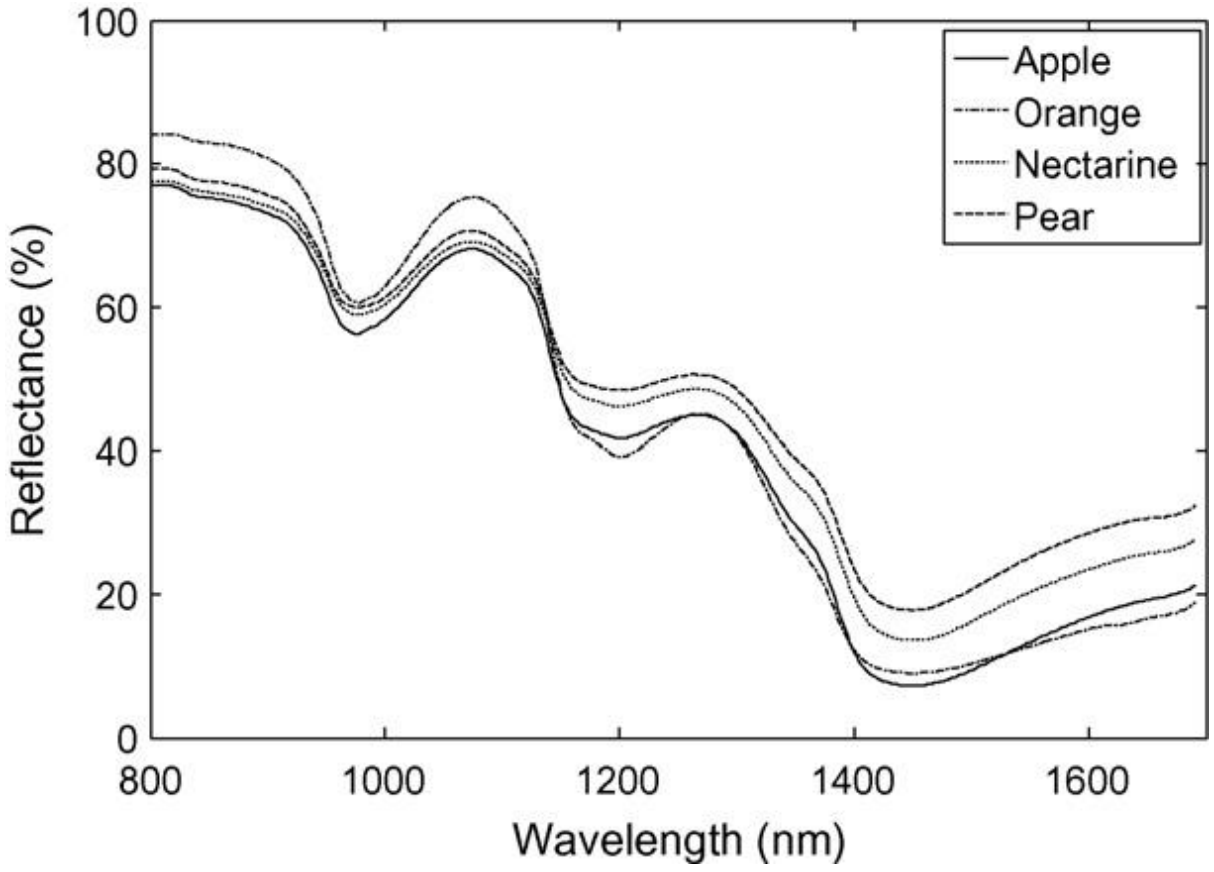

Fig. 4: Typical NIR reflectance spectra (Nicolai et al., 2007).

2.6.1. Instrument measurement setup

Three ways in which the instrument can be set up for obtaining near infrared spectra are shown in (Fig. 5). The difference in the three setups is the positioning of the light source and detector. The most important thing to note when selecting a certain measurement setup, is to make sure that the penetration of NIR radiation into fruit tissue decreases exponentially with the depth (Lammertyn et al., 2000). The illumination of the sample and detector system must allow fast spectral data acquisition of regions representing the whole sample for the attribute of interest (Greensill and Walsh, 2000a).

In the reflectance mode, both the light source and detector are mounted under a specific angle and this is to avoid specular reflection. In this mode, the light penetrates the sample and is re-emergent. The specularly reflected light is an important portion of the detected signal (Green and Walsh, 2000b). The transmittance mode is represented by the positioning of the light source opposite to the detector (Fig. 5b). The light easily penetrates the sample and is re-emergent into a non-illuminated area of detection (Greensill and Walsh, 2000a). The interactance mode is almost similar but different in that the positon of the light source and detector are parallel to each other in a way that the light due to specular reflection is not able to penetrate into the detector (Nicolai et al., 2007).

Both the reflectance and transmittance modes can be used to collect light and increase the signal to noise ratio (SNR) and they have been previously applied to in-line fruit sorting (Moomkesh et al., 2017; Ncama et al., 2017). Full transmittance optics are only possible with citrus fruit which have relatively high transmittance and low signal to noise ratio. SNR below 5000:1 was found to result in relatively poor calibration performances for sucrose in a water- cellulose matrix (Greensill and Walsh, 2000a, 2000b). The choice of an appropriate measurement setup mode to use highly depends on the sample and the light penetrating properties.

For apple, the penetration depth was found at 4mm in the 700-900 nm range while in the 900-1900 nm range it was between 2 and 3 mm (Lammertyn et al., 2000). For the same fruit, Fraser et al. (2000) reported a penetration depth of at least 25mm in the 700-900 nm range, which changes as the range changes. The skin of the fruit (thickness) may play a role in blocking light penetration even at 808 nm which results in poor predictions (Fraser et al., 2003). In grapefruit, a poor SSC prediction was obtained by Miller and Zude-Sasse (2004). The major problem

associated with the limited penetration depth is that it restricts the potential of reflectance or interactance measurements for detecting internal defects and in citrus fruit, it decreases the accuracy of NIR based measurements of internal quality attributes (Nicolai et al., 2007).

Schaare and Fraser (2000) compared the three modes (reflectance, interactance and transmittance) on kiwi fruit to measure soluble solids content, density and internal flesh colour and found interactance mode spectra to provide the most accurate estimates with a standard error of prediction (SEP) of ± 0.80 °Brix and correlation coefficient (R^2) of 0.93. While in mandarin fruit, transmittance mode was found to be more accurate with coefficient correlation of 0.96 and RMSEP of 0.32 °Brix. The success of transmittance mode in determining internal parameters or evaluating disorders can be aligned with the position of light source where the light passes through the fruit from side to side which covers the whole fruit and not miss any important information (Fig. 5) (Ncama, 2016). Research has shown that each mode has its own advantages and disadvantages so the use of one mode depends on the fruit being analyzed as well as the researcher's preference after weighing the available options

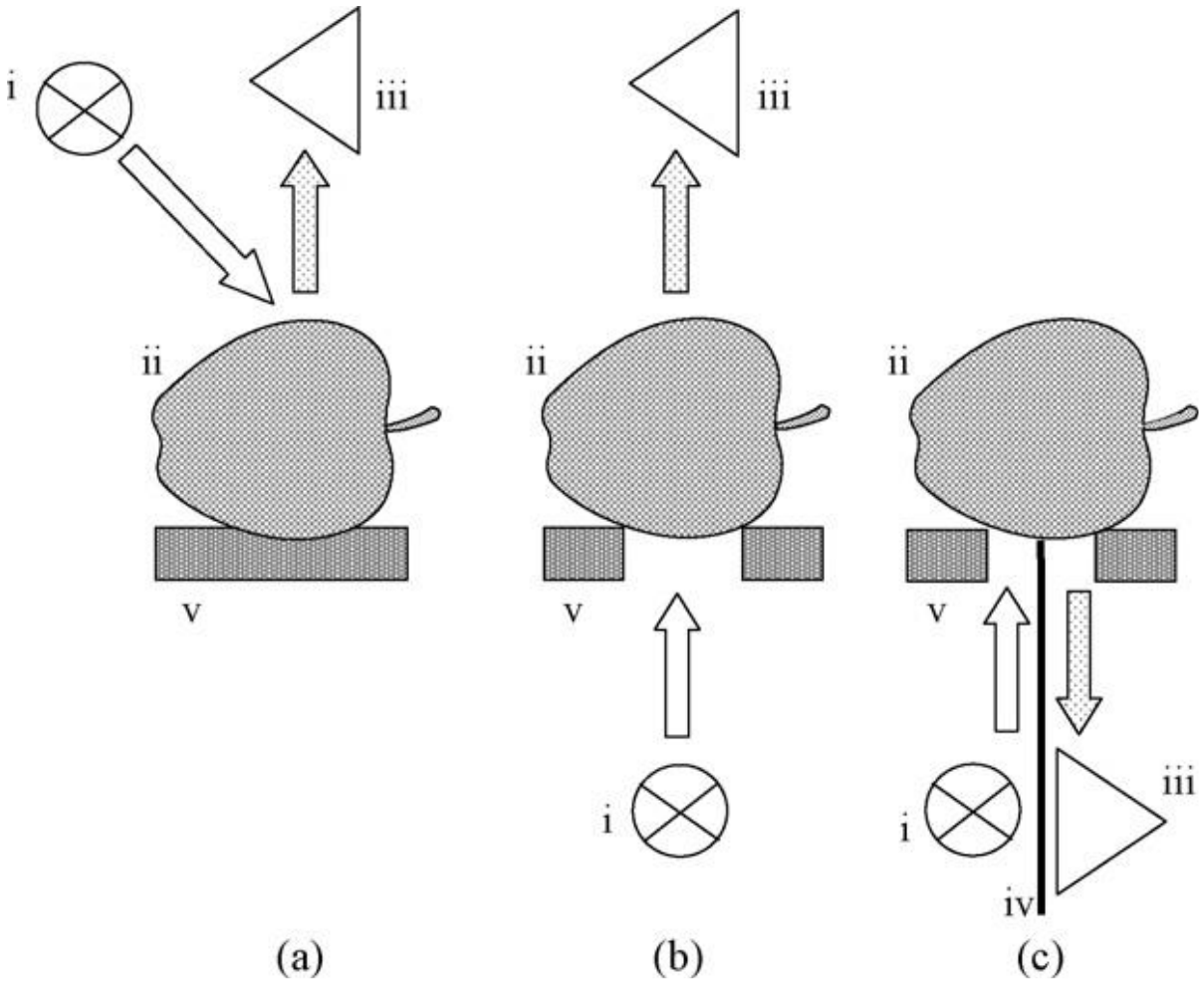

Fig. 5: Measurement setup for the acquisition of (a) reflectance, (b) transmittance, and (c) interactance spectra, with (i) the light source, (ii) fruit, (iii) monochromator/ detector, (iv) light barrier, and (v) support (Nicolai et al., 2007).

2.6.2. Common processing techniques

Sometimes the spectra show unnecessary information which is influenced by physical properties such as shape and size of the fruit resulting in data outliers (Leonardi and Burns, 1999). Pre-processing techniques are employed to remove such data and also for the extraction of important information. According to Ncama (2016), the spectral preprocessing methods are used to remove any error caused by the system and also used for data smoothing. They are applied to remove any irrelevant information which cannot be handled properly by the regression techniques (Nicolai et al., 2007). The suitable selection of pre-processing or pre-treatment method is an essential step in spectral analysis and calibration because of the baseline shifts and noises that exist in the spectra with broad wavelength regions (Cen et al., 2006).

For the facilitation and handling of robustness and easy to use models, statistical procedures are commonly used for pre-processing the complex spectral data. The most common smoothing types used for denoising the spectra are moving smoothing, first and second derivatives, de-trending, normalization, multiplicative scatter correction (MSC), Savitzky-Golay polynomial smoothing (first and second derivatives) (Næs et al., 2002), standard vector normalisation (SNV) and smoothing using moving average (Blanco and Villarroya, 2002). To increase the spectral resolution, the first and second derivatives are used. Other researchers have mentioned available data preprocessing methods which include orthogonal signal correction and standard normal variate.

Nicolai et al. (2007) explained Averaging, Centering, Smoothing, Standardization, Transformation and Normalization as methods that have been developed for data smoothing. Smoothing and MSC pre-processing techniques were used by Gomez et al. (2006) to correct the additive and multiplicate effects of spectra. The study resulted in better models for MSC pre-processing compared to the ones without. Huishan et al. (2005) reported that the quality of models will most likely not be improved by different spectral pre-processing after analyzing 3 different types of pre-treatments (SNV, MSC and Constant). By far, MSC pre-processing has been reported to be superior method compared to other pre-processing methods.

During spectrum acquisition, averaging over spectra is performed so as to reduce the thermal noise of the detector. The number of scans that are done will be dependent on the application. For example, in online grading systems, the PDA spectrophotometer will operate at a typical acquisition time below 50ms which gives it little time to acquire more than one scan (Nicolai et al., 2007). The main purpose for averaging over wavelength which is a common processing technique is to smooth the spectra by reducing the number of wavelengths. According to Greensill and Walsh (2000b), the optimum measurement precision for calibration development was lower than what was expected with 0.02 coefficient of variation and optimal signal to noise ratio of 5000:1.

The first step in pre-processing involves mean centering, which is to subtract the average from each variable in order to make sure that all the results are able to be interpreted in terms of variation around the mean (Nicolai et al., 2007). Another preprocessing method that is also common is standardization which means the spectrum is divided by the standard deviation at

every wavelength. In NIR spectroscopy, standardization should be avoided because the noise on a variable which has a small standard deviation can blow up resulting in the unreliable model (Næs et al., 2002).

There are a lot of chemometric software packages that offer a number of normalization methods of which multiple scatter correction is the most important technique. This functions as a compensation for additive and multiplicative effects in the spectral data. The baseline shifts and superposed peaks are often eliminated by the derivation of the last method called Transformation (Martens et al., 1991). NIR consists of two transformers which are Fourier and wavelet. They are used to enhance the signal to noise ratio in order to improve the signal quality, however, the wavelet transformer works better than Fourier because it has faster data decomposition due to its window width that varies with frequency (Nicolai et al., 2007).

2.6.3. Accuracy and robustness of vis/NIRS on citrus

The use of NIRS requires stable and reliable robust models in order to obtain accurate measurements (Magwaza et al., 2012). A robust model is a model that has high prediction accuracy that is relatively not sensitive to unknown changes in external factors (Nicolai et al., 2007). The value of the coefficient of determination R, (Equation 1) is usually used to describe the accuracy of the models for fruit quality prediction. The root mean square of calibration (RMSEC) (Equation 2) and root mean square error of prediction (RMSEP) (Equation 3) are also used. The coefficient of determination represents the measure of correlation between the calibrated and laboratory-based actual data and for a model to be a good one, this value should be high approaching 100% accuracy (Magwaza et al., 2012).

Both RMSEC (described as the error of calibration during the development of prediction formulae) and RMSEP (the error of the developed formulae to predict the validation data values) should be low, with a small difference between the two errors for the model to be robust and accurate (Magwaza and Opara, 2014). A low average difference between predicted and measured values is known as Bias (Equation 4) and it also explains a good model. Another characteristic of a good model is a very low number of latent variables or principal components (Huishan et al. 2005; Ncama, 2016). Lebotsa (2017) stated that the accuracy of the model is highly dependent on sample position during scanning and this is because of a variation in the distribution of quality parameters within a single fruit.

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

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

$$RMSEP = \sqrt{\sum(y_{pred} - y_{act})^2 / n}$$ (Equation 3)

$$Bias = \frac{1}{n}\sqrt{\sum(y_{pred} - y_{act})^2} / n$$ (Equation 4)

Where:

n = number of spectra

y_{act} = the actual value

y_{mean} = the mean value

y_{cal} = the calculated value

The robustness of the developed calibration model should be carefully assessed before vis/NIR technology is applied to any given fruit commodity that are grown under different conditions. The performance of the model is usually affected by variations within the tree which include factors such as the age of the tree, crop land, position of fruit on the tree and the effects caused by light. Not only is it affected by variations within the tree, but also within the orchard which is influenced by the location of the tree and light effects (Magwaza et al., 2012, 2014a).

A robust model should be accurate regardless of the instrument used. Wang et al. (1991) explained factors that affect model performance including changes in the use of an instrument in cases where calibration is done in one instrument but produces different results when another instrument is used. The authors also explained the effects caused by temperature fluctuations, electronic drift and changes in wavelength which may cause instrumental drift. Samples belonging to different batches due to tree position, within orchard variation, soil characteristics

and weather conditions may also affect the accuracy of a model (Peirs et al., 2002). These factors contribute to the difference in physicochemical properties of fruit from the same tree which affects model accuracy when measuring given parameters.

According to Golic and Walsh (2006), temperature differences between calibration and validation set mainly cause bias in fruit and vegetable applications. A bias of up to 0.3 °Brix was found by Peirs et al. (2003a) when the soluble solids content of apple was 10 °Brix, while in the same variety, Sánchez et al. (2003) found a bias of 0.4 °Brix. Model robustness was tested in kiwi fruit with different fruit sizes, origins and maturity stage. When the fruit were not categorized based on their differences, the results showed high validation errors (Kawano, 1998).

Peirs et al. (2002) also found a high validation error for soluble solids content for apples. The accuracy of calibration model for dry matter of apples developed for one season on another season was evaluated by Lovász et al. (1994). Guthrie et al. (1998) found fruits that were harvested in different times to result in lack of robustness of calibration. In order to reduce errors and obtain a good robust model, the use of a large sample size is crucial with fruit from different seasons and different canopy positions where variability is considered.

Table 3: An overview of previous use of Vis/NIRS in citrus fruit.

Cultivar	Acquisition mode	Spectral range (nm)	Attributes	Reference
Lemon	Reflectance, Transmittance,	400-1100	Fruit freeze damage	Moomkesh et al. (2017)
Lime	Reflectance	636-1236	Total soluble solids, titratable acidity and maturity index	Teerachaichayut and Ho (2017)
Sweet orange	Reflectance	325-1075	Oleocellosis	Zheng et al. (2010)
'Marsh' grapefruit	Reflectance	450-2500	Rind pitting	Ncama et al. (2018)
Mandarins	Reflectance	350-2500	Rind breakdown disorder, Rind hue angle (h°), rind dry matter, non-structural carbohydrates	Magwaza et al. (2014a)
Tangerine	Reflectance	768-960	Drying internal disorder	Peiris et al. (1998)
Orange	Reflectance	350-2500	Soluble sugar content, total acidity	Cayuela and Weiland (2010)
Valentia	Reflectance	800-2500	Soluble solids and total acidity	Cayuela (2008)
Grapefruit and orange	Reflectance	450-2500	Sweetness and flavor	Ncama et al. (2017)
Orange	Reflectance	700-1000	Soluble solids	Jamshidi et al. (2014)
Valencia orange	Reflectance	780-2500	External and internal quality	Magwaza, (2013)
Lemon	Reflectance	360-1250	Soluble solids, titrable acidity, vitamin C	Reddy et al. (2016)
Satsuma mandarin	Reflectance	800.44-2779.32	Mass, total soluble solids, total acidity	Lebotsa (2017)

2.6.4. Portable NIR F750 spectrophotometer

There has been a lot on ongoing research using NIR instruments for the prediction of certain physiological disorders and nondestructive analysis (Peirs et al., 2003b). Although these instruments are highly accurate, their application to field research, such as monitoring chemical changes of developing fruit on trees, are limited by their large size and weight. The availability of low-cost portable NIR has made it possible for fruit to be analyzed in the orchard which

gives it more advantages because fruit do not have to wait for a long time without being analyzed. The F-750 is a handheld, fully integrated system, allowing users to operate it out in the orchard or inside the lab. It's quite simple, the intuitive user interface makes it easy for anyone to use, while its model building software makes it customizable and versatile (Fraser et al., 2000).

Since this is usually done in an open space, there are external factors such as ambient light and fluctuating temperatures that should be accounted for by the use of appropriate data processing. The F-750 uses near-infrared (NIR) spectroscopy to estimate quality metrics such as dry matter, total soluble solids (TSS), titratable acidity, and colour (Tripathi, 2009). Working like a high-powered flashlight, the F-750 sends particles of light into a commodity, then measures the NIR light interactance with molecular components inside of the commodity to quantify user-selected traits (Miller and Zude-Sasse, 2004).

Portable NIR has many applications from determining optimal harvest timing by assessing fruit maturity to providing an objective analysis of produce quality of fruit in packing houses and upon import. The instrument was found to have a better potential for the determination of Brix value of apples (Temma et al., 2002). The instrument was also found to work better in other fruits like mangoes (Schmilovitch et al., 2000), in apples (Liu et al., 2007) and cherries (Lu, 2001). A miniature spectrometer with a range of 350–999 nm was used by Ventura et al. (1998) to measure the SSC of apple fruit. To minimize environmental stray light interference, the fruit was placed in a constructed box with a rubber ring and the results showed RMSEP of 1.1 °Brix. Various uses of portable instruments have been described (Temma et al., 2002; Miller and Zude-Sasse, 2004; Zude et al., 2013). However, there's a lack of research evaluating F-750 instrument on citrus fruit which could allow for the analyses of fruit while they are still in the orchard.

Fig. 6: Portable near infrared spectroscopy NIRF750spectroscopy.

2.7. Conclusion

The major citrus industries are still faced with a huge challenge with regard to exporting produce, due to postharvest losses caused by physiological disorders. The problem associated with these disorders is that they may not develop during packing and sorting but later after

harvest which coincides with the time the fruit reaches the international markets and the point of sale. To reduce the incidence of these disorders during postharvest storage, researchers have come up with a solution of using edible coatings. The use of edible coatings is increasingly becoming a key focus, mainly because of the characteristics they possess. Previous studies on edible coatings have focused more on improving the quality of fruit and extending shelf-life and there is still lack of understanding regarding the use of coatings on reducing the incidence of physiological disorders on citrus fruit.

Future research on the identification of pre-symptomatic biochemical markers that could be used to predict the disorders before the symptoms become visible is suggested. However, the methods used for measuring these biochemical attributes require a lot of sample preparation, which is time-consuming when working with a large sample number. For this reason, Vis/NIRS has been introduced, which is a rapid and simple way to predict internal and external parameters as well as physiological disorders in different fruits. The challenge, however, lies in the selection of a robust model that will give accurate predictions. The use of transmittance, reflectance and interactance modes together with pre-processing settings has been reviewed by many researchers in order to improve predictions. Vis/NIR spectroscopy has been previously used in most studies for predicting the internal quality, and although the focus has now shifted to predicting rind physiological disorders, more research and knowledge is still needed.

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Chapter 3: Determination of pre-symptomatic biochemical markers related to peteca spot in 'Eureka' lemon

Abstract

During export, South African citrus fruit must undergo cold quarantine treatment against two major phytosanitary pests, namely, fruit flies (*Ceratitis* sp.) and false codling moth (*Thaumatotibia leucotreta*). One of the phytosanitary requirements is to expose citrus fruit to cold disinfestation at -0.6 °C for 14 consecutive days. However, lemon fruit are sensitive to temperatures below 4 °C and tend to develop peteca spot (PS) which causes a major economic loss to the South African citrus industry. The biochemical profile or changes occurring in the rind of lemon fruit that could lead to the susceptibility to PS is not fully understood. The aim of this study was to identify pre-symptomatic biochemical markers related to PS in 'Eureka' lemon fruit. A total of 300 commercially mature 'Eureka' lemons were harvested from outside (OC) and inside (IC) positions of the tree canopy. In order to induce PS incidence, fruit were stored at 3 °C throughout the storage duration of 12 weeks. Sampling was done at 3 weeks interval for a period of 12 weeks. Each week, rind physico-chemical properties including PS incidence, colour, vitamin C, carotenoids, sucrose, glucose and fructose were measured. Data was subjected to analysis of variance using Genstat and correlation among variables was established using principal component analysis (PCA). Peteca spot incidence was significantly affected by canopy position ($p < 0.001$) with fruit from IC having more disorder than OC. The high PS incidence in the IC was related to high sucrose concentration, which was positively correlated with the disorder (r = 0.78). OC fruit were less susceptible to PS and this coincided with high ascorbic acid (AsA), total carotenoids, glucose and fructose concentrations and color index. Ascorbic acid, fructose and total carotenoids were negatively correlated with PS (r = -0.65, -0.48 and -0.53), respectively. The correlations (both negative and positive) between biochemical parameters such as AsA, sucrose, fructose, glucose, carotenoids and PS demonstrates the possibility of using these biochemical attributes as pre-symptomatic markers for the susceptibility of 'Eureka' lemons to PS.

Key words: Biochemical profile; citrus; physico-chemical properties, principal component analysis

3.1. Introduction

Citrus production in South Africa is a major contributor to the economy in terms of sustainable land management, food security, value addition, employments and export earnings (Citrus Growers' Association of South Africa, 2018). Citrus exports made up 2.46% of the gross value of agricultural products in 1996, which has increased to 25% by 2017. Of the total citrus production, lemons (*Citrus limon*) account for about 13% (9 781 ha) (National Department of Agriculture, 2018). The international trade of fruits and vegetables is faced with a phytosanitary concern of fruit flies, which has led to fruit consignments being rejected in the export market (Manrakhan et al., 2018). Jansen (2017) reported that approximately 8% of South African export shipments are rejected for not meeting temperature requirements prior to packing. Additionally, about 15% of the containers, vessels, cold storage facilities, and refrigerated road transport equipment used for cold chain management are rejected annually.

This has spurred a need for fruit coming from South Africa to undergo a period of cold sterilization, which necessitates fruit exposure to -0.6 °C for a prescribed period of at least 15 consecutive days according to the export market requirements (Ncama et al., 2018). There has been a rapid increase in lemon plantings in South Africa and exports have increased over the past decade, but markets that require cold treatment for fruit fly disinfestation have remained inaccessible for lemon fruit (Manrakhan et al., 2018). Although a recent study by Manrakhan et al. (2018) showed a 99.99% confidence level that commercial export grade 'Eureka' lemon produced in South Africa are not a host for fruit flies (*Ceratitis capitata, Ceratitis quilicii, Ceratitis rosa* and *Bactrocera dorsalis*), markets still require an assurance for fly free consignments.

The major problem associated with these requirements is that citrus fruits are sensitive to temperatures below 12 °C and they tend to develop physiological disorders (Alférez et al., 2003; Porat et al., 2004; Van Wyk et al., 2009). Chilling injury and PS are the main rind disorders in lemon that cause major economic losses to the citrus industry (Cronje et al., 2013; Cronje, 2015). Peteca spot is a postharvest physiological disorder that occurs in lemon fruit, its incidence is quite common on fruit stored under 3 °C (Undurraga et al., 2009).

This disorder occurs unpredictably which is, it may not manifest during harvest but later, about three weeks after harvest which concurs with the time that South African citrus reach international market and point of sale (Undurraga et al., 2009; Cronje, 2015). The incidence of peteca occurrence varies from year to year within fruit from the same tree, although this has not been fully evaluated. The symptoms of peteca can be seen in green fruit, but yellow fruit are more susceptible (Undurraga et al., 2009). They include light brown lesions that develop on the flavedo of the fruit which reduces the quality of the fruit (Cronje, 2015).

Factors that have been reported to influence the incidence of PS in citrus fruit include: changes in nutrition during fruit development relating to Calcium (Ca) and Boron (B) metabolism (Khalidy et al., 1969), the position of the fruit on the tree/ canopy position (Wild, 1991), harvest maturity (Undurraga et al., 2009), ethylene degreening (Cronje, 2015) and synthetic wax application (Wild, 1991). Canopy position, as well as orchard location are considered important in fruit production as they directly influence the accumulation of carbohydrates (Barry et al., 2003). They have been reported to have an influence in the biochemical compounds of the fruit, which affects the susceptibility of rind disorders (Magwaza et al., 2013a).

Cronje et al. (2011) and Magwaza et al. (2013b) reported Nules clementine from the inside canopy to be more susceptible to rind pitting disorder. However, there is still a gap with the knowledge of the relationship between biochemical changes within the fruit, antioxidant system and the development of rind disorders (Magwaza, 2013). Understanding this cause-response relationship is crucial in identifying potential pre-symptomatic markers that could lead to the development of these disorders (Ncama, 2016).

Researchers have reported that the breakdown of larger molecules, including carbohydrates, proteins and lipids during respiration form free radicals that can be aligned with citrus physiological disorders (Purvis and Shewfelt, 1993). These free radicals (reactive oxygen species) are species that contain one or more unpaired electrons (Gill and Tuteja, 2010), which are responsible for cell damage and result in a loss of cell compartmentalization which reduces cell respiration hence causing cell death (Ncama, 2016). Once the cell dies, the flavedo of the fruit start collapsing which is visually seen as rounded depressions/ lesions.

One of the most important carotenoids in citrus is lycopene, a red carotene that is characterized by a high antioxidant capacity because of the presence of double bonds (Aizawa et al., 2011).

Carotenoids play an important role in chloroplast and chromoplast stabilization and they also act as powerful scavengers of reactive oxidation species (ROS). Antioxidants in plant cells protect the plant against harmful effects of ROS including hydrogen peroxide, ozone and singlet oxygen which are known to be partially reduced forms of atmospheric oxygen (Mittler, 2002; Purvis, 2004).

Lado et al. (2015) conducted a study that assessed the association of carotenoids to chilling injury incidence in grapefruit. The authors found that the fruit susceptibility to chilling injury was linked with the presence of lycopene in the peel. Yellow marsh grapefruit and Red Star Ruby were subjected to a low temperature at 2 °C and the results showed a high susceptibility to chilling injury in the yellow fruit, while red fruit showed no symptoms. This is due to a high carotenoid content in the red grapefruit which was found to be 14 times higher than in the yellow fruit. This led to a conclusion that carotene, mostly lycopene plays a role in protecting citrus fruit from the development of chilling injury.

The biochemical profile or changes occurring in the rind of lemon fruit that could lead to the prediction and/or susceptibility to PS is not fully understood, which makes it important to identify potential biochemical markers of rind condition that are related to the occurrence of the disorder. Assessing these biochemical markers and correlation with the disorder constitute the principal framework of this research towards understanding different mechanisms that influence the incidence of PS, which may lead to a pre-symptomatic detection and prediction of the disorder. The objectives of this study was to determine pre-symptomatic biochemical markers that are related to peteca spot, determine the effect of canopy position on peteca spot development, and to determine changes in physico-chemical properties over 12 weeks cold storage at 3 °C .

3.2. Materials and methods

3.2.1. Reagents and standards

All the chemicals and other consumables used in this study were obtained from B&M Scientific CC (Cape Town, South Africa) and Prestige laboratory supplies (PTY) Ltd (Durban, KwaZulu-Natal, South Africa). They included 2, 6-dichloroindophenol dye, sodium hydroxide pellets (NaOH), metaphosphoric acid (MPA), ascorbic acid, acetone CP grade, methanol CP, filter

syringe nylon, vial clear glass and caps and sugars standards (sucrose, D-glucose, and D-fructose).

3.2.2. Fruit sampling

The study was conducted using 'Eureka' lemon fruit harvested from a commercial orchard at Malowe Farm located in KwaZulu-Natal, South Africa (Latitude: 30°14'S, Longitude: 29°56'E). 'Eureka' was selected as the lemon cultivar in this study as it is the most important lemon cultivar grown in South Africa, which constitutes 75 % of South African commercial lemon plantings (Citrus Growers' Association of South Africa, 2018). Trees where fruit were harvested from were selected on the basis of a randomized complete block design with five replicates and each block (replicate) consisting of five trees. Of the five trees in each block, three were used as net plot where fruit were sampled. A total of 300 commercially mature 'Eureka' lemon fruit were harvested in May 2017. The lemon rootstock was (C639) with 35% juice content at harvest, which is the minimum acceptable for South African lemon exported to the EU, according to Lado et al. (2014). Half (150) of the harvested fruit were from the outside (exposed to sunlight) and another from the inside (shaded) position of the tree canopy. The harvested fruit were transported in a well-ventilated vehicle to the Postharvest Technology Laboratory of the University of KwaZulu-Natal, where all experiments were conducted. Upon arrival in the laboratory, fruit were left overnight at 16 °C before experiments began the following day.

3.2.3. Storage temperature and data collection

After harvest, week 0 data was collected and quality parameters such as colour, mass and internal parameters were measured. The remaining fruit were transferred into a cold room at 3 °C, which has been reported to induce PS (Undurraga et al., 2009). Fruit were destructively analyzed every three weeks for the period of 12 weeks and PS incidence was scored. The PS index was calculated using Eq. 1 according to Cronje (2015).

$$\text{Peteca}_{\text{Index}} = \frac{\sum\{Peteca\ (0-2)\ x\ No.\ of\ fruit\ in\ each\ class\}}{Number\ of\ fruit\ in\ a\ rep} \qquad 1$$

On each sampling date, the rind was peeled by hand from the rest of fruit and immediately stored at -40 °C until further analysis. A VirTis Freeze dryer system (Model 6KBTES-55, SP industries, Warminster, PA, USA) used to dry the frozen samples for 7 days at > 250 millitor and -40 °C. Freeze dried samples were weighed and water content calculated from freeze-dried samples and expressed as a percentage of dry mass, after which samples were ground into fine powder using a pistil and mortar.

3.2.4. Fruit mass and colour index

Fruit mass was measured using a calibrated weighing balance (RADWAG Wagi Electronic Inc., Poland). Measurements of rind colour variables, L*, a*, b* and *h* of fresh lemon were carried out from three random spots on the equatorial position of a fruit using portable colourimeter (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. The parameters C* (chroma) and h* (hue angle) were calculated according to C*= (a*2−b*2)1/2 and h*= arctan (a*/b*), respectively. The total colour difference was expressed as a citrus colour index (CCI) calculated using Eq. 2, according to Pathare et al. (2013) and Vidal et al. (2013).

$$CCI = \frac{1000 * a}{L * b} \qquad 2$$

3.2.5. Determination of TSS and TA

Total soluble solids (TSS) analysis were measured from squeezed juice using a digital hand-held refractometer with a dynamic control system (RFM340+ BS®, Bellingham and Stanley Ltd, Basingstoke, Hants, UK). Titratable acidity (TA) was determined 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. 3 was applied to calculate TA, expressed as % citric acid (Ncama et al., 2017). The ration of TSS/TA was calculated according to (Eq. 4).

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

$$TSS\ to\ TA\ ratio = \frac{TSS}{TA} \quad 4$$

3.2.6. Extraction and quantification of rind ascorbic acid

The concentration of ascorbic acid was determined from a method described by Hernández et al. (2006) with slight modification. The extraction was carried out from 150 ± 0.5 mg of the dry sample using 5 mL of 3% (w/v) aqueous metaphosphoric acid (MPA). After homogenizing for 1 min using vortex, the sample was placed in ice cubes for 5 min. The sample was centrifuged for 20 min with lamp off using GeneVac (SP Scientific, Genevac LTD., Suffolk, UK) thereafter 0.5 mL of the extract was transferred into a test tube and 2.5 mL of 2.6-dichloroindophenol dye (0.015 g dye in 100 mL of H_2O) was added. After 10 min of incubation in the dark, the absorbance values of the supernatant was read at 515 nm in triplicates using spectrophotometer against 3% MPA.

The preparation of standard curve for ascorbic acid was carried out by dissolving 0.0352 g of ascorbic acid in 100 mL of metaphosphoric acid solution. From the stock solution, 0, 200, 400, 600; 800 and 1000 µL were positioned into test tubes and the volume was made up to 1 mL using metaphosphoric acid. Each test tube was added with 2.5 mL of 2, 6-dichloroindophenol dye and incubated for 10 minutes in the dark. The absorbance values were read at 515 nm to give a linear standard curve with $R^2 = 0.985$. The results were expressed as g/kg.

3.2.7. Determination of total carotenoid content

The total carotenoids were extracted using the method described by Lichtenthaler (1987) with slight modification. A 150 ± 0.5 mg of the lyophilized sample was added into a test tube together with 2 mL of 80% (v/v) acetone. The sample was centrifuged using GenVac® (SP Scientific, Genevac LTD., Suffolk, UK) for 10 min. For maximum detection of carotenoids, the supernatant was measured at 6 wavelengths (470, 646.8, 645, 505, 435 and 663.2 nm). The concentrations of chlorophyll *a* (Ca), chlorophyll *b* (Cb), total carotenoids (Cx) and β carotene were calculated using Eqs. 5, 6, 7 and 8 (Corrêa et al., 2011).

$$Ca = 12.25\ (A663.2 - 2.79\ A646.8) \quad 5$$

$$Cb = 21.5 (A646.8 – 5.10 A663.2) \quad (6)$$

$$Cx = (1000 (A470 – 1.82 Ca – 85.02 Cb) / 198 \quad (7)$$

Where Ca = Chlorophyll a, Cb = Chlorophyll b, Cx = total carotenoids

$$\beta \text{ carotene} = 0.216 A663.2 – 1.22 A645 = 0.304 A505 + 0.452 A453 \quad (8)$$

3.2.8. Determination of sugars

Nonstructural carbohydrates were determined using a method described by Magwaza et al. (2014) with slight modifications for lemons. Briefly, 150 ± 0.5 mg of lyophilized sample was mixed with 70% (v/v) aqueous methanol (3 mL) (v/v) and vortexed for a minute. After incubation for an hour in a hot shaking water bath (55 °C) as described by Terry et al. (2017), the sample was centrifuged for 20 min using GenVac® (SP Scientific, Genevac LTD., Suffolk, UK) and filtered through 0.4 micron nylon filter syringe into HPLC vial for high performance liquid chromatography (HPLC) analysis.

The concentrations of fructose, glucose, and sucrose were quantified using a Phenomenex® column (Rezex RCM - Monosaccharide) equipped with a refractive index detector by injecting 1 mL sample extracts into a Phenomenex- SecurityGuard™ cartridges C18 (4 x 3.0 mm) ID, 10/pk and column of 3.2 – 8.0 mm. Ultra-pure HPLC-grade water was used as mobile phase at 0.6 mL/min flow rate and the column compartment temperature set at 80 °C. The presence and concentration of fructose, glucose and sucrose were determined by comparing the peak detected in the samples with peak area and concentration of the standard curve. The linear reading of the standard curve was from 0.05- 2.5 mg/L and $R^2 = 0.996$. The results were expressed as g/kg.

3.2.9. Statistical analysis

All measured biochemical variables were analyzed using GenStat statistical software (GenStat®, 19th edition, VSN International, UK). The data was subjected to analysis of variance (ANOVA) and means were separated by least significance difference measured at $P \leq 0.05$. Correlations were performed using Pearson's correlation and the data was subjected

to principal component analysis (PCA) using Unscrambler 10.3 (Camo software, As Norway), which was done to find the relationship between parameters and disorder incidence in different canopy positions.

3.3. Results and discussion

3.3.1. The effect of canopy position and storage time on peteca spot development

The interaction between canopy position and storage time for PS incidence was significant ($p = 0.003$), which indicates that PS was affected by cold storage over time (Fig. 1). The incidence of PS was highly influenced by canopy position ($p < 0.001$), where inside canopy fruit had more disorder development than outside canopy fruit. The relationship between canopy position and PS incidence in 'Eureka' lemon has not been fully evaluated, but rather, the disorder has been previously associated with ethylene treatments (Cronje, 2015), changes in Calcium content in the fruit rind (Khalidy et al., 1969), fruit maturity and storage temperature (Undurraga et al., 2009). However, the significant difference between peteca incidence and canopy position observed in this study could indicate that these are not the only factors that affect the susceptibility of PS.

Comparable results have previously been reported (Cronje et al., 2013; Magwaza et al., 2013b; Olarewaju et al., 2017; Ncama et al., 2018). The authors found a high incidence of rind breakdown in the inside canopy of 'Nules Clementine' mandarin and in chilling injury of 'Marsh' grapefruit. The incidence of PS has not been reported to increase with time but the development of other physiological disorders such as rind breakdown in 'Nules Clementine' mandarin was found to increase over time (Magwaza et al., 2013). The increase in PS incidence over time also confirms the results by Undurraga et al. (2009), where 3 °C was found to cause a high PS development. It was also observed that for the first 3 weeks, in both canopy positions there was no disorder development. This could be because during this stage, the fruit were still green (Fig. 3B) and as reported by Undurraga et al. (2006), yellow fruit are more susceptible to PS than green fruit probably due to over maturity and also because the rate of biochemical attributes synthesis increases as the fruit matures.

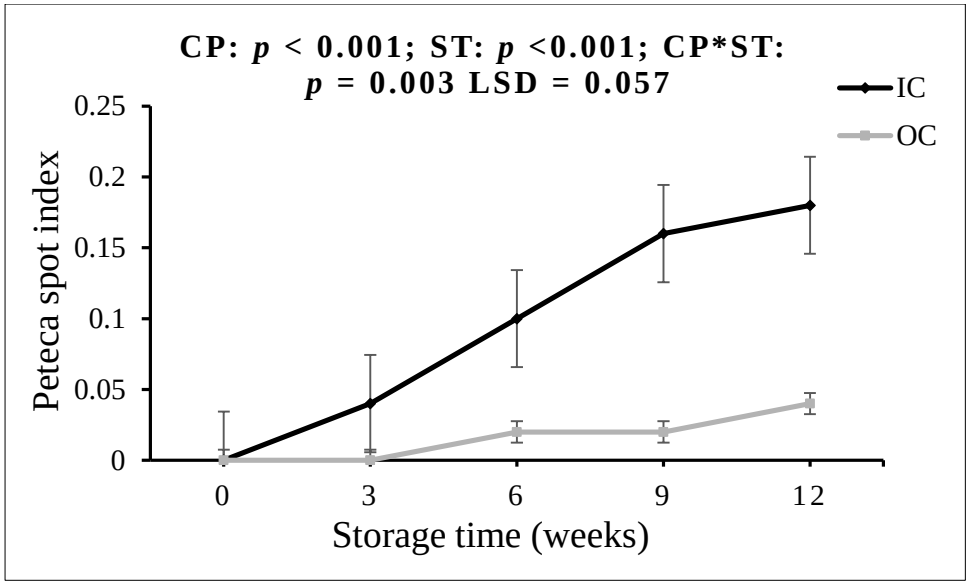


Fig. 1: The incidence of peteca spot in 'Eureka' lemons harvested from outside canopy (OC) and inside canopy (IC) positions over 12 weeks of cold storage at 3 °C. LSD: least significance difference; CP: canopy position; ST: storage time.

The biochemical parameters were significantly influenced by storage time. However, mass, and dry matter of the fruit were not significantly affected by storage time (Table 1). Sugars were expressed as TSS and acidity was based on titratable acids. TA showed a decrease over the period of 12 weeks and this could be due to an increase in sugars and carbohydrates as the fruit changed colour. Lycopene plays a role in the colour development of citrus fruit (Lado et al., 2016). An increase in lycopene concentration over time could be an indication that it played a direct role in the colour development of 'Eureka' lemons.

Table 1: The response of 'Eureka lemon fruit parameters and statistical analysis over 12 weeks of storage time in 3 °C.

	Mass (g)	***TA (%)***	***TSS (%)***	***Lycopene (g/kg)***	***Dry matter (g)***
Week 0	116.5 ab	4.9 a	7.7 a	63.3 a	7.3 a
Week 3	120.3 ab	5.2 ab	7.6 a	69.4 a	7.4 a
Week 6	113.0 ab	5.3 ab	7.7 a	73.3 b	7.5 a
Week 9	112.2 a	5.8 bc	8.1 ab	75.9 bc	7.6 a
Week 12	129.6 b	6.4 c	8.6 b	76.5 c	7.6 a
CV (%)	14.5	15.5	5.9	9.1	6.3
SED	7.701	0.380	0.297	2.843	0.212
P value	0.166	<0.001	0.029	0.010	0.481

3.3.2. The effect of canopy position and storage time on nonstructural carbohydrates

The nonstructural carbohydrates evaluated in this study include sucrose, glucose and fructose which have been found more abundant in citrus fruit (Tzur et al., 1992). These carbohydrates have been reported to play a role in the development of physiological disorders in citrus fruit (Cronje et al., 2013; Magwaza et al., 2013a). There were no significant interactions between storage time and canopy position for glucose ($p = 0.18$) and fructose ($p = 0.252$), however, the interaction between canopy position and storage time was found to be significant for sucrose ($p < 0.001$). This means that, the concentration of sucrose was affected by both storage time and the position of the fruit on the tree hence the different trend from the two sugars. The effect of canopy position was found to be highly significant in all the 3 sugars ($p < 0.001$) (Fig. 2) where non-structural carbohydrates were more concentrated in the outside canopy (Glucose: 56.18 g/kg; Fructose: 60.31 g/kg) than in the inside canopy (48.95 and 54.3 g/kg), respectively (Fig. 2). This is because outside canopy fruit have a higher photosynthetic and respiration rate so they are synthesizing and metabolizing carbohydrates at a higher rate compared to inside canopy fruit (Cronje et al., 2013).

These results agree with those found in other citrus cultivars by (Cronje et al., 2011b, 2013; Magwaza et al., 2013b; Olarewaju et al., 2018). However, the response of sucrose was contradictory with those found by these authors. Sucrose was found to be higher in the inside

canopy (59.4 g/kg) and Ncama et al. (2018) reported similar results in grapefruit. The least abundant sugar found in 'Eureka' lemons in the outside canopy was sucrose (43 g/kg) and the more abundant was fructose (60.31 g/kg) while in the inside canopy sucrose (59.4 g/kg) was abundant and glucose was the least (56.18 g/kg) (Fig. 3). A typical trend for sugars showed an inconsistency over time. From week 0 (after harvest), the concentrations were low and started increasing after 3 weeks of storage. Glucose and fructose reached a peak in week 6, and then started declining from week 9 (Figs. 2B and C).

A similar trend was found by Olarewaju et al. (2017) in 'Nules Clementine' mandarin, where the concentration of sugars started showing a decline after 9 weeks of storage. However, sucrose in this study showed a different trend, where the peak was only reached in week 9 (Fig. 2A). The initial low concentration of sugar content, followed by an increase over time could be because during the first three weeks, fruit were still green (Fig. 4B) and characterized with low acidity. The change in colour from green to yellow is an important quality trait for citrus fruit (Cronje et al., 2011a; Lado et al., 2016). For this to happen, the flavedo needs to accumulate adequate sugar concentration in order to supply energy required for the chloroplast to chromoplast conversion (Huff, 1984). This conversion, as the fruit changes colour could explain the decline in sugars after week 9 which is when the fruit had turned yellow and start reaching senescence.

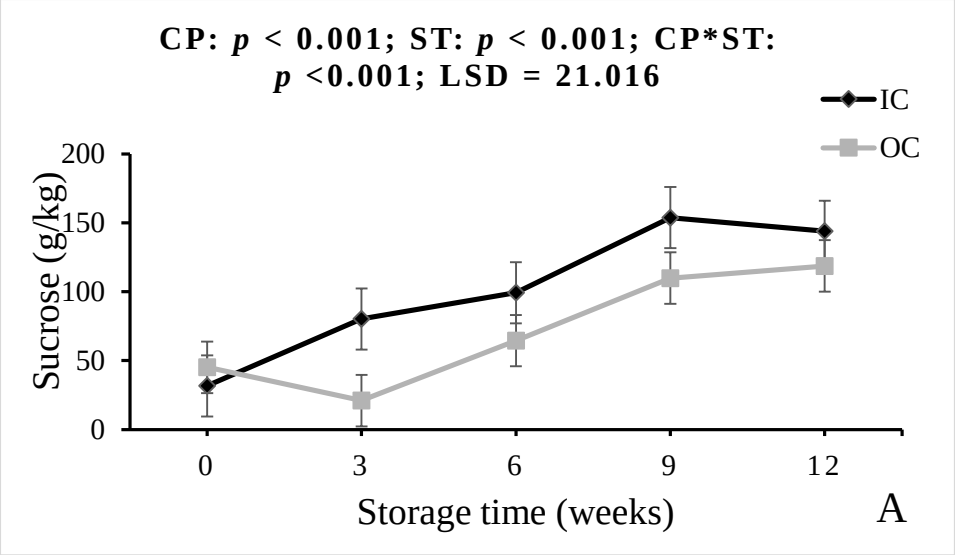
Non-structural carbohydrates (sucrose, glucose and fructose) in plants are known to play a protective role against stress. Gupta and Huang (2014) explained that carbohydrates play a major role as asmoprotectants that are present all over the fruit. Carbohydrates are able to effectively maintain osmotic balance, membrane and protein stabilization which protects the fruit from drought and stress related conditions. In citrus fruit, they are related to drought tolerance, heat stress, maturity of the fruit and changes in colour (Holland et al., 2005). The accumulation of non-structural carbohydrates has been associated with the ability of chilling tolerance in plants (King et al., 1988).

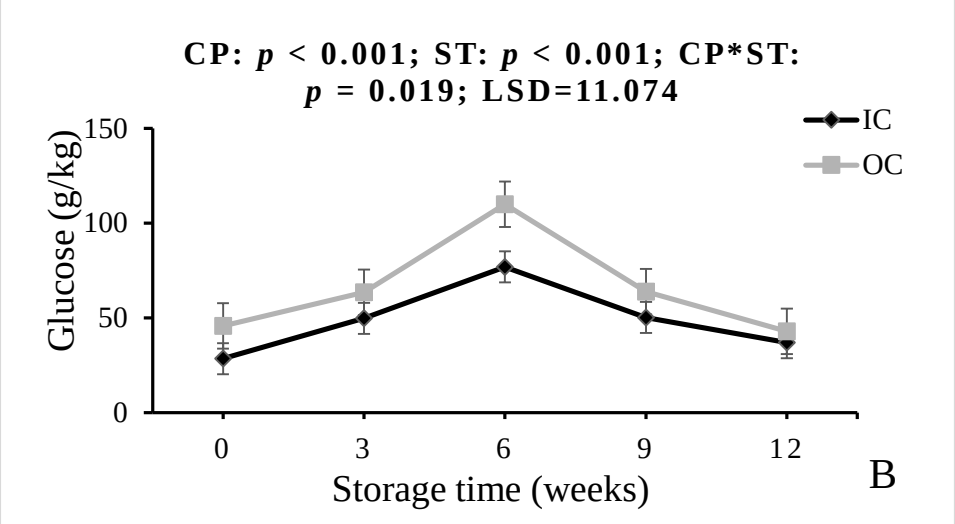
Cronje et al. (2013) stated that the rind condition of the fruit during postharvest is affected by the concentration of carbohydrates in the flavedo which leads to a high sensitivity of fruit to physiological disorders. It is therefore hypothesized that the accumulation of carbohydrates could lead to a less susceptibility of 'Eureka' lemon to PS development. This is because carbohydrates are able to stabilize proteins from stress denaturation because of their impact on

water structure and the degree of hydrophobic interactions applied between biomolecules. Moreover, carbohydrates give more prominent steadiness to chemical responses and biomembranes under environmental stresses because of the capacity to make metastable 'glassy' states with high viscosity (Roos, 1993).

The results observed in this study indicated that PS incidence was high in fruit harvested from the inside canopy which was associated with lower glucose and fructose compared to outside canopy. This suggests that the decreased level of these sugars in the inside canopy was not abundant enough to protect the fruit from developing PS which can also be related to a low photosynthetic rate in the inside canopy. This could be true because the outside canopy fruit with high concentration of the sugars did not develop the disorder. An increase in sucrose concentration has been reported to be a representation of fruit defense mechanism which balances abscisic acid deficiency that supports harsh conditions that could lead to the dehydration of the fruit, hence leading to the development of disorders (Gupta and Huang, 2014). However, the increased level of sucrose that was observed in the inside canopy was not sufficient enough to protect the fruit against the development of PS. Comparable results were reported by Holland et al. (2005) in panalate fruit where sucrose was found to be not abundant enough to protect the fruit from developing rind staining.

It has previously been reported that the variation in photosynthetic active radiation due to canopy position affects the rind of the fruit consequently the chlorophyll concentration, photosynthetic rate and carbon fixation (Magwaza et al., 2013). If these processes are not optimally occurring, the rind physiological condition is reduced which can result in the development of physiological disorders (Cronje et al., 2013). The amount of light that the fruit is exposed to (canopy position) during growth and development is therefore, an important factor that affect the concentration of these 3 sugars and hence the development of PS. This is mainly due to a lack of resources for developmental respiration caused by low carbohydrates in the inside canopy fruit hence the inability of the fruit to tolerate stress exposure during postharvest storage conditions (Cronje et al., 2011b).





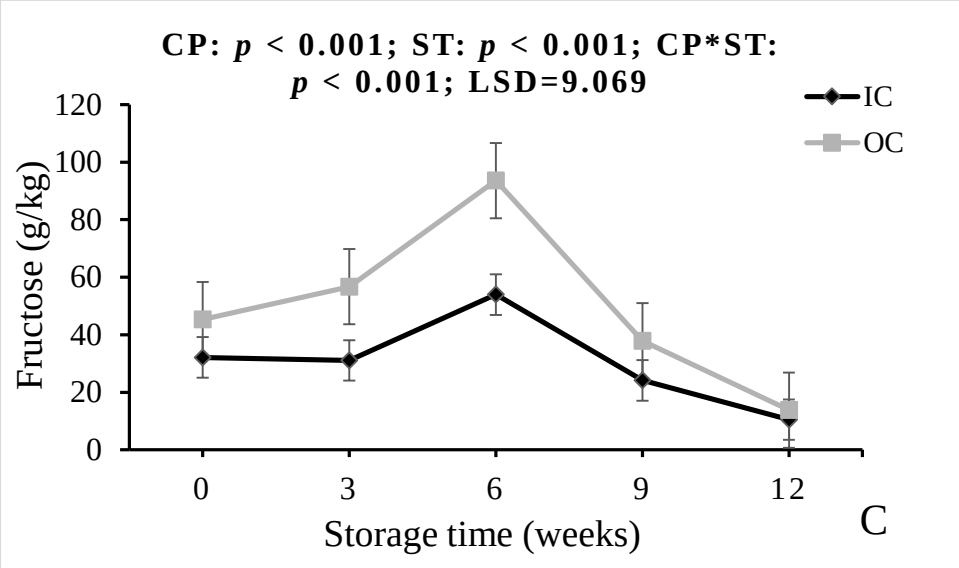


Fig. 2: The effect of canopy position and storage time on sucrose (A), glucose (B) and fructose (C) of ‘Eureka’ lemons harvested from outside canopy (OC) and inside canopy (IC) over 12

weeks of storage at 3 °C. LSD: least significance difference; CP: canopy position; ST: storage time.

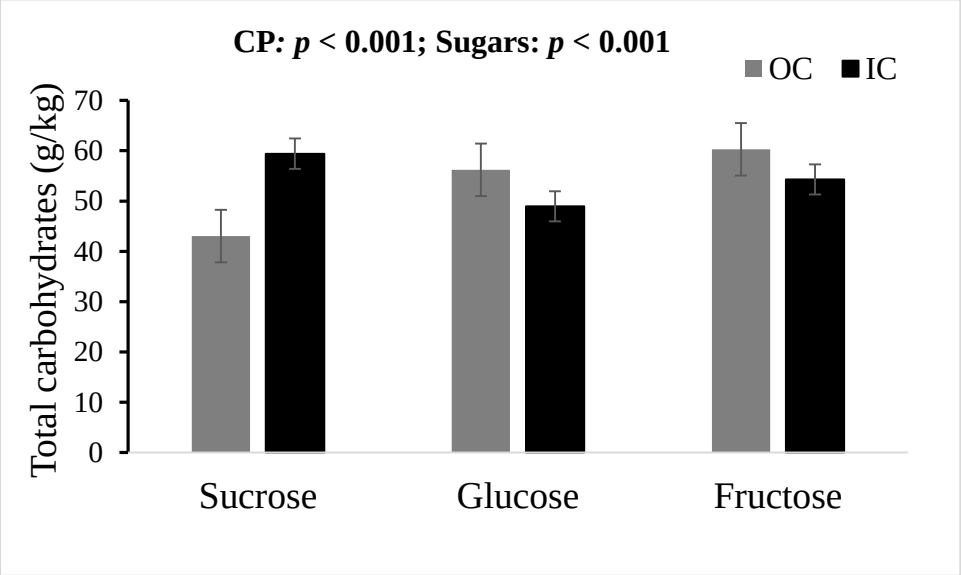


Fig. 3: The effect of canopy position on total carbohydrates (sucrose, glucose and fructose) of 'Eureka' lemons harvested from outside canopy (OC) and inside canopy (IC) positions on over 12 weeks of storage at 3 °C.

3.3.3. The effect of canopy position and storage time on colour development and carotenoids

The results for carotenoids cannot be discussed independently because their accumulation is dependent on rind colour. Carotenoids are known to play an important role in scavenging reactive oxygen species (Mittler, 2002). In citrus fruit, carotenoids are responsible for giving citrus fruit their characteristic colour and they range from yellow coloured lemon to white and red grapefruit and the orange colour of sweet orange and mandarin (Britton, 2008). The interaction between canopy position and storage time was highly significant ($p < 0.001$). This suggests that both canopy position and storage time influenced the concentration of total carotenoids. Canopy position significantly affected the total carotenoids of 'Eureka' lemons with ($p = 0.017$) (Fig. 4B).

The inside canopy fruit had low carotenoids (Fig. 4A) and poor rind colour compared to the outside canopy fruit. Carotenoid concentration was affected in the inside canopy because light is an important (but not only) factor for carotenoid synthesis (Steyn, 2012). After harvest, total carotenoids were significantly higher, and the concentration decreased with cold storage time.

The outside canopy fruit had higher (119.5 g/kg) carotenoids compared to the inside canopy (88.4 g/kg). The lower concentration in the inside canopy fruit could be that while the fruit was still attached to the plant, it received low amount of light during fruit development, therefore, the concentration of chlorophyll needed for photosynthesis process to provide carbon dioxide fixation for the development of the flavedo was limited. When the photosynthesis of the fruit is reduced, respiration rate also reduces which results in low concentration of chlorophyll hence total carotenoids (Steyn, 2012).

β carotene is a precursor for vitamin A and it plays a role in colour development in fruit (Bogacz-Radomska and Harasym, 2018). It was interesting to note that β carotene, which is one of the carotenoids found in the rind of lemon fruit, was probably less influential in the development of the yellow colour of the fruit as seen by the low average concentration of (8. 90 g/kg). The pigment had low concentrations compared to lycopene and total carotenoids, however, the degradation pattern was similar to that of total carotenoids. β carotene was not significantly affected by canopy position ($p = 0.985$) and during the first week of cold storage, the concentration reached values between (11 – 19 g/kg) with outside canopy having the bigger value (Fig. 4B). That was followed by a decrease over the weeks which shows that it barely contributed to the colour development of the fruit.

Colour is the most important attribute in the fruit industry. The yellow coloured lemon is used as an indication of quality fruit in the trade market. The interaction between canopy position and storage time was not significant for all colour parameters, although storage time independently showed a highly significant difference ($p <0.001$) (Fig. 5A, B, C, D). The results for colour showed that both the greenness (a*) and yellowness (b*) were significantly affected by canopy position with ($p = 0.0026$) and ($p = 0.05$), respectively. This shows that inside canopy fruit had a greener colour during the first weeks of storage compared to outside canopy fruit and the colour change was slower than outside canopy colour change (Fig. 5B, C). This could be attributed to reduced light received by the fruit which altered colour development. The slow colour change possibly contributed to high disorder incidence in the inside canopy because of slow metabolic changes occurring in the fruit. The low colour index (Fig. 5D) which was observed in the inside canopy could be a visual symptom which indicates that there was a reduced rind colour which has been reported to contribute to higher incidence of physiological disorders in citrus fruit (Cronje et al., 2013).

Luminosity was not significantly affected by canopy position, but outside canopy fruit were yellower compared to inside canopy fruit. Fruit from the outside canopy were more luminous with values ranging from (77.86 – 92.61) than fruit coming from the inside canopy (71.08 – 82.08). Khalid et al. (2012) when working on 'Kinnow' mandarin and Olarewaju et al. (2018) on 'Marsh' grapefruit reported comparable results with fruit coming from the outside canopy showing a more yellow colour compared to those in the inside canopy.

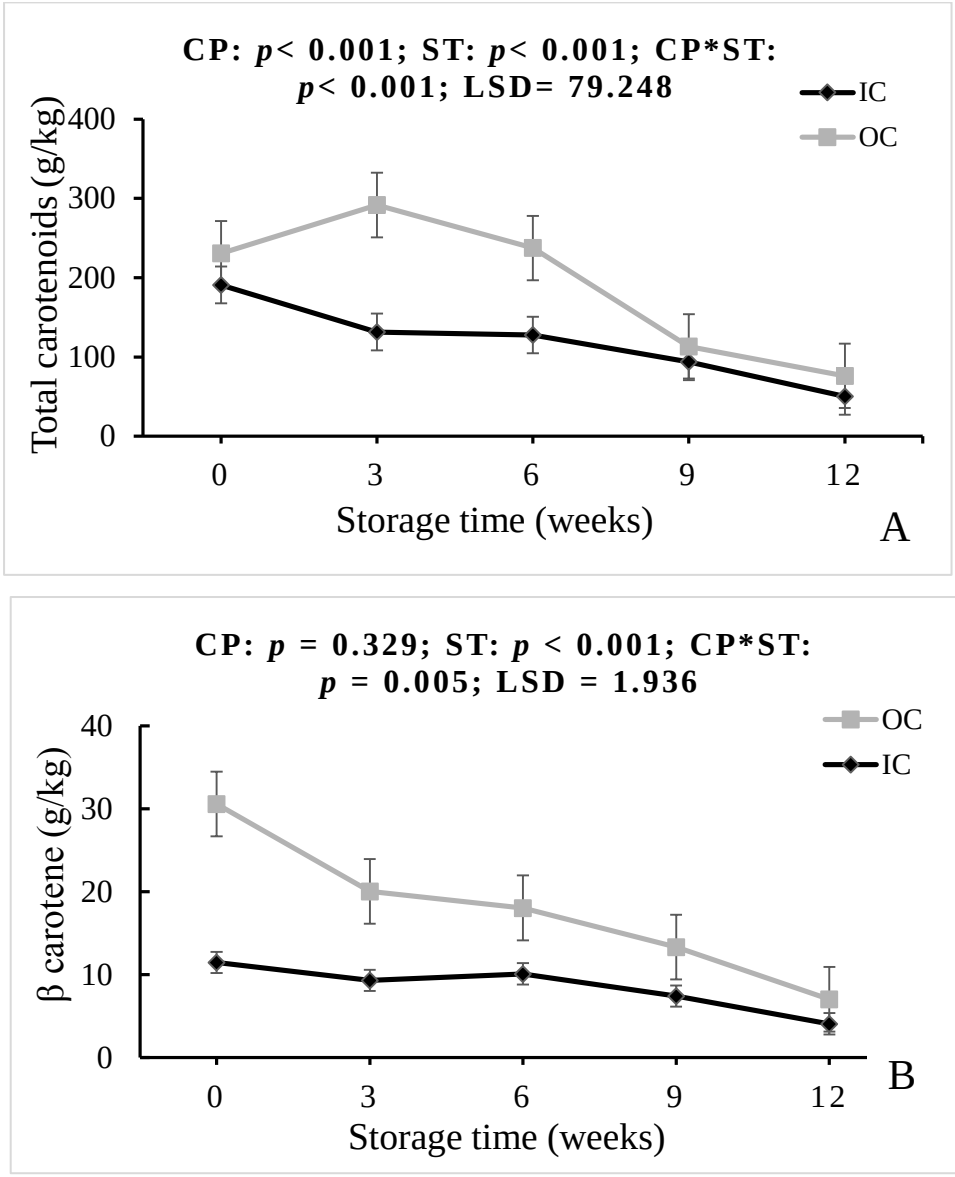
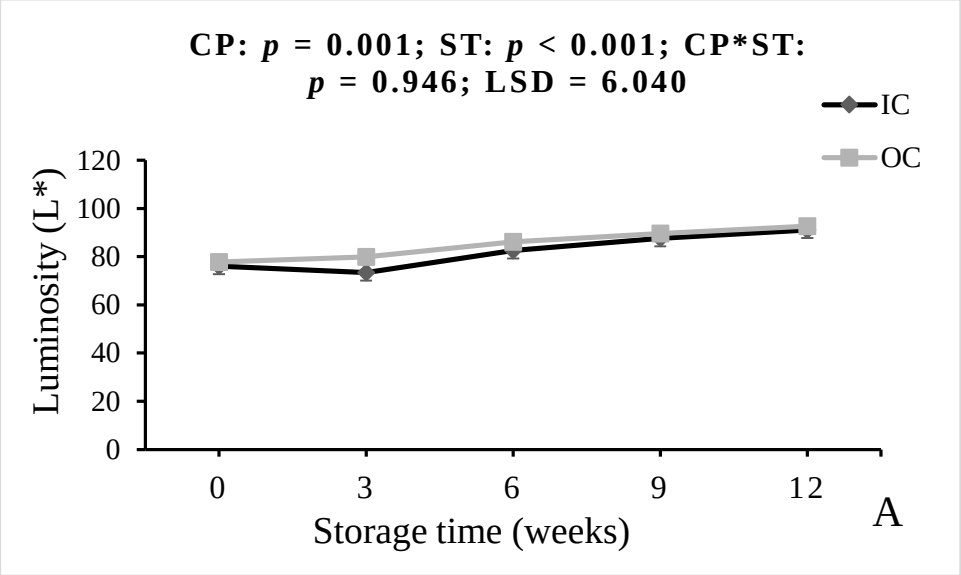
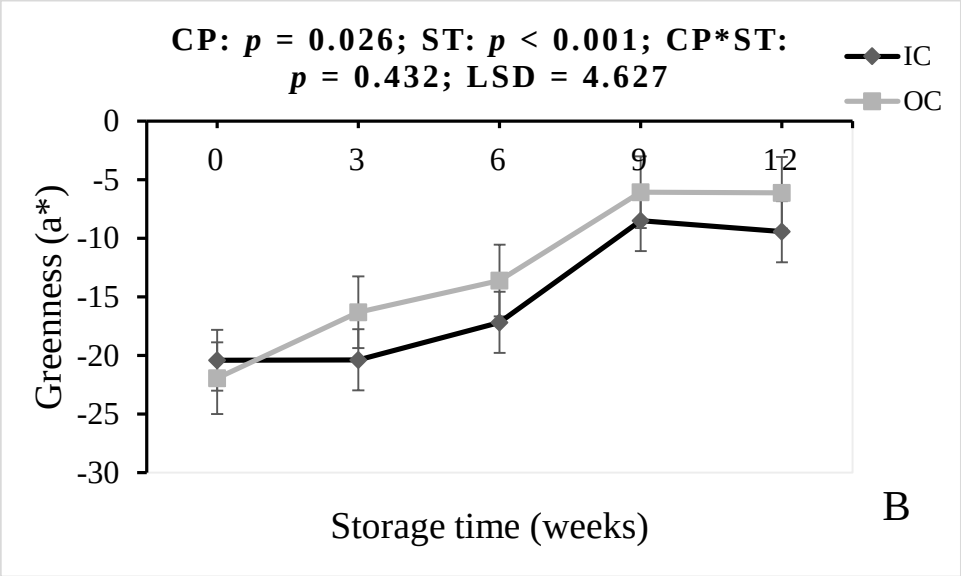
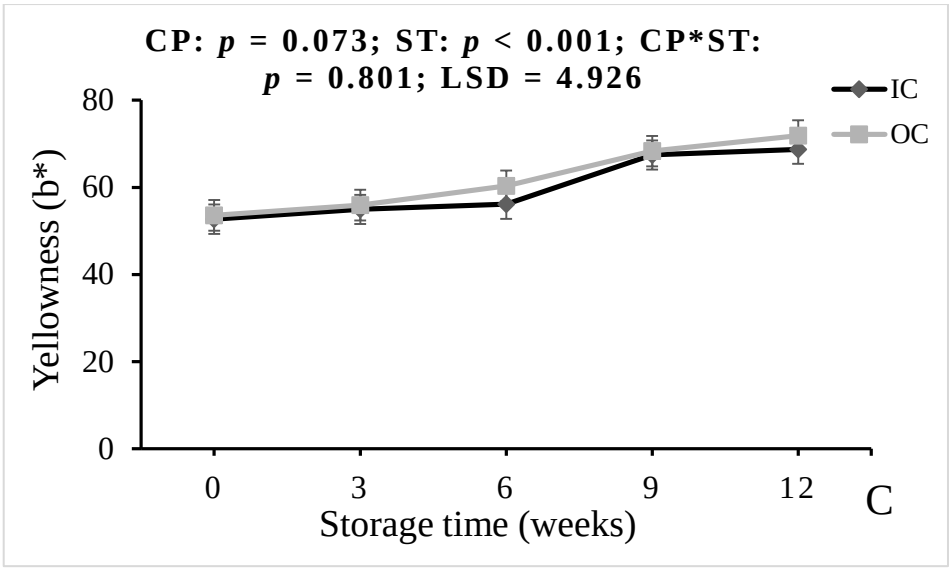


Fig. 4: The effect of canopy position and storage time on total carotenoids (A) and β carotene (B) of 'Eureka' lemons harvested from outside canopy (OC) and inside canopy (IC) positions over 12 weeks cold storage at 3 °C. LSD: least significance difference; CP: canopy position; ST: storage time.


CP: p = 0.001; ST: p < 0.001; CP*ST: p = 0.946; LSD = 6.040
IC
OC
Luminosity (L*)
120
100
80
60
40
20
0
0
3
6
9
12
Storage time (weeks)
A


CP: p = 0.026; ST: p < 0.001; CP*ST: p = 0.432; LSD = 4.627
IC
OC
Greenness (a*)
0
-5
-10
-15
-20
-25
-30
0
3
6
9
12
Storage time (weeks)
B



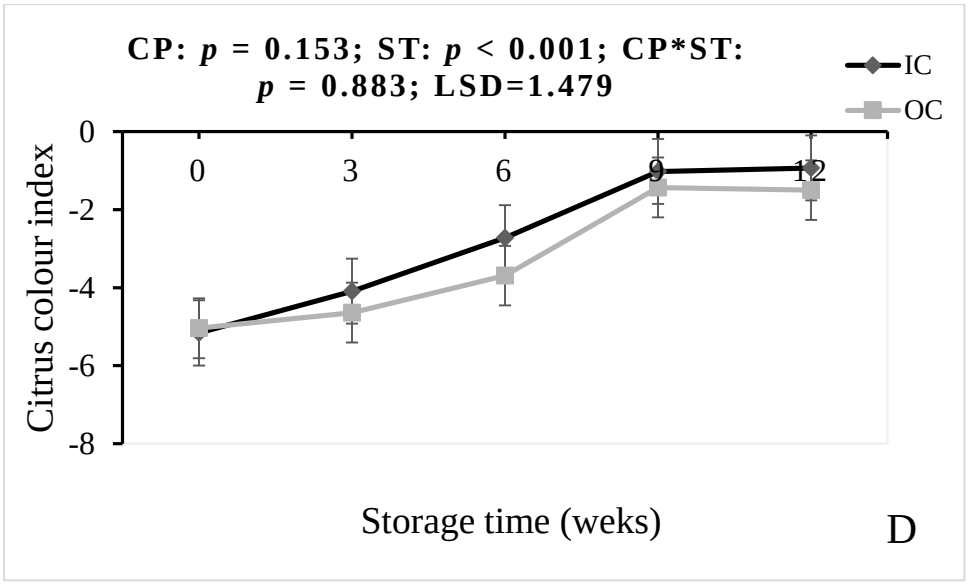


Fig. 5: The effect of canopy position and storage time on colour parameters (luminosity (A); greenness (B); yellowness (C); citrus colour index (D)) of 'Eureka' lemons harvested from outside canopy (OC) and inside canopy (IC) positions over 12 weeks cold storage at 3 °C. LSD: least significance difference; CP: canopy position; ST: storage time.

3.3.4. The effect of canopy position and storage time on ascorbic acid

Ascorbic acid is one of the non-enzymatic antioxidants that has been reported to influence the development of physiological rind disorders in citrus fruit (Foyer, 1993). Ascorbic acid is synthesized from a precursor of hexose sugar in plants (Loewus, 2012) and its important function in fruit is in protecting cells from substances that damage DNA (Blokhina et al., 2003).

The results obtained in this study showed that the interaction between canopy position and storage time did not significantly influence ascorbic acid (p = 0.187). However, a significant difference (p <0.001) between canopy position and ascorbic acid concentration was observed (Fig. 6). According to Izumi et al. (1992), the concentration of ascorbic acid is affected by canopy position, which is closely associated with the formation of chlorophyll and photosynthetic activity.

Fruit from the outside canopy had high ascorbic acid (1.89 g/kg) compared to fruit in the inside canopy (1.51 g/kg) at harvest. The high ascorbic acid found in the outside canopy where there was less PS occurrence suggests a defense mechanism of the fruit against stress conditions that may have led to the disorder development. This might be linked to the fact that the outside canopy fruit were exposed to enough sunlight that may have led to the up-regulation of the ascorbate glutathione cycle, which is an important pathway for the recycling of ascorbic acid (Valpaesta and Bottella, 2004).

Storage time was also found to significantly influence ascorbic acid concentration (p < 0.001) which showed that ascorbic acid was decreasing with time, although the rate of ascorbic acid loss in the outside canopy was lower than inside canopy. This is probably because the concentration of ascorbic acid is influenced by light and temperature (Klein and Perry, 1982), which is typically high in the outside canopy positon of the tree. According to Zhan et al. (2013), ascorbic acid is synthesized from carbohydrates, therefore, the lower sugar content that was observed in the inside canopy could be related to low levels of ascorbic acid in the inside canopy, compared with outside canopy fruit. The negative correlation between sucrose and ascorbic acid (r^2 = -0.88) could explain that, although sucrose was high in the inside canopy, the disorder still developed because of the suppressed ascorbic acid (Valpuesta and Botella, 2004).

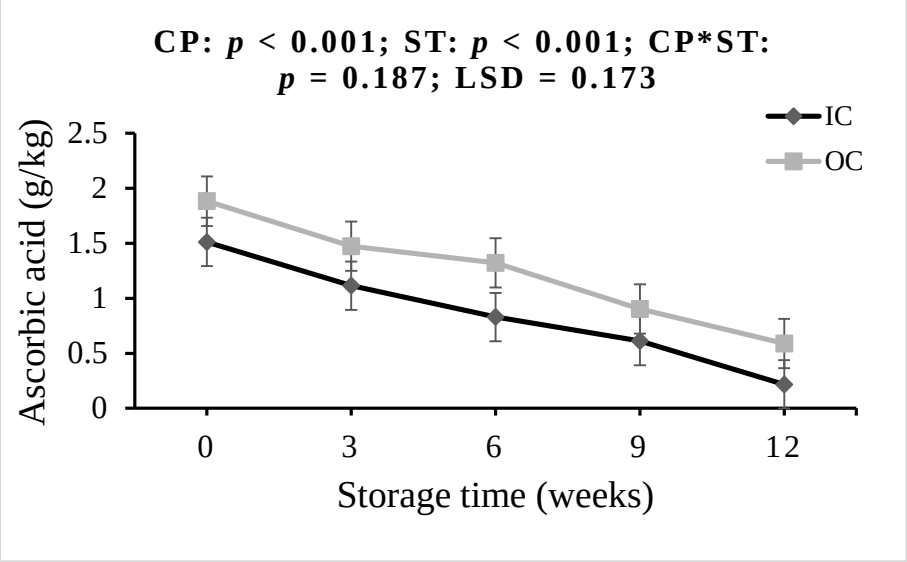


Fig. 6: The effect of canopy position and storage time on ascorbic acid of 'Eureka' lemons harvested from outside canopy (OC) and inside canopy (IC) positions over 12 weeks cold storage at 3 °C. LSD: least significance difference; CP: canopy position; ST: storage time.

3.3.5. Correlation between peteca spot and biochemical parameters

A strong correlation (both negative and positive) was observed between the studied parameters and PS (Table 2). This points to a possible relationship between rind biochemical attributes and the susceptibility of fruit to PS. Since the incidence of the disorder was significantly affected by canopy position, the correlation was performed for each canopy position. The upper (right) quadrant represents correlations in the outside canopy while bottom (left) quadrant represents the inside canopy (Table 2). Even though the correlations within canopy positions differed, the difference was not significant.

According to Liu et al. (2003), correlation is considered strong, moderate and weak when the obtained r^2 values corresponds to r^2 values of (0.75, 0.75-0.50 and 0.50-0.30) respectively. In the inside canopy, sucrose, total acidity, luminosity, greenness, yellowness and colour index showed a positive correlation to PS with r^2 values of 0.84, 0.51, 0.62, 0.60, 0.64 and 0.67, respectively. Sucrose showed a strong correlation to PS while glucose and colour parameters were moderately correlated. On the other hand, a strong negative correlation was observed with total carotenoids (r^2 = -0.76) and ascorbic acid (r^2 = -0.76) and β carotene was moderately correlated with PS (r^2 = -0.58) while fructose had a weak correlation (r^2 = -0.46). Ncama et al. (2018) also reported sucrose to be positively correlated to chilling injury in 'Marsh' grapefruit.

Outside canopy fruit showed a similar trend although with different values (Table 2). The difference in correlations amongst same parameters in the inside and outside canopy positions shows that the development of the disorder was affected by the position of the fruit in the canopy.

Table 2: Peteca spot and biochemical parameters correlations of 'Eureka' lemons. T caro= total carotenoids; AsA= ascorbic acid; TA= titratable acidity; TSS= total soluble solutes; DM= dry matter; L*= luminosity; a*= greenness; b*= yellowness; CCI= citrus colour index; PI= Peteca index. The upper (right) quadrant represents outside canopy while the lower (left) represents inside canopy.

	Sucrose	*Glucose*	*Fructose*	*T caro*	*Lycopene*	*AsA*	*βcarotene*	*Mass*	*TA*	*TSS*	*DM*	*L**	*a**	*b**	*CCI*	*PI*
Sucrose		-0,20	-0,49	-0,72	-0,14	-0,70	-0,64	-0,37	0,52	0,05	-0,01	0,74	0,74	0,81	0,80	**0,66**
Glucose	0,07		0,83	0,19	0,04	-0,07	-0,11	-0,27	0,07	0,10	-0,31	0,03	0,01	-0,10	-0,14	-0,22
Fructose	-0,49	0,68		0,49	0,16	0,27	0,21	-0,09	-0,23	0,11	-0,42	-0,36	-0,40	-0,53	-0,45	**-0,48**
T caro	-0,81	-0,18	0,37		0,21	0,50	0,38	-0,01	-0,26	-0,04	-0,32	-0,50	-0,51	-0,63	-0,71	**-0,45**
Lycopene	-0,10	-0,01	0,01	-0,07		-0,05	-0,10	0,13	0,03	0,11	0,12	-0,07	0,04	-0,10	-0,13	-0,21
AsA	-0,88	-0,24	0,37	0,80	0,12		0,88	0,35	-0,72	-0,04	-0,12	-0,61	-0,70	-0,68	-0,82	**-0,55**
βcarotene	-0,54	0,06	0,50	0,63	0,03	0,57		0,38	-0,75	-0,08	0,02	-0,65	-0,69	-0,69	-0,75	**-0,57**
Mass	0,15	0,31	0,13	-0,07	-0,18	-0,08	-0,09		-0,36	-0,21	0,43	-0,40	-0,36	-0,32	-0,30	-0,02
TA	0,59	-0,07	-0,47	-0,40	-0,21	-0,64	-0,41	0,07		0,07	-0,02	-0,50	0,68	0,65	0,51	**0,60**
TSS	-0,01	-0,53	-0,35	0,03	-0,11	0,11	-0,08	-0,07	0,05		-0,28	-0,05	0,06	0,00	0,00	-0,26
DM	0,10	-0,34	-0,51	-0,11	0,03	-0,14	-0,13	0,03	0,32	0,20		0,02	0,11	0,10	0,24	0,19
L*	0,73	0,07	-0,37	-0,58	0,00	-0,80	-0,43	0,06	0,58	-0,04	0,14		0,87	0,89	0,61	**0,60**
a*	0,68	-0,06	-0,47	-0,59	-0,09	-0,78	-0,45	-0,09	0,61	-0,04	0,27	0,87		0,94	0,65	**0,66**
b*	0,77	-0,12	-0,58	-0,62	-0,06	-0,81	-0,48	0,07	0,67	0,09	0,32	0,89	0,95		0,69	**0,78**
CCI	0,71	0,18	-0,36	-0,64	-0,45	-0,71	-0,42	0,31	0,58	0,01	0,17	0,47	0,52	0,60		**0,57**
PI	**0,84**	0,07	**-0,46**	**-0,76**	-0,22	**-0,76**	**-0,58**	0,00	**0,51**	-0,12	-0,01	**0,62**	**0,60**	**0,64**	**0,67**	

3.3.6. The Principal Component Analysis

In order to further understand the correlation between measured parameters and PS, the data was subjected to PCA, which shows variability in a given sample size. PC-1 and PC-2 of all 3 PCA showed 57% variability (Fig. 7, 8, 9) the contribution of PC-1 was 42% while PC-2 contributed 15% of the total variation during mapping.

Fig. 7 shows a variation in storage time and canopy position and this shows a change in the biochemical parameters that was affected by cold storage of 3 °C. There was a clear separation between storage time and canopy position which shows that storage time had a significant influence on physico-chemical properties (Fig. 7). A loss in variability during storage time could be associated with a decrease in chlorophyll and carotenoids, colour change (Cronje et al., 2011a) and an increase in carbohydrates (Magwaza et al., 2013) which may lead to fruit senescence.

Fig. 8 shows two different clusters of samples harvested from the inside and outside canopy positions. These clusters allowed for the differentiation between the two canopy positions with only a few data points misclassified. This is a visual indication of the previously mentioned data showing inside canopy fruit to be highly susceptible to peteca development compared to outside fruit. Furthermore, PC-2 showed a high contribution to sample distribution seen by the clear separation between inside and outside canopy. Magwaza et al. (2014) and Ncama et al. (2018) also showed the possibility of PCA clustering data according to the two different canopy positions.

The main contributors to the susceptibility of PS as seen in Fig. 9 were found to be sucrose, TA, colour index and all the colour parameters (L*, a*, b*), which were closely related to PS. These were positively correlated with the disorder meaning that they significantly contributed to its occurrence, however, there was a negative correlation between PS and ascorbic acid, β carotene, total carotenoids and fructose, which is seen by the variables further away from peteca index. The PCA shows the possibility of fruit sorting according to the susceptibility of fruit to peteca spot.

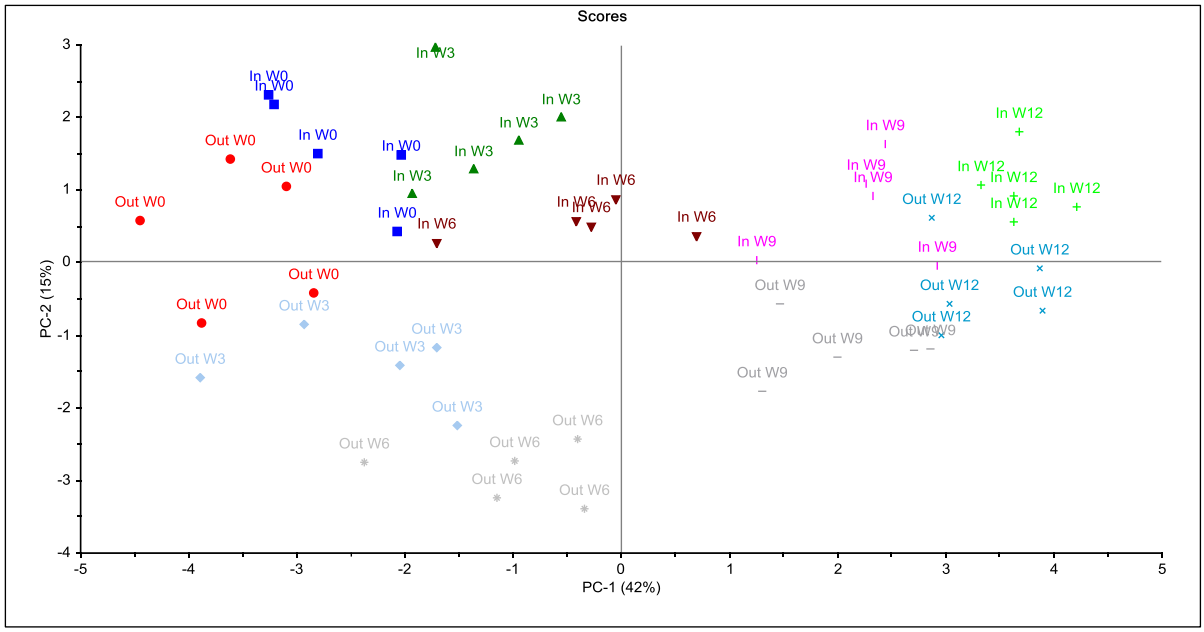


Fig. 7. Principal component analysis (PCA) plot determined by two principle components (PC-1 and PC-2) showing clear clusters of fruit harvested from inside (In) and outside (Out) canopy positions over a period of 12 weeks storage time. W0 to W12 represents week 0 to week 12.

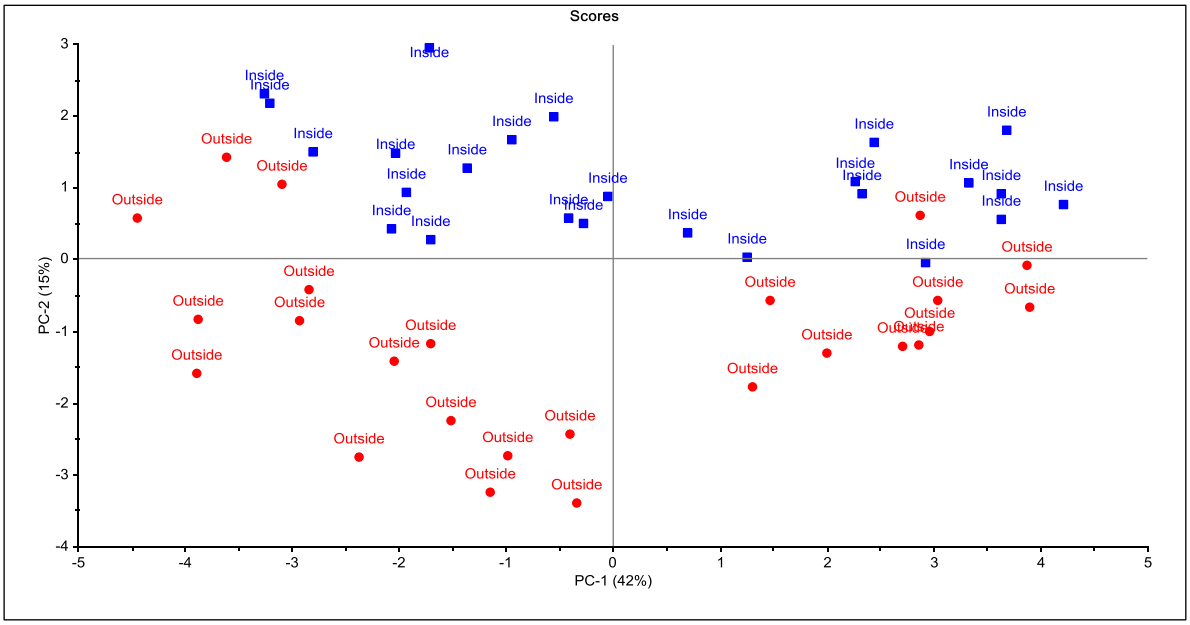


Fig. 8. Principal component analysis (PCA) plot determined by two principle components (PC-1 and PC-2) representing the ability of the spectra to sort factors according to inside (blue) and outside (red) canopy position.

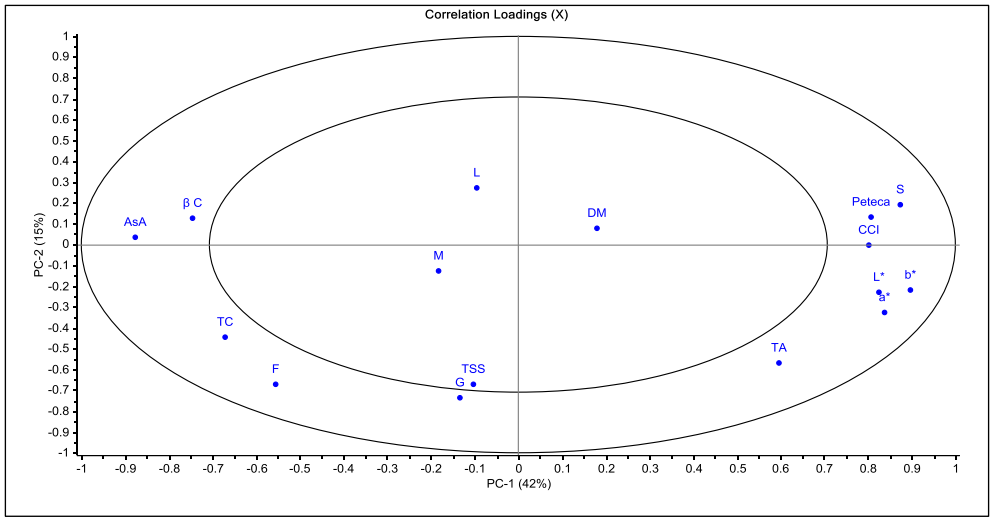


Fig. 9. Principal component analysis (PCA) plot determined by two principle components (PC-1 and PC-2) showing a correlation between measured rind physico-chemical and biochemical properties and susceptibility of 'Eureka' lemons to peteca spot. DM = dry matter content; L* = luminosity; a* = greenness; b* = yellowness; S= sucrose; F= fructose, G= glucose; CCI= colour index, TA= total acidity, L= lycopene; M= mass; TSS= total soluble solutes; AsA= ascorbic acid; βC= beta carotene, TC= total carotenoids.

3.4. Conclusion

Canopy position was found to have a significant influence on the postharvest quality of 'Eureka' lemons. Fruit from the inside canopy which is often characterized by low sunlight exposure were found to be more susceptible to PS compared to fruit in the outside canopy. Similarly, the biochemical parameters were generally low in the inside canopy. Pearson's correlation showed a positive correlation between PS and sucrose, glucose, total acidity, luminosity, greenness, yellowness and colour index and a negative correlation between fructose, total carotenoids, ascorbic acid and β- carotene. A high concentration of these parameters in the outside canopy where the fruit were less susceptible suggests a high chance of protection of fruit from the development of PS.

The ability of PCA to separate inside and outside canopy fruit suggests that parameters clustered together had a positive relationship with PS hence they have a possibility of being used as pre-symptomatic biochemical markers. Secondly, it could further be argued that the PCA could be useful in classifying fruit according to their susceptibility to PS, which would reduce losses in the packhouses. For example, since fruit from the outside canopy are less

susceptible, they could be used for international markets, while those form the inside canopy go to local markets.

The weak and none correlations found in some of the parameters shows that a wide range of factors may influence the development of PS. In spite of the fact that carbohydrates have been reported to play a protective role against stress conditions that may lead to the development of physiological disorders in fruit, their concentration/ response is highly influenced by reduced sunlight. This could indicate that the level of carbohydrate alone might not be the primary cause for PS, but a combination of other environmental and biochemical factors. The significant correlations (both negative and positive) between biochemical parameters and PS proves the possibility of using these biochemical attributes as pre-symptomatic markers for the susceptibility of 'Eureka' lemons to peteca spot.

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Chapter 4: Evaluating the efficacy of Chitosan and CMC incorporated with Moringa leaf extracts on reducing peteca spot incidence on 'Eureka' lemon

Abstract

Lemon (*Citrus limon* L.) is one of the most cultivated citrus fruit in South Africa. In citrus packhouses, fruit are coated with commercial synthetic waxes to enhance shelflife and reduce water loss. However, application of these waxes has been linked to the development of physiological disorders such as peteca spot (PS) in lemons. The aim of this research was to evaluate the efficacy of chitosan (CH) and carboxymethyl cellulose (CMC) incorporated with moringa leaf extracts (M) on reducing the incidence of peteca spot on 'Eureka' lemons. A total of 500 'Eureka' lemons were harvested from Malowe orchard farm, a commercial orchard in the midlands of KwaZulu-Natal. Half of the fruit were harvested from the outside position of the tree and another half from the inside canopy position. Fruit were assigned to 5 coating treatments, namely; control, 1% M+CMC, 1% CMC, 1% CH and 1% M+CH. After coating, fruit were transferred into a cold room with delivery air temperature set at 3 °C to induce the disorder. Sampling was done at 3 weeks interval for a period of 12 weeks. At each sampling week, peteca spot incidence, fruit physico-chemical properties including colour, mass, vitamin C, carotenoids, carbohydrates, TSS, TA, total phenolics and flavonoids, were measured. The results showed that coating treatments and canopy position significantly affected the incidence of peteca spot. Fruit coated with CMC, CH, M+CMC were less susceptible to PS development in both inside and outside canopy, while control and fruit coated with M+CH showed a high susceptibility in the inside canopy position. Coating tratments significantly affected total phenolic and flavonoid concentrations, but had no significant effect on carbohydrates. The most effective coating treatment was M+CMC followed by CMC and CH. These coating treatments were also able to reduce mass loss, ascorbic acid loss and delay in colour change of fruit. The results found in this study demonstrated that either M+CMC, CMC, or CH coating treatments can be used for reducing postharvest PS in 'Eureka' lemons.

Keywords: Canopy position; edible coatings; physico-chemical properties, citrus, peteca spot

4.1. Introduction

Lemon (*Citrus limon* L.), belonging to the citrus family Rutaceae, is one of the most cultivated citrus cultivars in South Africa (Citrus Growers' Association of South Africa, 2018). Lemon plantings and exports have significantly increased in the past decade due to a global high demand of the fruit. This is largely attributed to the high nutritive and therapeutic characteristics of lemons (Iglesias et al., 2007). The 2018 estimated export production for lemon in South Africa is over 20 million tons (Citrus Growers' Association of South Africa, 2018). The most important lemon cultivar planted in South Africa is 'Eureka'. During 2017 export season, the total area used for 'Eureka' plantings was 9097 ha which represents 83% of the total lemon planted in Eastern Cape and Limpopo provinces (Citrus Growers' Association of South Africa, 2018).

Lemons are characterized by high vitamin C content (0.53 g/kg in juice and 1.29 g/kg in peel) and other vitamins such as vitamin B, riboflavin and minerals, which are related to the prevention of various non-communicable illnesses such as cancer and cardiovascular disease (Silalahi, 2002; Baradaran et al., 2014; United States Department of Agriculture, 2018). After harvest, citrus fruit are prone to the development of rind physiological disorders which are caused by internal and external factors. For this reason, the fruit are usually coated with commercial synthetic waxes like thiabendazole (TBZ), imazalil (IMZ) and sodium ortho-phenil phenate (SOP) in packhouses with the purpose of enhancing shelflife, reduce water loss, and fruit shrinkage (Petracek et al., 1998; Palou et al., 2015).

Wax coating is applied on the surface of the fruit, which makes the probability of consuming the coating with the fruit high. This has raised concerns regarding health and environmental effects which are associated with chemical residues (Palou et al., 2015). Some waxes have been found to impair fruit quality and cause rind physiological disorders by restricting gas exchange through the peel, which causes anaerobic conditions in the internal atmosphere of the fruit (Porat et al., 2005; Arnon et al., 2015). Carnauba and shellac coatings caused rind pitting in grapefruit (Petracek et al., 1998) and chilling injury in 'Satsuma' mandarin (Kellerman et al., 2014). Polyethylene-based waxes aggravated the incidence of PS in lemon (Wild, 1991).

Peteca spot is a physiological disorder that causes a major loss in all citrus producing provinces in South Africa (Cronje, 2015). The disorder occurs from time of harvest until cold storage and

the symptoms can be seen 3 to 4 weeks after packing and sorting (Khalidy et al., 1969). A major problem relating to this disorder is that fruit are shipped to distant markets and the time of disorder development can coincide with the time the lemon reach the international market which can result in the rejection of the whole fruit consignments. This makes it important to find ways to reduce the PS incidence without impairing fruit quality and posing threat to human health. Consumer's demands for healthy and high quality fruit has led to many countries changing their regulations and imposing limitations to the use of agrochemicals and synthetic waxes (Palou et al., 2015), which has now led to a switch from synthetic waxes to edible coatings.

The use of edible coatings is increasingly becoming a core focus in postharvest handling, however, most of the edible coatings that have been evaluated on citrus fruit focused on hydroxypropyl methylcellulose, beeswax and shellac composites which require using powerful organic solvents like ammonia to dissolve resulting in restricted gas exchange (Sánchez-González et al., 2011). Some of the edible coatings based on plant extracts that have been found to have positive results in reducing postharvest physiological disorders include salicylic and methyl jasmonate, which reduced chilling injury in lemon (Siboza et al., 2014), aloe vera, for reducing rachis browning in grapes (Castillo et al., 2010). Among a wide variety of edible coatings, moringa, chitosan and CMC have been reported to dominate the food industry, which is seen by the increasing published research on their use.

Moringa, incorporated with CMC, was found to extend shelflife and maintain the quality of oranges (Adetunji et al., 2013). Gol et al. (2013) applied CMC, Hydroxypropylmethyl-cellulose (HPMC) and chitosan coatings on strawberries and found that they were able to maintain higher concentrations of total phenolics and anthocyanins which extended shelflife and postharvest quality of the fruit. Ahmed et al. (2018) reported a prolonged shelflife and quality of grapefruit coated with chitosan. Extended shelflife was also reported in guava which was treated with chitosan-cassava starch coating before storage (de Aquino et al., 2015). Tesfay and Magwaza (2017) evaluated the efficacy of CH and CMC incoported with moringa on postharvest quality of avocados, their findings showed animproved quality and extending shelf-life after 21 days of storage at 5.5 °C. Notably, the authors also found that CMC containing moringa extract was particularly able to suppress postharvest diseases and maintain the quality of avocados.

Chitosan and CMC coatings have previously been evaluated in various citrus fruit including 'Navel' oranges, 'Star Ruby' grapefruit as well as mandarins, and the coatings have been found to increase fruit firmness while sensory evaluations showed that fruit flavor was not impaired (Arnon et al., 2015). The success of moringa, CMC and CH to be used as edible coatings is mainly based on the fact that they are very affordable and preparation is quite simple. However, there is currently no research that has evaluated the potential of edible coatings as postaharvest treatment of 'Eureka' which is the most important lemon cultivar. The aim of this research study was therefore, to evaluate the efficacy of CH and CMC and their combinations with moringa leaf extracts on reducing the incidence of PS on 'Eureka' lemons.

4.2. Materials and methods

4.2.1. Reagents and standards

The chemicals and standards used for all analyses in this study were purchased from two companies, B&M Scientific CC (Cape Town, South Africa) and Prestige laboratory supplies PTY Ltd (Durban, KwaZulu-Natal, South Africa). Chemicals used in this study included Folin-Ciocalteu reagent; 2, 6-dichloroindophenol dye; sodium carbonate anhydrous; sodium hydroxide pellets; sodium nitrate; metaphosphoric acid; ascorbic acid; acetone; aluminum chloride anhydrous; methanol; nylon syringe filters; clear glass vials and caps; sugars standards (sucrose, D-glucose, and D-fructose); gallic acid and quercertin standards.

4.2.2. Fruit sampling

A total of 500 'Eureka' lemons with an average mass ranging from (106.7 – 130.7 g) at harvest were harvested from a commercial orchard at Malowe orchard farm located in uMzimkhulu, KwaZulu-Natal, South Africa (Latitude: 30º14'S, Longitude: 29º56'E). Fruit were systematically harvested from two canopy positions, namely, the sun exposed outside canopy position (OC) and inside canopy (IC). Harvesting was done using a completely randomized design and fruit were selected randomly from 10 trees. After harvesting, the fruit were transported to the University of KwaZulu-Natal research laboratory using a well-ventilated car.

4.2.3. Treatments and storage

Upon arrival to the laboratory, fruit were assigned to 5 coating treatments:
T_1: Control (untreated lemons); T_2: Lemons treated with 1% M+CMC (moringa + carboxymethyl cellulose); T_3: Lemons treated with 1% CMC (carboxymethyl cellulose); T_4: Lemons treated with 1% CH (chitosan); T_5: Lemons treated with 1% M+CH (moringa + chitosan)

The preparation of coating treatments was done according to a method described by Tesfay et al. (2017). Fruit were immersed in assigned coating treatments for 1 min and left at room temperature for about 30 min for the coating to dry. The fruit were then packed and transferred to 3 °C to induce the development of PS (Undurraga et al., 2009). Each treatment consisted of 5 replications with 50 fruit per treatment and sampling was done for a period of 12 weeks at 3 weeks intervals.

4.2.4. Postharvest quality measurements

After coating treatments, the data for week 0 was collected and physico-chemical variables such as colour, mass, TSS and TA, ascorbic acid, and carbohydrates were measured. The same sampling procedure was repeated with fruit in cold storage. Fruit were destructively analyzed every three weeks for the period of 12 weeks and PS incidence was scored. The PS index was calculated using Eq. 1 according to Cronje (2015).

$$\text{Peteca}_{\text{Index}} = \frac{\sum\{Peteca\ (0-2)\ x\ No.\ of\ fruit\ in\ each\ class\}}{Number\ of\ fruit\ in\ a\ rep} \qquad 1$$

On each sampling date, the rind was peeled using a peeler separating the flavedo and albedo and immediately stored at -40 °C until further analysis. The frozen samples were freeze-dried using a VirTis Freeze dryer system (Model 6KBTES-55, SP industries, Warminster, PA, USA) for 7 days at (150 - 250 millitor) and -40 °C. The dry samples were weighed and water content was calculated from freeze-dried samples and expressed as a percentage of dry mass, after which samples were ground into fine powder using a laboratory blender (OmniBlend (PTY) Ltd. Cape Town, South Africa).

4.2.5. Fruit mass and colour index

The measurement of fruit mass was carried out using a calibrated weighing balance (RADWAG Wagi Electronic Inc., Poland). Fruit colour was objectively quantified based on L*, a*, b* and *h* parameters measured using a portable colourimeter (Chroma Meter, Konica Minolta Sensing, INC., Japan). Colour sampling was done from three random spots on the equatorial position of a fruit using. Calibration of the instrument was done by scanning a 100% white reference brick with Y= 91.59, X = 0.3167 and y = 0.3315 prior fruit scanning. The parameters C* (chroma) and h* (hue angle) were calculated according to C*= (a*2−b*2)1/2 and h*= arctan (a*/b*), respectively. The total colour difference was expressed as a citrus colour index (CCI) using Eq. 2 (Pathare et al., 2013; Vidal et al., 2013).

$$CCI = \frac{1000*a}{L*b} \qquad 2$$

4.2.6. Determination of total carotenoids

The concentration of total carotenoid was determined and quantified using a method described by Lichtenthaler (1987) with slight modifications. A 150 mg ± 0.5 of the powdered sample was added into a test tube, and 2 mL of 80% (v/v) acetone was added. The sample was centrifuged for 10 minutes using GenVac® (SP Scientific, Genevac LTD., Suffolk, UK). For maximum detection of carotenoids, six wavelengths (470, 646.8, 645, 505, 435, 663.2 nm) were used to measure the supernatant. The calculations for the concentrations of chlorophyll *a* (Ca), chlorophyll *b* (Cb), total carotenoids (Cx), β carotene and lycopene were done using Eqs. 3, 4, 5, 6 and 7 (Corrêa et al., 2011).

Ca = 12.25 (A663.2 – 2.79 A646.8) 3

Cb = 21.5 (A646.8 – 5.10 A663.2) 4

Cx = (1000 (A470 – 1.82 Ca – 85.02 Cb) / 198 5

Where Ca = Chlorophyll a, Cb = Chlorophyll b, Cx = total carotenoids

$$\beta \text{ carotene} = 0.216\ A663.2 - 1.22\ A645 = 0.304\ A505 + 0.452\ A453 \quad (6)$$

$$\text{Lycopene} = -0.0458^{*}\ A663 + 0.204^{*}\ A645 + 0.372^{*}\ A505 - 0.0806^{*}\ A435 \quad (7)$$

4.2.7. Determination of TSS and TA

Total soluble solids (TSS) and total acidity (TA) was determined using juice which was squeezed by hand from the fruit. TSS was measured using a digital hand-held refractometer with a dynamic control system (RFM340+ BS®, Bellingham and Stanley Ltd, Basingstoke, Hants, UK) and TA was determined 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 TA was calculated using the citrus acid formula (Eq. 8) and was expressed as % citric acid (Ncama et al., 2017).

$$TA\ (\%\ citric\ acid) = \frac{0.0064\ \text{X titre (NaOH) mL X } 100}{10\ \text{mL juice}} \quad (8)$$

4.2.8. Quantification of nonstructural carbohydrates

The analysis of nonstructural carbohydrates was carried out using a method described by Magwaza et al. (2014) with slight modifications for lemon. The analysis was done in both the flavedo and albedo separately. In short, 150 ± 0.5 mg of the powdered sample was added into a test tube followed by the addition of 3 mL 70% (v/v) aqueous methanol (v/v) and vortexed for 1 minute. The sample was incubated for an hour in hot water shaking bath (55 °C) as described by Terry et al. (2017), and then centrifuged for 20 min using GenVac® (SP Scientific, Genevac LTD., Suffolk, UK). A 0.4 micron nylon syringe filter was used to filter the sample into HPLC vial for high performance liquid chromatography (HPLC) analysis.

The concentrations of fructose, glucose, and sucrose were quantified using a Phenomenex® column (Rezex RCM - Monosaccharide) equipped with a refractive index detector by injecting 1 mL of the sample extracts into a Phenomenex- SecurityGuard™ cartridges C18 (4 x 3.0 mm) ID, 10/pk and column of 3.2 – 8.0 mm. Ultra-pure HPLC-grade water was used as mobile phase at 0.6 mL/min flow rate and the column compartment temperature was set to 80 °C. The

concentrations of fructose, glucose and sucrose were determined by comparing the detected peak in the samples with peak area and concentration of the standard curve.

The preparation of calibration standards was done by weighing 250 ± 0.5 mg fructose, glucose, and sucrose each into a 100 ml volumetric flask. Ultra-pure water (HPLC grade) was used for dissolving the sugar standards and added up to volume giving a concentration of 2.5 mg/mL. The final concentrations of 0.05, 0.25, 0.5, 1.25 and 2.5 mg/mL of the solution were used for the reading of linear standard curve with $R^2 = 0.97$ and the data was expressed as g/kg.

4.2.9. Extraction of total phenolics and flavonoids

Phenolic concentration was spectrophotometrically determined using a method described by Ilahy et al. (2011) with modifications. Briefly, 150 ± 0.5 mg was extracted with 3 mL of 80% methanol. The solution was vortexed for 1 min followed by centrifuging for 10 minutes using GenVac® (SP Scientific, Genevac LTD., Suffolk, UK).

For extraction of total phenolics, 50 µL was poured into a test tube and diluted with 950 µL of distilled water. This was followed by the addition of 125 µL Folin-Ciocalteu reagent. After 3 minutes, the addition of 1250 µL 7% sodium carbonate solution followed, and distilled water was used to make up the final volume to 4 mL. The solution was incubated in the dark at room temperature for 90 minutes. The absorbance was measured at 760 nm against blank. The same procedure was followed for the reading of the linear standard curve using gallic acid solution (100 µg/mL) with R^2 value of 0.995. The results were expressed as g gallic acid equivalent per kg (g/kg GAE).

For the determination of total flavonoid concentration, the aluminum chloride colourimetric assay described by Kamtekar et al. (2014) was used. Briefly, 500 µL of the diluted solution was added with 4 mL and 300 µL of 5% sodium nitrate solution. This was allowed to stand for 5 minutes then followed by the addition of 300 µL 10% aluminum chloride. After 6 minutes, 2 mL of 1M sodium hydroxide was added. Distilled water was added to give a final volume of 10 mL and this gave rise to orange to yellowish colour. Using a spectrophotometer, the absorbance was measured at 510 nm against the blank. Quercetin stock solution (1000 µg/mL) was used for the calibration of standard curve with R^2 of 0.996. All samples were measured in

triplicates and results for flavonoids were expressed as g of quercetin equivalents per kg of dry mass (g/kg QE).

4.2.10. Extraction and quantification of rind ascorbic acid

Ascorbic acid was extracted from the freeze dried sample using a method described by Hernández et al. (2006) which was slightly modified for citrus. Briefly, 150 ± 0.5 mg of the dry sample was mixed with 5 mL of 3% (w/v) aqueous metaphosphoric acid. The solution was homogenized for 1 min using a vortex, and then placed in ice cubes for 5 min. The sample was thereafter centrifuged for 20 min using GeneVac (SP Scientific, Genevac LTD., Suffolk, UK) with lamp off. Subsequently, 0.5 mL was incubated in the dark using 2.5 mL of 2, 6-dichloroindophenol dye (0.015 g dye in 100 mL of H_2O). The reading of the absorbance was at 515 nm in triplicate using spectrophotometer against 3% MPA.

The standard curve for ascorbic acid was quantified by dissolving 0.0352 g of ascorbic acid in 100 mL of metaphosphoric acid solution followed by a serial dilution leading to the following concentrations; 0, 200, 400, 600; 800 and 1000 µL. Each concentration was left for 10 min in the dark to react with 2.5 mL of 2, 6-dichloroindophenol dye. The absorbance values were read at 515 nm to give a linear standard curve with the R^2 value of 0.992. The results were expresses as g/kg.

4.2.11. Statistical analysis

The data for all variables measured was subjected to the analysis of variance (ANOVA) using GenStat statistical software (GenStat®, 18th edition, VSN International, UK). Means were separated using least significance differences measured at $p \leq 0.05$.

4.3. Results and discussion

4.3.1. Effect of coating treatment and canopy position on peteca spot

The development of peteca spot was significantly affected by the interaction between coating treatment and canopy position ($p < 0.001$) suggesting that the differences in the peteca incidence observed was influenced by the type of coating treatment and the position of fruit on

the tree (Fig. 1). The effectiveness of the coating treatments was demonstrated by the difference in peteca spot development between the coated and control fruit in the same canopy position. The control had the highest incidence of the disorder (16.5%) compared to fruit coated with CMC (1.6%), CH (4%) and M+CMC (0.6%) and this was worse on fruit harvested from the inside position of the tree canopy compared to the outside canopy. Fruit coated with M+CH were also found to be susceptible to peteca spot (6.5%) (Fig. 1). Fruit coated with CH, CMC and M+CMC were less susceptible to peteca spot development throughout the storage time irrespective of canopy position.

The effectiveness of CH coating in reducing chilling injury and preserving quality of cucumber was also demonstrated by Zhang et al. (2015). This is because the coating has the ability to activate plant's defense responses (Chien and Chou, 2006). Another observation made was that fruit harvested from the outside canopy had less peteca spot development compared to fruit in the inside canopy. Comparable results were also reported by Magwaza et al. (2013) and Cronje et al. (2013) where 'Nules Clementine' mandarin harvested from shaded position inside the tree canopy had higher rind breakdown than those from outside sunexposed position. This has been largely attributed to sunlight exposure of the fruit while they are still attached to the tree which photosynthesis processes hence increasing fruit's biochemical attributes that protect the fruit from any stress that leads to the development of disorders.

The interaction between storage time and canopy position was not significant ($p = 0.054$), however, storage time significantly affected peteca spot development ($p < 0.001$). This could be due to the fact that of the all coating treatments, only control had a high incidence of the disorder while CMC, M+CMC and CH showed less susceptibility throughout storage time. There was less peteca spot incidence during the first 3 weeks of cold storage followed by a rapid increase from week 6 (Fig. 2). This shows that peteca spot may not occur directly after harvest and its development may coincide with the time the fruit reach the international market causing a huge economic loss for the citrus industry.

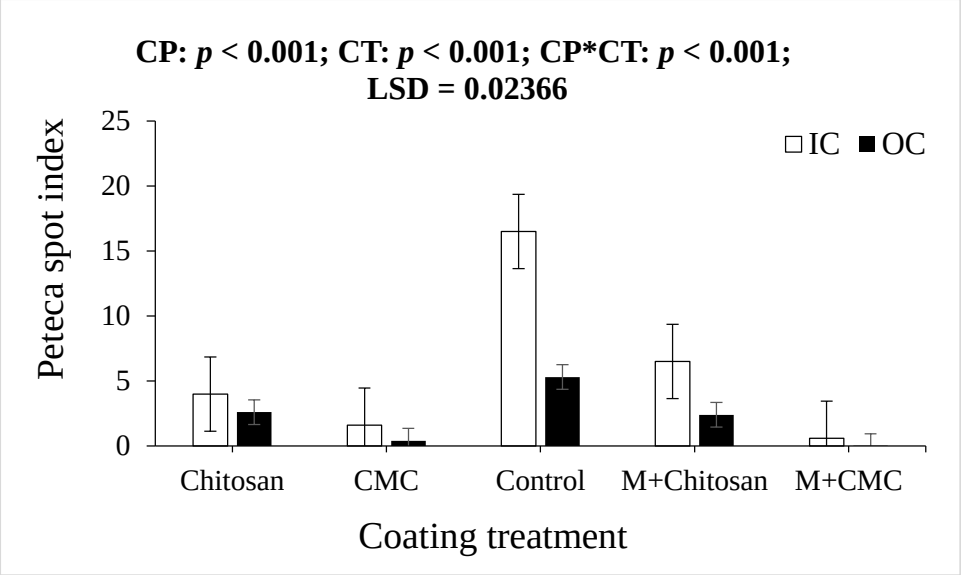


Fig. 1: The effect of edible coatings on peteca spot development of 'Eureka' lemon in fruit harvested from inside canopy (IC) and outside canopy (OC) positions during postharvest cold storage of 3 °C. LSD: least significance difference; CP: canopy position, CT: coating treatment.

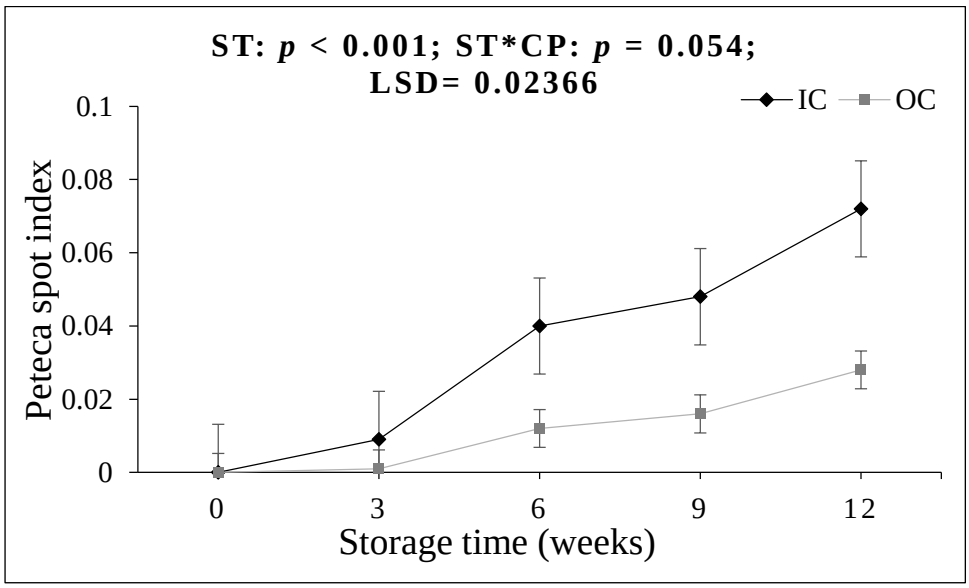


Fig. 2: The effect of canopy position on the development of peteca spot in 'Eureka' lemons in fruit harvested from inside canopy (IC) and outside canopy (OC) over 12 weeks postharvest cold storage at 3 °C. LSD: least significance difference; CP: canopy position; ST: storage time.

4.3.2. The effect of coating treatments and storage time on mass loss of 'Eureka' lemons

The significant interaction between coating treatment and storage time ($p < 0.001$) defined the changes in mass loss over 12 weeks of cold storage. From the results observed, coating

treatments were effective in reducing mass loss over time compared to the control (Fig. 3). Fruit coated with M+CMC had the least percentage of mass loss (9.770 %) after the storage period followed by CH and CMC, with mass loss of 10.6 and 14.0%, respectively. This shows the ability of the coating to maintain the initial mass of fruit during cold storage. The explanation for the coating's ability to reduce mass loss could be because coatings, such as chitosan when applied to the fruit, have the ability to form a film on the fruit surface, which blocks water vapor exchange hence reducing water loss through the process of transpiration (Shao et al., 2015).

Carboxymethyl cellulose coating has been reported to form a semipermeable layer on the fruit surface, which reduces moisture loss, respiration as well as the movement of solutes across membranes (Arnon et al., 2014). A delay in mass loss is one of the main benefits of applying coatings in fruit mainly because of the barrier they form, which reduces oxygen supply (Gao et al., 2018). The importance of fruit mass is seen on a daily basis in local stores where the mass of the fruit determines its price. Fruit are usually sold per kg and fruit that show shrinkage are discarded. This makes it important for the fruit to maintain its mass throughout storage time and shelf-life in order to minimize losses, not only in the local markets but in the export market as well.

Although the coatings were found effective in reducing mass loss, fruit coated with M+CH had a high increase in mass loss following the trend for control (uncoated fruit). At the end of storage, M+CH coated fruit lost about 21.63 % of the initial mass while control fruit loss about 24.17 %. The results found in this study corroborate with those reported by other researchers who evaluated the effectiveness of edible coatings on the reducing mass loss in various citrus fruit (Galed et al., 2004; Chien et al., 2007; Hosseini et al., 2018). The ability of M+CMC coating to reduce mass loss in citrus was reported by Adetunji et al. (2013). Toğrul and Arslan (2004) reported that uncoated mandarin fruit lost about 30 % of the initial mass after storage period of 25 days. 'Oronules' mandarin fruit coated with chitosan (1.2 %) at 5 °C showed a low decrease in mass loss (Contreras-Oliva et al., 2012).

Canopy position had no significant effect on the initial mass of fruit, however, the mass started changing with time (Table 1). This could be due to water loss by the fruit through active metabolic processes like respiration and transpiration under cold storage. A change in the water status of the rind has been reported as one of the determining factors for fruit susceptibility to

rind pitting disorder (Alférez and Burns, 2004) hence control fruit that showed a higher percentage of mass loss were more susceptible to peteca spot.

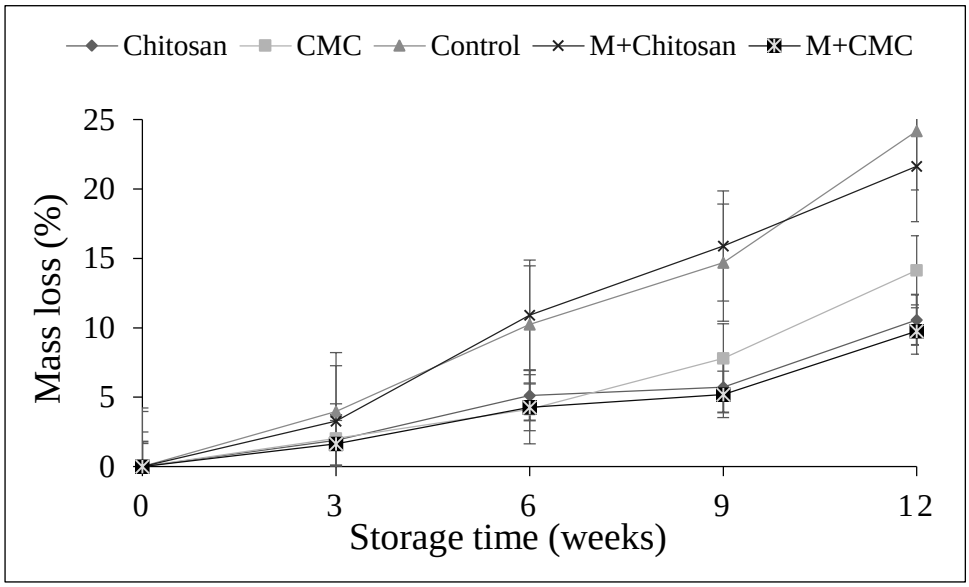


Fig. 3: The effect of coating treatment on mass loss of 'Eureka' lemons in fruit harvested from inside canopy (IC) and outside canopy (OC) positions during postharvest cold storage of 3 °C. LSD: least significance difference; CP: canopy position, CT: coating treatment.

4.3.3. The effect of coating treatments and canopy position on colour and total carotenoids

Coating treatments significantly affected the fruit colour parameters ($p < 0.001$). Amongst the treatments, control fruit were more luminous with average values of 87.94 in the OC and 85.36 in the IC (Fig. 4A). This was followed by M+CH coated fruit with average values of 87.35 in the OC and 83.89 in the IC position. The results showed that control and M+CH treatments had a rapid loss of the initial green colour such that by the third week of cold storage, the fruit had already developed shades of yellow colour on the rind which turned completely yellow by the end of storage time.

These results demonstrated the effectiveness of edible coatings in improving the visual appearance of citrus fruit by maintaining the lower values of L*, a* and b*. The other treatments (CH, CMC and M+CMC) were effective in maintaining the initial green colour for the first 6 weeks of storage by inhibiting a fast chlorophyll degradation (Fig. 5). Ali et al. (2011)

ascribed a delay in colour change to low respiration and reduced ethylene which results in a modified fruit atmosphere. This could explain the very low peteca spot incidence in coated fruit since the colour change was slow, because yellow fruit are more susceptible to PS than green fruit (Undurraga et al., 2009).

Edible coatings are known to create a semipermeable film which is able to delay ripening and senescence and inhibits colour alterations (Han et al., 2014) of which the three treatments (CH, CMC and M+CMC) were able to do. The loss in green colour as the fruit changes to yellow can be related to ethylene production which causes a natural ripening process which is also related to chlorophyll breakdown and an increase in carotenoids content. The relationship between coating treatments and colour change in fruit is through delaying metabolic processes that lead to a rapid increase in colour change. Han et al. (2014) reported a retention in colour after the application of chitosan which was also seen in pepper (Ali et al., 2015). Chitosan coating was also found to delay ripening in guava (de Aquino et al., 2015).

The colour of the fruit was significantly affected by canopy position ($p < 0.001$). Sun exposed fruit from outside canopy had high luminosity (L*), greenness (a*) and yellowness (b*) which shows a better colour development compared to the fruit harvested from inside position of the tree canopy. However, a rapid increase in colour change for the control and M+CH was observed with L* values reaching 87.94 and 87.35, respectively and yellowness 62.59 and 61.64, respectively, while in the other coatings luminosity ranged from 83.01 - 85.67 and yellowness 58.41 – 59.49. The low values of greenness in the inside canopy fruit coated with (CH, CMC and M+CMC) (Fig 4B) demonstrate the ability of coating to delay breakdown of chlorophyll and the synthesis of carotenoids. In comparison of the outside and inside canopy positions, the low colour change in the inside canopy could be caused by the reduced intensity of sunlight reaching the fruit hence reducing metabolic processes in the fruit.

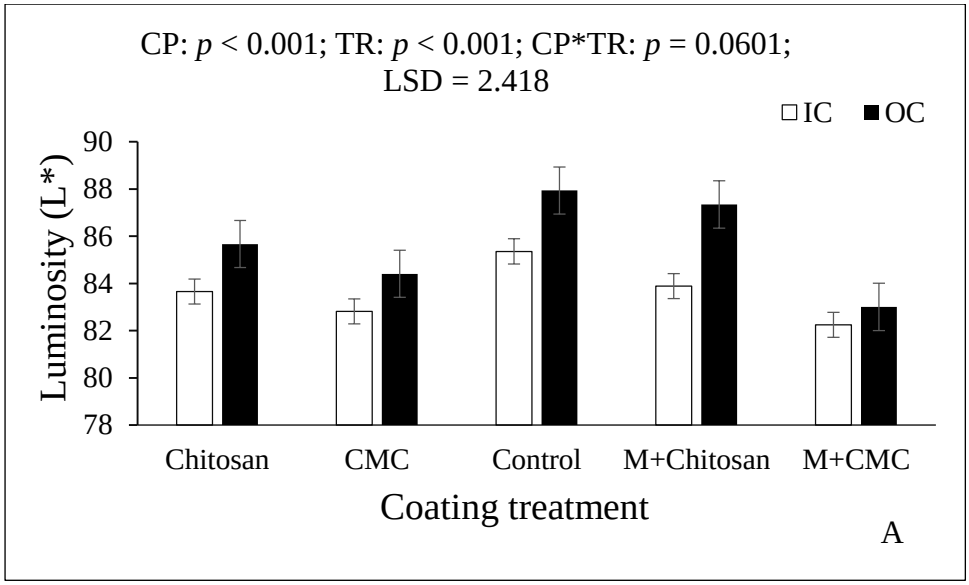
Ulluh et al. (2017) reported that applying coatings in fruit delays colour breakdown and the synthesis of carotenoids. The loss in pigment content, which contributes to a change in green colour is due to the conversion of chloroplast to chromoplasts leading to a fast ripening and the formation of lycopene and β carotene. Mature citrus fruit are known to contain chromoplasts with the ability to store huge amounts of carotenoids, which could explain why carotenoids were increasing as the fruit ripens (Table 1). The chromoplast usually shows natural variations in the type and level of accumulation of carotenoids amongst same species. Coating treatments

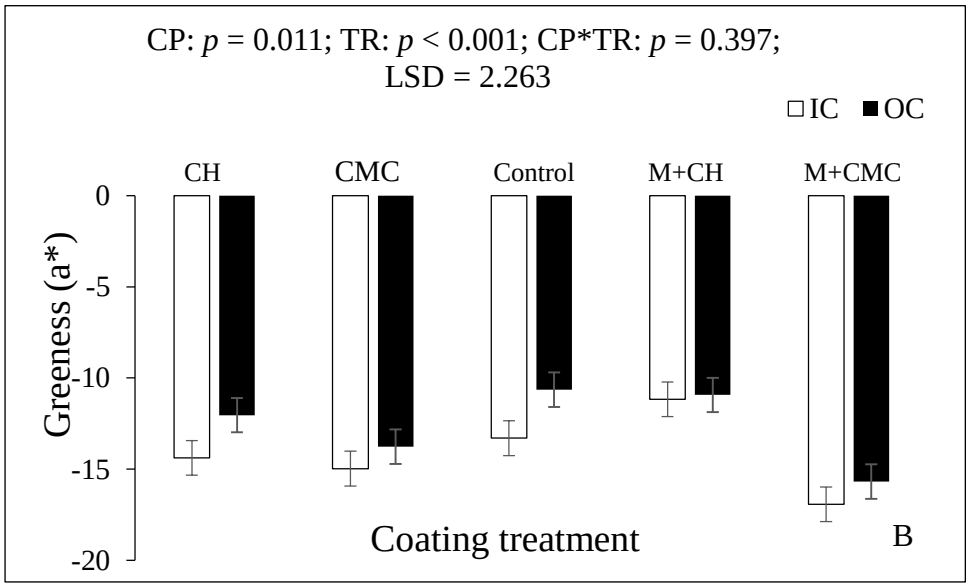
caused a significant effect on total carotenoids ($p = 0.002$). Fruit coated with M+CMC showed high carotenoids in the outside canopy compared to the rest of the treatments, which can be related to the colour index (Fig. 6).

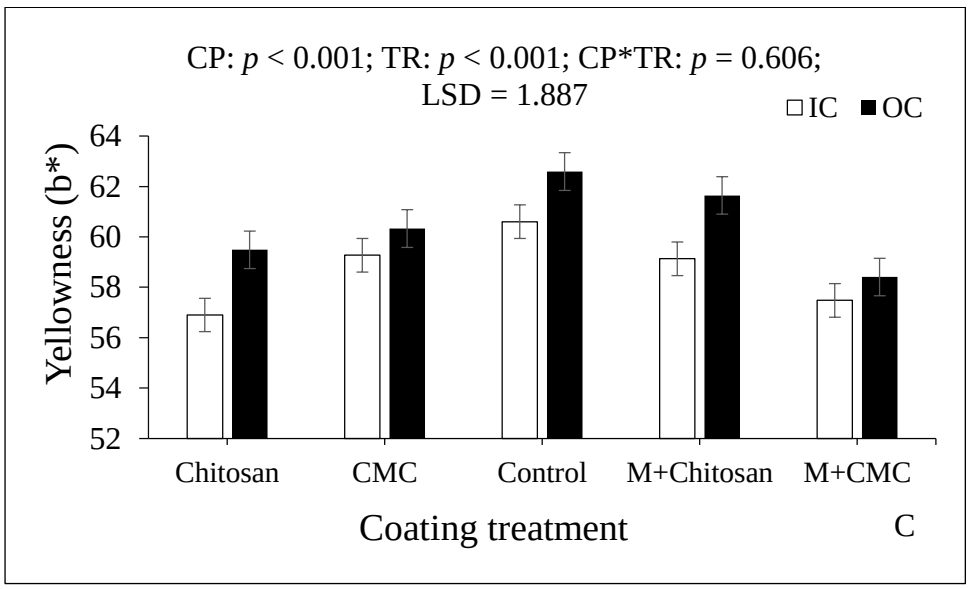
Carotenoids have an advantageous ability to prevent membrane damage because they play a role as photoprotective compounds in fruits (Cronje et al., 2011). This could be the reason why coatings that had high carotenoids were less susceptible to PS incidence. Canopy position also showed a significant effect in total carotenoids ($p < 0.001$). Fruit harvested from the outside canopy had more carotenoids compared to those in the inside canopy with values ranging from 118 – 145 g/kg in the inside canopy and 75 – 114.5 g/kg in the inside canopy (Fig 6A). This is because light is an important factor in the synthesis of carotenoids.

Coating treatments had no significant effect on β carotene, however, canopy position significantly affected the concentrations of β carotene and lycopene ($p < 0.001$) (Fig 6B, C). Fruit harvested from outside canopy had the highest β carotene ranging from 5.1 to 5.8 g/kg while fruit from the inside canopy had 3.9 – 4.6 g/kg B carotene. β carotene is one of the pigments found in citrus fruit that plays a role in plant's metabolism and photosynthesis. The pigment absorbs light energy from chlorophyll and transmit it so as to protect the fruit from oxidative damage (Pons et al., 2014). An increase in lycopene concentration is dependent on the colour of the fruit. The ripeness, as well as the developmental conditions of the fruit is associated with an increase in lycopene (Abdel-Salam, 2016).

The control treatment showed the lowest average concentation of lycopene compared to the other treatments (Fig. 6C). This could be due to the fact that control fruit started changing colour from the early stages of storage and the fruit were reaching senescence at a faster rate. The synthesis of lycopene is affected by storage time and modification of the atmosphere and this is because under cold storage conditions, the metabolic activity of the fruit is reduced resulting in decreased physiological changes in the fruit (Abdel-Salam, 2016). An increase in β carotene and lycopene in the sun-exposed fruit can be related to increased radiation and temperature which plays a role in pigment production. Lycopene is known to play a role in the colour development of citrus fruit meaning that fruit with high lycopene concentration in the outside canopy position had a better colour development.


CP: p < 0.001; TR: p < 0.001; CP*TR: p = 0.0601;
LSD = 2.418
IC
OC
Luminosity (L*)
90
88
86
84
82
80
78
Chitosan
CMC
Control
M+Chitosan
M+CMC
Coating treatment
A


CP: p = 0.011; TR: p < 0.001; CP*TR: p = 0.397;
LSD = 2.263
IC
OC
CH
CMC
Control
M+CH
M+CMC
Greeness (a*)
0
-5
-10
-15
-20
Coating treatment
B


CP: p < 0.001; TR: p < 0.001; CP*TR: p = 0.606;
LSD = 1.887
IC
OC
Yellowness (b*)
64
62
60
58
56
54
52
Chitosan
CMC
Control
M+Chitosan
M+CMC
Coating treatment
C

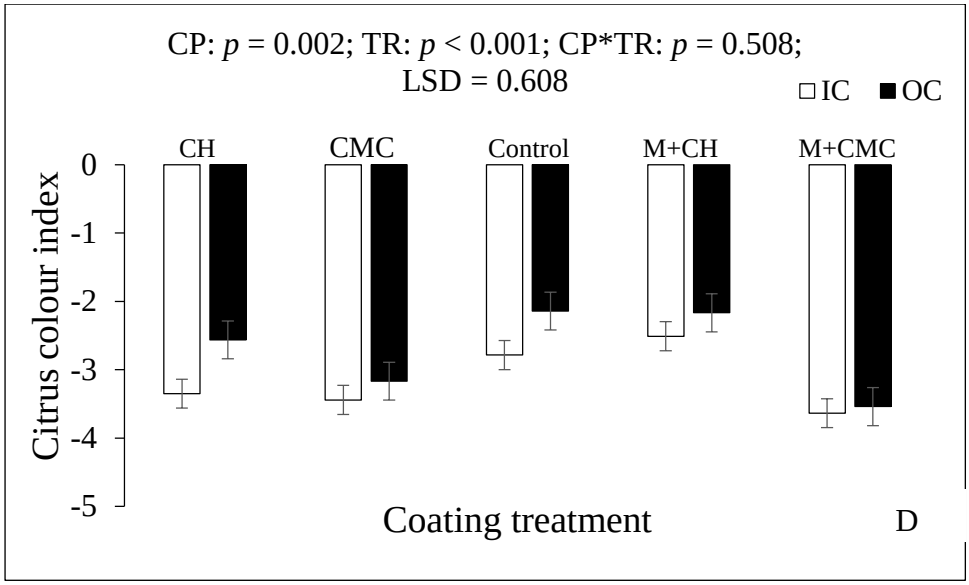


Fig. 4: The effect of coating treatment on colour parameters (luminosity (A); greenness (B); yellowness (C); citrus colour index (D)) of fruit harvested from outside canopy (OC) and inside canopy (IC) positions over 12 weeks of postharvest cold storage at 3 °C. LSD: least significance difference; CP: canopy position; CT: coating treatment.

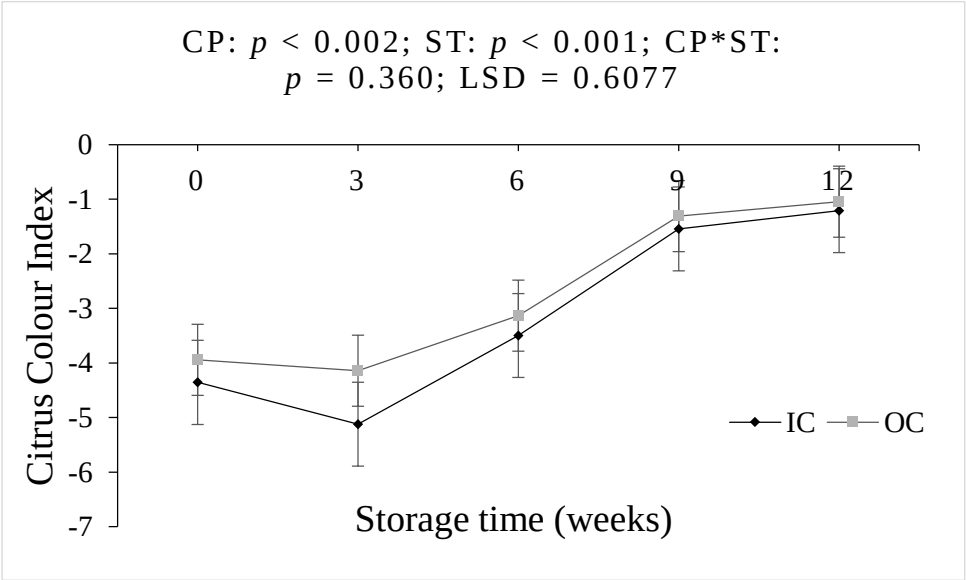
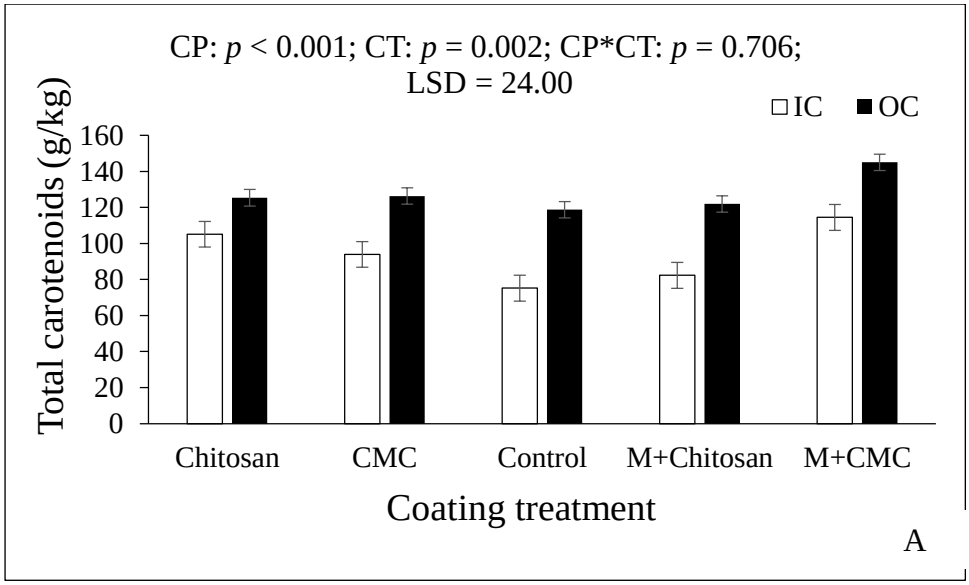
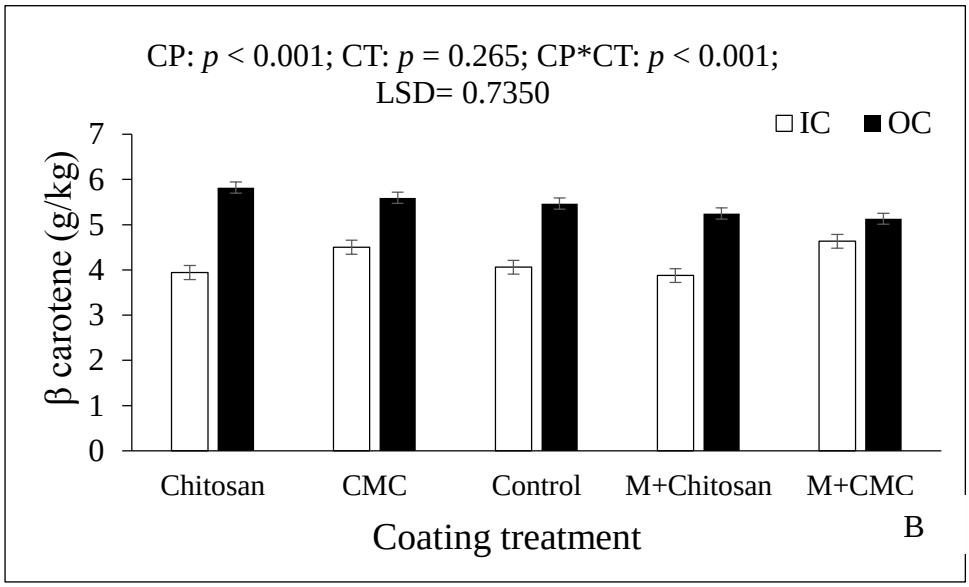


Fig. 5: The effect of canopy position on colour parameters (luminosity (A); greenness (B); yellowness (C); citrus colour index (D) of fruit harvested from outside canopy (OC) and inside canopy (IC) positions over 12 weeks of postharvest cold storage at 3 °C. LSD: least significance difference; CP: canopy position; ST: storage time.


CP: p < 0.001; CT: p = 0.002; CP*CT: p = 0.706;
LSD = 24.00
□IC ■OC
Total carotenoids (g/kg)
160
140
120
100
80
60
40
20
0
Chitosan
CMC
Control
M+Chitosan
M+CMC
Coating treatment
A


CP: p < 0.001; CT: p = 0.265; CP*CT: p < 0.001;
LSD= 0.7350
□IC ■OC
β carotene (g/kg)
7
6
5
4
3
2
1
0
Chitosan
CMC
Control
M+Chitosan
M+CMC
Coating treatment
B

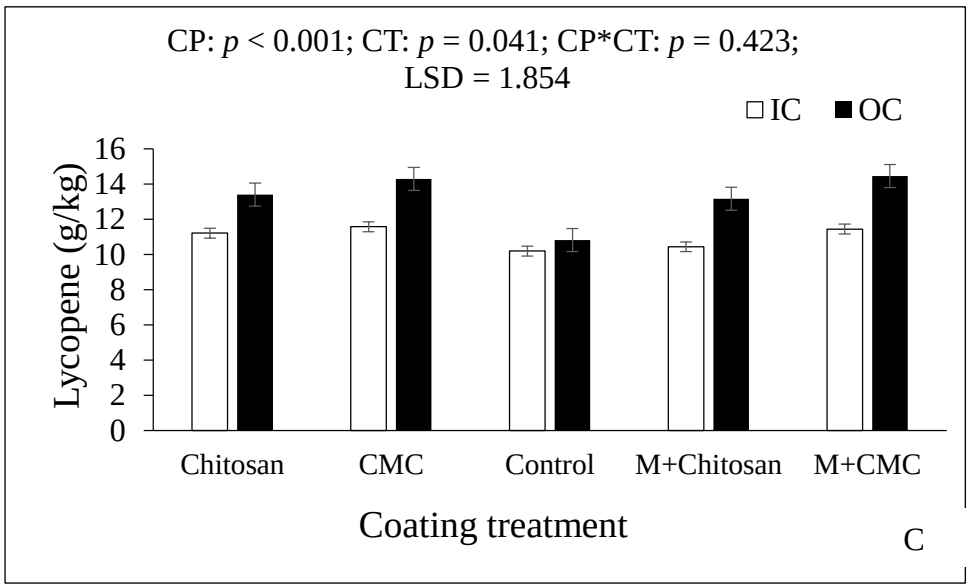

CP: p < 0.001; CT: p = 0.041; CP*CT: p = 0.423;
LSD = 1.854
□IC ■OC
Lycopene (g/kg)
16
14
12
10
8
6
4
2
0
Chitosan
CMC
Control
M+Chitosan
M+CMC
Coating treatment
C

Fig. 6: The effect of edible coatings on total carotenoids (A), β carotene (B) and lycopene (C) of 'Eureka' lemons in fruit harvested from inside canopy (IC) and outside canopy (OC) positions during postharvest cold storage of 3 °C. LSD: least significance difference; CP: canopy position, CT: coating treatment.

4.3.4. The effect of coating treatments on TSS and TA

The results showed that neither coating treatment nor canopy position had a significant effect on total soluble solutes (TSS) and titratable acidity (TA) (Table 1). This coincides with the observation that was made by Machado et al. (2012), where the application of coatings had no effect on physicochemical properties and in cases where there was a difference, the difference was not significant between the coated and uncoated fruit. Olarewaju et al. (2018) also found canopy position to cause no significant effect on TSS of 'Marsh' grapefruit, however, the authors reported TA and TSS/TA to be significantly affected by canopy position where outside canopy fruit had high TA compared to inside canopy fruit.

Total acidity can be related to the ripening of the fruit. For non-climacteric fruit like lemon, there are no considerable alterations after harvesting the fruit, which could be the reason why there were no significant chemical variations during storage time hence coatings and canopy position caused no difference. Obenland et al. (2008) also reported that edible coatings were most likely to cause any significant effect in TSS and TA when they were evaluated in 'Navel' orange. The titratable acidity in this study was found to be highly influenced by storage time ($p < 0.001$) where it was showing an increase in all the treatments. The values of TA during storage increased from 2.63 to 5.25 % in the inside canopy and 3.52 to 5.11 % in the inside canopy at the end of storage.

Some authors have observed a decline in TSS and TA of fruit that were not coated which was also related to weight loss and respiration rate (Toğrul, and Arslan, 2004). The decrease is caused by a reduction in respiration rate which decreases the synthesis and the use of metabolites thereby resulting to a decrease in TSS through slower hydrolysis of carbohydrates to sugars (Obenland et al., 2008). In a study conducted in guavas, de Aquino et al. (2015) found an increase in TSS of uncoated fruit meaning the fruit ripened faster hence the quality of the fruit was altered. This was also found to be true in uncoated grapes (Sánchez-González et al., 2011).

4.3.5. The effect of coating treatments and canopy position on non-structural carbohydrates

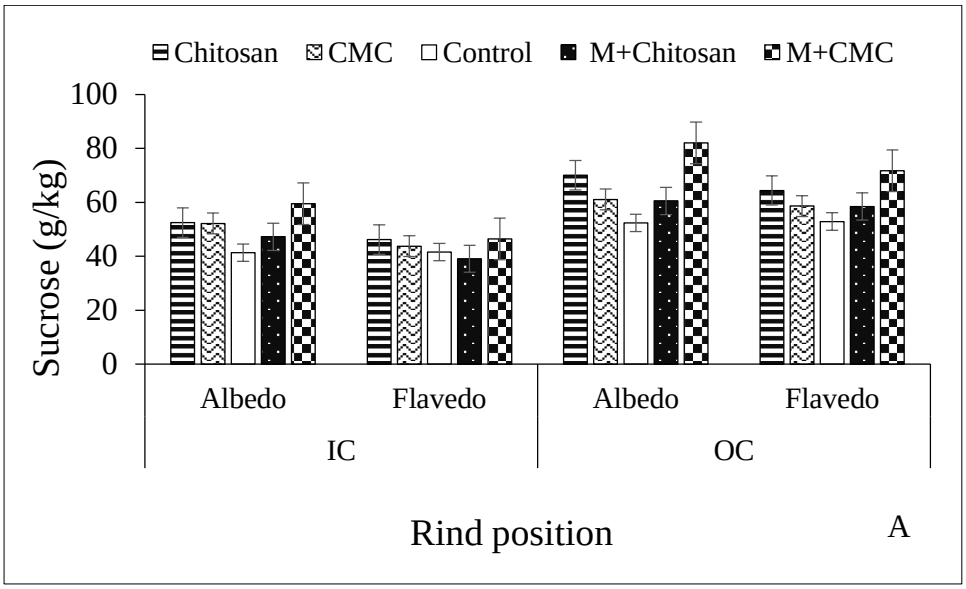
The application of coating treatments before cold storage had no significant effect on the concentrations of sucrose ($p = 0.056$), glucose ($p = 0.470$) and fructose ($p = 0.738$) (Fig. 7A, B, C). However, control fruit had high concentrations of sugars at the end of storage period which was also seen by an increase in TSS (Table 1). This could be due to the rapid ripening of control fruit during storage meaning sugars were metabolizing and synthesizing at a faster rate which was controlled in coated fruit. Although M+CMC coating had higher sucrose, no significant differences were found in the carbohydrates of 'Eureka' lemons when comparing the coating treatments.

Canopy position showed a significant effect on all the 3 sugars ($p < 0.001$). Sucrose, glucose and fructose were found to be more concentrated in fruit harvested from the outside canopy of the tree compared to inside canopy with higher values of 76.86; 80.35 and 40.51 g/kg, respectively, while fruit from the inside canopy had 52.97; 67.96 and 30.64 g/kg, respectively. Comparable results to these were found in other studies done by Cronje et al. (2011); Magwaza et al. (2013) in 'Nules Clementine' mandarin. Many studies have shown sun-exposed fruit from the outside canopy to have a high concentration of non-structural carbohydrates and less susceptibility of rind disorders. The explanation according to Cronje et al. (2013) is that the process of photosynthesis and respiration occurring in these fruits is high which results in a faster synthesis and high metabolic processes of carbohydrates. Cronje et al. (2011) also hypothesized that the reduced carbohydrate content in the inside canopy could cause a higher susceptibility of 'Nules Clementine' mandarins to rind breakdown and this hypothesis was strengthened by the results found in this study.

The relationship between non-structural carbohydrates and the development of physiological disorders in citrus fruit has been reported by Cronje et al. (2013). This suggests that the accumulation of carbohydrates by the fruit can help in preventing the development of physiological disorders and this is also related to the osmoprotective role that carbohydrates play in defending the fruit from external stress conditions (Holland et al., 2005). Although the coatings had no significant effect on carbohydrates, the increased concentrations in the outside canopy for all (sucrose, glucose and fructose) suggests that the sugars were abundant enough

to protect the fruit from PS development hence fruit from the outside canopy position were found to be less susceptible to the disorder. The low concentrations of carbohydrates in the inside canopy can also be related to the low in colour development that was observed which is related to low TSS and TA.

The concentrations of carbohydrates in this study were evaluated in both the flavedo and the albedo of the fruit. This is becoming a key focus in most citrus fruit because the rind has been the most ignored part yet it contains high amounts of antioxidants and carbohydrates (Lü et al., 2016). Rind position was found to have a significant effect on all the three sugars ($p < 0.001$), sucrose and fructose were more concentrated in the albedo 65.21 and 40.54 g/kg, respectively than in the flavedo 43.41 and 33.12 g/kg, respectively while glucose was found more abundant in the flavedo (79.35 g/kg) compared to the albedo (66.8 g/kg). This means that both the flavedo and albedo parts of the fruit are important as they contain a significant amount of non-structural carbohydrates.



CP: $p < 0.001$

RP: $p < 0.001$

CT: $p < 0.056$

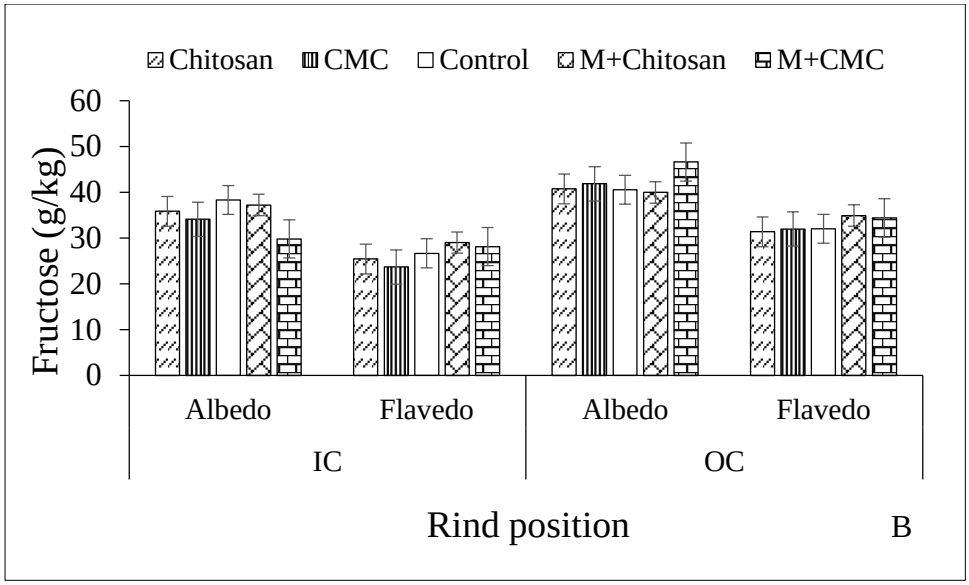
CP*RP: $p = 0.228$

CP*CT: $p = 0.012$

RP*CT: $p = 0.064$

CP*RP*CT: $p = 0.909$

LSD= 7.909



CP: $p < 0.001$

RP: $p < 0.001$

CT: $p < 0.738$

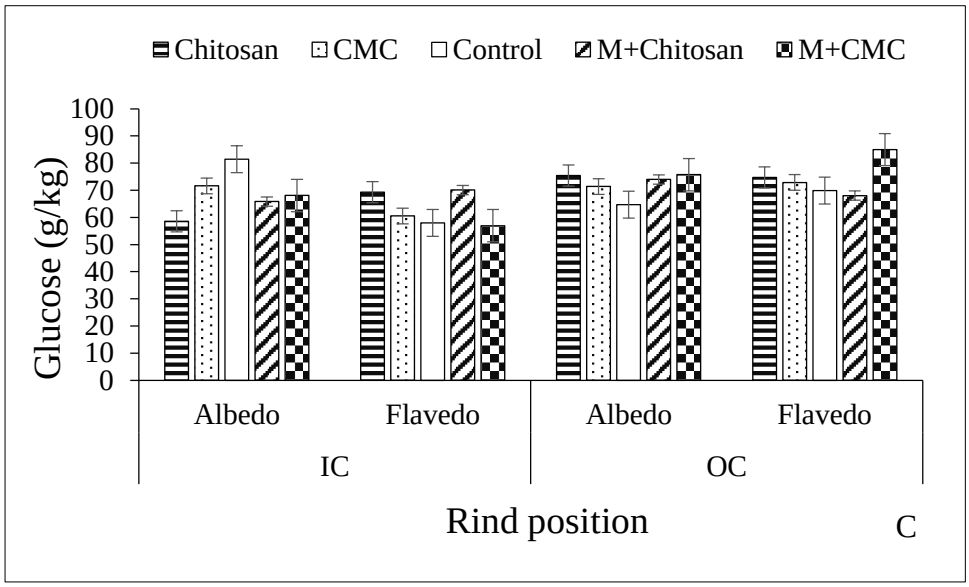
CP*RP: $p = 0.828$

CP*CT: $p = 0.267$

RP*CT: $p = 0.800$

CP*RP*CT: $p = 0.387$

LSD= 7.784



CP: $p < 0.001$

RP: $p < 0.001$

CT: $p = 0.470$

CP*RP: $p = 0.469$

CP*CT: $p = 0.063$

RP*CT: $p = 0.116$

CP*RP*CT: $p = 0.438$

LSD= 11.652

Fig. 7: The effect of coating treatment on sucrose (A), fructose (B) and glucose (C) of 'Eureka' lemons in the albedo and flavedo of fruit harvested from outside canopy (OC) and inside canopy (IC) positions over 12 weeks of postharvest cold storage at 3 °C. LSD: least significance difference; CP: canopy position; RP: rind position; CT: coating treatment.

4.3.6. The effect of coating treatment and canopy position on total phenolic and flavonoid concentrations

One of the important roles of phenolic compounds in plants is helping the plant defend itself against pests and diseases. The health promoting characteristics that have been reported in citrus fruit are known to be dependent on phenolics and flavonoids concentrations (Lü et al., 2016). The results showed that total phenolics were significantly affected by coating treatment

($p < 0.001$) (Fig. 8A). Fruit coated with M+CMC had high total phenolic concentration, while the control showed low concentrations of total phenolics. Total phenolics were also significantly affected by storage time ($p < 0.001$) (Table 1). This could be due to the polyphenol oxidase activity, which causes oxidation of phenolics after a certain period of time, turning them into quinones (Stanley, 1998). According to Klimczak et al. (2007), the polyphenols concentration in citrus fruit depend on the storage conditions such as temperature and the length of storage.

This study evaluated total phenolics and flavonoids in 2 different rind positions, the flavedo and the albedo which were found to be significantly different ($p < 0.001$) (Fig. 8A, 9A). To the best of my knowledge, the amount of phenolic and flavonoid concentrations in the flavedo and albedo of 'Eureka' lemons has not been evaluated before. From the results, it was found that total phenolics were more abundant in the albedo while flavonoids were more in the flavedo. Similar results were observed in Pumelelo fruit (Lü et al., 2016; Rahman et al., 2018). However, Escobedo-Avellaneda et al. (2014) found that both phenolics and flavonoids were more abundant in the albedo than in the flavedo of 'Valencia' orange. The concentration of phenolics in the albedo had values ranging from 2.153 to 2.691 g/kg GAE and 1.830 to 2.152 g/kg GAE in the flavedo.

There was a slight difference in coating treatments with M+CMC, M+CH, CH, CMC and the control reaching values of 2.691, 2.528, 2.491, 2.354 and 2.153 g/kg GAE, respectively, at the end of storage time (Fig. 8A). However, control fruit showed a rapid decrease in total phenolics, which started 6 weeks after storage (Fig 8B). Dong et al. (2019) evaluated the total phenolics in 'Eureka' lemon peels and found that the highest concentration was 7.960 g/kg GAE, while the lowest was 4.300 g/kg GAE, which is higher than what was found in this study, probably because their extraction was done on fresh weight. Irkin et al. (2015) found the highest concentration in lemon to be 5.810 g/kg GAE while Ye et al. (2011) reported 4.710 – 7.870 g/g GAE in mandarin fruit.

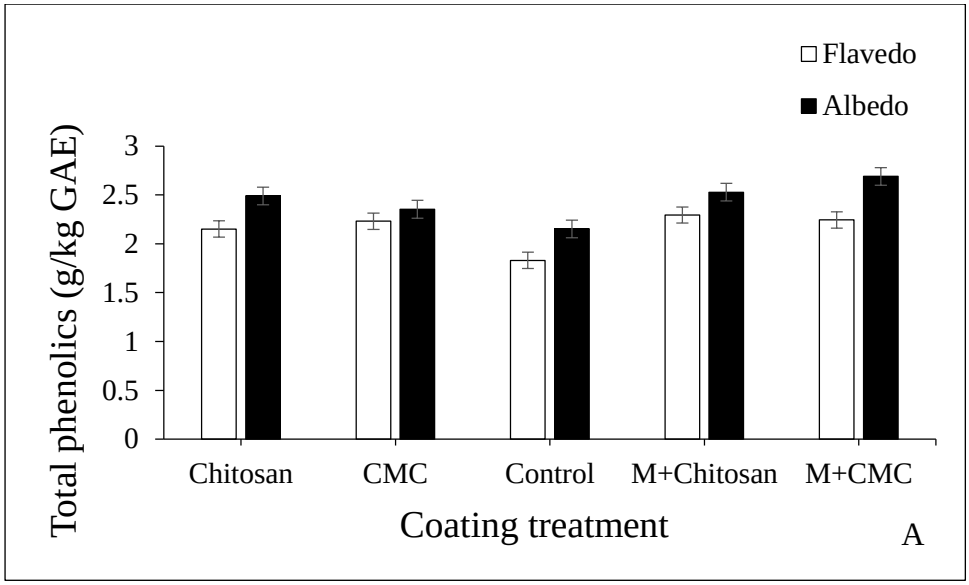
Rind position caused a significant effect on total flavonoid concentration ($p < 0.001$). Unlike phenolics, total flavonoids were found to be more abundant in the flavedo than in the albedo. The total flavonoid concentration in the flavedo ranged from 2.104 to 2.907 g/kg QE and 1.872 – 2.104 g/kg QE in the albedo (Fig. 9A). Total flavonoids were also found to be significantly

affected by coating treatments ($p < 0.001$) and high values were observed in the flavedo of fruit coated with CMC and M+CMC, while low values were found in fruit coated with M+CH.

The type of fruit and storage conditions may have a huge influence on the concentration of phenols and flavonoids, which is, they may either increase or decrease it (Singleton et al., 1999). This partially explains why there was a huge variation amongst the concentration of phenolic and flavonoids in citrus fruit. Phenolics are known as secondary metabolites that play a role in functioning of living cells and are important scavenging free radicals. The high variation in these concentrations could be due to the difference in cultivars, extraction methods, coating treatments, environmental and growing conditions. Storage period has also been reported to influence the concentration of phenolics and flavonoids which could also explain the results found in this study. This is because under low storage conditions, the activities and transcriptional activators that play a role in the synthesis of phenolics and flavonoids are affected (Zou et al., 2016).

Both phenolic and flavonoid concentrations were significantly affected by canopy position ($p < 0.001$). More concentrations were found in the outside canopy than inside canopy. Comparable results were reported by Olarewaju et al. (2017) in 'Nules Clementine' mandarin. Ben-Yehoshua et al. (1992) explained the reason of high concentration of phenolics in the outside canopy to be related to the high radiation that stimulates the production of phenylalanineaminialyase inducing the production of phenolic compounds. This was also related to the high photosynthetically active radiation in the outside canopy which initiates phytoalexins synthesis, a phenolic compound that helps the fruit defend itself against stress conditions.

Hagen et al. (2007) also related a high production of phenolic concentration to high radiation from the sunlight in the outside canopy position of the tree. Similarly, the synthesis of flavonoids has also been reported to be related to light intensity and temperature. The enzymes that are involved in the production of flavonoids are stimulated by light hence the concentration increases in sun-exposed fruit (Treutter, 2001). This could explain the high concentration of phenolics and flavonoids that were observed in fruit harvested from outside canopy.

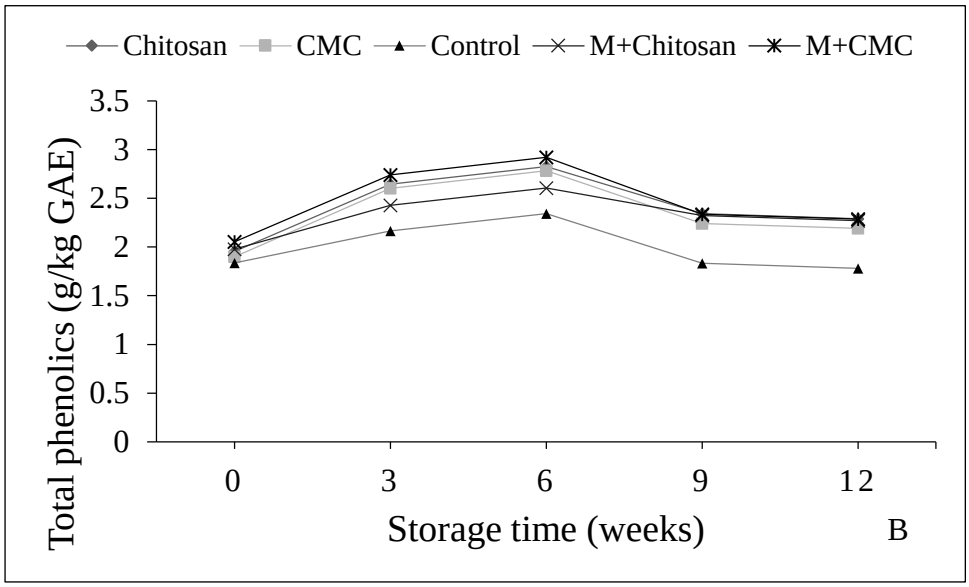


CT: $p < 0.001$

RP: $p < 0.001$

CT*RP: $p = 0.009$

LSD= 0.169



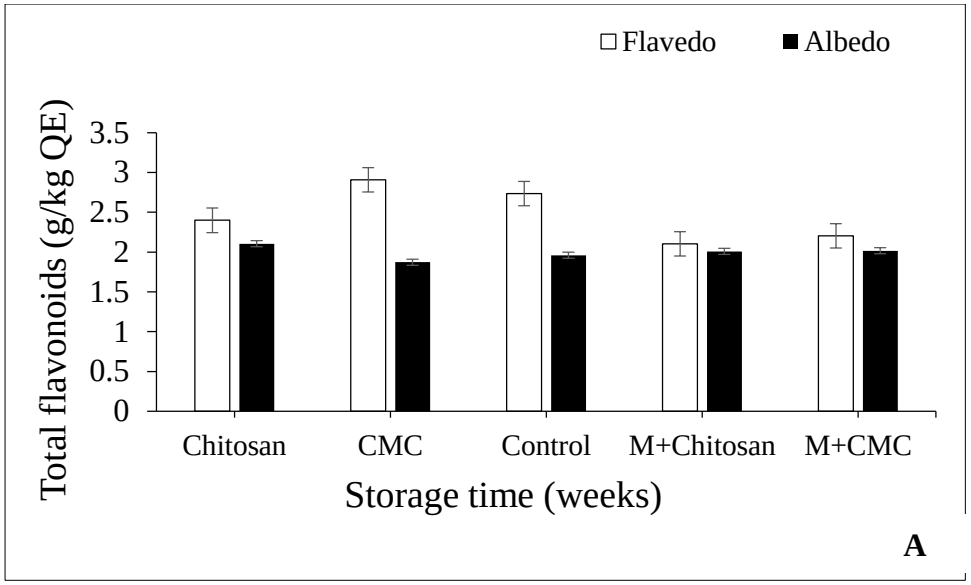
CT: $p < 0.001$

ST: $p < 0.001$

CT*ST: $p = 0.438$

LSD= 0.267

Fig. 8: The effect of coating treatments on phenolic concentration of 'Eureka' lemon in fruit harvested from inside canopy (IC) and outside canopy (OC) positions during 12 weeks postharvest cold storage at 3 °C. LSD: least significance difference; CP: canopy position, CT: coating treatment; RP: rind position; ST: storage time.

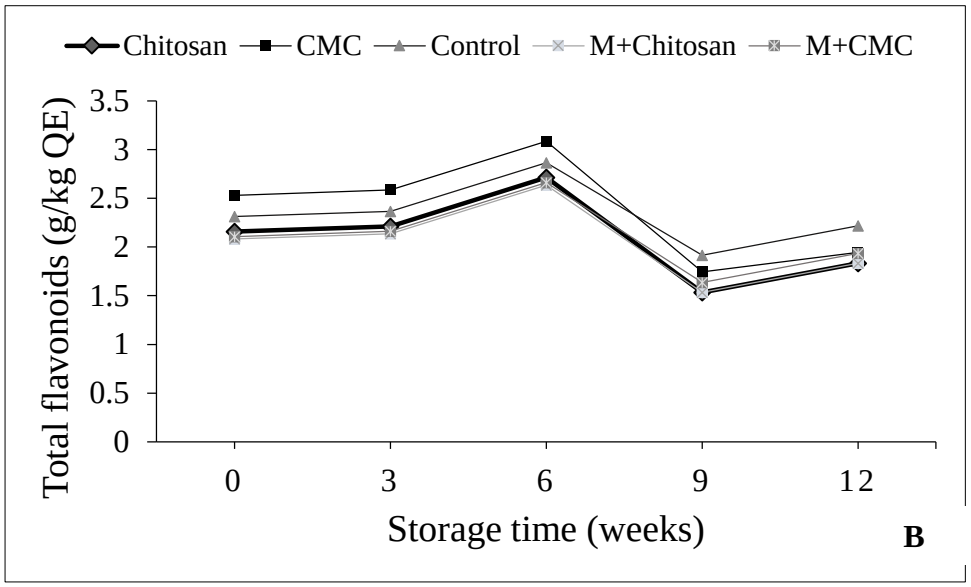


CT: $p < 0.001$

RP: $p < 0.001$

CT*RP: $p < 0.001$

LSD= 260.3



CT: $p < 0.001$

ST: $p < 0.001$

CT*ST: $p = 0.982$

LSD= 411.6

Fig. 9: The effect of coating treatments on flavonoids of 'Eureka' lemons in fruit harvested from inside canopy (IC) and outside canopy (OC) positions during 12 weeks postharvest cold storage at 3 °C. LSD: least significance difference; CP: canopy position, CT: coating treatment; RP: rind position; ST: storage time.

4.3.7. The effect of coating treatments and canopy position on ascorbic acid

The loss of ascorbic acid (AsA) in citrus fruit is a determining factor for the shelflife of the fruit (Laing et al., 1978). The stored fruit reaches senescence more quickly when there is a rapid loss of ascorbic acid and this makes it important to find ways to delay the loss which is done through the application of edible coatings. Coating treatments had a significant effect on ascorbic acid of 'Eureka' lemons ($p < 0.001$). Amongst the treatments, the control and M+CH

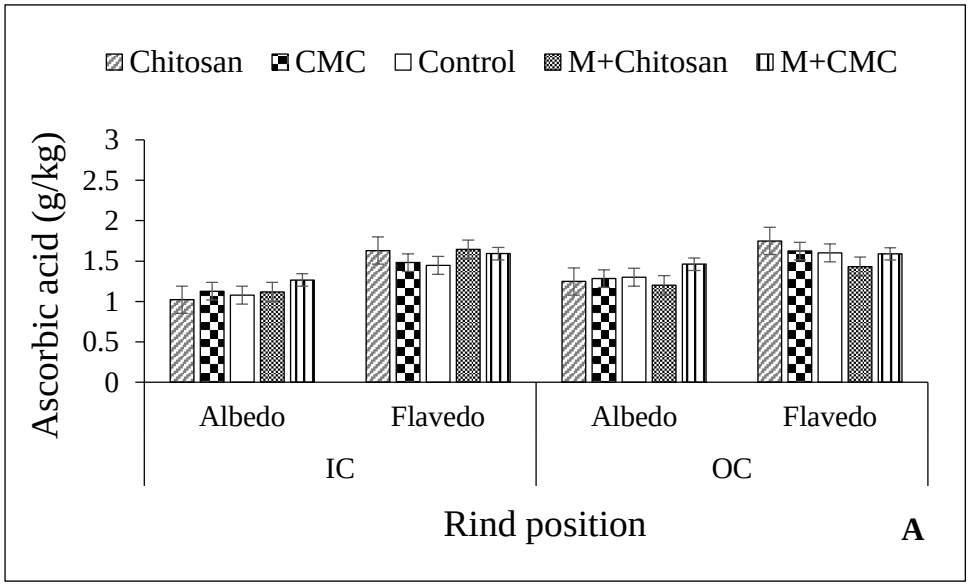
had low concentrations of ascorbic acid, 1.08 and 1.12 g/kg, respectively, and the loss rate was faster during storage time compared to the other treatments (Fig. 10A). This was seen by the fast decline in the concentration after 6 weeks of cold storage (Fig. 10B). Fruit coated with CH, CMC and M+CMC had high AsA (1.75, 1.60 and 1.59 g/kg), respectively. These coating treatments were effective in reducing AsA loss which could partly explain the lower susceptibility of coated fruit to peteca spot.

The results showed that ascorbic acid was significantly affected by canopy position; rind position and storage time (p <0.001). The outside canopy fruit had high concentration of ascorbic acid compared to the inside canopy fruit mainly because of the high exposure of the fruit to sunlight during growth and development (Fig 10A). Ascorbic acid content has been previously reported to be highly influenced by light and temperature during growth and development of the fruit (Lee and Kader, 2000). The high concentration of AsA in the outside canopy can be related to the high carbohydrates that were also observed in the outside canopy because AsA is synthesized from carbohydrates (Valpuesta and Botella, 2004). A high AsA can also be related to defense mechanism of fruit against stress conditions.

The average concentration of ascorbic acid was more in the flavedo (1.60 g/kg) than the albedo (1.30 g/kg) part of the fruit in the outside canopy position. While fruit in the inside canopy had an average of 1.56 k/kg in the flavedo and 1.12 g/kg in the albedo. The separation of the flavedo and albedo was done to compare the difference in the two tissues since PS is known to not only affect the flavedo but also the albedo. The interaction between canopy position and rind position was found to be significant ($p < 0.001$), indicating that the difference in concentrations within the rind position was affected by the position of the fruit while still attached on the tree.

Ascorbic acid was also found to change over time during cold storage. Storage time, storage temperature and light are some of the reported factors that lead to the degradation of ascorbic acid (Robertson and Samaniego, 1986). Burdula et al. (2006) found about 52.8% AsA loss in lemon after 8 weeks of storage, which was also determined by storage temperature. Lee and Coates (1999) also reported the instability of ascorbic acid which easily decomposes and changes over time, especially during unfavorable storage conditions. The loss of AsA during storage time was explained to be caused by the activity of two major enzymes that are involved in AsA oxidation (phenoloxidase and ascorbic acid oxidase) (Salunkhe et al., 1991).

These results confirm those found by Adetunji et al. (2013) where moringa incorporated with CMC was effective in reducing AsA loss during cold storage in citrus fruit. The mechanism behind this, as explained by the authors is due to the low oxygen permeability of the coating treatment which was able to lower the activity of the enzymes that are responsible for the oxidation of AsA. Shao et al. (2015) evaluated the effectiveness of chitosan and clove oil edible coatings on citrus green mould and found that pure chitosan prevented the growth of green mould while the combination of the two was not effective and as found in this study, fruit coated with M+CH coating was not effective in delaying or reducing AsA loss. A delay in AsA loss by chitosan was also observed by Ali et al. (2011) in papaya fruit. Similarly, the application of CMC was also found to reduce AsA loss in mandarin fruit (Toğrul, and Arslan, 2004) which is due to the gas barrier of the coating that inhibits oxygen from entering the fruit hence decreasing the possibility of AsA autoxidation in aerobic conditions.



CT: $p < 0.001$

RP: $p < 0.001$

CP: $p < 0.001$

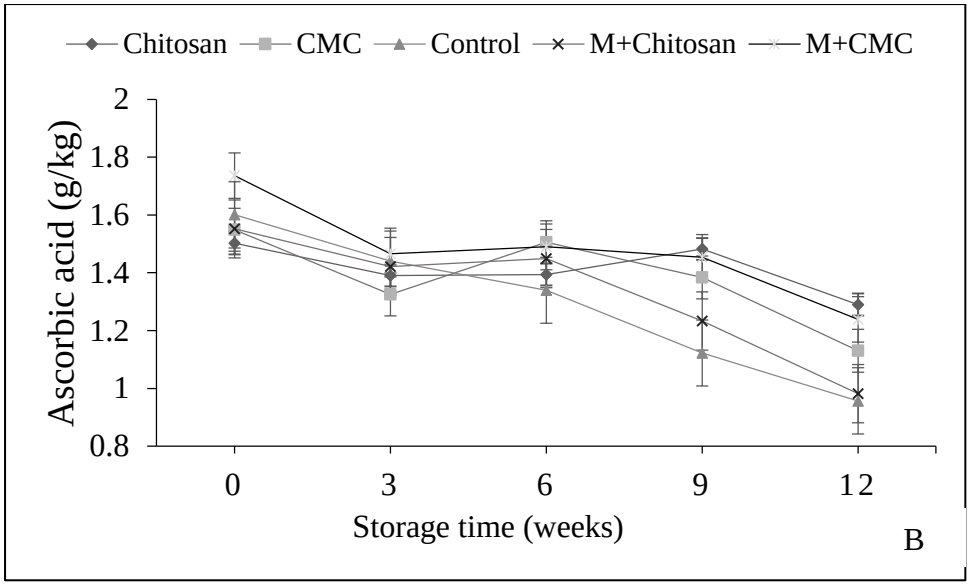
CP*RP: $p < 0.001$

CP*TR: $p = 0.706$

RP*TR: $p = 0.003$

CP*RP*TR: $p = 0.663$

LSD= 0.107



CT: $p < 0.001$

ST: $p < 0.001$

CT*ST: $p < 0.001$

LSD= 0.120

Fig. 10: The effect of coating treatments on ascorbic acid of 'Eureka' lemons in fruit harvested from inside canopy (IC) and outside canopy (OC) positions during 12 weeks postharvest cold storage at 3 °C. LSD: least significance difference; CP: canopy position, CT: coating treatment; RP: rind position; ST: storage time.

Table 1: The fruit parameters of ‘Eureka’ lemons measured over 12 weeks storage period in fruit harvested from inside canopy and outside canopy positions.

	INSIDE CANOPY						***OUTSIDE CANOPY***					
Storage time (weeks)	**Mass (g)**	**TSS (%)**	**TA (%)**	**Tcar (g/kg)**	**Phenols (g/kg GAE)**	**Flavonoids (g/kg QE)**	**Mass (g)**	**TSS (%)**	**TA (%)**	**Tcar (g/kg)**	**Phenols (g/kg GAE)**	**Flavonoids (g/kg QE)**
0	122.6 ab	5.23 a	2.63 a	41.52 a	1.65 a	2.13 b	127 cde	6.2 a	3.52 b	64.1 a	2.24 b	2.46 cd
3	106.71 a	5.31 b	3.25 ab	69 ab	2.88 cd	2.19 b	120.1 bcd	5.9 b	3.25 a	73.74 ab	2.15 b	2.49 cd
6	107.1 a	6.5 b	4.85 bc	91.21 abc	3.06 d	2.63 d	119 bc	6.8 ab	4.98 ab	133.62 cd	2.33 b	2.96 e
9	118.21 bc	6.07 ab	5.81 d	103.21 bc	1.63 a	1.98 b	130.7 e	7.2 c	5.79 bc	128.61 d	2.80 cd	1.37 a
12	110.81 ab	7.12 c	5.25 cd	157.12 d	1.58 a	2.28 bc	129.9 de	7.8 cd	5.11 b	237.41 e	2.75 c	1.63a

4.4. Conclusion

This study demonstrated the effectiveness of edible coatings in reducing the incidence of peteca spot of 'Eureka' lemons during cold storage. The results showed that fruit coated with M+CMC, CMC and CH were less susceptible to the development of the disorder in both inside and outside canopy positions, while the control showed a high incidence of the disorder followed by M+CH coating. The incidence of peteca spot was not only affected by coating treatments, but storage time and canopy position also played a role. Fruit from the inside canopy position were found more susceptible to the development of the disorder compared to fruit harvested from the outside canopy. Furthermore, the ability of the coating treatments to reduce mass loss, ascorbic acid loss and delay colour change was also observed. Coating tratments significantly affected total phenolic and flavonoid concentrations but had no significant effect on nonstructural carbohydrates. To conclude, the study demonstrated the potential of M+CMC, CH and CMC edible coatings as the best postharvest treatments for reducing peteca spot in 'Eureka' lemons. The most effective coating treatment was moringa incorporated with carboxymethyl cellulose (M+CMC) followed by pure CMC and pure chitosan (CH). These coating treatments were also able to reduce ascorbic acid loss, mass loss and a delay in colour change which prevented the fruit from ripening fast and reaching senescence. Either one of the coatings (M+CMC, CMC or CH) is therefore, recommended for coating fruit after harvest before fruit packing. The combination of moringa and chitosan (M+CH) was less effective in reducing the incidence of peteca spot, therefore the coating is not recommended.

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Chapter 5: The use of vis/NIR spectroscopy for non-destructive prediction of peteca spot in 'Eureka' lemons

Abstract

Peteca spot is a commercially important rind physiological disorder that affects the South African lemon industry during postharvest storage. The appearance of the disorder can take up to 5 weeks after harvest, therefore, detecting its occurrence during packing and sorting becomes difficult. The aim of this study was to test the feasibility of visible to near infrared spectroscopy (Vis/NIRS) as a non-destructive tool to predict susceptibility of 'Eureka' lemon fruit to peteca spot. The study was conducted at the University of KwaZulu-Natal with 520 fruit harvested from inside and outside canopy positions from Malowe commercial orchard farm located in KwaZulu-Natal, South Africa. Vis/NIR spectroscopy and chemometric methods in reflectance mode combined with appropriate pre-processing techniques and PLS regression was explored for determining rind biochemical properties of 'Eureka' lemons which can be used as presymptomatic biochemical markers related to the development of the disorder. The reflectance spectral data was obtained using a laboratory bench-top monochromator NIR Systems model XDS spectrometer System equipped with a quartz halogen lamp and lead sulfide detector. Physico-chemical parameters such as sucrose, glucose, fructose, total soluble solids (TSS) and ascorbic acid (AsA) were destructively measured and used as reference data for calibration and validation of partial least square (PLS) models. All measured variables showed good prediction models which includes sucrose $R^2 = 0.81$, glucose $R^2 = 0.77$, fructose $R^2 = 0.70$, TSS $R^2 = 0.78$ and AsA $R^2 = 0.86$. The result of PLS and principal component analysis (PCA) models developed in this study demonstrated that Vis/NIRS can be used as a non-destructive tool to pre-screen 'Eureka' lemon fruit for susceptibility of to peteca spot disorder.

Key words: biochemical attributes, partial least squares, physico-chemical parameters, presymptomatic biochemical markers, Principal Component Analysis

5.1. Introduction

External appearance is one of the important factors that determine the price and market for citrus fruit (Magwaza and Opara, 2015). Fruit that show a slight defect in the pack line are

removed and discarded because they are rejected in the international market, which causes a great economic loss (Blasco et al., 2007). Peteca spot (PS) is among several commercially important postharvest physiological disorders that affects the South African lemon industry (Cronje, 2015). The disorder results in the collapse of oil glands which is seen by dark brown lesions on the surface of the fruit (Cronje, 2005, Undurraga et al., 2006). The actual cause of the disorder is not clearly understood, but the incidence has been aggravated by low storage temperature of 3 °C (Undurraga et al., 2009).

This is a problem to South African lemon growers because fruit exported from South Africa have to undergo a period of cold sterilization which involves fruit exposure to -0.6 °C for ± 14 days followed by 5 °C during shipping for quarantine against fruit flies (Bassal and El-Hamahmy, 2011; Manrakhan et al., 2018) and these conditions can lead to the development of peteca spot. Apart from storage, factors that have been reported to increase the incidence of physiological disorders in citrus fruit include, canopy position, non-structural carbohydrates, rind dry matter, fruit maturity, antioxidants, carotenoids and flavonoids (Magwaza et al., 2013; Cronje et al., 2013; Nasirifar et al., 2018). Even with these factors known, the disorder is more likely to occur unpredictably, which makes it important to find means to predict rind postharvest physiological disorders during packing and sorting, before the fruit are shipped to international markets.

The possibility of physico-chemical properties to be used as pre-symptomatic biochemical markers has been recently studied by Ncama et al. (2018) in ‘Marsh’ grapefruit. The authors found inside canopy fruit to be more susceptible to chilling injury, with rind pitting incidence more in the outside canopy fruit. The inside canopy was characterized by low dry matter but had higher antioxidant activity, sucrose and fructose. These biochemical variables were positively correlated to rind pitting disorder and negatively correlated to chilling injury and the authors concluded that they could be successfully used as pre-symptomatic markers for predicting chilling injury and rind pitting disorders.

The development of peteca spot in lemon is unpredictable. The disorder can occur any time after harvest until postharvest storage. Cronje (2015) reported that peteca spot can occur as early as 3-5 days after harvest while Khalidy et al. (1969) observed the symptoms 3-4 weeks after postharvest storage. This problem has led to the objective of this study, which is to find

means to predict the individual fruit to the susceptibility to peteca spot and this will help in fruit packing and sorting prior shipping to international markets. Predicting factors that are related to peteca spot development may allow for the possibility of these factors to be used as presymptomatic markers to predict the disorder.

The major problem associated with determining pre-symptomatic markers, however, is that the currently used methods for the determination of physico-chemical attributes requires destructive measurements of a single fruit which is labour intensive and time consuming as they require specialized sample preparation (Arendse et al., 2018). For this reason, nondestructive measures have currently gained more interest and they are more preferred over destructive techniques because they are cost effective, timesaving and they allow for the measurement and analyses of an individual fruit (Nicolai et al., 2007).

Several nondestructive methods have been identified and reviewed by Magwaza et al. (2012) and one of the commonly used method that has gained interest in postharvest handling and technology is vis/NIR spectroscopy because of its instrumentation and commercial application. One of the main advantages of NIR is that it allows for the analyses of a large sample size at once which involves non-destructive measurements (Roggo et al., 2007). The chemometrics of NIR involve multivariate analyses which allows for the interpretation of large data sets. In order to extract required data from the vis/NIR spectrum, PLS (partial least squares) and PCA (partial component analyses) are used (Cozzolino et al., 2011). Research has shown PLS regression as the most commonly used and effective method by far (Shenk and Westerhaus, 1991; Norgaard et al., 2000).

The use of vis/NIR spectroscopy and chemometric methods has been previously reported to accurately predict internal and external quality parameters as well as rind physiological disorders in citrus fruit (Liu et al., 2010; Magwaza et al., 2012; Sánchez et al., 2013a). Near infrared spectroscopy was able to effectively predict rind biochemical profile of 'Nules' clementine mandarin (Magwaza et al., 2013) and drying disorder in tangerine (Piers et al., 1998). Sánchez et al. (2013b) also showed the ability of NIR to simultaneously evaluate the external and internal quality parameters of intact oranges. However, there are currently no studies reporting the prediction of peteca spot in 'Eureka' lemons. The objective of this study was to evaluate the use of vis/NIR spectroscopy for non-destructive prediction of peteca spot in 'Eureka' lemons.

5.2. Materials and methods

5.2.1. Fruit sampling

The study was conducted at the University of KwaZulu-Natal, Pietermaritzburg campus, South Africa. A total of 520 commercially mature fruit of the cultivar 'Eureka' lemon, which is the most important cultivar grown in South Africa were harvested from a commercial orchard at Malowe farm (Latitude: 30°14'S, Longitude: 29°56'E). At the time of harvest, fruit had 36% juice content. Fruit were harvested from two canopy positions, the inside (shaded part of the tree) and outside canopy (sun exposed part of the tree). After harvesting, a well-ventilated car was used to transport the fruit to the Postharvest Technology Laboratory of the University of KwaZulu-Natal where the experiment took place.

5.2.2. Measurement of internal parameters

After harvest, fruit were divided into two groups, where 260 fruit were scanned using a laboratory bench-top monochromator NIRSystems model XDS spectrometer (Foss NIR system, Inc, Maryland, USA) and destructively analyzed and the other half (260) were stored into a cold room with temperature set at 3 °C, a storage temperature that has been reported to induce the incidence of peteca spot (Undurraga et al., 2009). Internal quality parameter such as total soluble solids (TSS), ascorbic acid and non-structural carbohydrates were measured. Total soluble solids were measured from squeezed juice using a digital hand-held refractometer with a dynamic control system (RFM340+ BS®, Bellingham and Stanley Ltd, Basingstoke, Hants, UK). The rind was peeled by hand and directly stored -40 °C for the determination of ascorbic acid and carbohydrates. The frozen samples were transferred into a VirTis Freeze dryer system (Model 6KBTES-55, SP industries, Warminster, PA, USA) in order to remove moisture and dry the samples for a period of 7 days at -40 °C and > 250 millitor. The dried samples were then ground into fine powder using a pestle and mortar.

For fruit that were stored in cold room, the incidence of peteca spot was scored in 3 weeks intervals for the period of 12 weeks. The calculation of PS index was done using Eq. 1, according to Cronje (2015).

$$\text{Peteca}_{\text{Index}} = \frac{\sum\{Peteca\ (0-2)\ x\ No.\ of\ fruit\ in\ each\ class\}}{Number\ of\ fruit\ in\ a\ rep} \quad 1$$

5.2.3. Vis/NIR spectral acquisition

For lemon, a reflectance mode was used to acquire the vis/NIR data with the use of a laboratory bench-top monochromator NIRSystems model XDS spectrometer (Foss NIR system, Inc, Maryland, USA). The machine was equipped with a quartz halogen lamp and lead sulfide (pbs) detector. Fruit were scanned right after harvest and then destructively analyzed. The other fruit were stored in a cold room at 3 °C in order to induce the development of peteca spot. After 12 weeks of storage, most fruit had developed the disorder. The fruit were scanned and then destructively analyzed.

For calibration of the machine, a 100% white reference tile was scanned in order to give a background reference before the scanning of fruit samples according to Magwaza et al. (2016). This was also repeated every 30 minutes into fruit scanning so as to reduce baseline shift of spectral data since the sample size was large. The spectrum was acquired at (450-2500 nm) from 4 sides of the fruit. A maximum of 32 scans were obtained for each spectrum, which were automatically averaged and recorded as log 1/reflectance.

5.2.4. Extraction and quantification of ascorbic acid

A method described by Hernández et al. (2006) with a slight modification was used for the determination of ascorbic acid. Briefly, 150 ± 0.5 mg of the powdered sample was extracted using 5 mL of 3% (w/v) aqueous metaphosphoric acid (MPA). Using a vortex, the solution was homogenized for 1 min and placed in ice cubes for 5 min. This was followed by centrifuging for 20 min using GeneVac (SP Scientific, Genevac LTD., Suffolk, UK) with the lamp off. After pipetting 0.5 mL of the extract into a test tube, 2.5 mL of 2, 6-dichloroindophenol dye (0.015 g dye in 100 mL of H_2O) was added and incubated in the dark. The reading of absorbance values of the supernatant was done at 515 nm in triplicates using spectrophotometer against 3% MPA.

The standard curve for ascorbic acid was prepared from 0.0352 g of ascorbic acid which was dissolved in 100 mL of metaphosphoric acid solution. From the stock solution, 0, 200, 400, 600; 800 and 1000 µL were poured into test tubes and the volume was made up to 1 mL using metaphosphoric acid. In each test tube, 2.5 mL of 2, 6-dichloroindophenol dye was added and mixed well. After incubating for 10 minutes in the dark, the absorbance values were read at 515 nm to give a linear standard curve with $R^2 = 0.956$. The results were expressed as g/kg.

5.2.5. Extraction and quantification of nonstructural sugars

The determination of nonstructural sugars was carried out using a method described by Magwaza et al. (2014b) with slight modifications for lemons. Briefly, 150 ± 0.5 mg of the lyophilized sample was extracted with 70% (v/v) aqueous methanol (3 mL) (v/v) and vortexed for 1 minute. The sample was incubated for 60 min in hot shaking water bath set at 55 °C as described by Terry et al. (2017), followed by centrifuging for 20 min using GenVac® (SP Scientific, Genevac LTD., Suffolk, UK). In order to obtain a clear extract, 0.4 micron nylon filter syringe was used to filter the extraction into HPLC vial for high performance liquid chromatography (HPLC) analysis.

The quantification of fructose, glucose, and sucrose was done using a Phenomenex® column (Rezex RCM - Monosaccharide) which was equipped with a refractive index detector by injecting 1 mL sample extracts into a Phenomenex- SecurityGuard™ cartridges C18 (4 x 3.0 mm) ID, 10/pk and column of 3.2 – 8.0 mm. Ultra-pure HPLC-grade water was used as mobile phase at 0.6 mL/min flow rate and the column compartment temperature set at 80 °C. The linear reading of the standard curve was from 0.05 - 2.5 mg/L of the standard sucrose, glucose and fructose with $R^2 = 0.987$. The concentration of the sugars present in each sample was determined by comparing the peak detected in the samples with peak area and concentration of the standard curve. The results were expressed as g/kg.

5.2.6. Model performance evaluation

The regression statistics of prediction models were evaluated based on the following statistical terms: Pearson correlation coefficients (R; Eq. 2) between predicted and observed reference values, root mean square error of calibration (RMSEC; Eq. 3), root mean square error of

validation or prediction (RMSEP; Eq. 4), the residual predictive deviation (RPD; Eq. 5) and number of latent variables (LVs) (Olarewaju et al., 2019). For a model to be ideal, it should have higher R and RPD values and low values of RMSEC and RMSEP.

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

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

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

$$RPD = \frac{SD}{RMSEP} \quad (5)$$

Where:

N = number of spectra

y_{act} = the actual value

y_{mean} = the mean value

y_{cal} = the calculated value

5.2.7. Chemometric data analysis

The analyses of spectra was done using unscrambler chemometric software (version 10.3 CamoSoftware, AS Norway). Partial least squares regression (PLSR) was applied to spectral data for the development of prediction models for each measured variable. The development of PLS regression model was done using the obtained spectral data and the destructively measured variables. For each parameter, a different preprocessing method including Savitzky-Golay, smoothing using reducing average, Savitzky-Golay first and second derivative were tested, which was done to correct light scatter, remove outliers and make the data more suitable for prediction according to Magwaza et al. (2012).

The outliers were measured using Hoteling T^2 outlier detection technique for the improvement of the performance of the model in cross validation before external validation was done (Olarewaju et al., 2019). After testing the pre-processing methods, the best model was obtained using Savitzky-Golay smoothing and for other parameters, preprocessing was not necessary. Principal component analysis (PCA) was applied in order to compare spectral characteristics from the two different canopy positions as well as finding correlations between parameters. The scattering noise observed from a measured full range (400 – 2500 nm) was removed from the spectra before PCA was applied so all vis/NIRS analyzed in this study was performed on wavelengths from 600 – 2100 nm using full cross validation.

In order to establish the accuracy of the model, it was scored according to RPD, which is the ratio of the standard deviation of validation reference data to root mean square error of validation or prediction (Williams and Sobering, 1996). The RPD of < 1.5 suggests that the model is unstable. 1.5 < RDP < 2.0 means the model is suitable for rough prediction, 2.0 < RPD < 2.5 is suitable for quantitative predictions, RPD > 2.5 is good and RDP > 3 is excellent.

5.3. Results and discussion

5.3.1. The incidence of peteca spot in different canopy positions

Fruit stored for the period of 12 weeks for the development of peteca spot started showing symptoms 3 weeks after cold storage with the incidence increasing with time. The results showed that the incidence of peteca spot was significantly affected by canopy position (p < 0.001). Fruit harvested from the inside canopy position showed a high susceptibility to the disorder compared to fruit in the outside canopy (Fig. 1). These results agree with findings of other researchers reporting that canopy position has a direct influence on rind quality hence the development of rind disorders (Cronje et al., 2011a, b; Magwaza et al., 2013; 2014a). The high incidence of the disorder in the shaded inside canopy has been previously associated with the concentration and allocation of nonstructural carbohydrates, which can result in fruit's premature senescence (Magwaza et al., 2014a).

The concentration of glucose, fructose and sucrose in this study were high in the outside canopy position where there was less incidence of the disorder which confirms the findings of the authors (Magwaza et al., 2014a). The difference in susceptibility of citrus fruit harvested from

different canopy positions to physiological rind disorders could allow for the selection of fruit with higher chance of developing peteca spot to be sent to local markets or used for processing, which will reduce losses caused in the international markets.

The use of vis/NIR for prediction of peteca spot incidence resulted in a poor model with R^2 = 0.26. This could be due to the discrete nature of the disorder scores, which was also reported by Magwaza et al., 2014b for rind breakdown disorder of 'Nules Clementine' mandarin. The authors explained that the poor prediction could be due to the fact that data in NIR is complex, which becomes difficult to correlate to the disorder.

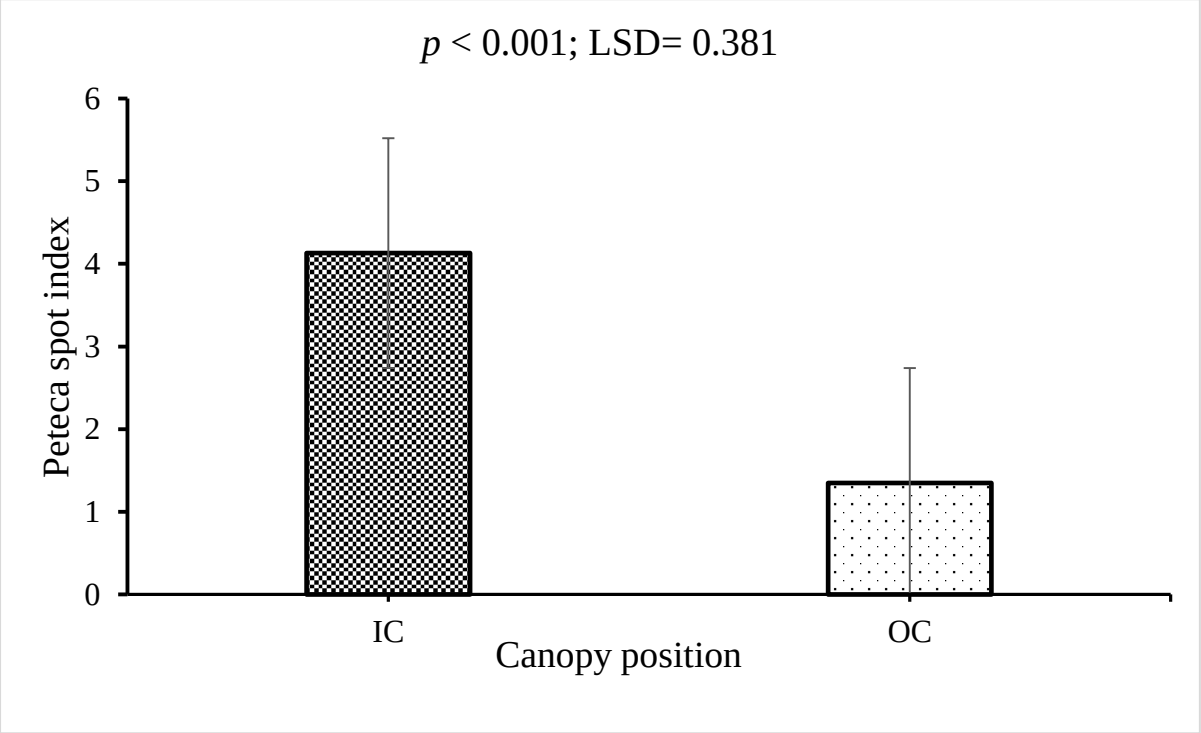


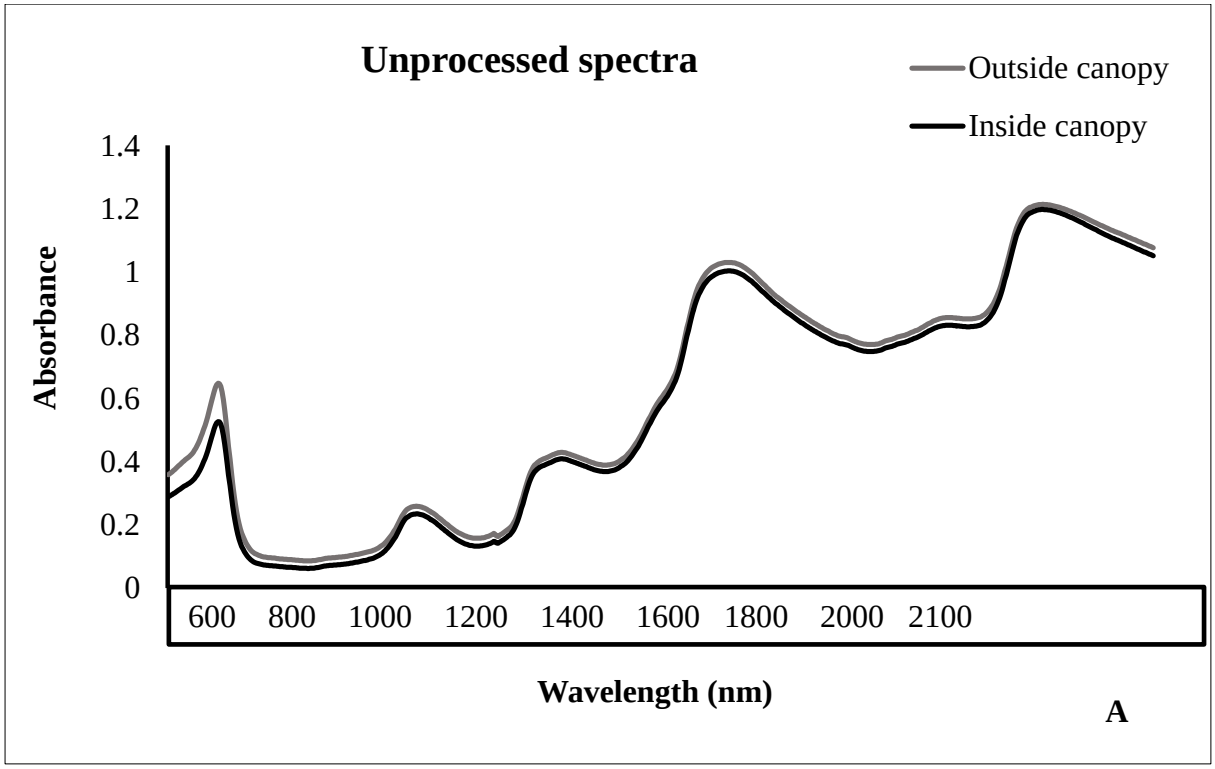
Fig. 1: The effect of canopy position on peteca spot incidence of 'Eureka' lemons after 12 weeks of cold storage at 3 °C.

5.3.2. Spectrum description

Fig. 2A shows the unprocessed typical spectra of 'Eureka' lemons from different canopy positions; inside and outside canopy represented by each line which was acquired from the FOSS NIRSystem. Some of the model development did not require any mathematical pre-treatments, however, SG (first derivative) was applied for the development of other models and the spectra is shown in Fig 2B. The spectral features in this study are similar to those obtained

by Olarewaju et al. (2019) in 'Marsh' grapefruit. Strong absorption bands were observed around 600, 1000, 1200, 1500 and 2050 nm. According to (Clément et al. 2008), absorption at these wavebands are a result of red absorbing pigments such as chlorophyll, third overtone of O-H stretching, second vibrational overtones that are of close association with H-O-H stretching, first and second overtones of C-H stretching and third overtone of O-H, C-H and C-H_2, respectively (Golic et al., 2003; Magwaza et al., 2012).

The region from 2000 nm and above has been related to the combinations of O-H, C-H and C-C stretches and vibrations, which is directly associated with carbohydrates (Tewari et al., 2008). The contribution of each variable to the model development is shown in Fig. 2B. Spectral peaks with higher absolute values of regression coefficient indicated that the contribution of the variable to the model was significant, while variables that had values closer to zero were not important to the model (Magwaza et al., 2014c).



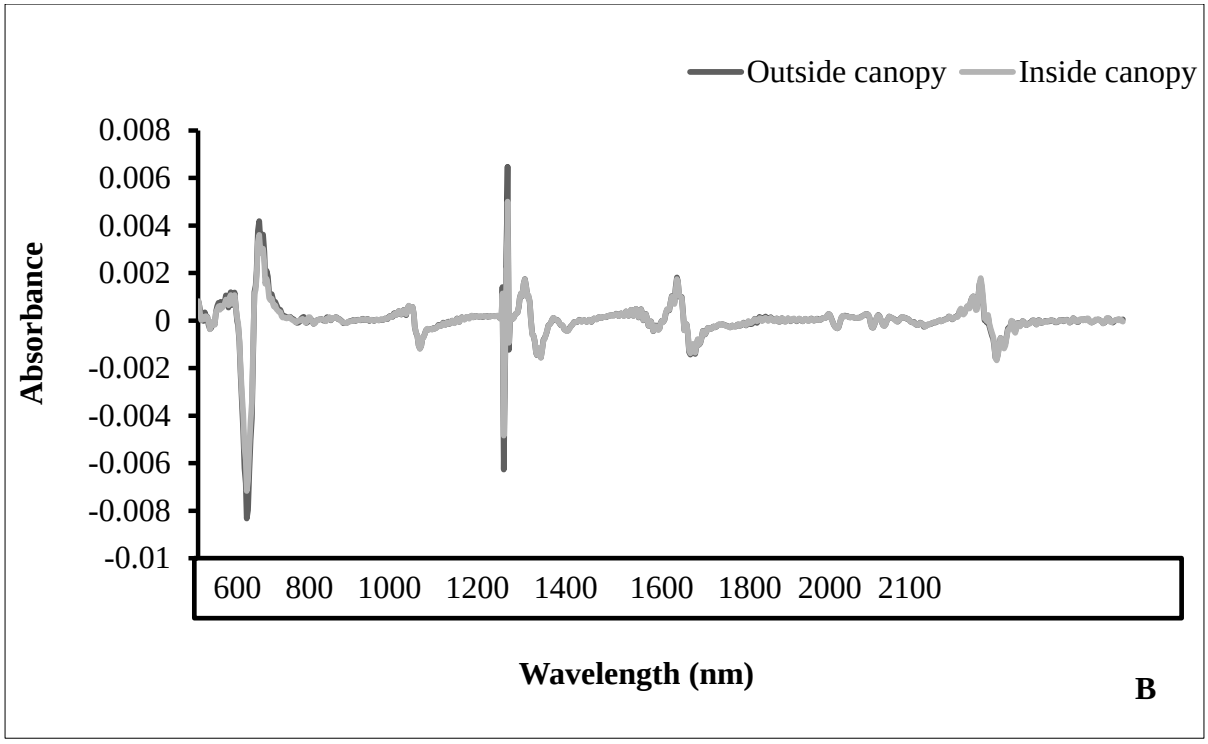


Fig. 2: Line plot for unprocessed (A) and processed (B) spectra for 'Eureka' lemons.

5.3.3. Principal component analysis

Principal component analysis was applied to understand the correlation between measured parameters and peteca spot. PC-1 and PC-2 showed 89% variability. PC-1 showed a high contribution of 73% and PC-2 contributed 16% of the total variation during mapping (Fig. 3). Variables that are located in the same plane as peteca index (PI) are positively correlated with the disorder, while those in the opposite direction show a negative correlation. Although sucrose, glucose and ascorbic acid were positively correlated to peteca spot, the correlation was not strong. Peteca spot was strongly correlated to fructose and TSS. This means that these variables were directly involved in the development of the disorder and this can also be seen by the low concentrations of fructose, TSS, sucrose, glucose and ascorbic acid in fruit harvested from the inside canopy of the tree, where the incidence of the disorder was high.

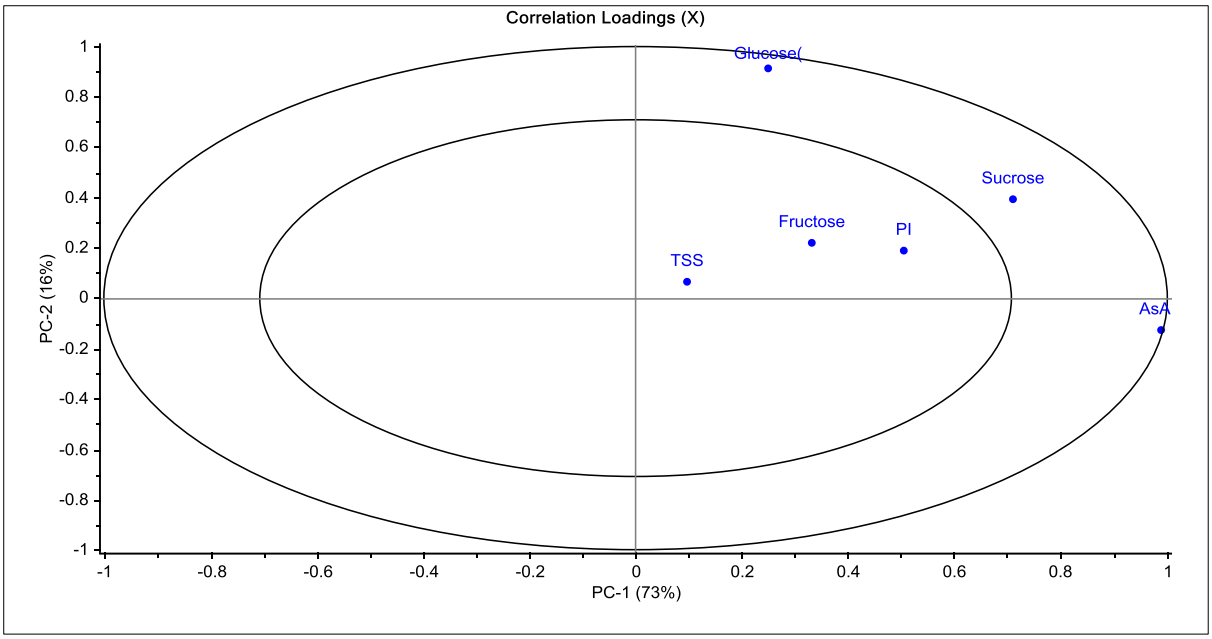


Fig. 3: Principal component analysis (PCA) plot determined by two principle components (PC-1 and PC-2) showing a correlation between measured variables and the susceptibility of 'Eureka; lemon to peteca spot. TSS= total soluble solutes; AsA= ascorbic acid; PI= peteca index.

5.3.4. PLS prediction models based on Vis/NIR

Table 1 shows statistical analysis for PLS calibration and validation models. The best prediction in terms of R-value was seen for ascorbic acid $R^2 = 0.86$ followed by sucrose $R^2 = 0.81$, TSS $R^2 = 0.78$; glucose $R^2 = 0.77$ and fructose $R^2 = 0.70$ with corresponding RMSEP values of 1.74 g/kg, 1.41 g/kg, 2.60 g/kg, 0.37% and 2.85 g/kg, respectively. Even though the correlation between predicted and reference values could be high, Saeys et al. (2005) suggested that the accuracy of the model be tested using RPD. Sucrose and glucose had high RPD values of 2.5 and 3.2, respectively compared to the other parameters, which is an indication of a good model according to Williams and Sobering (1996). TSS and AsA had RPD values of 2.3 and 2.2, respectively meaning the models are suitable for quantitative predictions, while the model for fructose was suitable for rough prediction with RPD = 1.7.

Different wavelength ranges were tested for each physico-chemical parameter in order to obtain the best model. Selection was done based on the observed peak, areas that resulted in too much noise in the peak were removed and the development of calibration models was done based on wavelength bands that gave the highest R^2, lowest RMSEC and RMSEP. The best

and improved model for TSS and AsA was obtained from wavelengths between (850 – 1200 nm) with the application of SG (first derivative) with second polynomial order. The models for sucrose, glucose and fructose did not require any preprocessing and were developed using wavelength range between (900 -1600 nm). Comparable results were reported in 'Nules Clementine' mandarin for sucrose, glucose and fructose, which had better model development at wavelengths between (900 -1800 nm) (Magwaza et al., 2012). Tewari et al. (2008) also reported wavelengths between 350-1800 nm as best for sucrose, glucose and fructose prediction.

Table 1: Model performance for 'Eureka' acquired from FOSS NIRSystem in reflectance mode.

			Calibration			Validation				
Parameter (g/kg)	Pre-processing	LV	R^2	RMSEC	Slope	R^2	RMSECV	Slope	RPD	Spectral range
TSS	SG (Smoothing)	17	0.77	0.36	0.73	0.78	0.37	0.72	2.3	850 - 1200 nm
Sucrose	None	18	0.84	1.34	0.82	0.81	1.41	0.81	2.5	900 - 1600 nm
Glucose	None	18	0.79	5.09	0.71	0.77	2.60	0.68	3.2	900 - 1600 nm
Fructose	None	19	0.71	2.68	0.69	0.70	2.85	0.67	1.7	900 - 1600 nm
AsA	SG (Smoothing)	9	0.87	1.86	0.71	0.86	1.74	0.65	2.2	850 - 1200 nm

Table 2: Descriptive statistical analysis for calibration and validation sets for biochemical properties of 'Eureka' lemons in the outside canopy (above) and inside canopy (below) positions. SD= standard deviation, max= maximum, min= minimum, CV= coefficient of variation.

Parameter	Calibration set						Validation set					
	Mean (g/kg)	SD	Max (g/kg)	Min (g/kg)	Range (g/kg)	CV (%)	Mean (g/kg)	SD	Max (g/kg)	Min (g/kg)	Range (g/kg)	CV (%)
TSS	6.41	0.47	7.60	5.26	2.34	7.33	6.81	0.83	7.44	6.21	1.23	4.84
AsA	2.86	0.90	4.96	1.02	3.94	31.47	2.81	3.8	4.12	0.98	3.14	27.05
Sucrose	54.47	1.71	59.28	51.51	7.78	3.14	54.04	3.51	60.02	51.94	8.07	3.07
Glucose	75.79	5.39	90.37	63.95	26.42	7.11	73.15	2.62	93.15	63.14	30.01	9.52
Fructose	48.32	3.51	56.36	41.86	14.51	7.26	45.32	4.90	52.17	42.07	10.10	5.66

Parameter	Calibration set						Validation set					
	Mean (g/kg)	SD	Max (g/kg)	Min (g/kg)	Range (g/kg)	CV (%)	Mean (g/kg)	SD	Max (g/kg)	Min (g/kg)	Range (g/kg)	CV (%)
TSS	5.43	0.33	6.25	4.58	1.67	6.12	5.47	0.39	6.44	4.76	1.68	7.09
AsA	1.25	0.56	2.95	0.45	2.50	44.8	1.34	0.62	2.92	0.27	2.65	46.27
Sucrose	48.84	1.18	51.49	46.35	5.14	2.42	48.25	0.95	50.24	46.66	3.58	1.97
Glucose	59.38	4.37	69.62	52.37	17.26	7.35	60.09	5.26	70.75	52.47	18.28	8.75
Fructose	40.14	3.16	50.23	36.86	13.36	7.88	40.74	2.58	45.99	36.57	9.42	6.32

5.3.5. Determination of rind physico-chemical properties using PLS regression

Non-structural carbohydrates measured in this study (sucrose, glucose and fructose) were significantly affected by canopy position (Table 2). Fruit harvested from the outside canopy had more sugars compared to fruit in the inside canopy. The most abundant sugar in 'Eureka' lemons was fructose (75.79 ± 5.39 g/kg) followed by sucrose (54.47 ± 1.71 g/kg). The least abundant sugar was fructose with the mean value of (48.32 ± 3.51 g/kg) observed in the outside canopy position. The relationship between carbohydrates and the occurrence of rind disorders has been reported by Cronje et al. (2011a). Carbohydrates have been linked to rind breakdown disorder in 'Nules Clementine' mandarin (Magwaza et al., 2012, 2013). Fruit that had high incidence of the disorder had a reduced concentration of carbohydrates.

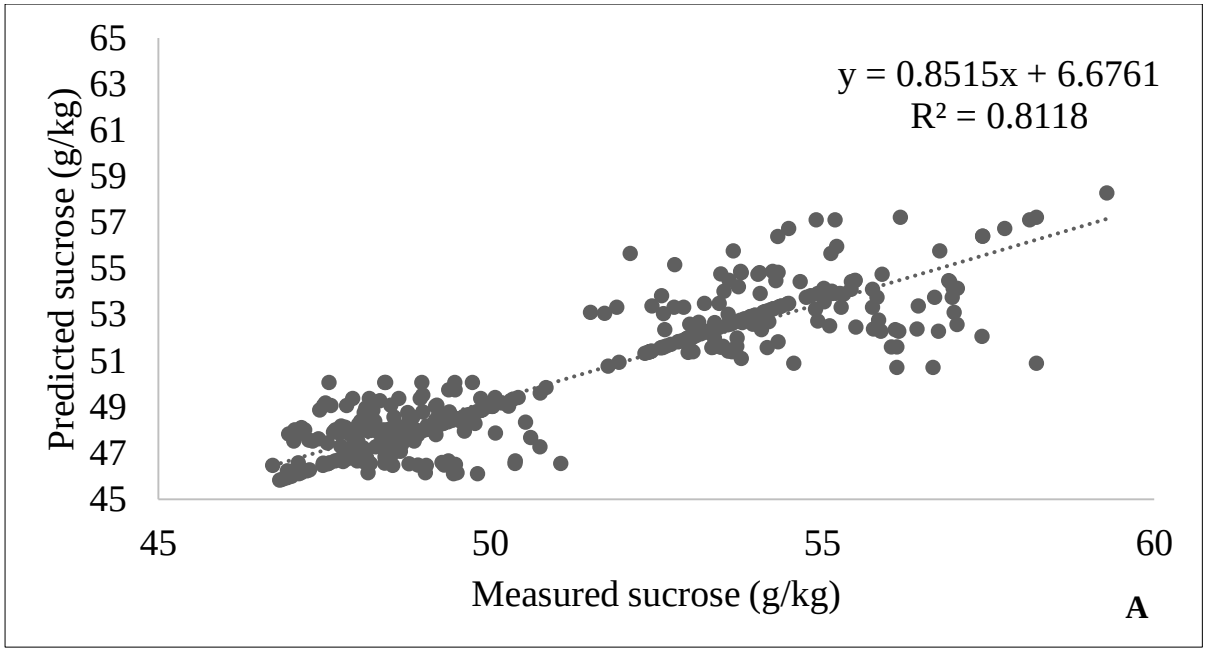
This is because carbohydrates are known to play a protective role against stress conditions that can lead to the development of rind disorders by maintaining osmotic balance, membrane and protect stabilization (Gupta and Huang, 2014). The ability of plants to obtain chilling tolerance has been linked to the accumulation of carbohydrates (King et al., 1988). Gupta and Huang (2014) explained that an increase in sucrose concentration could decrease the incidence of rind physiological disorders because sucrose has the ability to balance abscisic acid deficiency that supports harsh conditions that could lead to fruit dehydration.

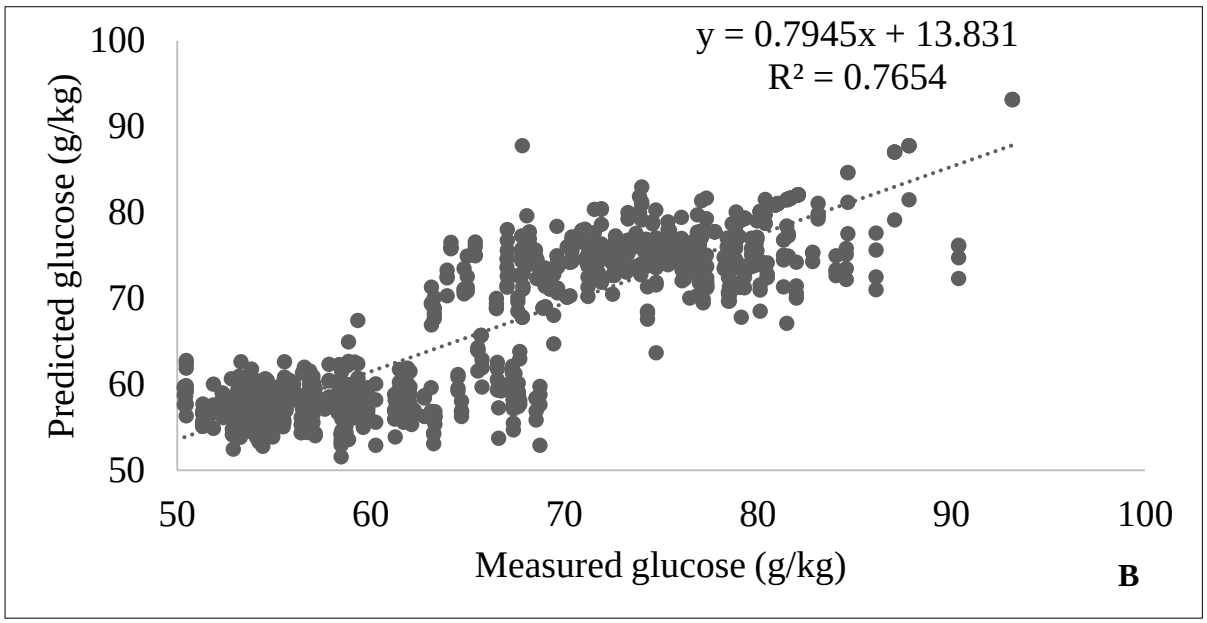
The increased incidence of peteca spot in fruit harvested from the inside canopy can therefore be related to the low glucose, sucrose and fructose observed in the inside canopy. This suggests that for outside canopy fruit, which were less susceptible to the disorder, the concentration of the sugars was abundant enough to protect the fruit from the development of the disorder. Vis/NIRS was able to predict sucrose, glucose and fructose. The reference values for sucrose ranged from 51.51 – 59.28 g/kg and predicted values ranging from 51.94 – 60.02 g/kg. For glucose, reference values ranged from 63.95 – 90.37 g/kg and 63.14 – 93.15 g/kg for prediction while glucose had values of 41.8 – 56.36 reference and 42.07 – 52.17 predicted (Fig. 4).

The reference values obtained for TSS from destructive analysis ranged from a minimum of 4.39 to a maximum of 8.1% while predicted values from NIR spectroscopy were in the range of 4.85 to 7.44% (Fig. 5). The PLS model for the determination of AsA was developed using SG (first derivative) with second polynomial order which improved the model from $R^2 = 0.75$ to $R^2 = 0.86$. The values for AsA content determined by destructive analysis ranged from a

minimum of 1.02 to maximum of 4.96 g/kg and those predicted from NIR ranged from 0.98 to 4.12 g/kg (Fig. 6). The results obtained from both inside and outside canopy positions are shown in Table 2.

The concentration of ascorbic acid was affected by the position of the fruit on the tree. The sunexposed fruit from the outside canopy had high AsA compared to fruit harvested from the shaded inside canopy position. This is because light is important for the up-regulation of the ascorbate glutathione cycle which is an important pathway for the recycling of ascorbic acid (Valpaesta and Bottella, 2004). Reddy et al. (2016) reported that the best model for predicting AsA in lemon was developed from first derivative spectral preprocessing technique which gave a correlation coefficient of regression $R^2 = 0.80$, which agrees with present findings.


y = 0.8515x + 6.6761
R² = 0.8118
Predicted sucrose (g/kg)
65
63
61
59
57
55
53
51
49
47
45
45
50
55
60
Measured sucrose (g/kg)
A


y = 0.7945x + 13.831
R² = 0.7654
Predicted glucose (g/kg)
100
90
80
70
60
50
50
60
70
80
90
100
Measured glucose (g/kg)
B

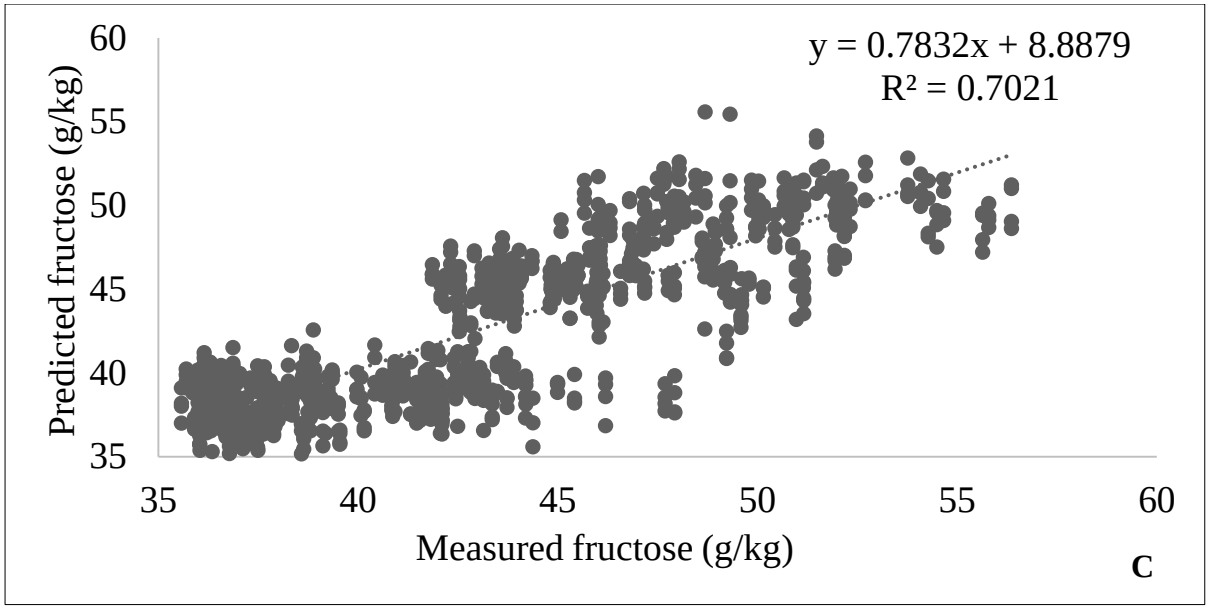


Fig. 4: Scatter plots of destructively measured nonstructural carbohydrates, sucrose (A), glucose (B) and fructose (C) against Vis/NIRS predicted sugars of 'Eureka' lemons in fruit harvested from inside and outside canopy positions.

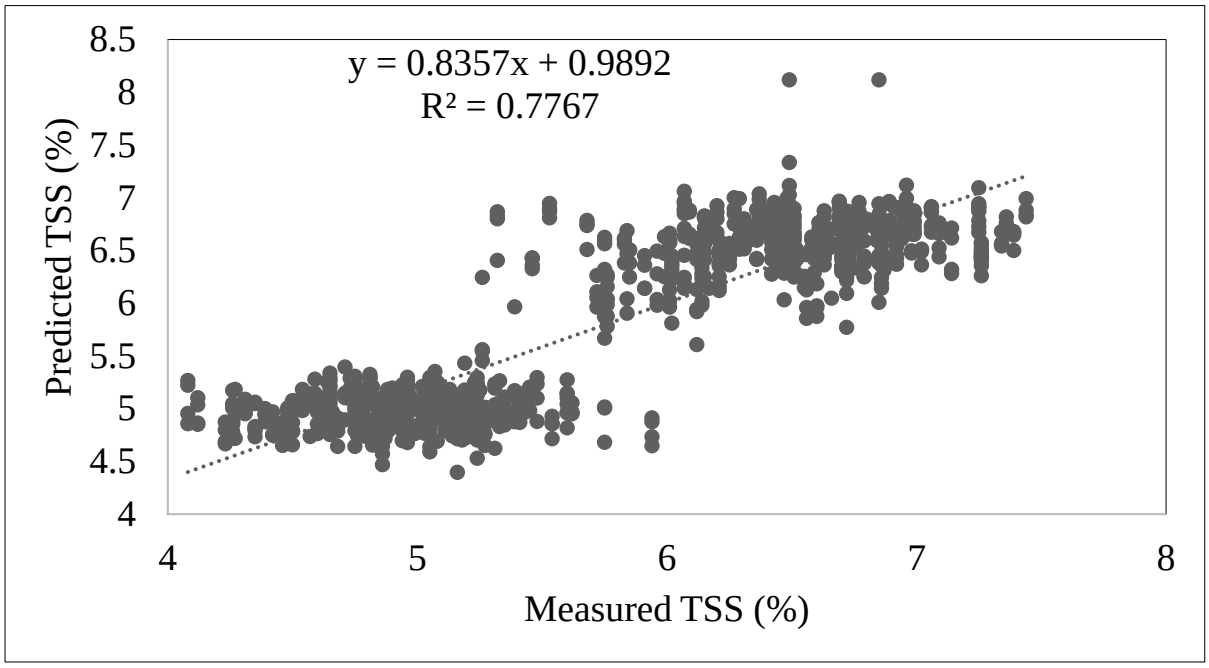


Fig. 5: Scatter plots of destructively measured TSS against Vis/NIRS predicted TSS of 'Eureka' lemons in fruit harvested from inside and outside canopy positions.

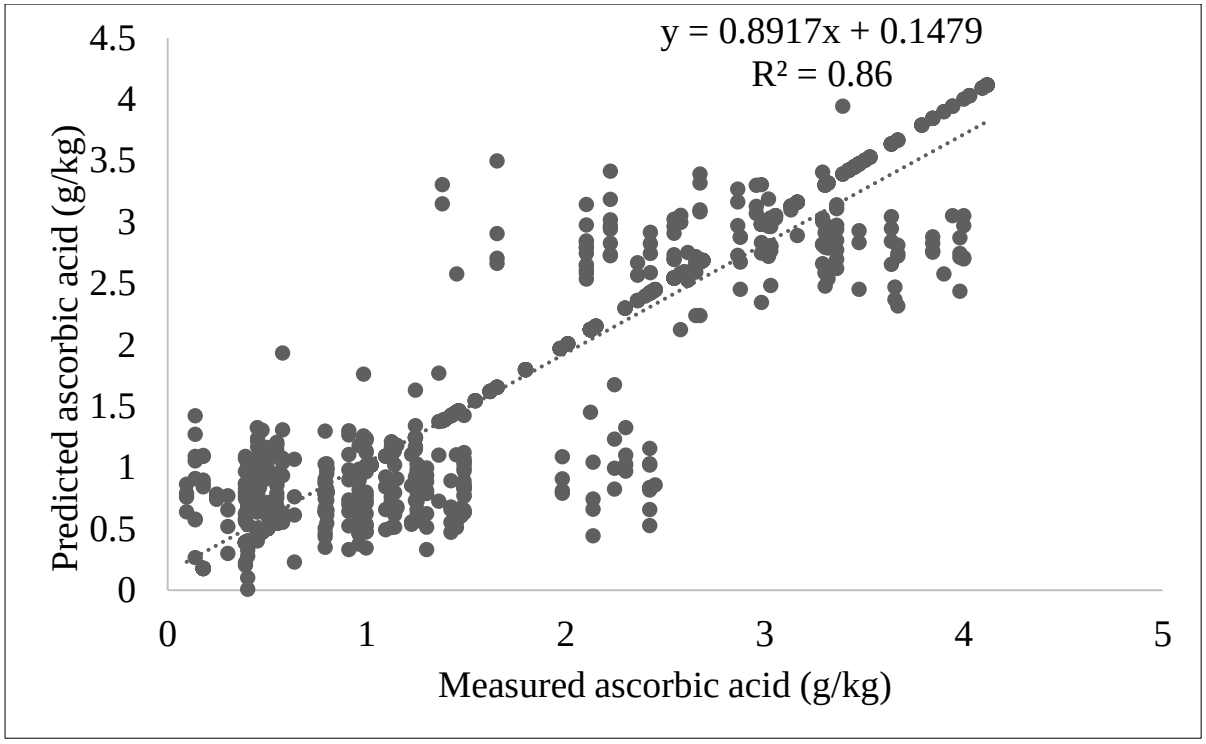


Fig. 6: Scatter plots of destructively measured AsA against AsA predicted from Vis/NIRS of 'Eureka' lemons in fruit harvested from inside and outside canopy positions.

5.4. Conclusion

The obtained results demonstrated that Vis/NIR spectroscopy in reflectance mode combined with appropriate pre-processing techniques and PLS regression is a good and reliable technique to determine rind biochemical properties of 'Eureka' lemons. Although peteca spot incidence was difficult to predict due to the discrete nature of the disorder, the positive correlation of physico-chemical attributes and peteca spot index suggests that accurately predicting these parameters could be related to the development of the disorder since these parameters have been previously reported as potential presymptomatic biochemical markers. From the results obtained, the concentration of sucrose, glucose and fructose, TSS and AsA can be predicted with R^2 values of 0.81, 0.77, 0.70, 0.78 and 0.86, respectively and root mean square errors of 1.41 g/kg, 5.37 g/kg, 2.85 g/kg, 0.37% Brix, and 1.74 g/kg, respectively. The high residual predictive deviation (RPD) values, indicating a high level of accuracy for the model was obtained for sucrose and glucose. The models for sucrose, glucose and fructose were developed without the use of any preprocessing method which means that spectral pre-processing algorithm is not always necessary for the development of a good model.

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Chapter 6: Overall discussions and conclusions

6.1. Literature review

The objective of this study was to non-destructively determine pre-symptomatic biochemical markers related to peteca spot of 'Eureka' lemons and to evaluate the efficacy of edible coatings on reducing the incidence of the disorder. The first chapter focused on general background introducing the postharvest rind physiological disorder, peteca spot (PS), the economic impact it has on South African citrus industry and factors that are related to the disorder occurrence in 'Eureka' lemons. A critical review of literature showed the possibility of using non-structural carbohydrates, ascorbic acid (AsA), TSS, TA as presymptomatic markers that are related to rind physiological disorders in citrus fruit (Ncama et al., 2018a; Magwaza et al., 2013).

As stated in the literature, the determination of these biochemical markers is time consuming and labor intensive hence researchers have introduced the use of visible to near infrared spectroscopy (vis/NIRS) to non-destructively predict and monitor postharvest losses during storage. NIR has been identified as the best method because of its chemometrics (Nicolai et al., 2007; Magwaza et al., 2014a, b). This is also because it allows for the measurement of large sample size at the same time and it involves non-destructive measurements. Previous uses for NIR was to predict sweetness and internal and external parameters of fruit (Piers et al., 2002; Sánchez et al., 2013) but the focus has shifted to its use in predicting rind physiological disorders (Ncama et al., 2018b, Olarewaju et al., 2019).

Vis/NIRS was found to accurately predict rind pitting disorder in 'Marsh' grapefruit (Ncama et al., 2018b), it was then hypothesized that it could be used to predict peteca spot in lemon. Rind physiological disorders of citrus fruit and factors that affect the susceptibility were discussed and it was seen that these factors play a direct role in the development of physiological disorders. In order to reduce postharvest losses in citrus packhouses, fruit are coated with synthetic waxes but there's a shift to the use of edible coatings because synthetic waxes have been reported to cause health and environmental issues.

The review of literature also indicated the wide use of edible coatings in reducing postharvest losses of citrus fruit. Most of previous uses for edible coatings were aimed at improving quality and extending shelflife and studies that focus on the use of edible coatings on reducing the

incidence of physiological rind disorders in citrus fruit, especially peteca spot in 'Eureka' lemons are limited. A wide range of edible coatings have been identified and are used in packhouses but what has been identified to dominate the industry is moringa (M), carboxymethyl cellulose (CMC) and chitosan (CH) (Tesfay and Magwaza, 2017). These coatings were found successful in reducing mass loss, and ascorbic acid loss in citrus fruit (Arnon et al., 2014), however, what motivated this study is the gap that was found in evaluating these edible coatings in reducing the susceptibility of 'Eureka' lemons to peteca spot.

6.2. Determination of pre-symptomatic biochemical markers related to peteca spot in 'Eureka' lemons

Physico-chemical parameters that have been identified in the review as potential markers for predicting the incidence of rind physiological disorders were evaluated in this study. Parameters such as colour, ascorbic acid, total carotenoids, non-structural carbohydrates, TSS and TA were measured. Principal component analysis (PCA) was performed in order to observe the correlation of physicochemical parameters with peteca spot incidence. Peteca spot was positively correlated with sucrose, colour parameters, and TA. A strong positive correlation between peteca spot and sucrose suggested that sucrose had a direct influence on the susceptibility fruit to peteca spot development. One of the important factors that was evaluated is canopy position because it was also found to have an influence on the development of rind physiological disorders in citrus fruit.

A high susceptibility of peteca spot was observed in fruit harvested from the inside canopy position compared to fruit from the outside canopy. Briefly, this was explained to be due to high sunlight exposure of the fruit in the outside canopy which increases the rate of metabolism and synthesis of rind parameters (Cronje et al., 2011). PCA was able to separate fruit from the inside and outside canopy which could allow for fruit classification according to their susceptibility to peteca spot development. Since fruit from the inside canopy had high susceptibility to peteca spot, they could be sent to local markers or used for processing in order to avoid cold storage that could lead to the development of the disorder. Both positive and negative correlation between physico-chemical parameters proved the possibility of using these variables as presymptomatic markers that are related to peteca spot. However, it also showed that a wide variety of internal and external factors could lead to fruit's susceptibility to the disorder, therefore more studies investigating these factors is recommended.

6.3. Evaluating the efficacy of Chitosan and CMC incorporated with Moringa leaf extracts on reducing peteca spot incidence on 'Eureka' lemons

Due to the increasing health and environmental concerns relating to the use of synthetic waxes on reducing the incidence of rind physiological disorders in citrus fruit, the efficacy of CMC and CH incorporated with moringa leaf extracts edible coatings was evaluated. Fruit were assigned to 5 treatments; control, M+CMC, CMC, CH, and M+CH. The effectiveness of these coating treatments in reducing peteca spot was evaluated in both inside and outside canopy position. The findings in the study showed that fruit coated with M+CMC, CMC and CH were less susceptible to the development of peteca spot in both inside and outside canopy positions while control and fruit coated with M+CH showed a high incidence of the disorder in the inside canopy position.

The ability to use M+CMC, CMC, CH coatings in postharvest handling for reducing the incidence of peteca spot was demonstrated in this study. These coatings were also able to delay colour change of fruit which resulted in less incidence of the disorder since yellow fruit have been reported to be more susceptible to peteca spot compared to green fruit (Undurraga et al., 2009). Furthermore, edible coatings reduced mass loss and ascorbic acid loss. The most effective coating treatment, which is recommended for commercial use is moringa incorporated with carboxymethyl cellulose (M+CMC) followed by pure CMC and pure CH. Further studies evaluating these edible coating treatments in other citrus fruit should also be considered. Since fruit coated with M+CH showed high susceptibility to peteca spot, the coating was therefore not recommended.

6.4. The use of vis/NIR spectroscopy for non-destructive prediction of peteca spot in 'Eureka' lemons

Presymptomatic biochemical markers that have been identified in chapter 3 were further evaluated using non-destructive measurements. The use of Vis/NIR spectroscopy in reflectance mode with PLS regression was found to be a good and reliable technique that was able to determine rind biochemical properties of 'Eureka' lemons. PCA was used to find correlations between sucrose, glucose, fructose, TSS, AsA and the development of peteca spot. These parameters were positively correlated with the peteca spot, however, the prediction of peteca spot was poor due to the nature of the disorder. The ability to accurately predict these

parameters which are related to peteca spot suggests that the models could be used for relating the parameters to the disorder. However, means to accurately predict peteca spot should be further investigated. This could be done by increasing sample size and using fruit from different locations. The ability to accurately predict rind pitting disorder was explored in 'Marsh' grapefruit (Ncama et al., 2018b), which proves the possibility of accurately predicting peteca spot.

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