EFFECT OF CANOPY POSITION AND NON-DESTRUCTIVE DETERMINATION OF RIND BIOCHEMICAL PROPERTIES OF CITRUS FRUIT DURING POSTHARVEST NON-CHILLING COLD STORAGE

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FOREWORD

The research contained in this thesis was completed by the candidate while based in the Discipline of Horticultural Sciences, School of Agricultural, Earth and Environmental Sciences of the College of Agriculture, Engineering and Science, University of KwaZulu-Natal, Pietermaritzburg, South Africa. The research was made possible by the funding received from National Research Foundation and Citrus Research International through Postharvest Innovation Programme (PHI 2/2014).

The contents of this work have not been submitted in any form to another university and, except where the work of others is acknowledged in the text, the results reported are due to investigations by the candidate.

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I, Olarewaju Olaoluwa Omoniyi, declare that:

1) The research reported in this dissertation, except where otherwise indicated or acknowledged, is my original work;
2) This dissertation has not been submitted in full or in part for any degree or examination to any other university;
3) This dissertation does not contain other persons’ data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons;
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5) Where I have used material for which publications followed, I have indicated in detail my role in the work;
6) This dissertation is primarily a collection of material, prepared by myself, published as journal articles or presented as a poster and oral presentations at conferences. In some cases, additional material has been included;
7) This dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.
8) The phytohormone analysis aspect of this study (Chapter 6) is a collaborative work with Professor Karel Dolezal of Olomouc University, Czech Republic. Samples were sent to Olomouc and analysis was carried out by his group.

_______________________
Signed: Olaoluwa Omoniyi Olarewaju

Date:
The research reported in these presentations is based on experiments conducted in 2015 and/or 2016 using fruit collected from farms in Eastern Cape, KwaZulu-Natal, Limpopo, or Mpumalanga provinces of South Africa. I designed the experiments, collected, analysed the data, and did the presentation at the indicated conferences. The * indicates corresponding author.


DECLARATION 3—PUBLICATIONS

The research reported in these publications is based on experiments conducted in 2015 and/or 2016 using fruit collected from farms in Eastern Cape, KwaZulu-Natal, Limpopo, and/or Mpumalanga provinces of South Africa. I designed the experiments, collected, analysed the data, and wrote the first draft of each chapter (or paper where publication has been successful). The * indicates corresponding author.


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DEDICATION

My father
My mother
My wife
My son
My siblings
My friends
This thesis is a compilation of manuscripts where individual chapter is an independent article/manuscript introduced disjointedly. Hence, some repetition between individual chapters has been inevitable. Each chapter, except where stated otherwise, is formatted to the requirements of Elsevier BV Publishers of Scientia Horticulturae.
# TABLE OF CONTENTS

FOREWORD................................................................................................................................. I
DECLARATION 1- PLAGIARISM ..................................................................................................... II
DECLARATION 2- CONFERENCE PRESENTATIONS ..................................................................... III
DECLARATION 3- PUBLICATIONS ................................................................................................ IV
ACKNOWLEDGEMENTS ................................................................................................................. V
DEDICATION .................................................................................................................................... VII
PREFACE ........................................................................................................................................ VIII
TABLE OF CONTENTS .................................................................................................................. IX
LIST OF FIGURES ............................................................................................................................. X
LIST OF TABLES ............................................................................................................................ XIX
OVERVIEW ...................................................................................................................................... XXI
CHAPTER 1 ...................................................................................................................................... 1
1. GENERAL INTRODUCTION AND RESEARCH AIMS .......................................................... 1
   1. INTRODUCTION .................................................................................................................. 1
   2. RESEARCH HYPOTHESIS ................................................................................................. 4
   3. RESEARCH AIMS AND OBJECTIVES ............................................................................. 4
REFERENCES ............................................................................................................................... 6
CHAPTER 2 ..................................................................................................................................... 8
CAUSES AND NON-DESTRUCTIVE METHODS FOR DETECTING PHYSIOLOGICAL RIND DISORDERS ASSOCIATED WITH NON-CHILLING COLD STORAGE IN CITRUS FRUIT – A REVIEW ................................................................. 8
ABSTRACT .................................................................................................................................... 8
   1. INTRODUCTION ................................................................................................................ 9
   2. ANATOMY OF CITRUS FRUIT RIND ............................................................................... 11
      2.1. RIND QUALITY OF CITRUS FRUIT .............................................................................. 12
      2.2. PHYSIOLOGICAL RIND DISORDER ........................................................................... 13
   3. NON-CHILLING PHYSIOLOGICAL RIND DISORDERS OF CITRUS FRUIT ..................... 14
      3.1. RIND BREAKDOWN/PEEL PITTING .......................................................................... 15
      3.2. OLEOCELLOSIS ............................................................................................................ 16
      3.3. NECROSIS .................................................................................................................... 17
      3.4. STEM-END RIND BREAKDOWN (SERB) ..................................................................... 18
   4. CAUSES OF NON-CHILLING PHYSIOLOGICAL RIND DISORDERS ................................. 18
      4.1. PREHARVEST FACTORS THAT IMPACT CITRUS RIND QUALITY ......................... 19
         4.1.1. SCION AND ROOTSTOCK CULTIVAR ...................................................................... 19
         4.1.2. CANOPY POSITION .............................................................................................. 20
         4.1.3. MINERAL NUTRITION .......................................................................................... 22
         4.1.4. FRUIT MATURITY .................................................................................................. 23
      4.2. POSTHARVEST FACTORS THAT IMPACT CITRUS RIND QUALITY ......................... 24
   5. RIND BIOCHEMICAL PROPERTIES OF CITRUS FRUIT: POTENTIAL USE AS BIOMARKERS .......................................................................................................................... 24
      5.1. NON-STRUCTURAL CARBOHYDRATES ...................................................................... 25
2. MATERIALS AND METHODS ........................................................................................................ 112
  2.1. REAGENTS AND STANDARDS .............................................................................................. 112
  2.2. PLANT MATERIALS ............................................................................................................... 113
  2.3 RIND COLOUR MEASUREMENT ............................................................................................ 113
  2.4. SAMPLE PREPARATION ........................................................................................................ 114
  2.5. DETERMINATION OF TOTAL CAROTENOID CONTENT ....................................................... 114
  2.6. EXTRACTION AND DETERMINATION OF RIND SOLUBLE SUGARS ............................... 115
  2.7. SAMPLE EXTRACTION FOR TOTAL PHENOLICS AND FLAVONOIDS ANALYSES ........... 116
  2.7.1 DETERMINATION OF TOTAL PHENOLIC CONCENTRATIONS .................................... 116
  2.7.2. DETERMINATION OF TOTAL FLAVONOIDS CONCENTRATIONS .............................. 117
  2.8. SAMPLE EXTRACTION FOR DETERMINATION OF VITAMIN C AND RADICAL-SCAVENGING ACTIVITIES ................................................................. 117
  2.8.1. DETERMINATION OF VITAMIN C ................................................................................. 117
  2.8.2. DETERMINATION OF RADICAL-SCAVENGING ACTIVITIES ...................................... 118
  2.9. STATISTICAL ANALYSIS ...................................................................................................... 118
3. RESULTS AND DISCUSSION ...................................................................................................... 119
  3.1. EFFECT OF CANOPY POSITION ON RIND COLOUR AND PIGMENTS .............................. 119
  3.2. EFFECT OF CANOPY POSITION ON NON-STRUCTURAL CARBOHYDRATES ................... 120
  3.3. EFFECT OF CANOPY POSITION ON PHYTOCHEMICALS, VITAMIN C AND RADICAL-SCAVENGING ACTIVITIES ................................................................. 122
  3.4. CORRELATION ANALYSES .................................................................................................. 124
4. CONCLUSION .............................................................................................................................. 125
ACKNOWLEDGEMENTS ................................................................................................................. 126
REFERENCES ..................................................................................................................................... 127
CHAPTER 5 ...................................................................................................................................... 139
ROLE OF CANOPY POSITIONS ON RIND BIOCHEMICAL CONCENTRATIONS AND RADICAL-SCAVENGING ACTIVITIES OF ‘NULES CLEMENTINE’ MANDARINS DURING NON-CHALING POSTHARVEST COLD STORAGE* .......................................................................................................................... 139
ABSTRACT ...................................................................................................................................... 140
1. INTRODUCTION ......................................................................................................................... 141
2. MATERIALS AND METHODS ...................................................................................................... 144
  2.1. REAGENTS AND STANDARDS ............................................................................................ 144
  2.2. PLANT MATERIALS ............................................................................................................ 144
  2.3 RIND COLOUR MEASUREMENT ........................................................................................... 145
  2.4. SAMPLE PREPARATION ...................................................................................................... 146
  2.5. DETERMINATION OF TOTAL CAROTENOID CONTENT .................................................... 146
  2.6. EXTRACTION AND QUANTIFICATION OF RIND SOLUBLE SUGARS ............................... 147
  2.7. SAMPLE EXTRACTION FOR TOTAL PHENOLIC AND TOTAL FLAVONOIDS CONCENTRATIONS ................................................................. 147
  2.7.1 DETERMINATION OF TOTAL PHENOLIC CONCENTRATIONS .................................... 148
  2.7.2 DETERMINATION OF TOTAL FLAVONOIDS CONCENTRATIONS .................................. 148
  2.8. EXTRACTION AND DETERMINATION OF DPPH RADICAL-SCAVENGING ACTIVITIES ................................................................. 148
  2.9. STATISTICAL ANALYSIS .................................................................................................... 149
3. RESULTS AND DISCUSSION ...................................................................................................... 150
  3.1. EFFECT OF CANOPY POSITION ON TOTAL CAROTENOIDS CONCENTRATION AND RIND COLOUR ................................................................. 150
LIST OF FIGURES

Chapter 2

Figure 1: Anatomy of citrus rind.................................................................76
Figure 2: Visual (A) and microscopic (B) images of rind breakdown (RBD).................77
Figure 3: Rind staining or peel pitting at non-chilling temperature in ‘Navelate’ (A) and ‘Navelina’ (B) oranges .................................................................77
Figure 4: Oleocellosis in ‘Washington Navel’ orange ............................................78
Figure 5: Superficial flavedo necrosis blemish known as ‘Noxan’ in ‘Shamouti’ oranges ........78
Figure 6: Stem-end breakdown of ‘Marsh’ grapefruit (A) and ‘Valencia’ orange (B)...........79
Figure 7: A schematic depiction of chlorophylls a and b having Mg atom at the centre (A), and β-carotene (B) ........................................................................80
Figure 8: A Schematic flow chart of the carotenoid biosynthesis pathway in plants ...............81
Figure 9: A schematic set-up of a typical computer vision system (A), hyperspectral imaging system (B) and multispectral imaging system (C) .................................................................82
Figure 10: A schematic representation set-up of a typical X-ray imaging (A), X-ray computed tomography (B) systems and optical coherence tomography system (C)..................83

Chapter 3

Figure 1: Rainfall (A), relative humidity (B), maximum temperature and minimum temperature (C) registered during 2015 and 2016 seasons in KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces.  ....................................................................................................................103
Figure 2: Effect of canopy position (inside canopy (IC) and outside canopy (OC)), on luminosity (L*) (A), greenness (a*) (B), yellowness (b*) (C) and colour index (D) of ‘Marsh’ grapefruit from KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9). ....................................................................................................................104
Figure 3: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on total soluble solids (A), titratable acidity (B), total soluble solids/titratable acidity (C) and BrimA (D) of ‘Marsh’ grapefruit from KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9). ....................................................................................................................105
Figure 4: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on fructose (A), glucose (B) and sucrose (C) of ‘Marsh’ grapefruit juice content from KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).........106

Figure 5: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on sweetness index (A) and total sweetness index (B) of ‘Marsh’ grapefruit from KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).................................107

Chapter 4

Figure 1: Rainfall (A), relative humidity (B), maximum temperature and minimum temperature (C) registered during 2015 and 2016 seasons in KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces. ............................................................................................................................................................................103

Figure 2: Effect of canopy position (inside canopy (IC) and outside canopy (OC)), on luminosity (L*) (A), greenness (a*) (B), yellowness (b*) (C) and colour index (D) of ‘Marsh’ grapefruit from KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9)..........................................................................................................................104

Figure 3: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on total soluble solids (A), titratable acidity (B), total soluble solids/titratable acidity (C) and BrimA (D) of ‘Marsh’ grapefruit from KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9)........................................................................................................................................105

Figure 4: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on fructose (A), glucose (B) and sucrose (C) of ‘Marsh’ grapefruit juice content from KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9)...........106

Figure 5: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on sweetness index (A) and total sweetness index (B) of ‘Marsh’ grapefruit from KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).................................107

Chapter 5

Figure 1: Rainfall (A), relative humidity (B), maximum temperature (C) and minimum temperature registered during the growing seasons in Eastern Cape (EC) and Western Cape (WC) provinces. ...167
Figure 2: Effect of canopy position (inside canopy (IC) and outside canopy (OC)), on rind total carotenoid (A) and hue angle (B) content of ‘Nules Clementine’ mandarin from Eastern Cape (EC) and Western Cape (WC) provinces harvested over two seasons during postharvest non-chilling storage (Weeks 0, 3, 6 and 9). ................................................................. 168

Figure 3: Total carotenoid contents of the flavedo and albedo of ‘Nules Clementine’ mandarin from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9). ....................................................................................................................... 169

Figure 4: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on rind fructose (A), glucose (B), and sucrose (C) of ‘Nules Clementine’ mandarin harvested from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9)............ 170

Figure 5: Concentration of fructose (A), glucose (B) and sucrose (C) of the flavedo and albedo of ‘Nules Clementine’ mandarin from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9). ....................................................................................................................... 171

Figure 6: Effect of canopy position (inside canopy (IC) and outside canopy (OC) on rind dry matter of ‘Nules Clementine’ mandarin from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9). ....................................................................................................................... 172

Figure 7: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on radical-scavenging activities (DPPH) of ‘Nules Clementine’ mandarin fruit rind from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9). ......................... 173

Figure 8: Concentration of radical-scavenging activities of the flavedo and albedo of ‘Nules Clementine’ mandarin fruit rind from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9). ....................................................................................................................... 174

Figure 9: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on rind total phenolic concentration and total flavonoid concentration of ‘Nules Clementine’ mandarin from Eastern Cape (EC) and Western Cape (WC) provinces harvested over two seasons during postharvest non-chilling storage (Weeks 0, 3, 6 and 9). ....................................................................................................................... 175

Figure 10: Total phenolic and total flavonoid concentrations of the flavedo and albedo of ‘Nules Clementine’ mandarin from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9). ....................................................................................................................... 176

Chapter 6
Figure 1: Comparison of *trans*- and *cis*-zeatin-type cytokinins of ‘Nules Clementine’ mandarin fruit harvested from two canopy positions (inside canopy (IC) and outside canopy (OC)) from Eastern Cape (EC) and Western Cape (WC) provinces over two seasons during postharvest non-chilling cold storage. Vertical bars represent standard error of the mean value (n = 3). Abbreviations: *Trans*-zeatin (tZ), *cis*-zeatin (cZ), O-glucoside (OG), riboside (R), riboside-O-glucoside (ROG), 7-glucosides (7G).

Figure 2: Comparison of *trans*- and *cis*-zeatin-type cytokinins of ‘Nules Clementine’ mandarin fruit with (disorder) and without (none) rind breakdown after 9 weeks of cold storage at non-chilling temperature. Vertical bars represent standard error of the mean value (n = 3). Abbreviations: *Trans*-zeatin (tZ), *cis*-zeatin (cZ), O-glucoside (OG), zeatin riboside (ZR), riboside-O-glucoside (ROG), 7-glucosides (7G).

Figure 3: Comparison of DHZ7G, iP, and iP9G of ‘Nules Clementine’ mandarin fruit harvested from two canopy positions (inside canopy (IC) and outside canopy (OC)) from Eastern Cape (EC) and Western Cape (WC) provinces over two seasons during postharvest non-chilling cold storage. Vertical bars represent standard error of the mean value (n = 3). Abbreviations: Dihydrozeatin 7-glucosides (DHZ7G), N6-isopentenyladenine riboside (iPR) and N6-isopentenyladenine-N9-glucoside (iP9G).

Figure 4: Comparison of DHZ7G, iP, and iP9G of ‘Nules Clementine’ mandarin fruit with (disorder) and without (none) rind breakdown after 9 weeks of cold storage at non-chilling temperature. Vertical bars represent standard error of the mean value (n = 3). Abbreviations: Dihydrozeatin 7-glucosides (DHZ7G), N6-isopentenyladenine riboside (iPR) and N6-isopentenyladenine-N9-glucoside (iP9G).

Figure 5: Comparison of auxin concentration of ‘Nules Clementine’ mandarin fruit harvested from two canopy positions (inside canopy (IC) and outside canopy (OC)) from Eastern Cape (EC) and Western Cape (WC) provinces over two seasons during postharvest non-chilling cold storage (A and B). Abbreviations: IAA, indole-3-acetic acid and IAAsp, IAA-aspartate.

Figure 6: Comparison of IAA (A) and IAAsp (B) of ‘Nules Clementine’ mandarin fruit with (disorder) and without (none) rind breakdown after 9 weeks of cold storage at non-chilling temperature. Vertical bars represent standard error of the mean value (n = 3). Abbreviations: Dihydrozeatin 7-glucosides (DHZ7G), N6-isopentenyladenosine (iPR) and N6-isopentenyladenine-N9-glucoside (iP9G). Abbreviations: IAA, indole-3-acetic acid and IAAsp, IAA-aspartate.
Chapter 7

Figure 1: An image showing the overview of FOSS NIRSystem...............................................236
Figure 2: Line plot of absorbance spectra for ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin fruit.
....................................................................................................................................................237
LIST OF TABLES

Chapter 2
Table 1: An overview of the application of computer vision system for detecting physiological disorders of citrus fruit .................................................................73
Table 2: Application of X-ray imaging in detecting physiological disorders of citrus fruit ..........74
Table 3: An overview of the application of visible to near infrared spectroscopy in citrus fruit ....75

Chapter 3
Table 1: An overview of the application of computer vision system for detecting physiological disorders of citrus fruit .................................................................73
Table 2: Application of X-ray imaging in detecting physiological disorders of citrus fruit ..........74
Table 3: An overview of the application of visible to near infrared spectroscopy in citrus fruit ....75

Chapter 4
Table 1: Climatological data registered during the growing seasons in KwaZulu-Natal (KZN) and Limpopo (LMP) provinces .................................................................133
Table 2: Pearson correlation coefficient matrix between biochemical properties measured in ‘Marsh’ grapefruit rind measured in 2015 and 2016 seasons .................................................................134

Chapter 5
Table 1: Correlation coefficients among measured ‘Nules Clementine’ mandarin fruit rind biochemical and antioxidant parameters .................................................................177

Chapter 7
Table 1: Descriptive statistics for calibration and validation subsets for biochemical properties of ‘Marsh’ grapefruit rind .................................................................238
Table 2: Descriptive statistics for calibration and validation subsets for biochemical properties of ‘Nules Clementine’ mandarin rind .................................................................239
Table 3: Pearson correlation coefficient matrix between biochemical properties of ‘Marsh’ grapefruit .................................................................240
Table 4: Pearson correlation coefficient matrix between biochemical properties of ‘Nules Clementine’ mandarin fruit. 

Table 5: Model performance for ‘Marsh’ grapefruit using spectral data acquired (from KwaZulu-Natal and Limpopo) before cold storage (at week 0) and after cold storage (at week 9) using FOSS NIRSystem in reflectance mode. 

Table 6: Model performance for ‘Marsh’ grapefruit using pooled spectral data acquired (from KwaZulu-Natal and Limpopo) before cold storage (at week 0) and after cold storage (at week 9) using FOSS NIRSystem in reflectance mode. 

Table 7: Calibration model performance developed for ‘Marsh’ grapefruit using pooled spectral data (from KwaZulu-Natal and Limpopo separately) acquired before cold storage (at week 0) and after cold storage (at week 9) using FOSS NIRSystem in reflectance mode. 

Table 8: Model performance for ‘Nules Clementine’ mandarin using spectral data acquired (from KwaZulu-Natal and Limpopo) before cold storage (at week 0) and after cold storage (at week 9) using FOSS NIRSystem in reflectance mode. 

Table 9: Model performance for ‘Nules Clementine’ mandarin using pooled spectral data acquired (from KwaZulu-Natal and Limpopo) before cold storage (at week 0) and after cold storage (at week 9) using FOSS NIRSystem in reflectance mode. 

Table 10: Calibration model performance developed for ‘Nules Clementine’ mandarin using pooled spectral data (from KwaZulu-Natal and Limpopo separately) acquired before cold storage (at week 0) and after cold storage (at week 9) using FOSS NIRSystem in reflectance mode.
OVERVIEW

The incidence of various forms of postharvest non-chilling physiological rind disorders on various species of citrus fruit remains a major challenge to the citrus industry. The expression of these disorders cannot be visually observed until about 3-5 weeks after harvest when the fruit have been transported to various market destinations. This is because the incidence of rind disorders such as peel pitting and rind breakdown (RBD), reduces fruit marketability which causes a significant financial loss to the citrus industry. This study evaluated the role of canopy position and non-destructive determination of postharvest rind biochemical properties of ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin in relation to its respective rind physiological disorder at non-chilling storage temperature. It was hypothesised that preharvest factor such as canopy position may influence rind biochemical properties of the fruit and hence, could trigger the incidence of rind physiological disorders on citrus fruit stored at non-chilling temperatures after harvest. Similarly, phytohormonal changes in ‘Nules Clementine’ mandarin fruit rind from different canopy positions, in relation to the incidence of RBD during postharvest non-chilling cold storage were investigated. Rind biochemical properties of citrus fruit could be determined non-destructively with the use of visible to near-infrared spectroscopy (VIS/NIRS) coupled with appropriate chemometrics. ‘Marsh’ grapefruit (from inside canopy [IC] and outside canopy [OC] positions) were harvested from the KwaZulu-Natal and Limpopo provinces while ‘Nules Clementine’ mandarins were harvested from Eastern Cape, Western Cape, and Limpopo. Harvested fruit were labelled, weighed, and placed in cold storage (7.5 ± 0.5ºC) for 9 weeks. Fruit were analysed at 3 week intervals while chemometric models were developed using randomised reference data from fruit analysed before and after cold storage. For both citrus cultivars, canopy position showed a highly significant ($p < 0.001$) effect on some biochemical properties, such as total carotenoid and total flavonoid concentrations, while a significant ($p < 0.05$) influence of production region was observed on
same properties. Non-destructive determination of fruit rind biochemical properties using VIS/NIRS and chemometrics with RPD values ranging from 0.28 to 14.23 demonstrated poor to excellent models. Overall, the study revealed that canopy position influences biochemical properties of the citrus fruit rind and that high concentrations of these properties hindered the development of postharvest rind physiological disorders during non-chilling cold storage.
1. Introduction

Citrus (Citrus spp.) fruit lead among the most economically important horticultural products, such as avocados, apples, bananas, and grapes, in both local and international trade markets (Ladanyia and Ladaniya, 2010; Magwaza et al., 2012). This is partly because they are a natural source vitamin C, carotenoids, and folic acids, amongst many other healthy and beneficial nutrients (Ladaniya, 2008). Globally, South Africa is the second largest exporter of fresh citrus fruit after Spain, exporting over 1.6 million tons (~9 billion rand [ZAR]; September 2017) annually (Citrus Growers’ Association of South Africa, 2016). Major export destinations include Northern Europe (22%), Middle East (21%), Asia (11%), United Kingdom (10%), Far East (9%), Russia (9%), South Europe (7%), United States (4%), Canada (3%) and other (4%) (CGA, 2016).

The fruit belong to the Rutaceae family and are hesperidia, (berries having a leathery peel and internal segments) (Soule and Grierson, 1986). However, citrus fruit are confronted with several types of postharvest physiological disorders, such as chilling injury and different categories of non-chilling physiological disorders. The manifestation of various forms of postharvest non-chilling physiological disorders on the rind of various species of citrus fruit is a major challenge posing significant threat to the financial gains of the growers at both local and international markets (Agustí et al., 2001). This is because the incidence of disorders such as rind breakdown (RBD), rind staining, puffiness, pateca spots, and peel pitting on the rinds of citrus fruit ward off potential buyers. Although, symptoms of rind physiological disorders of citrus fruit are similar, their terminologies differ among citrus cultivars and
varieties. For instance, peel pitting is mostly attributed to ‘Marsh’ grapefruit (Alférez and Burns, 2004) and oranges like ‘Navel’ fruit (Alférez and Zacarias, 2001), while rind breakdown is mostly associated with ‘Nules Clementine’ mandarin (Cronje et al., 2011) even though some literatures associate it to some other cultivar of citrus fruit.

Un fortunately, the causal factors triggering the incidence of postharvest non-chilling physiological rind disorders of citrus fruit are yet to be fully identified (Alférez and Zacarias, 2014). Nevertheless, preharvest fruit position on tree canopy (otherwise known as canopy position), that is inside canopy (IC), has previously been suggested as a possible factor favouring the occurrence of postharvest rind disorders in ‘Nules Clementine’ mandarin fruit at non-chilling temperature (Magwaza et al., 2013a). Other studies reported that different light levels within the canopy, that is exposure of fruit to high (outside) or low (inside) light levels, affected rind carbohydrates and mineral elements during fruit development (Cronje et al., 2011). Similarly, it has been suggested that loss of rind moisture content could foster the induction and development of the non-chilling physiological rind disorders observed in citrus fruit (Alférez and Zacarias, 2014). The incidence of these disorders poses as more critical problem to the industry because their expression cannot be visually detected until about 3-5 weeks after harvest, when the fruit have been sorted and transported to various marketing destinations (Cronje et al., 2011). Furthermore, it becomes problematic as this coincides with the period of commercial export to market destinations (Cronje et al., 2011). This means that the incidence of postharvest non-chilling physiological rind disorders would have become conspicuous on fruit with such potential of developing disorders before reaching the export markets. Hence, any fruit with the non-chilling physiological rind disorder(s) is/are prone to be rejected by the regulating body of importing markets. Thus, this leads to
more expending because the products are sent back to local markets, which invariably causes significant financial loss to the industry.

Therefore, to work towards mitigating the problem, it is important to investigate causal factor(s) related to the susceptibility of citrus fruit to postharvest physiological rind disorders at non-chilling temperatures. In a bid to identify these factors, the role of canopy position on rind biochemical properties in relation to rind physiological disorders in citrus fruit were investigated. Furthermore, phytohormonal changes in ‘Nules Clementine’ mandarin fruit rind from different canopy positions were investigated in relation to the incidence of RBD during postharvest non-chilling cold storage. Non-destructive method(s) of determining these properties with a view of being able to sort fruit based on their susceptibility levels could help the citrus industry to deliver fruit of appealing appearance to the market and hence increase their financial gains as each fruit can be analysed. The non-destructive approach means that the current use of ‘representative’ sample measurement of a batch of fruit, which is usually time-consuming, destructive, and expensive will no longer be required. Previous studies by Magwaza et al. (2014) and Magwaza et al. (2012) documented that visible to near infrared spectroscopy (Vis/NIRS) based non-destructive models have high potential of predicting the susceptibility of ‘Nules Clementine’ mandarin to RBD. However, the models were not robust enough because they were developed using fruit from only one production region in South Africa, Western Cape, which was not a representation of fruit grown in the country. Consequently, the present study included a wider production area of ‘Nules Clementine’ mandarin fruit, to develop more robust Vis/NIRS based models capable of determining the investigated rind biochemical properties. Models were also developed to determine the rind biochemical properties of another citrus cultivar – ‘Marsh’ grapefruit.
2. **Research hypothesis**

The hypothesis was that canopy position, a preharvest factor, affects the biochemical properties of the citrus fruit rind such as non-structural carbohydrates, total carotenoids, total phenolic and total flavonoid concentrations, and radical-scavenging activities and its susceptibility to postharvest physiological rind disorders at non-chilling temperature (7.5 °C ± 0.5). It was further hypothesised that these biochemical properties and other targeted metabolites can be used as bio-markers of citrus fruit susceptibility to postharvest physiological rind disorders. In this way, biochemical properties would be analysed non-destructively to determine rind disorders of citrus fruit such as ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin. Furthermore, a robust Vis/NIR based non-destructive model can be developed to determine the biochemical property(ies) triggering postharvest physiological rind disorder in freshly harvested citrus fruit.

3. **Research aims and objectives**

The overall aim was to investigate the effect of canopy position on rind biochemical properties in relation to postharvest physiological rind disorders of citrus fruit at non-chilling temperatures. In addition, non-destructive determination of such biochemical properties of the citrus fruit rind was investigated, which invariably could play a significant role in the overall quality and economic value of citrus fruit.

Specific Objectives included the following:

1. To investigate the role of canopy position on physicochemical properties of ‘Marsh’ grapefruit after harvest and after postharvest cold storage at non-chilling temperature.
2. To evaluate the relationship among canopy position, production region and rind biochemical properties in relation to postharvest physiological rind disorders of ‘Marsh’ grapefruit at non-chilling temperature.

3. To explicate the role of canopy position, production region on rind biochemical properties in relation to postharvest physiological rind disorders of ‘Nules Clementine’ mandarin at non-chilling temperature.

4. To develop robust Vis/NIRS based non-destructive models, to determine the rind biochemical properties of ‘Nules Clementine’ mandarin and ‘Marsh’ grapefruit.
References


Abstract

Delivering citrus fruit without the appearance of physiological rind disorders to the market is one of the major problems confronting citrus industries globally. The appearance of the disorder on fruit rind is mostly unprecedented. Although, various studies have been conducted to investigate the factors or mechanisms favouring the incidence of these rind disorders, the exact factors triggering the disorders remain unknown. This review highlighted some known factors contributing to the development of physiological rind disorders associated with non-chilling cold storage. The physiological and biochemical basis for development of the disorders were also examined. Various non-destructive techniques for rapid determination of rind biochemical properties (presented as potential biomarkers) associated with the disorders were also explicated. The prospects of using non-targeted metabolomics to gain a holistic understanding of the metabolites triggering the development of citrus rind disorders during postharvest storage at non-chilling temperatures were discussed.

Keywords: Citrus fruit, ‘Marsh’ grapefruit, Near-infrared spectroscopy, ‘Nules Clementine’, Postharvest non-destructive technologies, Rind breakdown, Rind pitting, Targeted and non-targeted metabolomics,
1. Introduction

Citrus (*Citrus* spp.) fruit belongs to the *Rutaceae* family and are scientifically called *Hesperidia*, which are berry fruit having a leathery peel and internal segments (Soule and Grierson, 1986). The fruit are the largest economically important subtropical fruit crop grown in more than 50 countries around the world including South Africa (Ladanyia and Ladaniya, 2010). They are widely grown and consumed because they are a natural source of antioxidants such as vitamin C, phytochemicals such as carotenoids, and folic acids amongst many other healthy and beneficial nutrients (Ladaniya and Ladanyia, 2008). However, the incidence of various rind physiological disorders such as chilling and non-chilling cold storage injuries limit the acceptability of the fruit by consumers which invariably results in economic losses for citrus industries globally.

The economic losses suffered by global industries due to physiological disorder can reach as much as 60% of the total citrus production (Alquezar et al., 2010; Kader and Arpaia, 2002) and yet the mechanisms underlying the incidence of some of these physiological disorders are still not fully understood. Hence, mitigating solutions to preserve the appearance of citrus fruit rind (or rind quality) cannot be developed. Although, non-chilling physiological rind disorders affect the marketability of citrus fruit, they do not compromise the internal quality of the fruit (Alquezar et al., 2010; Magwaza et al., 2013a). The inability to predict the susceptibility of citrus fruit to rind disorder at non-chilling temperature is mainly because the symptom(s) of incidence cannot be visually observed until about three to five weeks postharvest (Cronje et al., 2011a; Van Rensburg and Bruwer, 2000). It becomes even more problematic as this period coincides with the period of commercial export to market destinations from South Africa (Magwaza et al., 2014b; van
Rensburg et al., 2004). Hence, novel solutions for non-destructive monitoring or detecting physiological rind disorders are required.

Lee et al. (2015) indicated that the incidence of postharvest rind disorders is influenced by certain factors to which fruit are exposed during preharvest, harvest or postharvest. Rind biochemical properties such as rind dry matter, non-structural carbohydrates and radical-scavenging activities constitute some of the most important constituents which play vital roles in the predisposition of fruit to different postharvest non-chilling physiological rind disorders (Di Majo et al., 2005; Magwaza et al., 2014b, 2014c). For instance, Ezz and Awad (2009) reported a significant relationship between sugars and rind pitting disorder of ‘Marsh’ grapefruit. Therefore, this suggest that certain rind biochemical properties of citrus fruit could be used as biomarker(s) for predicting the susceptibility of fruit to physiological rind disorder at non-chilling storage temperatures. However, conventional methods of determining rind biochemical properties are destructive, non-representative of fruit consignments, laborious, time consuming and require specialized sample preparation. Hence, some non-destructive methods of analyses could address these shortfalls and are receiving wide acceptance among scientific communities around the worlds. Their benefits include the ability to rapidly determine the maturity status (Olarewaju et al., 2016), biochemical components such as non-structural carbohydrates, and rind dry matter (Magwaza et al., 2012a; Magwaza et al., 2014a) and also monitor/detect physiological rind disorders in individual fruit (Magwaza et al., 2012b). The ability to determine the rind biochemical properties of individual fruit would help in sorting fruit based on their biochemical status and make decisions about the appropriate market to send each consignment.
Therefore, the objective of this review was to discuss current knowledge regarding rind quality of citrus fruit, postharvest physiological rind disorders and their causes, rind biochemical properties and non-destructive methods for detecting them.

2. **Anatomy of citrus fruit rind**

Citrus fruit consists of different tissue layers including the pericarp (peel/rind), a non-edible portion but an immediate purchase determinant at the market, and the endocarp (carpels), which is the edible portion and future purchase determinant (Figure 1). The pericarp, also known as the rind, hosts two different tissue layers including flavedo (outmost coloured part of the rind) and albedo (whitish coloured part of the rind) tissues. According to Spiegel-Roy and Goldschmidt (1996), the flavedo tissue consists of epidermal cells (external layer) and inner tightly packed parenchyma cells with no intercellular air spaces followed by the epicarp, hypoderm and the outer mesocarp. Hence, a healthy fruit can first be characterized by a flavedo having its epidermis with an apolyhedral cell layout covered by a cuticle (Albrigo, 1972a; Medeira et al., 1999). The albedo, on the other hand, is an intricate knot of meristematic cells whereby each cell has a direct plasmodesmata connection with eight other adjacent cells (Alquezar et al., 2010; Storey and Treeby, 1994). This formation results in large intercellular air spaces between the cells, which provide the characteristic spongy morphology of the albedo tissue. The albedo tissue occupies between 60-90% of the fruit volume during its early stage of development, which later becomes thinner as the fruit matures (Spiegel-Roy and Goldschmidt, 1996).

The flavedo tissue also houses oil glands containing the mixture of acid esters, alcohols, aldehydes and hydrocarbons (essential oils), which are phytotoxic to surrounding cells, and breakage of these
essential oils can contribute to the development of non-chilling physiological rind disorders (Knight et al., 2002; Spiegel-Roy and Goldschmidt, 1996). According to Petracek et al. (1998a), the oil gland is the site where rupture of oil bodies begins and the release of the oil into the surrounding cells could cause rind pitting disorder to the fruit. The breakdown of oil glands was reported as the primary symptom of rind pitting of grapefruit (Alférez and Burns, 2004). Further review of literature regarding the anatomy of citrus rind can also be found in a publication by Magwaza et al. (2013b).

2.1. Rind quality of citrus fruit

Although difficult to define, the quality of a horticultural product such as citrus fruit at the fresh fruit market is basically determined by its appearance. Hence, the condition of the outermost part, the rind, of the fruit plays a critical role in its quality characteristics and marketability (Khalid et al., 2012; Magwaza et al., 2013a). However, the citrus industry is faced with the challenge of delivering citrus fruit of attractive quality, to either local or international retail markets. Basically, fruit quality can be described as some carefully worked out attributes evaluated by consumers/buyers who either wilfully or unconsciously determine the overall quality attributes of a product for immediate or future pre-purchase guidance (Sloof et al., 1996). Therefore, citrus fruit rind quality could be based on external properties such as colour (chromatic attributes), size, shape, glossiness, shininess and the absence of surface defects or damage (Nicolaï et al., 2014). The external appearance influences consumer acceptability of quality (attributes that can be visually assessed by the naked eyes) (Nicolaï et al., 2014; Pathare et al., 2013). However, some physiological rind disorders still pose threats to the economic gains of the industries around the world (Alférez and Burns, 2004; Lafuente and Zacarias, 2006; Magwaza et al., 2013b).
2.2. Physiological rind disorder

Generally, physiological disorders limit the full potential of any horticultural product in the markets as consumers tend to discard fruit with any form of physiological disorder(s). These disorders may be referred to as the breakdown of tissues caused by adverse effects of the environments under which a particular fruit is exposed during the preharvest, harvest or postharvest (Morrow and Wheeler, 1997; Verma, 2009). To prolong the postharvest shelf-life, fruit are mostly exposed to relatively low temperatures (above freezing point - 0 °C) and up to 12 °C, to reduce the process(es) causing fruit to decay (Miller, 1946). This cold treatment, depending on citrus cultivar and its threshold, may inflict physiological disorders at either chilling or non-chilling temperatures (Miller, 1946). Therefore, the occurrences of these disorders are critical phenomena compromising either internal (pulp) or external (rind) quality parameters of citrus fruit. For this review, focus will be towards the phenomena compromising rind quality (appearance) of citrus fruit stored at non-chilling temperatures.

There exist several factors contributing to incidences of rind physiological disorders, ranging from preharvest such as cultivar and microclimate to postharvest factors such as temperature and relative humidity. Other factors as highlighted by Morrow and Wheeler (1997) include irradiance (intensity, photoperiod, and spectral quality), humidity, CO₂ concentration, air temperature, air movement, moisture level and mechanical effects. Postharvest physiological rind disorders result from the alteration of metabolism in response to the imposition of stresses which manifest as cell death on citrus rind (Watkins, 2003). Consequently, the incidence of cell death on citrus rind
significantly affects its appearance. Hence, such fruit get rejected by buyers in the market which result to economic loss for the industry.

According to Magwaza et al., (2013b), several types of physiological rind disorders affect citrus fruit. The authors argued that rind disorders highlighted in literature such as peel pitting (Alférez et al., 2005; Cajuste and Lafuente, 2007) and rind breakdown (RBD) (Treeby et al., 1995; van Rensburg et al., 2004) could be categorized as ‘non-chilling storage physiological rind disorders’ and terminologies used interchangeably. Whereas, chilling injury (CI) is another physiological rind disorders occurring because of exposure to temperatures below some critical threshold but above 0 °C chilling injury is aggravated when fruit are relocated to room temperature (Alférez et al., 2005; Martínez-Téllez and Lafuente, 1997; Siboza et al., 2014). Chilling injury was described by Lyons and Breidenbach (1987) as an irreversible physiological damage to plants and its parts. Therefore, incidence of rind disorder occurring at either chilling or non-chilling temperatures relies mostly on factors such as species, cultivar, symptoms, and country in which a research is been carried out or produced (Alférez et al., 2005; Magwaza et al., 2013b). Mostly, the incidence of chilling injury on citrus rind occurs because of prolonged exposure to chilling temperature while the incidence of physiological rind disorder at non-chilling temperature are largely unpredictable.

3. **Non-chilling physiological rind disorders of citrus fruit**

This section describes some of the most economically important non-chilling physiological rind disorders affecting different cultivars of citrus fruit.
3.1. **Rind breakdown/peel pitting**

Rind breakdown (RBD), peel (rind) pitting or rind staining are physiological disorder occurring mostly during postharvest storage at non-chilling temperatures in of citrus fruit such as mandarins, grapefruit, and oranges (Alférez et al., 2005) (Figures 2 & 3). Although, the terminology is variety/cultivar specific and country specific, but symptoms of disorder are similar on the flavedo (coloured part) of respective fruit rind (Magwaza et al., 2013c). Therefore, the use of terminology may vary in this review. Cronje (2007) described the disorder as randomly distributed dark/brown spot resulting from the collapse of oil glands of the flavedo. Similarly, the disorder was described as the collapse of flavedo and albedo tissues resulting in the breakage of oil glands which invariably oxidizes and turns bronze in colour due to the release of oil into intercellular spaces (Agustí et al., 2001; Alférez and Zacarias, 2014; Alquezar et al., 2010). According to Lafuente and Zacarias (2006), the disorder first manifests on the flavedo as sunken areas just above and within the oil glands which ultimately turns dark-brown and becomes necrotic around the rind. Examples of such occurrences are found affecting fruit such as ‘Marsh’ grapefruit (Petracek et al., 1998), oranges (Alférez et al., 2003; Porat et al., 2004) and mandarins (Cronje, 2007; van Rensburg et al., 2004). Furthermore, Alquezar et al. (2010) described the occurrence of the disorder as erratic with unpredictable and high variation characteristics. However, the disorders might occur because of the cold intolerance of the previously mentioned cultivars but susceptible to dehydration possibly due to their inability to accumulate enough abscisic acid (Sala et al., 2005). Abscisic acid contributes significantly to transpiration rate and cell water maintenance of plants and its parts (Alférez et al., 2005). Previous studies have suggested that manipulation of relative humidity and application of wax during postharvest storage of citrus fruit such as ‘Marsh’ grapefruit at non-chilling temperature could encourage the development of rind pitting (Alférez et al., 2005; Alférez
and Burns, 2004). This is because high relative humidity increases the atmospheric vapour pressure which invariably decreases fruit respiration thereby causing the collapse of the rind cells due to abnormal water potential with the atmosphere (Alférez et al., 2005; Alférez and Burns, 2004). This implies that subjecting fruit to a low atmospheric vapour pressure condition could help to maintain the quality of the fruit rind.

### 3.2. Oleocellosis

Oleocellosis (Figure 4), which is also referred to as rind-oil spot or autotoxicity, is a physiological rind disorder that commonly affect all varieties of citrus fruit (Ladaniya and Ladanyia, 2008). However, scientific understanding of the mechanism underlying the development of the disorder is still lacking. Although, it is an example of non-chilling physiological rind disorder, the disorder appears while fruit are at higher temperatures (Ladaniya and Ladanyia, 2008). Its occurrence is sequel to the rupturing of the oil glands which after releasing their phytotoxic contents (terpenes) results in necrosis to the surrounding epidermal cells (Knight et al., 2002; Ladaniya and Ladanyia, 2008). The scarred areas firstly become yellow and the sunken spots then turn conspicuously dark-brown as the ruptured oil spreads and oxidizes (Lafuente and Zacarias, 2006). It is thought that fruit drops during harvesting, handling as well as packaging causes oleocellosis and incidence may be aggravated if fruit are harvested immediately after rainfall, irrigation or other climatic conditions that increases fruit turgidity (Lafuente and Zacarias, 2006). Oleocellosis can cause significant decrease in citrus fruit rind quality as well as fresh fruit export and high percentage of the fruit (80%) from a sensitive farm can be susceptible to the disorder (Zheng et al., 2010).
In different scientific studies, the relationship of some biochemical and physiological characteristics with the occurrence of oleocellosis has been explored. Biochemical characteristics such as antioxidant compounds have been suggested to be related with the development of the rind disorders. For instance, Yuzu fruit (*Citrus junos*) with rind spot showed less antioxidative activities than fruit without the disorder (Sawamura et al., 1988). Sawamura et al. (1988) reported that total tocopherol contents of 8.2 mg/100 g α-tocopherol and 1.0 mg/100 g γ-tocopherol were lower in the rind of fruit with rind spot than fruit without the disorder, which had 7.2 and 4.5 mg/100 g, respectively. Furthermore, physiological characteristics such as carbon-dioxide and ethylene production, as well as total non-structural carbohydrate content were found to be related with increased severity of rind-oil spot in jagada fruit (*Citrus hassaku*) (Kanlayanarat et al., 1988). Therefore, proper management of antioxidant compounds, carbon-dioxide and ethylene production of the citrus fruit rind could reduce this disorder.

### 3.3. Necrosis

Necrosis (Figure 5) is a physiological disorder that affect rind of citrus fruit such as ‘Shamouti’ orange and its occurrences cause significant economic losses to citrus industries (Golomb, 1983). The disorder appears mostly during the postharvest life of the fruit as superficial pits that grow in time and number to form a necrosis patch on the flavedo of citrus fruit rind (Ben Yehoshua et al., 2001). The collapsed hypodermis tissue of an affected fruit is morphologically identical to destroyed oil glands (Ben Yehoshua et al., 2001). The expression of superficial flavedo necrosis disorder on citrus fruit rind is similar to the expression of stem end rind breakdown of oranges described below and rind pitting of grapefruit (Petracek et al., 1998). This means that this rind disorder could easily be confused with stem-end rind breakdown (SERB).
3.4. Stem-end rind breakdown (SERB)

Stem-end rind breakdown (Figure 6) is a severe physiological rind disorder of citrus fruit involving the breakdown and subsequent darkening of epidermal tissues around the stem end of fruit (Dou et al., 2001; Lafuente and Zacarias, 2006). The disorder has been reported frequently on small, thin-skinned fruit stored under a non-chilling postharvest condition, which favours rapid dehydration of fruit rind (Porat et al., 2004). The formation of a thin circle of undamaged tissue from cells with no stomata and a thick layer of wax around the calyx is a typical characteristic of SERB (Dou et al., 2001). According to Lafuente and Zacarias (2006), the disorder is suggested to appear following an excessive dehydration of the rind around the stem-end of detached fruit. Other factors that could contribute to the incidence of the disorder around the calyx include wax cuticle thickness, water potential alteration in stomata number (Albrigo, 1972b). Specific causes of SERB are unknown, but some preharvest conditions such as nutritional imbalances influence fruit susceptibility to SERB which is more common in small and well-coloured fruit (Ritenour and Dou, 2003).

4. Causes of non-chilling physiological rind disorders

There are various preharvest, harvesting, and postharvest conditions that initiate the development of various non-chilling physiological rind disorders of citrus fruit (Magwaza et al., 2013b; Peiris et al., 1998). Alférez et al. (2003) and Davies and Albrigo (1994) suggested that the cause of most disorders is specific to individual fruit and may not be changed. Other disorders occur sequel to the influence of environmental conditions of the fruit which could, fortunately, be manipulated (Agustí et al., 2004; Alférez and Burns, 2004). However, the causal factor(s) triggering the
occurrence of non-chilling physiological rind disorders remain(s) unclear despite huge researches
invested on the topic (Agustí et al., 2001; Alférez et al., 2003; Magwaza et al., 2013b). Therefore,
to gain understanding of the cause(s) and mechanism(s) governing different forms of physiological
rind disorders of citrus fruit and their relationship, it could be important to consider possible factors
contributing to the development of non-chilling physiological rind disorders.

4.1. Preharvest factors that impact citrus rind quality

The impact of preharvest factors on citrus quality is increasingly receiving scientific interest
because many physiological disorders do not always need particular environmental conditions for
occurrences but share some relationship with the later stages of fruit ripening (Ferguson et al.,
1999). Therefore, the unpredictable nature of non-chilling physiological rind disorder suggests the
possible role of some preharvest factors on the susceptibility of citrus fruit to the rind disorders.
These factors may include choice of scion and rootstock cultivar, nutritional imbalances, maturity,
canopy position, and fruit moisture relations.

4.1.1. Scion and rootstock cultivar

The postharvest characteristics, including fruit sensitivity to physiological rind disorders, of most
fruit are genetically predetermined and vary with cultivar (Beverly et al., 1993). Therefore, the
choice of rootstock/cultivar may be a vital management strategy that growers interested in
delivering citrus fruit of quality rind (rind without disorder) to various retail markets may consider.
Although, non-chilling physiological rind disorder affect many citrus cultivars, level of
susceptibility differs from one fruit to the other. Citrus fruit like ‘Marsh’ grapefruit (Alférez and
Burns, 2004); ‘Nules Clementine’ mandarin (Cronje et al., 2011a); ‘Fallglo’ tangerine (Petracek
et al., 1998b); ‘Navelina’, ‘Navelate’ ‘Shamouti’ and ‘Lane late’ oranges (Agustí et al., 2004; Alférez et al., 2003; Establés-Ortiz et al., 2009; Lafuente and Sala, 2002) are known to be highly sensitive to non-chilling physiological rind disorders. Interestingly, while ‘Nules Clementine’ mandarin fruit are susceptible to postharvest RBD, ‘Oroval Clementine’ mandarin harvested from the same orchard and put under same postharvest conditions are RBD tolerant (van Rensburg et al., 2004; Van Rensburg and Bruwer, 2000). Similarly, ‘Pinalate’ orange which is susceptible to non-chilling physiological rind pitting disorder, is tolerant of CI (Magwaza et al., 2013b). Therefore, crop improvement methods such as breeding and grafting which allows for the integration of quality traits of two different plants into one may be an effective course towards the management of non-chilling physiological rind disorders.

4.1.2. Canopy position

Position of fruit in the tree canopy, otherwise regarded as canopy microclimate, is considered to play significant role in the susceptibility of fruit to physiological rind disorders (Arpaia et al., 1991; Duarte and Guardiola, 1995; McDonald et al., 2000; Vitor et al., 2001; Wild, 1991). Furthermore, the varying intensity of sunlight reaching fruit in different canopy positions affect rind biochemical properties behaviour differently (Cronje et al., 2011b). The authors showed that fruit shading from sunlight influence biological processes during growth and development. This is because response of photoreceptors mediates light responses, that is, different light colours activate different photoperiods which invariably activates different genes. Fruit receiving higher intensity of sunlight may accumulate more carbohydrate due to high photosynthetic rate than the ones receiving lower intensity of sunlight, but this response may not always be the case (Purvis, 1980).
Exposure of fruit to direct sunlight has been linked to development of non-chilling physiological rind disorders of citrus fruit by several authors as symptoms are mostly observed in the portion of the rind exposed (Chikaizumi, 2000; Medeira et al., 1999; Wild, 1991). Medeira et al. (1999) believed high radiation of sunlight may induce plasmolysis, cell collapse, and localised flavedo dehydration which invariably form rind pitting of citrus fruit. The level of injury is dependent on temperature, period of exposure and cultivar. It is noteworthy that direct exposure of fruit to sunlight could result in fruit temperatures exceeding 38 °C (Chikaizumi, 2000). High temperature could induce oxidative stress in the rind and increased phenylamine ammonia-lyase (PAL; EC 4.3.1.5) activity in the rind which is correlated with one form of fruit rind injury or the other (McDonald et al., 2000; Poiroux-Gonord et al., 2013; Vitor et al., 2001). Other impacts include degradation of cellular membranes, protein and nucleic acids damage and inhibition of pigment synthesis (Vogele, 1937). As demonstrated by researchers, direct exposure of fruit to sunlight stimulates the concentration of sugars (Cronje et al., 2011a), chlorophyll and carotenoids (Khumalo, 2006) during fruit development. Varying intensity of sunlight due to fruit position within tree canopy was found to influence citrus rind quality differently (Cronje et al., 2013, 2011a, 2011b; Magwaza et al., 2012b; Magwaza et al., 2013a). These authors demonstrated that fruit exposed to direct (outside canopy) sunlight had significantly higher contents of rind carbohydrates and lower incidence RBD than those from shaded sunlight (inside canopy). Therefore, fruit harvested from outside canopy position of the tree might not be susceptible to non-chilling physiological rind disorder during the period of export to international markets. Also, this means that exposing citrus fruit to direct sunlight could play a beneficial role in mitigating susceptibility of fruit rind to postharvest non-chilling physiological rind disorders.
4.1.3. Mineral nutrition

A host of varieties of mineral elements are utilised during growth and development to produce a high-quality citrus fruit just like any other horticultural fruit. These minerals play their respective roles during synthesis, heredity, enzyme activation, membrane permeability and osmotic regulation (Clarkson and Hanson, 1980; Mengel and Kirby, 1982; Tisdale et al., 1985). There are about thirteen essential elements that contribute significantly to structural development or metabolism of the plant or its part, resulting in dysfunctionality if unavailable during growth and development (Marschner, 1995; Taiz and Zeiger, 2010). These essential elements include macro- and micro-plant nutrients (Marschner, 1995) and their relative concentrations prior to fruit harvest are related to various physiological disorders emanating from prolonged postharvest storage life (Resnizky and Sive, 1993). However, calcium (Ca), Nitrogen (N), phosphorous (P), potassium (K), magnesium (Mg) and sulphur (S) are scientifically known to play key roles in the production of quality fruit.

Regarding rind physiology, Ca is widely known to play significant role in cell wall structure and membrane function and hence, physiological disorders of numerous fruit (Poovaiah et al., 1988). Different researchers have documented the role of Ca in reducing various incidences of physiological rind disorders such as creasing of oranges (Storey et al., 2002), albedo breakdown of oranges (Treeby and Storey, 2002) and peteca spot of lemon fruit (Storey and Treeby, 2002). Furthermore, insufficient amount of Ca has been implicated in the development of physiological disorders such as cuticle cracking of cherries (Sekse, 1995) and sweet pepper (Opara et al., 1997); bitter pit, internal breakdown and lenticel discoulouration in apples (Wills et al., 2004); and blossom-end rot in pepper (Li et al., 2004; Morley et al., 1993). Similarly, high concentration of
K was thought to be associated with stress response because of reduced respiration conditions of citrus fruit since the element is known for stomatal regulations (Storey et al., 2002). Hence, exposing fruit to postharvest condition(s) where the concentrations of mineral nutrients of citrus rind can be maintained might play a beneficial role in the susceptibility of citrus fruit to non-chilling physiological rind disorders.

4.1.4. Fruit maturity

Various studies have linked citrus fruit maturity to physiological rind disorders (Alférez and Zacarias, 2001; Almela et al., 1992; Wild, 1991). For instance, Cronje (2009) indicated that mature citrus fruit are more susceptible to non-chilling physiological rind disorders. The susceptibility of citrus fruit to non-chilling physiological rind disorder are mostly determined at the moment of fruit colour break during pigmentation until time of harvest (Assimakopoulou et al., 2009). Similarly, the incidence of non-chilling physiological rind disorders has been documented to increase quantitatively with citrus fruit maturity (Alférez and Zacarias, 2001). However, mutual relationship between peteca spot and fruit maturity have been established (Duarte and Guardiola, 1995; Undurraga et al., 2009). According to Duarte and Guardiola (1995), fruit harvested green was more tolerant to peteca spot, a non-chilling physiological rind disorder, than fruit harvested at yellow stage. Therefore, to reduce the incidence of the disorder, the authors applied gibberellic acid (a plant growth regulator) to delay colour break due to its ability to retain chlorophyll and delay synthesis of carotenoid for a long period of time.
Clearly, it could be deduced that fruit susceptibility to non-chilling physiological rind disorders is cultivar and maturity dependent. It could also be indicated that rind disorders increase concomitantly with rind colour development.

4.2. Postharvest factors that impact citrus rind quality

The susceptibility of citrus fruit to non-chilling physiological rind disorder as affected by preharvest factors have been highlighted above. However, the condition of the fruit during its postharvest life could also play significant role in the development of the disorder. Hence, some postharvest factors have been implicated in fostering the development of the disorder include fruit water loss (Alférez et al., 2010; Alquezar et al., 2010) and wax application (Petracek et al., 1998a; 1998b; Wild, 1991) while ethylene application has been reported to play protective role against the development of the disorder (Porat et al., 2004).

5. Rind biochemical properties of citrus fruit: potential use as biomarkers

Besides the physical appearance of citrus fruit, other features such as biochemical properties could serve as pre-symptomatic biomarkers of physiological rind disorders of citrus fruit (Magwaza et al., 2013a). Biomarkers, by definition, are features that are accurately measured and assessed as indicators of normal biological processes (Naz et al., 2014).

Meanwhile, biochemical properties such as antioxidants are commonly known for their increased activities or existence in citrus fruit rind to be protective against rind physiological disorders (Janská et al., 2010; Rivera et al., 2007; Zhu et al., 2011). Furthermore, rind biochemical properties of citrus fruit such as carbohydrate, rind dry matter and antioxidants are known to play significant
role in the susceptibility of citrus fruit to postharvest physiological disorder as indicated by Di Majo et al., (2005). This role was further shown in studies conducted on ‘Nules Clementine’ mandarins as there were differences in biochemical properties based on susceptibility of the fruit to RBD (Magwaza et al., 2014a, 2014b). Also, other studies have reported relationship between vitamin C and CI of ‘Marsh’ grapefruit (Ezz and Awad, 2009). Therefore, using these biochemical properties as biomarkers to predict the susceptibility of citrus fruit to non-chilling physiological rind disorders are promising. Hence, understanding the behaviour of rind biochemical properties of fruit with or without non-chilling physiological rind disorder could provide pivotal direction towards unveiling the underlying mechanism(s) of rind disorder at non-chilling temperature.

Furthermore, vulnerability of fruit to postharvest physiological rind disorders at non-chilling temperature varies amongst fruit of same cultivar and even among fruit harvested from the same tree in the same orchard. This variation is mostly influenced by the position of the fruit within tree canopy (canopy position) because different levels of micro-climates reach different position of the tree canopy (Magwaza et al., 2013a).

### 5.1. Non-structural carbohydrates

Non-structural carbohydrates (NSC) such as glucose, fructose and sucrose have been suggested to play key role as regulatory molecules in crop plants because of their ability to control gene expression related to plant metabolism and chilling tolerance (Bolouri-Moghaddam et al., 2010). These sugars metabolise from starch (Cameron, 1932) which is the most crucial storage carbohydrate in most fruit and other tree organs (Goldschmidt et al., 1992).
The involvement of NSC in flavedo tissues have been suggested to influence citrus rind adaptation to the constantly changing postharvest environmental conditions and sensitivity to the expression of physiological damage due to exposure to low temperature (Cronje et al., 2013; Holland et al., 2005, 2002, Purvis, 1989, 1980). Lower osmotic potential of citrus rind due to high concentration of sucrose (an osmoregulatory property of plant cells) has also been linked to the incidence of rind disorder (Cronje et al., 2011a). Moreover, NSC have been found to form glass-like layer which prevent intracellular compartment from mechanical collapse and enable cells to avoid formation of intracellular ice crystals (Ingram and Bartels, 1996; Kosová et al., 2007).

Citrus fruit (‘Nules Clementine’ mandarin) with low concentration of rind NSC was found to be more susceptible to non-chilling physiological rind disorder compared to fruit with high concentration of the carbohydrates (Cronje et al., 2013, 2011a). Similarly, the concentration of carbohydrate in citrus flavedo was thought to affect citrus rind condition during its postharvest life and its susceptibility to physiological rind disorders (Holland et al., 2002; Purvis and Grierson, 1982; Purvis and Rice, 1983). The carbohydrate also affects chloroplast-chromoplast conversion during rind colour development (Barry and le Roux, 2010; Huff, 1984).

Therefore, the use of these biochemical properties (metabolites) could be explored as biomarkers of non-chilling physiological rind disorders of citrus fruit.

5.2. Pigments

Chlorophyll and carotenoids constitute the most crucial pigments in citrus fruit rind. Chlorophyll characterises the rind of an immature citrus fruit (Ladaniya, 2008), while carotenoid may indicate
fruit maturity. As fruit ripens, chromoplast synthesis more of carotenoids while chlorophyll disintegrates as chloroplast transform into carotenoid-rich chromoplast.

According to Mauzerall (1977) and Reinbothe and Reinbothe (1996), chlorophyll molecules synthesis occurs in the C5 pathway of an intact carbon structure of amino acid glutamate where a magnesium (Mg) atom occupies the centre of the chlorophyll molecules. Chlorophyll is essentially classified into chlorophyll a and chlorophyll b. While chlorophyll a contains a methyl group at the third position of chlorophyll structure, chlorophyll b contains a formyl group illustrated in Figure 7A (Jones, 1973). Chlorophyll breakdown are said to occur in three stages. First is catalysis by chlorophyllase followed by Mg-dechelatase and then pheophorbide a oxygenase stage where the porphyrin macrocycle accompanies the loss of green colour (Hörtensteiner and Kräutler, 2011; Matile et al., 1996).

On the other hand, carotenoids (50-75%) are mostly dominated in the rind of fruit such as citrus and comprises xanthophylls and carotenes (Curl and Baily, 1956). β-carotene (Figure 7B) is the most abundant naturally occurring carotene and are pure hydrocarbons. Although, both xanthophylls and carotenes comprise of 40 carbon atoms made from eight isoprene units, xanthophylls contains additional oxygen molecules (Salisbury and Ross, 1992). The processes for the biosynthesis of carotenoids have been widely covered in literature (Alquézar et al., 2008; Kato, 2012; Kato et al., 2004; Rodríguez-Concepción et al., 2004). However, the metabolic pathway (Figure 8) is mostly shared by some other crucial isoprenoids such as gibberellins and plastoquinone. Carotenoids, as reported by Rodríguez-Concepción et al. (2004) can serve as precursor for the synthesis of abscisic acid (ABA), a plant hormone known to induce heat
(Robertson et al., 1994) and chilling (Rikin et al., 1976) tolerance in various plants. However, many factors such as genetic, environmental, nutritional, and hormonal factors influence the synthesis of these pigments (CRI, 1995; Iglesias et al., 2001; Richardson and Cowan, 1995; Sites and Reitz, 1949).

The pigments which are majorly responsible for the colouration of rind and pulp of most citrus species functions by preventing photosensitised oxidation of photosynthetic (Harberlandt, 1965; Stanier and Cohen-Bazire, 1957) and non-photosynthetic (Goodwin, 1980) tissues and also aids photosynthesis. According to Merzlyak and Chivkunova (2000), pigments could protect fruit against various pre- and postharvest stresses. For instance, a yellow fruit (with high concentration of carotenoid) was reported to be more tolerant to physiological rind disorder compared with green grapefruit (Grierson, 1974). Furthermore, carotenoids protect plant cells including rind cells from photooxidative damage by terminating singlet oxygen ($^1$O$_2$) synthesised from the chlorophyll triplet in the reaction centre of photosystem II (Takano et al., 2005; Telfer, 2005). Therefore, these pigments might play positive role in preventing the susceptibility of citrus fruit to the unprecedented incidence of non-chilling physiological rind disorders. As a result, could be used as a biomarker that can be analysed non-destructively to monitor the rind disorder.

5.3. Phenolic compounds

Phenolic compounds are part of the most crucial secondary metabolites found in plants (Sharma et al., 2012). These compounds play key role as defence mechanism in plants and plant parts against reactive oxygen species (ROS) when exposed to low temperature (Lattanzio et al., 2012) and stabilises membrane by decreasing its fluidity and inhibiting diffusion of free radicals.
Recently, the role of phenolic compounds on the antioxidant capacity of citrus fruit rind has been of scientific interest because of their high importance (Li et al., 2006; Manthey and Grohmann, 1996; Sun et al., 2010; Xu et al., 2008). The compounds possess the ability to emit electromagnetic waves after ultraviolet excitation as well as offer well-designed means of exposing indicators of physiological anomalies within the internal structure of horticultural produce (Dixon and Strack, 2003; Hahn, 2009; Lichtenhaler and Schweiger, 1998).

Compounds such as coumarins, psoralens, phenolic acids and flavonoids are among the many phenolic compounds naturally occurring in citrus fruit (Benavente-García et al., 1997; Bocco et al., 1998). Flavonoids, found majorly in citrus fruit, are species and variety specific. The compounds are categorised as polymethoxylated flavones and flavanone glycosides (FGs) and are both associated with the postharvest physiology of the fruit. Citrus rind hosts FGs and phenolic acids as the primary groups of phenolics compounds (Simonne and Ritenour, 2011; Ye et al., 2011). These group of phenolic compounds play physiological role in citrus fruit and also function as antimicrobial activity against bacteria and fungus (Mathur et al., 2011). However, the concentration of these compounds may be affected by fruit maturity, environmental conditions during growth and development, and storage conditions (Abad-García et al., 2012). According to Manthey (2004) and Xu et al. (2008), phenolic compounds contribute significantly to the total antioxidant capacity of citrus fruit rind. Similarly, the antioxidant enzymatic system has been implicated in the chilling and non-chilling conditions triggering physiological rind disorders (Cajuste and Lafuente, 2007; Lafuente et al., 2003; Sala et al., 2005; Sala and Lafuente, 1999). Magwaza et al. (2013b) reported that boosting the FGs contents of citrus fruit rind could positively
influence the defence mechanism of the fruit due to stressful postharvest storage conditions. Therefore, the potential use of these compounds as biomarkers to determine the susceptibility of citrus fruit to non-chilling physiological rind disorders is promising.

5.4. Non-volatile organic acids

Ascorbic, citric, malic, and tartaric acids constitute the major non-volatile organic acids in citrus fruit (Kelebek, 2010). The high levels of antioxidant activities in citrus fruit are largely concomitant to the presence of organic acids with special reference to vitamin C (ascorbic acid) (Mathur et al., 2011; Sdiri et al., 2012). However, the role of citric, malic and tartaric acids towards the susceptibility of ‘Nules Clementine’ mandarin to non-chilling physiological rind disorder is still unclear (Magwaza et al., 2013b). Vitamin C, on the other hand, is known to be one of the most prolific and effective antioxidants freely available in plants and plant parts (Davey et al., 2000; Ioannidi et al., 2009). According to Sdiri et al. (2012), vitamin C is a strong antioxidant which accounts for a large proportion of citrus fruit antioxidant capacity. Its high concentration in outside canopy fruit (sun-exposed fruit) was found to be associated with citrus fruits’ (‘Nules Clementine’ mandarin) tolerance to RBD (Magwaza, 2013). Similarly, its role as a defence mechanism against most oxidative damage resulting from aerobic metabolism or ROS and as an enzyme co-factor makes it an important biochemical property existing in plants (Badejo et al., 2009; Smirnoff, 1996). Therefore, scientific understanding of the role of vitamin C in citrus fruit and its relationship with physiological rind disorder could be useful in preventing postharvest loss in citrus industry.
6. **Hormone involvement in non-chilling physiological rind disorder**

6.1 **Ethylene**

The potential involvement of phytohormone in the tolerance of citrus fruit to non-chilling physiological rind disorder have been reported. For instance, a sudden increase in ethylene production occurred prior to incidence of rind staining disorder of ‘Navel’ orange stored at non-chilling temperatures (Alférez et al., 2003). Similarly, artificial application of ethylene to ‘Navelina’ and Navelate’ orange fruit stored at non-chilling temperature inhibited the incidence of rind staining disorder (Cajuste and Lafuente, 2005; Lafuente and Sala, 2002). According to Cajuste and Lafuente (2005), the efficiency of ethylene in promoting tolerance of fruit against rind staining was connected to its effect on the stimulation of phenyalanine ammonia-lyase (PAL, rate-controlling enzyme in the synthesis of phenylpropanoids) activity. A change in phenylpropanoid metabolism is a crucial response to stress. Rapid increase in this enzyme activity occurred instantly after increase in ethylene and was concomitant with the incidence of non-chilling peel pitting disorder of citrus fruit (Sala et al., 2005). The increased enzyme activity indicated that PAL contribute to the protection of citrus fruit against non-chilling physiological rind disorder (Sala et al., 2005). Similarly, increased PAL and peroxidase activities due to ethylene conditioning was shown to protect citrus fruit against the development of non-chilling physiological rind disorder. Hence, ethylene could aid citrus fruit tolerance to postharvest storage conditions known to stimulate the development of non-chilling physiological rind disorders. Therefore, its analysis and probably with other rind phytohormones could help to unravel the crucial effect and link between hormone type and their involvement in protecting or subjecting citrus fruit to postharvest non-chilling physiological rind disorders.
6.2 Abscisic acid

The phytohormone abscisic acid (ABA) controls the stomata opening and contributes significantly to transpiration rate and cell water maintenance (Alférez et al., 2005). The role of the phytohormone in protecting citrus fruit against environmental stress is still unknown but its modulation levels enable the adaptation of plant cells to environmental stresses (Alférez et al., 2005). Studies by Kawada et al. (1979) reported seasonal levels of ABA in the flavedo of ‘Marsh’ grapefruit to be correlated with CI tolerance. However, Lafuente et al. (1997) could not establish any relationship between changes in ABA and incidence of CI in a highly susceptible ‘Fortune’ mandarin fruit during its maturation stage. Although, dehydration of fruit rind has been reported to stimulate the synthesis of ABA (Lafuente and Sala, 2002), the role of the hormone in the susceptibility of citrus fruit to non-chilling physiological rind disorders is still unknown (Alférez et al., 2005). Therefore, the possible involvement of ABA in the susceptibility of citrus fruit to postharvest non-chilling physiological rind disorder should be investigated.

7. Non-destructive methods for determining rind biochemical properties

The postharvest technology aspect of the horticultural industry has been neglected in the time past until recently, leaving the problems relating to detection of postharvest physiological rind disorders, amongst others, of citrus fruit unsolved (Gao et al., 2010). Identifying the exact cause(s) or mechanism(s) underlying postharvest physiological rind disorders of citrus fruit has remained a scientific challenge for decades and consumers are quick to discard fruit with rind defects (Cronje et al., 2011a; Magwaza et al., 2012a; Magwaza et al., 2013a). Therefore, meeting consumer demands for quality fruit and need for rapid and cost-effective innovative means of detecting or
monitoring physiological rind disorders non-destructively has necessitated scientific and technological research into the subject (Magwaza et al., 2012a).

With the meaningful advancement in science and technology to the improvement of the agricultural industry, innovative non-destructive methods of assessing fruit physiology and quality have been developed (Gao et al., 2010; Olarewaju et al., 2016). Hahn (2009) explicated that the structure and physiological status of a plant or plant parts such as fruit are represented by reflectance patterns of light which depends on certain factors. These factors include external morphology, internal structure, and distribution of internal metabolites or biochemical components (Hahn, 2009). Hence, various non-destructive techniques presented in the literature can be employed to detect physiological rind disorders affecting citrus fruit (Raghavendra and Rao, 2016). These innovative techniques include visible to near infrared (Vis/NIR) spectroscopy (Vis/NIRS), hyperspectral imaging, computed tomography imaging, chlorophyll fluorescence imaging, X-ray imaging, optical coherence tomography, and magnetic resonance imaging (MRI) (Gao et al., 2010; Hahn, 2009).

7.1. Use of computer vision imaging

Non-visible imaging of horticultural products including citrus fruit has been found immensely useful in fruit quality analysis (Bennedsen et al., 2005; Blasco et al., 2009; Mehl et al., 2002). The technique can measure the appearance of produce to guarantee acceptable external quality standards (Blasco et al., 2009). Computer vision illustrated in Figure 9A is an example of a non-visible imaging system capable of detecting external characteristics such as skin defects in apples, citrus, or peaches. The system is capable of separating external features of fruit based on pre-
programmed quality guidelines (Blasco et al., 2003). However, automatic identification of skin defects remains major challenge since some defects may only imply slight economic loss while others translate to huge losses (Blasco et al., 2009). Moreover, predicting or monitoring rind disorders before expression could also be a challenge with the use of computer vision as the technique only discriminate fruit based on the assessments of fruit appearance (Batchelor and Whelan, 1995).

Computer vision systems for citrus fruit identification based on the type of disorder affecting the fruit on a real-time operation are commercially unavailable since the machine only make use of colour information from acquired images (Blasco et al., 2009). It is also difficult to differentiate between various types of rind disorders since many disorders have similar colour expression. Nonetheless, due to the differences of shape and size of different disorders, the features have the potential to distinguish between various rind disorders. As a result, it makes it possible to produce automatic systems to sort fruit based on severity of disorders (Blasco et al., 2009).

Ariana et al. (2006) established a method for detecting rind defects such as black rot and decay. The authors reported that integrating Vis/NIR reflectance and ultraviolet induced fluorescence was more efficient in detecting most rind disorders than using the same method independently. However, the use of ultraviolet light is not encouraged due to its complexity of use and potential danger of the system to users (Gómez-Sanchis et al., 2008). Therefore, with ultraviolet light excluded and only Vis/NIR reflectance was used to detect damage caused by rot in citrus fruit (Gómez-Sanchis et al., 2008). These authors further analysed the spectra of the damage using hyperspectral image acquisition technique illustrated in Figure 9B.
Hyperspectral imaging technique involves conventional imaging techniques and spectroscopy to measure both spatial and spectral information from horticultural produce for the analysis of important biochemical parameters (Lu and Chen, 1999; Mehl et al., 2004, 2002; Polder et al., 2000). Multispectral imaging (Figure 9C) is another technique that produce images using multiple spectral part of electromagnetic radiation at the same scale from the same region of an agricultural product. Hyperspectral images consist hundreds of continuous spectra for individual spatial site of a sample and the strengths of pixels at specific wavelength of a spectrum denote a grey scale image of the sample at that wavelength (Gómez-Sanchis et al., 2008; Mehl et al., 2004). These imaging techniques have been successfully developed and applied to access internal quality of various horticultural products. The techniques could successfully be used as tools to analyse rind biochemical concentrations and assist to segregate fruit into quality grades. An overview of its application in citrus fruit is presented in Table 1.

7.2. Use of X-ray imaging

X-ray imaging techniques (Figure 10A) are majorly used in medical diagnosis amongst other applications such as industrial components inspection and security (Kotwaliwale et al., 2014). Its use for evaluation of quality in agricultural products is still uncommon but has high advantages as it compliments current quality evaluation techniques (Casasent et al., 1998). The technique has been employed efficiently in recent years for assessing internal quality of agricultural products. Its non-contact sensor abilities make it one of the most promising imaging techniques (Kotwaliwale et al., 2014). The use of X-ray in measuring internal quality of horticultural produce can be divided into three techniques. These are two-dimensional (2D) radiography, line scan radiography and X-
ray computed tomography (CT, Figure 10B) (Hahn, 2009). This technique is advantageous due to its simplicity, accessibility, accuracy, and cost efficiency.

Its application in detecting various forms of internal and external properties of some horticultural products have been demonstrated by many scientists (Hahn, 2009; Lin et al., 2008; Njoroge et al., 2002; Ogawa et al., 2003). The technique has been used for detection of water-core in apple, pits in processed olives and cherries, chilling injury in citrus and mechanical bruises (Brosnan and Sun, 2004; Lin et al., 2008; Ogawa et al., 2003; Reyes et al., 2000). Moreover, Barcelon et al. (1999a) successfully used X-ray imaging technique to monitor internal physiological changes during ripening of peach fruit at different maturity level. Similarly, an internal ripening disorder, ‘spongy tissue’, of ‘Alphonso’ mangoes detected as dark grey patches against the light grey areas of a healthy flesh in X-ray images showed the potential use of this technology for on-line sorting (Thomas et al., 1993).

Barcelon et al. (1999b) used X-ray absorption as index to detect mango fruit quality based on its association with quality indices such as density, moisture content, soluble solids, titratable acidity, and pH. Consequently, it was suggested that the technique could be used to non-destructively assess the quality of an intact mango fruit. A recent study by van Dael et al. (2016) also demonstrated the efficiency of X-ray imaging technique combined with image processing algorithms to detect granulation non-destructively and rapidly and endoxerosis internal disorders of oranges and lemons, respectively (Table 2). The authors concluded that the classification method was fast, robust to noise and could be applied to any existing on-line X-ray radiograph equipment.
Therefore, the use of this technique to determine and monitor rind biochemical changes and, hence, the susceptibility of citrus fruit to rind disorders is suggested.

### 7.3. Use of magnetic resonance imaging

Magnetic resonance imaging (MRI) is a non-destructive and non-invasive technique capable of penetrating and visually monitoring of internal structure of biological tissues (Gonzalez et al., 2001; Nicolaï et al., 2014). The technique can be used to determine the concentration, diffusivity, and movement of nuclei (McCarthy, 1994). Magnetic resonance which is commonly referred to as nuclear magnetic resonance (NMR) is a phenomenon occurring between atomic particles and external magnetic field (McCarthy, 1994).

The technique is mostly used for biomedical research and as radiological diagnostic tool (McCarthy, 1994) and its use in the field of horticultural science is speedily gaining scientific interest. MRI has been successfully applied as non-destructive tool for detection of various form of physiological disorders such as core breakdown in ‘Bartlett’ pears (Yang and Wang, 1989), water-core dissipation in ‘Braeburn’ and ‘fuji’ apples (Clark and Burmeister, 1999; Clark and Enza, 1999) and bruises in fruit (Chen et al., 1989). The technique has also been found useful in monitoring ripening and identification of seeds in mandarin and orange citrus fruit (Galed et al., 2004; Hernández-Sánchez et al., 2006). Furthermore, Gentili and Horowitz (1968) used MRI to determine flavonoids (6-C-β-D-Glucopyranosyldiosmetin and 8-C-β-D-glucopyranosyldiosmetin) in citrus fruit.
Therefore, its use in detecting, monitoring and prediction of possible metabolites associated with physiological rind disorder in citrus fruit may be achievable. However, high cost of instrument and low speed of image acquisition are some of the disadvantages associated with the technique which could limit its on-line implementation or use (Clark et al., 1997; Hahn, 2009).

7.4. **Use of optical coherence tomography**

Optical tomography (OT) is another non-destructive analytical technique appropriate for assessing the internal structure of biological tissues (Magwaza et al., 2012a). The technique basically recreates the three-dimensional (3D) images of a targeted object through transmission and scattering of light. Diffraction tomography, diffuse optical tomography, and optical coherence tomography (OCT, Figure 10C) are the three existing central approaches to OT. However, OCT provides a more suitable approach for the evaluation of internal structures of biological tissues than other approaches (Lasser, 2003; Lu et al., 2004).

Optical coherence tomography is a novel imaging technology, capable of providing visual analyses of internal microstructure of biochemical components of agricultural products (Fujimoto, 2003; Huang et al., 1991; Magwaza et al., 2012a). The technique can provide non-destructive high-resolution (1-15µm) cross-sectional tomographic imaging of objects (to the depth of 1-1.5mm) in a real-time mode and its image acquisition time is 1-3 sper tomogram (Magwaza et al., 2012a; Meglinski et al., 2010). Previously, OCT has only been applied to the biomedical industries for diagnosis of tumours and monitoring of blood sugars in diabetic patients, (Sapozhnikova et al., 2006, 2003; Zagaynova et al., 2005). It has also been used to visualize lipid distribution within arterial vessel walls (Hirano et al., 2014). Similarly, it is currently explored for image analyses of
internal and external quality attributes of horticultural products such as apples (Verboven et al., 2013) and onions (Ford et al., 2012; Landahl et al., 2012).

The technique also has high depth in scattering media such as horticultural fruit as it offers means to detect and monitor physiological activities and changes of biological tissues during growth and development (Magwaza et al., 2012a). The results of these studies have been promising and potential use of this technique in detecting and monitoring rind physiological disorder in citrus fruit should be explored at a commercial scale.

7.5. Use of Vis/NIR technology

The application of Vis/NIR for determining external and internal quality parameters of citrus fruit was extensively reviewed by Magwaza et al. (2012a). These authors indicated that biological materials are opaque to radiation in the Vis/NIR regions of the electromagnetic spectrum and the tissue structures composed of cells such as nuclei, mitochondria, membranes, vesicles and cell walls contribute significantly to the scattering of Vis/NIR waves by fruit and vegetable tissues (Nicolaï et al., 2014). Usually, absorption and scattering of the electromagnetic waves usually steers the association between light and matter in the Vis/NIR regions of the electromagnetic spectrum (Nicolaï et al., 2014). The intricate chemistry between absorption and scattering depend on the spectral and spatial changes at the microstructure level of the complex refractive index (Bohren and Huffman, 1983). Basically, biological materials are opaque to radiation in the Vis/NIR regions of the electromagnetic spectrum and the tissue structures composed of cells such as nuclei, mitochondria, membranes, vesicles and cell walls contribute significantly to the scattering of Vis/NIR waves by fruit and vegetable tissues (Nicolaï et al., 2014). Absorption of
electromagnetic wave is primarily caused by the C-H, O-H, and N-H bonds of metabolites which can be absorbed provided the right energy is excited on one of the vibrational states of the molecules which has its own explicit absorption spectrum (Magwaza et al., 2012a; Nicolaï et al., 2014; Olarewaju et al., 2016). This absorption, which occur in the infrared region, is caused by overtones and combinations caused by the fundamental bonds of different metabolites (Nicolaï et al., 2014).

Table 3 displays an overview of the application of Vis/NIR in citrus fruit. Quality features of ‘Satsuma’ mandarin was assessed and tested using Vis/NIR spectra by Gómez et al. (2006). The authors then established the association amongst acquired spectra and the fruit physiological properties such as firmness, total soluble solids, and acidity. Absorption of pigments of citrus peel is high in the ultraviolet and visible electromagnetic spectral range of about 500 nm while low reflectance values are mainly originating from scattering in this range (Sighicelli et al., 2005). The authors argued that citrus fruit rind has NIR reflectance values of about 80% which decreased within two days because of pre-necrosis disorder. Furthermore, a team of researchers from Japan used Vis/NIRS to non-destructively and automatically grade fruit such as pear and apple fruit effectively based on colour and maturity (Gao et al., 2010; Han et al., 2006). Hence, Vis/NIRS has the capacity to absorb the peaks of interested metabolites that could be used to monitor and predict rind physiological disorder (Magwaza et al., 2012a).

Considering most of the available non-destructive methods for determining biochemical properties related to the susceptibility of citrus fruit to non-chilling physiological rind disorders, Vis/NIRS is possibly the most innovative technique in terms of instrumentation, accessories, chemometric tools
and applications (Nicolaï et al., 2007). This is because the technology is fast, objective and can accurately determine biomarkers of interest thereby providing an online classification of fruit based on its rind quality among other quality parameters for either local or export markets.

8. Future prospect and conclusions

The physical appearance of citrus fruit in the market plays a major role in its acceptability by consumers. A citrus fruit with no rind disorder will be attractive and mostly be of good quality. Hence, the significance of rind quality in horticultural (citrus) industries is crucial as it constitutes a major decisive factor in global consumer-producer relationship (Lehnert et al., 2014; Schütz et al., 2014). Some of the limiting factors affecting fresh citrus fruit market is the physiological rind disorders including the ones that occur because of low temperature (Cronje et al., 2011a). However, the major biochemical metabolite(s) responsible for these disorders are still unknown. Therefore, future scientific research should include non-targeted metabolomics approach for possible identification of responsible metabolite(s) triggering physiological rind disorders of citrus fruit. Once identified, non-destructive analytical technologies for detecting incidence of physiological rind disorders of citrus fruit would be a lot easier to develop or investigate.

Metabolomics is an unbiased identification and quantification or exploration of the entire metabolite profile of cell, tissue, or organism (biological system) at a given time under a definite set of conditions (Fiehn, 2002; Heyman and Dubery, 2016). It is a recently discovered technological methodology offering the potential of providing holistic understanding or detecting the minutest of variation in immensely complex biochemical systems of living organisms (Ernst et al., 2014; Heyman and Dubery, 2016; Naz et al., 2014; Rochfort, 2005). Moreover,
metabolomics plays a key role in screening metabolites which correlate with specific type of physiological disorder. Hence, they could be used as early indices of disorder in citrus industries (Naz et al., 2014). The metabolomics approached in analysing bio-markers could be targeted or non-targeted (Naz et al., 2014).

Targeted metabolomics involve exact quantification of known metabolite(s) using accurate analytical standards and concentrating on the changes of the quantitated metabolites which mostly correlates to a biological activity (Chen et al., 2008; Fiehn, 2002; Naz et al., 2014). However, non-targeted metabolomics approach involves the analysis of all probable metabolites existing in each sample without having foreknowledge of the metabolite which often leads to the generation of enormous data sets (Naz et al., 2014; Scholz et al., 2004).

Non-targeted metabolomics analysis of plant or fruit are particularly thought-provoking because of the increased intricacy of the system due to diversity of unknown secondary metabolites (up to 200,000 metabolites) included in the metabolome (Dixon and Strack, 2003; Ernst et al., 2014). However, this comprehensive approach carries the potential of identifying the mechanism(s) underlying the physiological rind disorders of citrus fruit which has been a long-term problem in citrus industries globally (Heyman and Dubery, 2016; Magwaza et al., 2013b).

Although, rind dry matter (DM) and non-structural carbohydrates were suggested as postharvest bio-markers triggering the incidence of physiological rind disorder of citrus fruit. Based on these suggestions, possible relationships with non-chilling physiological disorders are worth scientific evaluation to proffer solutions to the incidence of rind disorders. This investigation will help to
establish if the earlier mentioned bio-markers are responsible for the occurrences of various forms of non-chilling postharvest disorders expressed on other citrus fruit cultivars.
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Zagaynova, E. V, Abelevich, A.I., Zagaynov, V.E., Gladkova, N.D., Denisenko, A.N., Feldchtein,


Table 1: An overview of the application of computer vision system for detecting physiological disorders of citrus fruit

<table>
<thead>
<tr>
<th>SN</th>
<th>Fruit Type</th>
<th>Physiological Disorder</th>
<th>System Description</th>
<th>Wavelength Range (nm)</th>
<th>Model Type</th>
<th>Success Rate (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Grapefruit</td>
<td>Canker</td>
<td>Multispectral imaging system (3-CCD RGB, 640x320 pixels)</td>
<td>200-2500</td>
<td>Discriminate analysis</td>
<td>100</td>
<td>Balasundaram et al. (2009)</td>
</tr>
<tr>
<td>2</td>
<td>Mandarin and Orange</td>
<td>Oleocellosis Scales Stem-end injury Thrips scarring Wind scarring</td>
<td>Multivariate imaging system (3-CCD RGB, 768x576 pixel, 0.17mm resolution)</td>
<td>-</td>
<td>Principal component analysis and Multiple image analysis</td>
<td>92.8 91.5 87.2 90.5 100.0 100.0 92</td>
<td>López-García et al. (2010)</td>
</tr>
<tr>
<td>3</td>
<td>Lemon and mandarin</td>
<td>Rind defects</td>
<td>Machine vision systems (2 CCDs; one for RGB and the other for monochromatic) (768x576 pixels &amp; 0.17 mm/pixel resolution)</td>
<td>Centred at 750</td>
<td>Bayesian discriminant analysis</td>
<td>99</td>
<td>Aleixos et al. (2002)</td>
</tr>
<tr>
<td>4</td>
<td>Mandarin and orange</td>
<td>Chilling injury Medfly Oleocellosis Phytotoxicity Scales Scarring Sooty mould Stem-end injury Thrips scarring</td>
<td>Computer vision system - combination of Vis, NIR and fluorescence (monochromatic RGB camera for Vis and fluorescence, and Hamamatsu BeamFinder III, C5332-01 camera for NIR, 768-576 pixels &amp; 0.17 mm/pixel resolution)</td>
<td>RGB (350-400) Vis/NIR (400-1800)</td>
<td>Linear discriminate analysis</td>
<td>86</td>
<td>Blasco et al. (2009)</td>
</tr>
<tr>
<td>5</td>
<td>Mandarin and orange</td>
<td>Chilling injury Medfly Oleocellosis Phytotoxicity Scales Scarring Sooty mould Stem-end injury Thrips scarring</td>
<td>Computer vision systems (CCD camera, fluorescence tube, polarizing filter, Matrox Meteor II, 768x576 pixels &amp; 0.17 mm/pixel resolution)</td>
<td>-</td>
<td>-</td>
<td>100 98 100 100 93 100 85 99 100 100 94</td>
<td>Blasco et al. (2007)</td>
</tr>
<tr>
<td>6</td>
<td>Ruby red grapefruit</td>
<td>Canker</td>
<td>Hyperspectral imaging system (electron-multiplying charge-coupled-device, 658x496 pixels)</td>
<td>400-900</td>
<td>Principal component analysis</td>
<td>93</td>
<td>Qin et al. (2008)</td>
</tr>
</tbody>
</table>
Table 2: Application of X-ray imaging in detecting physiological disorders of citrus fruit

<table>
<thead>
<tr>
<th>SN</th>
<th>Fruit Type</th>
<th>Physiological Disorder</th>
<th>System Description</th>
<th>Model Type</th>
<th>Success Rate (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Orange</td>
<td>Granulation</td>
<td>Computer tomography X-ray scanner (2P-CCD camera, Nikon metrology 160 Xi Gun set, 1024x1024 pixel, 75 kV, 468 mA, 128.9 um, 60 ms</td>
<td>Naïve Bayesian method and K-nearest neighbour (kNN) approach.</td>
<td>96</td>
<td>Van Dael et al. (2016)</td>
</tr>
<tr>
<td>2</td>
<td>Lemon</td>
<td>Endoxerosis</td>
<td>Computer tomography X-ray scanner (2P-CCD camera, Nikon metrology 160 Xi Gun set, 1024x1024 pixel, 75 kV, 468 mA, 128.9 um, 60 ms</td>
<td>Naïve Bayesian method and K-nearest neighbour (kNN) approach.</td>
<td>94</td>
<td>Van Dael et al. (2016)</td>
</tr>
</tbody>
</table>
Table 3: An overview of the application of visible to near infrared spectroscopy in citrus fruit

<table>
<thead>
<tr>
<th>SN</th>
<th>Fruit Type</th>
<th>Physiological Quality parameter</th>
<th>System Description</th>
<th>Wavelength Range (nm)</th>
<th>Optimum Wavelength (nm)</th>
<th>Pre-processing Method</th>
<th>Model Type</th>
<th>Accuracy (R²)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mandarin</td>
<td>Rind total sugar (mg/g DW)</td>
<td>Mobile fibre-optic spectrophotometer (LabSpec2500® near infrared analyser in diffuse reflectance mode with SI array and two Peltier cooled InGaAs detectors)</td>
<td>350-2500</td>
<td>900-1700</td>
<td>Multiple scatter correction</td>
<td>Partial least square</td>
<td>0.93</td>
<td>Magwaza et al. (2012b)</td>
</tr>
<tr>
<td>2</td>
<td>Orange</td>
<td>TSS (°Brix)</td>
<td>Fibre optic probe and integrating sphere multipurpose analyser spectrometer</td>
<td>780-2500</td>
<td>900-1800</td>
<td>First derivative</td>
<td>Partial least square</td>
<td>0.83</td>
<td>Magwaza et al. (2013c)</td>
</tr>
<tr>
<td>3</td>
<td>Orange</td>
<td>Vitamin C (mg/100 mL)</td>
<td>Fibre optic probe and integrating sphere multipurpose analyser spectrometer</td>
<td>780-2500</td>
<td>900-1800</td>
<td>Multiple scatter correction</td>
<td>Partial least square</td>
<td>0.72</td>
<td>Magwaza et al. (2013c)</td>
</tr>
<tr>
<td>4</td>
<td>Gannan</td>
<td>SSC (°Brix)</td>
<td>FT- NIR spectrometer equipped with interferometer InGaAs detector</td>
<td>800-2500</td>
<td>-</td>
<td>Standard normal variate and Multiple scatter correction</td>
<td>Partial least square and principal component regression</td>
<td>0.99</td>
<td>Lu et al. (2006)</td>
</tr>
<tr>
<td>5</td>
<td>Mandarin</td>
<td>Acidity (pH)</td>
<td>Spectrophotometer (FieldSpec Pro FR with Lowell pro-lam 14.5 V Bulb/128690 tungsten halogen)</td>
<td>350-2500</td>
<td>400-2350</td>
<td>Moving average method and multiplicative scatter correction</td>
<td>Partial least square and principal component regression</td>
<td>0.84 and 0.81</td>
<td>Gómez et al. (2006)</td>
</tr>
</tbody>
</table>
Figure 1: Anatomy of citrus rind
Source: (Donald Blake; Slideplayer.com)
Figure 2: Visual (A) and microscopic (B) images of rind breakdown (RBD)
*Source:* (Cronje et al., 2011a; Magwaza et al., 2012a)

Figure 3: Rind staining or peel pitting at non-chilling temperature in ‘Navelate’ (A) and ‘Navelina’ (B) oranges
*Source:* (Alférez et al., 2003)
Figure 4: Oleocellosis in ‘Washington Navel’ orange
Sources: (A) Óscar Mario Castro Solano, The American Phytopathological Society
(B) (Knight et al., 2002)

Figure 5: Superficial flavedo necrosis blemish known as ‘Noxan’ in ‘Shamouti’ oranges
Source: (Ben Yehoshua et al., 2001)
Figure 6: Stem-end breakdown of ‘Marsh’ grapefruit (A) and ‘Valencia’ orange (B)
Source: (Ritenour and Dou, 2003)
Figure 7: A schematic depiction of chlorophylls a and b having Mg atom at the centre (A), and β-carotene (B).
Sources: (Jones, 1973; Salisbury and Ross, 1992)
Figure 8: A Schematic flow chart of the carotenoid biosynthesis pathway in plants

ABA, abscisic acid; E-CHX, E-carotene hydroxylase; β-CHX, carotene hydroxylase; CMK, 4-diphosphocytidyl-methylerythritol kinase; CMS, 4-diphosphocytidyl-methylerythritol synthase; CRTISO, carotene isomerase; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, DXP reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate-synthase; GGPP, geranylgeranyl diphosphate; GGPS, geranylgeranyl diphosphate synthase; G3P, D-glyceraldehyde 3-phosphate; HDR, hydroxymethylbutenyl 4-diphosphate reductase; HDS, hydroxymethylbutenyl 4-diphosphate synthase; E-LCY, lycopene cyclase; β-LCY, lycopene cyclase; MCS, methylerthritol 2,4-cyclodiphosphate synthase; MEP, 2-C-methyl-D-erythritol 4-phosphate; NCED, 9-cis-epoxycarotenoid dioxygenase; NSY, neoxanthin synthase; PDS, phytoene desaturase; PSY, phytoene synthase; PTOX, plastid terminal oxidase; VDE, violaxanthin de-epoxidase; ZDS, α-carotene desaturase; ZEP, zeaxanthin epoxidase.

Source: (Alquézar et al., 2008)
Figure 9: A schematic set-up of a typical computer vision system (A), hyperspectral imaging system (B) and multispectral imaging system (C)

Sources: (Leiva-Valenzuela et al., 2014; Yoo et al., 2010; Zhang et al., 2014)
Figure 10: A schematic representation set-up of a typical X-ray imaging (A), X-ray computed tomography (B) systems and optical coherence tomography system (C).
Sources: (Uchida et al., 2001; Vidhya et al., 2017)
CHAPTER 3

COMPARATIVE STUDY ON THE ROLE OF CANOPY POSITION ON PHYSICOCHEMICAL PROPERTIES OF ‘MARSH’ GRAPEFRUIT DURING POSTHARVEST NON-CHILLING COLD STORAGE*

* Submitted and formatted according to Scientia Horticulturae (Under review)
Abstract

The physicochemical properties of citrus fruit play a critical role in its maturity and quality determination. Hence, this study investigated the effect of canopy position and production region on physicochemical properties of ‘Marsh’ grapefruit at harvest and after storage at 7.5 °C for 3, 6, and 9 weeks. The study also evaluated the use of BrimA as an adoptable internal quality and maturity parameter for 'Marsh' grapefruit. Fruit from inside canopy (IC) and outside canopy (OC) were harvested from KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces in South Africa. Colour indices were measured using calibrated colorimeter while sugars were measured using high performance liquid chromatography. At harvest, IC fruit from MP province were more luminous than the OC fruit while inverse results were recorded for fruit from KZN. At harvest, IC fruit had higher percentage of titratable acidity (TA) (2.73%) than OC fruit (2.40%) from MP, with opposite results from KZN. Overall, our result suggested that canopy position affected some physicochemical properties of ‘Marsh’ grapefruit. However, harvested fruit displayed a higher level of some physicochemical properties over the period of cold storage. BrimA could potentially be used as an index of internal quality of grapefruit but further studies are needed.

Keyword: Acidity, BrimA, Citrus spp., Citrus paradisi, Fruit quality, Rind colour.
1. Introduction

‘Marsh’ grapefruit (*Citrus paradisi* Macfadyen) is an economically important citrus cultivar hybridized from an orange and a shaddock during the early 1700s in the West Indies (Kiani and Imam, 2007; Agustí et al., 2014). The fruit is widely cultivated in many parts of the world, including South Africa, United States of America, and Israel (Vacante, 2010) because they are highly nutritional and have a number of medicinal properties (Kiani and Imam, 2007).

In the fresh produce market, physicochemical properties such as colour, shape and size constitute initial factors influencing consumers’ decision to purchase (Opara and Pathare, 2014; Magwaza and Opara, 2015). Rind colour is perceived to be a major external quality factor as consumer preference is largely determined by fruit appearance in both local and international markets before any purchase is done (Singh and Reddy, 2006). Furthermore, previous studies have shown that colour and overall appearance are important quality attributes affecting acceptability of citrus and other kinds of fruit (Pathare et al., 2013). From a consumer purchase perspective, this suggests that good looking citrus fruit will most likely exhibit quality taste experience that ultimately translate to financial gains for citrus fruit growers. However, consumer choice of subsequent procurements is dependent on fruit internal chemical properties such as total soluble solids (TSS), titratable acidity (TA) as well as the ratio of total soluble solids to titratable acidity (TSS/TA) (Opara and Pathare, 2014; Magwaza and Opara, 2015). Further indices of flavour quality include sweetness index (SI), determined by the quantity of individual non-saturated sugar components (Beckles, 2012; Magwaza and Opara, 2015), and total sweetness index (TSI), which is determined based on the contribution of main sugar components in relation to sucrose (Baldwin et al., 1998; Magwaza
and Opara, 2015). Being a non-climacteric fruit, flavour quality of citrus fruit generally decreases after harvest (Baldwin, 2009).

Although TSS/TA ratio is mostly used as determinant factor for citrus fruit maturity and internal quality, Jordan et al. (2001) reported that sometimes it does not share any relationship with organoleptic internal quality perception of fruit. As a result, it has been suggested that a quality measurement parameter that is more associated with citrus fruit internal quality is BrimA (difference of TSS and TA). BrimA, has been reported to be a better internal quality parameter for measuring internal quality or maturity of horticultural products such as grapes (Jordan et al., 2001), pomegranate (Fawole and Opara, 2013) and oranges (Obenland et al., 2009) than other industry standards including TSS, TA and TSS/TA (Magwaza and Opara, 2015). In view of this, California Department of Food and Agriculture set BrimA as industry standard for measuring internal quality of navel oranges (Ross, 2012). BrimA, increasingly becoming an international standard of horticultural fruit maturity and quality, was well introduced, and adequately discussed in a recent review of literature by Magwaza and Opara (2015).

Fruit position within tree canopy, based on the level of exposure of fruit to sunlight, is an important preharvest factor, which has been identified as a possible contributor to the postharvest quality of horticultural crops. Canopy positions have long been found to affect vitamin C content of grapefruit (Harding and Thomas, 1942), and physicochemical properties of ‘Nules clementine’ mandarin fruit thereby influencing its outward appearance (Cronje et al., 2011a; Cronje et al., 2013; Magwaza et al., 2013a). However, very limited research has been conducted to study the effect of canopy position on physicochemical properties of ‘Marsh’ grapefruit. Hence, the aim of
this study was to evaluate the effect of canopy position and production region on physicochemical properties of ‘Marsh’ grapefruit at harvest and after 3, 6, and 9 weeks of cold storage at 7.5 °C. The study also evaluated the use of BrimA as an adoptable internal quality parameter for ‘Marsh’ grapefruit.

2. Materials and methods

2.1. Reagent and standards

All chemicals including Sodium Hydroxide (NaOH), phenolphthalein, Folin-Ciocalteu reagent, metaphosphoric acid (MPA), sodium carbonate, gallic acid, quercetin, vitamin C, 2, 6 dichloroindophenol dye, 2,2-diphenyl-1-picrylhydrazyl (DPPH), acetone, ethanol (HPLC grade) and sugars standards (sucrose, D-glucose, and D-fructose) were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK). A Phenomenex® column (Rezex RCM - Monosaccharide) was used in the analyses. Water was purified in a Milli-Q Integral Water Purification System (Merck Millipore corporation, Billerica, MA, USA; $\sigma = 18$ M $\Omega$ cm$^{-1}$).

2.2. Fruit harvesting, sampling, and postharvest handling

Experiments were conducted using ‘Marsh’ grapefruit budded on ‘Troyer’ Citrange ([Poncirus trifoliata (L.) Raf.] × [C. sinensis]) and x639 ([Poncirus trifoliata (L.) Raf.] × [C. reshni]) rootstocks planted in 1993 at Bolton Citrus Farm, KwaZulu-Natal (KZN) (31° 34′ 44″ S, 28° 44′ 59″ E) and Unifruitti Farm, Mpumalanga (MP) (24° 22′ 24.39″ S, 30° 42′ 17.67″ E) provinces, respectively. Three fruit per canopy position were harvested from a height of 1-2 m from 50 uniform sized trees, from each farm, at commercial maturity during 2015 and 2016 sessions. The canopy positions were inside canopy (IC), i.e. fruit receiving less than 80% of full sunlight, and
outside canopy (OC), fruit exposed to 90-100% of full sunlight, of a fruit tree according to Cronje et al., (2011a). The rainfall (mm), relative humidity (%), maximum and minimum temperature (°C) registered during the growing season in KZN and MP provinces are displayed in Figure 1A-D. After harvesting, fruit were transported within 48 h at ambient temperature in ventilated cartons to the horticultural research laboratory where fruit were washed and sorted for blemishes and fruit damage. Upon arrival at the laboratory, fruit were left for 24 h at room temperature (20 ± 1ºC) to equilibrate after which each fruit was labelled, weighed, and transferred into cold storage (7.5 ± 0.5ºC) for 9 weeks. Ten individual fruit using one fruit per replicate were analysed at 3 weeks interval for 9 weeks (weeks 0, 3, 6 and 9).

2.3. Rind colour

Colour parameters (luminosity [L*], greenness [a*], and yellowness [b*]) were measured at three equidistant points around the equatorial axis of each fruit during each sampling. A Minolta NR-4000 colorimeter (Minolta NR 4000, Osaka, Japan) was used after calibration with a standard white tile (CR-A43; Y = 93.1, x = 0.3203) (Terry et al., 2007; Pathare et al., 2013). The colour index (CI) was calculated using Eq. 1 (Magwaza et al., 2013b). From consumer point of view, a fruit with high CI is found acceptable.

\[
CI = \frac{(1000 \times a)}{(L \times b)} \tag{1}
\]

Where CI is colour index, a is greenness, L is luminosity and b is the yellowness of the fruit.
2.4. **Citrus fruit juice extraction**

Fruit were cut into two halves after which the juices were extracted using a commercial juicer (Mellerware, South Africa). Extracted juices were then sieved through 4-fold muslin clothes into specimen containers which were stored in -20 °C for future analysis.

2.4.1. **Determination of TSS, TA, TSS to TA ratio and BrimA**

Total soluble solids were measured using a digital hand-held refractometer (Palette, Atago, Co. Limited, Japan). Titratable acidity was determined according to the method described by Rekha et al. (2012) with slight modification. Briefly, 20 mL of fruit juice was placed inside a beaker with 5 drops of phenolphthalein added and then titrated with NaOH until a change of colour to pink, which signifies the endpoint was noticed. The ratio of TSS to TA and BrimA indices were calculated using Eqs, 2 and 3, respectively.

\[
\text{TSS: TA ratio} = \frac{\text{TSS}}{\text{TA}} \quad 2 \\
\text{BrimA} = \text{TSS} - k (\text{TA}) \quad 3
\]

Where \(k\) is tongue sensitivity index which normality ranges between 2 and 10 (Jordan et al., 2001). \(k = 2\) was used in this study which also avoided of negative values (Fawole and Opara, 2013). All destructive analyses were done on individual fruit samples.

2.4.2. **Extraction and quantification of soluble sugars**

Soluble sugars were extracted from fruit juice samples diluted 1:10 (v/v) with ultra-pure water as described by Zielinski et al. (2014) with modifications and determined according to Olarewaju et
al. (2017). Briefly, 2 mL of diluted sample juice was centrifuged at 15000 rpm (Himac Centrifuge, Hitachi Koki Co., Ltd., Tokyo, Japan) for 20 min. Concentration of sucrose, glucose and fructose was then quantified using an isocratic HPLC system equipped with a refractive index detector. A 1 mL of diluted extract was injected into a Rezex RCM monosaccharide Ca\(^+\) (8%) column of 7.8 mm diameter × 300 mm (Phenomenex, Torrance, CA, USA) with a SecurityGuard\textsuperscript{TM} cartridges of 4 mm × 3 mm (Phenomenex). The mobile phase used was ultra-pure HPLC-grade water at a flow rate of 0.6 mL/min with the column compartment temperature set at 80 °C using a thermos-stated column compartment (G1316A, Agilent). The presence and concentration of individual sugars were determined by comparing peak area of samples with peak area and concentration of a known sugar standard curve (0.05 – 1.25 mg/L; \(R^2 = 0.99\)).

2.4.3. Determination of sweetness index and total sweetness index

Sweetness index and TSI were calculated using Eqs. 4 and 5, respectively according to Magwaza and Opara (2015).

\[
\text{SI} = (1.00 \text{[glucose]}) + (2.30 \text{[fructose]}) + (1.35 \text{[sucrose]})
\]

\[
\text{TSI} = (1.00x\text{[sucrose]}) + (0.76x\text{[glucose]}) + (1.50x\text{[fructose]})
\]

2.5. Experimental layout and data analysis

Experiments were laid out using a completely randomised design (CRD) with individual fruit as replicate. All statistical analyses were performed using GenStat® 18\textsuperscript{th} Edition (VSN International, Hemel Hempstead, UK). Data was subjected to analysis of variance (ANOVA) with canopy position, production region and cold storage duration as factors. Season two (2016) data from MP
were treated as missing values and least significant difference (LSD) at 5% level was considered significant.

3. **Results and discussion**

3.1. **Comparative effect of canopy position, cold storage, and production region on fruit appearance**

There were significant interactions between canopy position, production region and postharvest storage time for all colour parameters ($p \leq 0.05$) except the interaction between canopy position and production region for $L^*$ ($p = 0.511$) and interaction between canopy position and production region for CI ($p = 0.112$) (Figure 2). Rind colour is an important attribute affecting fruit external appearance, consumer appeal and purchase decision (Khalid et al., 2012; Pathare et al., 2013). The $L^*$ values ranged between 79.2 and 80.0 for outside canopy (OC) fruit and between 78.9 and 81.8 for inside canopy (IC) fruit from KZN province. Luminosity values ranged between 81.4 and 83.1 for IC fruit and 80.0 and 81.1 for OC fruit from MP. Our data indicated that there was significant difference between canopy positions with IC fruit from MP province being more luminous than the OC fruit, which had steady decline in $L^*$ during cold storage (Figure 2A). However, this was contrary with fruit from KZN province. The $a^*$ (Figure 2B) and CI (Figure 2D) values of the fruit followed the same trends, with $a^*$ values increasing towards positive axis of the colour chart suggesting that the fruit colour were consistently changing from green to yellow colour. The greener rind colour of IC fruit at harvest indicated a reduced expression of carotenoids during colour development (Khalid et al., 2012; Cronje et al., 2013). Similar findings were reported in earlier studies (Cronje et al., 2011b; Khalid et al., 2012; Magwaza et al., 2014), where OC fruit from KZN had higher CI compared to that recorded for IC fruit, but lower in OC fruit from MP with gradual
declining trend in both orchards after postharvest cold storage (Figure 2D). It is suggested that the reduced intensity of sunlight reaching IC fruit caused the delayed colour change.

External appearance in terms of fruit colouration is the first quality parameter assessed by potential consumers who have colour preference for a specific product (Crisosto et al., 2003; Leon et al., 2006; Pathare et al., 2013) The yellowness (b*) (Figure 2C) values, which ranged from 58.3 to 63.5 for IC fruit and 53.6 to 64.7 for OC fruit from KZN; and 51.9 to 62.5 for IC fruit and 54.1 to 63.5 for OC fruit from MP province is an indication of fruit quality in the trade markets. The IC fruit from MP were less yellow compared to OC fruit. Similar observations were reported by Cronje et al. (2011a) who investigated the effect of canopy position on rind colour of ‘Nules Clementine’ mandarin and suggested the varying results could be related to the role of radiation and temperature during pigment production. Similar results of development of desirable yellow colour during cold storage were also reported by Magwaza et al. (2013a) who investigated how canopy position affected rind biochemical profile of ‘Nules Clementine’ mandarin. Despite the variability observed, rind colour of all fruit developed more desirable yellow colour during postharvest storage with no incidence of non-chilling rind physiological disorders.

3.2 Effect of canopy position, cold storage, and location on physicochemical properties

Physicochemical properties such as TSS, TA and TSS/TA are vital indices commonly used to determine citrus fruit maturity and quality in the industry (Cheong et al., 2012). Contrary to expectation, canopy position had no significant effect on TSS ($p = 0.914$) but had highly significant effect on TA and TSS/TA ($p < 0.001$) (Figure 3). Postharvest storage time and production region had highly significant effect on TSS, TA and TSS/TA ($p < 0.001$). A significant interaction
occurred between canopy position, postharvest storage time and production region for the internal quality parameters \( p < 0.001 \). Generally, the flavour of a fruit relies greatly on TSS, TA and TSS/TA because they determine the overall sensory quality of stored fruit (Mattheis and Fellman, 1999; Sun et al., 2012). Titratable acidity was higher in OC fruit (3.02%) than IC fruit (2.78%) at week 0 for fruit from KZN. However, at weeks 3, 6 and 9, TA in OC fruit became lower than IC fruit from KZN, while IC fruit generally had higher TA than OC fruit throughout the entire period of cold storage for fruit from MP province (Figure 3B).

The TSS/TA ratio is a suitable means of determining fruit maturity and quality, since TA usually degrades (because of the catabolism of citric acid) when there is concurrent accumulation of sugars (TSS) (Legua et al., 2011; Navarro et al., 2015). In this study, canopy position influenced the TSS/TA ratio such that fruit from OC generally had higher TSS/TA than those from IC for fruit from both production regions after cold storage (Figure 3C). However, IC fruit had a higher TSS/TA (3.88%) than OC fruit (3.24%) at week 0 for fruit from KZN. However, as storage progressed the differences shifted to a higher TSS/TA ratio in OC fruit compared to IC fruit as shown in Figure 3C.

### 3.3 BrimA as a measure of internal fruit quality

The study also evaluated the potential of BrimA as an adoptable parameter for measuring internal quality of grapefruit. For fruit from MP, BrimA was consistently higher in OC fruit than in IC fruit throughout cold storage. This suggests better flavour in OC fruit than IC fruit (Figure 3D). However, for fruit from KZN, results were more inconsistent throughout cold storage. BrimA was higher in IC fruit than OC fruit, with a reversed pattern at weeks 6 and 9 (Figure 3D). The
inconsistent results could be attributed to exposure of fruit to different regional climates as indicated in Figures 1A and 1B. The amount of rainfall (Figure 1A) was exceptionally higher with lower relative humidity (Figure 1B) in KZN than in MP during the month of harvest (May 2016).

The correlation factors between BrimA and other internal quality parameters of ‘Marsh’ grapefruit from both production regions were tested and as expected, there was a strong positive correlation between TSS/TA and BrimA ($r = 0.94$) (Table 1). There was also a moderate positive correlation between BrimA and TSS ($r = 0.65$) while there was a negative relationship between BrimA and TA ($r = -0.65$). These results suggest the potential of BrimA as a parameter for measuring quality of ‘Marsh’ grapefruit. However, further research involving sensory (especially flavour) evaluation would be required to confirm the association between BrimA and consumer acceptability for grapefruit as earlier suggested by Jordan et al. (2001) and Obenland et al. (2009), who found BrimA to be more related to flavour.

### 3.4 Effect of canopy position, cold storage, and production region on soluble sugars

The interaction between the three factors, canopy position, production region and postharvest storage time was also significant ($p < 0.001$). The effect of canopy position was evaluated on three major non-structural carbohydrate components of ‘Marsh’ grapefruit juice. The carbohydrates included fructose, glucose, and sucrose. Similarly, Canopy position had significant effects on both glucose and sucrose ($p < 0.001$) but not on fructose ($p = 0.955$) (Figure 4). Results indicated that glucose was more concentrated for IC fruit than OC fruit from both regions at week 0. However, concentration was inconsistent over the storage period. At week 6, glucose concentrations in fruit from KZN were higher in OC fruit than in IC fruit, whereas in fruit from MP, glucose
concentrations were higher in IC fruit than OC fruit (Figure 4B). An early report shares some similarities on sugar concentrations in ‘Pumelo’ grapefruit by Sun et al. (2012), in which fructose and glucose concentrations in the juice of ‘Marsh’ grapefruit were fundamentally steady at different stages of postharvest cold storage. However, concentrations of sucrose in the juice declined over the storage periods, especially on week 9 (Figure 4C), which agrees with previous reports by Magwaza et al. (2013a) in the rind of ‘Nules Clementine’ mandarin and Holland et al. (2005) in two orange cultivars, ‘Navelate’ and ‘Pinalate’, stored at 2 and 12 °C.

3.5 Effect of canopy position, cold storage, and location on sweetness index and total sweetness index

Consumer preference for quality fruit is largely determined by organoleptic properties especially taste. Determining consumer acceptability of horticultural produce using sweetness index (SI) and total sweetness index (TSI), which are proportion of individual sugar components (sucrose, fructose, and glucose) has been suggested (Beckles, 2012). The canopy position, production region and postharvest storage time had significant effect on SI and TSI of ‘Marsh’ grapefruit \( (p < 0.001) \). Similarly, interaction between these factors also had significant effect on the parameters \( (p \leq 0.05) \).

At week 0, the SI value for IC fruit (12.45, 12.50) was higher than OC fruit (11.50, 11.53) from KZN while the opposite was found for fruit from MP province (Figure 5A). Fruit from MP consistently increased in taste quality as indicated by SI during postharvest for IC and OC fruit with sudden increase at week 6 to its peak and then a decline at week 9 suggesting the beginning of senescence. The TSI followed similar trends with SI (Figure 5). Therefore, it could be hypothesised that production region plays a significant role in the physiological response of
‘Marsh’ grapefruit in either canopy position. Although OC fruit are potentially higher in quality due to their exposure to higher levels of sunlight (Rosales et al., 2011).

4. Conclusions

This study has shown the effects of canopy position on physicochemical properties of ‘Marsh’ grapefruit at harvest and after 3, 6 and 9 weeks of cold storage. Canopy position effects appeared highly dependent on production region both at harvest and after cold storage. Physicochemical properties affected by canopy position included rind colour (L*, a*, b*), TA, TSS/TA, BrimA, concentrations of glucose and sucrose, SI and TSI. The effect of canopy position effect on TSS and fructose concentration was negligible. Also, there were strong interactions between canopy position, region, and storage time. Production region also influenced the physiological performances of fruit and there were strong interactions between canopy position, production region, and storage time. BrimA also demonstrated potential for use as a maturity indicator for grapefruit. With such contrasting results, which could possibly be attributed to rootstock and climate differences from the two regions further studies are suggested to validate these findings.

Acknowledgements

This work was made possible by the funding received from National Research Foundation (Thuthuka Project Number: 94022) and Citrus Research International through Postharvest Innovation Programme (PHI 2/2014). The authors are grateful to Dr Paul Cronje for his technical assistance. We appreciate the fruit donations from Bolton Citrus Farm (KwaZulu-Natal), and Unifruitti Farm f(Mpumalanga) towards the success of this study. We also express our gratitude to the South African Weather Services for providing us with weather information.
References


Figure 1: Rainfall (A), relative humidity (B), maximum temperature and minimum temperature (C) registered during 2015 and 2016 seasons in KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces.
(Source: South African Weather Services)
Figure 2: Effect of canopy position (inside canopy (IC) and outside canopy (OC)), on luminosity (L*) (A), greenness (a*) (B), yellowness (b*) (C) and colour index (D) of ‘Marsh’ grapefruit from KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD: least significant difference; P: canopy position; T: postharvest storage time; S: production region; * stands for an interaction between factors, vertical bars represent standard error.
Figure 3: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on total soluble solids (A), titratable acidity (B), total soluble solids/titratable acidity (C) and BrimA (D) of ‘Marsh’ grapefruit from KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

*LSD: least significant difference; P: canopy position; T: postharvest storage time; S: production region; * stands for an interaction between factors, vertical bars represent standard error.*
Figure 4: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on fructose (A), glucose (B) and sucrose (C) of ‘Marsh’ grapefruit juice content from KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD: least significant difference; P: canopy position; T: postharvest storage time; S: production region; * stands for an interaction between factors, vertical bars represent standard error.
Figure 5: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on sweetness index (A) and total sweetness index (B) of ‘Marsh’ grapefruit from KwaZulu-Natal (KZN) and Mpumalanga (MP) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD: least significant difference; P: canopy position; T: postharvest storage time; S: production region; * stands for an interaction between factors, vertical bars represent standard error.
Table 1: Correlation coefficients between internal quality indices of both inside and outside canopy fruit from two production regions (KwaZulu-Natal and Mpumalanga Provinces) of citrus fruit in South Africa

<table>
<thead>
<tr>
<th>Parameters</th>
<th>BrimA (%)</th>
<th>TSS (°Brix)</th>
<th>TSS/TA (%)</th>
<th>TA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BrimA (%)</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSS (°Brix)</td>
<td>0.6488</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSS/TA (%)</td>
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<td>0.3925</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>TA (%)</td>
<td>-0.6534</td>
<td>0.1521</td>
<td>-0.8257</td>
<td>-</td>
</tr>
</tbody>
</table>

Bold value indicates the strong correlation between BrimA and TSS/TA. TSS: total soluble solids; TA: titratable acidity.
CHAPTER 4
EFFECT OF CANOPY POSITION ON RIND BIOCHEMICAL PROPERTIES OF 'MARSH' GRAPEFRUIT DURING POSTHARVEST COLD STORAGE AT NON-CHILLING TEMPERATURE*

* Submitted and formatted according Scientia Horticulturae (Under review).
Abstract
A two-year trial was conducted to evaluate the influence of fruit position within tree canopy on biochemical properties of ‘Marsh’ grapefruit rind. The study was done in relation to the development of rind pitting during postharvest non-chilling cold storage. Fruit from inside canopy (IC) and outside canopy (OC) were harvested at commercial maturity in May 2015 and 2016 from KwaZulu-Natal (KZN) and Limpopo (LMP) in South Africa. Analyses were performed on grapefruit rind after harvest (week 0) and after 3, 6 and 9 weeks of non-chilling cold storage at 7.5 ± 0.5 °C. Colour indices were measured using calibrated chromameter while total phenolic concentration was determined by Folin Ciocalteu method. Canopy position showed a significant (p < 0.001) effect on parameters such as colour index (CI), total carotenoid content, fructose, sucrose, total flavonoid concentration, and radical-scavenging activities. Reducing sugars (fructose and glucose) were more concentrated in IC fruit harvested from both production region than OC fruit at weeks 0 and 3 but inverse results occurred at weeks 6 and 9. Canopy position showed no significant (p > 0.05) effect on vitamin C content of the fruit rind. However, production region significantly (p < 0.001) influenced vitamin C (14.4, 14.2, 14.4, 14.8 mg /g DW) and (12.3, 16.1, 15.9, 14.6 mg /g DW) of fruit rind at weeks 0, 3, 6, and 9 from KZN and LMP, respectively. Furthermore, vitamin C had a strong negative but significant correlation with fructose (r = 0.68), glucose (r = 0.66) and sucrose (r = 0.63). This study revealed that both canopy position and production region can influence biochemical properties of ‘Marsh’ grapefruit rind.

Keywords: Citrus, Colour, Physiological rind disorder, Radical-scavenging activities, Sugars, Vitamin C.
1. **Introduction**

‘Marsh’ grapefruit (*Citrus paradisi* Macfadyen) are widely cultivated in many part of the world including South Africa, United States of America and Israel (Vacante, 2009) for its high nutritional and medicinal properties (Kiani and Imam, 2007). However, incidence of rind physiological disorder at non-chilling temperatures such as rind pitting causes severe economic losses to fruit growers because the disorder affects the sub-epidermal cells of the fruit surface which invariably compromise the external appearance of the fruit (Agustí et al., 2001; Alférez et al., 2005, 2003; Lafuente and Sala, 2002). Fruit appearance plays a crucial role in the fresh fruit market because it influences consumer buying decision (Pathare et al., 2013). That is, a fruit without rind pitting disorder is largely preferred compared to fruit with the disorder. More importantly, rind disordered fruit is often rejected by consumers in the market even though its internal quality is not compromised by the disorder (Agustí et al., 2001).

Biotic and abiotic factors during preharvest and postharvest life influence rind disorders, but it has been problematic to associate a definite inductive factor to a postharvest rind disorders. This is because different factors can induce similar symptomology in fruit (Alférez and Burns, 2004; Grierson, 1986). Despite various scientific studies by researchers to understand the primary factor triggering the disorder, the subject is still unknown. Meanwhile, rind biochemical properties such as carbohydrates and phytochemicals such as total phenolic and total flavonoids concentrations, and vitamin C have been reported to play critical roles in the response of citrus fruit rind to postharvest physiological stresses (Cronje et al., 2011a; Magwaza et al., 2013a). These stresses manifest as various types of physiological disorders on different citrus cultivars under various postharvest storage conditions. These include peel or rind pitting (Alférez and Burns, 2004), rind
breakdown (Magwaza, 2013) and chilling injury (Alférez et al., 2005). Similarly, canopy position, which determines varying intensity of sunlight and other abiotic factors reaching different positions of tree canopy affects the physiological activities and biochemical properties of the fruit rind (Cronje et al., 2011a; Magwaza et al., 2014; Syvertsen and Albrigo, 1980). Canopy positions have been found to affect the biochemical properties of citrus fruit rinds such as ‘Nules clementine’ mandarin fruit thereby influencing its outward appearance (Cronje et al., 2013; Magwaza et al., 2013a). Therefore, possible link among canopy position, rind soluble sugars and rind breakdown of mandarin citrus fruit was suggested by Cronje et al. (2011b). However, limited research has been conducted to investigate the effect of canopy position on rind biochemical properties of ‘Marsh’ grapefruit in relation to the incidence of rind pitting disorder. Therefore, the aim of this study was to investigate the effect of canopy position and production region on the biochemical properties of ‘Marsh’ grapefruit rind during postharvest cold storage in relation to rind pitting.

2. Materials and methods

2.1. Reagents and standards

All chemicals including Sodium Hydroxide (NaOH), Folin-Ciocalteu reagent, metaphosphoric acid (MPA), sodium carbonate, gallic acid, quercetin, vitamin C, 2, 6 dichloroindophenol dye, 2,2-diphenyl-1-picrylhydrazyl (DPPH), acetone, ethanol (HPLC grade) and sugars standards (sucrose, D-glucose, and D-fructose) were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK). A Phenomenex® column (Rezex RCM - Monosaccharide) was used in the analyses. Water was purified in a Milli-Q Integral Water Purification System (Merck Millipore corporation, Billerica, MA, USA; σ = 18 M Ω cm⁻¹).
2.2. Plant materials

Experiments were conducted using ‘Marsh’ grapefruit budded on ‘Troyer’ Citrange ([*Poncirus trifoliata* (L.) Raf.] × [*C. sinensis*]) and × 639 ([*Poncirus trifoliata* (L.) Raf.] × [*C. reshni]*) rootstocks planted in 1993 on Bolton Citrus Farm, KwaZulu-Natal (KZN) (31° 34′ 44″ S, 28° 44′ 59″ E) and Olifant Rivers Farm, Limpopo (LMP) (32° 75′ 28″ S, 35° 89′ 31″ E) provinces, respectively. Three fruit per canopy position were harvested from a height of 1-2 m of 50 uniform sized trees, from each orchard, at commercial maturity over two 2015 and 2016 seasons. The canopy positions were inside canopy (IC), that is fruit exposed to less than 80% of full sunlight, and outside canopy (OC), fruit exposed to 90-100% of full sunlight, of a fruit tree as described by Cronje et al. (2011b). The rainfall (mm), relative humidity (%), maximum and minimum temperature (°C) registered during the growing seasons in KZN and LMP provinces are displayed in Table 1. After harvesting, fruit were transported within 48 h at ambient temperature in ventilated cartons to horticultural research laboratory where fruit were washed and sorted for blemishes and fruit damage. Fruit were left for 24 h at room temperature 20 ± 1 °C to equilibrate after which fruit were labelled, weighed, and transferred into cold storage (7.5 ± 0.5°C) for 9 weeks. Ten individual fruit using one fruit per replicate were analysed at 3 weeks interval for 9 weeks (weeks 0, 3, 6 and 9).

2.3 Rind colour measurement

Rind colour of remainder fruit was measured at three equidistant points around the equatorial axis of each fruit using a chromameter (Konica Minolta NR 4000, Osaka, Japan) after calibration using a standard white tile (CR-A43; Y = 93.1, x = 0.3138; y = 0.3203) (Pathare et al., 2013; Terry et al., 2007). Measurements were performed at three weeks’ interval for nine weeks of cold storage.
Colour indices measured included luminosity ($L^*$), greenness ($a^*$), and yellowness ($b^*$) while colour index (CI) was calculated using Eq. 1 (Magwaza et al., 2013b). From consumer point of view, a fruit with high CI is found acceptable.

\[
CI = \frac{1000 \times a}{L \times b}
\]

Where CI is colour index, $a$ is greenness, $L$ is luminosity and $b$ is the yellowness of the fruit.

### 2.4. Sample preparation

The rind was manually peeled off the fruit using table knife, snap frozen in liquid nitrogen and stored at -80 °C before freeze-drying over a period of three days using Virtis Benchtop freeze drier system (ES Model, SP Industries Inc., Warmister, USA) at 0.015 kPa and -75 °C. Dry matter (DM) was calculated by subtracting the mass of the freeze-dried samples from fresh samples and expressed as percentage DM. Dried samples were then milled into a fine powder using pestle and mortar and stored in -20 °C for further analysis.

### 2.5. Determination of total carotenoid content

Total carotenoid content was determined according to Lichtenthaler (1987) with slight modifications. The lyophilized sample (150 mg ± 0.5) was weighed into test tube followed by the addition of 2 mL of 80% (v/v) acetone before centrifugation for 10 min using GenVac® (SP Scientific, Genevac LTD., Suffolk, UK). The absorbance values of the supernatants were read at 470, 646.8, and 663.2 nm using Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific
Instruments, Inc., Columbia, USA) for maximum detection of carotenoids, chlorophyll a and b and total carotenoid content were calculated using Eqs. 1, 2, and 3, respectively

\[
C_a = 12.25 \, A_{663.2} - 2.79 \, A_{646.8} \quad 1 \\
C_b = 21.50 \, A_{646.8} - 5.10 \, A_{663.2} \quad 2 \\
C_c = (1000 \, A_{470} - 1.82 \, C_a - 85.02 \, C_b)/198 \quad 3
\]

Where \(C_a\) is chlorophyll a, \(C_b\) is chlorophyll b and \(C_c\) is total carotenoid content.

2.6. Extraction and determination of rind soluble sugars

Soluble sugars were extracted from 150 ± 0.5 mg of the lyophilized sample using 62.5% (v/v) aqueous methanol as described by Magwaza et al. (2014b) and modified by Olarewaju et al. (2017). Extracts were then diluted in ultra-pure water (1:10). The concentration of fructose, glucose, and sucrose were then quantified using an isocratic HPLC binary pump system (Agilent Technologies, UK) equipped with a refractive index detector. Diluted extract (1 mL) was injected into a Rezex RCM monosaccharide Ca⁺ (8%) column of 7.8 mm diameter × 300 mm (Phenomenex, Torrance, CA, USA) with a SecurityGuard™ cartridges of 4 mm × 3 mm (Phenomenex). The mobile phase used was ultra-pure HPLC-grade water at a flow rate of 0.6 mL/min with the column compartment temperature set at 80 °C using a thermos-stated column compartment (G1316A, Agilent). The presence and concentration of individual sugars were determined by comparing peak area of samples with peak area and concentration of a known sugar standard curve (0.05 – 1.25 mg/L; \(R^2 = 0.99\)).
2.7. Sample extraction for total phenolics and flavonoid analyses

Extraction was carried out according to modified method of Moo-Huchin et al. (2015) by Olarewaju et al. (2017). Briefly, a lyophilized sample of 150 mg ± 0.5 mg was weighed into a test tube and 3 mL of 50:50 (ethanol: water) v/v was added. The test tube, covered with aluminium foil, containing the mixture was subsequently placed in a shaking water bath (Gesellschaft für (GFL), Labortechnik mbH, Burgwedel, Germany) at 70 °C for 2 hrs, while a sample was removed and vortexed for 20 s at every 30 min interval. Sample were left to cool to room temperature, then centrifuged for 10 min using a GenVac® (SP Scientific, Genevac LTD., Suffolk, UK), and filtered using 0.45 µm nylon filter. Extracts were stored at -20 °C for further analysis of total phenolic and total flavonoid concentrations.

2.7.1 Determination of total phenolic concentrations

Extracts were analysed according to modified method of Moo-Huchin et al. (2015) by Olarewaju et al. (2017) for total phenolic concentration. Briefly, sample extract (10 µL) was measured into a 4.5 mL disposable cuvette in triplicate followed by the addition of 1.6 mL of distilled water, Folin-Ciocalteu reagent (100 µL) and 300 µL of sodium carbonate solution. The solution was mixed and incubated in the dark at room temperature for 2 h before the absorbance was measured at 765 nm using Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, USA). Gallic acid was used to generate a standard curve and total phenolic concentrations were expressed as mg gallic acid equivalent (GAE) /g DM.
2.7.2. **Determination of total flavonoid concentrations**

Extracts were analysed for total flavonoid concentration according to modified method of Lin and Tang (2007) by Olarewaju et al. (2017). Briefly, extract (100 µL) was measured into a 4.5 mL disposable cuvette followed by the addition of 3 mL of sodium hydroxide solution. The mixture was agitated and incubated at room temperature for 10 min. Absorbance was measured in triplicate at 420 nm using a Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, USA). Quercetin was used to generate a standard curve and total flavonoid concentration was expressed as mg quercetin equivalent (QTE) /g DM.

2.8. **Sample extraction for determination of vitamin C and radical-scavenging activities**

Extraction was carried out according to a method of Karioti et al. (2004) and Olarewaju et al. (2017). A lyophilized sample of 150 mg ± 0.5 mg was measured into a test tube followed by the addition of 5 mL of 3% MPA and incubated on ice cubes for 5 min. The extract was centrifuged for 20 min using GenVac® (SP Scientific, Genevac LTD., Suffolk, UK). The supernatant was stored in -20 °C for further analysis of vitamin C and DPPH radical-scavenging activities.

2.8.1. **Determination of Vitamin C**

Vitamin C was determined according to Olarewaju et al. (2017). Briefly, supernatant (0.5 mL) was measured into a test tube followed by the addition of 2.5 mL of 0.005% of 2, 6 dichloroindophenol dye. The mixture was incubated in the dark for 10 min at room temperature. Absorbance was read in triplicate at 515 nm against a 3% MPA solution blank under dim light using Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, USA) and the amount of vitamin C was calculated from a linear standard curve (0.00 – 100.00 µg/g; R² = 0.96).
2.8.2. **Determination of radical-scavenging activities**

Determination of radical-scavenging activities using DPPH assay was carried out according to Karioti et al. (2004) with modifications according to Olarewaju et al. (2017). Briefly, supernatant (20 µL) were measured into 4.5 mL disposable cuvette followed by the addition of 800 µL of methanol. One millilitre of 0.1 mM DPPH solution was added, vortexed and incubated in the dark at room temperature for 60 min. Absorbance was read in triplicate at 517 nm against a blank (absolute methanol) under dim light using Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, USA) against the 3% MPA and radical-scavenging activities were calculated by the percentage of DPPH that were scavenged using the Eq. 4.

Radical-scavenging activities (%) = \((1 - \frac{A_E}{A_D}) \times 100\)

Where \(A_E\) is the absorbance of the reaction mixture containing the standard antioxidant, or extract and \(A_D\) is absorbance of the DPPH solution only.

2.9 **Statistical analysis**

Experiments were laid out using a completely randomized design (CRD) with individual fruit as replicate. All statistical analyses were performed using GenStat® 18th Edition (VSN International, Hemel Hempstead, UK). Data were subjected to analysis of variance (ANOVA) with canopy position, season, production region and cold storage time as factors. Least significant difference (LSD) at 5% level was considered significant.
3. Results and discussion

3.1. Effect of canopy position on rind colour and pigments

The interaction between canopy position and production region was statistically not significant. However, the analysis of variance revealed that canopy position had a highly significant \((p < 0.001)\) effect on the greenness \((a^*)\), yellowness \((b^*)\), and colour index \((CI)\) while effect on luminosity of the rind during the entire period of cold storage \((L^*)\) was not significant \((p = 0.118)\). Production region showed significant \((p < 0.05)\) effect on rind colour indices during postharvest cold storage. Similarly, canopy position and production region significantly \((p < 0.05)\) affected rind pigments \((C_a, C_b\) and \(C_c)\) of the fruit during the period of cold storage. The \(a^*, CI\) and chlorophylls \(a\) and \(b\) followed similar pattern during the period of cold storage except at week 6 where inverse results occurred. These parameters, which measure the disappearance of chlorophylls \(a\) and \(b\), and hence, greenness \((a^*)\) during cold storage showed consistent transformation of the rind colour from green to yellow. The yellow colour encourages purchase of the fruit at the markets. Although not statistically significant, OC fruit \((-5.80\) and \(-4.27)\) from KZN were greener than IC fruit \((-5.38\) and \(-3.86)\) at week 0 and after week 3 of cold storage, respectively (Figure 1B). However, inverse results were obtained after weeks 6 and 9 of cold storage, with IC fruit \((-4.75\) and \(-2.50)\) being significantly greener in colour than OC fruit \((-4.06\) and \(-0.71)\), respectively (Figure 1B). The inconsistency nature of these results could be attributed to colour measurements being taken at different points along the equator axis of the fruit. For fruit from LMP, a progressive decline in greenness occurred during cold storage. Inside canopy fruit \((-8.44, -8.54, -6.37\) and \(-4.96)\) were significantly greener than OC fruit \((-7.96, -6.33, -4.38\) and \(-3.10)\) at weeks 0, 3, 6 and 9, respectively (Figure 1B) while OC fruit were yellower (Figure 1C). This concurred with report by Cronje et al. (2013) where OC fruit developed a more intense orange
colour (for ‘Nules Clementine’ mandarin) than IC fruit after the colour break. This indicates that sunlight contributes to rind colour development of the fruit. Like a* of the fruit rind, a significantly higher \( C_a \) pigment occurred in OC fruit (-6.00, -6.53, and -5.49 µg/g DW) than IC fruit (-5.30, -5.00 and -4.51 µg/g DW) from KZN at week 0 and after weeks 3 and 6, respectively (Figure 2A). Also, lower \( C_a \) pigment occurred in OC fruit (-5.08, -2.73, and -1.98 µg/g DW) than IC fruit (-5.63, -5.01, and -3.02 µg/g DW) from LMP at week 0 and after weeks 3 and 9 of cold storage, respectively (Figure 2A). These results further indicated that exposure of fruit to sunlight encourages better rind colouration. Generally, the disappearance of the chlorophyll is primarily due to the synthesis of carotenoids (Cronje et al. 2013). Total carotenoids, the pigment responsible for the development of the attractive colour (yellow) of the rind that encourages consumer purchase of grapefruit, followed an inconsistent pattern during the period of cold storage (Figure 2C). However, significantly higher results occurred in IC fruit (10.86 and 9.25 µg/g DW) than OC fruit (8.37 and 6.76 µg/g DW) from KZN at weeks 0 and 9, respectively (Figure 2C). Total carotenoids in inside canopy fruit (11.13 and 7.76 µg/g DW) from LMP were significantly higher than OC fruit (6.78 and 4.14 µg/g DW) at weeks 6 and 9 after cold storage, respectively (Figure 2C). This is contrary to the results of Cronje et al. (2013) who reported lower total carotenoid concentration of IC fruit than OC fruit. However, this could be because of the differences in eventual colour of the fruit investigated.

3.2. Effect of canopy position on non-structural carbohydrates

Sucrose, glucose, and fructose were the three main non-structural carbohydrates evaluated. The carbohydrates were significantly \((p \leq 0.05)\) affected by canopy position and production region. Overall, carbohydrates were significantly higher in IC fruit than OC fruit during the entire period.
of cold storage with some deviations (Figures 3A-C). In the case of fruit from KZN, reducing sugars (fructose and glucose) were more concentrated in IC fruit (284.5 and 205.9 mg/g DW) than OC fruit (238.9 and 156.4 mg/g DW), respectively at week 0 and barely increased during the period of cold storage. This was in contrast with Magwaza et al. (2013a) who reported higher concentration of the sugars in ‘Nules Clementine’ mandarin rind from OC than IC but agreed with the results obtained for fruit harvested from LMP, which indicated the influence of production region. These reducing sugars (fructose and glucose) became significantly more concentrated in OC fruit (263.0 and 91.9 mg/g DW) than IC fruit (222.9 and 177.5 mg/g DW), respectively from LMP region after week 9 of cold storage (Figures 3A and 3B). The contrasting results obtained for IC and OC fruit from both production regions indicated the influence of either agro-climatic conditions or rootstock used in the respective orchards from which fruit were harvested.

Generally, the non-reducing sugar (sucrose) declined regardless of production region over the period of cold storage in fruit from both canopy positions. This agreed with the declining trends in sucrose concentration reported in literature for ‘Nules Clementine’ mandarin stored at 7.5 °C (Magwaza et al., 2013a), ‘Navelate’ (stored at 2 °C), Pinalate’ (stored at 12 °C) oranges (Holland et al., 2002) and peach fruit stored at 5 °C (Yu et al., 2016). While difference in concentration of sucrose in IC and OC fruit from both regions were not statistically significant at week 0, concentration of sucrose in IC fruit, 74.4 and 55.4 mg/g DW, became significantly higher than OC fruit, 21.7 and 26.2 mg/g, from KZN at weeks 3 and 6 of cold storage, respectively (Figure 3C).

Previous studies have indicated that non-structural carbohydrates such as sucrose, fructose and glucose are important sources of energy that contribute to rind quality of fruit (Cai et al., 2015).
These carbohydrates also enhance resistance to fruit stress (Der Agopian et al., 2011) and neutralise oxidative challenge during abiotic stress in plants (Keunen et al., 2013). Further studies have also revealed that sugars play a role in biosynthesis of phytochemicals such as vitamin C which is a good antioxidant that protects plants against physiological disorders (Wei et al., 2017). Hence, it could be deduced from this study that the high concentration of sugars contributed towards the inhibition of rind pitting development on the fruit during the period of cold storage.

3.3. Effect of canopy position on phytochemicals, vitamin C and radical-scavenging activities

Figure 4 shows the effect of canopy position and production region on phytochemicals, vitamin C and radical-scavenging activities of grapefruit rind at week 0 and after weeks 3, 6 and 9 of non-chilling cold storage. Although production region showed significant ($p < 0.05$) effects on phytochemicals, vitamin C and radical-scavenging activities, canopy position had no significant effect on vitamin C ($p = 0.917$; Figure 4C). On the contrary, a higher concentration of vitamin C was reported in OC fruit than IC fruit by Magwaza et al. (2013a) and Magwaza (2013) which was speculated to increase the fruit tolerance to rind breakdown of ‘Nules Clementine’ mandarin. These discrepancies could be because different type of citrus fruit was used in the study. In this study, vitamin C was higher in fruit from KZN (14.41 mg/g DW, than fruit from LMP, 12.64 mg/g DW at week 0 but became lower in fruit from KZN, 14.19 and 14.43 mg/g DW) than LMP (16.13 and 15.85 mg/g DW) after cold storage at weeks 3 and 6, respectively (Figure 4C). It was speculated that storage temperature play a role in the response of fruit rind in the production of vitamin C concentration as observed in fruit from both regions.
The concentration of vitamin C was significantly higher in fruit from KZN (14.41 mg/g DW) than fruit from LMP (12.64 mg/g DW) at week 0 (Figure 4C). However, a reverse trend occurred postharvest with higher concentration occurring in fruit from LMP (16.13 and 15.85 mg/g DW) than fruit from KZN (15.85 and 14.43 mg/g DW) at weeks 3 and 6, respectively. The effects of canopy position on total flavonoid concentration (Figure 4B) and radical-scavenging activities (Figure 4D) were highly significant \( (p < 0.001) \). Total phenolic and total flavonoid concentrations followed similar trends with significant higher concentrations in IC fruit (2.96 mg GAE /g and 5.45 mg QTE /g) than OC fruit (2.31 mg GAE /g and 4.31 mg QTE /g) from LMP at week 0 while differences in the concentration of IC and OC fruit from KZN were not significant (Figures 4A and 4B). The former was in accordance with Magwaza et al. (2014b) who reported a higher concentration of total phenolics in the rind of bagged and IC ‘Nules Clementine’ mandarin fruit than OC fruit. However, this contradicts McDonald et al. (2000), who reported higher levels of phenols and flavanols in the rind of OC ‘Marsh’ grapefruit than IC fruit. The similarity in the result of total phenolic and flavonoid concentrations were not surprising since flavonoid is a major phenolic group contributing to the total phenolic concentration in the rind of a fruit (Fawole et al., 2012). The role of sunlight as contributing factor to the synthesis of phenolics was suggested by Awad et al. (2001). This could indicate that fruit from both canopy positions received enough sunlight for the synthesis of total phenolic concentrations, which could have acted as a defence mechanism against possible incidence of physiological disorder such as rind pitting. Furthermore, it was expected that total phenolic and total flavonoid concentrations would be higher in the OC fruit since the photosynthetically active radiation (PAR) in the OC is known to stimulate the synthesis of phenylalanine ammonialyase which induces the production of phenols such as phytoalexins (Ben Yehoshua et al., 1992). However, the reason for the inconsistencies was not
tested in this study but could possibly be attributed to preharvest cultural practices of the farm from which fruit were harvested or postharvest abiotic stress. As previously reported in literature (Contreras-Oliva et al., 2011; Del Caro et al., 2004), the concentration of total phenolics and total flavonoids generally increased during the postharvest cold storage of the fruit (Figures 4A and 4B). The increased concentrations could be due to the stimulation of phenylalanine ammonialyase activity during cold storage increasing the ability of the grapefruit rind to prevent the incidence of postharvest disorders such as rind pitting.

Using radical-scavenging activities of 50% as basis for good activity, good radical-scavenging activities were exhibited by IC and OC fruit from both production regions (Figure 4D). Although not significant at weeks 0 and 9, the mean values of IC fruit (56.38 and 74.96%) were higher than OC fruit (55.56 and 73.86%) from KZN while OC fruit (65.90 and 71.49%) were higher than IC fruit (59.49 and 67.32%) from LMP. At week 3, IC fruit (68.87%) were significantly higher than OC fruit (61.04%) from LMP while IC fruit (68.35%) were significantly lower than OC fruit (76.13%) from KZN at week 6 (Figure 4D). Previous studies have revealed that the antioxidant species plays a role in the incidence of different postharvest physiological rind disorders such as non-chilling rind pitting in ‘Navelate’ oranges (Cajuste and Lafuente, 2007). In retrospect, the lack of physiological rind disorders in IC and OC fruit from both production region suggests that the defence mechanisms of the fruit to stress were sustained during cold storage.

3.4. Correlation analyses

Correlation tests indicated a significant ($p < 0.05$) strong relationship between certain investigated parameters, as presented in Table 2. A strong and negative relationship was revealed between
sucrose and luminosity \((r = -0.845)\) while moderate but negative correlations related sucrose with greenness \((r = -0.537),\) yellowness \((r = -0.450),\) chlorophyll b \((r = -0.503),\) and total carotenoids \((r = 0.465).\) Correlation between reducing sugars (fructose and glucose) was positively strong \((r = 0.869)\) while sucrose had a moderate but positive correlation with fructose \((r = 0.515)\) and glucose \((r = 0.505).\) Furthermore, sucrose shared negative correlation with total phenolic concentration \((r = -0.533),\) vitamin C \((r = -0.631),\) and radical-scavenging activities \((r = -0.627),\) and had positive correlation with total flavonoid concentration \((r = 0.590).\) Strong and negative correlations were found between total flavonoids and vitamin C \((r = -0.708),\) radical-scavenging activities and total carotenoids \((r = -0.731),\) while positive correlations existed between total flavonoids and fructose \((r = 0.838),\) radical-scavenging activities and chlorophyll b \((r = 0.744).\) Radical-scavenging activities had poor correlations with vitamin C \((r = 0.414),\) total phenolic \((r = 0.369)\) and flavonoid \((r = -0.351)\) concentrations.

4. **Conclusion**

This study highlighted the role of canopy position and production region on rind biochemical concentrations in grapefruit stored at non-chilling temperature. The non-appearance of rind pitting during the study could suggest that biochemical properties of the fruit rind were in their optimal levels to defend the fruit against environmental stress. Generally, there was no clear trend in the role of canopy position among the measured parameters as fruit rind from the two production regions responded differently during postharvest cold storage. However, non-reducing sugars of IC and OC fruit from the production regions followed a similar pattern from week 0 to week 6 of cold storage. Correlation tests showed that sucrose is an important biochemical property of fruit rind which could have a direct or indirect impact on the performances of other biochemical
properties. Therefore, the role of rind sucrose in the defence mechanism of fruit against rind pitting should not be underrated.

Acknowledgements

This work was made possible by the funding received from National Research Foundation (Thuthuka Project Number: 94022) and Citrus Research International through Postharvest Innovation Programme (PHI 2/2014). The authors are grateful to Dr Paul Cronje for his technical assistance. We appreciate the fruit donations from Olifant Rivers Farms from Limpopo and Bolton Citrus Farm from KwaZulu-Natal towards the success of this study. We also express our gratitude to the South African Weather Services for providing us with weather information.
References


Lin, J.-Y., Tang, C.-Y., 2007. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. Food Chem. 101, 140–147. doi:http://dx.doi.org/10.1016/j.foodchem.2006.01.014


Table 1: Climatological data registered during the growing seasons in KwaZulu-Natal (KZN) and Limpopo (LMP) provinces.

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<th>SN</th>
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<th>MAR</th>
<th>APR</th>
<th>MAY</th>
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(Source: South African weather services)
Table 2: Pearson correlation coefficient matrix between biochemical properties measured in ‘Marsh’ grapefruit rind measured in 2015 and 2016 seasons

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<td>Colour index</td>
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<td>5</td>
<td>Chlorophyll a (µg/g DW)</td>
<td>0.219</td>
<td>0.084</td>
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<td>Chlorophyll b (µg/g DW)</td>
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<td>7</td>
<td>Total Carotenoid (µg/g DW)</td>
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<td>-0.287</td>
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<td>Rind fructose (mg/g DW)</td>
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<td>-0.239</td>
<td>-0.209</td>
<td>-0.281</td>
<td>0.000</td>
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<td>12</td>
<td>Total phenolic conc. (mg GAE/g DW)</td>
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<td>0.332</td>
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<td>Vitamin C (mg/g DW)</td>
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Values in bold are significantly different from 0 with a significance level alpha = 0.05
Figure 1: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) and production region (KwaZulu-Natal (KZN) and Limpopo (LMP)) on luminosity (A), greenness (B), yellowness (C) and colour index (D) of ‘Marsh’ grapefruit rind harvested over two seasons during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD least significant difference; P: canopy position; L: production region; T: storage time; * represent an interaction between factors.
Figure 2: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) and production region (KwaZulu-Natal (KZN) and Limpopo (LMP)) on rind chlorophyll a (A), chlorophyll b (B), and total carotenoid content (C) of ‘Marsh’ grapefruit harvested over two seasons during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD least significant difference; P: canopy position; L: production region; T: storage time; * represent an interaction between factors.
Figure 3: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) and production region (KwaZulu-Natal (KZN) and Limpopo (LMP)) on rind fructose (A), glucose (B), and sucrose (C) of ‘Marsh’ grapefruit harvested over two seasons during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD least significant difference; P: canopy position; L: production region; T: storage time; * represent an interaction between factors.
Figure 4: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) and production region (KwaZulu-Natal (KZN) and Limpopo (LMP)) on rind total phenolic concentration (A), total flavonoid concentration (B), vitamin C (C), and radical scavenging activities (D) of ‘Marsh’ grapefruit harvested over two seasons during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD least significant difference; P: canopy position; L: production region; T: storage time; * represent an interaction between factors.
CHAPTER 5

ROLE OF CANOPY POSITIONS ON RIND BIOCHEMICAL CONCENTRATIONS AND RADICAL-SCAVENGING ACTIVITIES OF ‘NULES CLEMENTINE’ MANDARINS DURING NON-CHILLING POSTHARVEST COLD STORAGE*

*Published in Scientia Horticulturae 226, 231-240 (2017)
Abstract

Rind biochemical concentrations and radical-scavenging activities of ‘Nules Clementine’ mandarin could play a significant role in the susceptibility of the fruit to various forms of physiological rind disorders. This study examined the effect of fruit position within tree canopy on biochemical concentrations and radical-scavenging activities of ‘Nules Clementine’ mandarin fruit rind after harvest at week 0 and after 3, 6 and 9 weeks of postharvest cold storage at 7.5 ± 0.5 °C. Biochemical concentrations and radical-scavenging activities of flavedo and albedo tissues of the rind were also examined. Fruit from inside canopy (IC) and outside canopy (OC) were harvested at commercial maturity in May 2015 and 2016 seasons from Unifruiti and Swartvelei Farms located at Eastern Cape (EC) (33° 27′ 32″ S, 25° 34′ 79″ E) and Western Cape (WC) (19° 02′ 33.8″ S, 33° 41′ 17.24″ E) provinces of South Africa, respectively. Results showed that canopy position played significant ($p < 0.001$) role on concentrations of total carotenoids, total phenolics and total flavonoids as well as rind dry matter, with OC fruit often characterised by higher biochemical concentrations than IC fruit during cold storage. The study further showed that production region influenced radical-scavenging activities and carbohydrate content of OC and IC fruit rind during cold storage. Radical-scavenging activities of OC fruit (61.2 %) were higher than IC fruit (52.5%) from EC whereas radical-scavenging activities of IC fruit (67.1 %) were higher than those of OC fruit (58.2 %) from WC at harvest. Overall, this study revealed that fruit position within tree canopy influenced its rind biochemical concentrations and radical-scavenging activities.

Keywords: Albedo, Antioxidants, Citrus fruit, Flavedo, Mandarin, Physiological rind disorder.
1. Introduction

‘Nules Clementine’ mandarin (*Citrus reticulata* Blanco) fruit is one of the major economically important citrus cultivar widely produced in South Africa (Barry and Rabe, 2004). However, the incidence of rind physiological disorders such as rind breakdown (RBD) is problematic to the industry as it affects fruit appearance, acceptability and ultimately, purchase in both local and international markets (Cronje et al., 2011a; Magwaza et al., 2014a). Hence, the financial gains of the growers are threatened (Alférez et al., 2003; Cajuste and Lafuente, 2007; Porat et al., 2004). The major problem surrounding the disorders is their characteristic development at about three to five weeks postharvest (Cronje et al., 2011; Magwaza et al., 2012; van Rensburg et al., 2004). In view of these, studies are increasingly focusing on identifying biomarker(s) or factors triggering the incidence of rind physiological disorders of citrus fruit (Alférez et al., 2003; Magwaza et al., 2014c; Porat et al., 2004). The incidence of these disorders, for instance, rind breakdown (RBD) of ‘Nules Clementine’ mandarin, does not necessarily compromise the consumable fleshy part of the fruit but mostly prevent consumers from purchasing the fruit thereby decreasing the economic value of the fruit (Agustí et al., 2001). The rind, also known as pericarp, is a morphological part of citrus fruit consisting of flavedo, the outermost orange-coloured part of citrus rind, and albedo, the inner whitish part of the rind (Iglesias et al., 2007). The flavedo, which consists of oil glands and orange pigments when ripe, plays a significant role in consumer acceptability and eventual purchase of the fruit. Considering several reports, the process of non-chilling physiological rind disorders is ignited in the cell membrane structure and continues through the epidermal and sub-epidermal tissues to the flattening of the surrounding cell layers to the eventual collapse cells and oil glands of the rind (Agustí et al., 2001; Magwaza et al., 2013b; Medeira et al., 1999; Vercher et al., 1994). Furthermore, some authors considered oil glands as the primary sites of injury where
ruptured oil bodies are released into the surrounding cells which then causes rind pitting disorder to citrus fruit (Petracek et al., 1998).

In various attempts to investigate the incidence of rind physiological disorders, the impact of canopy position, a preharvest factor, on rind biochemical profile, and hence, RBD have been reported (Cronje et al., 2011a; Magwaza et al., 2014b, 2013). Microclimates, such as temperature, vapour pressure deficit and photosynthetically active radiation (PAR), reaching different positions of citrus tree canopy in varying intensity affect the physiological activities and biochemical composition of fruit and its parts, including the rind (Cronje et al., 2011b; Greene and Gerber, 1967; Jahn, 1979; Magwaza et al., 2014c; Syvertsen and Albrigo, 1980). For instance, fruit exposure to different sunlight levels in citrus tree canopy affects rind concentration of carbohydrates and mineral elements during fruit development (Cronje et al., 2011a, 2011b). Hence, reduced transpiration due to lower temperature and higher humidity of fruit exposed to low light level (inside canopy) could hinder the build-up of carbohydrate (Cronje et al., 2013). Furthermore, limited PAR penetrating inside canopy (IC) could favour a reduced rate of photosynthesis and osmotic potential of fruit resulting in lower rind biochemical concentrations which could encourage the incidence of rind physiological disorders (Cronje et al., 2011a, 2011b, Magwaza et al., 2013a, 2013b).

Rind biochemical concentrations and radical-scavenging activities of citrus rind play significant roles regarding its susceptibility to physiological disorders and its overall quality (Magwaza et al., 2013a). Therefore, exploring rind biochemical concentrations such as carbohydrate, carotenoids, phenolic and flavonoid concentrations, and radical-scavenging activities of the rind (flavedo and
albedo) could proffer a better understanding of the relationship among canopy position, biochemical concentrations, and radical-scavenging activities of the fruit rind. This could give new knowledge towards the identification of possible biomarker(s) of rind physiological disorder. Previous studies have shown that rind carbohydrates of IC fruit were significantly lower than fruit from outside canopy (OC) and that greater incidence of RBD occurred on IC fruit rind than OC fruit (Cronje et al., 2013, 2011b; Magwaza et al., 2013a). Furthermore, high concentration of rind sucrose, an osmoregulatory compound in plant cells, has been implicated for lower osmotic potential in plant cells (Huang et al., 2000; Yakushiji et al., 1998). These reports suggested potential biochemical link among rind carbohydrates, canopy position and development of rind disorder. In addition, inadequate supply of rind carbohydrate for postharvest respiration has been implicated in the development of series of rind physiological disorders (Cronje et al., 2011b; Holland et al., 1999).

Citrus fruit rinds are naturally high in non-volatile organic acids such as phenolic compounds (Benavente-García et al., 1997; Manthey, 2004) which are the major constituents responsible for the antioxidant activities of the fruit. Scientific studies on how canopy position affect these variables in citrus fruit rind has earlier been reported (Magwaza et al., 2014c, 2013a). However, results from the studies were based on fruit harvested from a single production region, which was inadequate to confirm the proposed hypothesis regarding the effect of canopy position on rind biochemical concentrations of citrus fruit rind and its susceptibility to RBD. Therefore, an extensive study to include fruit from other production regions with different agro-climatic conditions is necessary to test the hypothesis on ‘Nules Clementine’ mandarins, which was the aim of this study. This study investigated the influence of canopy position on biochemical
concentrations and radical-scavenging activities of ‘Nules Clementine’ mandarin fruit rind (flavedo and albedo) harvested from two production regions at harvest and after postharvest cold storage in relation to the development of physiological rind disorders. To the best of the authors’ knowledge, the role of canopy position on the biochemical concentrations and radical-scavenging activities of the flavedo and albedo of citrus rind after harvest or after cold storage are unknown.

2. Materials and methods

2.1. Reagents and standards

All chemicals including Sodium Hydroxide (NaOH), Folin-Ciocalteu reagent, metaphosphoric acid (MPA), sodium carbonate, gallic acid, quercetin, 2, 6 dichloroindophenol dye, 2,2-diphenyl-1-picrylhydrazyl (DPPH), acetone, ethanol (HPLC grade) and sugars standards (sucrose, D-glucose, and D-fructose) were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK). A Phenomenex® column (Rezex RCM - Monosaccharide) was used in the analyses. Water was purified in a Milli-Q Integral Water Purification System (Merck Millipore corporation, Billerica, MA, USA; σ = 18 M Ω cm⁻¹).

2.2. Plant materials

A total number of 600 individual fruit were harvested from 50 uniform sized trees at commercial maturity over two seasons (2014/15 and 2015/2016) from Unifruiti and Swartvelei Farms located at Eastern Cape (EC) (33° 27′ 32″ S, 25° 34′ 79″ E) and Western Cape (WC) (19° 02′ 33.8″ S, 33° 41′ 17.24″ E) provinces of South Africa, respectively. ‘Nules Clementine’ mandarin fruit from EC were budded on ‘Carrizo’ citrange (((Poncirus trifoliata (L.) Raf.) × (C. sinensis)) rootstock and planted in 1997 at a spacing of 5.5 x 2 m. Fruit from WC were budded on Rough lemon (C.
*jambhiri* Lush.) rootstock planted in 2001 at a spacing of 5.5 x 2.5 m. To investigate the influence of canopy position on biochemical concentrations and radical-scavenging activities of the fruit rind, two fruit were harvested per canopy position per tree. The canopy positions were inside canopy (i.e. fruit exposed to less than 80% of full sunlight), and outside canopy (i.e. fruit exposed to 90-100% of full sunlight) of a fruit tree as described by Cronje et al. (2011a). Each fruit was harvested from a height of 1-2 m and was used as an individual replicate comprising a total of 100 fruit replicates per canopy position. The rainfall (mm), relative humidity (%), maximum and minimum temperature (°C) registered during the growing seasons in EC and WC provinces are displayed in Figure 1A-D, respectively. After harvesting, fruit were transported within 48 h at ambient temperature in ventilated cartons to postharvest research laboratory where the fruit were washed and sorted for blemishes and fruit damage. Fruit were left for 24 h at room temperature 20 ± 1 °C to equilibrate, sorted for physical blemishes and damages, washed, labelled, weighed, and transferred into cold storage (7.5 ± 0.5°C) for 9 weeks. Ten individual fruit using one fruit per replicate were analysed at 3 weeks interval for 9 weeks (weeks 0, 3, 6 and 9).

### 2.3 Rind colour measurement

Rind colour of individual fruit was measured in the CIE L*, C* and hue angle (h°) space using a chromameter (Konica Minolta NR 4000, Osaka, Japan) after calibration using a standard white tile (CR-A43; Y = 93.1, x = 0.3138; y = 0.3203) (Pathare et al., 2013; Terry et al., 2007). Readings at three equidistant points around the equatorial axis of each fruit were recorded.
2.4. Sample preparation

Individual fruit was manually peeled followed by separation of flavedo part from the albedo using lemon zester. Both parts were snap frozen in liquid nitrogen and stored at -80 °C before freeze-drying over a period of three days using Virtis Benchtop freeze drier system (ES Model, SP Industries Inc., Warmister, USA) at 0.015 kPa and -75 °C. Dry matter (DM) was calculated by subtracting the mass of the freeze-dried samples from fresh samples and expressed as percentage DM. Dried samples were then milled into fine powder using pestle and mortar and stored in -20 °C for further analysis.

2.5. Determination of total carotenoid content

Total carotenoid content was determined according to Lichtenthaler (1987) with slight modifications. The lyophilized sample (150 mg ± 0.5) was weighted into a test tube followed by the addition of 2 mL of 80% (v/v) acetone before centrifugation for 10 min using GenVac® (SP Scientific, Genevac LTD., Suffolk, UK). The absorbance values of the supernatants were read at 470, 646.8, and 663.2 nm using a Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, USA) for maximum detection of carotenoids. Chlorophyll a and b and total carotenoid content were calculated using Eqs. 1, 2, and 3, respectively.

\[
C_a = 12.25 A_{663.2} - 2.79 A_{646.8} \quad 1
\]

\[
C_b = 21.50 A_{646.8} - 5.10 A_{663.2} \quad 2
\]

\[
C_c = (1000 A_{470} - 1.82 C_a - 85.02 C_b)/198 \quad 3
\]

Where \(C_a\) is chlorophyll a, \(C_b\) is chlorophyll b and \(C_c\) is total carotenoid content.
2.6. Extraction and quantification of rind soluble sugars

Soluble sugars were extracted from 150 ± 0.5 mg of lyophilized sample using 62.5% (v/v) aqueous methanol as described by Terry et al. (2007), with slight modifications for citrus (Magwaza et al., 2014b). Concentration of fructose, glucose, and sucrose were then quantified using an isocratic HPLC binary pump system (Agilent Technologies, UK) equipped with a refractive index detector. A 1 mL of diluted extract was injected into a Rezex RCM monosaccharide Ca\(^+\) (8%) column of 7.8 mm diameter × 300 mm (Phenomenex, Torrance, CA, USA) with a SecurityGuard™ cartridges of 4 mm × 3 mm (Phenomenex). The mobile phase used was ultra-pure HPLC-grade water at a flow rate of 0.6 mL/min with the column compartment temperature set at 80 °C using a thermos-stated column compartment (G1316A, Agilent). The presence and concentration of individual sugars were determined by comparing peak area of samples with peak area and concentration of a known sugar standard curve (0.05 – 1.25 mg/L; \(R^2 = 0.99\)).

2.7. Sample extraction for total phenolic and total flavonoid concentrations

Extraction was carried out according to Moo-Huchin et al. (2015) with modifications. A lyophilized sample of 150 mg ± 0.5 mg was measured into a test tube and 3 mL of 50:50 (ethanol: water) v/v was added. The test tube, covered with aluminium foil, containing the mixture was subsequently placed in a shaking water bath (Gesellschaft für (GFL), Labortechnik mbH, Burgwedel, Germany) at 70 °C for 2 h with intermittent sample vortexing for 20 s at 30 min interval. The sample was left to cool down followed by centrifugation for 10 min using GenVac® centrifuge (SP Scientific, Genevac LTD., Suffolk, UK) and filtered using a 0.45 µm nylon filter. Extracts were stored at -20 °C for further analysis.
2.7.1 Determination of total phenolic concentrations

Extracts were analysed according to Moo-Huchin et al. (2015) for total phenolic concentration with some modifications. Briefly, sample extract (10 μL) was measured into a 4.5 mL disposable cuvette in triplicate followed by the addition of 1.6 mL of distilled water, 100 μL of Folin-Ciocalteu reagent and 300 μL of sodium carbonate solution. The solution was mixed and incubated in the dark at room temperature for 2 h before absorbance was read at 765 nm using Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, USA). Gallic acid was used to generate a standard curve and total phenolic concentrations were expressed as mg gallic acid equivalent (GAE) /g DM.

2.7.2. Determination of total flavonoid concentrations

Extracts were analysed for total flavonoid concentration according to Lin and Tang (2007) with modifications. Briefly, extract (100 μL) was measured into a 4.5 mL disposable cuvette followed by the addition of 3 mL of sodium hydroxide solution. The mixture was agitated and incubated at room temperature for 10 min. Absorbance was measured in triplicate at 420 nm using a Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, USA). Quercetin was used to generate a standard curve and total flavonoid concentration was expressed as mg quercetin equivalent (QTE) /g DM.

2.8. Extraction and determination of DPPH radical-scavenging activities

Extraction was carried out according to Karioti et al. (2004) with some modifications. A lyophilized sample of 150 mg ± 0.5 was measured into a test tube followed by the addition of 5
mL of 3% aqueous metaphosphoric acid and incubation on ice for 5 min. Extracts was centrifuged for 20 min using GenVac® centrifuge (SP Scientific, Genevac LTD., Suffolk, UK). Supernatant (20 μL) were measured into 4.5 mL disposable cuvette followed by the addition of 800 μL of methanol. One millilitre of 0.1 mM DPPH solution was added, vortexed and incubated in the dark at room temperature for 60 min. Absorbance was read in triplicate at 517 nm against a blank (absolute methanol) under dim light using Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, USA) and radical-scavenging activities were calculated by the percentage of DPPH that were scavenged using the Eq. 4.

Radical-scavenging activities (%) = (1 – A_E/A_D) x 100

Where A_E is the absorbance of the reaction mixture containing the standard antioxidant, or extract and A_D is absorbance of the DPPH solution only.

2.9 Statistical analysis

Experiments were carried out using a completely randomized design (CRD) with individual fruit as replicate. All statistical analyses were performed using GenStat® 18th Edition (VSN International, Hemel Hempstead, UK). Data were subjected to analysis of variance (ANOVA) with canopy position, season, production region and cold storage time as factors. Season two (2016) data from WC were treated as missing values and least significant difference (LSD) at 5% level was considered significant.
3. Results and discussion

3.1. Effect of canopy position on total carotenoids concentration and rind colour

The analysis of variance revealed that canopy position had a highly significant $(p < 0.001)$ effect on rind total carotenoid content (Figure 2A) and rind colour measured as hue angle (Figure 2B) in both seasons. The rind total carotenoid content of ‘Nules Clementine’ mandarin from EC and WC at harvest and after cold storage were similar in both seasons. Significant higher total carotenoid content occurred in rind of OC fruit than IC fruit at week 0 and after weeks 3, 6 and 9 of cold storage in both seasons. These results could explain the converse result of the rind colour (hue angle) been significantly higher in IC fruit than OC fruit at week 0. However, a non-significant difference occurred after week 6 of cold storage for fruit from WC and after week 9 of cold storage for fruit from both EC and WC in 2015 (Figure 2A). The rind colour fairly remained constant after weeks 3, 6 and 9 of cold storage in both seasons (Figure 2B) which could be attributed to the effect of cold storage in preserving the colour of the fruit for a longer period. A similar result of the inverse relationship between total carotenoid content and hue angle had previously been reported and the difference had been attributed to varying sunlight levels reaching the two canopy positions (Cronje et al., 2013; Hiratsuka et al., 2012). The fruit rind colour in this study further share similarities with results reported in literature by Cronje et al. (2011a) and Khalid et al. (2012). Inside canopy fruit showed yellower appearance (hue angle, EC = 93.1° and WC = 100.8°) than OC fruit which expressed a more orange appearance (hue angle, EC = 85.2° and WC = 78.7°) at week 0.

Total carotenoid concentration was significantly higher in flavedo than albedo of both IC and OC fruit after harvest and after 3, 6 and 9 weeks of cold storage (Figure 3). These results were not
surprising as flavedo tissue bears the colour expression (yellow or orange) of the fruit as against
the white colour of the albedo. Furthermore, the flavedo is more exposed to sunlight than the
albedo tissue and sunlight is well known to play an influential role in total carotenoid content
accumulation in plant tissues (Botella-Pavía et al., 2004; Pizarro and Stange, 2009; Simkin et al.,
2003; Toledo-Ortiz et al., 2010). This premise also explained the increased total carotenoids
content in OC fruit than IC fruit from EC and WC provinces which agreed with previous authors
who reported the enhancement of total carotenoid content accumulation in citrus fruit such as sweet
oranges and mandarins due to light exposure (Cronje et al., 2013, 2011b).

3.2. Effect of canopy position on rind soluble sugars and dry matter

The use of carbohydrates as a global biochemical marker of rind physiological disorder was
previously hypothesised to be impossible due to citrus fruit exposure to different preharvest and
postharvest factors (Magwaza et al., 2014c). This study supported this hypothesis as fructose,
glucose, and sucrose of citrus rind respond differently to canopy position from which the fruit were
harvested. Canopy position significantly affected fructose ($p < 0.001$), glucose ($p < 0.001$), and
sucrose ($p < 0.05$) concentrations of citrus fruit rind (Figures 4A, 4B and 4C). The significance
interaction between canopy position, production region and storage time were also noted. At week
0, rind sugars from both production region followed a similar pattern which corresponded to the
ones reported earlier in literature where the concentration of these sugars were higher in IC fruit
than OC fruit (Magwaza et al., 2014c; Rosales et al., 2011; Ting and Deszyck, 1961). However,
rind sucrose of IC fruit from EC were lower than OC fruit which corresponded to the results
reported for rind sucrose of ‘Nules Clementine’ mandarin fruit from WC by Cronje et al. (2013)
and Thorpe (1974). It was explained that exposure of fruit to reduced sunlight (IC) have a reduced
sink strength effect on fruit. In 2015, fructose, glucose, and sucrose of IC fruit (313.6, 317.2 and 210.8 mg/g DW, respectively) were significantly higher than OC fruit (277.1, 160.8, 79.5 mg/g DW, respectively) from WC at week 0 (Figures 4A, 4B and 4C). However, significantly higher concentration of rind glucose occurred for OC fruit than IC fruit while no significant difference was observed for fructose in 2016. The postharvest behaviour of fructose, glucose, and sucrose at weeks 3, 6 and 9 followed a similar pattern for both canopy positions and was further observed that these sugars were generally high which could partly explain why rind physiological disorder did not develop during cold storage (Figure 4). This is because high concentration of sugars is known to serve as a source of energy reserves and contribute to the sustenance of rind cell structures (Dennis and Blakeley, 2000; Kays and Paull, 2004) and protect plants against possible stressful conditions such as chilling injury (Der Agopian et al., 2011; Purvis and Grierson, 1982).

Investigating carbohydrate concentrations in flavedo and albedo, fructose was significantly higher in the flavedo than in the albedo while both glucose and sucrose were higher in the albedo at week 0 (Figure 5). A contrast result occurred after week 6 of cold storage where the concentration of fructose became higher in the albedo than flavedo while there were no significant differences after weeks 3 and 9 of cold storage (Figure 5A). Lower concentration of sucrose in flavedo remained unchanged throughout the period of cold storage while concentration in albedo increased consistently and peaked at week 6 before a decline at week 9 (Figure 5B). This suggested the possible influence of cold storage in reducing the rate at which sucrose is used in flavedo and albedo of the fruit rind. Previous studies have also shown that sucrose concentration in the flavedo of fruit attached to its mother plant does not change during extreme cold stress condition, but fructose and glucose does change (Holland et al., 1999). Similarly, the concentration of glucose
was lower in the flavedo at week 0 but became higher and declined gradually after weeks 3, 6, and 9 of cold storage (Figure 5C). This could be because of the translocation of glucose from the albedo to the flavedo part of the rind influenced by cold temperature (Purvis and Yelenosky, 1983) or could be the effect of rind moisture loss.

Although, carbohydrates provide energy to fruit during postharvest life but are also known to be responsible for the build-up of DM in fruit (Hiratsuka et al., 2012). In 2015 and 2016 seasons, rind DM followed similar trends throughout the period of cold storage, with rind DM ranging from 23.7 to 29.3% for OC fruit and 10.1 to 35.1% for IC fruit. Highly significant \( p < 0.001 \) difference was observed between the canopy positions where OC fruit had higher rind DM than IC fruit at weeks 3, 6 and 9 for both production regions (Figure 6). These results agreed with previous reports where rind DM of fruit from OC were higher than IC fruit (Cronje et al., 2011b; Magwaza et al., 2014c, 2013a). It was noteworthy that the rind DM of IC fruit from WC was significantly higher than its counterpart from EC while no significant difference occurred in IC and OC fruit from WC before cold storage at week 0 in both seasons (Figure 6).

3.3. Effect of canopy position on rind radical-scavenging activities

DPPH is a reliable assay for measuring antioxidant activities of a fruit (Shahidi et al., 2006; Villano et al., 2007). Its ability to donate hydrogen is an established mechanism for anti-oxidation (Babbar et al., 2011). Canopy position showed highly significant \( p < 0.001 \) effect on radical-scavenging activities of the fruit rind (Figure 7). There was significant interaction amongst the main factors (canopy position, production region, season, and storage time) for the radical-scavenging activities of the fruit rind (flavedo + albedo). In both seasons, the radical-scavenging activities followed a
similar pattern throughout the period of cold storage of the fruit from both production regions. In 2015, radical-scavenging activities of OC fruit (week 0 = 61.2 and week 3 = 75.1 %) were significantly higher than IC fruit (week 0 = 52.5 and week 3 = 67.2 %) from EC (Figure 7A). Similar results of higher radical-scavenging activities by DPPH in the OC fruit have been reported by Drogoudi and Pantelidis (2011) who reported higher antioxidant capacity in OC apple fruit than fruit exposed to shaded canopy position. This could suggest the influence of higher levels of temperature on radical-scavenging activities of the fruit which invariably inhibits the development rind breakdown on fruit stored at non-chilling temperature. Other preharvest factors such as relative humidity could be responsible for the radical-scavenging activities of fruit rind as observed at week 0 for WC (Figure 4A). There were no significant differences between fruit from EC after weeks 6 and 9 of cold storage in 2015 but 2016 indicating seasonal effect on the scavenging activities of the fruit rind. In comparison, the radical-scavenging activities between flavedo and albedo differed significantly ($p < 0.001$), with flavedo having higher radical-scavenging activities (59.7, 69.5, 59.8 and 53.3%) than albedo (49.5, 51.9, 51.3 and 47.7%) at weeks 0, 3, 6 and 9, respectively (Figure 8). These results explained the ability of the rind to resist the incidence of rind physiological disorder initiated from either flavedo or albedo in citrus fruit during postharvest cold storage.

3.4. Effect of canopy position on total phenolic, and flavonoid concentrations

Canopy position significantly ($p < 0.001$) affected rind antioxidant activities of total phenolic concentration and total flavonoid concentration on ‘Nules Clementine’ mandarin fruit rind (Figure 9). Although, the total phenolic concentration of citrus rind from both production regions in 2015 was significantly higher than that of 2016, but results followed a similar pattern during the period
of the experiments in both seasons. In 2015, the total phenolic concentration of fruit rind sampled at week 0 was significantly higher in the rind from OC fruit (4.29 mg GAE /g) than IC fruit (3.62 mg GAE /g) from WC (Figure 9A). Similar trends were observed at weeks 3 and 6 with an inverse result at week 9, where IC fruit became significantly higher than OC fruit (Figure 9A). Although, mean concentrations of total phenolic of OC fruit (3.02 and 4.63 mg GAE /g) were higher than IC fruit (2.89 and 4.25 mg GAE /g) from EC at weeks 0 and 3, respectively, albeit not statistically significant. Reverse results were recorded after 6 and 9 weeks of cold storage, where total phenolic concentration of IC fruit was significantly higher than OC fruit from EC after weeks 6 but not significant after weeks 9 (Figure 9A). These results are buttressed by Magwaza, (2013) who reported higher total phenolic concentrations in the rind of OC fruit than IC fruit at harvest. In addition, total phenolic concentration of IC fruit was significantly higher than OC fruit after cold storage which agreed with the above-mentioned author. It has been previously suggested that production of total phenolic concentrations increases due to radiation from sunlight (Awad et al., 2001; Hagen et al., 2007). Hence, could explain why there were higher concentrations of total phenolic concentrations in OC fruit in this study. Another probable explanation is the production of phenylalanine aminialyase which occur because of the availability of photosynthetically active radiation (PAR) on the OC position of citrus tree. This PAR usually initiates the synthesis of phytoalexins, a phenolic compound considered as a line of defence against stress (Ben Yehoshua et al., 1992).

Like the behaviour of total phenolic concentration, total flavonoid concentration followed similar trends with slight deviations. For fruit sampled at week 0 in 2015, total flavonoid concentration was significantly higher in the rind from OC fruit (1.85 mg QTE /g) than IC fruit (1.32 mg QTE
Similar trends were observed at weeks 3 and 6 with inverse results at week 9, where IC fruit became significantly higher than OC fruit. Although, the mean concentration of total flavonoids for OC fruit (1.17 mg QTE /g) were higher than IC fruit (1.09 mg QTE /g) from EC at week 0, results are not statistically significant. However, total flavonoid concentration of OC fruit (1.75 and 1.67 mg QTE /g) were significantly higher than the IC fruit (1.21 and 1.22 mg QTE /g) from EC after 3 and 9 weeks of cold storage, respectively (Figure 9B). Reverse results were recorded after 6 weeks of storage, where IC fruit (1.58 mg QTE /g) was not significantly higher than OC fruit (1.47 mg QTE/g) (Figure 9B). Saure (1990) and Treutter (2001) explained the synthesis of antioxidants, such as total flavonoid concentration, could be influenced by genetic background and developmental stage of the fruit. However, temperature and light play a significant role in the synthesis. It was further suggested that most enzymes involved in flavonoid production are stimulated by sunlight (Lancaster et al., 2000; Treutter, 2001) and total flavonoid concentration is higher in sun-exposed apple fruit (Awad et al., 2001). These statements agreed with the results obtained in the current study. The general high concentrations of both total phenolic concentration and total flavonoid concentration in the rind tissues of ‘Nule Clementine’ mandarin could be implicated in the ability of fruit to repel the development of non-chilling rind physiological disorders associated with fruit as no disorder was observed on the rind of the fruit used in the current study.

Total phenolic concentration and total flavonoid concentration were analysed separately on flavedo and albedo tissues of the fruit rind to investigate where the components were more concentrated. It was, therefore, revealed that these components were significantly ($p < 0.001$) concentrated, regardless of the canopy position, in the flavedo part of the rind than albedo part.
(Figures 10A and 10B). This could further explain the significant effect of sunlight radiation deposits on the flavedo part of the rind since it is the receptor (Awad et al., 2001; Lancaster et al., 2000; Treutter, 2001).

3.5. Correlation analysis of investigated parameters

Pearson’s correlation analysis was used to study the relationship between biochemical concentrations and radical-scavenging activities of ‘Nules Clementine’ mandarin fruit rind (Table 1). A significant ($p < 0.05$) correlations were revealed. For instance, a strong positive and significant relationship that radical-scavenging activities by DPPH exhibited with total phenolic concentration ($r = 0.73$), sucrose ($r = 0.62$) and fair correlation with total flavonoid concentration ($r = 0.46$) and total carotenoid content ($r = 0.48$). This agreed with Fawole and Opara (2013) where significant and strong correlation occurred between radical-scavenging activities measured by DPPH and total phenolic concentrations in pomegranate fruit. Similarly, further studies by Prior et al. (1998) and Wang et al. (1997) highlighted the important contribution of phenols and flavonoids to antioxidant activities of fruit which might be responsible for preventing the rind of ‘Nules Clementine’ mandarin from possible physiological disorder during the cold storage. Significant ($p = 0.05$) and positive correlation were also revealed between total phenolic concentration and sucrose ($r = 0.58$); total phenolic concentration and total flavonoid concentration ($r = 0.65$). This further suggests the significant role of sucrose in protecting the rind of the fruit during postharvest cold storage.
4. Conclusion

This study revealed the influence of canopy position on rind biochemical concentrations and radical-scavenging activities on ‘Nules Clementine’ mandarin fruit. Fruit from OC showed higher concentrations of total carotenoid, total phenolics and total flavonoids which were induced by sunlight than IC fruit despite the production region. This study confirmed the hypothesis that exposing citrus fruit to sufficient light increases total carotenoids content, total phenolic and total flavonoids concentrations of the fruit rinds. The results also showed that reducing sugars (glucose and fructose) increased, in nearly equal amounts in the rind of fruit from both canopy positions throughout the period of cold storage. It was also observed that rind biochemical concentrations in flavedo and albedo parts of citrus rind differs from one another and is mostly influenced by environmental factors of the orchard. Overall, this study suggests that the rind biochemical concentrations and radical-scavenging activities of the rind were high during cold storage and could be implicated in the mitigation of the development of rind physiological disorder as no disorder was recorded in the study.

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Figure 1: Rainfall (A), relative humidity (B), maximum temperature (C) and minimum temperature registered during the growing seasons in Eastern Cape (EC) and Western Cape (WC) provinces. (Source: South African weather services)
Figure 2: Effect of canopy position (inside canopy (IC) and outside canopy (OC)), on rind total carotenoid (A) and hue angle (B) content of ‘Nules Clementine’ mandarin from Eastern Cape (EC) and Western Cape (WC) provinces harvested over two seasons during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD least significant difference; P: canopy position; S: season; L: production region; T: storage time; * stands for an interaction between factors.
Figure 3: Total carotenoid contents of the flavedo and albedo of ‘Nules Clementine’ mandarin from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD: least significant difference; R: Rind tissue; T: storage time; L: production region; * stands for an interaction between factors.
Figure 4: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on rind fructose (A), glucose (B), and sucrose (C) of ‘Nules Clementine’ mandarin harvested from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD: least significant difference; P: canopy position; S: season; L: production region; T: storage time; * stands for an interaction between factors
Figure 5: Concentration of fructose (A), glucose (B) and sucrose (C) of the flavedo and albedo of ‘Nules Clementine’ mandarin from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD: least significant difference; R: Rind tissue; T: storage time; L: production region; * stands for an interaction between factors.
Figure 6: Effect of canopy position (inside canopy (IC) and outside canopy (OC) on rind dry matter of ‘Nules Clementine’ mandarin from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD: least significant difference; P: canopy position; S: season; L: production region; T: storage time; * stands for an interaction between factors.
Figure 7: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on radical-scavenging activities (DPPH) of ‘Nules Clementine’ mandarin fruit rind from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD: least significant difference; P: canopy position; S: season; L: production region; T: storage time; * stands for an interaction between factors.
Figure 8: Concentration of radical-scavenging activities of the flavedo and albedo of ‘Nules Clementine’ mandarin fruit rind from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD: least significant difference; R: Rind tissue; T: storage time; L: production region; * stands for an interaction between factors.
Figure 9: Effect of canopy position (inside canopy (IC) and outside canopy (OC)) on rind total phenolic concentration and total flavonoid concentration of ‘Nules Clementine’ mandarin from Eastern Cape (EC) and Western Cape (WC) provinces harvested over two seasons during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD least significant difference; P: canopy position; S: season; L: production region; T: storage time; * stands for an interaction between factors.
Figure 10: Total phenolic and total flavonoid concentrations of the flavedo and albedo of 'Nules Clementine' mandarin from Eastern Cape (EC) and Western Cape (WC) provinces during postharvest non-chilling storage (Weeks 0, 3, 6 and 9).

LSD: least significant difference; P: rind part; L: production region, T: storage time; * stands for an interaction between factors.
Table 1: Correlation coefficients among measured ‘Nules Clementine’ mandarin fruit rind biochemical and antioxidant parameters

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<th>Parameters</th>
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<th>2</th>
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<tr>
<td>2</td>
<td>DM (%)</td>
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<td>3</td>
<td>Fructose (mg/g DW)</td>
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<td>Glucose (mg/g DW)</td>
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<td>0.199</td>
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<td>5</td>
<td>Sucrose (mg/g DW)</td>
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<td>T. flavonoid concentration (mg QTE /g)</td>
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<td>0.140</td>
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<td>T. carotenoid content (µg /g DW)</td>
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<td>0.232</td>
<td>0.225</td>
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<td>8</td>
<td>T. phenolic concentration (mg GAE /g)</td>
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<td>0.645</td>
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Values in bold are significantly different from 0 with a significance level alpha = 0.05
CHAPTER 6
PHYTOHORMONAL CHANGES IN ‘NULES CLEMENTINE’ MANDARIN FRUIT RIND FROM DIFFERENT CANOPY POSITIONS DURING POSTHARVEST NON-CHILLING COLD STORAGE IN RELATION TO PHYSIOLOGICAL RIND DISORDER*

* Formatted to be submitted to Scientia Horticulturae
Abstract

The role of phytohormones in mitigating or enhancing the incidence of non-chilling rind physiological disorder of citrus fruit is largely unknown. Therefore, this study attempted to elucidate phytohormonal changes in ‘Nules Clementine’ mandarin fruit rind during postharvest cold storage in relation to rind breakdown. Fruit from inside canopy (IC) and outside canopy (OC) positions of citrus trees were harvested at commercial maturity from Unifruit and Swartvelei Farms located at Eastern Cape (EC) and Western Cape (WC) provinces of South Africa, respectively. Endogenous levels of cytokinins and auxins isomers and conjugates were analysed by using ultra-performance liquid chromatograph coupled to a triple quadrupole mass spectrometer (MS/MS) equipped with an electrospray interface. Analyses were performed on ‘Nules Clementine’ mandarin fruit rind after harvest (week 0) and after 3, 6 and 9 weeks of non-chilling cold storage at 7.5 ± 0.5 °C. The result revealed the presence of cytokinins: N6- isopentenyladenine, the two Zeatin isomers (trans-zeatin (tZ) and cis-zeatin (cZ), and cytokinin conjugates such as glucosides and ribosides (cZ7G and iP9G) and O-glucoside forms (cZROG, cZOG and tZOG) and zeatin metabolite glucoside (dihydrozeatin-7-glucoside (DHZ7G)). Cis-zeatin-type cytokinins including cZ riboside (cZR), cZ riboside-O-glucoside (cZROG), cis-zeatin-7-glucosides (cZ7G) concentrations were mostly higher in the OC fruit from both EC and WC provinces during postharvest non-chilling cold storage. Whereas dihydrozeatin concentration (the most abundant CK) were mostly higher in IC (326.10 and 29.38 pmol/g DW) fruit than OC (65.75 and 17.72 pmol/g DW) fruit from EC and WC before cold storage, respectively. Fruit without rind disorder had lower cZOG, cZR, cZROG and DHZ7G than fruit with the disorder. In fruit with rind disorder, tZOG, cZ7G and iP9G were below the limit of detection. The indole-acetic acid (IAA) concentration was higher in the IC fruit (344.15 pmol/g DW) than OC fruit (194.20 pmol/g DW).
from EC at week 0 while IAA concentration of OC fruit from WC were below the LOD and IC fruit had 53.20 pmol/g DW at week 0. Fruit without the disorder had more IAA concentration than fruit with RBD while IAAsp were higher in fruit with RBD than fruit without the disorder. The study revealed the preventive role of auxins in the incidence of the rind disorder. This study is the first to record the negative effect of IAAsp in a physiological disorder of citrus fruit. This study further revealed the crucial role and synergistic effect of cZ7G, tZOG, iP9G and IAA in the prevention of RBD of ‘Nules Clementine’ mandarin fruit while and cZOG, cZR, cZROG and IAAsp aided its incidence.

**Keywords:** *Cis*-zeatin, Cytokinins, Dihydrozeatin, Indole-acetic acid, Rind physiological disorder, Rind breakdown, *Trans*-zeatin.
1. Introduction

Physiological rind disorders of citrus fruit (*Citrus* spp.), which occurs during postharvest storage conditions, contribute significantly to the economic losses experienced by the global citrus industry (Alférez et al., 2003; Cajuste and Lafuente, 2007; Porat et al., 2004; Cronje et al., 2011a; Magwaza et al., 2014). The development of physiological rind disorders in citrus fruit such as rind breakdown (RBD) of ‘Nules Clementine’ mandarins at non-chilling postharvest cold storage is one of the many factors affecting the financial gains of the industries.

Although various studies have shown that preharvest factor such as canopy position influence the susceptibility of fruit to the development of postharvest RBD (Cronje et al., 2011a, 2011b; Magwaza et al., 2013; Magwaza et al., 2012), the exact factor(s) triggering the incidence of the disorders is(are) still unknown. Canopy position significantly affects rind carbohydrates and mineral elements during fruit development (Cronje et al., 2011b). The flavedo of fruit exposed to sunlight was reported to have a significantly higher concentration of carbohydrates than those from the inside canopy position (Cronje et al., 2011b). Increased transpiration potential by higher temperatures and lower relative humidity outside the tree canopy was thought to have induced the increased accumulation of carbohydrate (Cronje et al., 2011b). Hence, it was hypothesised that increased photosynthetically active radiation (PAR) of exposed region of the tree increased osmotic potential and photosynthetic rate of exposed fruit thereby increasing the rind quality and tolerance to RBD (Cronje et al., 2011a, 2011b; Magwaza et al., 2013a; Magwaza et al., 2012). Chapter 5 demonstrated that exposure of fruit to high or low sunlight within tree canopy affected rind biochemical concentrations and radical-scavenging activities of the fruit during postharvest cold storage at non-chilling temperature. The radical-scavenging activities of outside canopy fruit
rind were reported to be significantly higher than fruit from inside canopy (Olarewaju et al., 2017) indicating the crucial role of sunlight in the production of a higher antioxidant activity in the fruit rind.

Apart from the continual biochemical changes that occur in harvested fruit, little is known about the role of phytohormones during postharvest physiology despite their crucial roles in regulating plant physiological processes during growth and development. Phytohormones are known to play significant roles in plant responses to environmental conditions (He et al., 2009; Xu and Li, 2006). They also play important roles in signal transduction pathways during stress responses while regulating the internal and external stimuli (Kazan, 2015). Preharvest abiotic stresses modify the endogenous concentrations of phytohormones such as auxins, cytokinins and abscisic acid (ABA), which cause plants growth perturbations (Egamberdieva and Kucharova, 2009; Khan et al., 2014). Hence, the relationship between canopy position and behaviour of phytohormones during the postharvest life of citrus fruit could be an important step that will give impetus to a deeper understanding of the factors underlying fruit susceptibility or tolerance to physiological rind disorders at non-chilling temperature. This is because canopy position may influence stress resistance of the fruit through up-regulation of genes and pathways, which renders tissues cross-tolerant to many stresses (Bowler and Fluhr, 2000; Leshem and Kuiper, 1996). This may subsequently occur during postharvest cold storage or shelf life (Toivonen and Hodges, 2011).

The phytohormone, auxin is known to play crucial roles in different aspects of plant growth and development including vascular differentiation (Davies, 1995). Its involvement in improving the tolerance of plant (wheat and Arabidopsis) to various preharvest abiotic stresses such as heat, salt
and water stresses is widely reported (Hu et al., 2013; Iqbal and Ashraf, 2007; Jung and Park, 2011; Kazan, 2013). Similarly, cytokinins (CK) has been reported to be involved in maintaining cellular proliferation and differentiation as well as prevention of senescence (Schmülling, 2002). Cytokinins reduce concentration results in the closure of stomata induced by ABA, which reduces carbon uptake and assimilation. The upregulation of CK oxidase could also reduce carbon metabolism under stressful conditions (Egamberdieva et al., 2017). Hence, phytohormones could play significant role in the susceptibility of ‘Nules Clementine’ mandarin fruit to RBD, which mostly become visible only around three to five weeks postharvest as fruit tend towards senescence (Cronje et al., 2011a; Magwaza et al., 2012; van Rensburg et al., 2004). Therefore, the aim of this study was to investigate phytohormonal changes in ‘Nules Clementine’ mandarin fruit rind from different canopy positions in relation to the incidence of RBD during postharvest non-chilling cold storage.

2. Materials and methods

2.1 Chemicals

Original standards of cytokinins (isoprenoid, aromatic and 2-MeS CKs), auxins, and their corresponding isotopically labelled analogues were purchased from Olchemim Ltd. (Olomouc, Czech Republic) and Chemiclones (Waterloo, Canada). Chromatographic solvents (acetonitrile and methanol (MeOH)) of hyper grade quality, eluent additives (FA and NH₄OH) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Potassium hydroxide (KOH), sodium and potassium acetate (CH₃COONa, CH₃COOK), formic acid (CH₂O₂), sodium bicarbonate (NaHCO₃) and 99.8% ethanol (EtOH) were purchased from Lach-Ner, s.r.o.
Deionised (Milli-Q) water prepared by a Simplicity 185 water system (Millipore, Bedford, MA, USA) was used for all aqueous solutions.

2.1. Plant materials

‘Nules Clementine’ mandarin fruit (Citrus reticulata Blanco) were harvested from 50 uniformly sized trees at commercial maturity during 2014/15 season from Unifruiti and Swartvelei Farms located at Eastern Cape (EC, 33° 27′ 32″ S, 25° 34′ 79″ E) and Western Cape (WC, 19° 02′ 33.8″ S, 33° 41′ 17.24″ E) provinces of South Africa, respectively. Fruit from EC were budded on ‘Carrizo’ citrange ((Poncirus trifoliata (L.) Raf.) × (C. sinensis)) rootstock and planted in 1997 at a spacing of 5.5 x 2 m. Fruit from WC were budded on Rough lemon (C. jambhiri Lush.) rootstock planted in 2001 at a spacing of 5.5 x 2.5 m. Fruit were harvested from inside canopy and outside canopy positions as described elsewhere (Cronje et al., 2011a; Olarewaju et al., 2017). Harvested fruit were transported within 48 h under ambient temperature in ventilated cartons to postharvest research laboratory where the fruit were washed and sorted for blemishes and fruit damage. Fruit were left for 24 h at room temperature 20 ± 1 °C to equilibrate, sorted for physical blemishes and damages, washed, labelled, weighed, and transferred into cold storage (7.5 ± 0.5°C) for 9 weeks. Four replicates of 20 fruit per canopy position were analysed at 3 weeks interval for 9 weeks (weeks 0, 3, 6 and 9).

2.2 Rind breakdown rating

Fruit were evaluated for RBD incidence prior to cold storage and after 3, 6, and 9 weeks after cold storage. The incidence of RBD on fruit was visually inspection according to Alférez et al. (2003) and Lafuente et al. (1997) on a subjective scale from 0 = no breakdown to 3 = severe
breakdown. RBD was expressed as RBD index, after calculations according to equation 1 (Alférez et al., 2003; Lafuente et al., 1997).

\[
RBD \text{ index} = \frac{(\text{Peel pitting (0–3)} \times \text{number of fruit in each class})}{\text{Total number of fruit}}
\]  

(1)

2.3. Sample preparation

Individual fruit was manually peeled, snap-frozen in liquid nitrogen and cold-stored at -80 °C before freeze-drying over a period of three days using Virtis Benchtop freeze dryer system (ES Model, SP Industries Inc., Warmister, USA) at 0.015 kPa and -75 °C. Dried samples were then hand-milled into fine powder using pestle and mortar and stored in -40 °C for future analysis of phytohormones.

2.4. Quantitative analysis of endogenous CKs and auxins

Three replicates of lyophilized samples were used for analysis of endogenous levels of auxins and CKs. 2 mg of each sample were extracted in 0.5 mL of modified Bieleski buffer (60% methanol, 10% \( \text{CH}_2\text{O}_2 \) and 30% distilled water (\( \text{H}_2\text{O} \))) (Hoyerova et al., 2006) with isotope-labelled CK (0.25 pmol per sample of B, R, 9G, 7G and 0.5 pmol per sample of OG, NT) and auxin (5 pmol per sample) internal standards for control of purification step and to validate determination (Novak et al., 2008). Adjusted purification protocol by Dobrev and Kamińek (2002) was using for the joint purification of cytokinins and auxins by MCX cartridges. Auxins were obtained from MCX cartridges eluting with 80% MeOH, 0.35M \( \text{NH}_4\text{OH} \) solution was used for elution of cytokinin nucleotides and 0.35M \( \text{NH}_4\text{OH} \) in 60% MeOH for elution of cytokinin bases and \( \text{O-} \)glucosides.
The individual cytokinin elution was collected together. All of auxin and cytokinin elution were evaporated to dry by SpeedVac concentrator (CentriVap ® Acid-Resistant benchtop concentrator, Labconco Corp. MO, USA, 2015) and dissolved in 30 µL of 10% MeOH. The samples were analysed by ultra-performance liquid chromatograph (Acquity UPLC® I-class System; Waters, Milford, MA, USA) coupled to a triple quadrupole mass spectrometer (MS/MS) equipped with an electrospray interface (Xevo™ TQ-S, Waters, Manchester, UK) using the analytical separation described by Svačinová et al. (2012) for CKs and Pěnčík et al. (2009) for auxins. Quantification was obtained by multiple reaction monitoring of [M+H]⁺ and the appropriate product ion. Optimal conditions, dwell time, cone voltage, and collision energy in the collision cell, corresponding to the exact diagnostic transition, were optimized for each CK and auxin for selective MRM experiments (Pěnčík et al., 2009; Svačinová et al., 2012). Quantification was performed by MassLynx™ software package (versions 4.0 and 4.1, Waters, Milford, MA, USA) using a standard isotope dilution method.

2.5. **Statistical analysis**

The data collected were subjected to the analysis of variance (ANOVA) using GenStat 18.0 (VSN International, Hemel Hempstead, United Kingdom). Fischer’s least significant differences were calculated and used to separate means at 5% significance level
3. Results and discussion

3.1. Canopy position influenced cytokinins levels during postharvest cold storage

3.1.1. Trans- and cis-zeatin-type cytokinins

The concentrations of most of the trans-zeatin-type CKs (data not shown) were below the limit of detection (LOD, less than 0.05 pmol/g DW) except trans-zeatin-O-glucoside (tZOG). Temperature and/or sunlight exposure could play a crucial role in the inhibition or production of phytohormones and its conjugates in plant tissues. The concentration of tZOG was generally affected by canopy position ($p < 0.05$) and was significantly higher in IC fruit (15.80, 8.58, 11.59, 0.00) pmol/g DW than OC fruit (0.00, 0.00, 4.85, 0.00) pmol/g DW from EC after week 0, 3, 6, 9 of postharvest non-chilling cold storage, respectively (Figure 1A). Similarly, in fruit from WC, the concentration of tZOG were higher in IC fruit (12.15, 12.72, 15.96, 8.67) pmol/g DW than OC fruit (0.00, 7.97, 8.29, 0.00) pmol/g DW from WC region after week 0, 3, 6, 9 of postharvest non-chilling cold storage, respectively (Figure 1A). This indicated that exposure of fruit to sunlight could inhibit the production of the hormone. Although, there was no significant difference in cis-zeatin-O-glucoside (cZOG) concentration of IC and OC fruit from EC, cis-zeatin-type cytokinins including cis-zeatin riboside (cZR), cis-zeatin riboside-O-glucoside (cZROG), cis-zeatin-7-glucosides (cZ7G) were mostly higher in the OC fruit than IC fruit from both EC and WC provinces during postharvest non-chilling cold storage (Figures 1B-E). This indicated that the fruit exposure to cold storage triggered the in vitro production of these cytokinin conjugates. The cZR concentrations of IC and OC fruit harvested from EC increased from 4.82 and 2.80 pmol/g DW after week 0 and 9 to 6.76 and 46.20 pmol/g DW, respectively, during postharvest cold storage at non-chilling temperature (Figure 1C). Similar trend occurred for fruit harvested from WC.
The incidence of RBD only occurred on fruit harvested from EC during 2016 season and was observed on IC fruit after week 9 of cold storage. Hence, the result comparing the phytohormonal levels of fruit with and without rind disorder was based only on IC fruit from EC after week 9 of non-chilling cold storage at 7.5 ± 0.5 °C. Fruit without RBD had higher concentration of \( \text{tZOG} \) (12.86 pmol/g DW) and \( \text{cZ7G} \) (6.59 pmol/g DW) than fruit with rind disorder (0.00 pmol/g DW) and (0.00 pmol/g DW), respectively which were below LOD (Figure 2A & 2E). Other \( \text{cis} \)-type cytokinins including \( \text{cZOG} \), \( \text{cZR} \) and \( \text{cZROG} \) were significantly higher in disordered (118.13, 24.14 and 6.97 pmol/g DW) fruit than fruit without disorder (76.52, 11.57 and 4.86 pmol/g DW; Figures 2B-D).

Phytohormones are responsible for many physiological responses in plants including prevention of diseases and physiological disorders. The crucial function of CKs in the regulation of plant immunity against pathogens has been identified (Schäfer et al., 2015). Cytokinins control plants’ immune system by modulating salicylic acid signalling and play crucial role in the defence against pathogens and insects (Choi et al., 2011; Giron et al., 2013). Several active CKs, such as kinetin, 6-benzylaminopurine (6-BAP), and \( \text{rZ} \) have been demonstrated to be capable of increasing resistance against hemi-/biotrophic pathogens in Arabidopsis and tobacco (Argueso et al., 2012; Choi et al., 2011; Egamberdieva and Kucharova, 2009; Großkinsky et al., 2013, 2011). Scientific report of the phytohormone concentration of citrus fruit rind in relation to the prevention or manifestation of physiological rind disorder is not available in literature. The cytokinins reported in this study could have been involved in the prevention of physiological rind disorder in the mandarin fruit used in the study. \( \text{cZOG} \), \( \text{cZR} \), and \( \text{cZROG} \) concentrations were higher in fruit with RBD than on fruit without RBD. \( \text{rZOG} \) and \( \text{cZ7G} \) were absent in fruits with disorder. This
indicated that lower concentration of \( cZOG, cZR, \) and \( cZROG \) and absence of \( iZOG \) and \( cZ7G \) synergistically brought about the immunity in fruits without disorders.

Dihydrozeatin 7-glucosides (DHZ7G) had higher concentration than other measured CKs and were mostly higher in IC fruit than OC fruit from both EC and WC provinces during cold storage (Figure 3A). However, the concentration of DHZ7G became higher in the OC (222.91 pmol/g DW) fruit than IC (183.51 pmol/g DW) fruit at week 3 (Figure 3A). \( N^6 \)-isopentenyladenine riboside (iPR) and \( N^6 \)-isopentenyladenine-N9-glucoside (iP9G) concentrations were generally higher in the OC fruit (1.85, 0.67, 2.63, 6.68 and 8.92, 9.07, 6.72, 7.37 pmol/g DW) than IC fruit (1.48, 0.63, 1.31, 6.36 and 5.86, 4.66, 5.91 pmol/g DW, Figure 3B & C). The iP9G was below the LOD in fruit harvested from EC. Comparing the DHZ7G, iPR and iP9G concentration of fruit with and without rind disorder, DHZ7G concentration was significantly higher in fruit with RBD than those without (Figure 4). There was no significant difference in the concentration of iPR of fruit with and without RBD while iP9G concentration was higher in fruit without RBD than fruit with the disorder (Figures 4B & C). The presence of iP9G in the rind of IC and OC fruit harvested from WC and absence in fruit from EC could be because of the orchard practice, rootstock and agroecological conditions of fruit before harvest as these factors could greatly influence the production of endogenous hormones and their conjugates in plant tissues.

Zeatin is considered as an essential cytokinin in higher plants because of its ubiquitous nature and high activity (Rodo et al., 2008). Other free bases with cytokinin activity, \( cZ, N6-(\Delta2\)-isopentenyl) adenine and dihydrozeatin are also present in most plant tissues (Rodo et al., 2008). Derivatives of these bases include the corresponding ribosides and nucleotides, as well as glucosides with the
sugar moiety at the, O- of the side chain or at the N7, N9, or N3 of the adenine ring (Rodo et al., 2008). The result of this study revealed the presence of DHZ7G, iPR and iP9G in mandarin fruit. In CK-mediated resistance, interactions with other phytohormones, such as abscisic acid (Großkinsky et al., 2014) or salicylic acid (Argueso et al., 2012; Choi et al., 2010; Großkinsky et al., 2011) have previously been reported. However, information about the synergistic role of cZ glucoside and iP9G with auxin is not yet documented in literature. In this study, the CK conjugates, cZ7G, tZOG and iP9G synergistically acted with the auxin IAA to prevent RBD in mandarin fruit rind.

3.2. Canopy position affected auxin concentrations of fruit rind during postharvest cold storage

The influence of canopy position on rind concentration of indole-3-acetic acid (IAA) and IAA-aspartate (IAAsp) were examined during postharvest non-chilling cold storage. The IAA concentration was higher in the IC fruit (344.15 pmol/g DW) than OC fruit (194.20 pmol/g DW) from EC at week 0 while IAA concentration of OC fruit from WC were below the LOD and IC fruit had 53.20 pmol/g DW at week 0 (Figure 5A). The IAA concentration of OC fruit from EC fairly remained constant throughout postharvest period while a significant drop from 344.15 pmol/g DW at week 0 to 177.14 pmol/g DW at week 3 occurred in IC fruit from EC. The IAAsp concentration were below the LOD at week 0 and 3 but increased to 11.83 and 12.17 pmol/g DW at week 6 and 9, respectively (Figure 5B).

IAA is an important growth regulator that has been reported to have potent physiological regulatory properties. These properties include delaying ripening and softening of fruits, inducing
defence responses in plant tissues and inhibition of postharvest physiological disorders (Baldwin, 2003; Rojo et al., 2003). In this study, fruits without disorder had a high composition of IAA which is more than double the value recorded in fruits with RBD. This indicated that the presence of IAA could be responsible for the prevention of rind disorder. It was observed that the IAA concentration in both IC positions in EC and WC decreased significantly at the end of the cold storage period (week 9) compared to the concentration at the initial stage before storage (week 0; Figure 5A). The reverse is the case in fruits from OC position of both locations as there were increase in IAA concentration at week 9 compared to week 0 (Figure 5A). This suggested that the endogenous concentration of IAA during non-chilling postharvest cold storage is dependent on their canopy position. The IAA concentration in fruit with disorder is much higher than in fruit without disorder indicating the potent ability of IAA to prevent or block the manifestation of physiological disorders (Figure 6A). Figure 6 shows the IAA and IAAsp concentrations of fruit with and without RBD. Fruit without RBD had higher concentration of IAA than fruit with the disorder while IAAsp were significantly higher in fruit with RBD than fruit without the disorder (Figures 6A & B).

This crucial activity of IAA could be an outcome of its synergistic interaction with other hormones and or metabolic processes in the fruit during its postharvest life. Plant hormones interact in complex networks to balance the response to developmental and environmental cues (Denancé et al., 2013). The mechanisms governing these hormonal networks are unknown (Denancé et al., 2013). The ability of phytohormones to play this crucial role could be dependent on other factors such as the presence of coenzymes, seasonal variation, orchard or agricultural practices, temperature, and environmental stress.
However, the presence of IAAsp, a derivative of IAA resulted in physiological rind disorder in this study. The presence of the aspartate group in the IAAsp could be responsible for the manifestation of the disorder. The involvement of IAAsp in RBD disorders has not been established. This study will be the first to record the negative effect of IAAsp in the physiological disorder of citrus fruit. The processes involved in the conversion of IAA to IAAsp could have triggered the conditions involved in the manifestation of physiological rind disorder.

5. Conclusion

The phytohormonal concentration of ‘Nules Clementine’ mandarin fruit rind during postharvest non-chilling cold storage shows that different phytohormones are present in the fruit. Phytohormonal concentrations from week 0 to week 9 during postharvest cold storage followed inconsistent trends. The mechanism responsible for the inconsistency in the fruit rinds is unknown. The results also revealed the role of temperature (cold storage and inside canopy environment) and exposure to sunlight on the production of phytohormones and their conjugates. The results highlighted the positive effect of cZ7G, tZOG, iP9G and IAA in the prevention of physiological disorder and the negative effect of cZOG, cZR, cZROG and IAAsp. However, detailed study involving molecular physiology, biochemical pathways of phytohormones and enzymes, interactions of auxins and cytokinins during postharvest cold storage and effect of postharvest storage conditions on phytohormone concentration is suggested to have a better and in-depth understanding of the contribution of phytohormones to physiological rind disorder of citrus fruit.

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Figure 1: Comparison of *trans*- and *cis*-zeatin-type cytokinins of ‘Nules Clementine’ mandarin fruit harvested from two canopy positions (inside canopy (IC) and outside canopy (OC)) from Eastern Cape (EC) and Western Cape (WC) provinces over two seasons during postharvest non-chilling cold storage. Vertical bars represent standard error of the mean value (n = 3). Abbreviations: Trans-zeatin (tZ), cis-zeatin (cZ), O-glucoside (OG), riboside (R), riboside-O-glucoside (ROG), 7-glucosides (7G)
Figure 2: Comparison of trans- and cis-zeatin-type cytokinins of ‘Nules Clementine’ mandarin fruit with (disorder) and without (none) rind breakdown after 9 weeks of cold storage at non-chilling temperature. Vertical bars represent standard error of the mean value (n = 3). Abbreviations: Trans-zeatin (tZ), cis-zeatin (cZ), O-glucoside (OG), zeatin riboside (ZR), riboside-O-glucoside (ROG), 7-glucosides (7G)
Figure 3: Comparison of DHZ7G, iPR, and iP9G of ‘Nules Clementine’ mandarin fruit harvested from two canopy positions (inside canopy (IC) and outside canopy (OC)) from Eastern Cape (EC) and Western Cape (WC) provinces over two seasons during postharvest non-chilling cold storage. Vertical bars represent standard error of the mean value (n = 3). Abbreviations: Dihydrozeatin 7-glucosides (DHZ7G), $N^6$- isopentenyladenine riboside (iPR) and $N^6$-isopentenyladenine-N9-glucoside (iP9G).
Figure 4: Comparison of DHZ7G, iPR, and iP9G of ‘Nules Clementine’ mandarin fruit with (disorder) and without (none) rind breakdown after 9 weeks of cold storage at non-chilling temperature. Vertical bars represent standard error of the mean value (n = 3). Abbreviations: Dihydrozeatin 7-glucosides (DHZ7G), N6-isopentenyladenine (iPR) and N6-isopentenyladenine-N9-glucoside (iP9G).
Figure 5: Comparison of auxin concentration of ‘Nules Clementine’ mandarin fruit harvested from two canopy positions (inside canopy (IC) and outside canopy (OC)) from Eastern Cape (EC) and Western Cape (WC) provinces over two seasons during postharvest non-chilling cold storage (A and B). Abbreviations: Dihydrozeatin 7-glucosides (DHZ7G), N6-isopentenyladenine (iPR) and N6-isopentenyladenine-N9-glucoside (iP9G). Abbreviations: IAA, indole-3-acetic acid and IAAsp, IAA-aspartate.
Figure 6: Comparison of IAA (A) and IAAsp (B) of ‘Nules Clementine’ mandarin fruit with (disorder) and without (none) rind breakdown after 9 weeks of cold storage at non-chilling temperature. Vertical bars represent standard error of the mean value (n = 3). Abbreviations: Dihydrozeatin 7-glucosides (DHZ7G), $N^6$-isopentenyladenosine (iPR) and $N^6$-isopentenyladenine-$N^9$-glucoside (iP9G). Abbreviations: IAA, indole-3-acetic acid and IAAsp, IAA-aspartate.
CHAPTER 7
MODEL DEVELOPMENT FOR NON-DESTRUCTIVE DETERMINATION OF RIND BIOCHEMICAL PROPERTIES OF ‘MARSH’ GRAPEFRUIT AND ‘NULES CLEMENTINE’ MANDARINS USING VISIBLE TO NEAR-INFRARED SPECTROSCOPY AND CHEMOMETRICS*

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Abstract

Rind biochemical properties play major roles in defence mechanism against the incidence of rind physiological disorders of citrus fruit during cold storage. However, conventional methods of analysis are destructive, time-consuming, and also expensive. Consequently, non-destructive techniques were developed to rapidly determine the biochemical properties of ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin fruit rinds using FOSS NIRSystems which acquired the electromagnetic spectral from 400 to 2500 nm. Pre-processing algorithms were used to correct light scattering properties of the spectra. Methods with best results in terms of higher coefficient of determination (R²), residual predictive deviation (RPD), lower root mean square error of prediction (RMSEP) values were used for model development. Fruit rinds were analysed for biochemical properties using visible to near infrared spectroscopy (Vis/NIRS) and reference methods before and after 9 weeks of cold storage, respectively. Results obtained using partial least square regression algorithms showed good to excellent prediction models for rind biochemical properties such as sucrose (R² = 0.99, RMSEP = 0.11, RPD = 11.42), glucose (R² = 0.99, RMSEP = 0.77, RPD = 11.35), total flavonoids (R² = 0.99, RMSEP = 0.07, RPD = 12.37), vitamin C (R² = 0.79, RMSEP = 0.06, RPD = 2.01) and radical-scavenging activities (R² = 0.91, RMSEP = 0.17, RPD = 3.07) of grapefruit. Similarly, excellent models were developed for determination of rind biochemical properties of ‘Nules Clementine’ mandarin fruit. This study reported first application of Vis/NIR and chemometrics in determining of ‘Marsh’ grapefruit. The precision of the developed models to determine rind biochemical properties of ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin fruit rapidly and non-destructively were demonstrated.
Keyword: Antioxidants, Carbohydrates, Chemometrics, Multivariate analysis, Phytochemicals, Vitamin C.
1. Introduction

Consumer preference for citrus fruit without rind disorder influences the purchase of fruit at both local and international markets. Therefore, purchase for any citrus fruit, is largely depended on appearance and rind biochemical properties (Khalid et al., 2012). External appearance, which plays a major role in consumer acceptability, is closely linked with rewarding internal sensory quality (Pathare et al., 2013). Therefore, evaluation and determination of biochemical properties of citrus fruit rind is important in delivering quality fruit to the fresh fruit market. One of the limiting factors affecting the citrus industry is the sudden incidence of postharvest physiological rind disorders. Most rind disorders manifest only after harvesting and sorting has been completed at the pack-house and/or when fruit are ready to be delivered to the market, that is, after about three to five weeks postharvest (Cronje et al., 2011; Magwaza et al., 2012; van Rensburg et al., 2004).

In the market, rind colour of a fruit constitutes the initial factor that repels or attract potential buyers. Moreover, rind biochemical properties such as non-structural carbohydrates constitute some of the most important constituents that play vital roles in the predisposition of fruit to different postharvest non-chilling physiological rind disorders (Di Majo et al., 2005; Magwaza et al., 2014a, 2014b). For instance, Ezz and Awad (2009) reported a significant relationship between sugars and rind pitting disorder of ‘Marsh’ grapefruit. This suggests that certain rind biochemical properties of citrus fruit could be used as biomarker(s) for predicting the susceptibility of fruit to physiological rind disorder. Since the incidence of non-chilling physiological rind disorder of citrus fruit is unpredictable, it is important to monitor fruit rind biochemical properties such as carbohydrates, dry matter (DM), phytochemicals, vitamin C, and radical-scavenging activities and its correlation with the incidence of rind disorder of the fruit. This knowledge could guide growers.
to ensure that fruit with good quality rind is delivered to the markets irrespective of destinations. However, these rind parameters are mostly analysed using destructive, laborious, time-consuming, and expensive methods. In view of this, a non-destructive method to determine these rind properties could unveil new approach(es) to predicting the overall quality of the fruit rind and enhance the delivery of quality fruit at both local and international markets.

Visible to near-infrared spectroscopy (Vis/NIRS) is a non-destructive analytical tool that has gained wide recognition in the citrus industry for its suitability in the assessment of quality attributes of citrus fruit (Cayuela and Weiland, 2010; Gómez et al., 2006; Magwaza, 2013). Its use in assessing the quality of horticultural products such as avocado (Olarewaju et al., 2016) and pomegranate (Arendse et al., 2017) is well documented. However, its use in non-destructive assessment and determination of biochemical properties of ‘Marsh’ grapefruit is currently unknown. The aim of this study was to develop a robust and non-destructive calibration models for rapid assessment and determination of biochemical properties of ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin rind using Vis/NIRS.

2. **Materials and methods**

2.1. **Reagents and standards**

All chemicals including Sodium Hydroxide (NaOH), Folin-Ciocalteu reagent, metaphosphoric acid (MPA), sodium carbonate, gallic acid, quercetin, vitamin C, 2, 6 dichloroindophenol dye, 2,2-diphenyl-1-picrylhydrazyl (DPPH), acetone, ethanol (HPLC grade) and sugars standards (sucrose, D-glucose, and D-fructose) were purchased from Sigma-Aldrich Company Ltd. (Dorset, UK). A Phenomenex® column (Rezex RCM - Monosaccharide) was used in the analyses. Water was
purified in a Milli-Q Integral Water Purification System (Merck Millipore corporation, Billerica, MA, USA; σ = 18 M Ω cm\(^{-1}\)).

### 2.2. Plant materials

Experiments were conducted during 2015/16 seasons using ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin fruit. ‘Marsh’ grapefruit were budded on ‘Troyer’ Citrange (\([Poncirus trifoliata\) (L.) Raf.] \(\times\) [C. sinensis]) and x 639 (\([Poncirus trifoliata\) (L.) Raf.] \(\times\) [C. reshni]) rootstocks planted in 1993 on Bolton Citrus Farm, KwaZulu-Natal (KZN) (31° 34′ 44″ S, 28° 44′ 59″ E) and Olifant River Farm, Limpopo (LMP) (32° 7′ 28″ S, 35° 89′ 31″ E) provinces, respectively. ‘Nules Clementine’ mandarin fruit harvested from Unifruiti Farm, Eastern Cape (33° 27′ 32″ S, 25° 34′ 79″ E) were budded on ‘Carrizo’ citrange (\([Poncirus trifoliata\) (L.) Raf.] \(\times\) [C. sinensis]) while fruit harvested from Swartvelei Farm, Western Cape (25° 04′ 42″ S, 29° 23′ 09″ E) were budded on Rough lemon (\(C. jambhiri\) Lush.) rootstocks. A total number of 600 individual fruit (per cultivar) were harvested from 50 uniform sized trees at commercial maturity where six fruit were randomly selected from inside and outside canopy positions of the tree. After harvesting, fruit were transported within 48 h at ambient temperature in ventilated cartons to a horticultural research laboratory where fruit were washed and sorted for blemishes and fruit damage. Fruit were left for 24 h at room temperature 20 ± 1 °C to equilibrate after which fruit were labelled, weighed, and transferred into cold storage (7.5 ± 0.5°C) for 9 weeks. The fruit was then analysed for biochemical properties before and after cold storage.
2.3. Near infrared spectra acquisition

Visible to near infrared spectra of ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin fruit were acquired using a method described by Sabatier et al. (2013) with modifications according to Olarewaju et al. (2016). Briefly, spectral data were acquired in reflectance mode using a laboratory bench-top monochromator NIRSystems Model XDS spectrometer (FOSS NIRSystems, Inc., Silver Spring, Maryland, USA) equipped with a quartz halogen lamp and lead sulphide (PbS) detector (Figure 1). The spectra were acquired with a circular sample cup with a quartz window (38 mm in diameter and 10 mm in thickness). The equatorial region of each fruit was carefully positioned on the instrumental sample cup and placed in an enclosed window before scanning to avoid light leakage. The NIRSystem was operated on Vision software (VisionTM, version 3.5.0.0, Tidestone Technologies Inc., KS, USA). Spectra were obtained at 2 nm intervals over a full spectral range (450 2500 nm) and each spectrum consisted of 32 scans which were automatically averaged and recorded as log 1/reflectance (log 1/R). Line plot representing absorbance spectra for grapefruit and mandarin fruit are depicted in Figure 2. The integration time was less than 500 ms per spectrum collected. Each fruit was scanned two times along the equatorial region after rotating the fruit 180° and the two spectra were averaged.

2.4. Reference analysis

2.4.1. Sample preparation

Reference measurements were taken from the area of the fruit rind where spectra were acquired using conventional destructive methods. Scanned area of the fruit rind was manually peeled off using table knife, snap frozen in liquid nitrogen and stored at -80 °C before freeze-drying over a period of three days using Virtis Benchtop freeze drier system (ES Model, SP Industries Inc.,
Warmister, USA) at 0.015 kPa and -75 °C. Dry matter (DM) was calculated by subtracting the mass of the freeze-dried samples from fresh samples and expressed as percentage DM. Dried samples were then milled into a fine powder using pestle and mortar and stored in -20 °C for further biochemical analysis.

2.4.2. Rind pigments analyses

The total carotenoid concentration was determined using a modified method of Lichtenthaler (1987) according to Olarewaju et al. (2017). Briefly, lyophilized sample (150 mg ± 0.5) was weighted into a test tube followed by the addition of 2 mL of 80% (v/v) acetone before centrifugation for 10 min using GenVac® (SP Scientific, Genevac LTD., Suffolk, UK). The absorbance values of the supernatants were read at 470, 646.8, and 663.2 nm for maximum detection of carotenoids, chlorophyll a and b and total carotenoid concentration were calculated using Eqs. 1, 2, and 3, respectively.

\[
C_a = 12.25 A_{663.2} - 2.79 A_{646.8} \quad 1
\]

\[
C_b = 21.50 A_{646.8} - 5.10 A_{663.2} \quad 2
\]

\[
C_c = (1000 A_{470} - 1.82 C_a - 85.02 C_b)/198 \quad 3
\]

Where \(C_a\) is chlorophyll a, \(C_b\) is chlorophyll b and \(C_c\) is total carotenoid concentration.
2.4.3. Extraction and quantification of rind soluble sugars

Soluble sugars were extracted and analysed according to Olarewaju et al. (2017). Concentration of fructose, glucose, and sucrose were then quantified using an isocratic HPLC binary pump system (Shimadzu Corporation, Kyoto, Japan) equipped with a refractive index detector. A 1 mL of diluted extract was injected into a Rezex RCM monosaccharide Ca\(^{+}\) (8%) column of 7.8 mm diameter × 300 mm (Phenomenex, Torrance, CA, USA) with a SecurityGuard\textsuperscript{TM} cartridges of 4 mm × 3 mm (Phenomenex). The mobile phase used was ultra-pure HPLC-grade water at a flow rate of 0.6 mL/min with the column compartment temperature set at 80 °C using a thermos-stated column compartment (Faculty of Science Workshop, University of Natal, Pietermaritzburg). The presence and concentration of individual sugars were determined by comparing peak area of samples with peak area and concentration of a known sugar standard curve (0.05 – 1.25 mg/L; \(R^2 = 0.99\)).

2.4.4. Sample extraction for phenolics and flavonoid analyses

Extraction was carried out according to Moo-Huchin et al. (2015) as modified by Olarewaju et al. (2017). Briefly, 150 mg ± 0.5 mg lyophilized sample was measured into a test tube and 3 mL of 50:50 (ethanol: water) v/v was added. The test tube, covered with aluminium foil, containing the mixture was subsequently placed in a shaking water bath (Gesellschaft für (GFL), Labortechnik mbH, Burgwedel, Germany) at 70 °C for 2 h with intermittent sample vortexing for 20 s at 30 min interval. The sample was left to cool down followed by centrifugation for 10 min using GenVac\textsuperscript{®} centrifuge (SP Scientific, Genevac LTD., Suffolk, UK) and filtered using a 0.45 µm nylon filter. Extracts were stored at -20 °C for further analysis.
2.4.4.1. **Determination of total phenolic concentrations**

Extracts were analysed according to Moo-Huchin et al. (2015) as modified by Olarewaju et al. (2017) for total phenolic concentration. Briefly, sample extract (10 μL) was measured into a 4.5 mL disposable cuvette in triplicate followed by the addition of 1.6 mL of distilled water, 100 μL of Folin-Ciocalteu reagent and 300 μL of sodium carbonate solution. The solution was mixed and incubated in the dark at room temperature for 2 h before absorbance at 765 nm using Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, USA). Gallic acid was used to generate a standard curve and total phenolic concentrations were expressed as mg gallic acid equivalent (GAE) /g DM.

2.4.4.2. **Determination of total flavonoid concentrations**

Extracts were analysed for total flavonoid concentration according to Lin and Tang (2007) with modification by Olarewaju et al. (2017). Briefly, extract (100 μL) was measured into a 4.5 mL disposable cuvette followed by the addition of 3 mL of sodium hydroxide solution. The mixture was agitated and incubated at room temperature for 10 min. Absorbance was measured in triplicate at 420 nm using an Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, USA). Quercetin was used to generate a standard curve and total flavonoid concentration was expressed as mg quercetin equivalent (QTE) /g DM.

2.4.5. **Sample extraction for determination of vitamin C and radical-scavenging activities**

Extraction was carried out according to the method of Brand-Williams et al. (1995) with slight modification by Olarewaju et al. (2017). A lyophilized sample of 150 mg ± 0.5 mg was measured into a test tube followed by the addition of 5 mL of 3% aqueous metaphosphoric acid and
incubation on ice cubes for 5 min. The extract was centrifuged for 20 min using GenVac® (SP Scientific, Genevac LTD., Suffolk, UK). Supernatant were stored in -20 °C for further analysis of vitamin C and DPPH radical-scavenging activities.

2.4.5.1. **Determination of Vitamin C**

Vitamin C were determined according to Barros et al. (2007) and Klein and Perry (1982) with modifications by Olarewaju et al. (2017). Briefly, supernatant (0.5 mL) was measured into a test tube followed by the addition of 2.5 mL of 0.005% of 2, 6 dichloroindophenol dye. The mixture was incubated in the dark for 10 min at room temperature. Absorbance was read in triplicate at 515 nm against a 3% MPA solution under dim light using Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, USA) and the amount of vitamin C was calculated from a linear standard curve (0.00 – 100.00 µg/g; R² = 0.96).

2.4.5.2. **Determination of DPPH radical-scavenging activities**

Determination of radical-scavenging activities were carried out according to Olarewaju et al. (2017). The Supernatant (20 µL) were measured into 4.5 mL disposable cuvette followed by the addition of 800 µL of methanol. One millilitre of 0.1 mM DPPH solution was added, vortexed and incubated in the dark at room temperature for 60 min. Absorbance was read in triplicate at 517 nm against a blank (absolute methanol) under dim light using Ultraspec UV-1800 Spectrophotometer (Shimadzu Scientific Instruments, Inc., Columbia, USA) and radical-scavenging activities were calculated by the percentage of DPPH that were scavenged using the Eq. 4.

\[
\text{Radical-scavenging activities (\%) = (1 − \frac{A_E}{A_D}) \times 100}
\]
Where $A_E$ is the absorbance of the reaction mixture containing the standard antioxidant, or extract and $A_D$ is absorbance of the DPPH solution only.

2.6. Chemometric data analysis

Principal component analysis (PCA), a chemometric tool that determines effective wavelengths and detects outliers, and partial least squares (PLS) regression analysis, a tool usually used for calibration model development, were performed using the Unscrambler chemometric software (The Unscrambler™ Version 10.3, CAMO, Oslo, Norway). As a common practice before calibration model development, the spectral variation, effective wavelengths and outliers were first evaluated using full cross validation (leave-out-one) of PCA (Magwaza et al., 2014a). Consequently, identified outliers were removed from the calibration model development (Kuang and Mouazen, 2011; Magwaza et al., 2014a, 2014b).

After PCA analyses, spectral data (X) were then related with reference biochemical data (Y) using PLS (Sáiz-Abajo et al., 2005) regression (with wide kernel algorithm) which ensured that latent variables (LV) were ordered based on their relevance for predicting the Y – variable (Nicolaï et al., 2007). The spectral datasets were grouped into various calibration/training (60%) and prediction/test (40%) sets, including spectral data of fruit from each and combinations of production regions before storage, after storage and merger of overall data. Where necessary, spectral data were subjected to several mathematical pre-processing algorithms to smooth spectral data, correct light scattering and reduce the changes of light path length during regression analysis (Magwaza et al., 2012a). Pre-processing methods investigated either individually or in
combination with others included Smoothing using Savitzky-Golay (SG, with segment size of seven for averaging at first and second polynomial order of derivative), derivative using Gap, Gap Segment and Savitzky-Golay (first and second polynomial order of derivative). Other algorithms used were baseline, standard normal variate (SNV), multiplicative scatter correction (MSC), and mean normalization. Method(s) that gave the best results in terms of highest coefficient of determination ($R^2$, Eq. 7) and residual predictive deviation (RPD, Eq. 8), least root mean square error of calibration (RMSEC, Eq. 9) and root mean square error of prediction (RMSEP, Eq. 10) were used for calibration model development to predict interested biochemical properties. The RPD were rated based on model’s reliability check described in previous studies (Magwaza et al., 2012b; Zimmermann et al., 2007). That is RPD values greater than 3.0 were regarded as excellent models, those between 2.5 and 3.0 were considered good models, those between 2.0 and 2.5 are fit for quantitative predictions, those between 1.5 and 2.0 are appropriate for rough predictions while value less than 1.5 means that the model is unreliable.

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

$$RPD = \frac{SD}{RMSEP}$$  

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

$$RMSEP = \sqrt{\frac{\sum(y_{pred} - y_{act})^2}{n}}$$
Where, $n$ is the number of fruit samples used in model development; $y_{\text{act}}$ is the actual value measured by a destructive method; $y_{\text{mean}}$ is the average value of predicted data; $y_{\text{pred}}$ is the Vis/NIR predicted the value of fruit rind variables and SD is the standard deviation of reference data values.

### 2.6 Statistical analysis

Experiments were laid out using a completely randomized design (CRD) with individual fruit as replicate. All statistical analyses were performed using GenStat® 18th Edition (VSN International, Hemel Hempstead, UK). Data were subjected to analysis of variance (ANOVA) with canopy position, season, production region and cold storage time as factors. Mean separation between treatments were performed using least significant difference (LSD) at 5% level of significance.

### 3. Results and discussion

#### 3.1. Distribution of reference data sets

A significant amount of distribution existed among the samples (grapefruit and mandarin) and reference data were normally distributed around the mean which, together with range, were similar for calibration and validation data sets shown in Tables 1 and 2. For calibration set in grapefruit, reference values for sucrose, glucose, and fructose (rind carbohydrates) ranged from 68.50 to 427.40 mg/g DW, 205.00 to 311.30 mg/g DW, and 105.80 to 268.90 mg/g DW with a mean of 179.82, 252.27 and 304.53 mg/g DW, respectively (Table 1). Reference values ranged from 68.55 to 427.30 mg/g DW, 205.20 to 311.20 mg/g DW and 268.90 to 374.60 mg/g DW with a mean of 178.90, 252.58, and 304.80 mg/g DW, respectively for validation set (Table 1). Percentage coefficient of variation (CV%) for other reference parameters including total phenolics, total flavonoids, vitamin C, radical-scavenging activities, chlorophyll a, chlorophyll b and total
carotenoids were 25.12, 20.92, 6.61, 24.22, 52.71, 60.20, 61.72% (Table 1). Similarly, for
calibration set in mandarin fruit, reference values for sucrose, glucose, and fructose ranged from
46.50 to 269.40 mg/g DW, 113.45 to 312.30 mg/g DW, and 152.00 to 332.00 mg/g DW with a
mean of 92.17, 206.91 and 231.70 mg/g DW, respectively (Table 2). Reference values ranged from
46.80 to 269.40 mg/g DW, 113.45 to 312.50 mg/g DW and 152.00 to 331.90 mg/g DW with a
mean of 92.17, 206.79, and 231.64 mg/g DW, respectively for validation set (Table 2). The CV%
for other reference parameters including total phenolics, total flavonoids, vitamin C, radical-
scapeng activities, chlorophyll a, chlorophyll b and total carotenoids were 35.92, 18.97, 10.89,
21.45, 36.18, 75.44 and 46.75%, respectively (Table 2).

Wide variations, which could result from production locations or position of the fruit within tree
canopy, of reference datasets can contribute significantly to model accuracy and reliability as
discussed in previous studies (Davey et al., 2009; Magwaza et al., 2013; Olarewaju et al., 2016).
Similarly, various authors have suggested a wide variation of calibration and validation refere
data sets which enhanced predictive models (Clément et al., 2008; Lu et al., 2006; Magwaza et al.,
2013; Pérez-Marín et al., 2005). Therefore, the wide distribution of reference data was needed for
effective model development.

The relationship among measured rind biochemical properties (reference data) of citrus fruit rind
were studied using Pearson’s correlation algorithm for ‘Marsh’ grapefruit and ‘Nules Clementine’
mandarin, respectively (Tables 3 and 4). Tests showed no correlation among carbohydrates
(sucrose, glucose, and fructose), total phenolics and flavonoids concentrations for both citrus
cultivars (Tables 3 and 4). On the other hand, in grapefruit, vitamin C had a strong correlation with
sucrose \( (r = -0.72) \), glucose \( (r = -0.83) \), radical-scavenging activities \( (r = 0.84) \), chlorophylls a \( (r = 0.86) \) and b \( (r = 0.93) \), and total carotenoids \( (r = -0.84) \). Vitamin C also showed moderate relationships with fructose \( (r = -0.53) \) and total phenolic concentrations \( (r = 0.50) \) while no correlation existed with total flavonoid concentrations. However, in mandarin, vitamin C showed no relationship with sucrose, total phenolic and flavonoid concentrations but had strong to moderate correlations with glucose \( (r = -0.83) \), fructose \( (r = -0.71) \), radical-scavenging activities \( (r = 0.70) \), chlorophylls a \( (r = 0.68) \) and b \( (r = 0.62) \), and total carotenoids \( (r = -0.67) \). These relationships may indicate the abundant presence of vitamin C in the rind of the fruit as widely known as a predominant constituent of antioxidant in citrus fruit.

### 3.2. Spectral characteristics

Figure 1 shows the average absorbance spectra, without application of mathematical pre-treatments, of ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin fruit acquired by FOSS NIRSystem. Although mathematical pre-treatments were not applied on spectra for the development of some models, SG and SNV were employed during the development other models. The spectra were similar with to those acquired in previous studies for other citrus cultivars such as ‘Satsuma’ mandarin (Gómez et al., 2006), and ‘Valencia’ orange (Magwaza et al., 2011). The spectral curves of respective citrus cultivars were similar having notable absorbance peaks around 450, 650, 970, 1200, 1400, 1800 and 1956 nm. Spectra for ‘Nules Clementine’ mandarin were more pronounced at regions 450, 650, and 1876 while peaks at 970 and 1200 were more prominent in ‘Marsh’ grapefruit than ‘Nules Clementine’ mandarin. Spectral peaks at 970 and 1200 nm match the first and second vibrational overtones that are of close association with the H-O-H stretching
modes of H₂O absorption (Clément et al., 2008; Lestander and Geladi, 2005; Olarewaju et al., 2016).

The absorption bands between 1362 and 1388, and at 1402 could be related to the frequencies of the first overtones of C-H-C stretching and combinations while wavebands between 1680 and 1754 nm could be allocated to the first overtones of the C-H stretching and combinations modes (Tewari et al., 2008). The sharp spectral curve noticed at 1800 nm could be related to the second overtone of C-H stretch mode while the information-rich region from 1956 nm and above could be related to the combinations of O-H, C-H and C-C stretches and vibrations associated with carbohydrates (Golic et al., 2003; Tewari et al., 2008).

The wavelength absorption bands between 1415 and 2035 nm are mostly associated with phenolics and flavonoids (Cozzolino et al., 2004; Dykes et al., 2014; Frizon et al., 2015; Zhang et al., 2008). Light absorption by pigments (chlorophylls a and b, and carotenoids) dominates the reflectance spectra between 400 and 700 nm because of the electronic transitions occurring in part of the photoactive molecule (Toledo-Martín et al., 2016). Carotenoids absorption in the spectral range close to 500 nm are convincing and are responsible for the colouration of plants and its organs, such as fruit, including plant leaves without chlorophyll (Merzlyak and Gitelson, 1995; Thomas and Gausman, 1977). Pigments such as β-carotene, β-cryptoxanthin, lutein, zeaxanthin, capsanthin and capsorubin in pepper have been found to be associated with wavebands near 470 nm (Wall et al., 2001) while chlorophylls a and b, fruit green colour, could be absorbed in the visible region of the Vis/NIRS (498-568 and 670 nm) (Font et al., 2007; Gómez et al., 2006; Tkachuk and Kuzina, 1982).
One factor that mostly makes interpretation of Vis/NIR models in relations to various properties of horticultural products difficult is spectra co-linearity. The co-linearity of spectra could allow a pooled effect of many wavelengths where each contributes to the model development and not necessarily few range(s) of spectra (McGlone and Kawano, 1998). Hence, making use of the whole spectra regions during calibration could provide improved models development compared to utilising a few parts of the spectra or individual wavebands (Ozaki and Christy, 2007).

3.3. Determination of rind biochemical properties through PLS models

Multivariate calibration models were developed to determine rind biochemical properties of citrus fruit from Vis/NIR spectra using PLS regression algorithm. Although, principal component regression algorithm was examined during modelling (data not shown), PLS regression yielded better results and was employed. To achieve optimum models for determination of each rind biochemical properties, several mathematical pre-processing methods were explored coupled with no pre-treatment. However, special emphases were given to the best model statistics (in terms of high $R^2$ and RPD, and least RMSEP) which are presented in Tables 5, 6, and 7 for grapefruit and Tables 8, 9, and 10 for mandarins. Models were performed by critical examination of different wavelength ranges and combinations of fruit harvested from different production regions and acquired before (week 0) and after (week 9) cold storage. Tables 6, 7, 9 and 10 contains model statistics using pooled spectral data of fruit from different production region and cold storage time.

Often, pre-processing algorithms are used to correct light scattering and reduced changes of light pathlength (Magwaza et al., 2012a). The SG algorithm usually improves spectra definitions that
are superimposed, eliminate noise in the spectral line and correct baseline region where necessary (Osborne, 2000). Therefore, regression models were developed one after the other in a way that LVs were placed along directions of maximal covariance between the spectral matrix X and the response vector Y (Naes et al., 2002). Hence, spectral data were correlated with reference data obtained by conventional methods.

Similar to Magwaza (2013), cold storage had significant influence on the calibration and validation model performances for both grapefruit (Tables 5 and 6) and mandarins (Tables 8 and 9). Models based on spectra acquired after storage (week 9) performed better for determining rind biochemical properties than those based on spectral acquired before cold storage. Similar trends were observed for fruit from the two distinct production regions but for grapefruit harvested from KZN, rind biochemical properties including sucrose ($R^2 = 0.99$, $RMSEP = 0.11$, $RPD = 11.42$), glucose ($R^2 = 0.99$, $RMSEP = 0.77$, $RPD = 11.35$), fructose ($R^2 = 0.99$, $RMSEP = 0.99$, $RPD = 14.23$), total phenolics ($R^2 = 0.94$, $RMSEP = 0.07$, $RPD = 3.85$), total flavonoids ($R^2 = 0.99$, $RMSEP = 0.07$, $RPD = 12.37$), vitamin C ($R^2 = 0.79$, $RMSEP = 0.06$, $RPD = 2.01$), radical-scavenging activities ($R^2 = 0.91$, $RMSEP = 0.17$, $RPD = 3.07$), chlorophylls a ($R^2 = 0.86$, $RMSEP = 0.08$, $RPD = 2.53$) and b ($R^2 = 0.97$, $RMSEP = 0.14$, $RPD = 5.67$) were better determined using PLS models based on spectra acquired after week 9 of cold storage than those acquired at week 0 (Table 5). These excellent models were developed using full spectra range (400-2500 nm) as suggested by Olarewaju et al. (2016), 2 LVs and without any application of mathematical pre-processing algorithms.
Similarly, for mandarins harvested from LMP, sucrose ($R^2 = 0.97$, RMSEP = 0.79, RPD = 5.36), glucose ($R^2 = 0.97$, RMSEP = 6.86, RPD = 5.40), fructose ($R^2 = 0.97$, RMSEP = 1.14, RPD = 5.36), total flavonoids ($R^2 = 0.80$, RMSEP = 0.14, RPD = 3.27), vitamin C ($R^2 = 0.97$, RMSEP = 0.16, RPD = 5.19), radical-scavenging activities ($R^2 = 0.94$, RMSEP = 2.80, RPD = 3.91), chlorophylls a ($R^2 = 0.94$, RMSEP = 0.27, RPD = 3.85) / b ($R^2 = 0.97$, RMSEP = 5.35, RPD = 5.33), and total carotenoids ($R^2 = 0.97$, RMSEP = 0.60, RPD = 5.30) were better determined with models developed using spectral data acquired after week 9 than the ones acquired prior to cold storage (Table 8). Models for determining rind sugars and pigments (except chlorophyll a, 400-2500 nm, pre-processed with SG first order derivative using 4 LVs) were developed using the NIR region (700-2500) with SNV pre-treatment algorithm and 7 LVs. The whole spectral range was used to develop models that determined total phenolics (SNV applied, 7 LVs), total flavonoids (SNV applied, 2 LVs) and radical-scavenging activities (SNV applied, 1 LVs).

Putting agronomic and climatic factors, associated with different production regions and storage conditions, into consideration, PLS models were developed from samples of various sources (KZN and LMP for grapefruit; and EC and LMP for mandarins) and postharvest conditions (before and after cold storage) to build more robust models (Tables 7 and 10). As a result, for grapefruit, excellent models were developed to determine sucrose ($R^2 = 0.94$, RMSEP = 35.65, RPD = 3.76), vitamin C ($R^2 = 0.95$, RMSEP = 0.20, RPD = 3.33) and chlorophyll b ($R^2 = 0.94$, RMSEP = 0.79, RPD = 3.61) while good models determined glucose ($R^2 = 0.77$, RMSEP = 18.81, RPD = 1.80), radical-scavenging activities ($R^2 = 0.85$, RMSEP = 5.81, RPD = 2.45), chlorophyll a ($R^2 = 0.87$, RMSEP = 0.79, RPD = 2.31), and total carotenoids ($R^2 = 0.80$, RMSEP = 2.86, RPD = 1.71) (Table 7). Similarly, for mandarins, good models were developed to determine glucose ($R^2 = 0.73$, RMSEP = 6.86, RPD = 5.40), sucrose ($R^2 = 0.97$, RMSEP = 0.79, RPD = 5.36).
RMSEP = 35.46, RPD = 1.66), vitamin C \( (R^2 = 0.76, \text{RMSEP} = 0.72, \text{RPD} = 1.80) \), radical-scavenging activities \( (R^2 = 0.70, \text{RMSEP} = 6.89, \text{RPD} = 1.50) \), chlorophylls a \( (R^2 = 0.80, \text{RMSEP} = 0.78, \text{RPD} = 1.91) \) and b \( (R^2 = 0.87, \text{RMSEP} = 8.82, \text{RPD} = 2.48) \), and total carotenoids \( (R^2 = 0.82, \text{RMSEP} = 4.47, \text{RPD} = 2.10) \).

In comparison with previous studies, similar results have been reported for non-destructive determination of sucrose \( (R^2 = 0.90, \text{RMSEP} = 16.5) \), glucose \( (R^2 = 0.94, \text{RMSEP} = 11.41) \), and fructose \( (R^2 = 0.95, \text{RMSEP} = 11.58) \) in ‘Nules Clementine’ mandarin (Magwaza et al., 2012), total phenols in coffee \( (R^2 = 0.94, \text{RMSEP} = 1.48; \) Páscoa et al., 2013), yerba mate \( (R^2 = 0.81, \text{RMSEP} = 0.12; \) Frizon et al., 2015), and olives \( (R^2 = 0.87, \text{RMSEP} = 6.33; \) Bellincontro et al., 2012). Also, total flavonoids \( (R^2 = 0.94, \text{SEP} = 140) \) and vitamin C \( (R^2 = 0.80, \text{SEP} = 4.89) \) were determined in apples (Pissard et al., 2013) while similar results were reported for determination of radical-scavenging activities \( (R^2 = 0.95, \text{RMSEP} = 0.23; \) Moncada et al., 2013) in quinoa.

However, in some instances, models (using pooled data) developed to determine some rind biochemical properties such as fructose \( (R = 0.39, \text{RMSEP} = 26.22 \text{ and } \text{RPD} = 0.84) \) and total flavonoids \( (R = 0.37, \text{RMSEP} = 0.79 \text{ and } \text{RPD} = 0.68) \) of grapefruit from KZN and LMP (Table 7) were poor. It affected the strength of its determination when data were pooled with the aim of increasing model robustness. Similar trend was observed for total phenolics \( (R = 0.10, \text{RMSEP} = 0.77 \text{ and } \text{RPD} = 0.15) \) and flavonoids \( (R = 0.29, \text{RMSEP} = 0.19 \text{ and } \text{RPD} = 0.66) \) concentrations of mandarins from EC and LMP (Table 10). The poor models might be attributed to difference in the cultural practices among orchards used. Grapefruit and mandarins from different production regions were grown on various rootstocks which is known to influence fruit biochemical
compositions such as carbohydrates (Barry et al., 2004) and flavonoids (Gil-Izquierdo et al., 2004), and fruit response to biotic and abiotic stresses (Cimen and Yesiloglu, 2016; Treeby et al., 1995).

4. Conclusion

This study demonstrated that Vis/NIR spectroscopy combined with the appropriate pre-processing algorithm(s) can be used to develop models for the determination of rind biochemical properties of ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarins. The study also revealed that spectral pre-processing algorithm is not always required in developing excellent predictive model as demonstrated in determining rind biochemical properties of ‘Marsh’ grapefruit harvested from KZN. Rind biochemical properties such as non-structural carbohydrates, vitamin C and radical-scavenging activities were determined with a high level of accuracy as reflected by their respective high RPD values. Although earlier studies have reported high predictive models for some biochemical properties such as rind non-structural carbohydrates of mandarin fruit, the application of Vis/NIR spectroscopy to determine rind biochemical properties of ‘Marsh’ grapefruit is the first and it provided relatively new information.

Acknowledgements

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flavonoid contents, and antioxidant capacity of rice grain using near-infrared spectroscopy.

J. Agric. Food Chem. 56, 8268–8272. doi:10.1021/jf801830z

Figure 1: An image showing the overview of FOSS NIRSystem
Figure 2: Line plot of absorbance spectra for ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin fruit.
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Table 2: Descriptive statistics for calibration and validation subsets for biochemical properties of ‘Nules Clementine’ mandarin rind

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Table 3: Pearson correlation coefficient matrix between biochemical properties of ‘Marsh’ grapefruit

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Values in bold are significantly different from 0 with a significance level alpha = 0.05
Table 4: Pearson correlation coefficient matrix between biochemical properties of ‘Nules Clementine’ mandarin fruit

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Values in bold are significantly different from 0 with a significance level alpha = 0.05
Table 5: Model performance for ‘Marsh’ grapefruit using spectral data acquired (from KwaZulu-Natal and Limpopo) before cold storage (at week 0) and after cold storage (at week 9) using FOSS NIRSystem in reflectance mode.

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242
Table 6: Model performance for ‘Marsh’ grapefruit using pooled spectral data acquired (from KwaZulu-Natal and Limpopo) before cold storage (at week 0) and after cold storage (at week 9) using FOSS NIRSystem in reflectance mode.

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Legend:
- **SN**: Sample Number
- **Parameters**: Nutrient parameter
- **Production region**: Location of production
- **Time**: Time of measurement
- **Pre-treatment**: Sample pre-treatment
- **Spectral range**: Range of spectral data
- **LV**: Number of latent variables in the model
- **Calibration Model**: Model performance metrics
- **Validation Model**: Model performance metrics
- **R²**: Coefficient of determination
- **RMSEP**: Root mean square error of prediction
- **SEP**: Standard error of prediction
- **Bias**: Bias of prediction
- **Slope**: Slope of the validation model
- **RPD**: Relative prediction deviation

FOSS NIRSystem in reflectance mode.
Table 7: Calibration model performance developed for ‘Marsh’ grapefruit using pooled spectral data (from KwaZulu-Natal and Limpopo separately) acquired before cold storage (at week 0) and after cold storage (at week 9) using FOSS NIRSystem in reflectance mode.

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<td>R²</td>
<td>RMSEC</td>
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<td>Sucrose (mg/g DW)</td>
<td>KZN + LMP</td>
<td>Wk 0+Wk 9</td>
<td>SG (2nd order) 400-2500</td>
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Table 8: Model performance for ‘Nules Clementine’ mandarin using spectral data acquired (from KwaZulu-Natal and Limpopo) before cold storage (at week 0) and after cold storage (at week 9) using FOSS NIRSystem in reflectance mode.

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245
Table 9: Model performance for ‘Nules Clementine’ mandarin using pooled spectral data acquired (from KwaZulu-Natal and Limpopo) before cold storage (at week 0) and after cold storage (at week 9) using FOSS NIRSystem in reflectance mode.

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Table 10: Calibration model performance developed for ‘Nules Clementine’ mandarin using pooled spectral data (from KwaZulu-Natal and Limpopo separately) acquired before cold storage (at week 0) and after cold storage (at week 9) using FOSS NIRSystem in reflectance mode.

<table>
<thead>
<tr>
<th>SN</th>
<th>Parameters</th>
<th>Production region</th>
<th>Time</th>
<th>Pre-treatment Spectral range</th>
<th>LV</th>
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<th>Validation Model</th>
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<td>R²</td>
<td>RMSEC</td>
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<td>Sucrose (mg/g DW)</td>
<td>EC + LMP</td>
<td>Wk 0+Wk 9</td>
<td>SG (2nd order)</td>
<td>400-2500</td>
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<td>Glucose (mg/g DW)</td>
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<td>Wk 0 + Wk 9</td>
<td>SG (2nd order)</td>
<td>400-2500</td>
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<td>3</td>
<td>Fructose (mg/g DW)</td>
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<td>Wk 0 + Wk 9</td>
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<td>Total Phenolic (mg GAE/g)</td>
<td>EC + LMP</td>
<td>Wk 0 + Wk 9</td>
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<td>DPPH (%)</td>
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<td>Wk 0 + Wk 9</td>
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<td>400-2500</td>
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<td>10</td>
<td>Total Carotenoids (mg/g DW)</td>
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<td>Wk 0 + Wk 9</td>
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<td>400-2500</td>
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CHAPTER 8
SUMMARY AND FUTURE DIRECTION

1. Introduction

The South African citrus industry is globally recognised and ranks 11th in terms of world fresh citrus production and 2nd after Spain in terms of world fresh citrus exports in 2014/2015 seasons (Citrus Growers Association, 2016). The industry is the largest exporter of grapefruit (~225 000 tons) and 5th largest exporter of soft fruit (including mandarins, ~250 000 tons) to different destinations around the world (Citrus Growers Association, 2016). However, incidence of rind pitting disorder of ‘Marsh’ grapefruit and rind breakdown (RBD) of ‘Nules Clementine’ mandarin fruit at non-chilling temperature affects consumer acceptability of the fruit. This effect invariably leads to reduced financial gains to the growers. An array of studies has previously been done to understand the mechanism(s) underlying the development of these physiological disorders during non-chilling postharvest storage in other to design mitigating methods but success has been limited. In this study, the overall aims were (a) to investigate the role of canopy position on rind biochemical properties in relation to the postharvest physiological rind disorders of citrus fruit at non-chilling temperature, (b) to non-destructively determine rind biochemical properties of selected cultivars of citrus fruit.

Consequently, this thesis was structured into chapters, with each chapter addressing specific objectives;

- Chapter 1: Introduced the thesis by stating the motivation, research hypothesis and research aims and objectives.
Chapter 2: Presented a detailed review of literature of existing knowledge regarding rind quality of citrus fruit, non-chilling physiological rind disorders and their causes, rind biochemical properties and non-destructive methods for detecting rind biochemical properties.

Chapter 3: Investigated the role of canopy position on physicochemical properties including maturity indices of ‘Marsh’ grapefruit after harvest and after postharvest cold storage at non-chilling temperature.

Chapter 4: Evaluated the relationship among canopy position, production region and rind biochemical properties in relation to postharvest physiological rind disorders of ‘Marsh’ grapefruit at non-chilling temperature.

Chapter 5: Explicated the role of canopy position and production region on rind biochemical properties in relation to postharvest physiological rind disorders of ‘Nules Clementine’ mandarin at non-chilling temperature.

Chapter 6: Explicated phytohormonal changes in ‘Nules Clementine’ mandarin fruit rind from different canopy positions in relation to postharvest physiological rind disorders of ‘Nules Clementine’ mandarin at non-chilling temperature.

Chapter 7: Developed a robust Vis/NIRS based non-destructive models to determine rind biochemical properties such as non-structural carbohydrates, total carotenoids, total phenolic and total flavonoid concentrations, and radical-scavenging activities of ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin.
2. General discussion

2.1. Literature review on causes and non-destructive methods for detecting non-chilling physiological rind disorders

This chapter reviewed the literature on non-chilling physiological rind disorders and non-destructive methods for detecting their incidence. The objective was to discuss current knowledge on the susceptibility of citrus fruit to non-chilling physiological rind disorders, possible causes and non-destructive methods of detecting the delayed incidence of the disorder. The chapter also reviewed current knowledge on rind biochemical properties of citrus fruit and their potential use as pre-symptomatic biomarkers for predicting the incidence of non-chilling physiological rind disorders. Non-chilling physiological rind disorder of citrus fruit such as rind pitting or rind breakdown (RBD) are critical problems affecting the financial gains of citrus fruit growers (Agustí et al., 2001). This is because, the incidences of the disorders are usually delayed until about 3-5 weeks after fruit have been sorted in the pack house and transported to various marketing destinations (Cronje et al., 2011a). Examples of such disorder were highlighted in the review and included RBD, rind pitting, necrosis (Alférez et al., 2005; Ben Yehoshua et al., 2001; Magwaza, 2013), and oleocellosis (Ladaniya and Ladanyia, 2008). While some of the possible factors, including canopy position, causing incidences of the disorder in citrus fruit were discussed, the potential use of rind biochemical properties as biomarkers of physiological rind disorders was also discussed.

A study by Magwaza (2013) revealed that rind physiology, behaviour of biochemical properties and susceptibility of fruit to non-chilling physiological rind disorders differ from one fruit to the other, depending on conditions to which the fruit was exposed. Therefore, discovering exact
cause(s) or mechanism(s) underlying the incidence of rind disorder of citrus fruit would be a major breakthrough for the industry. This is because rapid and cost-effective innovative means to non-destructively detect or monitor the disorders can now be developed owing to the meaningful advancement in science and technology (Gao et al., 2010; Olarewaju et al., 2016). Consequently, various non-destructive techniques with the ability to detect physiological rind disorders of citrus fruit were discussed. The techniques included visible to near infrared (Vis/NIR) spectroscopy, hyperspectral imaging, computed tomography imaging, chlorophyll fluorescence imaging, x-ray imaging, optical coherence tomography, and magnetic resonance imaging (MRI) (Gao et al., 2010; Hahn, 2009).

The prospect of non-targeted metabolomics approach towards the identification of biochemical property(ies) triggering the incidence of physiological rind disorders at non-chilling temperatures were also discussed. This was done based on its potential to provide holistic understanding or determination of the minutest variation in complex biochemical systems of living organism (Ernst et al., 2014; Heyman and Dubery, 2016; Naz et al., 2014; Rochfort, 2005).

2.2. **Experimental findings**

The role of fruit position within tree canopy (canopy position) on maturity and quality properties of ‘Marsh’ grapefruit stored at non-chilling cold temperature for 9 weeks postharvest was reported in chapter 3. Results for maturity and quality indices suggested that fruit used for the experiment were matured and of good quality. The results further indicated that canopy position, production region and postharvest storage time affected some physicochemical properties such as colour indices, glucose, sucrose, sweetness index and total sweetness index. In agreement with
previous studies, outside canopy (OC) fruit had higher colour index (CI) than inside canopy (IC) fruit from KZN while CI was lower in fruit from MP with gradual declining trends in both orchards after postharvest cold storage. Reduced intensity of sunlight reaching IC fruit was suggested to have caused the delayed colour change (Cronje et al., 2011a; Khalid et al., 2012a; Magwaza et al., 2014a). The yellowness (b*) values ranged from 58.3 to 63.5 for IC fruit and 53.6 to 64.7 for OC fruit from KZN; and 51.9 to 62.5 for IC fruit and 54.1 to 63.5 for OC fruit from MP province. This provided an indication of fruit quality in the trade markets. Canopy position, production region and postharvest storage time had a significant effect on total soluble solid/ titratable acidity (TSS/TA, maturity and quality index) for fruit from both production regions. IC fruit had higher TSS/TA (3.88%) than OC fruit (3.24%) at week 0 for fruit from KZN. However, as storage progressed the differences shifted to a higher TSS/TA ratio in OC fruit compared to IC fruit. These results showed the influence of production region and postharvest cold storage time on the fruit studied.

The role of canopy position on rind biochemical properties of ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin fruit was also investigated. Results were related to non-chilling physiological rind disorders and presented in chapters 4 and 5, respectively. This approach was employed based on the premise that fruit exposure to various intensity of sunlight affect the postharvest quality of fruit rind and its appearance (Bramlage, 1993; Cronje et al., 2013; Hamadziripi, 2012; Khalid et al., 2012b). It was also suggested that canopy position affect the physiological activities and biochemical composition of fruit rind (Cronje et al., 2011a, 2011b; Magwaza et al., 2013). Similarly, the concentration of carbohydrates and mineral elements during fruit development have been reported to be affected by canopy position (Cronje et al., 2011b).
The study further investigated the use of non-destructive methods to determine the biochemical properties of fruit rind and results were presented in chapter 6. In this case, Vis/NIR spectroscopy was evaluated as an innovative tool for rapid and cost-effective determination of rind biochemical parameters of interest as existing analytical methods are time consuming, expensive and require specialised sample preparation (Magwaza, 2013; Olarewaju et al., 2016). Fruit (‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin) were harvested at commercial maturity from inside canopy (IC) and outside canopy (OC) of 50 trees from two production regions and cold stored at non-chilling temperature (7.5 °C ± 0.5) for 9 weeks postharvest. Fruit were scanned for acquisition of spectral information before and after cold storage with FOSS NIRSystem for model development as described in chapter 7.

The findings revealed that fruit rind biochemical properties from different canopy positions and production regions responded differently after harvest and after cold storage. There was no clear trend with respect to the effect of canopy position on rind biochemical properties of the fruit as inconsistent trends were observed for fruit from both production regions. Chapter 4 reported the effect of canopy position on rind pigments, non-structural carbohydrates, vitamin C and radical-scavenging activities of ‘Marsh’ grapefruit. Lower Ca pigment occurred in OC fruit (-5.08, -2.73, and -1.98 µg/g DW) than IC fruit (-5.63, -5.01, and -3.02 µg/g DW) from LMP at week 0 and after weeks 3 and 9 of cold storage, respectively. This indicated the influence of sunlight on rind colour development. Furthermore, significant higher total carotenoids occurred in IC fruit (10.86 and 9.25 µg/g DW) than OC fruit (8.37 and 6.76 µg/g DW) from KZN at weeks 0 and 9, respectively. Inside canopy fruit (11.13 and 7.76 µg/g DW) from LMP were significantly higher than OC fruit (6.78 and 4.14 µg/g DW) at weeks 6 and 9 after cold storage, respectively (Figure
This was contrary to the results reported by Cronje et al. (2013) who reported lower carotenoid concentration in IC fruit than OC fruit. This deviation was hypothesised to be due to differences in the eventual colour of the fruit investigated. Also, non-reducing sugars of IC and OC fruit from the two production regions followed similar pattern from week 0 to week 6 of cold storage. Correlation tests showed that sucrose is an important biochemical property of fruit rind which could have a direct or indirect impact on the performances of other biochemical properties. Therefore, the role of rind sucrose in the defence mechanism of fruit against rind pitting should not be underrated.

Chapter 5 reported the role of canopy position on rind biochemical properties of ‘Nules Clementine’ mandarin fruit. Significant higher total carotenoid content occurred in rind of OC fruit than IC fruit at week 0 and after weeks 3, 6 and 9 of cold storage in both seasons. This was contrary to what was reported for grapefruit in chapter 4 but supported previous findings by Cronje et al. (2013) who also reported higher total carotenoids in OC fruit than IC fruit. Combining the findings from these two cultivars of citrus fruit regarding total carotenoids, it could be deduced that the effect of canopy position on total carotenoids is cultivar specific.

At week 0, rind sugars from both production regions followed similar pattern which corresponded to the ones reported earlier in literature where the sugar concentrations were higher in IC fruit than OC fruit (Magwaza et al., 2014b; Rosales et al., 2011; Ting and Deszyck, 1961). However, rind sucrose of IC fruit from EC were lower than OC fruit which corresponded to the results reported for rind sucrose of ‘Nules Clementine’ mandarin fruit from WC by Cronje et al. (2013) and Thorpe (1974). It was explained that exposure of fruit to reduced sunlight (IC) have a reduced
sink strength effect on fruit. It was further observed that sugars were generally high in ‘Nules Clementine’ mandarin and could partly explain why RBD did not develop during postharvest cold storage. This is because high concentration of sugars is known to serve as source of energy reserves which also contribute to the sustenance of rind cell structures (Dennis and Blakeley, 2000; Kays and Paull, 2004) and protect plants against possible stressful conditions such as chilling injury (Purvis and Grierson, 1982).

For radical-scavenging activities, OC fruit (week 0 = 61.2 and week 3 = 75.1 %) were significantly higher than IC fruit (week 0 = 52.5 and week 3 = 67.2 %) from EC with similar reports by Drogoudi and Pantelidis (2011). The authors reported higher antioxidant capacity in OC apple fruit than fruit exposed to shaded canopy position. This suggested that high temperature of OC fruit influenced radical-scavenging activities which possibly inhibited the development of rind breakdown on fruit during non-chilling cold storage. It is recommended that future research should attempt to identify the threshold for the important parameters that may be involved in RBD etiology.

In chapter 6, the study investigated the phytohormonal changes in ‘Nules Clementine’ mandarin fruit rind from different canopy positions in relation to incidence of RBD during postharvest non-chilling cold storage. It was hypothesised that phytohormones play significant role in the susceptibility of ‘Nules Clementine’ mandarin fruit to RBD which mostly become visible only around 3-5 weeks postharvest as fruit tend towards senescence (Cronje et al., 2011a).
The incidence of RBD only occurred on fruit harvested from EC during 2016 season and was observed on IC fruit after week 9 of cold storage. Hence, the result comparing the phytohormonal levels of fruit with and without rind disorder was based only on IC fruit from EC after week 9 of non-chilling cold storage at 7.5 ± 0.5 °C. Fruit without RBD had higher concentration of \( iZOG \) (12.86 pmol/g DW) and \( cZ7G \) (6.59 pmol/g DW) than fruit with rind disorder (0.00 pmol/g DW) and (0.00 pmol/g DW), respectively which were below the limit of detection (LOD). Other cis-type cytokinins including \( cZOG \), \( cZR \) and \( cZROG \) were significantly higher in disordered (118.13, 24.14 and 6.97 pmol/g DW) fruit than fruit without the disorder (76.52, 11.57 and 4.86 pmol/g DW).

The IAA concentration was higher in the IC fruit (344.15 pmol/g DW) than OC fruit (194.20 pmol/g DW) from EC at week 0 while IAA concentration of OC fruit from WC were below the LOD and IC fruit had 53.20 pmol/g DW at week 0. The IAA concentration of OC fruit from EC fairly remained constant throughout postharvest period while a significant drop from 344.15 pmol/g DW at week 0 to 177.14 pmol/g DW at week 3 occurred in IC fruit from EC. The IAAsp concentration were below the LOD at week 0 and 3 but increased to 11.83 and 12.17 pmol/g DW at week 6 and 9, respectively.

The result of this study revealed the presence of dihydrozeatin 7-glucosides (DHZ7G), iPR and iP9G in mandarin fruit. In CK-mediated resistance, interactions with other phytohormones, such as abscisic acid (Großkinsky et al., 2014) or salicylic acid (Argueso et al., 2012; Choi et al., 2010; Großkinsky et al., 2011) have been reported. However, information about the synergistic role of \( cZ \) glucoside and iP9G in combination with auxin is not documented in literature. In this study,
the CK conjugates, cZ7G, tZOG and iP9G synergistically acted with the auxin IAA to prevent RBD in mandarin fruit.

Chapter 7 focused on using non-destructive approach (visible to near infrared spectroscopy) for rapid assessment and determination of rind biochemical properties of ‘Marsh’ grapefruit and ‘Nules Clementine’ mandarin. This approach was considered due to laborious, time-consuming, and expensive nature of conventional methods. The non-representative nature of few sample analysis to make decisions on all fruit in consignments for either local or international markets also necessitated the need to non-destructively monitor the biochemical status of each fruit rind. This was hypothesised to help in the delivery of quality fruit to the fresh fruit markets.

Rind biochemical properties of grapefruit such as sucrose ($R^2 = 0.99$, RMSEP = 0.11, RPD = 11.42), glucose ($R^2 = 0.99$, RMSEP = 0.77, RPD = 11.35), fructose ($R^2 = 0.99$, RMSEP = 0.99, RPD = 14.23), total phenolics ($R^2 = 0.94$, RMSEP = 0.07, RPD = 3.85), total flavonoids ($R^2 = 0.99$, RMSEP = 0.07, RPD = 12.37), vitamin C ($R^2 = 0.79$, RMSEP = 0.06, RPD = 2.01), radical-scavenging activities ($R^2 = 0.91$, RMSEP = 0.17, RPD = 3.07), Ca ($R^2 = 0.86$, RMSEP = 0.08, RPD = 2.53) and Cb ($R^2 = 0.97$, RMSEP = 0.14, RPD = 5.67) were determined using PLS models based on spectra acquired after week 9 of cold storage than those acquired at week 0. This suggested the influence of cold storage on the predictive model performance. Similar excellent results were achieved for determining rind biochemical properties of ‘Nules Clementine’ mandarin fruit. These excellent models were developed using full spectra range (400-2500 nm) as suggested by Olarewaju et al. (2016), 2 LVs and without any application of mathematical pre-processing algorithms.
3. **General conclusion**

In conclusion, this study attempted to attribute rind biochemical properties to non-chilling physiological rind disorder of ‘Marsh’ grapefruit and ‘Nules clementine’ mandarin fruit but the substantial absence of the rind disorder did not allow for such correlations. However, non-destructive models were developed to determine the biochemical properties of the citrus fruit using FOSS NIRSystem. Furthermore, this study revealed the positive effect of phytohormones cZ7G, tZOG, iP9G and IAA in the prevention of RBD and the negative effect of cZOG, cZR, cZROG and IAAsp. However, further studies harnessing molecular physiology, biochemical pathways of phytohormones and enzymes, interactions of auxins and cytokinins during postharvest cold storage and effect of postharvest storage conditions on phytohormone content are suggested in order to have a better and in-depth understanding of the contribution of phytohormones to RBD of citrus fruit.

**References**


