Phytochemical analysis, antioxidant and antimicrobial effects of extracts from grape (*Vitis vinifera*) by-products and mandarin orange (*Citrus reticulata* Blanco)

by

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DECLARATION

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

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ABSTRACT

The increase in level of customer sophistication, motivated by a general interest in healthier food options, has seen growing focus on fruit by-products processing and value addition as a potential source of natural preservatives. In this study, the phytochemical composition, pH, titratable acidity, antioxidant and antimicrobial properties of extracts from orange peel and pulp (OPE), grape pomace (GPE) and seeds (GSE) grown in South Africa were analysed. Spectrophotometric methods were used to quantify total phenols, total tannins, flavonoids, anthocyanins, proanthocyanidins, as well as ascorbic acid and total carotenoids. The pH was measured using a laboratory pH meter while a titrosampler was used to measure the titratable acidity. Antioxidant properties were evaluated using the 2,2-diphenyl-1-picrylhydrazyl radical-scavenging method, ferric reducing-antioxidant power test, oxygen radical absorbance capacity assay and the lipoxygenase inhibition assay. Comparisons were made against ascorbic acid used commercially as an antioxidant preservative. The antimicrobial properties were evaluated against five bacteria (*Listeria monocytogenes*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*) and one yeast (*Candida albicans*) using the broth microdilution method with comparisons against tetracycline (positive indicator) and sodium metabisulphite (artificial antimicrobial preservative). Total phenols and carotenoids were highest in GPE followed by GSE and OPE ($p \leq 0.05$). Flavonoids and anthocyanins were higher ($p \leq 0.05$) in GPE and GSE compared to OPE. The GSE had highest proanthocyanidins followed by GPE and OPE ($p \leq 0.05$). Ascorbic acid was only detected in OPE, which also had the highest titratable acidity and lowest pH ($p \leq 0.05$). The GSE had the highest antioxidant activity based on all four antioxidant assays, as evident in GSE having the highest antioxidant potency composite index followed by GPE and OPE ($p \leq 0.05$). The extracts showed less antimicrobial activity compared to the positive indicator and artificial antimicrobial preservative. Greatest antimicrobial activity among the extracts, however, was shown by OPE.

The order of antimicrobial activity of the extracts was OPE > GSE > GPE ($p \leq 0.05$). Current findings show that GSE is a potential antioxidant while OPE holds promise as an antimicrobial for the food industry. Overall, valorisation of fruit processing by-products is a promising avenue for enhancing food preservation and shelf life stability while offsetting environmental problems due to waste dumping.

OPSOMMING

Die toename in die vlak van verbruikers se gesofistikeerdheid, gemotiveer deur 'n algemene belangstelling in gesonder kos-opsies, het toenemende fokus begin plaas op die verwerking van en dus waardetoevoeging tot vrugtebyprodukte wat as 'n potensiële bron van natuurlike preserveermiddels gebruik kan word. In hierdie studie is die fitochemiese samestelling, pH, titreerbare suurheid, antioksidante en antimikrobiese eienskappe van ekstraksies van lemoenskil en -pulp (OPE), druiwepulp (GPE) en -pitte (GSE) verbou in Suid-Afrika, ontleed. Spektrofotometriese metodes is gebruik om totale fenole, totale tanniene, flavonoïede, antosianiene, pro-antosianidiene, sowel as askorbiensuur en totale karotenoïede te kwantifiseer. Die pH is gemeet met behulp van 'n laboratorium pH meter, terwyl 'n *titrosampler* gebruik is om die titreerbare suurstof te meet. Antioksidant eienskappe is geëvalueer deur gebruik te maak van die 2,2-difenyl-1-pikrylhidrasiel radikaal-opruimingsmetode, die reduksie-antioksidant kragtoets, die suurstof-radikaal absorbansiekapasiteitsassessering en die lipoksigenase-inhibisie-toets. Vergelykings is gemaak teen askorbiensuur wat kommersieel gebruik word as 'n antioksidant preserveermiddel. Die antimikrobiese eienskappe is geëvalueer teen vyf bakterieë (*Listeria monocytogenes*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*) en een gis (*Candida albicans*) met behulp van die sous-mikroverdunningsmetode met vergelykings teen tetrasiklien (positiewe aanwyser) en natrium metabisulfiet (kunsmatige antimikrobiese preserveermiddel). Totale fenole en karotenoïede was die hoogste in GPE, met laer vlakke in GSE en OPE. Flavonoïede en antosianiene was hoër in GPE en GSE, wanneer vergelyk met OPE. Die GSE het die hoogste pro-antosianidien inhoud, met laer vlakke gevind in GPE en OPE. Askorbiensuur is slegs in OPE gevind, wat ook in die bron die hoogste titreerbare suurheid en laagste pH gehad het. Die GSE het die hoogste antioksidant aktiwiteit op grond van al vier antioksidant-toetse gehad, soos blyk uit GSE wat die hoogste saamgestelde indeks van antioksidant potensiaal het, gevolg

deur GPE en OPE. Die onderskeie ekstraksies het minder antimikrobiese aktiwiteit getoon in vergelyking met die positiewe indikator en kunsmatige antimikrobiese preserveermiddel. Die grootste antimikrobiese aktiwiteit onder die ekstraksies is egter deur OPE getoon. Die volgorde van antimikrobiese aktiwiteit van die uittreksels was OPE> GSE> GPE. Huidige bevindinge toon dat GSE 'n potensiële antioksidant is, terwyl OPE belofte as 'n antimikrobiese verbinding vir die voedselbedryf inhou. In geheel, die waardetoevoeging tot die byprodukte van verwerkte vrugte het potensiaal om by te dra tot die verbetering van voedselbehoud en rakleeftydstabiliteit, terwyl die omgewingsprobleme weens afvalstorting ook hiermee aangespreek kan word.

This thesis is dedicated to my family

Biographical sketch

Trust Mukudzei Pfukwa was born on the 10th of April 1986 in Mutare, Zimbabwe. He graduated from the University of Zimbabwe with a BSc Food Science in 2008 after which, he worked for a hospitality company in Zimbabwe for six years. In 2017, he enrolled for the degree MSc in Food Science at Stellenbosch University.

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Last but not least, I thank my family and friends for being a source of motivation, strength, inspiration and love.

Research contributions

- **Conferences**

  o Pfukwa, T.M., Fawole, O.A., Gouws, P.A., Manley, M., Mapiye C. (2018). Phytochemical content, in-vitro antimicrobial and antioxidant activity of grape pomace, grape seed and orange pulp extracts, RMAA Conference and Congress 2018, Cape Town, South Africa.

Notes

This thesis is presented in the format prescribed by the Department of Food Science at Stellenbosch University. The structure is in the form of one research document and is prefaced by an introduction chapter with the study objectives, followed by a literature review chapter, materials and methods chapter and culminating with a chapter for elaborating a general discussion and conclusion. The language, style and referencing format is in accordance with the requirements of the International Journal of Food Science and Technology.

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Chapter 1

Background

1.1 Introduction

Globally, about 1.6 billion tonnes of food are lost or wasted annually (FAO, 2013). In South Africa, for example, annual postharvest food losses and wastes amount to 10 million tonnes (WWF-South Africa, 2017), with about 95% of the food wastage occurring along the value chains prior to reaching the consumer. Food spoilage due to oxidative degradation and microbial action is an important contributor to wastage through reduction in quality and shelf-life, which in turn result in reductions in nutrition value and safety of foods (Kumar *et al.*, 2015). These degradative processes are exacerbated by particular food processing steps such as comminution, which increases surface area of products such as meat and promotes interaction between pro-oxidants, enzymes, lipids and proteins (Hugo and Hugo, 2015). Preservatives, which are mostly synthetic, are used to inhibit and or delay these processes and enhance the quality, safety and shelf stability of foods (Kumar *et al.*, 2015).

Consumer health concerns and sentiments against synthetic preservatives have renewed fear and avoidance of processed foods (Bedale *et al.*, 2016). For example, cancer has historically been associated with nitrate and nitrites used in processed foods (Bedale *et al.*, 2016), while the use of benzoates has been linked to hyperactive behaviour in children, allergies, asthma and skin rashes (Bateman *et al.*, 2004; Hugo and Hugo, 2015; Lee and Paik, 2016). Access of such information to the public influences their purchasing behaviour towards trust and preference of foods preserved with natural preservatives (Bedale *et al.*, 2016; Hung *et al.*, 2016). These developments have encouraged the research industry to pursue natural preservatives especially from edible and medicinal plants, which can be demonstrated to be nutritional, safe and healthful (Kumar *et al.*, 2015).

The global fruit processing industry creates large amounts of waste usually composed of stems, skin, peels, pulp, seed and oilseed meals (Djilas *et al.*, 2009). For example, at least 40% of the 1 million tonnes of oranges produced annually in South Africa are channelled to juice production with fruit waste accounting for between 50 to 70% of the fresh orange weight and comprised of peels (60 - 65%), pulp (30 - 35%) and seeds (<10%) (Sharma *et al.*, 2017). In addition, according to SAWIS (2016), 1.5 million tonnes of grapes are produced for wine production with grape pomace making up 20 to 25% (w/w) of the pressed grapes on dry matter basis. Grape pomace is made up of stalks (~ 2%), seeds (~47%), skin and pulp (~51%) (Beres *et al.*, 2017; Zhang *et al*., 2017). These fruit by-products are treated as waste and generally disposed of in landfills (Siles *et al.*, 2016). The high moisture content and organic content of these fruit by-products upon putrefaction, poses a significant risk to the environment through production of greenhouse gasses and contamination of water bodies (Siles *et al.*, 2016). In addition, transportation to disposal sites is a significant economic cost to the fruit industry.

Interestingly, orange and grape by-products contain valuable bioactive compounds, which provide plant protection through defence against ultraviolet radiation, stress, pathogens, and predators (Pourcel *et al.*, 2007; M'hiri *et al.*, 2017). Bioactive compounds also play important roles in maintaining food quality, contributing to colour, taste and potentially contributing in the prevention of chronic diseases due to their antioxidant properties (Stintzing and Carle, 2004). These bioactive compounds include ascorbic acid, tocopherols and tocotrienols, carotenoids, and several phenolic compounds with potential for valorisation of the waste (M'hiri *et al.*, 2017). Such an approach towards agro-industrial by-products helps to unlock new value chains for the fruit processing industry and contribute immensely towards sustainable production practices.

In South Africa, systematic research investigating preservative capabilities of extracts from fruit by-products of commercial cultivars is rare. *In vitro* studies with some plant extracts

rich in phenolic compounds have shown synergistic effects in antioxidant and antimicrobial tests because of their mixture of metabolites, which present multiple points of intervention (Gyawali and Ibrahim, 2014; Hamza *et al.*, 2018). For successful adoption of natural fruit by-product-based preservatives in food systems, key parameters such as antioxidant and antimicrobial efficacy, safety and stability during food processing, warrant investigation (Lee and Paik, 2016).

1.2 Objectives

The objective of the current research was to determine the phenolic composition, antioxidant and antimicrobial properties of extracts from grape pomace (*Vitis vinifera L.* var. Pinotage), grape seed and orange peel and pulp (*Citrus reticulata* Blanco).

1.3 Hypothesis

Red grape pomace (*Vitis vinifera L.* var. Pinotage), grape seed and orange peel and pulp (*Citrus reticulata* Blanco) extracts have neither *in vitro* antioxidant capacity nor *in vitro* antimicrobial properties.

Chapter 2

Literature review

Abstract

The increase in the level of customer sophistication, motivated by general interest in healthier food options, has seen growing focus on plant derived food preservatives with fruit processing by-products a potential source. Plant phytochemicals, in this case from grape pomace (GP), orange peel and pulp (OP) and grape seed (GS), are being sought to replace synthetic food preservatives. The latter has been associated with adverse effects in human health such as the link between benzoates and attention deficit disorder. The target compounds in the fruit processing by-products include phenolic compounds, flavonoids, hydroxycinnamic acids, hydroxybenzoic acids, ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E) and carotenoids (provitamin A). Previous research has elaborated on the structure activity relationship of flavonoids in the antioxidant and antimicrobial properties displayed. Nevertheless, the phytochemical profile of the final extract, itself is dependent on the fruit genus and cultivar, vinification method (in case of grape) and extraction conditions is the most significant determinant of *in vitro* activity. Evidence from previous *in vitro* studies has shown the potential antimicrobial and antioxidant properties of fruit by-product extracts. It is therefore important to determine the phenolic contents of the locally available fruit by-products and evaluate their *in vitro* bioactivity to assess the potential for valorisation.

2. Introduction

Researchers are pushing the boundaries to provide solutions to problems associated with food waste and loss (Khan *et al.*, 2015). This is an important issue considering there is a drive towards sustainable food production. The food wastes involved, span from fruits and vegetables, cereals to roots and tubers as well as meat. These are important to address as there

is a significant quantity of inputs used for production and processing value chain (Hardersen and Ziolkowska, 2018). With 95% of the losses occurring before the food reaches the consumer, preservative use is imperative to curb oxidative degradation and microbial proliferation which negatively influence food shelf life stability. Considering that there is increased aversion to synthetic preservatives (Brewer, 2011), progressive knowledge on novel meat processing techniques based on antimicrobial and antioxidant potential of plant derived phytochemicals, has helped push consumer trends towards natural food ingredients to replace synthetics (Kumar *et al*., 2015; Velasco & Williams, 2011). The use of by-products from fruit or vegetable processing as a source of phytochemicals incorporates the values of sustainability to the food production chain, as it adds value to products which would have been wasted for little to no value (Troy *et al*., 2016).

Valuable phytochemicals still remain within the matrix of the fruit by-products after primary processing, with potential for valorisation. The target compounds include phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids), carotenoids, flavonoids (flavanols or flavan-3-ols, proanthocyanidins, flavones, and flavonols), and stilbenes (Teixeira *et al.*, 2014). These are secondary metabolites synthesised by plants in response to stress, either from ultraviolet radiation or pathogenic attack while some also contribute to sensory and organoleptic properties (M'hiri *et al.*, 2017). However, the qualitative and quantitative composition is also influenced by a variety of factors including environmental in addition to physico-chemical conditions of the industrial process, and the combination of solvents and the extraction procedures employed (Teixeira *et al.*, 2014). More importantly, this review will focus on the studies surrounding red grape pomace and orange pulp and peel.

Grape and orange by-products are in abundance in the Western Cape region of South Africa, creating significant waste streams from their processing which makes their exploitation a logical step to take towards the path of sustainable food production (Khan *et al*., 2015). As

such, an understanding of the phytochemical content present in the fruit by-products generated locally, can inform the decision making process on whether or not sufficient quantities are present for valorisation.

An understanding of the mechanisms behind the antioxidant and antimicrobial effect of the extracts is essential to find ways to achieve the best performance, which approximates or performs better than the synthetic preservatives currently in use. In terms of the oxidative degradation process, it has been found to be propelled by varying mechanisms that is chemical, photochemical and by enzymatic processes (Min and Boff, 2002). As such, previous researchers have employed different assays which evaluate the antioxidant capacity of the extracts; the merits and demerits of the methods are articulated by Huang *et al.* (2005); Pinchuk *et al.* (2012); Alam *et al.* (2013) and Shahidi and Zhong (2015). The selection of microorganisms for *in vitro* studies must be representative of the most prevalent food pathogenic and spoilage microflora. *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus* are common pathogenic bacteria and also exist as natural flora of certain food products such as meat, milk and eggs (Cerveny *et al.*, 2009). *Pseudomonas aeruginosa* and *Enterococcus faecalis* are spoilage microorganisms that reduce the aesthetic appeal of food by producing slime, undesirable colour changes and off odours (Cerveny *et al.*, 2009; Gyawali and Ibrahim, 2014). *Candida albicans* is found as common flora on some unprocessed/ minimally processed foods, and has the ability to grow at chiller temperatures and can produce harmful toxins (Gyawali and Ibrahim, 2014).

Most countries are realising that future opportunities lie in a bio-economy where economic growth can be reconciled with environmentally responsible activities. Research on ways to channel fruit processing by-products, therefore, towards retrieval of valuable plant phytochemicals contributes to resource efficiency and waste beneficiation in the economy (Khan *et al.*, 2015).

2.1 Current status of preservative usage in South Africa

The use of preservatives introduces an important hurdle that helps to reduce food losses while increasing food security, enhancing safety, shelf life and increasing profitability for retailers. In line with the Government Notice No. R. 965, which falls under the Foodstuffs, Cosmetics and Disinfectants Act, 1972 (The Ministry of Health, 1977), food grade preservatives are allowed for use to extend shelf-life and enhance safety. This particular regulation also outlines the type of preservative to use in a particular food product. The preservative effect of the artificial ingredients is centred on the control of at least one of the spoilage mechanisms for example; prevention of oxidative degradation by antioxidants such as citric acid, erythorbic acid, butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA), as well as propyl gallate; and inhibition of microbes by antimicrobials which include nitrites, nitrates, benzoates, sodium metabisulphite and sulphur dioxide (Anand and Sati, 2013). As such, the preservatives are used in a binary system where an antioxidant is paired with an antimicrobial to give the product the desired shelf life. An example of such is where sulphur dioxide (antimicrobial) is paired with sodium erythorbate (antioxidant) in meat products.

2.1.1 Challenges with synthetics

Most of the preservatives in use have been implicated in causing adverse health conditions in a segment of consumers. A study by Bateman *et al.* (2004), evaluating the effects of artificial benzoate preservatives and food colourings on three-year-old children, revealed that the preservative had an adverse impact on the behaviour of some children in the population under investigation. Findings from the study indicated that there were significantly greater increases in hyperactive behaviour during the active treatment as compared to subjects administered a placebo. These findings are also supported by Beezhold *et al.* (2014), who examined the relationship between benzoate-rich beverage consumption and attention deficit hyperactivity

disorder (ADHD) symptoms in college students. The researchers concluded that benzoate-rich beverages contributed significantly to ADHD–related symptoms.

The adverse reactions in sensitive individuals related to consumption of foods containing sulphite additives have been studied by Vally and Misso (2012). The reactions were found to range from dermatitis, urticaria, flushing, hypotension, abdominal pain and diarrhoea to life-threatening anaphylactic and asthmatic reactions. The risk of these reactions is particularly pronounced among asthmatics with a 3-10% prevalence (Vally and Misso, 2012). Sulphites are also reported to have anti-nutritional effects due to their ability to cleave thiamine into 4-methyl-5-hydroxyethyl thiazole and the sulfonic acid of 2, 5-dimethyl-4-aminopyrimidine causing the destruction of the vitamin (Prabhakar, 2014). This can be prevented, however, by avoiding the use of sulphites in foods that are major sources of thiamine.

Other epidemiological studies have investigated the potential relationship between nitrate, nitrite, and N-nitroso compounds and the risk of cancer (Alahakoon *et al.*, 2015). However, contrary to general consumer belief, Bedale *et al.* (2016) stated that processed meat contribute less than 5% of the ingested nitrate and nitrites, with the bulk being contributed by nitrate reduction in saliva and the rest coming from vegetables. Bedale *et al.* (2016) highlighted benefits associated with nitrate/nitrite including effects on blood pressure, exercise capacity, protection of the gastrointestinal tract from bacterial pathogens as well as inhibition of common oral pathogens. Nevertheless, customer concern of the health risk of synthetic preservatives continues to grow thereby increasing the demand for alternative natural products.

2.2 Fruit by products and current uses

The processing of fruits creates large amounts of waste usually composed of peels, stems, seed and oilseed meals (Djilas *et al.*, 2009). According to SAWIS (2016), 1.5 million tonnes of

grapes were produced locally for wine production in 2015 with grape pomace making up 20-25% of the weight of the grapes. On the other hand, at least 40% of the 1 million tonnes of oranges produced annually in South Africa are channelled to juice production with citrus by-products accounting for 50% of the wet mass of the fruit (Sharma *et al.*, 2017). Common methods used in the disposal of waste from fruit processing include composting, anaerobic digestion, incineration, thermolysis and gasification (Khan *et al.*, 2015; Sharma *et al.*, 2017). However, the quantities of by products from both lines of processing present the processors with a challenge in terms of disposal. The wastes have a high content of organic matter such as sugars, tannins, polyphenols, polyalcohols, pectins and lipids which increase the chemical oxygen demand (COD) and the biochemical oxygen demand (BOD) (Djilas *et al.*, 2009).

The increasing production of wine and disposal of increasing amounts of waste led to the introduction of regulations to curb the negative effects resulting from such practices (European Council Regulation (EC) 479/2008). Such regulations have pushed for greater sustainability in the European fruit and vegetable processing industry, opening the way for opportunities for value addition. The high content of organic matter in the waste offers an opportunity for valorisation, not only in terms of extraction of phytochemicals. Pectin and other mucilages are obtained by acid extraction from citrus waste and used as food ingredients (Sharma *et al.*, 2017). Dried orange peel and grape pomace are also used in the production of livestock feeds. These include molasses which are processed from orange processing waste and can be further fermented to produce biogas, ethanol, citric acid, volatile flavouring compounds, and microbial biomass (Djilas *et al.*, 2009).

Processing of grape pomace also produces a great range of products such as ethanol, tartrates, citric acid, grape seed oil, hydrocolloids and dietary fibre (Djilas *et al.*, 2009). Grape seed oil is rich in unsaturated fatty acids, particularly linoleic acid (Beres *et al.*, 2017). Anthocyanins are another valuable product obtained from processing grape pomace, for use as

natural colouring of food (Nardoia, 2016). Their excellent colouring properties finds use in products such as dairy desserts, ice creams, drinks and juices. Grape pomace has also been used as plant fertiliser, however, according to Nardoia (2016), such uses can have a negative impact on plant germination due to the high content of phenolic compounds.

2.3 Phytochemicals with emphasis on phenolics

Phytochemicals are plant metabolites produced during normal development and under stressful conditions to provide defence for the plant (Haminiuk *et al.*, 2012; Belščak-Cvitanović *et al.*, 2018). Plant secondary metabolites can be divided into three major groups: phenols, terpenoids, and nitrogen-containing compounds (Veberic, 2016). Phenols are comprised of phenolic acids, flavonoids, stilbenes, curcuminoids, coumarins, quinones and lignans, which allow plant adaption to fluctuating environments (Durand-Hulak *et al.*, 2015). Phenols represent the third most abundant constituent in grapes after carbohydrates and fruit acids (Gođevac *et al.*, 2010). They play a role in plant development (participating in plant hormone signalling or pollen germination), reproduction (pigments attracting pollinators) and defence (protecting from Ultraviolet light, competitors, pathogens and predators) (Durand-Hulak *et al.*, 2015). Thus, the constitutive levels of phenols, including those with chemo-protective properties, depends on their specific role in the plant, on the plant's age and reproductive status, and on various environmental factors (Kalt, 2005). In fruit, by-product processing techniques and parameters are also an important determinant (Balasundram *et al.*, 2006; Melo *et al.*, 2015).

2.3.1 Factors affecting phenolic content

Variables that influence phytochemical content in fruit by-products can be separated into pre-processing and post-processing factors for the purposes of this review. The 'processing' being recognised as the step that generates the fruit by-products. The pre-processing factors include the genetic variation within plant species, and varieties as well as the environmental and climatic conditions under which the fruits were grown and processed (Ncube *et al.*, 2008;

Atkinson and Urwin, 2012; Ibrahim *et al.*, 2013; Butelli *et al.*, 2017). The post-processing factors include the drying methods, extraction methods, solvent used for extraction, and duration of extraction and choice of plant material (Ncube *et al.*, 2008; Chikwanha *et al.*, 2018).

2.3.1.1 Pre-processing factors

In terms of the pre-processing factors, the primary source of variation in phenolic content emanates from species and varietal differences (Veberic, 2016). This observation is supported by Durand-Hulak *et al.* (2015) who reported that polyphenol profiles are correlated with gene expression. It has been observed that various biotic (Pavarini *et al.*, 2012) and abiotic (Ramakrishna and Ravishankar, 2011; Sampaio *et al.*, 2011; Ibrahim *et al.*, 2013) stresses may induce the accumulation of a particular group of phytochemicals, the signalling and specificity of stress response is under the control of certain gene products. These include transcription factors (important in generating specificity in stress responses), mitogen-activated protein kinase (MAPK; cascades for transducing the perception of environmental stimuli into internal signalling pathways), heat shock factors, reactive oxygen species (stress signal transduction molecules) and small RNAs (Atkinson and Urwin, 2012). Butelli *et al.* (2017) investigated the activity a regulatory gene encoding a myeloblastosis (MYB) transcription factor controlling anthocyanin biosynthesis in a range of citrus species. It was shown that the presence of different combinations of deletions, frame shifts, and stop mutations in the genetic material can account for the natural variations in phytochemicals observed in fruit species and varieties.

As such, the investigation of grape pomace from Pinotage, a locally bred South African grape cultivar, becomes important as there is limited information on its phytochemical composition. Furthermore, the contribution of the agro-climate to accrual of phytochemicals makes it necessary to evaluate their content in locally grown orange cultivars of commercial importance. This helps to gauge their applicability for the purpose of retrieval of valuable bioactive compounds.

2.3.1.2 Post-processing factors

The extraction process is an important step in the recovery, identification and use of bioactive components from by-products of fruit processing. Processing steps preceding the actual extraction such as the drying method (Chikwanha *et al.*, 2018) and choice of solvent have also generated much research interest (Agustin-Salazar *et al.*, 2014; Bosso *et al.*, 2016). Quality of the extract is influenced by parameters which include the plant part used as starting material, the solvent used for extraction and the extraction technology (Gil-chavez *et al.*, 2013). Emphasis is placed on the parameters that retain extracts with the greatest yield of active compounds, which also give high antioxidant power with the least impurity (Spigno and De Faveri, 2007). Influence exerted by type of plant material is dependent on the nature of the plant material; its origin; the degree of processing; moisture content and particle size (Ncube *et al.*, 2008).

Although freeze drying has been reported to give high retention of bioactive compounds in fruit by-products, oven drying at 40-60 °C is more acceptable as it is more cost effective for bulk processing than freeze drying (Chen *et al.*, 2011; Tseng and Zhao, 2012). However, it has been observed that processing of orange peel and pulp involving heat treatment, for example during drying, can lead to the formation of "melanoidins" which are complex polyphenols resulting from Maillard reactions. The Maillard reaction products have been found to have antioxidant effects, thereby increasing the antioxidant activity of extracts obtained (M'hiri *et al.*, 2017), and this is in agreement with a study by Chen *et al.* (2011).

In response to the fluctuating environment and developmental stage, fruits synthesise and localise phenolic compounds differently in various internal structures (i.e., cells, organelles) (Kalt, 2005). The organelle chosen for storage is dependant on function and need, which then becomes important when the fruits are harvested for the retrieval of phenolics (Kalt, 2005). The effect of choice of plant material is also highlighted by Wang *et al.* (2017), in their

research on the flavonoid composition of 35 different varieties of citrus belonging to 5 types (pummelos, tangerines, oranges, mandarins and hybrids). Four parts of each fruit had been examined and analysed, namely the flavedo, albedo, segment membrane and juice sacs. The juice sacs were found to have the lowest total phenolics content followed by the segment membrane in all 35 varieties. Six pummelos, three hybrids and three tangerines were found to have higher total phenolic content in the flavedo than in the albedo. These findings pertaining to differences in the localisation of phenolics also agree with Veberic (2016) and Farhadi *et al.* (2016) who stated that diverse plant organs or even tissues can be characterised by different phenolic composition. For example, flavonol localisation in fruit skins is because their biosynthesis is stimulated by light, such that concentration differences in fruits on the same tree will occur based degree of exposure to light (Manach *et al.*, 2004).

Variations in extraction method include extraction technique (microwave assisted extraction, supercritical fluid extraction, enzyme-assisted extraction, ultrasonic assisted extraction), time of extraction and temperature (Beres *et al.*, 2017). On one hand, traditional methods including Soxhlet extraction, solid-liquid, and liquid-liquid extraction are becoming less favoured due to their association with longer extraction time, higher solvent consumption and a higher risk of degradation of thermo-labile components (Kumar *et al.*, 2015). On the other hand, methods such as ultrasonic assisted extraction have been noted as being faster and more efficient than conventional solvent solid/liquid extraction because of the cell wall disruption caused by the ultrasonic waves, which increases the solvent access to cell contents and increases mass transfer of desired materials (Teixeira *et al.*, 2014). Based on the mentioned traits, ultrasonic extraction is considered an ideal method for efficient cost effective extraction.

The nature of solvent, as well as solvent-solid ratio, also influence the extraction yield and the composition of the extracts (Bosso *et al.*, 2016). Acidified methanol and ethanol are the most common extractants, with methanol being reported in several texts as the most

efficient for phenolic extraction. However, ethanol is preferred in the food industry due to the toxicity of methanol (Ignat *et al.*, 2011; Agustin-Salazar *et al.*, 2014). It is further highlighted by Spigno and De Faveri (2007) that solvent type dictates the purity of extract, with methanol and ethanol promoting significant co-extraction of concomitant substances, compared to ethyl-acetate, but with higher yield, nevertheless, obtained with ethanol.

2.4 Phytochemical composition of fruit by-products

2.4.1 Grape pomace extract

Knowledge of the phenolic profile is important since bioactivity of extracts is not attributed to one compound, but the result of synergistic and antagonistic effects among different polyphenols with other components of the medium (Baydar *et al.*, 2004; Özkan *et al.*, 2004; Lingua *et al.*, 2016). Contents of different phytochemicals of orange peel and pulp, grape pomace and seed are summarised in **Table 2.1**. In their work on winery by-products, Melo *et al.* (2015) and Ignat *et al.* (2011) identified phenolic compounds from four classes namely hydroxybenzoic acids, flavonoids (comprised of flavan-3-ols, flavonols and anthocyanins), stilbenes and lignans.

In the grape berry, the flavonoids such as the anthocyanins and resveratrol are mainly localised in the skins, while the flavan-3-ols (catechins and proanthocyanidins) are present both in the skins and in the seeds (Yang *et al.*, 2009). The three fractions that make up grape pomace (seeds, stems and skins) contain different amounts of phenolic compounds. Distribution of polyphenols in the fresh grape is approximately 1% in the pulp, 5% in the skin, and 62% in the seeds (Nardoia, 2016). Red grape pomace anthocyanin content has been studied by Negro *et al.* (2003), Melo *et al.* (2015) and Xu *et al.* (2016), with varying results which can be attributed to cultivar differences.

Proanthocyanidins occur in grapes as dimers, oligomers, and polymers of catechins made up of flavan-3-ol monomer units with the structures varying depending on the constitutive subunits, the degree of polymerization, and the linkage position (Teixeira *et al.*, 2014). Proanthocyanidins represent a quantitatively important value of the pomace composition (21%–52% of the dry weight matter), with the proanthocyanidin oligomers reported to be linked through covalent bonding to cell wall polysaccharide materials and may explain the high tannin content of the leftover pomace (Teixeira *et al.*, 2014). The total proanthocyanidin content of pomace (*Vitis vinifera* L) shown in Table 1, was reported by Drosou *et al.*, (2015) and Antoniolli *et al.* (2015). Catechin and epicatechin are the main flavanols in fruits, whereas gallocatechin, epigallocatechin, and epigallocatechin gallate are found in seeds of certain leguminous plants and more importantly in grapes (Manach *et al.*, 2004).

While they are located in all the parts of a grape, the proanthocyanidin content of skins is lower than that of the seeds and their structural characteristics also differ (Nardoia, 2016). Grape seed proanthocyanidins comprise only procyanidins (catechin (C) and epicatechin (EC) subunits) with 28.4% galloylated units, whereas grape skin proanthocyanidins include both procyanidins and prodelphinidins (epigallocatechin (EGC subunits) having only 3.8 % galloylated units (Brossaud *et al.*, 2001; Lorrain *et al.*, 2013; Nardoia, 2016). Galloylation of the units has a positive influence in the effectiveness of radical trapping, as highlighted by Guitard *et al.* (2016). Skin proanthocyanidins have a higher degree of polymerisation and a lower proportion of galloylated subunits than seeds (Lorrain *et al.*, 2013).

Table 2.1: Phytochemical composition of grape and orange fruit by-products

	Grape		Orange	References
	Pomace	Seed	Pulp and pomace	
Total phenolics (mg GAE/g)	985 - 2122[1]; 32.16 ± 1.05[2] ; 55.5 – 153.8[3]	88.11 - 667.98[4] 858 ± 3[5] ; 44.5[6]; 213-1652[7*]	27.18[8]; 196.2 ± 2.7[9#] ; 67–1962[10*] 10.216[13]	[1]-(Lingua *et al.,* 2016); [2]-(Andrés *et al., 2017)* [3]-Xu *et al.,* 2016); [4]-Teixeira *et al.,* 2014) [5]-(Negro *et al.,* 2003) [6]-(Ky *et al.,* 2014)
Total tannins (mg GAE/g)	54.5-152.2[3] 66.1-175[11]	39.1-105.8[6]	-	[7]-(Rockenbach *et al.,* 2011) [8]-(Zhang *et al.,* 2018)
Flavonoids (mg CE/g)	32.8-91.7[3] 66.8-187[11]	26.2-30.5[12]	38.97[8§]; 2.28 ± 0.22[9§]; 0.3-31.1[14£]	[9]-(Goulas and Manganaris 2012) [10]-(M'hiri *et al.,* 2017) [11]-(Chikwanha *et al.*, 2018) [12]-(Doshi *et al.*, 2015)
Proanthocyanidins (mg CyE/g)	15.3437 – 43.469[15]; 5.15[16] ; 54.9-87.0[11]	129 ± 16[5]	-	[13]-(Peixoto *et al.*, 2018) [14]-(Ghasemi *et al.*, 2009) [15]-(Drosou *et al.*, 2015)
Anthocyanins (mg Cy-3-glc E/g)	9.8 ± 0.4[5Φ]; 0.99 - 1.41[17Φ] ; 1.38 – 10.7[3]	1.4-6.8[18]; 0.034[13Φ]; 0.27-2.4[19]	-	[16]-(Antoniolli *et al.* (2015) [17]-(Melo *et al.*, 2015) [18]-(Rababah *et al.,* 2008)
Carotenoids (mg β-carotene/g)	0.00696 ± 0.00433[20Θ]	-	108[21] ; 0.042.7 ± 0.0012[9] 2.04 ± 0.036[22]	[19]-(Farhadi *et al.*, 2016) [20]-(Andrés *et al.*, 2017) [21]-(Wang *et al.*, 2008)
Ascorbic acid (mg/g)	0.071 ± 0.0139[20]; 0.263[13]	-	4.57-11.53[9] 0.3[22]	[22]-(Zou *et al.*, 2016)

[*](CE)- Chatechin Equivalents; [#](GAE) - Gallic acid Equivalents; [§](RE) -Rutin Equivalents; [£](QE) -Quercetin Equivalents; [Φ](ME) - Malvidin Equivalents; [Θ](LE) -Lycopene

Phenolic acids are present in plants in the free and bound forms with ester, ether, or acetal bonds linking the bound phenolics to various plant components (Ignat *et al.*, 2011). The phenolic acid content of *Vitis vinfera* L cv. Malbec was found to be 1984.5 µg/g and 104.6 µg/g for hydroxybenzoic acids and hydroxycinnamic acids, respectively (Antoniolli *et al.*, 2015).

Stilbenes found in grapes have two isomeric forms such as *cis*-astringin, *trans*-astringin; *cis*-resveratrol, *trans*-resveratrol; *cis*-piceid, *trans*-piceid (Mattos *et al.*, 2016). The most abundant stilbene in grape is resveratrol, which also plays a role as a phytoestrogen, based on its ability to activate estrogen receptors (Nunes *et al.*, 2017). Ramirez-Lopez and DeWitt (2014) reported a *trans*-resveratrol content in the range 0.28 – 0.86 mg/kg, while Rockenbach *et al.* (2011) did not detect any *trans*-resveratrol in red grape (*Vitis vinifera* and *Vitis labrusca*) skins but only in the seed fraction in a range of 1.11 – 3.75 mg/100 g. With respect to these results, Rockenbach *et al.* (2011) commented that most of the resveratrol from the skins may have been transferred to the wine, leaving the content below the detection limit. Grape skins are reported to contribute 65% of carotenoid content while pulp contributes 35%. In their research, Andrés *et al.* (2017) evaluated the total carotenoid and ascorbic acid content of grape pomace and reported values of 6.96 ± 4.33 µg lycopene g^{-1} extract and 71.00 ± 13.85µg ascorbic acid g^{-1} extract, respectively.

2.4.2 Grape seed extract

Grape seeds are by-products of the winery and grape juice processing. About 60-70 % of the grape extractable polyphenols are present in the grape seed (Shi *et al.*, 2003). Depending on the grape cultivar and extraction method used, there is variation in the range of grape seed phenolic content. Du *et al.* (2007) and (Shi *et al.*, 2003) found a grape seed phenolic range of 5-8 % (w/w), which was similar to that reported by Negro *et al.* (2003). Genetic variation, in this case within species, could have been the other source of variation in the phenolic content

highlighted by Ky *et al.* (2014) and Rockenbach *et al.* (2011). Nevertheless, the values reported showed that substantial quantities of polyphenols can be recovered from the extracts.

The polyphenolic composition of grape seeds is comprised of anthocyanins, phenolic acids, simple flavonoids and complex flavonoids (Shi *et al.*, 2003). According to Yu and Ahmedna (2013) and Passos *et al.* (2010), the principal phenolic compounds of grape seed are monomers and polymers of flavan-3-ols such catechin, epicatechin as well as epicatechin-3-O-gallate which easily condense to form monomeric, oligomeric and polymeric procyanidins. It was further reported by Lorrain *et al.* (2013) that grape seed proanthocyanidins comprised only of catechin and epicatechin procyanidin subunits with a mean degree of polymerisation of 2-11 (Passos *et al.*, 2010).

Anthocyanins also occur in grape seeds although their quantities were reported as being less than in than in the skins. In their research, Rababah *et al.* (2008) reported that grape seeds had anthocyanin contents in the range of 0.14 to 0.68 g /100g extracts dry matter (DM) basis. The observed variation in the range of values was attributed to the cultivar differences. Negro *et al.* (2003), on the other hand, did not detect any anthocyanins in seeds from the Negro amaro variety. Phenolic acids also make up the grape seed phenolic profile. Jara-Palacios *et al.* (2016) evaluated the composition of seeds from white grape *Vitis vinifera* cv. Zalema using near-infrared hyperspectral imaging and found total phenolic acid content of 30.1 ± 6.3 mg/100 g. Gallic acid was the main phenolic acid with lesser amounts of protocatechuic, caffeic, caftaric, *cis*-coutaric *trans*-coutaric acid.

2.4.3 Orange peel and pulp extract

Orange peels are a rich source of bioactive compounds including natural antioxidants such as phenolic acids and flavonoids (Tumbas *et al.*, 2010). In their research using reversed-phase high-performance liquid chromatography, Abad-García *et al.* (2014) reported that the citrus

polyphenolic profile is composed of flavanones (81-97%), flavones (0.3-13.6%), flavonols (0.1-6.0%), hydroxycinnamic acids (0.6-9.6%) and coumarins (0.2-0.4%). The total phenolic content of orange peels has been reported to be 15% higher than that in the edible portion of the fruit (Balasundram *et al.*, 2006; Goulas and Manganaris, 2012; Zhang *et al.*, 2018). This is in agreement with studies by Zhang *et al.* (2018) on the total phenolic and flavonoid content of different components of citrus by products (peels, pulp residues, seeds, and juices). The highest quantities were reported in the peels with total phenolics of 27.18 mg gallic acid equivalent (GAE) g^{-1} DW and total flavonoids of 38.97 mg rutin equivalent (RE) g^{-1} DW. In a separate study, Goulas and Manganaris (2012) reported the total phenolics and total flavonoids content of 196.2 ± 2.7 mg garlic acid/g d.m. and 2.28 ± 0.22 mg rutin/g d.m., respectively in orange (*Citrus sinensis*, cv. 'Valencia').

Orange flavonoids also have the same basic diphenylpropane (C6-C3-C6) skeleton and are present mainly as glycosides, with aglycones (flavonoids lacking sugar moieties) being less frequent. The main sugar moieties of flavonoids can either be monosaccharides (D-glucose and L-rhamnose) or disaccharides (neohesperidosides and rutinosides) and are glycosylated by a disaccharide at the C7 hydroxyl group position (Gattuso *et al.*, 2006; Tripoli *et al.*, 2007; M'hiri *et al.*, 2017).

Flavanones are the main orange flavonoids and exist mostly in two types namely neohesperidosides and rutinosides (Tripoli *et al.*, 2007). The flavanone neohesperidoside forms include naringin, neohesperidin and neoeriocitrin are composed of a flavanone bonded with neohesperidose (rhamnosyl-a-1,2 glucose) moiety. The rutinoside forms include hesperidin, narirutin and didymin, and have a flavanone attached to a disaccharide residue e.g. rutinose (ramnosyl-a-1,6 glucose) (Tripoli *et al.*, 2007). The main citrus flavanones are naringenin, hesperetin, eriodictyol, and isosakuranetin (Ignat *et al.*, 2011; M'hiri *et al.*, 2017). According to M'hiri *et al.* (2017), hesperidin (0.06–66.09 mg/g) and narirutin (0.03–26.5 mg/g) are the

most abundant flavanones in fresh orange peel regardless of variety. Goulas and Manganaris (2012) also reported the narirutin and hesperidin content of peels from orange (*Citrus sinensis*, cv. 'Valencia') to be 1280 ± 25 and 66095 ± 1642 µg/g DM, respectively.

Flavones are characterized by the presence of a 4-oxo group and a double bond in position 2–3 (M'hiri *et al.*, 2017). According to Bocco *et al.* (1998), flavones can be grouped into either of two groups, that is glycosylated flavones (luteolin, apigenin, and diosmin glucosides) or polymethoxylated flavones. Orange peel contains large quantities of polymethoxylated flavones such as sinensetin, nobiletin, tangeretin, and heptamethoxyflavone, which are scarce in other species. Compared to flavones, other flavonoids such as flavonols (quercetin, rutin, myricetin, kaempferol) and anthocyanins, occur in smaller amounts in citrus peel. However, if present, quercetin and kaempferol are the most common flavonols found in citrus peel, mainly in the glycosidic form. Anthocyanins are minor compounds in citrus fruits (Butelli *et al.*, 2017), whose content is determined by the level of orange maturity with higher contents, however, being found in blood oranges. This is also true for proanthocyanidins, as none were detected in a study by Hellstro *et al.* (2009).

In terms of orange phenolic acids, hydroxycinnamic acids are generally present in larger quantities than hydroxybenzoic acids with caffeic, p-coumaric, ferulic, and sinapic acids being the most common (Xu *et al.*, 2007). These have been reported to be present in small amounts (µg/g db), mostly in orange peel (M'hiri *et al.*, 2017) with ferulic acid being the most abundant phenolic acid, while caffeic acid is found in small amounts (Xu *et al.*, 2007). However, Wang *et al.* (2008) analysed eight citrus peel cultivars and showed that the most abundant phenolic acid is p-coumaric acid. This difference between phenolic profiles could be attributed to varietal characteristics and other factors which include growing area, weather conditions, and the ripeness of the fruit.

2.5 Mechanism of phenolic antioxidant action

Oxidative degradation, which is one of the main causes of shelf life reduction in foods, is brought about through various mechanisms. The reactions include those that generate reactive oxygen species that target different structures (lipids, proteins, and carbohydrates), and Fenton reactions, where transition metal ions play a vital role (Brewer, 2011). The generation of primary radicals is facilitated by accidental or intentional presence of oxidation initiators such as transition metals, oxidants, various homolysis-prone substances such as polyunsaturated fatty acids or enzymes (Kanner and Rosenthal, 1992). An example of a primary radical is singlet oxygen, which is a highly reactive, electrophilic, and non-radical molecule formed either chemically, enzymatically or photochemically (Min and Boff, 2002).

The photochemical pathway involves photosensitisers such as chlorophyll, riboflavin, myoglobin and synthetic food colorants, which have a natural ability to absorb energy from light where it can be transferred to triplet oxygen to form singlet oxygen (Min and Boff, 2002; Papuc *et al.*, 2017a). More importantly, the reactions of singlet oxygen are characterised by its ability to directly react with the electron-rich double bonds of unsaturated molecules (Min and Boff, 2002). Transition metals also contribute to pro-oxidant activity especially in meat; firstly through the reaction of ferrous iron with H_2O_2 to generate the highly oxidizing hydroxyl radical, and secondly, decomposition of lipid hydroperoxides in free radicals able to initiate or propagate lipid peroxidation (Papuc *et al.*, 2017a).

The effect of oxidative enzymes, particularly lipoxygenases, is of importance due to their ability to directly initiate generation of free radicals that can attack other constituents such as vitamins, colours, phenolics, and proteins (Eskin and Ronbinson, 2000). These enzymes, containing a non-heme iron, catalyse the stereo-specific dioxygenation of polyunsaturated fatty acids containing at least one 1-cis, 4-cis-pentadiene system such as linoleic, linolenic, or arachidonic acid and may also catalyse β-carotene oxidation. (Eskin and Ronbinson, 2000;

Papuc *et al.*, 2017a). Oxidation in meat products occurs enzymatically and non-enzymatically (Papuc *et al.*, 2017a). The lipoxygenase enzyme has been highlighted as a participant in the enzymatic pathway by Papuc et al. (2017). In live subjects, lipoxygenases generate peroxyl radicals which play a role in inflammatory disorders (Ribeiro *et al.*, 2014). However, in meat, these peroxyl radicals lead to oxidative instability of the meat. Therefore the inhibition of lipoxygenases would give greater shelf stability to meat products. It is these processes that initiate the oxidative degradation of foods rendering them unpalatable.

Antioxidants, as defined by Brewer (2011), are compounds or systems that delay autoxidation by inhibiting formation of free radicals or by interrupting propagation of the free radical by one (or more) of several mechanisms. The mechanisms include scavenging of reactive species that initiate peroxidation, chelating metal ions such that they are unable to generate reactive species or decompose lipid peroxides, quenching singlet oxygen preventing formation of peroxides, breaking the autoxidative chain reaction, and/or reducing localized O_2 concentrations (Brewer, 2011). Antioxidants can be classified into two groups based on their mode of action. The first being primary antioxidants, acting by donating a labile hydrogen atom and secondary antioxidants which may act by binding transition metal ions (Fe^{2+}, Fe^{3+}, and Cu^{2+}) able to catalyse oxidative processes, by scavenging oxygen, by absorbing UV radiation, by inhibiting enzymes, or by decomposing hydroperoxides (Rice-Evans *et al.*, 1996; Kumar *et al.*, 2015). The key mechanism is their reaction with free radicals to form relatively stable inactive products (Kumar *et al.*, 2015). Their structure confers stability to the radical intermediate because of resonance delocalization and absence of appropriate sites for attack by molecular oxygen (Maqsood *et al.*, 2014).

Phenolic compounds, flavonoids, in particular, exhibit a structure-function relationship hinged on three structural features. Firstly, the metal-chelating potential that is facilitated by the vicinal hydroxyl and carbonyl group around the molecule. Secondly, the presence of

electron-donating substituents; and lastly their ability to delocalize the unpaired electron, leading to the formation of stable phenoxyl radicals (Rice-Evans *et al.*, 1996; Balasundram *et al.*, 2006; Guitard *et al.*, 2016). The B ring hydroxyl configuration is reported to be the most significant determinant of scavenging of reactive oxygen species (ROS). This is based on its ability to donate hydrogen and an electron to hydroxyl, peroxyl, and singlet oxygen radicals, stabilizing them with the formation of a stable flavonoid radical (Kumar and Pandey, 2013).

It has also been observed that the chemical nature of phenolic compounds and thus their antioxidant potential, will vary based on their structural class, degree of hydroxylation (number and position on aromatic rings), other substitutions and conjugations, and degree of polymerization (Tripoli *et al.*, 2007). This was illustrated by Guitard *et al.* (2016) who investigated the use of the bond dissociation enthalpy (BDE) and a number of hydrogen atoms released per molecule of phenol as tools to select the most promising antioxidants. Ten synthetic phenols and sixty natural antioxidants were employed. The theory behind the investigation was based on three assumptions that firstly, the low value of bond dissociation enthalpy (BDE) of the phenolic bond favours the transfer of the phenolic hydrogen to free radicals (i.e., R•, RO• , and ROO•). Secondly, a high value of ionization potential (IP) avoids the transfer of an electron from phenols to oxygen thereby reducing the prooxidant potential of the antioxidant. Lastly, a high solubility of the phenol into the protected medium improves the antioxidant power (Zhang *et al.*, 2003; Guitard *et al.*, 2016). Epigallocatechin gallate, also abundant in tea extracts, was found to be the most effective antioxidant which was attributed to its pyrogallol and galloyl moieties which decrease bond dissociation energy and increase the number of radicals trapped per molecule (Guitard *et al.*, 2016). Dimeric and trimeric proanthocanidins found mostly in grape seed, have a structure similar to that of epigallocatechin gallate, thereby retaining the properties that give high antioxidant activity (Hellstro *et al.*, 2009). The chelating ability of polyphenols is provided by vicinal hydroxyl

groups thereby preventing the transition metal from participating in Fenton reactions (Kalt, 2005; Brewer, 2011; Kumar *et al.*, 2015).

Teixeira *et al.* (2014) found that anthocyanins contribute more to the antioxidant capacity of the fruits (90%) than flavonols, flavan-3-ols, and phenolic acids (10%). However, this was disputed by Andrés *et al.* (2017), who points out that antioxidant activity cannot be attributed to a single compound but to the action and interactions between the various phenolic compounds. In support of the latter, Lingua *et al.* (2016) found that the antioxidant capacity of wine was related to phenol profile rather than the content. Not every polyphenolic compound has the ability to delay autoxidation, more so, through the same mechanism and some compounds may be more effective than others (Brewer, 2011). For example, Brewer (2011) and Guitard *et al.* (2016) reported that flavonoids with multiple hydroxyl groups were more effective than antioxidants with a lower degree of hydroxylation. This could explain the higher activity of hydroxycinnamic acids compared to hydroxybenzoic acids. The CH=CH–COOH group of hydroxycinnamic acids ensures greater H-donating ability and radical stabilisation than the –COOH group in the hydroxybenzoic acids (Rice-Evans *et al.*, 1996; Balasundram *et al.*, 2006).

2.5.1 *In vitro* antioxidant activity

2.5.1.1 Grape pomace extracts

The antioxidant activity of grape pomace extracts and fractions, in comparison with butylated-hydroxytoluene (BHT), was investigated by Negro *et al.* (2003). The antioxidant activity was reported as a percentage of β-carotene protection against oxidation. The extracts were found to have antioxidant activity that was concentration dependent, with higher activity of extracts seen at 160 ppm comparable to BHT. This was attributed to the proanthocyanidin content of the extracts which was greater in the seed than the skin fraction of the pomace.

Tournour *et al.* (2015) determined the antioxidant properties of pomace extracts obtained from three red grape varieties (*Vitis vinifera* L. grape) namely Touriga Nacional, Touriga Franca and Tinta Roriz. It was established that all the pomace extracts had high oxygen radical absorbance capacity (ORAC), peroxyl radical scavenging and iron (II) chelating ability. The total phenolic content of the pomaces correlated strongly with 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, indicating that the phenolic content could be used to predict the strength of radical scavenging. However, the total phenolic content had a low correlation with ORAC and iron (II) chelating ability.

The antioxidant activity of pomace extract from red grape (*Vitis vinifera* L.) cv. Malbec was evaluated by Antoniolli *et al.* (2015). The ORAC assay was employed to measure the peroxyl-radical scavenging capacity. Pomace extracts were found to have significant peroxyl radical scavenging activity, which was attributed to the presence of polymeric procyanidins in the seed fraction of the pomace. Xu *et al.* (2016) investigated the antioxidant properties of pomace from two red grape (Cabernet Franc and Chambourcin) and white grape (Vidal Blanc and Viognier) varieties using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) radical scavenging assays were used in the analysis. The ABTS•+ scavenging capacity had a significant correlation with total phenolic content, total flavonoid content and condensed tannins. Total anthocyanin content and DPPH, on the other hand, had a significant positive correlation in agreement with a study by Chikwanha *et al.* (2018).

2.5.1.2 Grape seed extracts

Rockenbach *et al.* (2011) investigated the *in vitro* antioxidant properties of grape seed from seven grape cultivars grown in Brazil. There was a significant antioxidant activity from all the extracts, with high positive correlation between total phenolic content and DPPH radical-scavenging ability. The high antioxidant activity of the grape seed extracts is attributed to their

high content of gallolytated flavanols. The results obtained showed a significant correlation between the DPPH and ferric reducing antioxidant power (FRAP) methods, as both are based on the same mechanism of single-electron transfer.

Research was done by Yilmaz *et al.* (2015) on the: FRAP; 2,2-azino-bis(3-ethylbenzothiazoline- 6-sulfonic acid) diammonium salt (ABTS); and DPPH on seed fraction from 22 grape cultivars grown in Turkey. The study highlighted the superior antioxidant properties of grape seed extracts compared to pulp and skins. The results also highlighted the seasonal variations in phenolic content, translated into differences in antioxidant activity.

2.5.1.3 Orange peel and pulp extracts

Methanolic extracts of pulp and peel sections from two sweet orange cultivars (*Citrus sinensis* cv. Pera and cv. Lima), two species of limes (*C. latifolia* Tanaka cv. Tahiti and *C. limettioides* Tanaka cv. Sweet lime) and one cultivar of mandarin (*Citrus reticulata* Blanco cv. Ponkan) were assayed by De Moraes Barros *et al.* (2012) for DPPH and ferric reducing antioxidant power (FRAP) antioxidant activity. The DPPH radical scavenging capacity and FRAP activity were observed for the Pera and Lima oranges. The research also showed a strong correlation between the ascorbic acid content and *in vitro* antioxidant capacities values for the citrus peels and pulp separately. Negative correlation were also observed on the same parameters for pulp and peel together.

Studies by Chen *et al.* (2017) showed that orange (*C. reticulata*) peel extract had a significant antioxidant function based on the DPPH, ABTS free radical scavenging activity, oxygen radical absorbance capacity (ORAC) and nitric oxide (NO) inhibitory activity. The total phenolic content of the orange peels was found to be positively correlated with DPPH and FRAP antioxidant activity.

Malterud and Rydland (2000) investigated the inhibitory activity of orange (*Citrus sinensis* Osbeck; Rutaceae) peel toward soybean 15-lipoxygenase. The strongest inhibition was shown by 3,5,6,7,3′,4′-hexamethoxyflavone with half maximal inhibitory concentration (IC_{50}) of 49 ± 5 µM, while other flavonoids such as sinensetin, nobiletin, tangeretin, and tetramethylscutellarein showed less activity. Polymethoxylated flavones were also found to be inactive as scavengers of the diphenylpicrylhydrazyl radical. Malterud and Rydland (2000) concluded that while the citrus extracts had only modest radical scavenging activity, they may, however, counteract *in vitro* enzymatic lipid oxidation catalysed by 15-lipoxygenase. Research done by Ghasemi *et al.* (2009) concluded that there was no correlation between total flavonoids and radical scavenging activity. However, since only one assay (DPPH) was performed in the study, it warrants further investigation with more methods such as the ORAC and lipoxygenase assays to conclusively evaluate whether locally available fruit by-products have significant antioxidant activity. An interrogation of the by-products from locally grown cultivars will open avenues for adding value to fruit processing wastes while optimising food processing technology.

2.6 Mechanism of antimicrobial action

Control of spoilage and pathogenic microorganisms is essential to the provision of safe palatable food. Microbes of concern, especially in meat include *Escherichia coli*, *Salmonella spp.*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Yersinia enterocolytica*, *Clostridium perfringens*, *Clostridium botulinum*, and *Campylobacter jejuni, Pseudomonas pp* as well as certain yeast and mould species (Lee and Paik, 2016). Antimicrobial mechanisms of plant extracts manifest through disrupting the phospholipid bilayer of the cell membrane, disrupting enzyme systems and compromising the genetic material of bacteria (Wu *et al.*, 2013; Aziz and Karboune, 2016). The outer cell wall on Gram negative bacteria plays an important

role in bacterial cell wall viability and is an important site for interactions with antimicrobial compounds (Papuc *et al.*, 2017a).

The hydroxyl (–OH) groups on phenolic compounds have been observed to interact with membrane proteins thereby disrupting the lipid bilayer (Gyawali and Ibrahim, 2014). This disruption has an effect on bacterial cells through increased membrane permeability, compromising membrane fluidity, inhibiting respiration, altering ion transport processes, acting as proton exchangers and reducing the electron gradient across bacterial cell membranes (Papuc *et al.*, 2017b). These membrane disruptions cause breakdown of proton motive force and reduction in ATP generation, leading to cell death (Gyawali and Ibrahim, 2014). The –OH groups also bind the active site of bacterial enzymes, thereby altering the metabolism of the microorganism (Gyawali and Ibrahim, 2014). The inhibition of microbial enzymes is possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Cowan, 1999). *In vitro* studies have also shown polyphenols to have a propensity to bind protein, thus conferring an ability to inhibit enzyme activity (Haslam, 1996).

Research by Wu *et al.* (2013) showed that flavonoids had an inhibitory effect on *E. coli* DNA gyrase enzyme with IC_{50} values ranging from 0.037 to 1.89 mg/ml. DNA gyrase is an ATP-dependent bacterial enzyme involved in the transcription, replication of DNA and chromosome segregation process (Khan *et al.*, 2018). Flavonoids with hydroxyl groups such as kaempferol, had a greater inhibitory effect than those containing methoxyl groups such as nobelitin (Wu *et al.*, 2013). Flavonoids were also shown to have an inhibitory effect on *E. coli* with kaempferol, owing to its hydroxyl group substitutions, having the highest activity while nobelitin, a polymethoxylated flavone, demonstrated the least (Wu *et al.*, 2013). The strong correlation between the IC_{50} and minimum inhibitory concentration (MIC) values were found by Wu *et al.* (2013) to suggest that the inhibition of DNA gyrase could be an important mechanism in the antibacterial activity of flavonoids

Another mechanism observed, includes the inhibition of biofilms formed by spoilage and pathogenic bacteria. This could involve the inhibitory activity of polyphenols such as gallic acid and oligomeric proanthocyanidins on bacterial fructosyltransferase and glucosyltransferase enzymes involved in the synthesis of insoluble polymeric fructans and glycans needed to create an adhesion surface (Slobodníková *et al.*, 2016). The chelating ability of phenolic compounds can deprive microbes of essential iron required for growth (Scalbert, 1991). Tannins have also been known to inhibit the activity of fungal metalloenzymes such as peroxidase and laccase with lower activity against other enzymes such as pectinase and protease (Scalbert, 1991).

Research by Lin *et al*. (2004) concluded that exposure of bacterial cells to the low pH of grape extracts causes sub-lethal injury to cell membranes, causing disruption of proton motive force, and loss of H+-ATP-ase. Fruit extracts also contain organic acids which possess antimicrobial activity. The mode of action of organic acids in inhibiting microbial activity is thought to involve un-dissociated organic acids entering the bacterial cell, causing bacteria membrane disruption (leakage, transport mechanisms), inhibition of essential metabolic reactions such as glycolysis, stress on intracellular pH homeostasis (normal bacteria pH is $\pm$ neutral), accumulation of toxic anions and energy stress response to restore homeostasis (Indresh, 2008).

2.6.1 *In vitro* antimicrobial properties

2.6.1.1 Grape pomace extracts

Grape pomace extracts (GPE) from four grape varieties were evaluated for antibacterial activity by Xu *et al.* (2016). The agar well diffusion method was used against the test organisms, which included two species of pathogenic Gram positive bacteria (*Listeria monocytogenes* and *Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli* O157:H7 and *Salmonella typhimurium).* The pomace extracts exhibited antibacterial activity against *L.*

monocytogenes and *S. aureus*, with no activity detected against *E. coli* O157:H7 and *S. typhimurium.* The researchers attributed the lack of susceptibility of the Gram negative bacteria to the presence of a thick, highly hydrophobic outer membrane that acts as a permeability barrier to hydrophilic antibiotics and the presence of efflux pumps, target site modification and antibiotic inactivation mechanisms (Stavri *et al.*, 2007). However, the reduced sensitivity could have been due to the method used (Valgas *et al.*, 2007). Greater sensitivity was observed when Xu *et al.* (2016) used the same pomace extracts in a broth microdilution to obtain the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). From the pomace extracts, one red (Cabernet Franc) and one white (Voignier) had the greatest activity, especially against *L. monocytogenes.*

The susceptibility of Gram positive bacteria to pomace extracts was also demonstrated by Oliveira *et al.* (2013). Extracts of grape pomace derived from Merlot and Syrah (*Vitis vinifera*) wine production were tested against *S. aureus*, *B. cereus*, *E. coli*, *P. aeruginosa* and *C. albicans*. The observed antimicrobial activity was attributed to presence of flavanols, anthocyanins, phytosterols and triterpenes in the extracts.

2.6.1.2 Grape seed extracts

Antimicrobial activity of grape seed extracts (GSE) against *Bacillus cereus*, *Bacillus coagulans*, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* was investigated by Jayaprakasha *et al.* (2003) using the agar dilution method. Gram positive *B. cereus*, *B. subtilis, B. coagulans* and *S. aureus* had an MIC in the range 900-1000 ppm when using a methanol: water: acetic acid (90:9.5:0.5) extract. The Gram negative *E.coli* and *P. aeruginosa* were inhibited at an MIC in the range 1250-1500 ppm.

The disk diffusion method was used by Baydar *et al.* (2004) to investigate the antibacterial effects of GSE on food spoilage and pathogenic bacteria. *Aeromonas hydrophila,*

Bacillus amyloliquefaciens, Bacillus brevis, Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Enterobacter aerogenes, Enterococcus feacalis, Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Mycobacterium smegmatis, Proteus vulgaris, Pseudomonas aeruginosa and *Staphylococcus aureus* were used in the assay. The GSE at 20% inhibited all bacteria except *B amyloliquefaciens*. Gram positive bacteria *S. aureus*, *B. cereus* and *B. subtilis* were found to be more susceptible than the Gram negative *P. aeruginosa* and *E. coli,* and this was in agreement with studies by Jayaprakasha *et al.* (2003).

Having reviewed results from agar dilution and disk diffusion methods, it is important to consider those from broth microdilution method. Antimicrobial activity of GSE using the broth microdilution assay was evaluated by Peixoto *et al.* (2018). Organisms used included Gram negative bacteria (*Escherichia coli* ESBL (extended spectrum of beta-lactamase), *Klebsiella pneumoniae*, *K. pneumoniae* ESBL, *Morganella morganii* and *Pseudomonas aeruginosa*) and Gram positive bacteria (MRSA-methicillin-resistant *Staphylococcus aureus*, MSSA-methicillin- susceptible *S. aureus*, *Listeria monocytogenes* and *Enterococcus faecalis*) The antimicrobial activity of the extracts was found to be strongly correlated to the total flavonols, total phenolic acids, total phenolic compounds and total anthocyanins content of the GSE. Peixoto *et al.* (2018) also reported low MIC values against Gram positive bacteria compared to Gram negative.

2.6.1.3 Orange peel and pulp extracts

Ethanolic extracts of *Citrus sinensis* together with *Citrus limon* and *Citrus limetta* were screened for *in vitro* antifungal properties by (Mohanka and Priyanka, 2014). Sodium benzoate was used as a positive control in the experiment. *Citrus sinensis* extracts were found to be the most effective, as they showed significant inhibition of mycelia growth. Klangpetch *et al.* (2016) investigated the antibacterial effects of citrus peels from Pomelo, Kaffir lime and Lime. There was no significant difference between the antimicrobial activity of Kaffir lime extracts

and the positive control, chloramphenicol, against *E. coli.* According to Klangpetch *et al.* (2016), the mode of action for antimicrobial properties of phenolic extracts includes degradation of the microbial cell wall, disruption of the cytoplasmic membrane, causing leakage of cellular components, changing fatty acid and phospholipid constituents, influencing the synthesis of DNA and RNA and destroying protein translocation. The results also showed greater susceptibility of Gram positive bacteria to citrus peel extracts compared to Gram negative bacteria. The demonstration of antimicrobial properties of extracts from local cultivars will, therefore, go a long way in informing the efforts to harness fruit waste streams to contribute towards the bio-economy.

2.7 Summary

Previous research has shown that fruit by-products contain viable amounts of phytochemicals with potential for valorisation. This could prove important in the steps towards channelling fruit waste in South Africa towards sustainable uses. Effective extraction and preparation of extracts play an important role in the retrieval of maximal viable amounts of phytochemicals. The *in vitro* antioxidant assays performed in previous research show that natural extracts retain an ability to scavenge radicals, act as reducing agents and inhibit lipoxygenase enzymes. Control of these radical generation pathways, therefore, indicates the potential of natural extracts as antioxidant preservatives. Previous studies also demonstrate the ability of natural extracts to inhibit microbial growth with the activity being reported to correlate highly with the flavonoid groups. Based on this information, it could be important to identify the polyphenols classes, concentrations and preservative potential of by-products from fruit processing in South Africa. Therefore, the objective of the current research was to determine the phenolic composition, antioxidant and antimicrobial properties of extracts from grape pomace (*Vitis vinifera L.* var. Pinotage), grape seed and orange peel and pulp (*Citrus reticulata* Blanco).

Chapter 3

Materials and Methods

3.1 Chemicals and reagents

Folin-Ciocalteau reagent, sodium carbonate, aluminium chloride, dimethyl sulfoxide (DMSO), butylated hydroxytoluene (BHT), hexane, sodium nitrite and sodium hydroxide (Merck, Darmstadt, Germany); the standards gallic acid, catechins, L-ascorbic acid, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and β-carotene, the reagents polyvinylpyrrolidone, 2,2-diphenyl-1-picryl-hydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), fluorescein sodium salt, 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH), sodium tetraborate, boric acid, xylenol orange, linoleic acid, 15-lipoxygenase enzyme, nordihydroguaiaretic acid (NDGA), Mueller-Hinton agar, Mueller Hinton broth, iodonitrotetrazolium chloride (INT), sodium metabisulphite and tetracycline used in the current study were purchased from Sigma-Aldrich (Steinheim, Germany).

3.1.1 Sample preparation

The fruit by-products used in the current study were grape pomace, grape seed and orange peel and pulp. Fresh grape (*Vitis vinifera* cv. Pinotage) pomace was obtained in January from Stellenbosch University's Welgevallen Cellar (Stellenbosch, South Africa; S 33° 56' 22.38" E 018° 52' 1.92"). Mandarin orange (*Citrus reticulata* cv. Blanco) peel and pulp were sourced in September from Citrusdal (Western Cape, South Africa; 32°34'41.2"S 19°00'36.2"E). Before drying, the orange peel and pulp was washed. Grape seed was a donation from Brenn-O-Kem Pty Ltd (Wolseley, South Africa; 33.4181° S, 19.2385° E). All fruit by-products were collected in batches over six consecutive days (6 batches). Each day, about eight tons of grape and about 10 tons of oranges were harvested, pressed and a representative sample (2 kg) of fresh by-

product collected (n = 6 pressings). The sample from each day's pressing for each variety was divided into three fractions of 500 g. Fruit by-products were oven dried at 60 °C for 72 h and subsequently ground into fine powders using a Wiley mill (Model 4, Thomas Scientific, Swedesboro, NJ, USA) with a 1-mm sieve and stored at -20 °C, pending extraction.

3.1.2 Polyphenol extraction

Solid–liquid extraction of the three fruit by-products was performed at Brenn-O-Kem (Pty) Ltd, Wolseley, South Africa. The ground samples were defatted by a washing step in hot water, before extraction using 62% ethanol (1:10, w/v). The extract was recovered using a filter press (Model KFP639/20, Schenk, South Africa) and the filtrate was vacuum-dried using a single stage, liquid ring vacuum pump (Model MHF-150, Nash, Wisconsin, USA) to eliminate the solvent. The dried extracts were then ground (0.1 µm) into a powder. Before the determination of phytochemicals, the powdered extracts were separately weighed and re-suspended in 62% ethanol.

3.2 Phytochemical analyses

3.2.1 Determination of total phenols and tannins

Total phenolics and tannins of the extracts were determined by the Folin-Ciocalteu colorimetric method (Makkar, 2000). Absorbance was read at 725 nm using a UV-visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). Tannin content was calculated as the difference between the total phenolics before and after polyvinylpyrrolidone treatment. Measurement was carried out in triplicate and calculated from a calibration curve obtained with gallic acid as the standard. The content of total phenolics was expressed as g gallic acid equivalents (GAE)/100 g extract dry matter (DM).

3.2.2 Determination of total flavonoids

Total flavonoid content of the extracts was determined with the procedure described by Yang, Martinson, & Liu (2009). Total flavonoid content was expressed as g of catechin equivalents (CE)/100 g extract, DM).

3.2.3 Total monomeric anthocyanins

Total monomeric anthocyanin content was determined using the pH-differential method described by Giusti and Wrolstad (2001). The total monomeric anthocyanin content was expressed as cyanidin- 3- glucoside equivalent (g/100 g extract, DM).

3.2.4 Proanthocyanidins

Determination of proanthocyanidins was performed according to the method of Porter, Hrstich, & Chan (1986). Proanthocyanidin concentration was expressed as cyanidin equivalent (g CyE/100 g extract DM).

3.2.5 Total carotenoids

Total carotenoids were determined following the method of Hess, Keller, Oberlin, Bonfanti, & Schüep (1991). The absorbance of the samples was measured at 470 nm using a UV-visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin), against an ethanol: hexane, BHT blank. Estimates were calculated from a calibration curve obtained with β-carotene as the standard and expressed in g/100 g DM.

3.2.6 Ascorbic acid

Ascorbic acid content of extracts was determined using a colorimetric method as described by Mphahlele, Stander, Fawole, & Opara (2014). Estimations were calculated from a calibration

curve obtained with ascorbic acid as the standard and the content expressed as g ascorbic acid (AA)/100 g DM.

3.3 Titratable acidity and pH

Titratable acidity of extract samples was determined using an automatic titrator (Metrohm Compact Titrosampler, Herisau, Switzerland). Triplicate samples (2 g) were diluted with 70 mL of deionised water and titrated against 0.1 M sodium hydroxide solution until the end-point pH of 8.1 was reached. The results were expressed as tartaric acid equivalent (%, g/100 g solids). The pH of extract samples was measured in triplicate with a standard pH meter after calibration with pH 4.0 and 7.0 buffers.

3.4 Antioxidant activity assays

Multiple antioxidant evaluation assays namely; 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH), ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC) and lipoxygenase (15-LOX) inhibition were employed to sufficiently evaluate the antioxidant activity of the fruit by-product extracts. The DPPH and FRAP assays are electron transfer-based assays, while the ORAC and lipoxygenase (15-LOX) assays are hydrogen atom transfer reaction assays.

3.4.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The assay was performed according to the method of Tolic, Jurcevic, Krbavcic, Markovic, & Vahcic (2015), based on the reduction of stable DPPH radical in the presence of antioxidants. Absorbance measurements were made at 517 nm against a methanol blank using a UV-visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). Absorption of a sample containing the same amount of methanol and DPPH solution acted as a negative control,

while L-ascorbic acid was used as positive control. The EC_{50} for each treatment, sample concentration providing 50% of antioxidant activity (Peixoto *et al.*, 2018), was calculated and the result expressed in mg/mL, for comparison against ascorbic acid, and as Trolox equivalents (TE)/g extract.

3.4.2 Determination of ferric reducing antioxidant power (FRAP)

The FRAP assay was conducted according to Benzie and Strain (1996). The method is based on the reduction of the Fe^{3+} -2,4,6-tripyridyl-s-triazine (TPTZ) complex to the ferrous form at low pH. This reduction is monitored by measuring the absorbance change at 595 nm. A standard curve was prepared using different concentrations (0–1800 µM) of Trolox. FRAP values were calculated according to the calibration curve and they were expressed as Molar Trolox equivalents (M TE)/g extract.

3.4.3 Oxygen radical absorbance capacity (ORAC)

The assay was performed according to Gillespie, Chae, & Ainsworth (2007) and measures the free radical oxidation of a fluorescent probe through the change in its fluorescent intensity. Fluorescence kinetic readings were measured with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Readings were taken using a Tecan Spark 10M multimode microplate reader (Männedorf, Switzerland). The assay temperature was kept at 37 1C and duration was 1 hr. The ORAC values were calculated using the area under the curve (AUC) for the standard and samples. A standard curve was obtained by plotting the Trolox standards against the average AUC. The half maximal effective concentration (EC_{50}) for each treatment was calculated and the result expressed in µg/mL, also for comparison against ascorbic acid, and as Trolox equivalents (TE)/g extract.

3.4.4 15-Lipoxygenase (15-LOX) inhibition assay

The 96-well microplate based-ferrous oxidation-xylenol (FOX) assay was carried out according to the method described by Waslidge and Hayes (1995). Extract samples were reconstituted in 10% dimethyl sulfoxide (DMSO) and filtered. Concentration range of the extracts ranged from 2.44×10^{-3} - 80 µg/mL. Blank wells were prepared by adding 70 µL of assay buffer (borate buffer, 0.2 M) in triplicate. Positive control wells contained 50 µL of 960 U/mL LOX and 20 µL of nordihydroguaiaretic acid (NDGA), which was used as the positive control inhibitor in the assay. One-hundred percent initial activity (IA) wells were prepared by adding 50 µL of LOX and 20 µL of 10% DMSO. Sample wells (inhibitor) contained 50 µL of LOX and 20 µL of extract. The reaction was then initiated by adding 50 µL of substrate (Linoleic acid; 356 µM) to all the wells of a 96-well plate with further incubation for 10 min after which 100 µL of FOX was added to each well to terminate the reaction. The Fe^{3+}-dye complex was allowed to develop for 30 min at room temperature and the absorbance read at 570 nm on a 96-well Multiskan Microplate Photometer (Thermo Scientific, China). All data points were measured in triplicate and the results are presented as mean ± SD. The percent lipoxygenase inhibition for each extract was determined as follows:

$$Inhibition = \left[1 - \frac{IA - Inhibitor}{IA}\right] \times 100$$

The IC_{50} values for each extract were calculated and the result expressed in µg/mL of solvent.

3.5 Antimicrobial susceptibility testing

3.5.1 Culture of microorganisms and suspension preparation

Antibacterial activity was tested against three Gram positive bacteria (*Listeria monocytogenes* ATCC 7644, *Enterococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 29213), two Gram negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853) and one yeast (*Candida albicans* ATCC 14053) obtained from ThermoFisher Scientific

(Johannesburg, South Africa). Each strain was separately grown on Muller-Hinton (MH) agar (24 h at 35 °C for bacteria, and 48 h at 25 °C for yeast), after which a fresh working suspension was then prepared by suspending in 0.85% saline solution. Regulation of the initial suspension turbidity for the microbes was conducted by comparison of initial bacterial and yeast suspension in sterile 0.85% saline with 0.5 McFarland's standard. The initial bacterial suspensions contained about 10^8 CFU/mL. In addition, 1:100 dilutions of the regulated suspensions were made in sterile MH broth.

3.5.2 Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) of the extracts were determined using a modified broth microdilution method as described by Eloff (1998) and Oliveira *et al*. (2013). Test plates were covered with parafilm and incubated at 35 °C for 22 ± 2 h for bacteria and 28 °C for 48 h for yeast. Bacterial growth was detected after incubation by addition of 40 µL of an iodonitrotetrazolium (INT) ethanolic solution (0.2 mg/mL). The trays were again incubated at 35 ºC for 1 h, and in those wells, where bacterial growth occurred, INT changed from yellow to purple. The MIC values were defined as the lowest concentration of each natural product, which completely inhibited microbial growth. The results were expressed in milligrams per millilitres.

3.6 Data analyses

The EC_{50} values for DPPH, ORAC and IC_{50} values for lipoxygenase were calculated from the logarithmic non-linear regression curve derived from the plotted data using GraphPad Prism software version 4.03 (GraphPad Software, Inc., San Diego, USA). For each of the antioxidant method, an antioxidant index score of the sample was calculated according to the formula:

$$Antioxidant\ Index\ score = \left[\left(\frac{Sample\ score}{Best\ score}\right) \times 100\right]$$

For DPPH and ORAC two scores were calculated based on EC_{50} and Trolox equivalence values. Antioxidant potency composite (APC) index was then calculated as the average of the antioxidant index score of each method (Wang et al., 2017). The extracts' overall antioxidant activities were ranked based on the APC index.

Data for phenolic composition, antioxidant and antimicrobial activity were analysed using a general linear model (GLM) procedure of SAS (SAS Institute Inc., Cary, NC, US). The total number of observations for phenolic content and antioxidant activity data were 54 (3 varieties $\times$ 6 pressings $\times$ 3 replications). After averaging the replications, the remaining 18 observations were subjected to analysis of variance using the following model:

$$y_{ij} = \mu + t_i + \varepsilon_{ij}$$

Where: y_{ij} is the response variable (phenolic content, antioxidant activity and antimicrobial activity), μ is the overall mean, t_i is the effect of the ith treatment and ε_{ij} is the error term. Treatment means were generated using the LSMEANS option and separated using Tukey's multiple comparison test. Differences were declared at the $p \leq 0.05$ significance level. Furthermore, principal component analysis was used to analyse the relationships between the phenolic content with antioxidant and antimicrobial activities using the XLSTAT version 2018.5 (Addinsoft, France).

Chapter 4

Results and Discussion

4.1 Phytochemical content, pH and titratable acidity

Phenolic content, pH and titratable acidity data are shown in **Table 4.1**. Total phenolic content of the extracts were significantly (p ≤ 0.05) influenced by type of fruit by-product. Total phenolic content was highest in GPE with intermediate and lowest contents in GSE and OPE, respectively. The observation that type of fruit by-product had an effect on the phenolic content is consistent with literature showing that the primary source of variation emanates from species differences (Veberic, 2016). This is supported by Butelli *et al.* (2017) who reported that polyphenol profiles of citrus fruits were correlated with gene expression.

The observed difference between GPE and GSE mainly stem from tissue differences in phenolic content. In response to fluctuating environmental conditions and developmental stage, fruits synthesise and localise phenolic compounds differently in various internal structures (i.e., cells, organelles) depending on function and need, which then becomes important when the fruit by-products are collected for the retrieval of phenolics (Kalt, 2005; Wang *et al.*, 2017). These findings pertaining to differences in the localisation of phenolics also agree with Veberic (2016) who reports that diverse plant organs or tissues can be characterised by different phenolic composition.

The total phenolic content of GPE observed in this study is similar to that reported by Katalinić *et al.* (2010) and slightly higher than that reported by Xu *et al.* (2016), which had a content in the range of 5.55 - 15.4 g GAE/100 g. The observed GSE phenolic content was within the range of 2.13-16,5 g CE/100 g DW reported by Rockenbach *et al.* (2011) and higher than the range of 2.12–6.77 g GAE/100 g reported by Tseng & Zhao (2012). The difference of observed values from the latter was attributed to the solvent system of 70% acetone /0.1% HCl

/29.9% water (v/v/v) at a solvent to pomace powder ratio of 4:1 (v/w) employed for extraction by the Xu *et al.* (2016). The observed phenolic content of OPE was in the range of 0.67–19,62 g CE/100 g from a variety of cultivars reported by M'hiri *et al.* (2017).

The GPE had higher ($p \leq 0.05$) tannin content than GSE and OPE, with no significant difference between the latter two ($p > 0.05$). The tannin content observed for GPE was similar to that reported by Xu *et al.* (2016), while that for GSE was in the range reported by Ky *et al.* (2014). Since oranges are not known to contain tannins (Hellstro *et al.*, 2009), it can be speculated that the observed value could have come from the reaction of Folin-Ciocalteau reagent with melanoidins formed during drying of orange extract (M'hiri *et al.*, 2017).

Compared to OPE, flavonoid content was higher ($p \leq 0.05$) in GPE and GSE, with no significant difference between the latter two ($p > 0.05$). The total flavonoid contents observed for GPE and GSE were consistent with previous studies (Negro *et al.*, 2003; Xu *et al.*, 2016). According to Yang *et al.* (2009), grape berry flavonoids such as the anthocyanins and flavanols (quercetin), are mainly localised in the skins while the flavan-3-ols (catechins) are present both in the skins and in the seeds. That partly explains why the content of flavonoids in the GPE and GSE was not significantly different. The lower flavonoid content of OPE could be explained in terms of species and genetic differences between grapes and oranges (Butelli *et al.*, 2017). The OPE had total flavonoid content in the range reported by M'hiri *et al.* (2017).

Proanthocyanidin content was higher in GSE, followed by GPE and OPE in that order ($p \leq 0.05$). According to Yu & Ahmedna (2013), proanthocyanidins found in GSE are soluble in the organic solvent used in the extraction process as they are small compounds which are mainly monomeric and oligomeric in nature. In contrast, GPE proanthocyanidins are of a high-molecular weight and represent what is termed the non-extractable polyphenols (Yu and Ahmedna, 2013; Beres *et al.*, 2017).

Table 4.1: Phenolic composition, pH and titratable acidity of grape pomace extract (GPE), grape seed extract (GSE) and orange peel and pulp extract (OPE)

Assay	Treatment		
	GPE	GSE	OPE
Total phenols[1] (g GAE /100 g)	17.3 ± 0.25^{a}	9.97 ± 0.34^{b}	6.49 ± 0.62^{c}
Total tannins[1] (g GAE /100 g)	11.5 ± 0.26^{a}	3.49 ± 0.33^{b}	4.73 ± 0.61^{b}
Flavonoids[2] (g CE /100 g)	7.76 ± 0.036^{a}	7.61 ± 0.61^{a}	1.00 ± 0.017^{b}
Proanthocyanidins[3] (g CyE /100 g)	1.04 ± 0.24^{b}	3.54 ± 0.13^{a}	0.130 ± 0.012^{c}
Anthocyanins[4] (g Cyd_3E /100 g)	0.173 ± 0.02^{a}	0.214 ± 0.011^{a}	0.0069 ± 0.000053^{b}
Total carotenoids[5] (g β-CE /100 g)	0.026 ± 0.000028^{a}	0.021 ± 0.00065^{b}	0.0050 ± 0.00001^{c}
Ascorbic acid[6] (g AA /100 g)	ND*	ND	2.69 ± 0.37
pH	3.82 ± 0.044^{b}	4.26 ± 0.089^{a}	3.41 ± 0.073^{c}
Titratable acidity (%)	6.15 ± 0.056^{b}	4.51 ± 0.03^{c}	8.11 ± 0.064^{a}

Means followed by a different letter within a row indicate significant ($P \leq 0.05$) difference among samples.
Units: [1] –value expressed as g Gallic Acid Equivalents/100 g extract (DM); [2] -value expressed as g Catechin Equivalents/100 g extract (DM); [3] –value expressed as g Cyanidin Equivalents/100 g extract (DM); [4] –value expressed as g Cyanidin-3-glycoside Equivalents/100 g extract (DM); [5] –value expressed as g β-carotene Equivalents/100 g extract (DM); [6] –value expressed as g Ascorbic acid/100 g of extract (DM); * -Not Detected

These non-extractable polyphenols remain complexed within the cellular matrix to proteins or cell wall polysaccharides rendering them insoluble and requiring hydrolysis to release them (Beres *et al.*, 2017). The low proanthocyanidin content of OPE could be that they exist in the *citrus* genus in an un-extractable form or that there is low to none, as reported by Hellstro *et al.* (2009).

The OPE had lower (p ≤ 0.05) anthocyanin content compared to GPE and GSE that had no significant (p > 0.05) difference in anthocyanin content. This finding agrees with M'hiri *et al.* (2017) who reported that anthocyanins are minor compounds in citrus fruits with further evidence provided by Butelli *et al*. (2017).

Grape seed extract had the highest content of total carotenoids, with moderate levels in GSE and the lowest for OPE (p ≤ 0.05). The distinction in carotenoids content between grape- and citrus-based by-products could be due to species and environmental differences, while the variation between GSE and GPE could be ascribed to tissue differences as explained earlier for phenolic compounds. There was no ascorbic acid detected in the GPE and GSE extracts. This is in contrast to previous research by Sousa *et al.* (2014) who found GPE to have 26.3 mg of acid ascorbic/100 g. The contrast can be attributed to the difference in grape cultivars used, with Sousa *et al.* (2014) having used grapes from the *Vitis vinifera* L. Benitaka variety. Ascorbic acid values for OPE were consistent with range given by M'hiri *et al.* (2017).

All the extracts were acidic, with pH ranging from 3.41 to 4.26. The extracts followed the order of OPE > GPE > GSE in decreasing acidity (p ≤ 0.05; **Table 4.1**). This order was also true for titratable acidity, with OPE, GPE and GSE having the highest, intermediate and lowest titratable acidity, respectively (p ≤ 0.05). Differences in acidity amongst the investigated samples were not surprising as genetic and environmental factors could strongly influence the accumulation of organic acid in plant (Kalt, 2005).

4.2 *In vitro* antioxidant activity

In this section we tested the hypothesis that red grape pomace (*Vitis vinifera L.* var. Pinotage), grape seed and orange peel and pulp (*Citrus reticulata* Blanco) extracts do not have *in vitro* antioxidant capacity. The results for antioxidant activity are presented in **Table 4.2**. The GSE exhibited the strongest radical scavenging activity (DPPH) followed by GPE and OPE in decreasing order. Ascorbic acid had the lower EC_{50} values compared to the three fruit extracts ($p \leq 0.05$). A similar trend for antioxidant activity of the extracts was observed for the FRAP assay (GSE > GPE > OPE; $p \leq 0.05$). These findings could be attributed to anthocyanins, flavonoids and proanthocyanidins contents of the respective extracts. This is supported by negative correlations between DPPH EC_{50} values with anthocyanin and flavonoid contents, suggesting low effective concentration (i.e. high potency) in samples with high contents of anthocyanin and flavonoid, albeit non-significant ($p > 0.05$). Similarly, there was a positive correlation between the reducing power (FRAP) and anthocyanin content in the investigated samples (**Table 4.3; Figure 4.1**). Xu *et al*. (2016) found similar results showing that the antioxidant activity is highly correlated with anthocyanins, flavonoids and proanthocyanidins, which act as hydrogen and electron donors, free radical scavengers, non-radical scavengers of reactive oxygen species, chelators of transitional metal ions and enzyme inhibitors (Papuc *et al.*, 2017b). The electron donating ability of these flavonoid derivatives is based on their structure, which allows hydrogen or electron donation capacity, ability to stabilize and delocalize the unpaired electron and their potential to chelate transition metal.

While the DPPH assay lacks the presence of oxygen radicals present in nature, it retains relevance based on the assumption that extract antioxidant capacity is equal to electron donating capacity or reducing power demonstrated (Tolic *et al.*, 2015).

Table 4.2: *In vitro* antioxidant activity of grape pomace (GPE), grape seed (GSE) and orange peel and pulp (OPE) extracts

Assay		Sample				
		GPE	GSE	OPE	Ascorbic acid	NDGA
DPPH[1]	EC_{50} (mg/ml)	0.11 ± 0.02^b	0.022 ± 0.0045^c	0.81 ± 0.33^a	1.07 ± 0.046^a	
	T Eq. (mM TEq/g)	179.47 ± 2.7^b	86.0 ± 0.72^c	292.33 ± 24^a	-	
FRAP[2]	T Eq. (M TEq/g)	4.57 ± 0.23^b	4.93 ± 0.0053^a	3.39 ± 0.011^c	-	
ORAC[3]	EC_{50} (µg/ml)	7.10 ± 0.56^b	1.30 ± 0.14^d	2.10 ± 0.21^c	14.0 ± 3.40^a	
	T Eq. (mM TEq/g)	9.84 ± 0.079^a	7.73 ± 0.018^c	8.27 ± 0.10^b	-	
Lipoxygenase[4]	IC_{50} (µg/ml)	96.87 ± 13.9^a	39.20 ± 3.82^c	52.06 ± 0.20^b	-	0.32 ± 0.065^d
APC Index[5]	-	286.99	100	748.64	-	
Rank[6]	-	2	1	3	-	

Means followed by a different letter within a row indicate significant (P ≤ 0.05) difference among treatments.

[1]- 2,2-diphenyl-1-picrylhydrazyl (DPPH) expressed as EC_{50} values mg extract/ml solvent and mM Trolox Equivalents/g extract

[2]- FRAP expressed as Molar Trolox Equivalents/g extract

[3]- Oxygen radical scavenging capacity (ORAC) expressed as EC_{50} values µg extract/ml solvent and mM Trolox Equivalents/g extract

[4] - Lipoxygenase expressed as IC_{50} values µg extract/ml solvent

[5]- Antioxidant index score = [(sample score/best score) × 100], averaged for all four tests for each extract for the antioxidant potency composite (APC) index.

[6]- Ranked according to the APC index; the lower the index score the higher the antioxidant activity

In the same manner, the FRAP assay results are interpreted based on the assumption that capability of antioxidants to reduce ferric ions, reflects their ability to reduce reactive oxygen species (Benzie and Strain, 1996). Both methods, therefore demonstrate that extracts from fruit by-products have antioxidant properties performing greater than ascorbic acid used commercially.

With regards to ORAC assay, GSE exhibited the greatest antioxidant capacity as shown by the lowest EC_{50} and Trolox equivalents values, followed by OPE and GPE with ascorbic acid having the lowest capacity. This was also the trend in the lipoxygenase (LOX) assay, where the order of antioxidant activity was GSE > OPE > GPE ($p \leq 0.05$). The extracts, however, had much higher IC_{50} compared to NDGA, a known inhibitor of 15-lipoxygenase enzyme. The ORAC and lipoxygenase assays had a strong correlation with total phenols and total tannins (**Table 4.3**; **Figure 4.1**). The high radical quenching ability, as well as lipoxygenase inhibition of GSE, agrees with previous research by Yilmaz & Toledo (2004), who attributed the high antioxidant capacity of GSE to the hydrogen donating activity of dimeric, trimeric, oligomeric, and/or polymeric procyanidins. Guitard *et al.* (2016) report that in procyanidins, the bonding of the pyrogallol and gallolyl moieties drastically reduces the bond dissociation enthalpy which greatly favours transfer of the phenolic hydrogen to free radicals as is demonstrated in hydrogen atom transfer-assays. These free radicals are most often in the form of reactive oxygen species which are known to result in unrestricted oxidation of DNA, proteins and membrane lipids (Gillespie *et al.*, 2007).

The observed high antioxidant capacity of GSE is also attributed to its proanthocyanidin fraction, which has a structure that allows them to trap more radicals per molecule of antioxidant, giving them greater antioxidant activity (Guitard *et al*., 2016). Brewer (2011) also points out that proanthocyanidins contain multiple hydroxyl groups that can donate hydrogen, quench oxygen radicals and chelate metals.

Table 4.3*:* Pearson correlation coefficients (r) between variables investigated in grape pomace extract (GPE), grape seed extract (GSE) and orange peel and pulp extract (OPE)

	Total phenolic compounds	Tannins	Flavonoids	Anthocyanins	Proanthocyanidins	Carotenoids	Ascorbic acid	pH	Acidity
DPPH[1]	-0.460	-0.142	**-0.728**	**-0.729**	-0.586	**-0.701**	**0.803**	**-0.710**	**0.684**
FRAP[2]	0.566	0.148	**0.943**	**0.938**	**0.838**	**0.891**	**-0.953**	**0.924**	**-0.957**
ORAC[3]	**0.892**	**0.984**	0.397	0.190	-0.376	0.568	-0.382	-0.147	0.073
LOX[4]	**0.837**	**0.967**	0.321	0.097	-0.449	0.489	-0.296	-0.224	0.158
Staphylococcus aureus	0.645	0.348	**0.847**	**0.778**	0.578	**0.815**	**-0.809**	**0.743**	**-0.721**
Listeria monocytogenes	**0.764**	**0.804**	0.431	0.198	-0.200	0.534	-0.366	-0.016	-0.034
Enterococcus faecalis	**0.902**	**0.886**	0.551	0.350	-0.128	**0.689**	-0.546	0.081	-0.150
Pseudomonas aeruginosa	**0.742**	0.365	0.988	**0.962**	**0.707**	**0.977**	**-0.991**	**0.847**	**-0.890**
Escherichia coli	0.472	0.066	**0.865**	**0.876**	**0.838**	**0.802**	**-0.877**	**0.903**	**-0.919**
Candida albicans	**0.977**	**0.918**	**0.695**	0.505	-0.046	**0.805**	-0.652	0.210	-0.255

-Values in bold statistically significant (p ≤ 0.05)
[1]DPPH - 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity
[2]FRAP - ferric reducing antioxidant power
[3]ORAC - oxygen radical absorbance capacity
[4]LOX - lipoxygenase inhibition

The greater ORAC and lipoxygenase antioxidant activity of OPE compared to GSE could have been from the high ascorbic acid content of OPE. The ascorbic acid antioxidant mechanism is through quenching of reactive oxygen species and enzyme reduction (Kalt, 2005). Overall, GSE had the most antioxidant activity of all the extract across all the assays employed as seen by its rank according to the calculated APC index in **Table 4.2**. It is therefore suggested that GSE could be regarded for application in the food matrix as an antioxidant based on its *in vitro* performance.

4.2 *In vitro* antimicrobial activity

The hypothesis that red grape pomace (*Vitis vinifera L.* var. Pinotage), grape seed and orange peel and pulp (*Citrus reticulata* Blanco) extracts do not have *in vitro* antimicrobial capacity was tested. Results obtained indicate that antimicrobial activity varied significantly ($p \leq 0.05$) amongst the investigated extracts (**Table 4.4**). Overall, the order of antibacterial activity for the extracts OPE > GSE > GPE. Tetracycline was used as the positive control while sodium metabisulphite is an antimicrobial preservative used in meat and other foods in South Africa. Both the positive control and the commercial preservative exhibited strong inhibitory activity, greater than that of the extracts, against *Listeria monocytogenes*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* in line with previous studies (Hasselmann and Diseases, 2003).

In terms of the extracts, OPE had the greatest antimicrobial activity as observed by the low MIC across all the microorganisms tested ($p \leq 0.05$). The antimicrobial activity of OPE could be due to its high acidity levels. This is indicated by the strong negative correlation between OPE, ascorbic acid, acidity levels with *S. aureus*, *P. aeruginosa*, and *E. coli* microbial MIC (**Table 4.3; Figure4.1**).

Table 4.4: *In vitro* antimicrobial activity of grape pomace extract (GPE), grape seed extract (GSE) and orange peel and pulp extract (OPE) against reference microorganisms

Microorganism	MIC (mg/ml)				
	GPE	GSE	OPE	Sodium metabisulphite	Tetracycline
Staphylococcus aureus	9.375 ± 3.6084[a]	9.375 ± 3.6084[a]	3.125[b]	0.391[c]	0.003 ± 0.001[d]
Listeria monocytogenes	4.688 ± 1.8042[a]	2.344 ± 0.9021[a]	2.344 ± 0.9021[a]	0.293 ± 0.1128[b]	0.017 ± 0.0303[c]
Enterococcus faecalis	9.375 ± 3.6084[a]	2.344 ± 0.9021[b]	1.563[b]	0.391[c]	0.016[d]
Pseudomonas aeruginosa	12.5[a]	12.5[a]	6.25[b]	1.172 ± 0.4511[c]	0.008[d]
Escherichia coli	9.375 ± 3.608[a]	12.5[a]	3.125[b]	1.563[c]	0.003 ± 0.0011[d]
Candida albicans	6.25[a]	2.344 ± 0.9021[b]	1.563[b]	0.037 ± 0.0141[c]	0.003 ± 0.0011[d]

Means followed by a different letter within a row indicate significant ($p \leq 0.05$) difference among treatments.

The antimicrobial activity of organic acids involves the un-dissociated organic acids entering the bacterial cell, causing membrane disruption (leakage, transport mechanisms), inhibition of essential metabolic reactions such as glycolysis, stress on intracellular pH homeostasis, accrual of toxic anions and energy stress response to restore homeostasis.

The inhibitory effect of the extracts against *S. aureus, E. coli* and *P. aeruginosa* was highly correlated with flavonoid, anthocyanin and proanthocyanidins content (**Table 4.3; Figure 4.1**), in agreement with the results of Wu *et al*. (2013) and Peixoto *et al*. (2018). Hydroxylated flavonoids such as kaempferol were observed by Wu *et al*. (2013) to have an inhibitory effect on *E. coli*, with a strong correlation between the DNA gyrase enzyme inhibition IC_{50} and antibacterial MIC values indicating the importance of bacterial enzyme inhibition effect.

As such, Wu *et al.* (2013) concluded that an important mechanism in the antimicrobial activity of flavonoids is the inhibition of microbial enzyme. An example of such is DNA gyrase which is involved in the transcription, replication of DNA and chromosome segregation process (Wu *et al.*, 2013). The correlations between flavonoid, anthocyanin and proanthocyanidins groups with bacterial inhibition are in support of previous research findings by Lee & Paik, (2016). Their work highlighted that the phenolic profile as opposed to individual phenolics, is essential in giving strong antimicrobial effect as different phenolics present multiple mechanisms.

Overall, the extracts were found to be more effective against the Gram positive bacteria (*S. aureus, E. faecalis* and *L. monocytogenes*) and yeast than against the Gram negative (*P. aeruginosa* and *E.coli*). This is consistent with studies done by Jayaprakasha *et al.* (2003) and Peixoto *et al*. (2018) showing that Gram positive bacteria are more susceptible than Gram negative bacteria to plant phenolic extracts.

The presence of a unique liposaccharide cell wall (Xu *et al.*, 2016), deactivation and metabolism of phenolic compounds such as hydroxycinnamic acids, as well as presence of efflux pumps on organisms like *E. coli* (García-Lomillo and González-Sanjosé, 2017), are reported as the mechanisms of reduced susceptibility by Gram negative bacteria. However, the antimicrobial activity demonstrated by OPE, has positive implications in that it presents a viable substitute to sulphites, thereby reducing the allergenic risk posed by the use of the synthetic preservative (García-Lomillo and González-Sanjosé, 2017).

Based on the observed *in vitro* performance, it is suggested that GSE and OPE could be regarded for application in the food matrix as antioxidants and antimicrobials, respectively. Utilization of bioactive-rich citrus and winery by-products could provide an efficient, inexpensive, easily available and environmentally friendly platform for the production of novel food preservatives.

Purification of the extracts can possibly increase bioactivity of these extracts (Ignat *et al.*, 2011). In-depth analysis of the phenolic profile of the extracts using methods such as HPLC, to understand composition and isolation of active ingredients, is important. In addition, research is also necessary to establish bioavailability and real benefits of these extracts obtained from fruit by-products in a food matrix. Further studies to validate the safety and efficacy of such products for human use are also warranted.
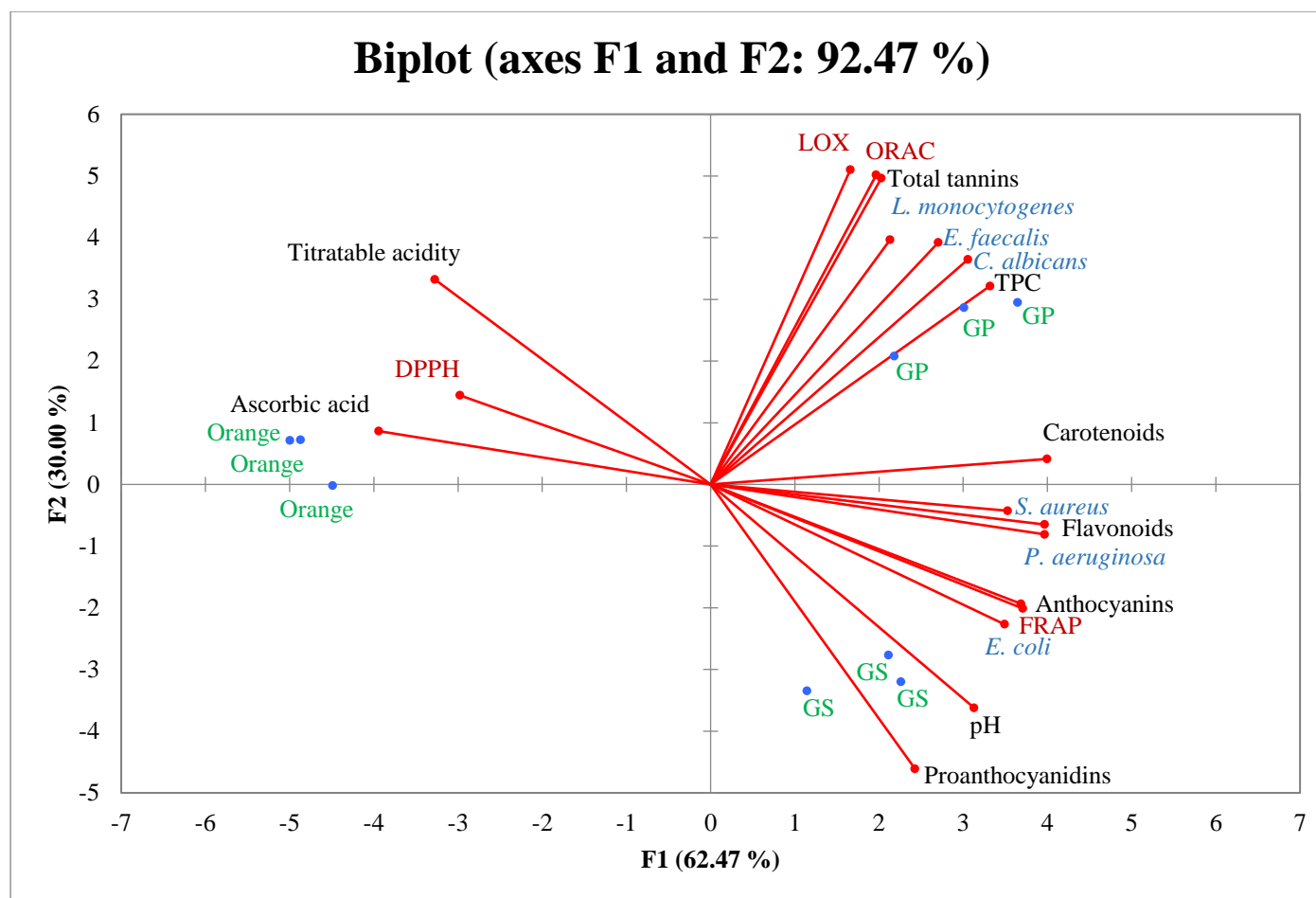
Figure 4.1*:* Biplot obtained from PCA illustrating the relationship between phenolic profile, antimicrobial activity and antioxidant activity of GPE, GSE and OPE

DPPH – 2,2-diphenyl-1-picrylhydrazyl; FRAP – ferric reducing antioxidant power; ORAC – oxygen radical absorbance capacity; LOX – 15-lipoxygenase.

4.3 Principal Component analysis

Principal component analysis was performed as an exploratory data analysis tool to visualize and differentiate the three extracts based on the composition and bioactivity profiles. The score plot of the PCA analysis showed that 92.5 % of the variability was explained by the first two components, which accounted for 62.5 % and 30.0 % of the total variance, respectively. The factor of analysis showed that the variables with higher contribution for the separation of the treatments on PC1 were total tannins, flavonoids, anthocyanins, carotenoids, ascorbic acid, pH, titratable acidity, *S. aureus*, *P. aeruginosa*, *E. coli*, *C. albicans*, DPPH and FRAP. Along PC1, ascorbic acid, titratable acidity and DPPH contributed to negative scores. For PC2, the variables that contributed on the separation were total tannins, proanthocyanidins, ORAC and LOX. Based on the separations obtained in PC1 and PC2, it was observed that the source of extract (type of fruit by-product) was the factor that contributed more to the phytochemical composition and level of bioactivity. The level of ascorbic acid was the main factor that influenced the pH level, titratable acidity and antimicrobial activity. On the other hand, the amount of proanthocyanidins, which makes up a large proportion of total tannins influenced the level of radical quenching (ORAC) and enzyme inhibition (LOX).

Chapter 5

Conclusions and recommendations

The highest contents of total phenols, tannins and carotenoids were found in GPE while GSE contained the highest content of proanthocyanidins and anthocyanins and OPE had the highest content of ascorbic acid. The GSE showed the best antioxidant activity whereas OPE had the strongest antimicrobial potential. While strong antioxidant activity which was greater than that of the commercial product was shown, there is need to improve the quality of extracts to match the performance of synthetic antimicrobials. Methods to improve selectivity of extraction solvents for targeted phytochemicals can help improve extract purity and increase antioxidant and antimicrobial properties. Thus, GSE and OPE extracts can be considered for potential food matrix applications either individually or in combination as potential sources of antioxidant and antimicrobial, respectively. Further research should be conducted to test the toxicity related to standardization of dose, of these fruit by-product extracts, before they can be commercialized in the form of natural food preservatives. Given that meat, during processing and marketing, is exposed to conditions under which chemical, photochemical and enzymatic radical generation processes occur, it could be used as a medium to evaluate the performance of extracts in a food matrix. In highlighting the potential in the fruit by-products, this research also contributes towards steps in value addition to waste and open new value chains for fruit processors.

References

Abad-García, B., Garmón-Lobato, S., Sánchez-Ilárduya, M.B., Berrueta, L.A., Gallo, B., Vicente, F. & Alonso-Salces, R.M. (2014). Polyphenolic contents in Citrus fruit juices: Authenticity assessment. *European Food Research and Technology*, **238**, 803–818.

Agustin-Salazar, S., Medina-Juárez, L.A., Soto-Valdez, H., Manzanares-López, F. & Gámez-Meza, N. (2014). Influence of the solvent system on the composition of phenolic substances and antioxidant capacity of extracts of grape (Vitis vinifera L.) marc. *Australian Journal of Grape and Wine Research*, **20**, 208–213.

Alahakoon, A.U., Jayasena, D.D., Ramachandra, S. & Jo, C. (2015). Alternatives to nitrite in processed meat: Up to date. *Trends in Food Science & Technology*, **45**, 37–49.

Anand, S.P. & Sati, N. (2013). Artificial preservatives and their harmful effects: Looking toward nature for safer alternatives. *International Journal of Pharmaceutical Sciences and Research (IJPSR)*, **4**, 2496–2501.

Andrés, A.I., Petrón, M.J., Adámez, J.D., López, M. & Timón, M.L. (2017). Food by-products as potential antioxidant and antimicrobial additives in chill stored raw lamb patties. *Meat Science*, **129**, 62–70.

Antoniolli, A., Fontana, A., Piccoli, P. & Bottini, R. (2015). Characterization of polyphenols and evaluation of antioxidant capacity in grape pomace of the cv. Malbec. *Food Chemistry*, **178**, 172–178.

Atkinson, N. & Urwin, P. (2012). The interaction of plant biotic and abiotic stresses: from genes to the field. *Journal of Experimental Botany*, **63**, 3523–3544.

Aziz, M. & Karboune, S. (2016). Natural antimicrobial/antioxidant agents in meat and poultry products as well as fruits and vegetables: A review. *Critical Reviews in Food Science and Nutrition*, **8398**, 1–25.

Balasundram, N., Sundram, K. & Samman, S. (2006). Phenolic compounds in plants and agri-industrial by-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, **99**, 191–203.

Bateman, B., Warner, J.O., Hutchinson, E., Dean, T., Rowlandson, P., Gant, C., Grundy, J., Fitzgerald, C., Stevenson, J. & Warner, J. (2004). The effects of a double blind, placebo controlled, artificial food colourings and benzoate preservative challenge on hyperactivity in a general population sample of preschool children. *Archives of Disease in Childhood*, **89**, 506–511.

Baydar, N.G., Özkan, G. & Sağdiç, O. (2004). Total phenolic contents and antibacterial activities of grape (Vitis vinifera L.) extracts. *Food Control*, **15**, 335–339.

Bedale, W., Sindelar, J.J. & Milkowski, A.L. (2016). Dietary nitrate and nitrite: Benefits, risks, and evolving perceptions. *Meat Science*, **120**, 85–92.

Beezhold, B., Johnston, C. & Notcha, K. (2014). Sodium benzoate–rich beverage consumption is associated with increased reporting of ADHD symptoms in college students: A pilot investigation. *Journal of Attention Disorders*, **18**, 236–241.

Belščak-Cvitanović, A., Durgo, K., Hudek, A., Bačun-Družina, V. & Komes, D. (2018). Overview of polyphenols and their properties. In: *Polyphenols: Properties, Recovery, and Applications* (edited by C.M. Galanakis). Pp. 1–429. Cambridge: Woodhead Publishing.

Benzie, I.F.F. & Strain, J.J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "'Antioxidant Power'": The FRAP assay. *Analytical Biochemistry*, **239**, 70–76.

Beres, C., Costa, G.N.S., Cabezudo, I., Silva-James, N.K. da, Teles, A.S.C., Cruz, A.P.G., Mellinger-Silva, C., Tonon, R. V., Cabral, L.M.C. & Freitas, S.P. (2017). Towards integral utilization of grape pomace from winemaking process: A review. *Waste Management*, **68**, 581–594.

Bocco, A., Cuvelier, M.-E., Richard, H. & Berset, C. (1998). Antioxidant activity and phenolic composition of citrus peel and seed extracts. *Journal of Agricultural and Food Chemistry*, **46**, 2123–2129.

Bosso, A., Guaita, M. & Petrozziello, M. (2016). Influence of solvents on the composition of condensed

tannins in grape pomace seed extracts. *Food Chemistry*, **207**, 162–169.

Brewer, M.S. (2011). Natural antioxidants: Sources, compounds, mechanisms of action, and potential applications. *Comprehensive Reviews in Food Science and Food Safety*, **10**, 221–247.

Brossaud, F., Cheynier, V. & Noble, A.C. (2001). Bitterness and astringency of grape and wine polyphenols. *Australian Journal of Grape and Wine Research*, **7**, 33–39.

Butelli, E., Garcia-Lor, A., Licciardello, C., Casas, G. Las, Hill, L., Recupero, G.R., Keremane, M.L., Ramadugu, C., Krueger, R., Xu, Q., Deng, X., Fanciullino, A.-L., Froelicher, Y., Navarro, L. & Martin, C. (2017). Changes in anthocyanin production during domestication of citrus. *Plant Physiology*, **173**, 2225–2242.

Cerveny, J., Meyer, J.D. & Hall, P.A. (2009). Microbiological spoilage of meat and poultry products. In: *Food microbiology and safety: Compendium of the microbiological spoilage of foods and beverages* (edited by W. Sperber & M. Doyle). Pp. 69–86. New York: Springer Science+Business Media.

Chen, M.L., Yang, D.J. & Liu, S.C. (2011). Effects of drying temperature on the flavonoid, phenolic acid and antioxidative capacities of the methanol extract of citrus fruit (Citrus sinensis (L.) Osbeck) peels. *International Journal of Food Science and Technology*, **46**, 1179–1185.

Chen, X.-M., Tait, A.R. & Kitts, D.D. (2017). Flavonoid composition of orange peel and its association with antioxidant and anti-inflammatory activities. *Food Chemistry*, **218**, 15–21.

Chikwanha, O.C., Raffrenato, E., Opara, U.L., Fawole, O.A., Setati, M.E., Muchenje, V. & Mapiye, C. (2018). Impact of dehydration on retention of bioactive profile and biological activities of different grape (Vitis vinifera L.) pomace varieties. *Animal Feed Science and Technology*, **244**, 116–127.

Cowan, M.M. (1999). Plant products as antimicrobial agents plant products as antimicrobial agents. *Clinical Microbiology Reviews*, **12**, 564–582.

Djilas, S., Čanadanović-Brunet, J. & Ćetković, G. (2009). By-products of fruits processing as a source of phytochemicals. *Chemical Industry and Chemical Engineering Quarterly*, **15**, 191–202.

Doshi, P., Adsule, P., Banerjee, K. & Oulkar, D. (2015). Phenolic compounds, antioxidant activity and insulinotropic effect of extracts prepared from grape (Vitis vinifera L) byproducts. *Journal of Food Science and Technology*, **52**, 181–190.

Drosou, C., Kyriakopoulou, K., Bimpilas, A., Tsimogiannis, D. & Krokida, M. (2015). A comparative study on different extraction techniques to recover red grape pomace polyphenols from vinification byproducts. *Industrial Crops and Products*, **75**, 141–149.

Du, Y.U., Guo, H. & Lou, H. (2007). Grape seed polyphenols protect cardiac cells from apoptosis via induction of endogenous antioxidant enzymes. *Journal of Agricultural and Food Chemistry*, **55**, 1695–1701.

Durand-Hulak, M., Dugrand, A., Duval, T., Bidel, L.P.R., Jay-Allemand, C., Froelicher, Y., Bourgaud, F. & Fanciullino, A.L. (2015). Mapping the genetic and tissular diversity of 64 phenolic compounds in Citrus species using a UPLC-MS approach. *Annals of Botany*, **115**, 861–877.

Eloff, J.N. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica*, **64**, 711–713.

Eskin, M. & Ronbinson, D. (2000). *Food Shelf Life Stability: Chemical, Biochemical, and Microbiological Changes*. New York: CRC Press.

FAO. (2013). *Food wastage footprint: Impacts on natural resources - Summary report*.

Farhadi, K., Esmaeilzadeh, F., Hatami, M., Forough, M. & Molaie, R. (2016). Determination of phenolic compounds content and antioxidant activity in skin, pulp, seed, cane and leaf of five native grape cultivars in West Azerbaijan province, Iran. *Food Chemistry*, **199**, 847–855.

García-Lomillo, J. & González-Sanjosé, M.L. (2017). Applications of wine pomace in the food industry: Approaches and functions. *Comprehensive Reviews in Food Science and Food Safety*, **16**, 3–22.

Gattuso, G., Caristi, C., Gargiulli, C., Bellocco, E., Toscano, G. & Leuzzi, U. (2006). Flavonoid glycosides in bergamot juice (Citrus bergamia Risso). *Journal of Agricultural and Food*

Chemistry, **54**, 3929–3935.

Ghasemi, K., Ghasemi, Y. & Ali Ebrahimzadeh, M. (2009). Antioxidant activity, phenol and flavonoid contents of 13 citrus species peels and tissues. *Pakistan Journal of Pharmaceutical Science*, **22**, 277–281.

Gil-chavez, G.J., Villa, A., Ayala-zavala, J.F., Heredia, J.B., Sepulveda, D., Yahia, E.M. & Gonz, G.A. (2013). Technologies for extraction and production of bioactive compounds to be used as nutraceuticals and food ingredients : An overview. *ComprehensiveReviews in Food Science and Food Safety*, **12**, 5–23.

Gillespie, K.M., Chae, J.M. & Ainsworth, E.A. (2007). Rapid measurement of total antioxidant capacity in plants. *Nature Protocols*, **2**, 867–870.

Giusti, M.M. & Wrolstad, R.E. (2001). Characterization and measurement of anthocyanins by UV - visible spectroscopy. *Current Protocols in Food Analytical Chemistry*, **F1.2.1-F1**, 0–13.

Gođevac, D., Tešević, V., Veličković, M., Vujisić, L., Vajs, V. & Milosavljević, S. (2010). Polyphenolic compounds in seeds from some grape cultivars grown in Serbia. *Journal of the Serbian Chemical Society*, **75**, 1641–1652.

Goulas, V. & Manganaris, G.A. (2012). Exploring the phytochemical content and the antioxidant potential of Citrus fruits grown in Cyprus. *Food Chemistry*, **131**, 39–47.

Guitard, R., Nardello-Rataj, V. & Aubry, J.-M. (2016). Theoretical and kinetic tools for selecting effective antioxidants: Application to the protection of omega-3 oils with natural and synthetic phenols. *International Journal of Molecular Sciences*, **17**, 1220.

Gyawali, R. & Ibrahim, S.A. (2014). Natural products as antimicrobial agents. *Food Control*, **46**, 412–429.

Haminiuk, C.W.I., Maciel, G.M., Plata-Oviedo, M.S.V. & Peralta, R.M. (2012). Phenolic compounds in fruits - an overview. *International Journal of Food Science and Technology*, **47**, 2023–2044.

Hamza, A.A., Heeba, G.H., Elwy, H.M., Murali, C., El-Awady, R. & Amin, A. (2018). Molecular characterization of the grape seeds extract's effect against chemically induced liver cancer: In vivo and in vitro analyses. *Springer Nature: Scientific Reports*, **8**, 1–16.

Hardersen, N. & Ziolkowska, J.R. (2018). Economic and environmental costs of meat waste in the US. In: *Handbook of Research on Social Marketing and Its Influence on Animal Origin Food Product Consumption* (edited by D. Bogueva, D. Marinova & T. Raphaely). Pp. 58–76. Oklahoma: IGI Global.

Haslam, E. (1996). Natural polyphenols (vegetable tannins) as drugs: possible modes of action. *Journal of Natural Products*, **59**, 205–215.

Hasselmann, C. & Diseases, I. (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clinical Microbiology and Infection*, **9**, ix–xv.

Hellstro, J.K.M., Riitta, A., Nen, O. & Mattila, P.H. (2009). Proanthocyanidins in common food products of plant origin. *Journal of Agricultural and Food Chemistry*, **57**, 7899–7906.

Hess, D., Keller, H.E., Oberlin, B., Bonfanti, R. & Schüep, W. (1991). Simultaneous determination of retinol, tocopherols, carotenes and lycopene in plasma by means of high-performance liquid chromatography on reversed phase. *International Journal for Vitamin and Nutrition Research*, **61**, 232–238.

Huang, D., Boxin, O.U. & Prior, R.L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, **53**, 1841–1856.

Hugo, C.J. & Hugo, A. (2015). Current trends in natural preservatives for fresh sausage products. *Trends in Food Science & Technology*, **45**, 12–23.

Hung, Y., Kok, T.M. de & Verbeke, W. (2016). Consumer attitude and purchase intention towards processed meat products with natural compounds and a reduced level of nitrite. *Meat Science*, **121**, 119–126.

Ibrahim, M.H., Jaafar, H.Z.E., Karimi, E. & Ghasemzadeh, A. (2013). Impact of organic and inorganic

fertilizers application on the phytochemical and antioxidant activity of Kacip Fatimah (Labisia pumila Benth). *Molecules*, **18**, 10973–10988.

Ignat, I., Volf, I. & Popa, V.I. (2011). A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. *Food Chemistry*, **126**, 1821–1835.

Indresh, H. (2008). Organic acids, plant extracts can be effective antibiotic alternatives [Internet document] . *WATTAgNet.com*URL https://www.wattagnet.com/articles/236-organic-acids-plant-extracts-can-be-effective-antibiotic-alternatives. Accessed 11/06/2018.

Jara-Palacios, M.J., Rodríguez-Pulido, F.J., Hernanz, D., Escudero-Gilete, M.L. & Heredia, F.J. (2016). Determination of phenolic substances of seeds, skins and stems from white grape marc by near-infrared hyperspectral imaging. *Australian Journal of Grape and Wine Research*, **22**, 11–15.

Jayaprakasha, G.K., Selvi, T. & Sakariah, K.K. (2003). Antibacterial and antioxidant activities of grape (Vitis vinifera) seed extracts. *Food Research International*, **36**, 117–122.

Kalt, W. (2005). Effects of production and processing factors on major fruit and vegetable antioxidants. *Journal of Food Science*, **70**, 11–19.

Kanner, J. & Rosenthal, I. (1992). An assessment of lipid oxidation in foods (Technical Report). *Pure and Applied Chemistry*, **64**, 1959–1964.

Katalinić, V., Možina, S.S., Skroza, D., Generalić, I., Abramovič, H., Miloš, M., Ljubenkov, I., Piskernik, S., Pezo, I., Terpinc, P. & Boban, M. (2010). Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 Vitis vinifera varieties grown in Dalmatia (Croatia). *Food Chemistry*, **119**, 715–723.

Khan, N., Roes-Hill, M. Le, Welz, P.J., Grandin, K.A., Kudanga, T., Dyk, J.S. Van, Ohlhoff, C., Zyl, W.H. Van & Pletschke, B.I. (2015). Fruit waste streams in South Africa and their potential role in developing a bio-economy. *South African Journal of Science*, **111**, 1–11.

Khan, T., Sankhe, K., Suvarna, V., Sherje, A., Patel, K. & Dravyakar, B. (2018). DNA gyrase inhibitors: Progress and synthesis of potent compounds as antibacterial agents. *Biomedicine &*

Pharmacotherapy, **103**, 923–938.

Klangpetch, W., Phromsurin, K., Hannarong, K., Wichaphon, J. & Rungchang, S. (2016). Antibacterial and antioxidant effects of tropical citrus peel extracts to improve the shelf life of raw chicken drumettes. *International Food Research Journal*, **23**, 700–707.

Kumar, S. & Pandey, A.. (2013). Chemistry and biological activities of flavonoids: An overview. *The Scientific World Journal*, **4**, 32–48.

Kumar, Y., Yadav, D.N., Ahmad, T. & Narsaiah, K. (2015). Recent trends in the use of natural antioxidants for meat and meat products. *Comprehensive Reviews in Food Science and Food Safety*, **14**, 796–812.

Ky, I., Lorrain, B., Kolbas, N., Crozier, A. & Teissedre, P.L. (2014). Wine by-products: Phenolic characterization and antioxidant activity evaluation of grapes and grape pomaces from six different French grape varieties. *Molecules*, **19**, 482–506.

Lee, N. & Paik, H. (2016). Status ,antimicrobial mechanism, and regulation of natural preservatives in livestock food systems. *Korean Journal Food Science of Animal Resources*, **36**, 547–557.

Lin, Y.T., Labbe, R.G. & Shetty, K. (2004). Inhibition of Listeria monocytogenes in fish and meat systems by use of oregano and cranberry phytochemical synergies. *Applied and Environmental Microbiology*, **70**, 5672–5678.

Lingua, M.S., Fabani, M.P., Wunderlin, D.A. & Baroni, M. V. (2016). In vivo antioxidant activity of grape, pomace and wine from three red varieties grown in Argentina: Its relationship to phenolic profile. *Journal of Functional Foods*, **20**, 332–345.

Lorrain, B., Ky, I., Pechamat, L. & Teissedre, P.L. (2013). Evolution of analysis of polyhenols from grapes, wines, and extracts. *Molecules*, **18**, 1076–1100.

M'hiri, N., Ioannou, I., Ghoul, M. & Mihoubi Boudhrioua, N. (2017). Phytochemical characteristics of Citrus peel and effect of conventional and nonconventional processing on phenolic compounds: a review. *Food Reviews International*, **33**, 587–619.

Makkar, H.P.S. (2000). *Quantification of tannins in tree foliage: A laboratory manual*. Vienna.

Malterud, K.E. & Rydland, K.M. (2000). Inhibitors of 15-lipoxygenase from orange peel. *Journal of Agricultural and Food Chemistry*, **48**, 5576–5580.

Manach, C., Scalbert, A., Morand, C., Rémésy, C. & Jiménez, L. (2004). Polyphenols: food sources and bioavailability. *American Journal of Clinical Nutrition*, **79**, 727–747.

Maqsood, S., Benjakul, S., Abushelaibi, A. & Alam, A. (2014). Phenolic compounds and plant phenolic extracts as natural antioxidants in prevention of lipid oxidation in seafood: A detailed review. *Comprehensive Reviews in Food Science and Food Safety*, **13**, 1125–1140.

Mattos, G.N., Tonon, R. V, Furtado, A. Al & Cabral, L.M. (2016). Grape by-products extracts against microbial proliferation and lipid oxidation: a review. *Journal of the Science of Food and Agriculture*, **97**, 1055–1064.

Melo, S.P., Massarioli, P.A., Denny, C., Santos, F.L. Dos, Franchin, M., Perreira, E.G., Souza Vieira, M.T.F. De, Rosalen, L.P. & Alencar, M.S. De. (2015). Winery by-products : Extraction optimization , phenolic composition and cytotoxic evaluation to act as a new source of scavenging of reactive oxygen species. *Food Chemistry*, **181**, 160–169.

Min, D.B. & Boff, J.M. (2002). Chemistry and reaction of singlet oxygen in foods. *Comprehensive Reviews in Food Science and Food Safety*, **1**, 58–72.

Mohanka, R. & Priyanka. (2014). Plant extract as natural food preservative against spoilage fungi from processed food. *International Journal of Current Microbiology and Applied Sciences* , **3**, 91–98.

Moraes Barros, H.R. De, Castro Ferreira, T.A.P. De & Genovese, M.I. (2012). Antioxidant capacity and mineral content of pulp and peel from commercial cultivars of citrus from Brazil. *Food Chemistry*, **134**, 1892–1898.

Mphahlele, R.R., Stander, M.A., Fawole, O.A. & Opara, U.L. (2014). Effect of fruit maturity and growing location on the postharvest contents of flavonoids, phenolic acids, vitamin C and antioxidant activity of pomegranate juice (cv. Wonderful). *Scientia Horticulturae*, **179**, 36–45.

Nardoia, M. (2016). *Effect of dietary polyphenol-rich grape by-products on growth performance, some physiological parameters, meat and meat products quality in chickens*.

Ncube, N.S., Afolayan, A.J. & Okoh, A.I. (2008). Assessment techniques of antimicrobial properties of natural compounds of plant origin : current methods and future trends. *African Journal of Biotechnology*, **7**, 1797–1806.

Negro, C., Tommasi, L. & Miceli, A. (2003). Phenolic compounds and antioxidant activity from red grape marc extracts. *Bioresource Technology*, **87**, 41–44.

Nunes, M.A., Rodrigues, F. & Oliveira, M.B.P.P. (2017). Grape processing by-products as active ingredients for cosmetic proposes. In: *Handbook of Grape Processing By-Products*. Pp. 267–292. Elsevier.

Nur Alam, M., Jahan Bristi, N. & Rafiquzzaman, M. (2013). Review on in vivo and in vitro methods evaluation of antioxidant activity. *Saudi Pharmaceutical Journal*, **21**, 143–152.

Oliveira, D.A., Salvador, A.A., Smânia, A., Smânia, E.F.A., Maraschin, M. & Ferreira, S.R.S. (2013). Antimicrobial activity and composition profile of grape (Vitis vinifera) pomace extracts obtained by supercritical fluids. *Journal of Biotechnology*, **164**, 423–432.

Özkan, G., Sagdiç, O., Baydar, N.G. & Kurumahmutoglu, Z. (2004). Antibacterial activities and total phenolic contents of grape pomace extracts. *Journal of the Science of Food and Agriculture*, **84**, 1807–1811.

Papuc, C., Goran, G. V., Predescu, C.N. & Nicorescu, V. (2017a). Mechanisms of oxidative processes in meat and toxicity induced by postprandial degradation products: A review. *Comprehensive Reviews in Food Science and Food Safety*, **16**, 96–123.

Papuc, C., Goran, G. V., Predescu, C.N., Nicorescu, V. & Stefan, G. (2017b). Plant polyphenols as antioxidant and antibacterial agents for shelf-life extension of meat and meat products: Classification, structures, sources, and action mechanisms. *Comprehensive Reviews in Food Science and Food Safety*, **16**, 1243–1268.

Passos, C.P., Cardoso, S.M., Barros, A.S., Silva, C.M. & Coimbra, M.A. (2010). Application of Fourier transform infrared spectroscopy and orthogonal projections to latent structures/partial least squares regression for estimation of procyanidins average degree of polymerisation. *Analytica Chimica Acta*, **661**, 143–149.

Pavarini, D.P., Pavarini, S.P., Niehues, M. & Lopes, N.P. (2012). Exogenous influences on plant secondary metabolite levels. *Animal Feed Science and Technology*, **176**, 5–16.

Peixoto, C.M., Dias, M.I., Alves, M.J., Calhelha, R.C., Barros, L., Pinho, S.P. & Ferreira, I.C.F.R. (2018). Grape pomace as a source of phenolic compounds and diverse bioactive properties. *Food Chemistry*, **253**, 132–138.

Pinchuk, I., Shoval, H., Dotan, Y. & Lichtenberg, D. (2012). Evaluation of antioxidants: Scope, limitations and relevance of assays. *Chemistry and Physics of Lipids*, **165**, 638–647.

Porter, L.J., Hrstich, L.N. & Chan, B.G. (1986). The Conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry*, **25**, 223–230.

Pourcel, L., Routaboul, J.-M., Cheynier, V., Lepiniec, L. & Debeaujon, I. (2007). Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends in Plant Science*, **12**, 29–36.

Prabhakar, K. (2014). Permitted Preservatives – Sulfur Dioxide. *Encyclopedia of Food Microbiology*, **3**, 108–112.

Rababah, T.M., Ereifej, K.I., Al-Mahasneh, M.A., Ismaeal, K., Hidar, A.-G. & Yang, W. (2008). Total phenolics, antioxidant activities, and anthocyanins of different grape seed cultivars grown in Jordan. *International Journal of Food Properties*, **11**, 472–479.

Ramakrishna, A. & Ravishankar, G.A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling and Behavior*, **6**, 1720–1731.

Ramirez-Lopez, L.M. & DeWitt, C.A.M. (2014). Analysis of phenolic compounds in commercial dried grape pomace by high-performance liquid chromatography electrospray ionization mass

spectrometry. *Food Science & Nutrition*, **2**, 470–477.

Ribeiro, D., Freitas, M., Tomé, S.M., Silva, A.M.S., Porto, G., Cabrita, E.J., Marques, M.M.B. & Fernandes, E. (2014). Inhibition of LOX by flavonoids: A structure-activity relationship study. *European Journal of Medicinal Chemistry*, **72**, 137–145.

Rice-Evans, C.A., Miller, N.J. & Paganga, G. (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biology and Medicine*, **20**, 933–956.

Rockenbach, I.I., Gonzaga, L.V., Rizelio, V.M., Souza, E. De, Gonçalves, S., Genovese, M.I. & Fett, R. (2011). Phenolic compounds and antioxidant activity of seed and skin extracts of red grape (Vitis vinifera and Vitis labrusca) pomace from Brazilian winemaking. *Food Research International*, **44**, 897–901.

Sampaio, B.L., Bara, M.T.F., Ferri, P.H., Da, S., Santos, C. & Realino De Paula, J. (2011). Influence of environmental factors on the concentration of phenolic compounds in leaves of Lafoensia pacari. *Revista Brasileira de Farmacognosia Brazilian Journal of Pharmacognosy*, **21**, 1127–1137.

SAWIS. (2016). *SA Wine Industry Statistics NR 40- 2016*. Paarl.

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry*, **30**, 3875–3883.

Shahidi, F. & Zhong, Y. (2015). Measurement of antioxidant activity. *Journal of Functional Foods*, **18**, 757–781.

Sharma, K., Mahato, N., Cho, M.H. & Lee, Y.R. (2017). Converting citrus wastes into value-added products: Economic and environmently friendly approaches. *Nutrition*, **34**, 29–46.

Shi, J., Yu, J., Pohorly, J.E. & Kakuda, Y. (2003). Polyphenolics in grape seeds-biochemistry and functionality. *Biochemistry and Functionality Article in Journal of Medicinal Food*, **6**, 291–299.

Siles, J.A., Vargas, F., Gutiérrez, M.C., Chica, A.F. & Martín, M.A. (2016). Bioresource technology integral valorisation of waste orange peel using combustion , biomethanisation and co-composting

technologies. *Bioresource Technology*, **211**, 173–182.

Slobodníková, L., Fialová, S., Rendeková, K., Kováč, J. & Mučaji, P. (2016). Antibiofilm activity of plant polyphenols. *Molecules*, **21**, 1–15.

Sousa, E.C., Athayde Uchôa-Thomaz, A.M., Osvaldo, J., Carioca, B., Maia De Morais, S., Lima, A. De, Martins, C.G., Alexandrino, C.D., Augusto, P., Ferreira, T., Livya, A., Rodrigues, M., Rodrigues, S.P., Do, J., Silva, N. & Rodrigues, L.L. (2014). Chemical composition and bioactive compounds of grape pomace (Vitis vinifera L.), Benitaka variety, grown in the semiarid region of Northeast Brazil. *Food Science and Technology*, **34**, 135–142.

Spigno, G. & Faveri, D.M. De. (2007). Antioxidants from grape stalks and marc: Influence of extraction procedure on yield, purity and antioxidant power of the extracts. *Journal of Food Engineering*, **78**, 793–801.

Stavri, M., Piddock, L.J. V & Gibbons, S. (2007). Bacterial efflux pump inhibitors from natural sources. *Journal of Antimicrobial Chemotherapy*, **59**, 1247–1260.

Stintzing, F.C. & Carle, R. (2004). Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. *Trends in Food Science & Technology*, **15**, 19–38.

Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D.A. & Garcia-Viguera, C. (2014). Natural bioactive compounds from winery by-products as health promoters: A review. *International Journal of Molecular Sciences*, **15**, 15638–15678.

The Ministry of Health, S.A. (1977). *REGULATIONS – PRESERVATIVES AND ANTIOXIDANTS*. *Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act 54 of 1972)*. South Africa: The Ministry of Health.

Tolic, M.T., Jurcevic, I.L., Krbavcic, I.P., Markovic, K. & Vahcic, N. (2015). Phenolic content, antioxidant capacity and quality of chokeberry (Aronia melanocarpa) products. *Food Technology and Biotechnology*, **53**, 171–179.

Tournour, H., Segundo, M., Magalhaes, L., Barreiros, L., Quieroz, J. & Cunha, L. (2015). Valorization

of grape pomace: Extraction of bioactive phenolics with antioxidant properties. *Industrial Crops and Products*, **74**, 397–406.

Tripoli, E., Guardia, M. La, Giammanco, S., Majo, D. Di & Giammanco, M. (2007). Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. *Food Chemistry*, **104**, 466–479.

Troy, D.J., Ojha, K.S., Kerry, J.P. & Tiwari, B.K. (2016). Sustainable and consumer-friendly emerging technologies for application within the meat industry: An overview. *Meat Science*, **120**, 2–9.

Tseng, A. & Zhao, Y. (2012). Effect of different drying methods and storage time on the retention of bioactive compounds and antibacterial activity of wine grape pomace (Pinot Noir and Merlot). *Journal of Food Science*, **77**, 192–201.

Tumbas, V.T., Ćetković, G.S., Djilas, S.M., Čanadanović-Brunet, J.M., Vulić, J.J., Knéz, Ž. & Škerget, M. (2010). Antioxidant activity of mandarin (citrus reticulata) peel. *Acta Periodica Technologica*, **41**, 195–203.

Valgas, C., Machado de Souza, S., A Smânia, E.F. & Smânia Jr, A. (2007). Screening methods to determine antibacterial activity of natural products. *Brazilian Journal of Microbiology*, **38**, 369–380.

Vally, H. & Misso, N. La. (2012). Adverse reactions to the sulphite additives. *Gastroenterol Hepatol Bed Bench*, **5**, 16–23.

Veberic, R. (2016). The impact of production technology on plant phenolics. *Horticulturae*, **2**, 1–8.

Velasco, V. & Williams, P. (2011). Improving meat quality through natural antioxidants. *Chilean Journal of Agricultural Research*, **71**, 313–322.

Wang, Y., Chuang, Y. & Hsu, H. (2008). The flavonoid, carotenoid and pectin content in peels of citrus cultivated in Taiwan. *Food Chemistry*, **106**, 277–284.

Wang, Y., Qian, J., Cao, J., Wang, D., Liu, C., Yang, R., Li, X. & Sun, C. (2017). Antioxidant capacity,

anticancer ability and flavonoids composition of 35 citrus (Citrus reticulata Blanco) varieties. *Molecules*, **22**, 1–20.

Waslidge, N.B. & Hayes, D.J. (1995). A colorimetric method for the determination of lipoxygenase activity suitable for use in a high throughput assay format. *Analytical Biochemistry*, **231**, 354–358.

Wu, T., Zang, X., He, M., Pan, S. & Xu, X. (2013). Structure−activity relationship of flavonoids on their anti- Escherichia coli activity and inhibition of DNA gyrase. *Journal of Agricultural and Food Chemistry*, **61**, 8185–8190.

WWF-South Africa. (2017). *Food Loss and Waste: Facts and Futures Taking steps towards a more sustainable food future (FOOD 2017 Report)*. Cape Town .

Xu, G., Ye, X., Chen, J. & Liu, D. (2007). Effect of heat treatment on the phenolic compounds and antioxidant capacity of citrus peel extract. *Journal of Agricultural and Food Chemistry*, **55**, 330–335.

Xu, Y., Burton, S., Kim, C. & Sismour, E. (2016). Phenolic compounds, antioxidant, and antibacterial properties of pomace extracts from four Virginia-grown grape varieties. *Food science & nutrition*, **4**, 125–33.

Yang, J., Martinson, T.E. & Liu, R.H. (2009). Phytochemical profiles and antioxidant activities of wine grapes. *Food Chemistry*, **116**, 332–339.

Yilmaz, Y., Göksel, Z., Erdoğan, S.S., Öztürk, A., Atak, A. & Özer, C. (2015). Antioxidant activity and phenolic content of eed, skin and pulp parts of 22 grape (Vitis vinifera L.) cultivars (4 common and 18 registered or candidate for registration). *Journal of Food Processing and Preservation*, **39**, 1682–1691.

Yilmaz, Y. & Toledo, R.T. (2004). Major flavonoids in grape seeds and skins: Antioxidant capacity of catechin, epicatechin, and gallic acid. *Journal of Agricultural and Food Chemistry*, **52**, 255–260.

Yu, J. & Ahmedna, M. (2013). Functional components of grape pomace: Their composition, biological

properties and potential applications. *International Journal of Food Science and Technology*, **48**, 221–237.

Zhang, H.-Y., Sun, Y.-M. & Wang, X.-L. (2003). Substituent effects on O-H bond dissociation enthalpies and ionization potentials of catechols: A DFT study and its implications in the rational design of phenolic antioxidants and elucidation of structure-activity relationships for flavonoid antioxidants. *Chemistry:European Journal*, **9**, 502–508.

Zhang, H., Yang, Y. & Zhou, Z. (2018). Phenolic and flavonoid contents of mandarin (Citrus reticulata Blanco) fruit tissues and their antioxidant capacity as evaluated by DPPH and ABTS methods. *Journal of Integrative Agriculture*, **17**, 256–263.

Zhang, N., Hoadley, A., Patel, J., Lim, S. & Li, C. (2017). Sustainable options for the utilization of solid residues from wine production. *Waste Management*, **60**, 173–183.

Zou, Z., Xi, W., Hu, Y., Nie, C. & Zhou, Z. (2016). Antioxidant activity of Citrus fruits. *Food Chemistry*, **196**, 885–896.