
Impacts of Preharvest and Postharvest Handling and Processing on Bioactive Compounds and Functional Properties of Pomegranate Fruit Fractions and By-products

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DECLARATION

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SUMMARY

Pomegranate fruit (*Punica granatum* L. Punicaceae) is highly valued owing to its high concentration of bioactive compounds found in the arils and peel. In fact, evidence from literature indicates that pomegranate fruit consumption has been associated with reduced risk of life threatening non-communicable diseases such as cancers and cardiovascular disorders. Although substantial amount of research has been reported on the effects of preharvest factors on phytochemical and functional properties of pomegranate, including cultivar and micro-climatic differences, little is known about the effects of postharvest and processing techniques on individual phenolic concentrations of fruit fractions such as arils and peel. The aim of this study was therefore to examine the impacts of preharvest and postharvest handling factors and processing methods on bioactive components and functional properties of pomegranate fruit and by-products. Drying characteristics and a thin-layer drying model for pomegranate peel over a wide temperature range were included in this study given the importance of drying as a commonly applied processing method in the processing of high-moisture products such as fruit.

The results showed that concentrations of total phenolic and total tannin as well as radical scavenging activity (RSA) by DPPH assay declined as fruit maturity advanced, while ferric reducing antioxidant power (FRAP), total anthocyanin, total flavonoid and vitamin C concentration increased significantly ($P < 0.01$). Principal component analysis (PCA) demonstrated that fruit grown in areas with lower altitude were associated with higher bioactive compounds at the full ripe stage. The study also showed significant ($P < 0.05$) interaction effect between fruit maturity and altitude of the growing location on the phenolic compounds concentration.

Studies on the effect of different extraction methods on phenolic compounds and antioxidant properties of pomegranate juice did not show significant influence ($P > 0.05$) on fructose and total soluble solid concentration of pomegranate juice. Juice obtained from arils plus seed had the lowest citric acid concentration (18.96 g/L) and high juice colour saturation (2.69). Juice obtained by pressing fruit cut in half along the longitudinal axis (halved fruit) had significantly higher total phenolics, total tannins, radical scavenging activity and ferric reducing antioxidant power, which highlights the impact of extraction method on the quality of pomegranate juice. The influence of packaging and long term cold storage of whole pomegranates on phenolic compounds and antioxidant properties of fruit fractions and by-

products thereof was also investigated. The result showed that total phenolics in pomegranate juice and peel decreased significantly ($P < 0.05$) with prolonged storage duration regardless of package type. Catechin increased by 65.43% under modified atmosphere package (MAP) while rutin increased by 139.39% in individual shrink wrap package after 4 months of cold storage. Rutin was the predominant flavonoid in peel (3446.24 mg/kg dry matter), and its concentration decreased by 65% in fruit peel stored in MAP at the end of the storage (4 months). The study showed that punicic acid constituted 68.09% of total fatty acids in the seed oil and the concentration did not change significantly after 4 months under MAP and individual shrink wrap packaging, respectively. Fruit peel of whole pomegranates stored in individual shrink wrap package showed poor inhibitory activity against Gram negative bacteria (*Klebsiella pneumonia*), with minimum inhibitory concentration (MIC) of 1.56 mg/mL while seed oil showed better activity against diphenolase with inhibitory concentration (IC_{50}) of 0.49 $\mu\text{g/mL}$ after 4 months of storage. The effects of drying on the phenolic concentration, antioxidant, antibacterial and anti-tyrosinase properties were also studied. Freeze dried peel extracts had the highest total phenolic, tannin and flavonoid concentration compared to oven dried peel at the temperatures studied (40°C, 50°C and 60°C). Pomegranate peel extracts dried at 50°C showed the highest inhibitory activity with MIC value of 0.10 mg/mL against Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) and monophenolase (22.95 mg/mL).

Drying behaviour of pomegranate peels showed that drying time decreased as the oven drying temperature increased. The effective moisture diffusivity of pomegranate peel ranged from 4.05×10^{-10} to $8.10 \times 10^{-10} \text{ m}^2/\text{s}$ over the temperature range investigated, with mean activation energy (E_a) of 22.25 kJ/mol. Empirical models were successfully applied to describe drying kinetics of pomegranate peel and these models could be used as analytical tools for future drying performance assessment.

OPSOMMING

Granate (*Punica granatum* L. Punicaceae) word hoog aangeskryf weens die hoë konsentrasie van bioaktiewe verbindings wat in die saadomhulsel en skille voorkom. Volgens literatuur is daar bewyse gevind dat granate kan bydrae tot verminderde risiko van lewensgevaarlike kwale soos kanker asook kardiovaskulêre siektes. Alhoewel 'n aansienlike hoeveelheid navorsing gerapporteer het oor die effek van voor-oes faktore op fitochemiese en funksionele eienskappe van granate, insluitend kultivar en mikroklimaat verskille, is daar nog min bekend oor die uitwerking van na-oes en verwerkings tegnieke op afsonderlike fenoliese konsentrasies op beide die saadomhulsels en skille. Die doelwitte van hierdie studie was dus om te toets wat die impak van voor-oes en na-oes hantering en verwerking op bioaktiewe verbindings en funksionele eienskappe van granate en neweprodukte is. Uitdroog eienskappe en 'n dunlaag drogings model vir granaat skil oor 'n wye temperatuur reeks was ook ingesluit in hierdie studie gegewe die belangrikheid van die droog as 'n algemeen toegepas verwerking metode in die verwerking van 'n hoë-vog bioprodukte.

Resultate het gewys dat konsentrasies van totale fenole en tanniene asook die "radical scavenging activity" (RSA) in die DPPH toets afneem tydens rypwording, terwyl "ferric reducing antioxidant power" (FRAP), totale antosianien, totale flavonoïede en vitamien C beduidend toeneem ($P < 0.01$). "Principal component analysis" (PCA) het getoon dat vrugte geproduseer word in areas op laer hoogtes bo seevlak areas geassosieer word met verhoogde bioaktiewe verbindings tydens die voryp stadium. The studie het 'n beduidende interaksie tussen vrug rypwording en verskille in hoogte bo seevlak op fenoliese verbindings getoon.

Studies oor die uitwerking van verskillende ekstraksie metodes op fenoliese verbindings en antioksidant eienskappe van granaatsap het nie 'n beduidende invloed ($P > 0.05$) op fruktose en totale oplosbare soliede inhoud van granaatsap getoon nie. Die laagste sitroensuur inhoud was waargeneem in saadomhulsels plus saad (18.96 g/L) en hoë sap kleur versadiging (2.69). Sap wat van gehalveerde vrugte verky is, het beduidende hoë totale fenole, totale tanniene, RSA en FRAP getoon wat die belangrikheid van ekstraksie metode op granaatsap kwaliteit uitwys. Invloed van verpakking en langtermyn koelstoring op fenoliese verbindings en antioksidant eienskappe van granate en neweprodukte was getoets. Die resultaat het gewys dat totale fenole in granaatsap en skil beduidend afneem ($P < 0.05$) met langdurige stoor, ongeag die tipe verpakking. Catechin het toegeneem met 65.43% onder veranderde atmosfeer verpakking terwyl rutin toegeneem het met 139.39% in afsonderlike

kleefplastiek verpakking na 4 maande van koelstoring. Rutin was die oorheersende flavonoïed (3446.24 mg/kg droëmateriaal) in skil, en die konsentrasie het afgeneem met 65% in vrug skil gestor in modified atmosphere packaging (MAP) aan die einde van stoor periode (4 maande). Die studie het gewys dat “puniceic” suur 68.09% van die totale vetsure in saadolie uitmaak en dat die inhoud nie beduidend verander het na 4 maande onder MAP en afsonderlike kleefplastiek verpakking nie. Granaatskil wat in afsonderlike kleefplastiek verpakking gestoor is, het swak inhiberende aktiwiteit teen Gram negatiewe bakterieë (*Klebsiella pneumonia*) getoon (met minimum inhiberende konsentrasie van 1.56 mg/mL) terwyl saadolie beter aktiwiteit teen difenolase met inhiberende konsentrasie (IC₅₀) getoon het met die konsentrasie van 0.49 µg/mL na 4 maande opberging. Uitwerking van uitdroging op die fenoliese konsentrasie, antioksidant, antibakteriële en anti-tyrosinase eienskappe was ook bestudeer (40°C, 50°C and 60°C). Granaat skil ekstrakte wat by 50°C gedroog is, het die hoogste inhiberende aktiwiteit getoon, met die minimum inhiberende konsentrasie waarde van 0.10 mg/mL teen Gram positiewe (*Staphylococcus aureus* en *Bacillus subtilis*) en monofenolase (22.95 mg/mL).

Uitdrogingsgedrag van granaat skille het getoon dat droogtyd afneem soos die onddroog temperatuur toeneem. Die effektiewe vog deurlaatbaarheid van die granaat skil het gewissel van 4.05×10^{-10} to 8.10×10^{-10} m²/s oor die temperatuur reeks wat ondersoek was; met gemiddelde aktiverings energie (E_a) van 22.25 kJ/mol. Empiriese modelle was suksesvol toegepas om die drogingskinetika van granaat skil te beskryf, en dit kan as 'n hulpmiddel vir toekomstige uitdroging werkverrigting gebruik word.

LIST OF PUBLICATIONS

List of papers published on international peer-reviewed journals

1. **Mphahlele R.R.**, Fawole O.A., Stander M.A., Opara U.L., 2014. Preharvest and postharvest factors influencing bioactive compounds in pomegranate (*Punica granatum* L.)- A review. *Sci Hortic.* 178 (1), 114–123.
2. **Mphahlele R.R.**, Fawole O.A., Stander M.A., Opara U.L., 2014. Effect of fruit maturity and growing location on the postharvest concentrations of flavonoids, phenolic acids, vitamin C and antioxidant activity of pomegranate juice (cv. Wonderful). *Sci. Hortic.* 179, 36–45.
3. Oluwafemi J.C., Fawole O.A., **Mphahlele R.R.**, Opara U.L., 2015. Impact of preharvest and postharvest factors on changes in volatile compounds of pomegranate fruit and minimally processed arils – Review. *Scientia Horticulture.* 188, 106–114.
4. **Mphahlele R.R.**, Caleb O.J., Fawole O.A., Opara U.L., 2015. Effects of different maturity stages and growing locations on changes in chemical, biochemical and aroma volatile composition of ‘Wonderful’ pomegranate juice. *J. Sci. Food and Agr.* 96, 1002–1009.
5. **Mphahlele R.R.**, Fawole O.A., Opara U.L., 2016. Effect of extraction method on chemical, volatile composition and antioxidant properties of pomegranate juice. *S. Afri. J. Bot.* 103, 135–144.

LIST OF CONFERENCE PRESENTATIONS

1. **Mphahlele R.R.**, Opara, U.L., 2012. Pre-and postharvest factors affecting functional properties of pomegranate- a review. CIGR Technical Symposium: 7th International CIGR Technical Symposium: Innovating the food value chain. Stellenbosch, South Africa, 25-29 November 2012, Stellenbosch University.
2. **Mphahlele R.R.**, Stander M.A., Fawole O.A., Opara U.L. 2014. Flavonoids and phenolic acids concentration of 'Wonderful' pomegranate from different growing locations in South Africa. 1st Annual Symposium in Analytical Sciences, 27 March 2014, Stellenbosch University.
3. **Mphahlele R.R.**, Mokwena, L., Tredoux, A.G.J., Caleb, O.J., Stander, M.A., Fawole, O.A., Opara U.L., 2014. Changes in chemical, biochemical and aroma volatile composition of pomegranate juice (cv. Wonderful) at different maturity stages and agro-climatic locations. 18th International Commission of Agricultural and Biosystems Engineering, 16-19 September 2014, Beijing.

NOTE

This dissertation presents a compilation of manuscripts where every chapter is an individual entity and some duplication between chapters, therefore, has been unavoidable.

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1. Introduction

Pomegranate (*Punica Granatum* L., Punicaceae) is one of the oldest edible fruit and is widely considered a 'superfruit' due to its high concentration of health-promoting compounds and functional properties such as antioxidant, antiinflammatory and antimicrobial activities (Jurenka, 2008; Stover and Mercure, 2007). Juice extraction from arils is the most common method of processing, but this generates huge waste (peel, pulp and seeds) which are rich in bioactive compounds and lipids. Although considerable amount of research has been reported on the effects of preharvest factors on antioxidant and functional properties of pomegranates, including cultivar and micro-climatic differences (Mditshwa et al., 2013), little is known about the effects of postharvest handling and processing techniques.

Pomegranate is widely grown in areas such as Iran, India, Egypt, Lebanon, China, Spain, France, USA, Oman, Syria, Tunisia, Italy, Greece, Cyprus, Israel, Turkey, Chile, Portugal and most recently South Africa (Al-Said et al., 2009; Holland et al., 2009; Fawole and Opara, 2013 a,b). During the past three years, the area under commercial production of pomegranates in South Africa has increased by nearly 6-folds to 4500 ha (Pomegranate Association of South Africa, 2015). Globally, pomegranate fruit consumption has gained popularity in recent times due to its valuable source of polyphenols which is often comparable to beverages such as wine and green tea (Gil et al., 2000).

Research on other types of fruit and processed foods show that postharvest and processing practices have substantial impacts on nutritional and functional properties (Rodrigues et al., 2010; Nicoli et al., 1997). This information is required to optimize postharvest handling and processing protocols and support value addition of pomegranates. The high concentration of antioxidant components in pomegranate peel (Ismail et al., 2012) has raised interest on methods of extracting juice from whole fruit. It is known that natural antioxidants contained in foods are lost during processing operations such as drying (Nicoli et al., 1997), and drying temperature can exert considerable influence on properties of dried products (Correia and Beirão-da-Costa, 2012). Research on phytochemical and functional properties of pomegranates has been reported from major growing areas in Asia and Middle East (Ozgen et al., 2008) and South Africa (Mditshwa et al., 2013), covering the effects of climatic and environmental factors, maturity and genotype.

Most postharvest studies did not examine the functional properties (Villaescusa et al., 2000), while others have reported opposing results such as the effects of packaging on loss of anthocyanin (Lopez-Rubira et al., 2005; Gil et al., 1996). The effects of fruit maturity stage, different packaging systems, long-term storage, methods of juice extraction and drying on bioactive components and functional properties of pomegranate have not been adequately investigated.

2. Research aim and objectives

The aim of this study was to examine the impacts of preharvest factors, postharvest handling and processing methods on bioactive components and functional properties of pomegranate fractions and waste. To achieve this aim, the study included the following specific objectives:

- 2.1. Investigate the effects of different maturity stages and growing locations on changes in biochemical and aroma volatile composition of 'Wonderful' pomegranate juice;
- 2.2. Evaluate the effects of maturity status and growing location on the postharvest concentrations of flavonoids, phenolic acids, vitamin C and antioxidant activity of pomegranate juice (cv. Wonderful);
- 2.3. Assess the impacts of extraction method on biochemical, volatile composition, antioxidant properties and antibacterial activity of pomegranate juice;
- 2.4. Determine the influence of packaging system and long term storage on pomegranate fruit. Part 1: Physiological attributes of whole fruit, biochemical quality, volatile composition and antioxidant properties of juice;
- 2.5. Determine the influence of packaging systems and long term storage on pomegranate fruit. Part 2: Bioactive compounds and functional properties of fruit by-products (peel and seed oil);
- 2.6. Evaluate the effect of drying on the bioactive compounds, antioxidant, antibacterial and anti-tyrosinase activities of pomegranate peel; and
- 2.7. Characterise the drying kinetics of pomegranate peels (cv. Wonderful).

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PAPER 1

Preharvest and postharvest factors influencing bioactive compounds in pomegranate (*Punica granatum* L.)

Abstract

Pomegranate fruit is a rich source of bioactive compounds such as flavonoids, phenolic acids and vitamin C and are attributed with diverse medicinal properties and health benefits that are highly desirable. Flavonoids, phenolic acids and vitamin C are found mainly in the peel, pith and juice (arils) of the pomegranate. The fruit is commonly consumed as fresh fruit or juice. In addition, the fruit is used in food industry in the manufacture of jellies, concentrates, and flavouring and colouring agents. Pomegranate juice is a rich source of antioxidants, found to be higher than other natural juices and beverages such as green tea and red wine. The stability and concentration of these functional properties are affected by preharvest factors such as cultivar, agro-climatic conditions, maturity, harvest season, irrigation and fertilization and postharvest factors such as storage, packaging and treatments. This review discusses the preharvest and postharvest factors influencing the functional properties of pomegranate fruit.

Keywords: Cultivar, Maturity, Packaging, Polyphenols, Pomegranate, Storage

1. Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest known edible fruit belonging to Punicaceae family. To date, pomegranate is widely grown in areas such as Iran, India, Egypt, Lebanon, China, Spain, France, USA, Oman, Syria, Tunisia, Italy, Greece, Cyprus, Israel, Turkey, Chile, Portugal and most recently South Africa (Al-Said et al., 2009; Holland et al., 2009; Fawole and Opara, 2013a,b). It is one of the oldest fruit making an appearance in the list of foods that contain some of the highest antioxidant values. Moreover, pomegranate fruit and its juice are a vast source of antioxidants, currently being graded together with blueberries and green tea for the nutritional health benefits that it can provide.

Pomegranate fruit is the most studied part and is reported to contain polyphenols in the peel, seed and juice. The major polyphenols in pomegranate fruit are flavonoids, condensed tannins and hydrolysable tannins (Gil et al., 2000; Van Elswijk et al., 2004; Seeram et al., 2008). Flavonoids including, flavonols, anthocyanins and phenolic acids are mainly found in the peel and juice of pomegranate while hydrolysable tannins including gallotannins and ellagitannins are found in the

peel and membrane. In addition, condensed tannins are mainly located in the peel and juice. Many of the compounds available are reported having various medicinal properties and health benefits.

Scientific studies have shown that various extracts of pomegranate possess a wide range of pharmacological properties such as antimicrobial (Duman et al., 2009), anti-inflammatory (Lee et al., 2010), cardioprotective (Davidson et al., 2009), free radical scavenging (Fawole et al., 2012a,b), hepatoprotective (Celik et al., 2009), tyrosinase inhibition property (Fawole et al., 2012b) and anti-diabetic effects (Xu et al., 2009). Pomegranate and its usage are intensely rooted in human history and its utilization is found in many prehistoric human cultures as food and medicinal remedy. Moreover, as a result of increased awareness of pomegranate as a medicinal fruit, consumers, researchers, and the food industries are more interested in how food products can help maintain health; and the role that it plays in the prevention of many illnesses has become widely accepted (Viuda-Martos et al., 2010). Consequently, the extent of pomegranate production has increased significantly in many regions and under diverse growth conditions (Shwartz et al., 2009). Several factors such as cultivar, agro-climatic condition, fertilizer, irrigation, maturity, storage and postharvest treatments influence the quality attributes of pomegranate fruit. The aim of this review is to discuss the preharvest and postharvest factors influencing the bioactive compounds in pomegranates.

2. Preharvest factors

2.1. *Genotype*

Several studies have shown that bioactive compounds in pomegranate fruit vary among cultivars. The influence of cultivar differences on functional properties of pomegranate fruit is summarized in Table 1. Fawole et al. (2012a) investigated the chemical and phytochemical properties and antioxidant activities of three pomegranate cultivars grown in South Africa. The authors reported total phenolic concentration ranging from 289 to 450 mg gallic acid equivalent /100 mL, with 'Bhagwa' having the highest amount of total phenolic concentration followed by 'Arakta' and 'Ruby'. The study further showed that total anthocyanin concentration found in 'Bhagwa' was 1.6-folds more than that found in Arakta cultivar. Similarly, Zaouay et al. (2012) found total phenolic concentrations ranging between 133.93 and 350.06 g/100 mL and between 50.5 and 490.4 mg/L of total anthocyanin in 13 Tunisian grown pomegranate cultivars. The authors reported higher concentrations of total phenolics and total anthocyanin in sour than in sweet cultivars. Similarly, studies conducted in Italy by Ferrara et al. (2011) found high concentration of polyphenols (97.1

mg/L) and vitamin C (236.3 mg/L) in sour cultivars compared to sweet cultivars. Total phenolic concentration between 41.01 and 83.43 mg/100 g was reported by Legua et al. (2012) for ‘Hamde’ and ‘Mesri’ pomegranates grown in Morocco. Ozgen et al. (2008) found monomeric anthocyanin concentration ranging between 6.1 and 219 mg cy3-Gluc/L for ‘Tatli’ and ‘Kan’ cultivars, respectively, grown in the Mediterranean region of Turkey. On the other hand, Tehranifar et al. (2010) found total anthocyanin concentration between 5.56 mg/100 g and 30.11 mg/100 g of juice in pomegranates grown in Iran. The authors also found a range of 295.79 mg/100 g to 985.37 mg/100 g for total phenolics and 9.91–20.92 mg/100 g ascorbic acid. Zarei et al. (2010) studied the physico-chemical properties and bioactive compounds of six pomegranate cultivars in grown in Iran and the authors reported concentration of ascorbic acid ranging from 8.68 to 15.07 mg/100 g for ‘Aghaye’ and ‘Shahvar’, respectively. Furthermore, the authors found total anthocyanin concentration ranging between 7.93 and 27.73 mg/100 g for ‘Aghaye’ and ‘Shirin-e-Bihaste’, respectively. Total phenolic concentration was reported to range between 526.40 mg tannic acid/100 g (‘Shahvar’) and 797.49 mg tannic acid/100 g (‘Aghaye’), while the concentration of total tannins was between 18.77 mg tannic/100 g (‘Shahvar’) and 38.21 mg tannic acid/100 g (‘Aghaye’). Furthermore, condensed tannins were between 12.14 and 12.57 mg catechin/100 g in cultivar ‘Shirin e-Bihaste’ and ‘Aghaye’, respectively. Jing et al. (2012) investigated the phytochemical composition of pomegranate seed oil from four cultivars (Suanshiliu, Tianhongdan, Sanbaitian and Jingpitian) grown in China and the results revealed significant differences in the levels of phenolics with 50% aqueous acetone, ranging from 1.29 to 2.17 mg of gallic acid equivalents per gram of dry seeds (mg GAE/g) in all tested cultivars. Total flavonoids ranging from 0.37 to 0.58 mg CAE/g was obtained by 80% aqueous methanol in pomegranate seeds of four cultivars. In addition, total proanthocyanidins significantly varied from 68 to 182 µg cyanidin equivalents (CyE) /g of the seeds of the four cultivars (Jing et al., 2012).

2.2. Agro-climate and seasonal variation

The effects of agro-climate and growing season on bioactive compounds in pomegranates are highlighted in Table 2. Different agro-climatic conditions and seasonal variation have been shown to influence bioactive compounds of pomegranate fruit. A study conducted by Schwartz et al. (2009) explained the variations in the compositions of bioactive compounds in the arils and peel of 11 accessions grown under Mediterranean and desert climate in Israel. The authors reported higher anthocyanin concentration in arils of most cultivars grown in the Mediterranean climate compared to those grown in desert climate. On the contrary, however, higher total phenolics, hydrolyzable

tannins, punicalagin, and punicalin were found in fruit peel grown in a desert climate (Schwartz et al., 2009). Fawole and Opara (2013c) found that total phenolic concentration and gallotannins varied between two harvest seasons for ‘Bhagwa’ and ‘Ruby’ pomegranates grown in South Africa. However, it was observed that total anthocyanin and total flavonoids concentration were not affected by growing season in both cultivars. Mditshwa et al. (2013) found different total phenolic concentrations of ‘Bhagwa’ pomegranates grown in agro-climatic locations characterized by different elevations and temperature. For instance, total phenolic concentration varied between 8.54 and 13.91 mg GAE/ mL crude juice and the authors concluded that factors related to altitude may have a strong effect on the biosynthetic pathway of phenolics.

2.3. *Maturity status*

Several reports have shown that the chemical properties of fruit are highly dependent on the stage of development and ripening (Borochoy-Neori and Shomer, 2001; Dumas et al., 2003; Toor et al., 2006; Raffo et al., 2006). Maturity status is one of the main factors determining the compositional quality of pomegranate fruit. A summary on the effects of fruit maturity status on bioactive compounds in pomegranates is presented in Table 3. Mirdehghan and Rahemi (2007) found that total phenolics levels increased in peel and arils of fruit at early stage of development; however, phenolic concentrations decreased with advancing maturation, reaching 3.70 and 50.22 mg/g dry weight in arils and peel, respectively, at harvest. Also, Zarei et al. (2011) observed a significant decline in ascorbic acid concentration, total phenolics, total tannins and condensed tannins during fruit maturation. However, the authors observed an increase in total anthocyanin concentration from 3.68 to 24.42 mg /100 g in pomegranate cv ‘Rabbab-e-Fars’ during fruit maturation. Weerakkody et al. (2010) reported a decline in total phenolic concentration during fruit development of pomegranate (cv. Wonderful) grown in Australia from 1706 to 117 mg GAE/ mL. According to Fawole and Opara (2013d), during fruit maturity of ‘Ruby’ pomegranate cultivar, total phenolics, total flavonoid concentration and total gallotannins concentration declined significantly from 1051.60 to 483.31 mg GAE /100 mL, 752.18 to 397.27 mg CE /100 mL and 64.80 to 29.07 mg GAE /100 mL, respectively, as fruit maturity advanced. More specifically, the authors reported significant decline in individual phenolics during the fruit maturity. For instance, with advancing maturity, gallic acid concentration decreased, epicatechin concentration increased while catechin concentration remained unchanged. Similarly, the study on changes in juice anthocyanin concentrations of Spanish cvs ME5, ME17, MO6 and MA4 showed that the amount of anthocyanin pigment increased during fruit development, with juice changing from colourless to dark colour (Legua et al., 2000). Recently, Fawole and Opara

(2013e) reported a decline in flavonols (+catechin, –epicatechin) and phenolic acid (phenolic acid, protocatechuic acid, gallic acid and ellagic acid) in ‘Bhagwa’ pomegranate fruit during maturation. However, in the case of ascorbic acid, protocatechuic acid and total anthocyanin, the concentration increased significantly at later maturity stages. Al-Maiman and Ahmad (2002) found that arils and peel of unripe pomegranate fruit contained higher polyphenols whereas ripe fruit contained the least. Shwartz et al. (2009) investigated changes in chemical constituents of pomegranate peel and arils during the maturation and ripening of two Israeli commercial accessions (‘Wonderful’ and ‘Rosh-Hapered’). In both cultivars, a reduction in total phenolics and hydrolyzable tannins was found in the peel during maturation while anthocyanin level increased. In addition, anthocyanin concentration in the arils significantly increased in ‘Wonderful’ whereas no changes were observed in ‘Rosh-hapered’. In another study, Borochoy-Neori et al. (2009) found that arils of fruit harvested early in the season had lower total phenolics (0.22–0.88 pyrogallol equivalents, g/L) than fruit harvested late (1.21–1.71 pyrogallol equivalents, g/L). Given this evidence, it can be seen that the concentration of bioactive compounds in pomegranate fruit are influenced by maturity status.

2.4. Cultural practices

Irrigation and fertilization, among other factors, can influence water and nutrient supply to the plant, which in turn may affect nutritional composition of pomegranate fruit.

2.4.1. Irrigation

Very little is known about the effects of irrigation on bioactive compounds of pomegranate. Response of pomegranate tree to different irrigation levels and the effect on vegetative growth and fruit quality were recently investigated by Khattab et al. (2010). The authors reported the effects of low irrigation levels (7, 9, 11, 13 or 15 m³/tree/year) on anthocyanin concentration in fruit. Among irrigation levels employed, 7 m³/tree/year resulted in increased anthocyanin concentration compared to other treatments. Although pomegranate tree is considered to be tolerant to soil water deficit (Holland et al., 2009), there are limited publications on pomegranate fruit response to different deficit irrigation conditions. Recently, Mellisho et al. (2012) investigated pomegranate (*P. granatum* L.) fruit response to different deficit irrigation conditions. Based on their findings, severe deficit irrigation (32%) improved fruit total phenolic concentration and total anthocyanin. Similarly, Mena et al. (2012) studied sustained deficit irrigation effects on color and phytochemical characteristics of pomegranate. The authors reported that moderate (43%) and severe (12%) water stress treatments resulted in lower total phenolic compounds, punicalagin and total anthocyanin in pomegranate juice

than that of the control. These results were contrary to that of Galindo et al. (2014), who reported that total anthocyanin and total phenolic concentrations did not change in pomegranate tree subjected to sustained irrigation of 105% and 33% from the beginning of the second half of rapid fruit growth period to the last harvest.

2.4.2. *Fertilization*

Studies have shown that bioactive compounds in pomegranate are strongly influenced by fertilization. For instance, the study by Khayyat et al. (2012) on the effects of spray application of potassium nitrate on fruit characteristics of ‘Malas yazdi’ pomegranate showed that fruit treated with 250 mg/L potassium nitrate had the highest vitamin C concentration compared to those treated with 500 mg/L potassium nitrate and control treatment. Similarly, a study on the effects of compost tea and some antioxidant applications on leaf chemical constituents, yield and fruit quality of ‘Manfalouty’ pomegranate tree showed that foliar application of compost tea with double combine antioxidants treatment (ascorbic acid plus citric acid) gave highest vitamin C and total anthocyanin concentration in fruit in the second season in comparison with other studied treatments in both seasons (Fayed, 2010).

3. Postharvest factors

Pomegranate is a non-climacteric fruit and like other fruits, it is subjected to continuous physiological and biochemical changes after harvest. These changes in pomegranate often lead to weight loss, husk scald and aril discoloration. Such changes cannot be stopped completely; however, they can be retarded within certain limits by applying diverse postharvest treatments and hurdle technologies (Lee and Kader, 2000). Application of postharvest treatments such as heat treatment, maintaining optimum storage temperature, modified atmosphere packaging, controlled atmosphere storage, shrink wrapping, coating and drying have been reported to affect both keeping and nutritional quality, as well as bioactive compounds in pomegranates (Artés et al., 2000; Sayyari et al., 2010).

3.1. *Storage temperature and relative humidity*

Various studies have shown that storage conditions have a notable influence on phytochemicals in pomegranates (Gil et al., 1996a; Ghafir et al., 2010). Temperature management procedures are important for maintenance of quality attributes including the nutrition components. However, available information on storage temperature and relative humidity effect is limited to

vitamin C and anthocyanin, as well as phytochemicals. Generally, anthocyanins are labile compounds and are easily susceptible to degradation in various environmental conditions. Temperature, storage period and time of processing after fruit harvest have been found to influence anthocyanin stability (Pilano et al., 1985; Markakis, 1982; García-Viguera et al., 1999; Martí et al., 2001; Stintzing and Carle, 2004). Furthermore, loss of anthocyanins has been attributed to many other factors such as pH and acidity, phenolic compounds, sugars and sugar degradation products, oxygen, ascorbic acid, fruit maturity and thawing time (Withy et al., 1993; García-Viguera et al., 1998). Biosynthesis of anthocyanin pigments in fruit during postharvest storage at low temperatures has been reported in pomegranates (Ben-Arie et al., 1984). Storage of pomegranate juice at low temperatures such as 5°C rather than 25°C reduced the rate of anthocyanin degradation. However, the addition of ascorbic acid treatment was found to increase the degradation of anthocyanin at both temperatures (Gil et al., 2000). Similarly, a study by López-Rubira et al. (2005) showed that arils stored at 1°C for 13 days had no significant change in anthocyanin concentration and antioxidant activity. Effects of storage time of unprocessed and pasteurized juices on anthocyanin concentration of four selected pomegranate varieties were investigated by Alighourchi et al. (2008). The authors observed that the average degradation percentage of anthocyanin ranged from 23.0 to 83.0% during 10 days of cold storage at 4°C. In pasteurized juice, however, the average degradation of anthocyanins was 42.8% after 10 weeks of storage at 4°C.

Long storage periods have been shown to influence anthocyanin concentration of pomegranate. The influence of storage temperature and ascorbic acid addition on pomegranate juice was investigated by Martí et al. (2001). The authors reported 1% loss in anthocyanin after storage of pomegranate juice at 25°C for 150 days, whereas 20% loss was found at 5°C after 5 months. This was similar to the findings reported by Alighourchi and Barzegar (2009), who reported 71.8%, 91.3%, and 96.9% degradation of total anthocyanin concentration at 4°C, 20°C, and 37°C, respectively. Fischer et al. (2011) reported pigment degradation and concomitant colour loss at 20°C and upon illumination. However, no significant differences were found in non-anthocyanin phenolics throughout the storage. It has been suggested that the degradation of anthocyanin is largely triggered by oxidation or cleavage of covalent bonds, which increases with an increase in temperature during storage or processing (Laleh et al., 2006). O'Grady et al. (2014) showed that anthocyanin concentration declined with increase in temperature from 4 to 8°C in 'Arakta' stored for 7 days. Mirsaeedghazi et al. (2014) examined the effects storage at -25°C on the anthocyanin and phenolic components of pomegranate juice and the authors found that total anthocyanin, phenolic concentration and total antioxidant of pomegranate juice decreased by 11%, 29% and 50% after 20

days storage. Among monomeric anthocyanin, pelargonidin 3, 5-diglucoside had the highest degradation, while ellagic acid decreased by 15%. The reported decrease in anthocyanin and phenolic concentration at -20°C was attributed to oxidation and storage at the temperature which could not preserve the nutritional concentration of pomegranate juice. On the contrary, however, according to Fawole and Opara (2013f) ‘Bhagwa’ and ‘Ruby’ stored at $5 \pm 0.3^{\circ}\text{C}$ and $92 \pm 3\%$ RH for 8 weeks exhibited no change in antioxidant activity. Vitamin C is another important component of pomegranate juice; however, its concentration is affected by storage temperature and extended storage period (Kader, 1988). Aarabi et al. (2008) investigated the concentration of ascorbic acid in selected pomegranate juices during storage at 4°C for 60 days and reported 100% loss of initial ascorbic acid concentration after 15 days at 4°C . Similarly, a significant loss in vitamin C concentration was observed in pomegranate fruit (‘Wonderful’) stored at 5°C and 7.5°C after 5 months of storage (Arendse et al., 2014). O’Grady et al. (2014) also observed that ascorbic acid concentration reduced over time in ‘Ruby’ arils stored at 1°C , 4°C and 8°C for 7 days.

3.2. Technological treatments

3.2.1. Controlled atmosphere storage

Controlled atmosphere (CA), in which the air composition is modified by increasing CO_2 and decreasing O_2 , offers several advantages in produce, including: (a) retardation of metabolic process such as of ripening and senescence in fruit, (b) retardation of loss of some nutritional components such as vitamins, (c) decay control, (d) insect control, and (e) alleviation of physiological disorders such as chilling injury in some fresh produce. However, very little information is available on the effect of CA storage on the bioactive compounds in pomegranates. Several researchers indicated that controlled atmosphere storage has the benefit of controlling postharvest decay of fruit; however, a CO_2 -enriched atmosphere with low O_2 concentration can affect total ascorbic and anthocyanin concentration adversely, with negative consequences on fruit colour and nutritional values (Holcroft and Kader, 1999).

Artés et al. (1996) investigated different controlled atmosphere conditions (21% O_2 and 0% CO_2 ; 10% O_2 and 5% CO_2 ; 5% O_2 and 5% CO_2 ; 5% O_2 and 0% CO_2 plus 2.3 ppm ethylene; 5% O_2 and 0% CO_2 plus ethylene-free (less than 0.2 ppm) on pomegranate cv. Mollar. A decrease in vitamin C in the pomegranate cultivar in all treatments during shelf life was reported. Furthermore, lower vitamin C was found in fruit stored at 21% O_2 and 0% CO_2 (5.1 mg/100 mL). Holcroft et al. (1998) studied the effects of CO_2 (10 or 20 kPa) on anthocyanins, phenylalanine ammonia lyase and glucosyl transferase in the arils of stored pomegranates. The authors observed that arils stored at 10

or 20 kPa CO₂ had lower anthocyanin concentration. However, anthocyanin was better maintained at 10 kPa CO₂ (283.0 µg/mL) compared to 20 kPa CO₂ (206.2 µg/mL). Based on their findings, the authors suggested that anthocyanin synthesis and or degradation might have been affected by CO₂ and O₂ concentration. In general, the lower the O₂ concentrations during storage, the lower the losses of ascorbic acid and other vitamins.

3.2.2. *Modified atmosphere packaging*

Modified atmosphere packaging (MAP) has been successfully used to extend the shelf life of minimally fresh processed pomegranate arils (Artés et al., 1995; Gil et al., 1996a, b; Villaescusa et al., 2000) but their effects on bioactive compounds is not well established. López-Rubira et al. (2005) investigated shelf life and overall quality of minimally processed pomegranate arils modified atmosphere packaging; polypropylene baskets sealed with bioriented polypropylene to create passive conditions and treated with UV-C. The authors observed that arils stored at 5°C for 13 or 15 days showed no significant change in anthocyanin as well as antioxidant activity. Gil et al. (1996a) investigated influence of modified atmosphere packaging; perforated polypropylene and oriented polypropylene (40 µm) on anthocyanin of minimally processed pomegranate ('Mollar de Elche') stored at 8°C, 4°C, and 1°C for 7 days. At the end of shelf life, total anthocyanin decreased in the samples stored at 8°C and 4°C, whereas significant increase was observed in seeds stored at 1°C under modified atmospheres. Furthermore, Artés et al. (2000) did some work on the modified atmosphere packaging of pomegranate cv. Mollar de Elche stored at 2°C or 5°C for 12 weeks in unperforated and perforated polypropylene film. Both perforated and unperforated films suffered decrease in total anthocyanin at the end of shelf life. However, arils stored in perforated polypropylene at 5°C showed an increase in total anthocyanin concentration after cold storage. The finding clearly explains the influence of extended storage periods on anthocyanin concentration.

3.2.3. *Coating and waxing*

Coating is known as an environment friendly technology that gives advantages for shelf life increase of pomegranate fruit during storage. Influence of coating on bioactive compounds and nutritional value of pomegranate fruit has been reported by several researchers (Table 4). Sayyari et al. (2011a) found that pomegranate coated with 0.1, 0.5, and 1.0 mM acetyl salicylic acid maintained total phenolics (270 mg /100 g) and anthocyanin (130 mg /100 g) concentration in fruit stored at 2°C for 84 days and the authors suggested that acetyl salicylic acid could have potential postharvest application for improving health benefits of pomegranate fruit. In another study, Sayyari and Valero

(2012) found that fruit coated with salicylic acid (2 mM) showed significant increase in anthocyanin and phenolics after storage at 2°C for 90 days when applied on Mollar de Elche cultivar. In addition, salicylic acid (2 mM) applied to sour pomegranate reduced the rate of the decline in ascorbic acid (vitamin C) losses compared to control fruit (Sayyari et al., 2009). On the contrary, Sayyari et al. (2010) found lower losses of total phenolic, increase in ascorbic concentration on Mollar de Elche pomegranate treated with oxalic acid concentrations (2, 4, and 6 mM). Higher anthocyanin was observed after storage particularly for fruit treated with 6 mM oxalic acid (Sayyari et al., 2010). Barmann et al. (2014) did some research on the influence of putrescine and carnauba wax on the bioactive compounds of pomegranate. Fruit treated with putrescine plus carnauba wax retained tannins concentration averaging 235.0 mg equiv. gallic acid /100 g 15 days after storage at 5°C. At the end of the storage period, total anthocyanins concentration were higher more especially at 3°C and found to be 175.07 mg equiv. delphinidin-3, 5-diglucoside /100 g for fruit treated with putrescine (2 mM) + carnauba while fruit treated with putrescine alone retain total anthocyanin averaging 152.54 mg equiv. delphinidin-3, 5-diglucoside /100 g as compared to control fruit (105.35 mg equiv. delphinidin-3, 5-diglucoside /100 g).

Mirdehghan et al. (2007) investigated the influence of putrescine and spermidine at concentration of 1 mM applied either by pressure infiltration or immersion on pomegranate arils and stored at 2°C for 60 days. The authors showed that polyamines applied by pressure infiltration resulted into significant increase in total phenolics (139.16 mg equiv. gallic acid /100 g) in spermidine treated arils compared to putrescine-treated arils (128.78 mg equiv. gallic acid /100 g). In addition, spermidine-infiltrated arils had higher total anthocyanin (229.86 mg equiv. cyanidin-3-glucoside /100 g) compared to putrescine-immersed pomegranate arils (198.57 mg equiv. cyanidin-3-glucoside /100 g). It was concluded that total phenolics were affected by the treatment method. Numerous previous studies have shown that chitosan coating had beneficial effects in maintaining the anthocyanin concentration of pomegranate (Varasteh et al., 2012; Alighourchi et al., 2008). Ghasemnezhad et al. (2013) found the highest anthocyanin concentration (71.78 mg /100 mL) of pomegranate arils coated with 1% chitosan following 12 days of storage at 4°C. Furthermore, arils coated with 1% or 2% chitosan delayed anthocyanin degradation and diglucoside anthocyanins were more stable than the monoglucosides (Varasteh et al., 2012). According to Zhang and Quantick (1998), applying chitosan film on the surface of the fruit could modify its endogenous CO₂ and O₂ levels, which could result in a reduction in O₂ supply for the enzymatic oxidation of anthocyanin. On the other hand, chitosan can also increase phenylalanine ammonia-lyase enzyme activity and lead to an increase in phenolic production (Liu et al., 2007).

3.2.4. Package films

Packaging is an important part of product preservation and has direct influence on the product with respect to physical and chemical changes. Several researchers have tested different materials and their effects on pomegranate phytochemicals. Packaging selection as well as processing influence fruit quality including chemical attributes during storage. Table 5 summarises the effects of applying different package films on the bioactive compounds of pomegranate. Artés et al. (2000) did some work on the impact of modified atmosphere technique on the quality attributes of sweet pomegranate stored at 2 or 5°C for 12 weeks. The authors observed that fruit packaged with unperforated polypropylene film of 25 µm thickness in modified atmosphere packaging and perforated polypropylene of 20 µm thicknesses showed a decline in total anthocyanin after storage at 2 or 5°C after 12 weeks. Furthermore, a general trend of decrease in individual anthocyanin (3-glucoside (Dp3) and delphinidin-3,5-diglucoside (Dp3-5) was observed at the end of cold storage and shelf life in all treatments (Artés et al., 2000). A significant increase in anthocyanin in packaged arils was also reported which is in agreement with other authors. For instance, Gil et al. (1996a) observed that storage in perforated polypropylene bags preserved pigments, with a slight increase in anthocyanin during storage in modified atmosphere at 1°C. D'Aquino et al. (2010) found that fruit wrapped with polyolephinic heat-shrinkable film treated with fludioxonil had lower total phenolic which decreased from 139.6 to 122.3 mg gallic acid /100 g while anthocyanin decreased from 32.1 to 29.4 mg Cya -3-gluc /100 g at the end of shelf life.

Loss in bioactive compounds of pomegranate fruit depends on the type of package material employed. Pérez-Vicente et al. (2004) found higher anthocyanin degradation in minibrik-200 (95%) than transparent and green glass bottle (77–78%). The high loss of anthocyanin in minibrik-200 is attributed to oxygen permeability of the material (Perez-Vincente et al., 2004). Pomegranate fruit ('Primsole') packaged with polypropylene (40 µm thick) caused reduction in total phenolic concentration from 1492 to 1393 mg/L at 5°C after 10 days (Palma et al., 2009). However, no significant difference was found in degradation of anthocyanin after 10 days storage at 5°C (Palma et al., 2009).

Higher total anthocyanin and vitamin C were retained in minimally processed seed of 'Shlefy' pomegranate fruit packaged in polyethylene bags stored at 5 and 7°C for 4 month compared to commercial packaging (Falcon) and vapour guard waxing (2%) (Ghafir et al., 2010). Abd-elghany et al. (2012) reported that fruit wrapped with polyolefin film and treated with calcium chloride (2%) retained higher anthocyanin concentration averaging 0.38 and 0.34 mg/100 g stored at 5°C and 20°C, respectively, than untreated fruit which gave 0.30 and 0.22 mg/100 g fresh weight at the end of cold

storage. A significant loss in vitamin C concentration of pomegranate fruit ('Gok Bahce') stored at higher temperatures (10°C) was also reported (Koksal, 1989). Polyolefin films plus skin coating with a sucrose polyester (SPE) Semperfresh retained vitamin C in fruit stored at 8°C and 15°C for a period of 12 and 9 weeks, respectively, compared to non-treated fruit (Nanda et al., 2001).

3.2.5. Effect of drying on the bioactive compounds of pomegranate

Drying may also affect the presence and stability of bioactive compounds such as polyphenols due to their sensitivity towards heat. Jaiswal et al. (2010) observed that cabinet-dried and sun-dried arils resulted in 61% (from 250.5 to 97.4 µg/g) and 83% (from 250.5 to 42.2 µg/g) loss of anthocyanin, respectively. It was concluded that inhibition of polyphenol oxidase by oven-drying at high temperature may be liable for protecting the anthocyanins from oxidation compared to sun-drying, resulting in enhanced anthocyanin degradation. According to Severini et al. (2003), anthocyanins are stable at high temperatures, while polyphenol oxidase is heat-labile and is considerably inhibited above 80°C. Similarly, Bchir et al. (2012) observed that anthocyanin and total phenolic concentration of pomegranate seeds decreased with an increase in temperature. Opara et al. (2009) found that sun-dried peels retained between 76.8 and 118.4 mg/100 g fresh weight of vitamin C in cultivars investigated. High vitamin C concentration in sun-dried fruit peel may be attributed to the slow and gradual moisture removal associated with low temperature drying for longer period in comparison with short-time high-temperature oven drying (Vega-Gálvez et al., 2008) while sun drying is weather dependent and in turn may affect the homogeneity and quality of the final product. Increase in individual phenolic compound by freeze drying was also reported. Calín-Sánchez et al. (2013) found that freeze drying of pomegranate rind (peel) resulted in higher punicalagin concentrations during drying.

4. Conclusion

Pomegranate fruit has been the focus of recent interest among researchers for their role in human health and prevention of chronic diseases. Pomegranates contain several bioactive compounds including phenolic acids, tannins, flavonoids, and vitamins which have been reported to have numerous health benefits. Studies have also demonstrated that pomegranate peel contains substantial amount of phenolic compounds compared to the arils (juice). Available evidence has shown that preharvest and postharvest factors influence the bioactive compounds of pomegranate fruit. However, recent findings are limited to the general screening of the total phenolic concentration. It is noteworthy that very few studies in this review reported information on the

influence of preharvest and postharvest factors on the individual bioactive compounds of both arils and peel. Future studies should focus on isolated phytochemicals as it will improve our understanding of the mechanism of action responsible for the various beneficial effects. The results may be important towards optimising postharvest handling and processing protocols of pomegranates.

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Table 1 Effect of cultivar differences on bioactive compounds in pomegranate fruit.

Factor levels	Country	Fruit part	Bioactive compounds	Key findings	References
Bhagwa, Arakta, Ruby	South Africa	Juice	Total phenolics, total anthocyanin, total flavonoids, gallic acid	Bhagwa had the highest total phenolics, total flavonoid, anthocyanin than Arkata and Ruby	Fawole et al. (2012a)
Sour vs. Sweet 13 cultivar	Tunisia	Juice	Total phenolic, total anthocyanin	Higher TP and AA, less delphinidin-3,5-diglucoside in sour cultivars, higher TP and anthocyanin (delphinidin-3,5-diglucoside) were recorded in sweet cultivars	Zaouay et al. (2012)
Sour vs. Sweet 8 cultivar	Italy	Juice	Polyphenols, vitamin C	Sour cvs. exhibited higher polyphenols and vit C than sweet cvs	Ferrara et al. (2011)
10 cultivars	Morocco	Juice	Total phenolics	Hamde gave highest total phenolic concentration than Mesri	Legua et al. (2012)
32 accessions	Egypt	Juice	Vitamin C total anthocyanin, ellagic acid	Vitamin C concentration ranged between 2.77 and 9.48 mg/100 mL; total anthocyanin (0.045-1.37 mg/mL); ellagic acid ranged between 0.84 and 10 mg/L	Hassan et al. (2012)
	Turkey	Arils	Total phenols, total monomeric anthocyanin	TP varied between 1245 and 2076 mg GAE/L while total monomeric anthocyanin ranged between 6.1 to 219 mg Cy3-gluc/L	Ozgen et al. (2008)

Table 1 (continued) Effect of cultivar differences on bioactive compounds in pomegranate fruit.

Factor level	Country	Fruit part	Bioactive compounds	Key findings	References
20 cultivars	Iran	Juice	Total phenolic, total anthocyanin, ascorbic acid	Total anthocyanin varied between (5.56 mg /100 g and 30.11 mg /100 g); TP (295.79 mg /100 g and 985.37 mg /100 g); ascorbic acid (9.91 mg /100 g and 20.92 mg /100 g)	Tehraniifar et al. (2010)
6 cultivars	Iran	Juice	Total anthocyanin, ascorbic acid, total phenolic, total Tannins, condensed Tannins	Higher TP, total anthocyanin, TTs, CTs were recorded in Aghaye but recorded the lowest ascorbic acid; Shahvar showed the lowest TP; Shiri-e-Bihaste had the lowest CTs but higher ascorbic acid concentration	Zarei et al. (2010)
4 cultivars	China	Seed oil	Total phenolic, total flavonoids concentration, proanthocynadins	Suanshiliu had the highest TP, TFC, and proanthocynidins followed by Tianhongdan, Sanbaitian and Jingpitian	Jing et al. (2012)

TP, total phenolics; AA, antioxidant activity; vit C, vitamin C; TTs, total tannins; CTs, condensed tannins; TFC, total flavonoids concentration; GAE, gallic acid equivalent; Cy3-gluc, cyanadin-3-glucoside .

Table 2 Effects of agro-climate and growing season on bioactive compounds in pomegranate fruit.

Pre-harvest factors	Factor levels	Country	Fruit part	Bioactive compounds	Key findings	References
Agro-climatic variation	Mediterranean climate (Newe Ya'ar) vs. Desert climate (Arava Valley); 11 cultivars	Israel	Aril	Total phenolic, anthocyanin concentration	Higher TP and AC in arils from Mediterranean climate than in the peel	Schwartz et al. (2009)
			Peel	Total phenolic, hydrolyzable tannins, punicalagin, punicalin	Higher AA, TP, HTs, punicalagin and punicalin in the peel from desert climate	
	Mediterranean climate (Newe Ya'ar) vs. Desert climate (Arava Valley)	Israel	Arils	Anthocyanins	Anthocyanin accumulation changed inversely to the season's temperatures	Borochov-Neori et al. (2011)
	Mediterranean climate (Porteville, Wellington, Piketberg)	South Africa	Whole fruit	Total anthocyanin, total phenolic, vitamin C	Total anthocyanin, total phenolic and vitamin C ranged between 0.07 to 0.16 mg CyE/ mL, 8.54 to 13.91 mg GAE/ mL and 0.67 to 1.41 mg AAE/ mL, respectively	Mditshwa et al. (2013)

TP, total phenolics; AC, anthocyanin; HTs, hydrolysable tannins; AA, antioxidant activity; AAE, ascorbic acid equivalent; CyE, cyanidin equivalent; GAE, gallic acid equivalent.

Table 2 (continued) Effects of agro-climate and growing season on bioactive compounds in pomegranates

Pre-harvest factors	Factor levels	Country	Fruit part	Bioactive compounds	Key findings	References
Seasonal variation	Early vs. late ripe	Israel	Arils	Soluble phenolics	Fruit harvested late in the season had higher soluble phenolics concentration than fruit harvested early	Borochoy-Neori et al. (2009)
	Summer vs. winter	Israel	Arils	Anthocyanin	Anthocyanin accumulation changed inversely to the season's temperatures	Borochoy-Neori et al. (2011)
	Two seasons	South Africa	Juice	Total phenolics, gallotannins	Higher total phenolic concentration and gallotannins were found in the first season than second season	Fawole and Opara, (2013a)

Table 3 Effects of fruit maturity status on bioactive compounds in pomegranate fruit.

Factor levels	Country	Fruit part	Bioactive compounds	Key findings	References
Early vs. Late maturation	Iran	Arils, Peel	Total phenolics	Total phenolics (TP) increased both in the peel and arils at an early maturity, decreased during maturation; TP was higher in the arils than in the peel; TP in arils and peel decreased during fruit growth and development	Mirdeghan and Rahemi (2007)
Unripe vs. ripe	Saudi Arabia	Juice	Polyphenols, ascorbic acid	PP and AA were higher in unripe fruit; PP and AA decreased with advance in maturity	Al-Maiman and Ahmad (2002)
Maturity stage ‘Rabbab-e-Fars’	Iran	Arils	Ascorbic acid, total anthocyanin, total phenolic, total tannins, condensed tannins	AA, TP, TT, CTs decreased while total anthocyanin increased during fruit maturation	Zarei et al. (2011)
Maturity stage- ‘Ruby’	South Africa	Arils	Total phenolics, total flavonoid, total gallotannins,	TP, TF, TG declined as fruit maturity progressed	Fawole and Opara (2013b)
Maturity stage- ‘Bhagwa’	South Africa	Arils	Ascorbic acid, (+) catechin, (–) epicatechin, phenolic acid, protocatechuic acid, gallic acid and ellagic acid, anthocyanin	(+) catechin, (–) epicatechin, phenolic acid, protocatechuic acid, gallic acid and ellagic acid declined while ascorbic acid, protocatechuic acid and total anthocyanin increased with fruit maturity	Fawole and Opara (2013c)

Table 3 (continued) Effects of fruit maturity status on bioactive compounds in pomegranates

Factor levels	Country	Fruit part	Bioactive compounds	Key findings	References
Maturity stage- 'Bhagwa', 'Ruby'	South Africa	Arils	Total phenolics, anthocyanin, gallotannins, total flavonoids,	Total phenolics, gallotannins, total flavonoid declined as fruit maturity advances while anthocyanin increased	
Maturity stage- 'Wonderful', 'Rosh-Hapered'	Israel	Arils, peel	Total phenolics, hydrolysable tannins, total anthocyanin	In both cvs, total anthocyanin increased whereas TP and HTs were reduced in the peel during maturation	Shwartz et al. (2009)

AA, ascorbic acid; PP, polyphenols; TTs, total tannins; CTs, condensed tannins; TF, total flavonoid; TG, total gallotannins, Hydrolysable tannins.

Table 4 Effect of different coating material on the bioactive compounds of pomegranate cultivar, fruit part stored under different temperature regimes.

Coating	Cultivar	Fruit part	Storage temperature and duration	Effects	References
(a) 0.1, (b) 0.5, (c) 1.0 mM acetyl salicylic acid (ASA),	Mollar de Elche	whole fruit	2°C, 84 days	Total phenolics and anthocyanins were maintained in all treatments	Sayyari et al. (2011a)
Methyl jasmonate or methyl salicylate (0.01 and 0.1 mM)	Mollar de Elche	whole fruit	2°C, 84 days	Both treatments increased total phenolics and anthocyanins	Sayyari et al. (2011b)
2 mM Salicylic acid	Mollar de Elche	Whole fruit	2°C, 90 days	Significant increase in anthocyanin and phenolic were observed	Sayyari and Valero (2012)
2 mM putrescine and 1:10 Carnauba wax (carnauba wax: water).	Mridula	Whole fruit	3 and 5°C, 60 days	Both treatments (combined) retained higher anthocyanin, ascorbic acid and tannins	Barmann et al. (2014)
Oxalic acid (2, 4, and 6 mM)	Molar de Elche	Whole fruit	84 days at 2°C	Lower losses of total phenolics were recorded; increase in ascorbic acid concentration, total anthocyanin was higher after storage for fruit treated with 6 mM oxalic acid	Sayyari et al. (2010)

Table 4 (continued) Effect of different coating material on the bioactive compounds of pomegranate cultivar, fruit part stored under different temperature regimes.

Coating	Cultivar	Fruit part	Storage temperature and duration	Effects	References
Sucrose polyester (SPE) Semperfresh™ + Polyolefin films	Garnesh	Whole fruit	8, 15 and 25°C, 12 weeks	SPE treatment failed to reduce loss of vitamin C concentration during storage	Nanda et al. (2001)
(a) 0.25, (b) 0.5 and (c) 1% (w/v) chitosan aqueous solutions and 1% (v/v) acetic acid for 1 min	'Tarom'	Arils	4°C and 95% RH, 12 days	Chitosan application delayed decrease in total phenolics and total anthocyanins during storage	Ghasemnezhad et al. (2013)
1% or 2% Chitosan	Rabbab-e-Neyriz	Arils	2°C or 5°C; 135 days	Delayed anthocyanin degradation, diglucoside anthocyanins were more stable than the monoglucosides, colour deterioration were prevented in the arils	Varasteh et al. (2012)
(a) Putrescine; (b) Spermidine (1 mM) either by pressure infiltration or by immersion	Mollar de Elche	Arils	2°C for 60 days	Ascorbic acid, total phenolic and total anthocyanins were maintained	Mirdehghan et al. (2007)

Table 5 Summary of the effects of different package films on the bioactive compounds of pomegranate fruit.

Package film	Cultivar	Fruit part	MA Composition O ₂ and CO ₂	Temperature and storage time	Effects	References
(b) Perforated polypropylene (PPP) 25 µm thickness-control			(6 holes of 1 mm dia per dm ²) 20 µm thickness		Anthocyanin deteriorated at the end of shelflife	
Oriented polypropylene (40 µm thickness)	Mollar de Elche	Aril	O ₂ permeability 290 cm ³ /m ² 24 hr. bar; CO ₂ permeability 1112 cm ³ /m ² 24 hr. bar (passive)	8°C, 4°C and 1°C, 7 days	Anthocyanin increased at 1°C	Gil et al. (1996a)
(a) Transparent glass bottle (b) green glass bottle (c) Paperboard carton with polyethylene layer (Minibrik-200)	Mollar	Juice	–	160 days	95% anthocyanin degradation in minibrik and less for those stored in transparent and green glass bottles (77-78%). Antioxidant activity was not affected by packaging employed	Pérez-Vincente et al. (2004)

Table 5 (continued) Summary of the effects of different package films on the bioactive compounds of pomegranate fruit.

Package film	Cultivar	Fruit part	MA Composition O ₂ and CO ₂	Temperature and storage time	Effects	References
Polypropylene (40µm thick)	Primosole	Whole fruit	O ₂ permeability 300 mL/m ² days bar; CO ₂ permeability 1,000 ml/m ² d bar/11.4 and 6.5	5°C, 10 days	Total phenolic deteriorated	Palma et al. (2009)
Polyolephinic heat-shrinkable film + fludioxonil	Primosole	Whole fruit		8°C for 6 or 12 weeks at 90% RH; 20°C shelflife	Total polyphenols, anthocyanin decreased after shelflife	D'Aquino et al. (2010)
a) Commercial packaging (Falcon), (b) Polyethylene bags 0.03 mm (c) Vapor guard waxing (2%)	'Shlefy'	Whole fruit		5 and 7°C at 85% RH for 4 months	Polyethylene bags retained anthocyanin and vitamin C at both temperature compared to commercial packaging and vapour guard waxing	Ghafir et al. (2010)
Polyolefin films (BDF-2001 and D-955) + coating (sucrose polyester (SPE) Semperfresh™)	Garnesh	Whole fruit	–	8, 15 and 25 °C, 12 weeks	Vitamin C was retained for 12 weeks at 8°C and for 9 weeks at 15°C in polyolefin films treated than non-treated fruit	Nanda et al. (2001)

Table 5 (continued) Summary of the effects of different package films on the bioactive compounds of pomegranate fruit.

Package film	Cultivar	Fruit part	MA Composition O ₂ and CO ₂	Temperature and storage time	Effects	References
Polyolefin Film + CaCl ₂ (0%, 2% and 3%)	Wonderful	Whole fruit	CO ₂ 33.000 and O ₂ 8.000 (cm / m / 24 hr bar at 23°C 0% RH)	5±1°C, 85±5 % RH, 2 months	Fruit treated with polyolefin film plus 2% CaCl ₂ retained Ascorbic Acid, no significant in anthocyanin changes in all treatments	Abd-elghany et al. (2012)

PAPER 2

Effects of different maturity stages and growing locations on changes in biochemical and aroma volatile composition of ‘Wonderful’ pomegranate juice

Abstract

This study investigated the changes in biochemical attributes of pomegranate fruit such as total soluble solids (TSS), titratable acidity (TA), TSS/TA ratio, pH, individual compounds (organic acids and sugars) and volatile composition as affected by fruit maturity status and growing location (Kakamas, Koedoeshoek and Worcester in South Africa). Headspace solid phase microextraction coupled with gas chromatography/mass spectrometry was used for volatile analysis. A significant increase in TSS from 14.7 ± 0.6 to 17.5 ± 0.6 °Brix was observed with advancement in fruit maturity, while TA decreased from 2.1 ± 0.7 to 1.1 ± 0.3 g citric acid per 100 mL across all agro-climatic locations investigated. Fruit TSS/TA ratio and pH increased from 7.8 ± 2.6 to 16.6 ± 2.8 and from 3.3 ± 0.1 to 3.6 ± 0.2 respectively during fruit maturation across all agro-climatic locations. Fructose and glucose concentrations increased continually with fruit maturity from 69.4 ± 4.9 to 91.1 ± 4.9 g/kg and from 57.1 ± 4.7 to 84.3 ± 5.2 g/kg, respectively. A total of 13 volatile compounds were detected and identified, belonging to five chemical classes. The most abundant volatile in unripe and mid-ripe fruit was 1-hexanol, while 3-hexen-1-ol was highest at commercial maturity. Knowledge on the impact of fruit maturity and agro-climatic locations (with different altitudes) on biochemical and aroma volatile attributes of pomegranate fruit provides a useful guide for selecting farm location towards improving fruit quality and the maturity stage best for juice processing.

Keywords: *Punica granatum*; maturity; organic acids; volatile compounds; sugars

1. Introduction

Pomegranate (*Punica granatum* L.) belongs to the Punicaceae family and is increasingly cultivated around the world in parts of Europe, Asia, North Africa, the Mediterranean basin and, most recently, South Africa (Holland et al., 2009; Fawole and Opara, 2013a; Fawole and Opara, 2013b). Widespread cultivation of pomegranate is highly related to its rich and unique source of phytochemical compounds found in the juice or arils (Gil et al., 2000). The major polyphenols in pomegranate fruit are flavonoids, condensed tannins and hydrolysable tannins (Van Elswijk et al., 2004; Seeram et al., 2008). Polyphenols are an essential source of protective compounds against the damaging effects of free radicals (Cao et al., 1996). Pomegranate fruit is largely consumed fresh or used in the preparation of

juices, canned beverages, jellies and jams and in flavourings and colourings for drinks (Opara et al., 2009).

Consumption of pomegranate fruit has been associated with reduced incidence of non-communicable diseases such as cancer, cardiovascular disease and diabetes owing to its high antioxidant capacity (Caleb et al., 2012). Pomegranate fruit has long been used in folk medicine, and its utilisation is found in many ancient human cultures as food and medicinal remedy (Longtin, 2003). The reported health benefits range from eliminating parasites and worms to treatment and cure of apthae, ulcers, diarrhoea, acidosis, dysentery, haemorrhage, microbial infections and respiratory pathologies (Opara et al., 2009). The antioxidant activity of pomegranate fruit has been attributed to its high levels of total phenolic concentration (Gil et al., 2000; Borochoy-Neori et al., 2009).

Pomegranate is a non-climacteric fruit and thus does not continue to ripen after harvest. In addition to the effects of postharvest factors such as storage conditions and packaging, the postharvest quality of pomegranate fruit is influenced by preharvest factors such as maturity status, climatic conditions, genotype and season (Mirdehghan and Rahemi, 2007; Mphahlele et al., 2014). Thus, the acceptability of pomegranate fruit by consumers and processors depends on a combination of several attributes, including physical appearance (colour and size) and biochemical constituents (sugar concentration, acidity and flavour) (Al-Said et al., 2009). The composition and concentration of chemical attributes have been of interest because of their important influence on sensory properties (Al-Said et al., 2009). Studies on the effects of maturity status, season and agro-climatic conditions on the quality attributes of pomegranate have been reported (Mditshwa et al., 2013; Fawole and Opara 2013a, b).

The aroma volatile composition at different maturity stages of 'Bhagwa' and 'Ruby' pomegranate fruit was reported recently by Fawole and Opara (2013c) who observed variations in the composition and relative proportions of the aroma volatiles among fruit maturity stages for both cultivars. However, there is limited information on individual organic acids and sugars in pomegranate, including their pattern of accumulation during fruit growth and development. In addition, no report exists on the impact of different maturity stages and agro-climatic locations (with different altitudes) on the biochemical and volatile organic composition of 'Wonderful' pomegranate fruit. Thus, to obtain a better understanding of the acclimatisation and adaptation of pomegranate fruit to different altitudes and climates, this study investigated the effects of different maturity stages and growing locations with different altitudes on changes in the biochemical and aroma volatile composition of 'Wonderful' pomegranate juice. This information is important in the juice-processing industry interested in improving the flavour characteristics of pomegranate products.

2. Materials and methods

2.1 Plant material

Fruit were collected from three growing locations in 2013 in South Africa at different altitudes and maturity stages. Fruit characteristics and harvesting periods are outlined in Table 1. Climatic conditions at the three growing locations are summarised in Table 2. Fruit trees sampled in all growing locations were between 5 and 7 years old, with drip irrigation. Healthy fruit were harvested at 100, 121 and 141 days after full bloom from orchards in each of the three agro-climatic locations, transported to the laboratory and stored for less than 2 weeks at 7.5°C and 95% relative humidity before processing.

2.2 Fruit biochemical properties

For each location, a random sample of 27 fruits of uniform size was used for juice extraction. Fruit were hand-peeled and the arils juiced using a LiquaFresh juice extractor (Mellerware, Cape Town, South Africa). Fruit juice samples were then analysed in triplicate for the following biochemical properties.

2.3 Total soluble solids, titratable acidity and pH

Pomegranate juice total soluble solids (TSS, °Brix) was measured using a digital refractometer (Atago, Tokyo, Japan) calibrated with distilled water at 20°C. An 862 Compact Titrosampler (Metrohm AG, Herisau, Switzerland) was used to determine titratable acidity (TA, g citric acid (CA) per 100 mL). About 2 ml of juice sample was diluted with 70 ml of distilled water and titrated with 0.1 mol/l NaOH to an end-point of pH 8.2. The pH of pomegranate juice was measured at room temperature with a pH meter (Crison, Barcelona, Spain). Fruit maturity index was determined as the ratio TSS/TA.

2.4 Determination of biochemical properties

2.4.1. Sugars and organic acids

An Arena 20XT random access chemistry analyser (Thermo Scientific, Madison, WI, USA) was used for enzyme robot assays. Organic acids (including L-malic, succinic and citric acids) and sugar (D-glucose and D-fructose) concentrations were determined using enzymatic test kits (R-Biopharm AG, Darmstadt, Germany) by measuring the formation of NADPH at 340 nm according to the described protocol of the kits. The following methods were used:

1. L-Malic acid:

Kit used:

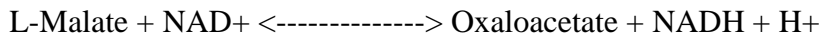
- Enzytec™ Fluid L-Malic acid Id-No: 5280

Manufacturer: Thermo Fisher Scientific Oy, Finland

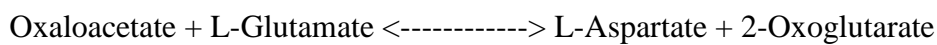
Distributed by: R-Biopharm AG, Germany.

Principle of the method:

L-MDH



GOT



L-MDH = L-Malate-dehydrogenase

GOT = Glutamate-Oxaloacetate-Transaminase

2. D-Glucose:

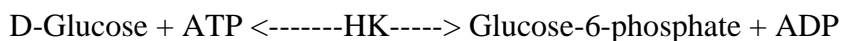
Kit used:

- Enzytec™ Fluid D-Glucose Id-No: 5140

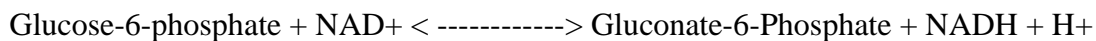
Manufacturer: Thermo Fisher Scientific Oy, Finland.

Distributed by: R-Biopharm AG, Germany.

Principle of the method:



G6P-DH



ATP = Adenosine-5'-triphosphate

HK = Hexokinase

ADP = Adenosine-5'-diphosphate

NAD⁺ = Nicotinamid-adenien-dinucleotide

3. D-Fructose:

Kit used:

- Enytec™ Fluid D-Fructose Id-No: 5120

Manufacturer: Thermo Fisher Scientific Oy, Finland.

Distributed by: R-Biopharm AG, Germany.

Principle of the method:

D-Fructose + ATP $\xrightarrow{\text{HK}}$ Fructose-6-phosphate + ADP

D-Glucose + ATP $\xrightarrow{\text{HK}}$ Glucose-6-phosphate + ADP

Fructose-6-phosphate $\xrightarrow{\text{PGI}}$ Glucose-6-phosphate

Glucose-6-phosphate + NAD⁺ $\xrightarrow{\text{G6P-DH}}$ Gluconate-6-Phosphate + NADH + H⁺

ATP = Adenosine-5'-triphosphate

HK = Hexokinase

ADP = Adenosine-5'-diphosphate

PGI = Phosphoglucose Isomerase

NAD⁺ = Nicotinamid-adenien-dinucleotide

G6P-DH = Glucose-6-phosphate dehydrogenase

4. Citric Acid:

Boehringer Mannheim / R-Biopharm Citric acid Roche Cat. No. 10139076035

Manufacturer: R-Biopharm AG, Darmstadt

Principle of the method:

Citric acid (citrate) $\xrightarrow{\text{CL}}$ oxaloacetate + acetate

Oxaloacetate + NADH + H⁺ $\xrightarrow{\text{L-MDH}}$ L-malate + NAD⁺

Pyruvate + NADH + H⁺ $\xrightarrow{\text{L-LDH}}$ L-lactate + NAD⁺

The amount of NADH oxidized in reactions is stoichiometric to the amount of citrate. NADH is determined by absorbance at 340 nm.

CL = Citrate lyase

L-MDH = L-Malate dehydrogenase

L-LDH = L-Lactate dehydrogenase

NADH = Reduced Nicotinamide-adenine dinucleotide

5. Succinic Acid:

Boehringer Mannheim / R-Biopharm Succinic acid Roche Cat. No. 10176281035

Manufacturer: R-Biopharm AG, Darmstadt

Principle of the method:

Succinate + ITP + CoA $\xrightarrow{\text{SCS}}$ IDP + Succinyl-CoA + Pi

IDP + PEP $\xrightarrow{\text{PK}}$ ITP + Pyruvate

Pyruvate + NADH + H⁺ $\xrightarrow{\text{L-LDH}}$ L-Lactate + NAD⁺

The amount of NADH oxidized in reactions is stoichiometric to the amount of succinic acid. NADH is measured by its light absorbance at 340nm.

ITP = Inosine-5'-triphosphate

CoA = Coenzyme A

SCS = Succinyl-CoA synthetase

IDP = Inosine-5'-diphosphate

Pi = Inorganic phosphate

PEP = Phosphoenolpyruvate

PK = Pyruvate kinase

NADH = Nicotinamid-adenine-dinucleotide

L-LDH = L-Lactate dehydrogenase

2.5 Aroma volatile composition

Volatile compounds were trapped and extracted from headspace vials using the headspace solid phase microextraction (HS-SPME) method described by Melgarejo et al. (2011). Aliquots of fresh pomegranate juice (10 mL) were placed in 20 mL headspace vials, followed by the addition of NaCl (300 g/L) to facilitate the evolution of volatiles into the headspace and inhibit enzymatic degradation (Caleb et al., 2013). 50 µL of 3-octanol was added into the vials as internal standard. SPME vials were equilibrated for 10 min at 50°C in an autosampler incubator at 250 × g. A 50/30 µm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS)-coated fibre was exposed to the sample headspace for 20 min at 50°C. After extraction, desorption of the volatile compounds from the fibre was carried out in the injection port of a gas chromatography/mass spectrometry (GC/MS) system for 10 min. The

fibre was inserted in a fibre-conditioning station for 15 min between samples for cleaning to prevent cross-contamination. Separation and quantification of the volatile compounds were performed using an Agilent 6890 N gas chromatograph (Palo Alto, CA, USA) coupled with an Agilent 5975 MS mass spectrometer detector (MSD). The GC/MS system was equipped with a polar DB-FFAP column (Model 122–3263, J&W Scientific, Folsom, CA, USA) with 60 m nominal length, 250 μm internal diameter and 0.5 μm film thickness. Analyses were carried out using helium as carrier gas at a flow rate of 1.9 mL/ min with nominal initial pressure of 216.3 kPa and average velocity of 36 cm/ s. The injector temperature was maintained at 250°C. The oven programme was set as follows: 70°C for 1 min, then ramped to 142°C at 3°C/ min and finally ramped to 240°C at 5°C/ min and held for 3 min. The MSD was operated in full-scan mode and the ion source and quadrupole temperatures were maintained at 230 and 150°C, respectively. The transfer line temperature was kept at 280°C. Compounds were tentatively identified by their retention time (RT) and Kovats index (KI) values and by comparison with mass spectral libraries (NIST, version 2.0) (Melgarejo et al., 2011; Caleb et al., 2013). For quantification, the calculated relative abundances were used.

3. Statistical analysis

Statistical analyses were carried out using Statistica Version 11.0 (StatSoft Inc., Tulsa, OK, USA). Data were subjected to analysis of variance (ANOVA) and means were separated by least significant difference (LSD, $P = 0.05$) according to Duncan's multiple range test. GraphPad Prism Version 4.03 (GraphPad Software, Inc., San Diego, CA, USA) was used for graphical presentations.

4. Results and discussion

4.1 Biochemical properties of pomegranate

The TSS concentration of pomegranate fruit harvested from the three agro-climatic locations is shown in Table 3. There were significant effects of maturity and altitude as well as their interaction on TSS concentration ($P < 0.001$). Fruit from semi-arid climate (Kakamas) did not exhibit any significant change in TSS concentration (15.5 ± 0.4 to 15.7 ± 0.6 °Brix) across all maturity stages. The lowest TSS concentration was observed in fruit from Koedoeshoek, characterised by subtropical climate, at unripe (14.7 ± 0.6 °Brix) and mid-ripe (14.1 ± 0.7 °Brix) stages, but it increased slightly at full-ripe stage (15.9 ± 0.6 °Brix). The highest TSS concentration was found in fruit from Mediterranean climate (Worcester) at mid-ripe (16.9 ± 0.5 °Brix) and full-ripe (17.5 ± 0.6 °Brix) stages. The TSS concentrations at full-ripe stage measured in this study (15.7 ± 0.6 to 17.5 ± 0.6 °Brix) were within the previous range

reported for cv. Wonderful at commercial harvest (14.6-17.6 °Brix) (Mena et al., 2011; Fawole and Opara, 2013d). An increase in TSS concentration with advancing fruit maturity is explained by a mechanism related to starch synthesis and sugar hydrolysis as fruit advances in maturation (Kulkarni and Aradhya, 2005). Besides, lower TSS concentration in fruit from subtropical and semi-arid climates at full-ripe stage could be driven by higher temperatures of 26.46 and 30.8°C, respectively (Table 2). In agreement with the findings in the present study, it has been reported that in cherry tomato higher temperatures (26–30°C) promoted higher fruit sugar levels; however, with increased sink competition, sugar concentration decreased, presumably owing to higher respiration at higher temperatures (Gautier et al., 2005). Lower TA was found in fruit from Mediterranean (222 m; 1.1±0.4 g CA per 100 mL) compared with fruit from semi-arid (662 m; 1.6±0.4 g CA per 100 mL) and subtropical (898 m; 2.1±0.7 g CA per 100 mL) climates at unripe stage. However, TA declined at mid-ripe stage across in fruit from semi arid and subtropical climate whereas those from mediterranean climate remained relatively stable, and did not vary significantly at full-ripe stage ($P>0.05$).

The maturity index TSS/TA is responsible for pomegranate juice taste and flavour, and some authors have used it as a reliable indicator of fruit maturity for classifying pomegranate cultivars (Martinez et al., 2006; Cam et al., 2009; Hasnaoui et al., 2011). There were significant interaction effect on TSS/TA across all maturity stages and agro-climatic locations ($P<0.05$) (Table 3). The TSS/TA ratio ranged between 7.8±2.6 and 10.2±2.2 at unripe stage and then increased gradually with advancing fruit maturation across all agro-climatic locations. Fruit from the semi-arid climate had the highest TSS/TA ratio (16.8±3.6) at mid-ripe stage, followed by fruit from Mediterranean (13.4±2.3) and subtropical (10.7±2.7) climates. However, a different pattern was observed at full-ripe stage, where a slight but notable difference in TSS/TA was observed in fruit from Mediterranean (16.6±2.8) and semi-arid (14.2±2.7) locations, with the lowest ratio found in fruit from subtropical (12.2±3.5) location. Fruit grown in Mediterranean location (222 m) exhibited significantly higher TSS and than fruit grown in semi-arid (662 m) and subtropical (898 m) locations at commercial harvest. A similar trend was observed in 11 Israeli accessions, with fruit grown in Mediterranean climate having significantly higher TSS than fruit grown in desert climate (Schwartz et al., 2009). This suggests that Mediterranean climate could favour rapid accumulation of TSS. Harvest period can also affect TSS, since TSS concentration was previously observed to increase during maturation and ripening of pomegranate fruit (Shwartz et al., 2009). The pH level, which characterises the acidic taste of fruit (Cemeroglu et al., 1992) did not vary at unripe stage across all agro-climatic locations, with values ranging between 3.3±0.1 and 3.4±0.1. Both maturity and altitude had a significant interactive effect on pH ($P<0.0001$).

At mid-ripe stage, the lowest pH was found in fruit from Mediterranean (2.8 ± 0.2) and subtropical (3.0 ± 0.09) climates, while the highest pH level (3.3 ± 0.1) was observed in fruit from semi-arid climate. Furthermore, fruit from subtropical climate did not exhibit significant variation in pH at full-ripe stage, while a slight increase was observed in fruit from Mediterranean and semi-arid climates, with pH values averaging 3.4 ± 0.4 and 3.6 ± 0.2 , respectively.

4.2 Concentrations of individual organic acids

Concentrations of individual organic acids in 'Wonderful' pomegranate juice are shown in Fig. 1. Citric, malic and succinic acids were the major organic acids identified at different maturity stages and across different agro-climatic locations, while acetic acid was below the detection limit in this study. The altitude, maturity, and their interaction had a significant effect on the citric acid concentration ($P < 0.0001$) (Fig. 1A). Citric acid concentrations ranged from 5.0 ± 1.5 to 5.7 ± 0.1 g/kg at the unripe stage and increased considerably during fruit maturation across all agro-climatic locations. At mid-ripe stage, the concentration of citric acid was higher in fruit from semi-arid (19.9 ± 3.6 g/kg) and Mediterranean (21.4 ± 4.2 g/kg) locations than in fruit from subtropical location (12.9 ± 3.6 g/kg). However, a subsequent decrease to 12.0 ± 3.4 and 14.3 ± 1.47 g/kg was observed in fruit from semi-arid and Mediterranean locations, respectively, which did not differ significantly from that in fruit from subtropical location (15.98 ± 4.31 g/kg) at full-ripe stage. In line with the findings of the present study, citric acid has been reported as the major organic acid in various pomegranate cultivars at commercial harvest (Fawole and Opara, 2013a; Fawole and Opara, 2013b; Gundogdu and Yilmaz, 2012; Poyrazoglu et al., 2012). Citric acid concentrations measured in this study (14.3 ± 1.5 to 16.0 ± 4.3 g/kg) were within the range reported for pomegranate cv. Wonderful at commercial maturity (3.85–18.54 g/L) (Mena et al., 2011). It was previously suggested that the major acid in pomegranate juice accounting for TA is citric acid (Melgarejo et al., 2000).

There was a significant effect of altitude and maturity, as well as their interaction ($P = 0.0022$) on the L-malic acid concentration (Fig. 1B). Malic acid concentration did not vary significantly ($P > 0.05$) at unripe and mid-ripe stages in fruit from subtropical and Mediterranean locations, while that in fruit from semi-arid location did not change across all maturity stages (Fig. 1B). However, malic acid concentration increased in fruit from subtropical (0.6 ± 0.1 g/kg) and Mediterranean (0.7 ± 0.2 g/kg) locations at full-ripe stage. This is contrary to Fawole and Opara (2013a) who reported a decrease in malic acid with advancing ripeness of cv. Bhagwa. The current finding, however, is supported by Shwartz et al. (2009) who observed an increase in malic acid concentration during fruit development in pomegranate accessions 121–2 (a landrace of 'Rosh-Hapered') and 101–2 (a landrace of 'Wonderful')

grown in Israel. With regard to malic acid concentration found in fruit from different agro-climatic locations with different altitudes, it has been reported that the activity of malic enzyme responsible for catalysing the conversion of malate to pyruvate is directly related to temperature, hence high temperatures would increase malic enzyme activity (Lakso and Kliewer, 1975). Therefore it is logical to suggest that the higher malic acid concentration found in fruit from lowest altitude could be attributed to its lower growth temperature ($\sim 24^{\circ}\text{C}$) compared with fruit from semi-arid ($\sim 30^{\circ}\text{C}$) and subtropical ($\sim 26^{\circ}\text{C}$) locations.

There were significant effects of altitude, maturity, and altitude x maturity interaction ($P < 0.0006$) on the succinic acid concentration (Fig. 1C). Succinic acid exhibited a steady increase with fruit maturation across all investigated agro-climatic locations. However, higher succinic acid concentration was found in fruit from subtropical and Mediterranean climates at full-ripe stage compared with fruit from semi-arid climate. Higher total acid concentration was observed in fruit from semi-arid and Mediterranean locations at mid-ripe stage, but it decreased significantly at full-ripe stage to a level similar to that found in fruit from subtropical location. Generally, the observed decrease in acidity with ripening could be attributed to an array of factors, such as increased respiration, reduced translocation of acids from leaves, transformation of acids to other compounds, dilution due to increased volume of fruit, and reduced ability of fruit to synthesise acids with maturity (Diakou et al., 2000; Moing et al., 2001).

4.3 Concentrations of individual sugars

Concentrations of individual sugars in pomegranate juice harvested at different maturity stages and agro-climatic locations are shown in Fig. 2. Fructose and glucose were detected at all maturity stages and agro-climatic locations, while sucrose was below the limit of detection in the investigated cultivar. The altitude, maturity and their interaction ($P < 0.05$) had significant effects on the concentration of fructose, glucose, total sugars and glucose and fructose ratio (G/F). Fructose concentration did not vary at unripe and mid-ripe stages across all locations. There was a gradual increase in fructose at full-ripe stage to levels ranging from 78.4 ± 2.0 to 91.1 ± 4.9 g/kg, with the highest concentration found in fruit from Mediterranean location (Fig. 2A). In the case of glucose, significantly higher concentration was observed in fruit from semi-arid location (63.69 ± 3.6 g/kg) at unripe stage compared with fruit from subtropical (58.7 ± 3.6 g/kg) and Mediterranean (57.1 ± 4.8 g/kg) locations. However, glucose concentration was stable at mid-ripe stage in fruit from semi-arid location (662 m), but did not differ from that in fruit from subtropical (898 m) and Mediterranean (222 m) locations. Higher glucose concentration was observed in fruit from Mediterranean location (84.2 ± 5.2 g/kg) at

full-ripe stage than in fruit from subtropical (73.2 ± 3.8 g/kg) and semi-arid (74.5 ± 3.31 g/kg) locations. Fructose and glucose were found to be the most dominant sugars in this study across the investigated agro-climatic locations. Higher concentrations of fructose and sucrose than those reported in this study were observed in ‘Bhagwa’ pomegranate grown in South Africa (Fawole and Opara, 2013a) and in Spanish cultivars ‘Mollar de Elche’ and ‘C25’ (Carbonell-Barrachina et al., 2012). Several studies reported slightly higher glucose than fructose concentration (Gautier et al., 2005; Ozgen et al., 2008). As can be observed in Fig. 2C, fruit from Mediterranean climate showed higher total sugar concentration (175.3 ± 10.1 g/kg) at full-ripe stage than fruit from subtropical (153.5 ± 7.1 g/kg) and semi-arid (152.9 ± 4.7 g/kg) climates, highlighting the significant influence of agro-climatic location on the level of sugars in the investigated cultivar. The higher TSS concentration in fruit from Mediterranean climate was in accordance with the glucose and fructose levels in juice, suggesting that sugars are the main soluble solids in pomegranate juice. Although the glucose/fructose ratio varied significantly across all investigated locations and maturity stages, it was higher in fruit from semi-arid location (1.0 ± 0.4) than in fruit from Mediterranean (0.9 ± 0.1) and subtropical (0.9 ± 0.1) locations at full-ripe stage. It has been reported that fructose is twice as sweet as glucose (Nookaraju et al., 2010), and could be used as a measure of degree of juice sweetness during fruit ripening (Al-Maiman and Ahmad, 2002).

4.4 Aroma volatile composition

The relative abundances (%) of volatile organic compounds (VOCs) are presented in Table 4. A total of 13 volatile compounds belonging to the chemical classes of aldehydes, alcohols and monoterpenes were detected. Different VOC profiles were observed during fruit maturation across all investigated growing locations. It has been shown that pomegranate fruit has low concentrations of volatile compounds, resulting in lower intensities of VOCs of the fruit parts (Carbonell-Barrachina et al., 2012). There were significant effects ($P < 0.05$) of maturity and altitude on the VOCs. Most of the identified VOCs were below 1% during fruit maturation across all agro-climatic locations. As can be observed, alcohols were in relatively higher abundance, representing 0.22–0.54% of the VOCs in pomegranate fruit. Generally, 1-hexanol was prominent at unripe stage, with a higher amount found in fruit from Mediterranean location at lower altitude (222 m; 0.54%) than in fruit from subtropical (898 m; 0.37%) and semi-arid (662 m; 0.29%) locations. A slight increase in 1-hexanol was observed in fruit from subtropical location (0.54%) at mid-ripe stage compared with fruit from semi-arid (0.22%) and

Mediterranean (0.32%) locations. However, 1-hexanol was not detected at full-ripe stage across all locations.

Less 3-hexen-1-ol was detected in fruit from Mediterranean location (0.01%) than in fruit from semi-arid (0.09%) and subtropical (0.14%) locations at unripe stage. A different pattern was observed at mid-ripe stage, with a higher level found in fruit from subtropical (0.21%) and Mediterranean (0.19%) locations than in fruit from semi-arid location (0.07%). However, 1-hexanol and 3-hexen-1-ol were not detected in fruit from Mediterranean location at full-ripe stage, while 3-hexen-1-ol remained relatively lower in fruit from subtropical location at full-ripe stage. In contrast to our study, 1-hexanol was reported to be the predominant volatile in Iranian cultivar 'Berit Kazeroon' at commercial harvest (Raisi et al., 2008). Limonene was detected across all maturity stages and agro-climatic locations in relative abundances between 0.004 and 0.01%. Alpha-Terpeneol was found in fruit from semi-arid location at unripe and mid-ripe stages, while it was found at mid-ripe and full-ripe stages in fruit from subtropical and Mediterranean locations. Relative abundances of α -terpeneol across locations and corresponding maturity stages ranged between 0.02 and 0.05%. Limonene and α -terpeneol belong to the monoterpene family. Generally, monoterpenes can be related to pine, lemon and citrus notes. Limonene and α -terpeneol were previously reported to be the main volatile compounds contributing to the aroma and odour profile of cv. Wonderful (Vázquez-Araújo et al., 2011). Andreu-Sevilla et al. (2013) found that limonene was the main compound in three pomegranate juices, representing about 55% of the total concentration of volatiles in the headspace of cultivars 'Wonderful' and 'Mollar de Elche'. Similarly, Caleb et al. (2012) found limonene and α -terpeneol to be present at very low concentrations in pomegranate cultivars 'Acco' and 'Herskawitz'. Generally, fruit from Mediterranean climate consists mainly of alcohols, ketones and monoterpenes at full-ripe stage, while fruit from semi-arid and subtropical locations contains only two chemical families (alcohols and monoterpenes).

5. Conclusions

This study showed that fruit maturity status and growing location had significant influences on the biochemical properties as well as composition and concentration of aroma volatile compounds in pomegranate cv. Wonderful. Only one alcohol, 1-hexanol, predominated the volatile profile, but it was not detected in fruit at full-ripe stage. On the other hand, monoterpenes (limonene and alpha-terpeneol) were detected across all fruit maturity stages and altitudes, indicating that they are key aroma volatiles in the investigated cultivar, though lower relative abundances of these volatiles were observed. This highlights the importance of harvesting pomegranate fruit at optimal maturity and the need for a comprehensive study of other cultivars. Furthermore, fruit from lower altitude (222 m), characterised

by Mediterranean climate, had higher TSS, glucose and fructose concentrations than fruit from semi-arid and subtropical climates at commercial harvest. The findings suggest that Mediterranean climate favours the synthesis and accumulation of biochemical attributes in pomegranate fruit. The concentration of citric acid was significantly higher than that of malic and succinic acids across all maturity stages which shows that citric acid form major component of pomegranate juice. The present study highlights the need to incorporate flavour attributes into traditional maturity assessments for pomegranate. Thus, further research is required in this area given the importance of flavour in consumer perception of the quality of pomegranate arils.

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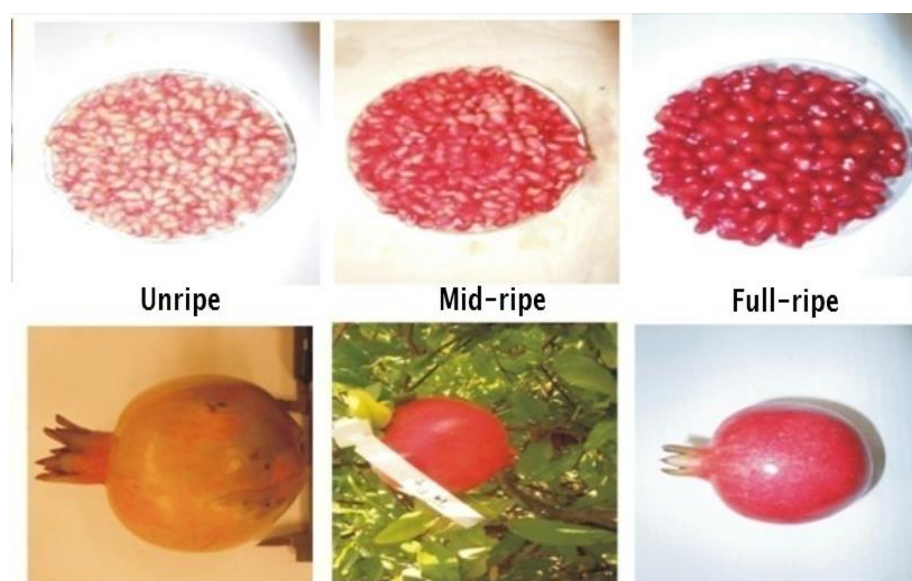
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Table 1

Description of the selected maturity stages of ‘Wonderful’ pomegranate fruit.

DAFB	Maturity stage	Fruit characteristics
100	Unripe	Mature: mature light-red arils with mature kernels
121	Mid-ripe	Mature: red skin, mature red arils with mature kernels
141	Full-ripe	Commercial harvest; deep-red skin, deep red arils with mature kernels

DAFB, Days after full bloom.



Schematic representation of maturity stages of ‘Wonderful’ pomegranate fruit.

Table 2

Climatic conditions at three different pomegranate (cv. Wonderful) growing locations in South Africa.

Altitude (growing location) (m)	Biome	Longitude E	Latitude S	Average rainfall (mm)	Minimum temperature (°C)	Maximum temperature (°C)	Light intensity (MJ/m ²)
662 (Kakamas)	Semi-arid	20°38' 00''	28° 45' 00''	0.34	11.08	30.80	23.14
898 (Koedoeshoek)	Sub-tropical	30° 30' 45.3''	25°23' 38.6''	41.47	11.07	26.46	13.40
222 (Worcester)	Mediterranean	19° 26' 00''	33° 39' 00''	1.19	9.72	24.55	19.06

Source: \\ http:www.arc.agric.za/arc-iscw; data were daily averages for the growing season.

Rainfall data were averages for the growing season.

Table 3

Biochemical properties of pomegranate (cv. Wonderful) juice harvested at three different altitudes and maturity stages.

Growing location	Altitude (m)	Maturity stage	TSS (°Brix)	TA (g citric acid 100/ mL)	TSS:TA	pH
Unripe						
Kakamas	662		15.5±0.4bc	1.6±0.4b	10.2±2.2cd	3.4±0.1ab
Koedoeshoek	898		14.7±0.6e	2.1±0.7a	7.8±2.6d	3.3±0.1b
Worcester	222		14.9±0.1cd	1.1±0.4a	7.8±1.3d	3.3±0.1b
Mid-ripe						
Kakamas	662		15.5±0.6bcd	1.0±0.21e	16.8±3.6a	3.3±0.1b
Koedoeshoek	898		14.1±0.7cde	1.5±0.4bc	10.7±2.7c	3.0±0.9c
Worcester	222		16.9±0.5a	1.3±0.2b-e	13.4±2.3b	2.8±0.2c
Full-ripe						
Kakamas	662		15.7±0.6b	1.1±0.3cde	14.2±2.7ab	3.6±0.2a
Koedoeshoek	898		15.9±0.6b	1.3±0.3bcd	12.2±3.5bc	2.8±0.1c
Worcester	222		17.5±0.6a	1.1±0.2de	16.6±2.8a	3.4±0.4ab
<i>P</i> -value						
Maturity (M)			<0.0001	<0.0001	<0.0001	<0.0001
Altitude (A)			<0.0001	<0.0004	<0.0001	<0.0001
M*A			<0.0001	0.4933	<0.0108	<0.0001

Mean ± Standard deviation are presented; values within a column followed by a different letter are significantly different ($P < 0.05$) according to Duncan's multiple range test. A, altitude; M= maturity; A1, 662 m (Kakamas); A2, 898 m (Koedoeshoek); A3, 222 m (Worcester).

Table 4

Volatile organic compounds of pomegranate (cv. Wonderful) juice at different maturity stage harvested from different agro-climatic locations.

Volatile compound(s)	RT (min)	Est. K index	Lit. K index	Kakamas (662 m)			Koedoeshoek (898 m)			Worcester (222 m)		
				Unripe	Mid ripe	Full ripe	Unripe	Mid ripe	Full ripe	Unripe	Mid ripe	Full ripe
3-Buten-2-ol, 2-methyl-	6.1	603	600	0.013 ± 0.003	–	–	0.015 ^a ±0.003	0.014 ^a ±0.003	–	0.03 ± 0.008	–	–
limonene	10.3	1018	1019	0.005 ^b ± 0.0003	0.009 ^a ± 0.006	0.01 ^a ± 0.004	0.004 ^b ± 0.0001	0.008 ^a ± 0.0004	0.006 ^b ± 0.002	0.004 ^b ±0.0001	0.008 ^a ±0.0005	0.007 ^a ±0.001
Eucalyptol	10.5	1059	1033	–	–	–	–	0.005 ± 0.0008	–	–	–	–
E-2-Hexenal	11.2	806	800	–	0.01 ± 0.003	–	0.01 ^b ± 0.0001	0.02 ^a ± 0.001	–	0.04 ^a ± 0.003	0.03 ^b ± 0.002	–
1-Pentanol	11.6	761	764	–	–	–	–	–	–	0.003 ^a ± 0.0001	–	0.002 ^a ± 0.001
2-Buten-1-ol, 2-methyl-	14.2	746	754	0.005 ± 0.0002	–	–	–	–	–	–	–	–
1-Hexanol	15.3	860	858	0.29 ^b ± 0.08	0.22 ^b ± 0.05	–	0.37 ^b ± 0.07	0.54 ^a ± 0.09	–	0.54 ^a ± 0.09	0.32 ^b ± 0.06	–
Z-3-Hexen-1-ol	15.7	868	857	0.09 ^a ± 0.009	0.07 ^b ± 0.01	0.1 ^a ± 0.009	0.14 ^{ab} ± 0.05	0.21 ^a ± 0.06	0.1 ^b ± 0.008	0.01b±0.003	0.19 ^a ± 0.05	–
2-Nonanone	16.9	1052	1069	–	–	–	–	0.04 ± 0.002	–	–	–	0.04 ± 0.004
1-Octanol	23.2	1061	1059	0.003 ± 0.0009	–	–	0.002 ± 0.0007	–	–	–	–	–
4-Terpineol	25.3	1137	1177	–	0.006 ^a ±0.0006	0.005 ^a ±0.0009	0.0013 ^a ±0.0001	0.005 ^b ±0.0007	–	–	0.003 ^a ± 0.0007	0.003 ^a ± 0.0007
p-Menthan-3-ol	26.5	1164	1150	–	0.01 ± 0.005	–	–	0.006 ± 0.0006	–	–	–	–
alpha-Terpineol	28.6	1143	1143	0.02 ^b ± 0.003	0.04 ^a ± 0.009	0.04 ^a ± 0.008	0.02 ^b ± 0.003	0.05 ^a ± 0.008	0.02 ^b ± 0.008	0.02 ^{ab} ± 0.006	0.03 ^a ± 0.009	0.03 ^a ± 0.008

*RT, retention time. Est. K. index, estimated kovats index. Lit. K. index, literature kovats index (NIST library, version 2). Mean ± Standard deviation presented. 3-octanol was used as a standard. Values within the same row followed by a different letter are significantly different ($P < 0.05$) according to Duncan's multiple range test (VOCs for the same location were compared). VOCs, volatile organic compounds.

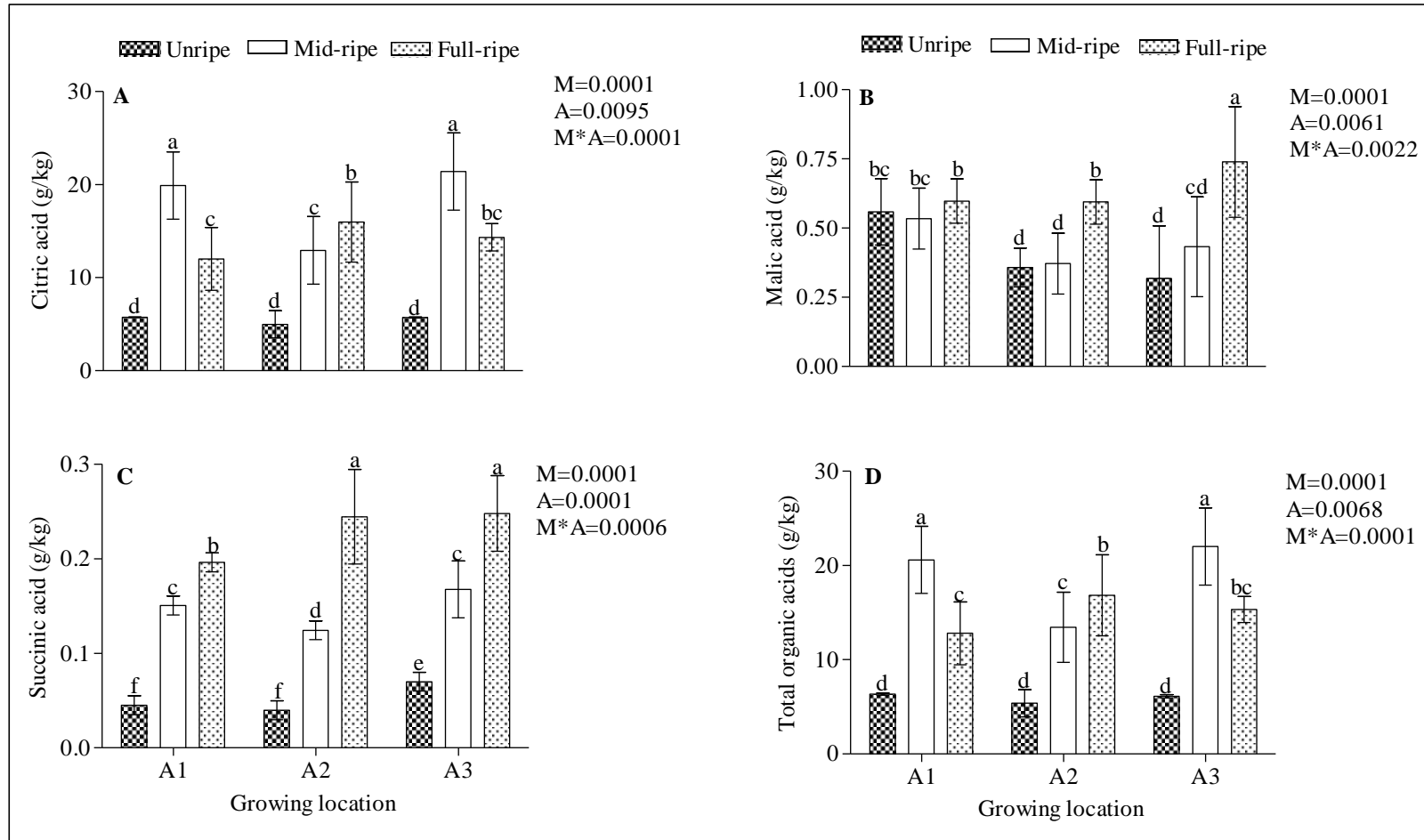


Fig. 1. Organic acid concentration of pomegranate (cv. Wonderful) juice harvested at three different altitudes and maturity stages: (A) citric acid; (B) malic acid; (C) succinic acid; (D) total organic acids. Values are presented as mean±standard deviation. Means with different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test. A, altitude; M, maturity; A1, 662 m (Kakamas); A2, 898 m (Koedoeshoek); A3, 222 m (Worcester).

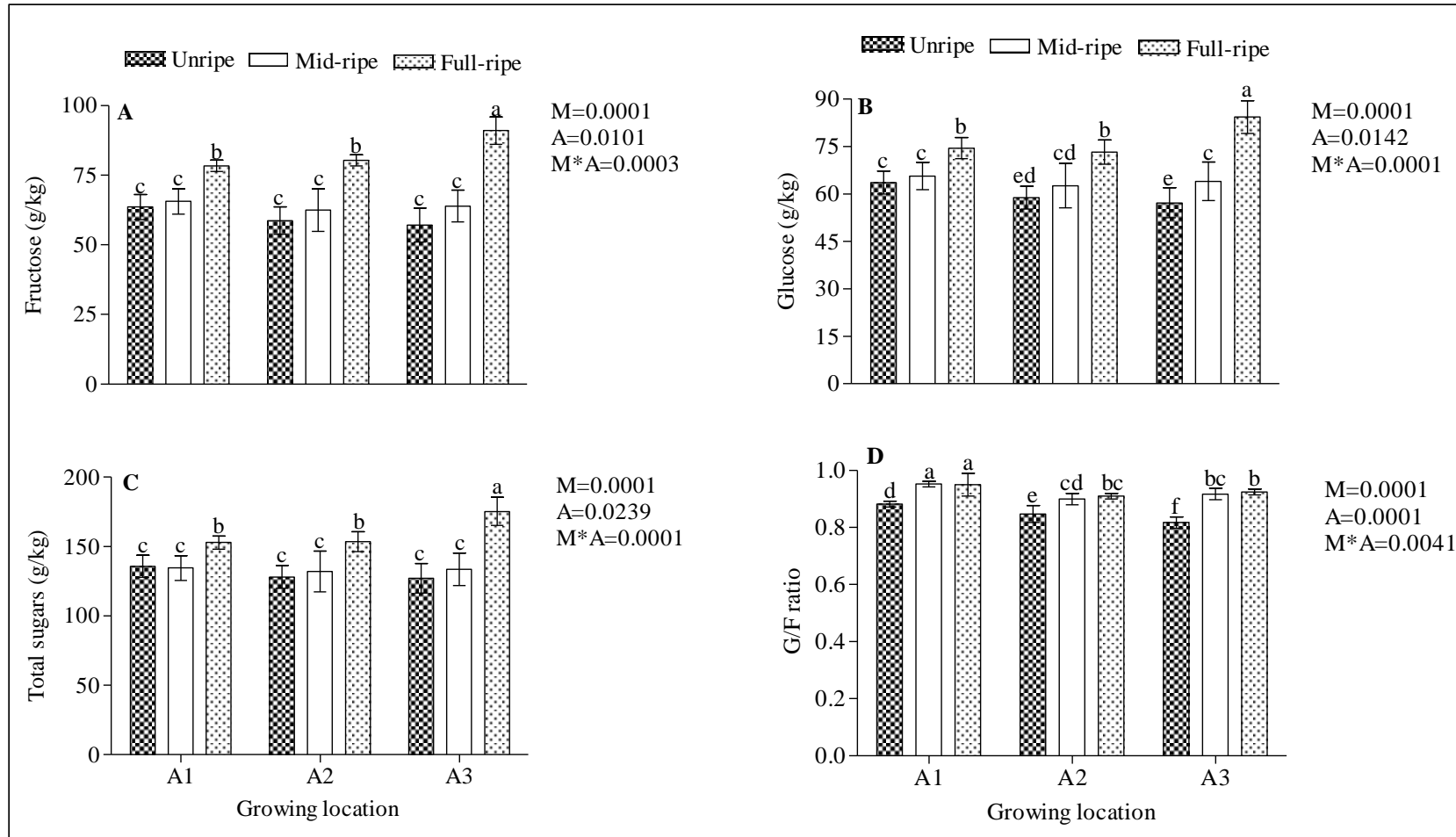


Fig. 2. Sugar concentration of pomegranate (cv.Wonderful) juice harvested at three different altitudes and maturity stages: (A) fructose; (B) glucose; (C) total sugars; (D) glucose/fructose (G/F) ratio. Values are presented as mean±standard deviation (n=3). Means with different letters are significantly different (P<0.05) according to Duncan's multiple range test. A, altitude; M, maturity; A1, 662 m (Kakamas); A2, 898 m (Koedoeshoek); A3, 222 m (Worcester).

PAPER 3

Effect of fruit maturity and growing location on the postharvest concentrations of flavonoids, phenolic acids, vitamin C and antioxidant activity of pomegranate juice (cv. Wonderful)

Abstract

Pomegranate fruit (*Punica granatum* L.) production and consumption have increased recently due to increasing scientific evidence on its high content of health beneficial compounds. This study was conducted to investigate the phytochemical concentrations and antioxidant activity of pomegranates (cv. Wonderful) as affected by fruit maturation and growing location. High performance liquid chromatography (HPLC) coupled with liquid chromatography mass-spectrometry (LC-MS) and liquid chromatography mass-spectrometry electrospray (LC-MS^E) were used to analyse phenolic composition at different maturity stages. Catechin, epicatechin and naringin were the most dominant flavonoids irrespective of maturity and altitude, while gallic acid was the dominant phenolic acid. The concentrations of total phenolics and total tannins as well as radical scavenging activity (RSA) in DPPH assay declined as maturity advanced while ferric reducing antioxidant power (FRAP), total anthocyanin, total flavonoid and vitamin C increased significantly ($P < 0.01$). There was a significant and negative correlation ($r = -0.64$) between total phenolic concentration and antioxidant activity in the FRAP assay. Principal component analysis (PCA) showed that fruit grown in area with lower altitude were associated with higher bioactive compounds at full ripe stage. Furthermore, PCA plot also revealed that fruit growing location had a significant and prominent impact on the bioactive compounds than maturity status.

Keywords: Antioxidant activity; HPLC; Altitude; Maturity; Pomegranate; Phenolic compound

1. Introduction

Pomegranate fruit (*Punica granatum* L.) is a good source of phenolic compounds including flavonoids (anthocyanins, flavonols), condensed tannins (proanthocyanadins) and hydrolysable tannins (ellagitannins and gallotannins) (Hernandez et al., 1999; Gil et al., 2000; Li et al., 2006). These compounds play a significant role in fruit colour, flavour, texture and antioxidant activities (Hernandez et al., 1999; Tomas-Barberan and Espin, 2001). Although pomegranate was used extensively in folk medicine, recent studies have demonstrated that high consumption of pomegranate fruit and other products is associated with reduced risk of chronic diseases such as

cancers and cardiovascular disease (Viuda-Martos et al., 2010; Facial and Ocalhau, 2011). This association is often attributed to the exceptionally high antioxidant capacity which is linked with high phenolic composition in the juice (Gil et al., 2000; Fischer et al., 2011).

Globally, pomegranate is popularly consumed as fresh aril or processed product (Opara et al., 2009), and there is an increasing interest among consumers because of the potential benefit in human diet. The biosynthesis and accumulation of phenolic compounds can be an endogenously controlled process during developmental differentiation (Strack, 1997). Also, differences in concentration and quantities of phenolic compounds in fruit depend on a number of factors such as genotype, pre-harvest environmental conditions as well as the degree of maturity at harvest (Mirdehghan and Rahemi, 2007; Caleb et al., 2012). For instance, during fruit maturation of 'Bhagwa' pomegranate, Fawole and Opara (2013a) reported decreases in catechin and epicatechin concentrations. On the other hand, Borochoy-Neori et al. (2009) reported an inverse relationship between anthocyanin accumulation in the pomegranate arils and season temperature. Other studies reported relationships between chemical concentration of pomegranate and other type of fresh produce and elevation of the growing location. Mditshwa et al. (2013) found that fruit grown in high altitude locations and high light intensity had significantly higher vitamin C concentration than those from low altitude locations and low light intensity conditions. Higher elevation and high light intensity were reported to increase soluble phenolic and flavonols and antioxidant capacity of grape berries and bilberry leaves (Pereira et al., 2006; Martz et al., 2010).

Antioxidant levels vary considerably among fruit maturity stages and cultivars. Considering quantitative changes in concentrations of total bioactive compound, unripe fruit have been reported having the highest levels of bioactivities, which decreased at the semi-mature stage, and remained relatively unchanged at commercial harvest maturity (Dragovic-uzelac et al., 2007). Change in antioxidant activity of pomegranate juice during fruit maturity is directly related to the concentrations of bioactive compounds in the juice (Shwartz et al., 2009; Fawole et al., 2012). 'Wonderful' pomegranate is the most widely cultivated pomegranate cultivar due to its best combination of yield and quality. However, little is reported on the composition of phenolic acids and flavonoids during fruit maturity in different growing locations. To date, previous studies focused on the phenolic compounds of 'Bhagwa' and 'Ruby' cultivars (Fawole and Opara, 2013a, b; Mditshwa et al., 2013). The aim of this study was to investigate the effect of maturity stages and growing locations on flavonoids, phenolic acids and antioxidant activity of pomegranate juice (cv. Wonderful). This study is important to the beverage industry and consumers looking for fruit juice with high functional qualities.

2. Materials and methods

2.1. Plant material

Fruit were collected from three growing locations in South Africa at different maturity stages and altitudes (Tables 1 and 2). Sampled trees in all growing locations were between 5 and 7 years, with drip irrigation. Healthy fruit per orchard were harvested during the same week (25–27 February, 18–21 March and 8–10 April 2013) at 21 days interval, transported to the laboratory and stored for less than two weeks at 7.5°C, 95% RH before processing.

2.2. Sample preparation

For each location a random sample of 27 fruit of uniform size was used for juice extraction. At each sampling date, juice was obtained individually from nine healthy fruit ($n = 9$). Fruit were hand-peeled and the arils juiced using a LiquetaFresh juice extractor (Mellerware, South Africa). All samples were centrifuged at $4000 \times g$ for 10 min and the supernatants were filtered through a 0.45 μm nylon membrane (Waters Corporation) filter before HPLC analysis. Phenolic compounds were measured in triplicate of juice and results were presented as mean \pm SE.

2.3. Phytochemical analysis

2.3.1. Determination of flavonoid and phenolic acids by LC–MS and LC–MS^E at different maturity stages

LC–MS and LC–MS^E analyses were conducted on a Waters Synapt G2 quadrupole time-of-flight mass spectrometer system (Milford, MA, USA). The instrument was connected to a Waters Acquity ultra-performance liquid chromatography (UPLC) and Acquity photo diode array (PDA) detector. Ionisation was achieved with an electrospray source using a cone voltage of 15 V and capillary voltage of 2.5 kV using negative mode for analysis of phenolic compounds. Nitrogen was used as the desolvation gas, at a flow rate of 650 L/h and desolvation temperature of 275°C. The separations were carried on a waters UPLC BEH C18 column (2.1×50 mm, 1.7 μm particle size), with injection volume of 3 μL at flow rate of 0.4 ml/min. The gradient for the analysis of phenolic compounds started with 100% using 0.1% (v/v) formic acid (solvent A) and kept at 100% for 0.5 min, followed by a linear gradient to 22% acetonitrile (solvent B) over 2.5 min, 44% solvent B over 4 min and finally to 100% solvent B over 5 min. The column was subjected to 100% solvent B for an extra 2 min. The column was then re-equilibrated over 1 min to yield a total run time of 15 min.

Reference standards (Sigma-Aldrich, South Africa) of flavonoids and phenolic acids were used for the quantification of individual compounds in pomegranate juice (PJ).

2.3.2. Determination of total phenolic concentration

Total phenolic concentration (TPC) was measured using the Folin–Ciocalteu (Folin–C) method as described by Makkar (2000) with slight modification (Fawole et al., 2012). In a test tube, diluted PJ extract (50 μ L) was mixed with 450 μ L of 50% methanol followed by the addition of 500 μ L Folin–C and then sodium carbonate (2%) solution after 2 min. The mixture was vortexed and absorbance read at 725 nm using a UV–visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). Gallic acid standard curve (0.02–0.10 mg/mL) was used and TPC was expressed as milligram gallic acid equivalent per 100 mL PJ (mg GAE/100 mL PJ).

2.3.3. Determination of total tannin concentration

Total tannin analysis was carried out using Folin–C method described by Makkar (2000). Polyvinylpyrrolidone (PVPP) was used to separate tannin from non-tannin compound in PJ by adding 100 mg of PVPP to 1.0 mL of distilled water and 1.0 mL PJ in a test tube. The mixture was vortexed and kept at 4°C for 15 min followed by centrifugation at 4000 \times g for 10 min. After the extraction, 50 μ L of supernatant was mixed with 450 μ L of 50% methanol followed by the addition of 500 μ L Folin–C and then sodium carbonate (2%) solution after 2 min. The absorbance was recorded at 725 nm using UV–visible spectrophotometer after incubation for 40 min at room temperature. Separate juice extract not treated with PVPP was measured for total phenolic concentration. Total tannin concentration was calculated as:

$$\text{total tannin concentration (TTC)} = \text{TPC}_{(\text{in juice without PVPP})} - \text{TPC}_{(\text{in juice treated with PVPP})} \quad (1)$$

where TPC referred to total phenolic concentration (mg GAE/100 mL PJ). Results were expressed as milligram gallic acid equivalent per 100 mL PJ (mg GAE/100 mL PJ).

2.3.4. Determination of total flavonoid concentration

Total flavonoid concentration was measured spectrophotometrically as described by Yang et al. (2009). PJ (1 mL) was extracted with 50% methanol (10 mL) and vortexed for 30 s. The mixture was sonicated in an ultrasonic bath for 10 min and centrifuged at 4000 \times g for 12 min at 4°C. Distilled water (1.2 mL) was added to 250 μ L of extracted PJ and then followed by 75 μ L of 5% sodium nitrite. After 5 min, freshly prepared 10% aluminium chloride (150 μ L) was added to the

mixture, followed by the addition of 500 μL sodium hydroxide after a another 5 min, and 775 μL distilled water bringing the final volume to 3 mL. The mixture was vortexed and absorbance was immediately read using spectrophotometer at 510 nm. Catechin (0.025–0.125 mg/mL) was used for the standard curve. The results were expressed as catechin equivalent per 100 ml PJ (mg CE/100 mL PJ).

2.3.5. Determination of total monomeric anthocyanin concentration

The pH differential method described by Giusti and Wrolstad (2001) was used to determine total monomeric anthocyanin concentration. PJ (1 mL) was extracted with of 50% methanol (14 mL) by sonication for 5 min and followed by centrifugation at $4000 \times g$ for 12 min. Juice supernatant (1 mL) was taken into vials and diluted with 7 mL of potassium chloride buffer (pH 1.0) and sodium acetate buffer (pH 4.5), separately. After 10 min absorbance values of each buffer mixture was measured at 510 nm and 700 nm in a UV–visible spectrophotometer. Results were expressed as milligram cyanidin-3-glucoside equivalent per 100 mL pomegranate juice (mg C3gE/100 mL PJ).

$$A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5} \quad (1)$$

$$\text{Monomeric Anthocyanin Concentration (MAC)} = \frac{(A \times MW \times DF)}{\epsilon \times L} \quad (2)$$

where A = absorbance values at 510 nm and 700 nm, ϵ = cyanidin-3-glucoside molar absorbance (26,900), MW = cyanidin-3-glucoside molecular weight (449.2 g/ mol), DF = dilution factor, L = cell path length (1 cm).

2.4.6. Determination of ascorbic acid concentration

Ascorbic acid was determined according to Klein and Perry (1982) with slight modifications (Barros et al., 2007). Briefly, PJ (1.0 mL) was mixed with 14 mL of 1% metaphosphoric acid followed by sonication on ice for 4 min and centrifugation at $4000 \times g$ for 12 min. Supernatant (1.0 mL) was pipetted into a tube and mixed with 9 ml of 2,6 dichlorophenolindophenol dye (0.0025%). The mixture was incubated in the dark for 10 min before absorbance was measured at 515 nm. Calibration curve of authentic L-ascorbic acid (0.01 – 0.1 $\mu\text{g/mL}$) was used to calculate ascorbic acid concentration. Results were expressed as ascorbic acid equivalents per millilitre crude juice (μg AAE/mL PJ).

2.4. Antioxidant assays

2.4.1. Radical scavenging activity (RSA)

The ability of PJ to scavenge the 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical was measured following the procedure described by Karioti et al. (2004) with slight modifications (Fawole et al., 2012). In Eppendorf tubes, PJ extract (15 μ L) was mixed with 735 μ L methanol and 0.1 mM solution of DPPH (750 μ L) dissolved in methanol. The mixture was incubated for 30 min in the dark at room temperature before measuring the absorbance at 517 nm using a UV–visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). The RSA was determined by ascorbic acid standard curve (0–2000 μ M). The results were presented as micro molar ascorbic acid (AA) equivalent per millilitre of crude pomegranate juice (μ M AAE/mL PJ).

2.4.2. Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power assay was performed according to the method of Benzie and Strain (1996). FRAP solutions contained 25 mL acetate buffer (300 mM acetate buffer, pH 3.6), 2.5 mL (10 mM of TPTZ solution), 2.5 mL (20 mM of FeCl_3 solution). Ten millilitre of aqueous methanol (50%) was added to PJ (1 mL), sonicated for 10 min in cold water and centrifuged for 5 min at 4°C. PJ (150 μ L) was mixed with 2850 μ L FRAP and the absorbance was read at 593 nm after 30 min incubation using a UV–visible spectrophotometer. Trolox (100–1000 μ M) was used for calibration curve, and results were expressed as trolox (μ M) equivalents per millilitre pomegranate juice (μ M TE/mL PJ).

3. Data analysis

Analysis of variance was performed using SPSS statistics for windows, version 20.0. (Armonk, NY, IBM Corp). Means were separated using Duncan multiple range test where there was statistical significance ($P < 0.05$). Relationship among the measured fruit parameters were determined by subjecting data to Pearson correlation test in SPSS and principal component analysis (PCA) using XLSTAT software version 2012.04.1 (Addinsoft, France). GraphPad Prism software version 4.03 (GraphPad Software, Inc., San Diego, USA) was used for graphical presentations.

4. Results and discussion

4.1. Concentrations of individual flavonoids at maturity stages and growing areas

Individual flavonoids including the catechin, epicatechin, taxifolin, rutin, eriodictyol 7-0- β -glucoside, kaempferol-3-D-glucoside, naringin, and hesperidin were identified (Table 3). Fruit harvested from the area with the highest (898 m) altitude (Koedoeshoek) had higher catechin concentration (29.01 mg/L crude juice) at unripe stage than those from lower altitudes (662 m (Kakamas) (16.16 mg/L crude juice) and 222 m (Worcester) (18.13 mg/L crude juice) altitude locations. However, the concentration was stable till the full ripe stage for fruit harvested from the highest and lowest altitudes. Previous studies have reported concentrations of catechin, epicatechin in pomegranate juice (Fawole and Opara, 2013a; Poyrazoglu et al., 2002) at commercial harvest maturity. A different pattern was observed for those harvested at medium altitude. For instance, fruit from medium altitude had significantly higher concentration (24.00 mg/L) at full ripe stage compared to those from higher (16.35 mg/L) and lower (15.22 mg/L) altitudes (Table 3). The altitude, maturity and their interaction had significant effects ($P < 0.001$) on epicatechin. Fruit harvested from the three different altitudes had epicatechin ranging from 8.82 to 16.55 mg/L at the unripe stage (Table 3). Fruit harvested from the investigated three different altitudes had epicatechin ranging from 8.82 to 16.55 mg/L at the unripe stage (Table 3). The epicatechin concentration did not change at mid ripe and full ripe maturity stages particularly for those harvested at higher altitudes (898 and 662 m) (Table 3). Fruit from lower altitude (222 m) had significantly ($P < 0.001$) higher epicatechin concentration averaging 28.72 mg/L crude juice at full ripe stage. Taxifolin did not vary significantly ($P > 0.05$) among all the altitudes and maturity stages. Rutin concentration was significantly ($P < 0.01$) higher at full ripe stage in pomegranate fruit harvested from the lower (4.28 mg/L) altitude as compared with those from higher (2.30 mg/L) and medium (1.95 mg/L) altitudes.

Eriodictyol 7-0- β -glucoside concentration varied significantly ($P < 0.001$) with maturity stage and altitude (Table 3). The concentration decreased considerably in mid ripe fruit harvested from medium (662 m) altitude and remained relatively unchanged at full ripe stage while those from lower altitude (222 m) decreased at full ripe stage. However, fruit from higher altitude (898 m) did not exhibit any considerable change in eriodictyol 7-0- β -glucoside concentration with maturity stages. Eriodictyol 7-0- β -glucoside was reported for the first time in cultivar 'Wonderful' in this study. Significantly lower kaempferol-3- β -d-glucoside concentration was found in fruit from medium altitude averaging 0.32 mg/L at unripe stage as compared to those from lower (1.16 mg/L) m and

medium (1.57 mg/L) altitudes; however, an increase was observed at mid-ripe and remained unchanged at full ripe stage for those harvested at locations with higher altitudes (662 and 898 m). In the case of fruit harvested at low altitude, significantly ($P < 0.001$) higher kaempferol 3- β -D-glucoside concentration was found at the full ripe stage compared to those harvested at higher altitude locations (898 and 662 m), irrespective maturity.

The interaction of altitude and maturity had significant effect on naringin ($P < 0.05$) (Table 3). For instance, fruit from higher altitude (898 m) had significantly lower naringin concentration (14.24 mg/L) at unripe stage than those from medium (36.68 mg/L) and lower (38.04 mg/L) altitudes. The naringin concentration did not change at mid ripe and full ripe for those harvested at lower and medium altitudes. Fruit for medium altitude (662 m) had significantly higher naringin concentration averaging 43.02 mg/L than those from higher (20.26 mg/L) and lower (26.56 mg/L) altitudes at full ripe stage. Hesperidin concentration was significantly ($P < 0.05$) influenced by maturity stage and altitude. Fruit from medium and lower altitudes had significantly higher hesperidin concentration averaging 5.54 and 6.00 mg/L, respectively, than those from higher altitude (3.25 mg/L) at full ripe stage. Both naringin and hesperidin were identified for the first time in cultivar 'Wonderful'. Pomegranate fruit harvested from the different altitudes had higher level of (-) epicatechin, naringin and (+) catechin irrespective of maturity stages. In this study, there is a pronounced variation in the levels of flavonoid derivatives of 'Wonderful' grown at different altitudes and at different maturity stages. It has been highlighted that the complexity of the biochemical profile and the variations in fruit could be attributed to maturity, growing season and geographical location. In addition, high temperatures and high exposure to sunlight have been reported to increase biosynthesis of phenolic components (Vinson et al., 2005; Al-Farsi et al., 2005). It is worth noting that the three locations which had different maximum temperatures during the fruit ripening which possibly could influence the individual flavonoids concentration hence the rate of developmental events such as fruit maturation is dependent on the temperature (Hurd and Graves, 1985). Based on this consideration, it is logical to hypothesize that many of the differences in flavonoids concentration might have been also affected by differences in maturity between locations.

4.2. Concentrations of phenolic acids

Gallic and protocatechuic acids were detected in this study and have been previously reported in juice of other pomegranate cultivars (Fischer et al., 2011; Fawole and Opara, 2013a). Concentrations of gallic acid and protocatechuic acid at different maturity stages and growing locations are presented in Table 4. Gallic acid concentration was significantly different ($P < 0.01$)

between maturity stages and different altitudes. Fruit harvested at higher (898 m) (28.91 mg/L) and lower (222 m) (33.62 mg/L) altitudes had higher gallic acid concentration at unripe stages than those harvested at medium (662 m) (14.99 mg/L) altitude. Furthermore, gallic acid concentration remained relatively unchanged in fruit harvested at higher altitude while those from lower altitude increased at mid ripe stage (Table 4). A slight increase with advancing maturity was observed in fruit harvested at 662 m averaging 39.31 mg/L at full ripe stage but did not differ with those harvested at 222 m (34.32 mg/L) altitude. Furthermore, fruit harvested at 898 m (17.92 mg/L) altitude had the lowest gallic acid concentration at full ripe stage. The values at full ripe stage ranged between 14.99 and 47.25 mg/L and were within the range (2.5 and 88.1 mg/L) reported by Ferrara et al. (2011) in pomegranate genotypes from Apulia region at commercial harvest, but higher than those reported by Poyrazoglu et al. (2002) in Turkish cultivars. In the case of protocatechuic acid, fruit harvested at 222 m altitude showed a slight increase at full-ripe stage but did not differ significantly with those at 898 m.

4.3. Concentration of total phenolics

Total phenolic concentration was higher in fruit from higher (898 m) and lower (222 m) altitudes than those from medium (662) altitude averaging 254.22, 366.87 and 205.33 mg GAE/100 mL at unripe stage, respectively (Table 5). The concentration of total phenolics declined at mid-ripe stage in fruit from low and high altitudes and remained relatively constant at full-ripe stage. A decreasing trend in total phenolic concentration during maturity was also reported for other pomegranate cultivars (Du et al., 1975; Al-Maiman and Ahmad, 2002). Total phenolic concentration in fruit harvested at medium altitude decreased significantly ($P < 0.001$) at mid-ripe stage but increased at full ripe stage. Higher solar radiation accompanied with higher altitudes has often been implicated as having an impact on secondary metabolite profiles (Liang et al., 2006; Germ et al., 2009). In the present study, accumulation of total phenolic concentration in fruit harvested at medium altitude could be in response to high light intensity (23.14 MJ/m²). In addition, increased total phenolic concentration could likely be related to the lower rainfall level at this location. Variations in the phenolic concentration of pomegranate juice have been reported to vary significantly due to geographical variation as well as exposure to extreme temperatures and maturity stage (Kondakova et al., 2009). Fruit harvested at lower altitude (222 m) associated with Mediterranean climate had higher total phenolic concentration compared to those from higher altitudes (662 and 898 m) at full ripe stage. Mditshwa et al. (2013) attributed the higher phenolic concentration of pomegranate to lower altitude of the growing area. On the other hand, it could be

suggested that fruit maturity among three locations varied due to climate effect, thus resulting in variation in the phenolic concentration of the investigated pomegranate cultivar.

4.4. Total tannin concentration

Significant difference ($P < 0.001$) in total tannin concentration was observed at unripe stage (Table 5). Higher concentration was recorded in fruit harvested at lower altitude (351.68 mg GAE/100 mL) followed by higher altitude 898 m (241.62 mg GAE/100 mL) and medium (186.07 mg GAE/100 mL) altitude. Total tannin concentration significantly decreased at mid ripe among different altitudes. A decline in the total tannins with maturity reduces the astringency of pomegranate, which is a desirable sensory attribute in fruit. However, an increase at full ripe stage was observed (Table 5). The highest total tannins were found in fruit harvested at lower altitude (275.57 mg GAE/100 mL) followed by medium altitude (198.48 mg GAE/100 mL) and higher altitude (186.03 mg GAE/100 mL). The results suggest that tannins are major components of total phenolic compounds in PJ investigated. Similar trend was also reported in pomegranate cultivar grown in Iran (Zarei et al., 2010).

4.5. Total flavonoids concentration

The accumulation of total flavonoids substantially increased in pomegranate juice during fruit maturity (Table 5). There was no significant difference found at unripe stage but slightly increased as the fruit advanced in maturity. Fruit harvested at higher and lower altitudes with the exception of medium altitude, recorded significantly ($P < 0.001$) higher total flavonoid concentration at full ripe stage. However, no variation was observed between higher and lower altitudes. Total flavonoid concentrations at full ripe were 458, 457 and 335 mg CE/100 mL at lower, higher and medium altitude, respectively. Contrary to our result, Fawole and Opara (2013a) reported a decrease in total flavonoids with advancing maturity in 'Bhagwa' pomegranate fruit. Higher concentrations of catechin, epicatechin and naringin were reported, suggesting that these compounds contributed to total flavonoid at full-ripe stage in this study. Flavonoids and other phenolics are considered to possess a light protective function because of their potent light-absorbing attribute (Liakoura et al., 2001; Hashiba et al., 2006), the compounds also mitigate the effects of free radicals (Spitaler et al., 2006). Therefore, lower total flavonoid concentration may be related to high temperatures which can inhibit biosynthesis and enhance degradation of flavonoids as observed in fruit harvested in medium altitude characterized by high maximum temperature of 30°C.

4.6. Concentration of total monomeric anthocyanin

The altitude, maturity and their interaction had a significant effect ($P < 0.01$) on total monomeric anthocyanin concentration (Table 5). Fruit from medium altitude (662 m) had lower total anthocyanin concentration (17.53 mg/100 mL crude juice) whereas there was no significant difference between fruit harvested at higher altitude (23.99 mg/100 mL crude juice) and lower altitude (23.85 mg/100 mL crude juice) at mid-ripe stage (Table 5). Full ripe fruit harvested at higher and lower altitudes had total anthocyanin concentration between 21.73 and 32.12 mg/100 mL crude juice, respectively, with those harvested at medium altitude had the lowest (17.53 mg/100 mL). Increase in anthocyanin pigment during fruit maturity was also observed for 'Bhagwa', 'Mollar' and 'Ganesh' pomegranate cultivars (Gil et al., 1995; Kulkarni and Aradhya, 2005; Fawole and Opara, 2013a). Anthocyanin concentration reported in this study is within the range with that reported by Schwartz et al. (2009) for accession 101-2 at commercial harvest. Pomegranate fruit grown under desert climate were reported to contain juice with lower anthocyanin concentration than the juice from Mediterranean climate (Schwartz et al., 2009). In this study, total anthocyanin concentration was found to be lower in fruit harvested at medium altitude (Kakamas, 662 m), which is characterized by arid to semi-arid climate.

Lower level of anthocyanin concentration might be ascribed to increased anthocyanin degradation at higher temperatures and inhibition of mRNA transcription genes involved in anthocyanin synthesis (Mori et al., 2007). In addition, enzymes involved in anthocyanin biosynthesis pathways operate at an ideal temperature between 17 and 26°C, beyond which anthocyanin synthesis is inhibited (Haselgrove et al., 2000). The maximum temperature (30°C) at medium altitude (Table 2) may contribute to the lower anthocyanin concentration of fruit from this area, which agrees with the result reported by Mditshwa et al. (2013) in 'Bhagwa' pomegranate. Maximum temperatures at higher (26°C) and lower (24°C) altitudes are suitable for higher anthocyanin accumulation (Haselgrove et al., 2000) leading to increased activity of enzyme responsible for anthocyanin synthesis. Several researchers have reported that high light intensity results in decreased anthocyanin levels (Bergqvist et al., 2001; Spayd et al., 2002). Fruit harvested at medium altitude (662 m) with higher light intensity (23.14 MJ/m²) exhibited lower anthocyanin concentration as compared to those from higher (898 m) (13.40 MJ/m²) and lower (222 m) (19.06 MJ/m²) altitudes. These results suggest that temperature, light intensity and fruit maturity status or the combination of three could influence anthocyanin biosynthesis in the investigated pomegranate cultivar.

4.7. Vitamin C concentration

Vitamin C concentration increased significantly ($P < 0.05$) with advancing maturity (Fig. 1A). Pomegranate fruit harvested from medium altitude (662 m) exhibited significantly ($P < 0.05$) higher vitamin C concentration (114.33 $\mu\text{g AAE/mL}$) than those harvested from higher (898 m) (92.55 $\mu\text{g AAE/mL}$) and lower (222 m) (84.31 $\mu\text{g AAE/mL}$) altitudes at full-ripe stage. Vitamin C, also known as ascorbic acid, plays a significant role in plant tissue due to its significant antioxidant activity (Kulkarni and Aradhya, 2005; Gomez and Lajolo, 2008). In contrary to our findings, a considerable decrease in vitamin C concentration with advancing maturation was observed in ‘Ganesh’ and ‘Taifi’ pomegranate accessions (Al-Maiman and Ahmad, 2002; Kulkarni and Aradhya, 2005) at commercial harvest (full ripe). Our results corroborate with the findings by Shwartz et al. (2009) for ‘Wonderful’ and ‘Rosh-hapered’ accessions and Fawole and Opara (2013a) for ‘Bhagwa’. According to Lee and Kader (2000), vitamin C accumulation in fruit is optimally synthesised from sugar during photosynthesis at higher light intensity. This suggests that increase in vitamin C concentration in fruit harvested at medium altitude (662 m) at full-ripe stage could be associated with higher light intensity (23.14 MJ/m^2) as compared to those harvested at lower (222 m) (19.06 MJ/m^2) and medium (898 m) (13.40 MJ/m^2) altitudes having lower light intensity during the same growing season (Table 2). Mditshwa et al. (2013) found similar results with the vitamin C concentration of pomegranate cv. Bhagwa being positively influenced by high light intensity.

4.8. Antioxidant activity

4.8.1. Radical scavenging activity (RSA)

There were significant effects ($P < 0.001$) of altitude, maturity, and their interaction on the radical scavenging activity (Fig. 1B). Fruit harvested at lower altitude significantly ($P < 0.01$) showed higher (775.60 $\mu\text{M AAE/mL}$) antioxidant activity at unripe stage than those harvested at higher (746.79 $\mu\text{MAAE/mL}$) and medium (690.82 $\mu\text{M AAE/mL}$) altitudes. There was a slight decrease in antioxidant activity of fruit harvested at higher and medium altitudes whereas those harvested at lower altitude remained unchanged at mid-ripe stage. However, antioxidant activity decreased to same level at full-ripe stage for three altitudes investigated. The decrease was concomitant with decrease in total phenolic concentration from unripe to full ripe stage in fruit harvested from lower and higher altitudes (Table 5). Our findings are in agreement with those previously reported for ‘Bhagwa’ cultivar (Fawole and Opara, 2013a). Higher antioxidant activity has been attributed to higher total phenolic compound present in pomegranate and many other fruit (Tzulker et al., 2007;

Solomon et al., 2006). Decrease in antioxidant activity measured by radical scavenging activity could be attributed to decline in total phenolic as the fruit matures. However, RSA at full ripe decreased while total phenolic concentration increased at medium altitude suggesting that total phenolic measured in this study was not active scavenging compounds.

4.8.2. Ferric reducing antioxidant power (FRAP)

The antioxidant activity as measured by the FRAP assay is presented in Fig. 1C. Antioxidant activity of fruit harvested at three altitudes increased significantly ($P < 0.05$) as the fruit maturity advanced. The FRAP values at unripe stage was 93.84, 89.05 and 90.40 $\mu\text{MTE/mL}$ for fruit harvested at lower, higher and medium altitudes, respectively and did not differ significantly from each other. However, at mid-ripe stage, the antioxidant activity decreased significantly irrespective of altitude and then increased at full-ripe stage with for those harvested at lower altitude having a significantly higher antioxidant activity (184.25 $\mu\text{M TE/g}$) than at higher (153.51 $\mu\text{M TE/g}$) and medium (148.48 $\mu\text{M TE/g}$) altitudes. Our findings contradict that of Fawole and Opara (2013a), where decline in FRAP in pomegranate juice with maturation was reported in 'Bhagwa'. The increase in antioxidant activity measured by FRAP assay at full-ripe stage may be attributed to an increased concentration of anthocyanin pigments.

4.9. Multivariate analysis

4.9.1. Pearson correlation test

Pearson correlation was conducted to determine the relationships among postharvest quality attributes associated with maturity stages of 'Wonderful' pomegranate (Table 6). Significant ($P < 0.05$) positive correlations were found between rutin and kaempferol ($r = 0.68$); rutin and (-)-epicatechin ($r = 0.66$); kaempferol-3- β -D-glucoside and epicatechin ($r = 0.67$), indicating the relationship between individual flavonoids. Protochatechuic acids correlated with kaempferol- β -D-glucoside ($r = 0.69$). Total phenolic had a significant positive correlation with total tannins ($r = 0.88$). Besides, antioxidant activity measured by FRAP assay correlated positively and significantly ($P < 0.01$) with vitamin C ($r = 0.75$), total anthocyanin concentration ($r = 0.56$) and total flavonoid ($r = 0.86$), highlighting their considerable contribution to antioxidant activity. Several studies have highlighted that pomegranate juice exhibits high antioxidant capacity due to its high level of flavonoids, phenolics and other polyphenol compounds (Gil et al., 2000; Kulkarni and Aradhya, 2005). Significant negative ($P < 0.05$) correlation were calculated between RSA and vitamin C ($r =$

-0.64) as well as RSA and total flavonoids ($r = -0.68$) whereas significant negative correlation between RSA and FRAP ($r = -0.65$) was also observed. These findings suggest that vitamin C and total flavonoids and total flavonoids concentration may not contribute significantly to the antioxidant activity of pomegranate juice measured by radical scavenging activity.

4.9.2. Principal component analysis

The investigated metabolites at different maturity stages and three altitudes were subjected to principal component analysis (PCA). The total variability is described by 8 factors (F1–F8), with the first two principal factors (F1 and F2) explaining 65.30% of the total variability (Fig. 2A). The first factor was responsible for 43.15% of the total variation, whereas factor two contributed 22.15%, indicating that the possible variation among metabolites at different maturity stages and altitudes was explained by the F1 (Fig. 2A and B). The observations (Fig. 2A and B) showed that fruit harvested at medium altitude (662 m) could be characterized with eriodictyol 7-0- β -glucoside and RSA which had high negative scores (Tables 7 and 8) along F1 at unripe stage (Fig. 2A and B). Positive scores (Tables 7 and 8) along F1 (Fig. 2A and B) corresponded to total flavonoids, total anthocyanin, epicatechin, rutin, kaempferol-3- β -D-glucoside and protocatechuic acids during full-ripe stage of fruit harvested at lower altitude. Total tannin had lower positive score in F1 (Tables 7 and 8) which is associated with those harvested at higher altitude during full-ripe stage (Fig. 2A and B). Higher positive scores along F2 (Fig. 2A and B) corresponded with those harvested at lower (222 m) during unripe stage (Tables 7 and 8). Fruit with lower negative scores were from medium altitude harvested at full-ripe stage (associated with FRAP). The results revealed that fruit of 'Wonderful' cultivar at different maturity stages and grown indifferent altitudes were successfully distinguished on the basis of polyphenol concentration variations.

5. Conclusion

The present study showed that differences in climatic conditions, altitudes and maturity stages have a profound influence on the bioactive compounds of pomegranate fruit (cv. Wonderful). The results also indicated that (+)-catechin, (-)-epicatechin, naringin, gallic acid had high concentrations regardless of maturity stages and altitudes. Pomegranate juice showed significantly higher antioxidant activity measured by FRAP which increased with advancing fruit maturity, whereas RSA decreased. Higher vitamin C concentration was found in fruit harvested from medium (662 m) altitude, with lower total anthocyanin concentration than those from lower (222 m) and higher (898 m) altitudes at full ripe stage. In addition, principal component analysis showed that fruit

harvested from three different altitudes varied significantly in concentration of metabolites. More specifically, fruit harvested at lower altitude characterized by the mediterranean climate had significantly higher metabolites especially total flavonoids, total anthocyanin, epicatechin, rutin, kaempferol-3- β -d-glucoside and protocatechuic acids followed by higher (subtropical climate) and medium (arid to semi-arid climate), highlighting the importance of altitude and climatic condition of growing area on the biochemical composition of pomegranate cv. Wonderful. In addition, significant variation in phenolic concentrations could be rooted in the maturity of the fruit during harvest since ripening is driven by temperature which varied significantly across the altitudes. It could be suggested that fruit did not mature simultaneously, thus resulting in variation in the phenolic compounds investigated.

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Table 1

Description of the selected maturity stages of ‘Wonderful’ pomegranate fruit.

DAFB	Maturity stage	Fruit characteristics
100	Unripe	Mature: mature light-red arils with mature kernels
121	Mid-ripe	Mature: red skin, mature red arils with mature kernels
141	Full-ripe	Commercial harvest; deep-red skin, deep red arils with mature kernels

DAFB, Days after full bloom

Table 2

Climatic conditions of pomegranate (cv. Wonderful) at three different altitudes in South Africa

Altitude (growing location) (m)	Biome	Longitude (E)	Latitude (S)	Average rainfall (mm)	Minimum Temperature (°C)	Maximum Temperature (°C)	Light intensity (MJ/m ²)
662 (Kakamas)	Semi-arid	20° 38' 00"	28° 45' 00"	0.34	11.08	30.80	23.14
898 (Koedoeshoek)	Suptropical	30° 30' 45.3"	25°23' 38.6"	41.47	11.07	26.46	13.40
222 (Worcester)	Mediterranean	19° 26' 00"	33° 39' 00"	1.19	9.72	24.55	19.06

Source: \\ <http://www.arc.agric.za/arc-iscw>; data were daily averages for the growing season.

Rainfall data were averages for the growing season.

Table 3

Individual flavonoid compounds (mg/L crude juice) in pomegranate juice harvested at three different altitudes and maturity stages.

Altitude (growing location)	Maturity stage	Catechin	Epicatechin	Taxifolin	Rutin	Eriodictyol 7-O - β -glucoside	Kaempferol-3- β -D-glucoside	Naringin	Hesperidin
Unripe									
662 (Kakamas)		16.16 ^{cd}	8.82 ^c	0.42 ^b	0.95 ^c	3.12 ^a	0.32 ^c	36.68 ^{ab}	3.61 ^c
898 (Koedoeshoek)		29.01 ^a	12.98 ^{bc}	0.86 ^{ab}	1.60 ^{bc}	2.48 ^{abc}	1.16 ^{bc}	14.24 ^c	4.36 ^{abc}
222 (Worcester)		18.13 ^{cd}	16.55 ^{bc}	1.51 ^a	2.10 ^{bc}	2.49 ^{abc}	1.57 ^{bc}	38.04 ^{ab}	4.41 ^{abc}
Mid-ripe									
662 (Kakamas)		15.33 ^{cd}	18.86 ^b	0.96 ^{ab}	2.59 ^b	1.77 ^{bc}	2.04 ^b	24.52 ^{cb}	4.59 ^{abc}
898 (Koedoeshoek)		20.30 ^{bc}	16.41 ^{bc}	0.84 ^{ab}	2.57 ^b	2.48 ^{abc}	2.11 ^b	16.22 ^c	4.24 ^{abc}
222 (Worcester)		12.35 ^d	11.40 ^{bc}	0.72 ^{ab}	1.86 ^{bc}	3.48 ^a	1.01 ^{bc}	27.18 ^{cb}	4.04 ^{bc}
Full-ripe									
662 (Kakamas)		24.00 ^{ab}	11.38 ^{bc}	1.15 ^{ab}	1.95 ^{bc}	2.78 ^{ab}	1.16 ^{bc}	43.02 ^a	5.55 ^{ab}
898 (Koedoeshoek)		16.35 ^{cd}	16.20 ^{bc}	0.59 ^b	2.30 ^{bc}	2.77 ^{ab}	1.54 ^{bc}	18.01 ^c	3.26 ^c
222 (Worcester)		15.22 ^{cd}	28.72 ^a	1.25 ^{ab}	4.28 ^a	1.56 ^c	4.05 ^a	20.66 ^c	6.00 ^a
<i>P-value</i>									
Altitude (A)		<0.001	<0.05	2.403	<0.01	0.097	<0.01	<0.0001	0.173
Maturity (M)		<0.01	<0.05	0.339	<0.01	0.494	<0.001	0.154	0.199
A * M		<0.001	<0.001	5.557	<0.01	<0.001	<0.0001	<0.05	<0.05

Means are presented. Values within a column followed by a different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test.

Table 4

Phenolic acids (mg/L crude juice) in pomegranate juice harvested at three different altitudes and maturity stages.

Altitude (growing location)	Maturity stage	Gallic acid	Protocatechuic acid
	unripe		
662 (Kakamas)		14.99 ^d	0.95 ^c
898 (Koedoeshoek)		28.91 ^{bc}	2.42 ^{ab}
222 (Worcester)		33.62 ^b	1.50 ^{bc}
	Mid-ripe		
662 (Kakamas)		30.51 ^{bc}	2.17 ^{ac}
898 (Koedoeshoek)		27.43 ^{bcd}	2.00 ^{ac}
222 (Worcester)		47.28 ^a	1.55 ^{bc}
	Full-ripe		
662 (Kakamas)		39.31 ^{ab}	1.55 ^{bc}
898 (Koedoeshoek)		17.92 ^{cd}	2.77 ^{ab}
222 (Worcester)		34.32 ^{ab}	3.13 ^a
	<i>P-value</i>		
Altitude A		<0.05	0.06
Maturity (M)		<0.01	<0.05
A * M		<0.01	0.07

Values within a column followed by a different letters are significantly different ($P < 0.05$) according to Duncan's multiple range test. Means are presented.

Table 5.

Phenolic compounds of pomegranate juice harvested at three different altitudes and maturity stages.

Altitude (growing location)	Total monomeric anthocyanin (mgC ₃ gE/100 mL PJ)			Total phenolic (mg GAE/100 mL PJ)			Total flavonoids (mg CE/100 mL PJ)			Total tannins (mg GAE/100 mL PJ)		
	Unripe	Mid-ripe	Full-ripe	Unripe	Mid-ripe	Full-ripe	Unripe	Mid-ripe	Full-ripe	Unripe	Mid-ripe	Full-ripe
	662 (Kakamas)	14.66 ^c	17.52 ^c	21.73 ^b	205.3 ^{3de}	158.88 ^e	207.81 ^{cd}	97.06 ^{cd}	83.94 ^d	335.62 ^b	186.07 ^d	96.20 ^e
898 (Koedoeshoek)	16.77 ^c	23.99 ^b	29.87 ^a	254.22 ^{bc}	227.82 ^{dc}	202.36 ^{de}	142.16 ^{cd}	161.36 ^c	457.30 ^a	241.62 ^{bc}	122.30 ^e	186.03 ^d
222 (Worcester)	15.08 ^c	23.85 ^b	32.11 ^a	366.87 ^a	285.38 ^b	288.02 ^b	129.69 ^{cd}	105.69 ^{cd}	458.44 ^a	351.68 ^a	183.59 ^d	276.57 ^b
Altitude (A)	<0.001			<0.001			<0.001			<0.001		
Maturity (M)	<0.001			<0.001			<0.001			<0.001		
A * M	<0.01			0.076			0.272			0.062		

Each value in the table is represented as mean. Different letters in the same column indicate significant difference (P<0.05) according to Duncan's multiple range test.

Table 6

Pearson correlation coefficients between variables investigated at different maturity stages.

No.		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
1	TAC	1																	
2	Vit C	.551**	1																
3	TP	.060	-.145	1															
4	TT	.031	.030	.885**	1														
5	TF	.721**	.710**	.025	.204	1													
6	RSA	-.434**	-.635**	.339**	.157	-.679**	1												
7	FRAP	.567**	.754**	.047	.318**	.862**	-.649**	1											
8	Catechin	-.081	.009	.061	.173	.036	.041	.021	1										
9	Epicate	.370**	.115	.190	.170	.349**	-.190	.295**	.023	1									
10	Taxifolin	-.028	.099	.166	.165	.104	-.039	.121	.098	.243*	1								
11	Rutin	.364**	.212	.113	.093	.371**	-.204	.323**	.087	.667**	.261*	1							
12	Eriodiglu	-.144	-.131	.034	-.010	-.168	.092	-.174	-.033	-.384**	-.192	-.272*	1						
13	Kaempgluc	.229*	.133	.113	.082	.292**	-.172	.294**	-.172	.677**	.277*	.628**	-.284*	1					
14	Naringin	-.090	.077	.047	.129	.013	-.025	.082	.083	-.201	.004	-.149	.123	-.467**	1				
15	Hesperidin	.152	.172	.067	.100	.190	-.105	.258*	.271*	.231*	.379**	.395**	-.346**	.162	.292**	1			
16	Gallic acids	-.122	-.093	.186	.152	-.108	.159	-.085	.003	-.175	.343**	-.238*	.006	-.108	.144	.079	1		
17	Protoc acids	.136	.130	-.018	.004	.256*	-.173	.195	-.151	.331**	.055	.308**	-.019	.695**	-.639**	-.250*	-.105	1	

TAC = total anthocyanin concentration, Vit C = vitamin C, TP = total phenolic, TT = Total tannin, TF = Total flavonoid, RSA = radical scavenging activity, FRAP = ferric reducing antioxidant power, Epicate = epicatechin, Kaempgluc = kaempferol glucose, eriodiglu = eriodictyol 7-O- β -glucoside, Protoc acids = protochatechuic acids.

* = P<0.05 and ** = P<0.01 (2-tailed).

Table 7

Factor loadings, eigenvalue, cumulative variance (%) for the first eight principal (F1-F8) components based on the polyphenol, vitamin C concentration and antioxidant activity of pomegranate (cv. Wonderful).

	Loadings							
	F1	F2	F3	F4	F5	F6	F7	F8
TAC	0.779	-0.324	0.055	-0.442	-0.003	-0.281	0.057	-0.089
Vit C	0.644	-0.248	0.677	-0.003	-0.029	-0.198	-0.145	0.070
TP	0.062	0.869	0.017	-0.458	0.117	0.018	0.058	-0.113
TT	0.198	0.779	0.290	-0.283	0.273	0.331	0.052	0.057
TF	0.822	-0.252	0.424	-0.214	0.176	0.051	-0.027	-0.041
RSA	-0.732	0.477	-0.359	-0.276	0.063	-0.088	0.092	0.108
FRAP	0.786	-0.081	0.540	-0.124	0.126	0.184	0.107	0.082
Cat	-0.085	0.119	0.141	0.551	0.797	-0.081	0.061	-0.103
Epicat	0.890	0.182	-0.365	-0.031	-0.158	0.128	-0.001	0.004
Tax	0.455	0.805	0.089	0.263	-0.076	-0.042	-0.220	-0.111
Rutin	0.930	0.134	-0.290	-0.024	-0.143	-0.094	0.040	-0.037
Eriodigluc	-0.729	-0.150	0.418	-0.447	0.071	-0.246	0.068	-0.051
Kaempgluc	0.899	0.212	-0.332	-0.001	-0.152	0.004	0.068	-0.090
Naringin	-0.317	0.338	0.718	0.194	-0.465	0.128	0.000	-0.010
Hesp	0.644	0.419	0.161	0.453	-0.150	-0.253	0.288	0.097
GA	-0.146	0.915	0.054	-0.072	0.042	-0.315	-0.145	0.104
Protoc acids	0.834	-0.177	-0.326	-0.103	0.338	-0.014	-0.131	0.160
Eigenvalue	7.335	3.765	2.325	1.455	1.208	0.547	0.235	0.130
Cumulative%	43.148	65.297	78.975	87.532	94.638	97.852	99.235	100.000

TAC = total anthocyanin concentration, Vit C = vitamin C, TP = total phenolic, TT = total tannin, TF = total flavonoid, RSA = radical scavenging activity, FRAP = ferric reducing antioxidant power, Cat = catechin, Epicat = epicatechin, Tax = Taxifolin, Eriodigluc = eriodictyol 7-O- β -glucoside, Kaempgluc = kaempferol glucose, Hesp = hesperidin, GA= gallic acids, Protoc acids = protochatechuic acids.

Table 8

Scores within each principal (F1-F8) of pomegranate (cv. Wonderful) from three growing locations.

Altitude	F1	F2	F3	F4	F5	F6	F7	F8
M1_A1	-3.818	-1.418	0.716	-0.064	-0.703	1.189	0.727	0.034
M1_A2	-1.240	0.690	-1.060	0.746	2.622	-0.067	0.179	0.354
M1_A3	-1.010	4.317	0.341	-0.444	-0.183	0.734	-0.537	-0.251
M2_A1	0.070	-0.987	-1.932	1.743	-1.425	0.084	-0.573	0.353
M2_A2	-0.180	-0.959	-1.731	0.312	0.139	-0.560	0.200	-0.856
M2_A3	-2.448	0.521	-0.183	-1.921	-0.773	-1.344	0.093	0.307
M3_A1	0.620	-0.078	3.405	1.668	-0.037	-0.744	-0.022	-0.066
M3_A2	1.720	-3.095	0.823	1.668	0.793	0.478	-0.699	-0.031
M3_A3	6.286	1.009	-0.379	-0.451	-0.434	0.229	0.632	0.156

M1= unripe, M2= mid-ripe, M3= full-ripe. Altitude A1= 662 m (Kakamas); A2 = 898 m (Koedoeshoek); A3 = 222 m (Worcester).

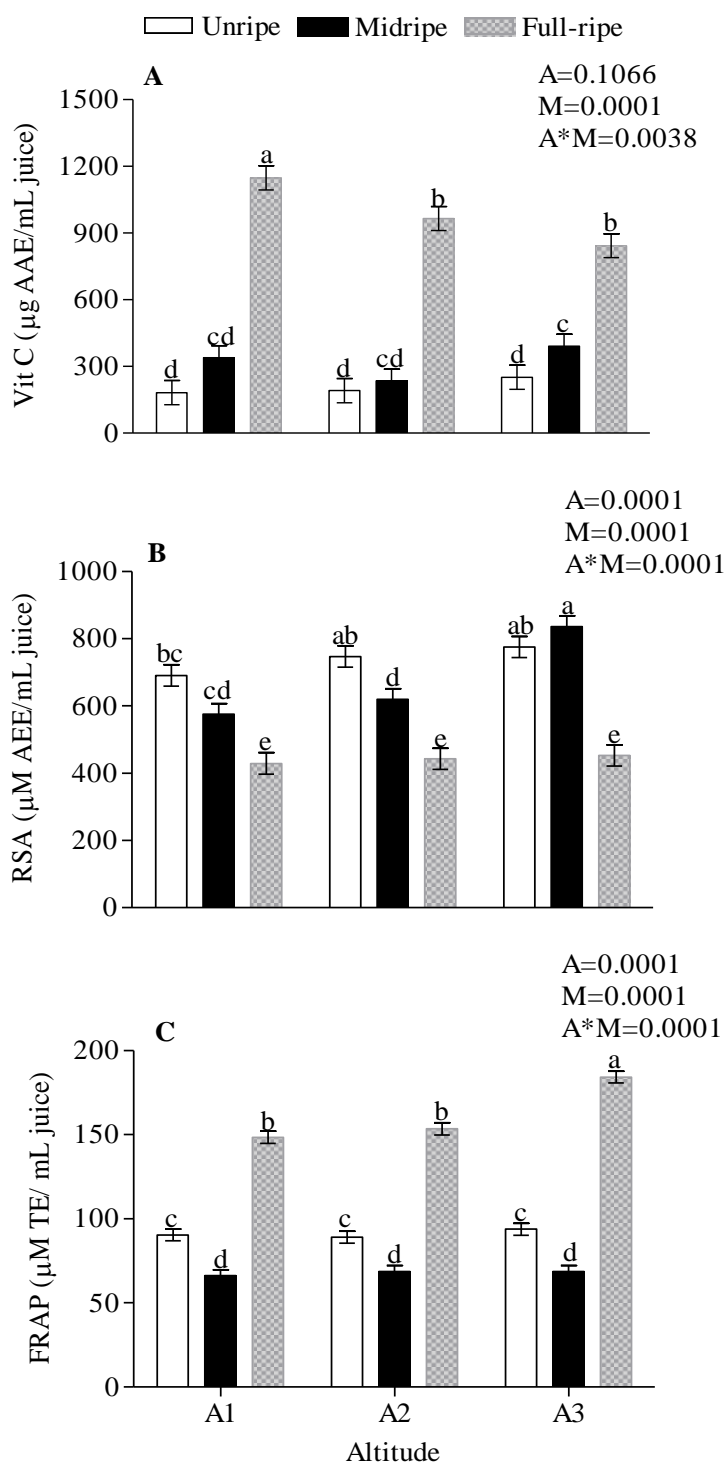


Fig. 1. Changes in vitamin C and antioxidant activities of pomegranate juice harvested at different maturity stages from three different altitudes. Bars with same letter are not significantly different ($P < 0.05$; Duncan's multiple range test). Means \pm SE presented. AAE = ascorbic acid equivalent, TE = trolox equivalent, FRAP = ferric reducing antioxidant power. Altitude A1= 662 m (Kakamas); A2= 898 m (Koedoeshoek); A3 = 222 m (Worcester).

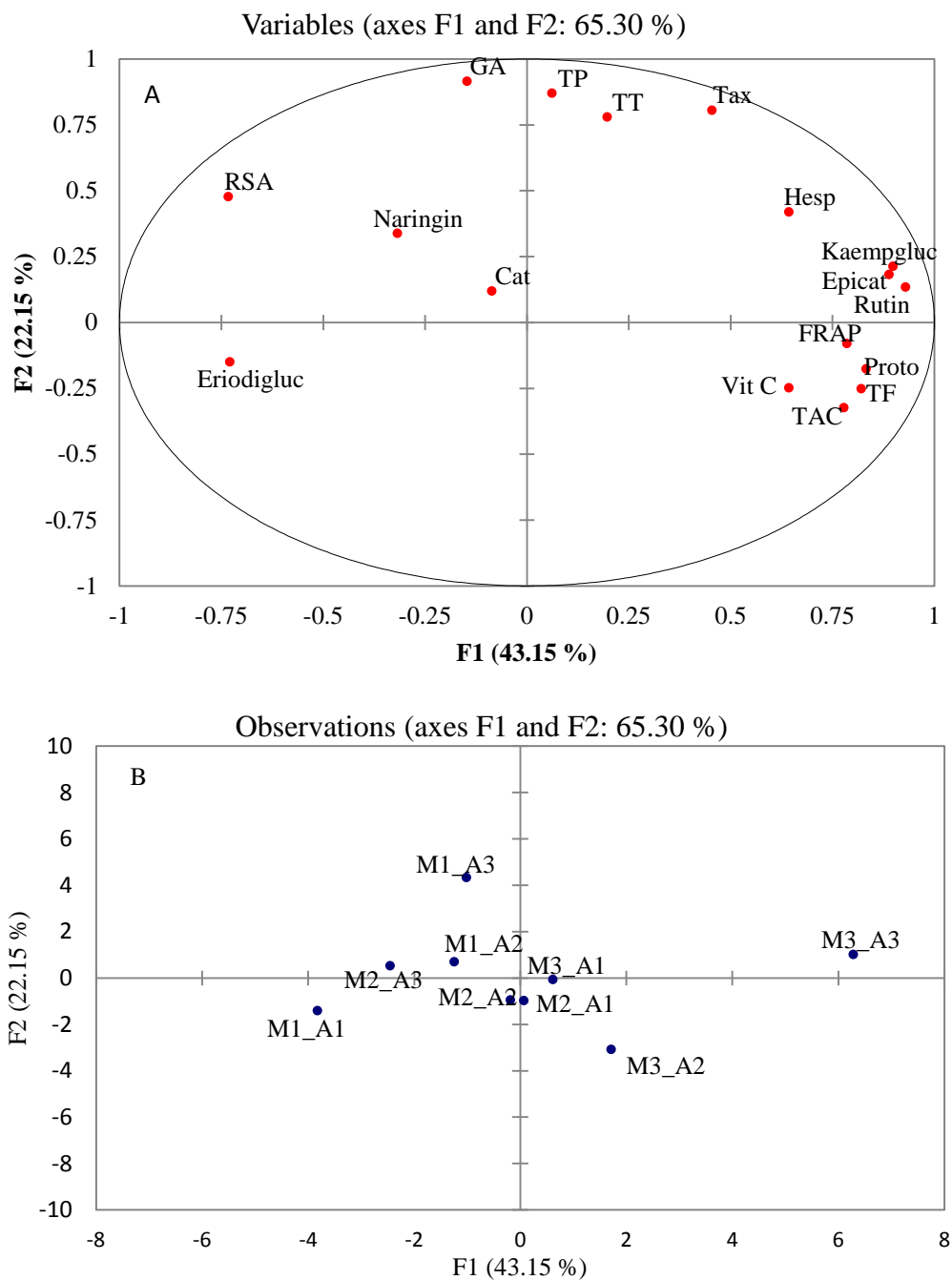


Fig. 2. Principal component analysis of the first two factors (F1 and F2) based on metabolites of pomegranate cv. Wonderful. Variable plot (A): TAC = total anthocyanin concentration, Vit C = vitamin C, TP = total phenolic, TT = total tannin, TF = total flavonoid, RSA = radical scavenging activity, FRAP = ferric reducing antioxidant power, Cat = catechin, Epicat = epicatechin, Tax = taxofolin, Kaempgluc = kaempferol glucose, Hesp = hesperidin, Eriodiglu = eriodictyol 7-O- β -glucoside, GA = gallic acids, Protoc acids = protochatechuic acids. Observation plot (B): M1 = unripe, M2 = Mid-ripe, M3 = full-ripe. Altitude A1 = 662 m (Kakamas), A2 = 898 m (Koedoeshoek), and A3 = 222 m (Worcester).

PAPER 4

Effect of extraction method on biochemical, volatile composition and antioxidant properties of pomegranate juice

Abstract

This study investigated the biochemical and volatile composition and bioactive compounds extracted from different fruit fractions of pomegranate (*Punica granatum* L.) cv. Wonderful. Juice variants evaluated included juice extracted without crushing the seeds using a juice extractor (arils), juice extracted by crushing the seeds using a blender (arils plus seed), juice extracted by pressing whole fruit using a squeezer (whole fruit) and juice extracted from halved fruit using a commercial handpress juicer (halved fruit). There were no significant differences ($P > 0.05$) in total soluble solids ($^{\circ}$ Brix) concentration in pomegranate juice obtained using different extraction methods; however, juice extracted using squeezer had higher titratable acidity (1.78 mg citric acid /100 mL), lower pH concentration (1.58) and juice yield (28.01%). The lowest citric acid concentration was observed in blended juice (18.96 g/L) and high juice colour (2.69). Fructose concentration did not vary in all extraction methods. Catechin and epicatechin were the most dominant flavonoids whereas gallic acid was the dominant phenolic acid identified in all extraction methods. The total phenolics, tannins, flavonoids and anthocyanin concentration in the investigated juice ranged from 185.73- 285.94 mg gallic acid equivalent /100 mL, 120.00- 267.10 mg gallic acid equivalent /100 mL, 103.05- 181.42 mg catechin equivalent /100 mL, 10.96 - 13.91 mg cyanidin 3-glucoside equivalent /100 mL crude juice, respectively. Furthermore, halved fruit juice had high radical scavenging activity and ferric reducing antioxidant power. The most abundant volatile compounds were ethyl acetate (21.35- 31.45%) and 3-Octanone (8.12-18.74%) in all the juice variants. Principal component analysis (PCA) also revealed that the biochemical, volatile and bioactive compounds separated the investigated juice extraction method. The results of the study provide information on the importance of methods of extraction on the quality of pomegranate juice.

Keywords: Antioxidant activity, Organic acids, Pomegranate, Volatile compounds, Extraction methods.

1. Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest recognized edible fruit belonging to Punicaceae family. To date, pomegranate is widely grown in areas such as Iran, India, Egypt, Lebanon, China, Spain, France, USA, Oman, Syria, Tunisia, Italy, Greece, Cyprus, Israel, Turkey, Chile, Portugal and South Africa (Al-Said et al., 2009; Holland et al., 2009; Fawole and Opara, 2013a, b). Currently, South Africa's commercial production of pomegranate fruit stands at 758 330 cartons (Hortgro, 2014). Pomegranate fruit has gained popularity in the past 15 years due to its valuable source of polyphenols when compared with other compound rich beverages such as wine and green tea (Gil et al., 2000; Fischer et al., 2013).

Pomegranate fruit is a rich source of bioactive compounds including phenolic acids, tannins, flavonols and anthocyanins (Viuda-Martos et al., 2010), and consumption has intensified because of their role in promoting health by reducing the risk of atherosclerosis, cancer, diabetes and neurodegenerative disorders (Miguel et al., 2010; Viuda-Martos et al., 2010). Moreover, these bioactive compounds (phenolic acids, flavonoids and hydrolysable tannins) were found to be present in higher amounts, in particular, high concentration of hydrolysable tannins. These are reported to be mainly located in the fruit peel and mesocarp (Fischer et al., 2011). Research has showed that these compounds may be scavengers of reactive species, thus exhibiting antioxidant activity (Fawole et al., 2012; Fischer et al., 2013).

Generally, pomegranate similar to any other fruit is not only available as fresh arils but also widely distributed as processed products such as juice, jams, anardana, carbonated drinks, garnish and deserts (Al-Maiman and Ahmad 2002; Opara et al., 2009). The edible parts of pomegranate fruit (50%) comprised 40% arils (juice sacs) and 10% seeds. Arils contain 85% water, 10% total sugars (fructose and glucose), organic acid (ascorbic acid, citric acid, and malic acid), and bioactive compounds such as phenolics and flavonoids (anthocyanins) (Viuda-Martos et al., 2010).

The desire of the consumers to maintain a diet which promotes better health has increased the demand of juices that preserve their natural nutritive value. Therefore, alternative processing methods which potentially increase nutritive properties are necessary. Bioactive concentration and composition of pomegranate juice are strongly influenced by cultivar, climatic conditions, maturity status and juice extraction methods (Turfan et al., 2011; Caleb et al., 2012; Rajasekar et al., 2012; Fawole and Opara, 2013a, b; Mphahlele et al., 2014a, b). More recently, several methods of juice extraction such as juice processing from the whole and separated aril sacs have been explored (Miguel et al., 2004; Muhacir-Güzel et al., 2014). These researchers have shown that high amount of

polyphenolic compounds were found in juice extracted from the whole fruit whereas juice from arils only had the least. Similarly, Fischer et al. (2011) found higher total polyphenol and hydrolysable tannins concentrations in juice from whole fruit than those from arils only due to migration of phenolic compounds from rind during pressing the fruit. However, the varietal differences on the polyphenol concentrations were also observed among the studies. Tzulker et al. (2007) reported 20 and 6.5-fold higher antioxidant activity in juice obtained from the whole fruit and aril only juice, respectively.

Pomegranate fruit has different fractions including pith, carpellary membrane and the peel. These non-edible fractions contain broad group of compounds with beneficial health effects than the part (aril) edible by consumers. There have been research findings on preharvest and postharvest management of pomegranate cv. Wonderful grown in South Africa, but less attention has been given to individual phenolic concentrations and volatile composition resulting from juice processing of pomegranate fruit. The objective of the study was to investigate the effect of different extraction methods on the biochemical properties, volatile organic compounds and bioactive compounds of pomegranate juice cv. ‘Wonderful’.

2. Materials and methods

2.1. Plant material

Pomegranate fruit (cv. Wonderful) were obtained in 2015 during commercial harvest from Sonlia Pack-house (33°34'851"S, 19°00'360"E) in the Western Cape, South Africa. Fruit were transported in an air-conditioned car to the Postharvest Technology Research Laboratory at Stellenbosch University. Fruit were stored at 7.5 ± 0.5 °C and $92 \pm 3\%$ RH for less than five days before processing.

2.2. Sample preparation

Fruit of the same size without any physical defects were randomly selected and washed with tap water before processing. Four extraction methods were employed as illustrated in Table 1. A total of 30 fruit were used for each extraction method. Fruit weight, peel, aril and seed proportion are highlighted in Table 2. All the extraction were performed three times and then immediately stored at -80 °C until analysis. Juice yield was calculated according to Türkyilmaz et al. (2013) using equation 1:

$$\text{Juice yield} = (\text{weight of unclarified pomegranate juice} \div \text{weight of pomegranate with rinds}) \times 100 \quad (1)$$

2.3. Biochemical composition

2.3.1. Total soluble solids (TSS), titratable acidity (TA), pH and juice color

Pomegranate juice total soluble solid (°Brix) was measured using digital refractometer (Atago, Tokyo, Japan, calibrated with distilled water). A metrohemn 862 compact titrosampler (Herisau, Switzerland) was used to determine titratable acidity (g citric acid (CA) / 100 mL). Juice sample of approximately 2 mL was diluted with 70 mL of distilled water and titrated with 0.1 N of NaOH to the end-point of pH 8.2. The pH was measured at room temperature with a pH metre (Crison, Barcelona, Spain). Juice colour absorbance was measured at a wavelength of 520 nm using spectrophotometer (Thermo Scientific, Madison, USA). Fruit maturity index was determined as the ratio between TSS and TA.

2.3.2. Sugars and organic acids (refer to Chapter 1)

A Thermo Scientific Arena 20XT random access chemistry analyser was used for enzyme robot assays. The organic acids including L-malic, succinic and citric and sugars (D-glucose, D-fructose and sucrose) concentrations were determined using enzymatic test kits (R-Biopharm AG, Germany) by measuring the formation of NADPH at 340 nm according to the described protocol of the kits.

2.3.3. Determination of phenolic acid, flavonoids and individual anthocyanin concentration

LC-MS and LC-MS^E analyses were conducted on a Waters Synapt G2 quadrupole time-of-flight mass spectrometer system (Milford, MA, USA). The instrument was connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC) and Acquity photo diode array (PDA) detector. The gradient for the analysis of phenolic compounds started with 100% using 0.1% (v/v) formic acid (solvent A) and kept at 100% for 0.5 min, followed by a linear gradient to 22% acetonitrile (solvent B) over 2.5 min, 44% solvent B over 4 min and finally to 100% solvent B over 5 min. The column was subjected to 100% solvent B for an extra 2 min. The column was then re-equilibrated over 1 min to yield a total run time of 15 min. Reference standards (Sigma-Aldrich, South Africa) of flavonoids and phenolic acids were used for the quantification of individual compounds in pomegranate juice (PJ). For anthocyanin, solvents that constituted a mobile phase were A (7.5% (v/v) formic in water) and B (7.5% (v/v) formic acid in acetonitrile). The gradient started with 1% B isocratically for 0.5 min followed by a linear increase to 15% at 15 min, 2% at 20 min and 28% at 25 min. Column precondition at 100% B subsequently followed for 1 min followed

by re-equilibration for 4 min (total run-time of 30 min). The injection volume of 3 μL at a flow rate of 0.1 mL/min was used. Anthocyanin was identified by comparison with mass spectra with those in the literature (Sentandreu et al., 2013). Proportion of individual anthocyanins was calculated and presented from the peak areas.

2.3.4. Determination of total phenolic concentration

Total phenolic concentration (TPC) was measured using the Folin-Ciocalteu (Folin-C) method as described by Makkar (2000) with slight modification (Fawole et al., 2012). Diluted PJ extract (50 μL) was mixed with 450 μL of 50% methanol followed by the addition of 500 μL Folin-C and then sodium carbonate (2%) solution after 2 min. The mixture was vortexed and absorbance read at 725 nm using a UV-visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin) after incubation for 30 min at room temperature. Gallic acid standard curve (0.02– 0.10 mg/mL) was used and TPC was expressed as milligram gallic acid equivalent per 100 mL PJ (mg GAE /100 mL PJ).

2.3.5. Determination of total tannin concentration

Total tannin analysis was carried out using Folin-C method described by Makkar (2000). Polyvinylpyrrolidone (PVPP) was used to separate tannin from non-tannin compound in PJ by adding 100 mg of PVPP to 1.0 mL of distilled water and 1.0 mL PJ in a test tube. The mixture was vortexed and kept at 4°C for 15 min followed by centrifugation at 4000 \times g for 10 min. After the extraction, 50 μL of supernatant was mixed with 450 μL of 50% methanol followed by the addition of 500 μL Folin-C and then sodium carbonate (2%) solution after 2 min. The absorbance was recorded at 725 nm using UV-visible spectrophotometer after incubation for 40 min at room temperature. Separate juice extract not treated with PVPP was measured for total phenolic concentration. Total tannin concentration was calculated using equation 2:

$$\text{Total tannin concentrations (TTC)} = \text{TPC}_{(\text{in juice without PVPP})} - \text{TPC}_{(\text{in juice treated with PVPP})} \quad (2)$$

where TPC refers to total phenolic concentration (mg GAE /100 mL PJ). Results were expressed as milligram gallic acid equivalent per 100 mL PJ (mg GAE /100 mL PJ).

2.3.6. Determination of total flavonoid concentration

Total flavonoid concentration was measured spectrophotometrically as described by Yang et al. (2009). PJ (1 mL) was extracted with 50% methanol (10 mL) and vortexed for 30 s. The mixture was sonicated in an ultrasonic bath for 10 min and centrifuged at $4000 \times g$ for 12 min at 4°C. Distilled water (1.2 mL) was added to 250 μ L of extracted PJ and then followed by 75 μ L of 5% sodium nitrite. After 5 min, freshly prepared 10% aluminium chloride (150 μ L) was added to the mixture, followed by the addition of 500 μ L sodium hydroxide after a another 5 min, and 775 μ L distilled water bringing the final volume to 3 mL. The mixture was vortexed and absorbance was immediately read using spectrophotometer at 510 nm. Catechin (0.025–0.100 mg/mL) was used for the standard curve. The results were expressed as catechin equivalent per 100 mL PJ (mg CE/100 mL PJ).

2.3.7. Determination of total monomeric anthocyanin concentration

The pH differential method described by Giusti and Wrolstad (2001) was used to determine total monomeric anthocyanin concentration. PJ (1 mL) was extracted with of 50% methanol (14 mL) by sonication for 5 min and followed by centrifugation at 4000 g for 12 min. Juice supernatant (1 mL) was taken into vials and diluted with 7 mL of potassium chloride buffer (pH 1.0) and sodium acetate buffer (pH 4.5), separately. After 10 min absorbance values of each buffer mixture was measured at 510 nm and 700 nm in a UV-Visible spectrophotometer. Results were expressed as milligram cyanidin-3-glucoside equivalent per 100 mL pomegranate juice (mg C₃g E /100 mL PJ) according to equations 3 & 4.

$$A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5} \quad (\text{eqn.3})$$

$$\text{Monomeric Anthocyanin Concentration (MAC)} = \frac{(A \times MW \times DF)}{\epsilon \times L} \quad (\text{eqn.4})$$

where A = Absorbance values at 510 nm and 700 nm, ϵ = Cyanidin-3-glucoside molar absorbance (26,900), MW = Cyanidin-3-glucoside molecular weight (449.2 g/mol), DF = Dilution factor, L = Cell path length (1cm).

2.3.8. Determination of ascorbic acid concentration

Ascorbic acid was determined according to Klein and Perry (1982) with slight modifications (Barros et al., 2007). Pomegranate juice (1.0 mL) was mixed with 14 mL of 1% metaphosphoric acid followed by sonication on ice for 4 min and centrifugation at $4000 \times g$ for 12 min. Supernatant (1.0

mL) was pipetted into a tube and mixed with 9 mL of 2,6 dichlorophenolindophenol dye (0.0025%). The mixture was incubated in the dark for 10 min before absorbance was measured at 515 nm. Calibration curve of authentic L-ascorbic acid (0.01 – 0.1 µg/mL) was used to calculate ascorbic acid concentration. Results were expressed as ascorbic acid equivalents per millilitre crude juice (µg AAE/mL PJ).

2.4. Antioxidant property

2.4.1. Radical scavenging activity (RSA)

The ability of PJ to scavenge the 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical was measured following the procedure described by Karioti et al. (2004) with slight modifications (Fawole et al., 2012). Pomegranate juice extract (15 µL) was mixed with 735 µL methanol and 0.1 mM solution of DPPH (750 µL) dissolved in methanol. The mixture was incubated for 30 min in the dark at room temperature before measuring the absorbance at 517 nm using a UV–visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). The RSA was determined by ascorbic acid standard curve (0–2000 µM). The results were presented as micro gram ascorbic acid (AA) equivalent per millilitre of crude pomegranate juice (µM AAE/mL PJ).

2.4.2. Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power assay was performed according to the method of Benzie and Strain (1996). FRAP solutions contained 25 mL acetate buffer (300 mM acetate buffer, pH 3.6), 2.5 mL (10 mM of TPTZ solution), 2.5 mL (20 mM of FeCl₃ solution). Ten millilitre of aqueous methanol (50%) was added to PJ (1 mL), sonicated for 10 min in cold water and centrifuged for 5 min at 4°C. PJ (150 µL) was mixed with 2850 µL FRAP and the absorbance was read at 593 nm after 30 min incubation using a UV–visible spectrophotometer. Trolox (100–1000 µM) was used for calibration curve, and results were expressed as trolox (µM) equivalents per millilitre pomegranate juice (µM TE/mL PJ).

2.5. Extraction and gas chromatographic analyses of volatile compounds

Volatile compounds were trapped and extracted from the vial headspace using headspace solid-phase micro-extraction (HS-SPME) method described by Melgarejo et al. (2011). Ten millilitre aliquot of fresh pomegranate juice was in a 20 mL SPME vial. Sodium chloride (30% mass/volume) was added to the juice to facilitate evolution of volatiles into the headspace and inhibit enzymatic

degradation and 10 μL of 3-octanol (at 1 ppm) was added as an internal standard. The SPME vials were equilibrated for 10 min at 50°C in the CTC autosampler incubator at 250 rpm. Subsequently, a 50/30 μm divinylbenzene/-carboxen/-polydimethylsiloxane (DVB/CAR/PDMS) coated fibre was exposed to the sample headspace for 20 min at 50°C. The desorption of the volatile compounds from the fiber coating was made in the injection port of CTC at 250°C during 5 min in splitless mode. Separation, identification and quantification of the volatile compounds were performed on a gas chromatograph using Agilent 6890 N (Agilent, Palo Alto, CA), coupled with an Agilent mass spectrometer detector Agilent 5975 MS (Agilent, Palo Alto, CA). The GC–MS system was equipped with a polar Agilent Technologies DB-FFAP capillary column (model J & W 122-3263) with dimensions 60m \times 250 mm i.d. and 0.50 μm film thickness. Analyses were carried out using helium as carrier gas with a flow of 1.9 mL min^{-1} with nominal initial pressure of 216.3 kPa and average velocity of 36 cm sec^{-1} . The injector temperature was maintained at 250°C. The oven temperature was as follows: 70°C for 1.00 min; and then ramped up to 142°C at 3°C min^{-1} and finally ramped up to 240 at 5°C min^{-1} and held for 3 mins. Compounds were tentatively identified by comparison of the retention times (RI); Kovats retention indices (KI); and, by comparison with mass spectral libraries (NIST, version 2.0). For quantification, the calculated relative percentages were used.

2.6. Statistical analysis

Statistical analyses were carried out using statistical software (STATISTICA, Vers. 12.0, StatSoft Inc., USA). Data was subjected to analysis of variance (ANOVA) and means were separated by least significant difference (LSD; $P < 0.05$) according to Duncan's multiple range test. Principal component analysis (PCA) was carried out using XLSTAT software version 2012.04.1 (Addinsoft, France). GraphPad Prism software version 4.03 (GraphPad Software, Inc., San Diego, USA) was used for graphical presentations. All samples were measured in triplicate and the values are reported as mean \pm standard error.

3. Results and Discussion

3.1. Biochemical properties of pomegranate juice

Methods of extraction did not significantly ($P > 0.05$) influence the TSS ($^{\circ}\text{Brix}$) concentration of the pomegranate juice (Table 3). Juice extracted by pressing the whole fruit had the highest TA concentration (1.78 g (CA)/ 100 mL) than the rest of the extraction methods (Table 3). Similar findings were also reported by Muhacir-Güzel et al. (2014) who found no significant variation in

pomegranate juices extracted from pomegranate fruit with rind and juicy sacs. TA concentration of 1.78 g (CA)/ 100 mL found in our study is inconsistent with those reported by (Rinaldi et al., 2013) who found lower TA concentration (0.47 g (CA)/ 100 mL) in juice from the whole fruit (cv. Wonderful), but higher than those reported by Beaulieu et al. (2015) in cv. Wonderful (DPun 81). The variation in TA concentration could be attributed to fruit maturity and agro-climatic conditions.

The ratio of TSS to TA value is an essential criterion for assessing the taste of pomegranate juice. The lowest TSS:TA concentration was found in the whole fruit (Table 3). In addition, the result was confirmed by significantly ($P < 0.001$) strong negative correlation ($r = -0.91$) between TSS:TA and TA which clearly highlights that TSS:TA ratio is influenced by TA concentration which is responsible for the extremely bitter or sour taste of pomegranate juice. This observation was in agreement with those reported in the literature (Mena et al., 2011; Rajasekar et al., 2012). Moreover, pH values clearly indicate varying strength in acidity (from 1.85 to 3.23) of the juice obtained using different extraction methods (Table 3). Juice obtained using a squeezer (whole fruit) had the lowest pH concentration (1.85) followed by juice obtained using handpress (halved fruit, 2.67). Since bacterial growth is influenced by pH of a medium, it is logical to suggest that whole and halved fruit juice could be stored longer as low pH has the ability to inhibit bacterial growth. Rajasekar et al. (2012) found a pH range of 2.66 and 2.50 in blender (where pith, carpellary membrane and the arils were juiced) and mechanical press (where arils were juice) in variety Haku-botan, respectively; this is in the range of those observed in our study.

High juice yield is a desirable quality for juice production. Hand pressed fruit (halved fruit) produced considerably higher percentage juice yield (96.58%) than arils (51.86%) and arils plus seed (44.61%) (Table 3). It is worth noting that juice extracted from the whole fruit had the lowest juice yield percentage (28.01%). This could be as a result of incomplete disruption of all arils in the fruit, as it was observed that some arils were trapped within pith and carpellary membranes during the extraction process. Colour absorbance for juice sample obtained using blender were significantly higher, having 3-fold red colouration than those obtained using other extraction methods (Table 3). The increased colour of juice obtained from crushing of the aril and seed is not well understood in this study.

The nature and concentration of the organic acids found in fruits are of interest because of their important influence on the organoleptic properties and stability of fruit juices (Kader, 2008). The main organic acids, sugars measured in the investigated juice variants are shown in Fig. 1. Slight but notable difference was observed in the total acid concentration, with blended juice (arils plus seeds) having the lowest total acids concentration (19.46 g/L) while the highest was obtained in arils

(22.07 g/L) (Fig. 1a). The lower total acids concentration in blended juice is in line with titratable acidity concentration observed in this study indicating that organic acids form major part of pomegranate juice.

With regards to the individual organic acids, the highest concentration of citric acid was observed in juice obtained using juice extractor (arils, 21.60 g/L) while the lowest was detected in blended juice (arils plus seeds, 18.96 g/L) (Fig. 1b). High amount of citric acid in juice (arils) is not surprising as citric acid is largely concentrated in the arils. Possible dilution effect in arils plus seeds could be as a result from inclusion of the seed concentration such oil. Similar to total acid concentrations, whole fruit and halved fruit juice had similar amount of citric acid concentrations as juice obtained from arils. It is suggested that inclusion of peel and the pith concentrations into the juice during extraction process was either minimal or did not result in dilution effect. Significantly higher L-malic acid and succinic concentrations were found in blended and handpressed fruit juice, respectively (Fig. 1c and 1d). Thus, it could be presumed that organic acids concentration was significantly influenced by the extraction methods.

Individual soluble sugars and total sugars concentrations of pomegranate crude juice are presented in Fig. 2. Juice obtained using blender had the lowest ($P < 0.05$) total sugar concentrations compared to the rest of the extraction method (Fig. 2a). Similarly, glucose concentration did not vary ($P > 0.05$) among the investigated juice variants (Fig. 2b). Furthermore, fructose concentration ranged from 60.70 g/L to 70.55 g/L with slightly lower concentration observed in blended juice (arils plus seeds) albeit significant (Fig. 2c). A possible cause could be crushing of arils which resulted in dilution due to addition of seed concentration such as oil into the juice. The ratio of glucose to fructose did not vary significantly ($P > 0.05$) as a result of similar amounts of glucose and fructose concentrations observed in the pomegranate juice (Fig. 2d).

3.2. *Flavonoids and phenolic acids concentrations*

Flavonoid compounds including catechin, epicatechin and rutin were identified in pomegranate crude juice, whereas gallic acid was the only phenolic acid found in all pomegranate juice investigated (Fig. 3). Catechin concentration was the highest flavonoid compound in all the juice, followed by epicatechin and rutin (Fig. 3a, 3b and 3c). Several researchers have reported catechin as the most dominant flavonoids in 'Wonderful' and several Chinese cultivars (Mphahlele et al., 2014b; Li et al., 2015). Significantly higher ($P < 0.05$) catechin concentration was found in juice obtained by blender (arils plus seeds) with an average of 1.67 mg/L which was 54.96, 137.37 and 94.27% higher than those of juice from arils, whole and halved fruit, respectively. This difference

may be because of the extraction of catechin concentration from pomegranate seed residue. He et al. (2011) showed that pomegranate seed residue is a rich source of catechin. In the case of epicatechin, halved fruit (1.23 mg/L) had the highest concentration than the arils, arils plus seed and whole fruit juice (Fig. 3b). Most of the phenolic compounds that are present in the pomegranate peel are passed onto the juice during pressing. de-Pascual-Teresa et al. (2000) found the presence of epicatechin in pomegranate peel. This suggests that the hand press method used in this study could facilitate in the increasing of epicatechin in pomegranate juice. Rutin concentration ranged from 0.9 to 0.14 mg/L with no significant ($P>0.05$) differences amongst the pomegranate juice (Fig. 3c). Notable difference was observed in the gallic acid concentration among the extraction method and followed the order arils > whole fruit > halved fruit > arils plus seeds (Fig. 3d). These results suggest that even though the phenolic compounds classes are similar among the methods of extraction, the specific compounds may be more abundant as results of different extraction methods.

3.3. Individual anthocyanin concentrations

It is well known that anthocyanins are responsible for the desirable red colour of pomegranate juices as well as many other red-coloured fruit juices (Li et al., 2010). In this study, eight individual anthocyanins were detected and identified including delphinidin-3,5-diglucoside, cyanidin-3,5-diglucoside, pelargonidin-3,5-diglucoside, delphinidin-3-glucoside, cyanidin-3-glucoside, pelargonidin-3-glucoside, cyanidin-pentoside and cyanidin-3,5-pentoside-hexoside (Fig. 4). Individual anthocyanin concentrations observed in pomegranate crude juice had the same anthocyanin profile. A similar anthocyanin profile was also detected by Fischer et al. (2011) in Peruvian pomegranate juice. It is noteworthy that the proportion (%) of the individual anthocyanin in the pomegranate juice are in the order: cyanidin-3,5-diglucoside > delphinidin-3,5-diglucoside > cyanidin-3-glucoside > pelargonidin-3,5-diglucoside > delphinidin-3-glucoside > pelargonidin-3-glucoside > cyanidin pentoside > pelargonidin-3-glucoside > cyanidin-3,5-pentoside-hexoside (Fig. 4a). Moreover, anthocyanin derivatives in the juices are in the order: cyanidin > delphinidin > pelargonidin > cyanidin pentoside > cyanidin-3,5-pentoside-hexoside (Fig. 4b). Diglucoside had relatively higher proportions (%) than monoglucoside in all the pomegranate juice (Fig. 4c).

3.4. Total phenolic concentration (TPC), total monomeric anthocyanin (TMA), total tannins and total flavonoids

Total phenolic concentration is in the order of arils plus seeds (138.36 mg GAE/100 mL) > whole fruit (185.37 mg GAE/100 mL) > arils (215.21 mg GAE/100 mL) > halved fruit (289.94 mg

GAE/100 mL) (Table 4). An increase in total phenolic concentration may be related to increased extractability of several phenolic compounds found in the exposed rind, arils and membrane of halved pomegranate fruit as compared to whole fruit in the present study. Therefore, higher TPC found in the hand pressed (halved fruit) juice could be a preferable option as healthy products. Rajasekar et al. (2012) found that juice containing pith together with the carpellary membrane and the seeds had higher total phenolic concentration than that of the arils only. Similarly, significantly higher ($P < 0.05$) total tannin concentrations was found in the juice obtained using handpress with the lowest observed in blended juice. Furthermore, total flavonoid concentration is in the order of arils plus seeds (50.39 mg CE/100 mL) > arils (36.67 mg CE/100 mL) > halved fruit (36.28 mg CE/100 mL) > whole fruit (23.35 mg CE/100 mL). On the other hand, whole fruit juice had 21.40, 23.72.91 and 19.22% abundant TMA concentrations than juice from arils, arils plus seed and halved fruit, respectively. Similar findings was also observed by Türkyilmaz et al. (2013), who reported that higher concentration of anthocyanin during extraction from the arils (pomegranate quarters) was attributable to easier lowest pressing pressure (1.2 bars, 5 min). According to Kalt et al. (2000) the high level of anthocyanin at low pH 1 is consistent with the presence of the flavylum cation which is most intensely colored, compared to the quinonoidal pseudobase, and chalcone forms, which are pale or colourless. Therefore, it could be argued that high anthocyanin found in juice obtained from squeezed juice resulted from low pH (1.85) contain therein.

3.5. Radical scavenging activity (RSA), ferric reducing antioxidant power (FRAP) and ascorbic acid concentration

Significantly higher ($P < 0.05$) antioxidant activity measured by radical scavenging activity was found in halved fruit juice whereas whole fruit juice had the least (Table 5). The study is consistent with the report of other investigators, which demonstrated higher antioxidant activities in the peel, pith and carpellary membrane than arils (Li et al., 2006; Fischer et al, 2011). In this study, arils and whole fruit juice did not vary significantly ($P > 0.05$) in radical scavenging activity. Additionally, hand press (halved fruit) mechanism was able to effectively extract phenolic compounds responsible for maximising the concentrations of antioxidant active substances.

Antioxidant activity measured by FRAP of pomegranate juice from halved fruit was 76.23, 40.94 and 71.76% higher than those of juice from arils, whole fruit and arils plus seeds, respectively. Of the four pomegranate juice, halved fruit juice had the highest ascorbic acid concentration (500.00 µg AAE/ mL juice). This could be due to the contribution of ascorbic acid from non-edible parts of the pomegranate (pith and carpellary membranes) by the hand press as previous study has reported

pomegranate that peels are rich source of ascorbic acid (Opara et al., 2009). In addition, it has been reported that higher concentration of citric acid prevents oxidation of ascorbic acid in fruit juices (Fernandez-Fernandez et al., 2010). Thus, it could be suggested that lower ascorbic acid concentration in the blended juice could be as a result of lower concentration of citric acid. Our results indicate that the antioxidant activities (RSA and FRAP) of the different juice were moderately correlated with total phenolics ($r = 0.70$). These results also show that most of the variation in antioxidant activity in the juice of pomegranate can be accounted for by the variation in phenolic compounds concentration.

3.6. Volatile organic composition (VOC) as influenced by method of extraction

A total of 10 VOCs were identified in pomegranate juice cv. 'Wonderful' which belong to the chemical classes of esters, ketones, alcohols, terpenes and monoterpenes (Table 6). Different from our study, Vázquez-Araújo et al. (2011) detected up to 23 volatile compounds in pomegranate juice homogenates (juice mixed with albedo and carpellary membrane of cv. Wonderful) belonging to aldehydes, alcohols and terpenes volatile organic compound groups. Fawole and Opara (2014) reported 14 VOCs in different pomegranate cultivars using similar method employed in this study. Caleb et al. (2013) detected and identified 13 VOCs in pomegranate juice. In this study, juice obtained from arils plus seeds had the highest total relative percentage VOCs (70.37%) while juice extractor (arils) had the least. Therefore, it could be suggested that extraction method increases the number of volatile aroma and this may be dependent on volatile composition of pomegranate fruit parts involved during extraction. According to Koppel et al. (2014) decreased volatile concentration could be linked to less intense flavour attributes in the juice. Therefore, it could be argued that juice obtained from arils plus seed characterized by higher total relative percentage had higher aroma compared to other extraction methods.

High proportions of ethyl acetate and 3-octanone compounds were found in this study irrespective of extraction methods used (Table 6). The average relative peak percentage of ethyl acetate (19.19%) found in the study is relatively higher than that reported by Andreu-Sevilla et al. (2013) in pomegranate cvs. 'Wonderful' (1.28%) and 'Mollar de Elche' (10.8%). Several other compounds including limonene, beta-pinene, alpha-pinene and 1-hexanol were relatively present in lower percentages. However, limonene and alpha-pinene did not vary significantly in all juice variants ($P > 0.05$). Limonene has been identified as important volatile component of pomegranate juice (Vázquez-Araújo et al, 2011; Mayuoni-Kirshnbaum and Parot, 2013) and the odour contribution has been described as lemon and orange. Besides, significantly higher beta-pinene

(1.99%) and 1-hexanol (5.52%) were observed in the arils plus seeds juice and has been shown to be associated with pine, resin, and turpentine odor (Càlin-Sánchez et al., 2011) whereas 1-hexanol is characterised by mint and grass odor (Hamouda et al., 2014). Alpha pinene is characterised by turpentine and pine flavour (Vázquez-Araújo et al., 2011; Mayouni-Kirshnbaum and Parot, 2013). The results suggest that this group of volatiles may be the main contributor to the general aroma in pomegranate juice in this study. To some extent higher relative peak percentage of limonene were reported by other authors. Andreu-Sevilla et al. (2013) found 55% limonene in three pomegranate juice in the headspace of the cvs. 'Wonderful' and 'Mollar de Elche'. Caleb et al. (2013) found 13.07% of limonene in cvs. 'Acco' and 'Herskawitz'. Significantly higher relative percentage of 2-methyl-1-propanol and ethanol were detected only in arils plus seed and whole fruit juice, respectively. Moreover, 2-methyl-1-propanol and ethanol VOCs contributed 23.97 and 19.60% of pomegranate crude juice, respectively. Ethanol percentage (19.60%) in this study was much higher than that reported by Beaulieu et al. (2015) in 11 cultivars grown in the USA which ranged from 0.07 to 0.19%.

3.7. Principal component analysis (PCA)

The biochemical and phenolic compounds and antioxidant activities of pomegranate crude juice obtained using different extraction methods were subjected to PCA. Overall, the total variability was described by 3 factors (F1–F3), with the first two principal factors (F1 and F2) explaining 85.00% of the total variability (Fig. 5a). PC1 explained 57.61% of the total variation while PC2 contributed only 24.56% of the total variability (Fig. 5a). This means that the variation among pomegranate juice obtained using different extraction method was explained by the F1 (Fig. 5a and b). As can be observed, the analysis (Fig. 5a and b) demonstrated that juice from halved fruit (handpress) could be relatively associated with cyanidin glucoside, delphinidin 3,5 diglucoside, total phenolic concentration, total tannins, fructose, vitamin C, and RSA which had high positive scores along F1 (Table 7). High negative scores (Table 7) along F1 (Fig. 5a and b) correspond to L -malic, TSS, pH juice color, total flavonoid concentration, cyanidin-3,5-diglucoside, pelargonidin-3,5-diglucoside, cyanidin pentoside, cyanidin-3,5-pentoside hexoside and catechin concentration of blended juice (arils plus juice). Moreover, lower positive score along the F1 (Table 7; Fig. 5a and b) is associated with titratable acid, citric acid, total acids, total anthocyanin concentration, juice yield, rutin, pelargonidin-3-glucoside, gallic acid concentration of whole fruit juice (squeezer).

Along F2 (Fig. 5a and b), high positive scores (as shown in Table 7) for squeezed fruit (whole fruit) could characterize the juice for having high titratable acidity, total anthocyanin concentration

and delphinidin-3-glucoside. In addition, lower negative scores (Fig. 4a and b) along F2 (Table 7) were from juice obtained using juice extractor (associated with succinic acid, pelargonidin 3,5-diglucoside, fructose, glucose, total sugars, total tannin concentration and total flavonoid concentration). High negative scores (Table 7) along F2 (Fig. 5a and b) corresponded with TSS:TA, total flavonoid concentration, TSS, fructose, glucose, total sugars, total phenolic concentration, pelargonidin-3,5-diglucoside, pH, succinic acid, and juice yield from handpressed fruit juice. The results demonstrated that pomegranate juice obtained using different extraction methods were successfully separated based on the biochemical, and phenolic compounds concentration.

Conclusions

The results indicated that method of extraction significantly influenced pH concentration, TA, TSS:TA, juice yield and colour, suggesting that the type of fruit fraction had an influence on juice biochemical attributes. Fructose and glucose were predominant sugars and citric acid was the predominant acid in all the pomegranate juice. The results also showed that catechin was the highest amongst flavonoid compounds whereas gallic acid was the highest phenolic acids detected irrespective of the juice extraction methods used. Likewise, VOCs including ethyl acetate and 3-octanone had relatively higher percentage which shows that they form part of the fruit fractions used in this study. Also, juice obtained from intact fruit could influence the final concentration of total phenolic and antioxidant activity. The results of the study provide information on the importance of methods of extraction on the quality of pomegranate juice.

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Table 1

Extraction methods used to obtain juice from pomegranate fruit cv. Wonderful.

Extraction technique	Description
1. Juice extractor	Suitable for processing arils, without crushing the seeds (kernels). Juice was extracted from aril by spinning at a minimum speed.
2. Blender	Suitable for processing arils with seeds. In this case, seeds are crushed. Arils plus seeds were blended at a maximum speed for approximately 30 s.
3. Squeezer	Suitable for squeezing whole fruit without blending the fruit. Juice was obtained by pressing the whole fruit at a force of 15 000 N for 5 min.
4. Handpress	Suitable for extracting juice by applying compression force on both side of half-fruit section. In this case, the pith, carpellary membrane and arils were consistently included during the extraction process.

Table 2

Average fruit fractions (n=30) of pomegranate cv. Wonderful.

Fruit fraction	Weight (g)	Peel proportion (%)	Aril proportion (%)	Seed proportion (%)
Arils	331.92±6.83	-	53.79±0.82	-
Arils plus seed	341.78±15.04	-	52.74±3.10	29.62±1.44
Whole fruit	367.82±22.63	50.38±0.25	48.89±0.50	-
Halved fruit	343.43±3.95	49.33±2.51	49.00±3.29	-

Table 3

Biochemical concentration of pomegranate juice extracted using different extraction technique.

Extraction technique	TSS (°Brix)	TA (mg CA 100/ mL)	TSS:TA	pH	Juice color	Yield (%)
Juice extractor	16.33±0.35a	1.55±0.04b	10.58±0.90a	3.23±0.01a	0.87±0.04b	51.86±1.54b
Blender	16.34±0.11a	1.53±0.05b	10.73±0.92a	3.23±0.03a	2.62±0.06a	44.61±1.64c
Squeezer	16.03±0.20a	1.78±0.02a	9.00±0.51b	1.85±0.02c	0.82±0.03b	28.01±0.84d
Handpress	16.16±0.39a	1.56±0.04b	10.39±1.07a	2.67±0.64b	0.83±0.06b	96.58±1.04a

The values are mean (n=3) ± SE; mean value followed by different letter within same column are significantly different (P<0.05) according to Duncan's multiple range test (DMRT). TSS, Total soluble solids; CA, Citric acid; TA, Titratable acidity.

Table 4

Phenolic concentrations of pomegranate juice extracted using different extraction technique.

Extraction technique	Total phenolics (mg GAE/100 mL PJ)	Total tannins (mg GAE/100 mL PJ)	Total flavonoids (mg CE/100 mL PJ)	Total monomeric anthocyanins (mg C3gE/100 mL PJ)
Juice extractor	215.21±21.90b	158.58±25.16bc	36.67±3.43ab	11.22±0.78b
Blender	138.36±2.27c	120.00±11.86c	50.39±6.93a	10.96±0.56b
Squeezer	185.73±3.89b	177.36±10.61b	23.35±2.07b	13.91±0.17a
Handpress	289.94±13.08a	267.10±14.87a	36.28±5.37ab	11.47±1.49ab

The values are mean (n=3) ± SE; mean value followed by different letter within same column are significantly difference (P<0.05) according to Duncan's multiple range tests (DMRT).

Table 5

Antioxidant activity of pomegranate juice extracted using different extraction technique.

Extraction technique	RSA ($\mu\text{M TE/ mL PJ}$)	FRAP ($\mu\text{M TE/ mL PJ}$)	Ascorbic acid ($\mu\text{g AAE/ mL PJ}$)
Juice extractor	877.75 \pm 159.56b	62.42 \pm 3.54c	395.17 \pm 12.06b
Blender	465.81 \pm 17.07b	91.97 \pm 1.55b	427.19 \pm 52.39ab
Squeezer	988.05 \pm 17.07c	65.74 \pm 3.40c	187.28 \pm 4.69c
Handpress	1337.84 \pm 43.90a	139.32 \pm 4.05a	500.00 \pm 13.08a

The values are mean (n=3) \pm SE; different letter in the same column indicate significant difference (P<0.05) according to Duncan's multiple range tests (DMRT).

Table 6

Volatile organic compounds (VOCs) of pomegranate juice extracted using different extraction technique and their sensory descriptors.

Volatile compound(s)	RT (min)	Est. K index	Lit. K index	Juice extractor	Blender	Squeezer	Hand press	Descriptors
Ethyl acetate	4.21			29.70±0.82a	21.35±1.90a	27.67±2.71a	31.45±3.94a	Anise, ethereal, pineapple
Alpha-pinene	5.95		861	1.66±0.03a	2.29±0.18a	1.35±0.04a	1.88±0.18a	Harp, pine
2-Methyl-1-propanol	7.18			nd	23.97±2.51	nd	nd	
Beta-pinene	7.40	932	980	1.38±0.036c	1.99±0.01a	nd	1.67±0.06b	Woody
Isoamyl acetate	7.70			nd	6.58±0.77	nd	nd	Banana, pear
3-Octanone	11.75			14.08±1.32a	8.12±0.95 a	14.71±0.97a	18.74±4.13a	
Ethanol	11.96			nd	nd	19.60±0.51	nd	
3-Octanyl acetate	14.37			0.31±0.01a	nd	0.27±0.06a	nd	
1-Hexanol	15.23	860	858	2.01±0.53b	5.52±0.00a	nd	1.52±0.40b	Mint, grass
Limonene	9.70	1018	1019	0.38±0.00a	0.55±0.00a	0.16±0.00a	0.67±0.14a	Lemon, orange
Total				49.52	70.37	63.76	55.93	

RT, retention time. Est. K. index, estimated kovats index. Lit. K. index, literature kovats index (NIST library, version 2). 3-octanol was used as a standard. The values are mean (n=3) ± SE of the relative percentage are presented. nd= not detected. Values within the same row followed by a different letter are significantly different (P<0.05) according to Duncan's multiple range test (DMRT).

Table 7

Factor loadings, eigenvalue, cumulative variance (%) and score for the first three principal (F1–F3) components based on pomegranate juice using different extraction technique.

Loadings	F1	F2	F3
TSS	-0.812	-0.536	0.229
TA	0.528	0.849	0.030
TSS:TA	-0.588	-0.809	-0.010
pH	-0.713	-0.677	0.183
Citric Acid	0.442	0.071	0.894
L-Malic acid	-0.953	-0.279	-0.118
Succinic Acid	0.797	-0.604	-0.016
Total acids	0.629	-0.125	0.767
Fructose	0.835	-0.519	0.182
Glucose	0.634	-0.648	0.422
Total sugar	0.682	-0.675	0.281
Vitamin C	0.965	-0.199	0.169
Juice yield	0.378	-0.865	-0.331
Juice color	-0.893	0.056	-0.446
TPC	0.842	-0.524	-0.125
TTC	0.849	-0.446	-0.284
RSA	0.948	-0.314	-0.047
FRAP	0.775	-0.317	-0.547
TAC	0.571	0.821	0.025
TFC	-0.823	-0.486	-0.295
Catechin	-0.955	-0.256	-0.149
Gallic acid	0.324	-0.016	0.946
Rutin	0.532	0.120	-0.838
Epicatechin	0.676	-0.389	-0.626
Dp3,5dG	0.984	-0.176	0.024
Cya-3,5-dG	-0.944	-0.316	0.093
Pel-3,5-dG	-0.825	-0.559	0.089
Del-3-G	-0.015	0.988	0.156

Cya-3-G	0.958	0.165	-0.234
Pel-3-G	0.562	0.261	-0.785
Cya-Pent	-0.880	0.068	-0.471
Cya-3,5-pent-hexo	-0.916	0.073	0.395
Eigenvalue	18.437	7.859	5.704
Cumulative variance (%)	57.615	82.174	100.000

Scores

Arils	-1.044	-1.599	3.863
Arils plus seeds	-6.565	0.350	-1.921
Whole fruit	3.254	4.364	0.107
Halved fruit	4.355	-3.116	-2.049

TSS= total soluble solids, TA, titratable acidity, TPC = total phenolic concentration, TTC= total tannin concentration, RSA = radical scavenging activity, FRAP = ferric reducing antioxidant power, TAC = total anthocyanin concentration, TFC = total flavonoid concentration, Del 3,5- dG= delphinidin 3,5- diglucoside, Cya 3,5-dG = cyanidin 3,5-diglucoside, Pel 3,5-dG= pelargonidin 3,5- diglucoside, Del-3-G= delphinidin 3-glucoside, Cya 3-G= cyanidin 3-glucoside, pel 3-G= pelargonidin 3-glucoside, Cya-pent = cyanidin pentoside, Cya 3,5-pent-hexo= cyanidin 3,5-pentoside-hexoside.

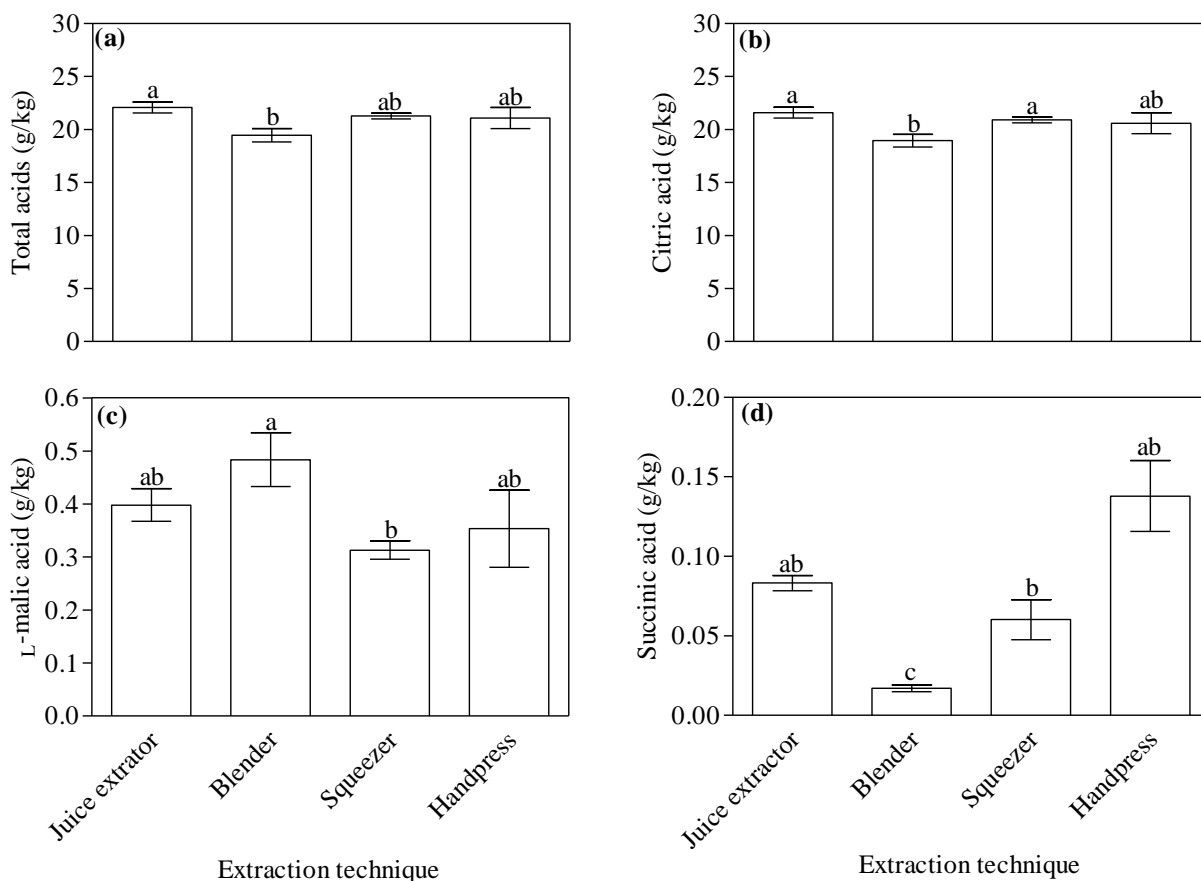


Fig.1. (a) Total acids, (b) Citric acid, (c) L-malic acid and (d) Succinic acid concentrations of pomegranate juice extracted using different extraction technique. Bars with same letter are not significantly different ($P < 0.05$; Duncan's multiple range tests). Data presented are mean \pm standard error of three replicates.

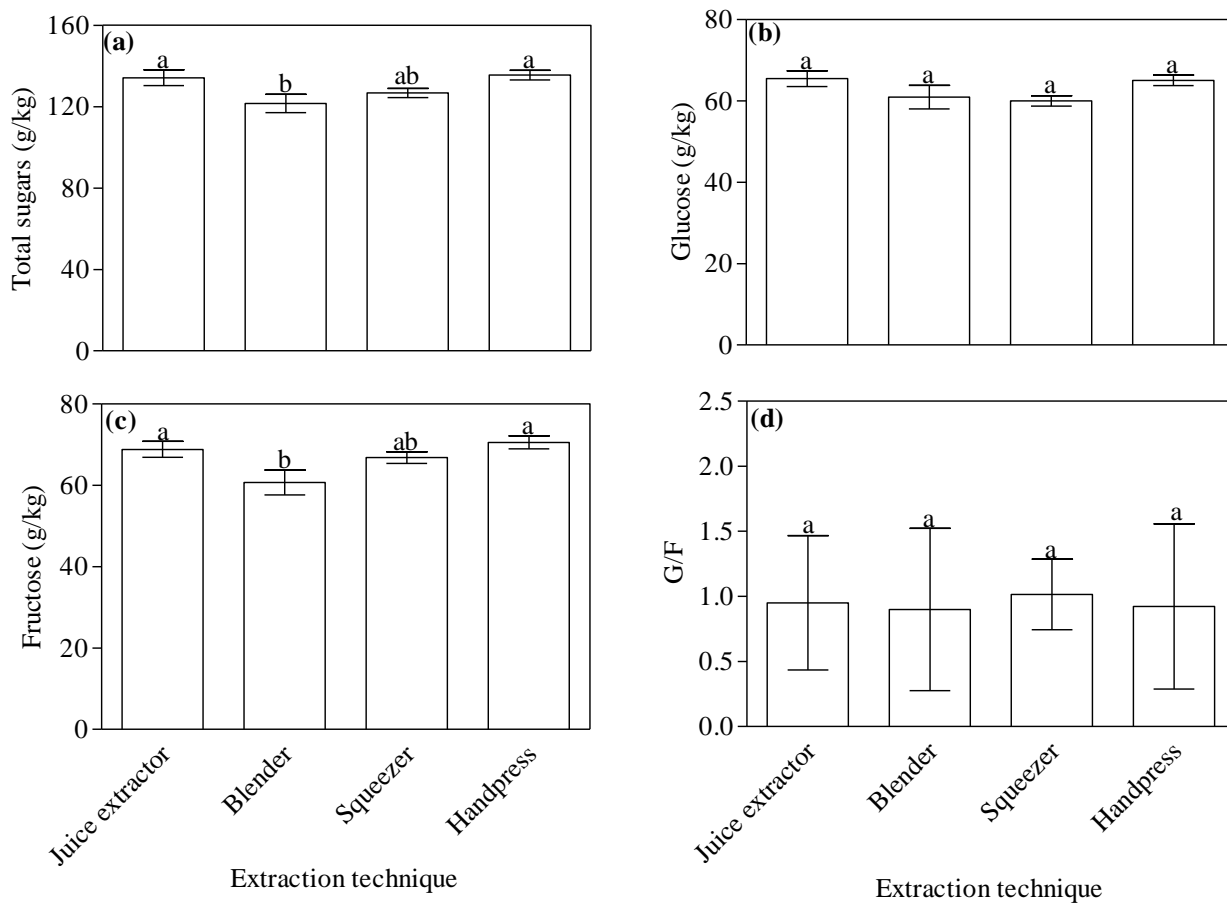


Fig. 2. (a) Total sugars, (b) Glucose, (c) Fructose and (d) G/F concentrations of pomegranate juice extracted using different extraction technique. Bars with same letter are not significantly different ($P < 0.05$; Duncan's multiple range tests). Data presented are mean \pm standard error of three replicates.

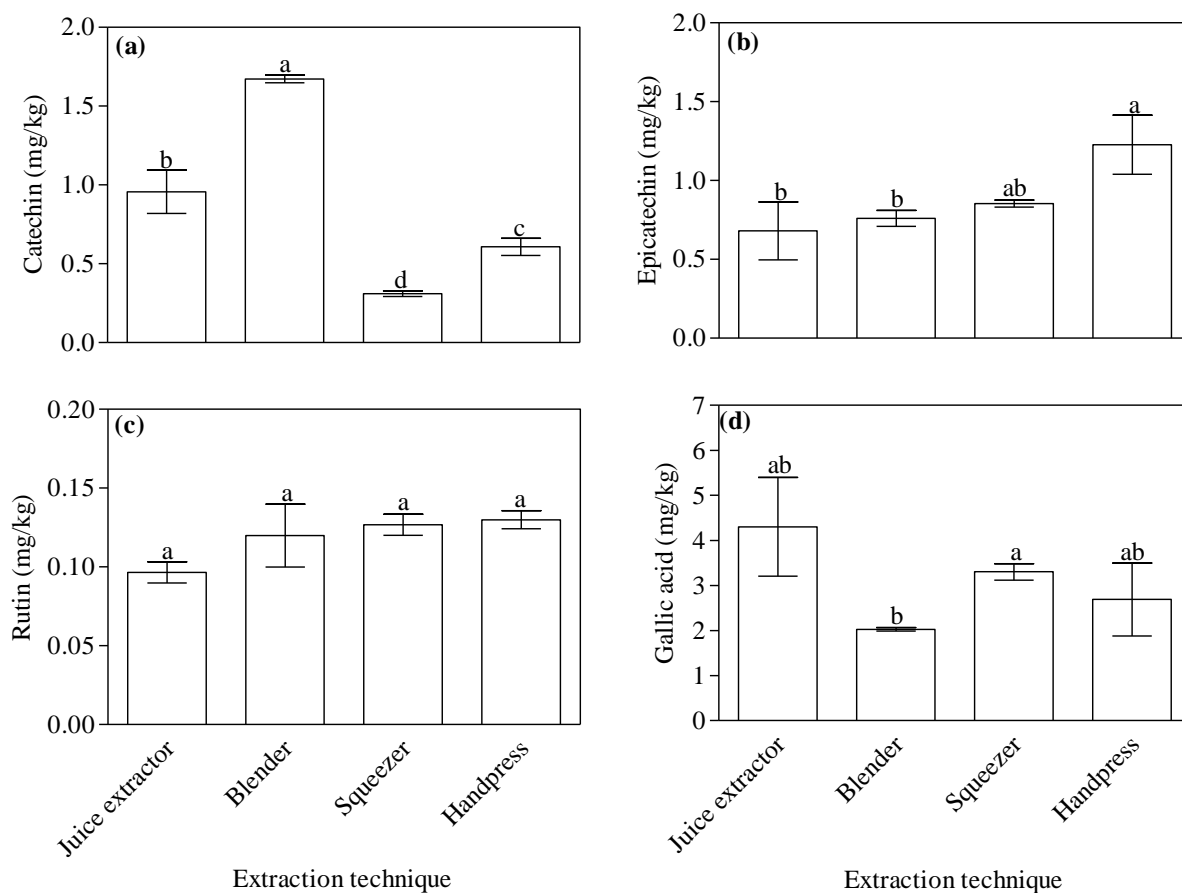


Fig. 3. (a) catechin, (b) epicatechin, (c) rutin and (d) gallic acid concentrations of pomegranate juice extracted using different extraction technique. Bars with same letter are not significantly different ($P < 0.05$; Duncan's multiple range tests). Data presented are mean \pm standard error of three replicates.

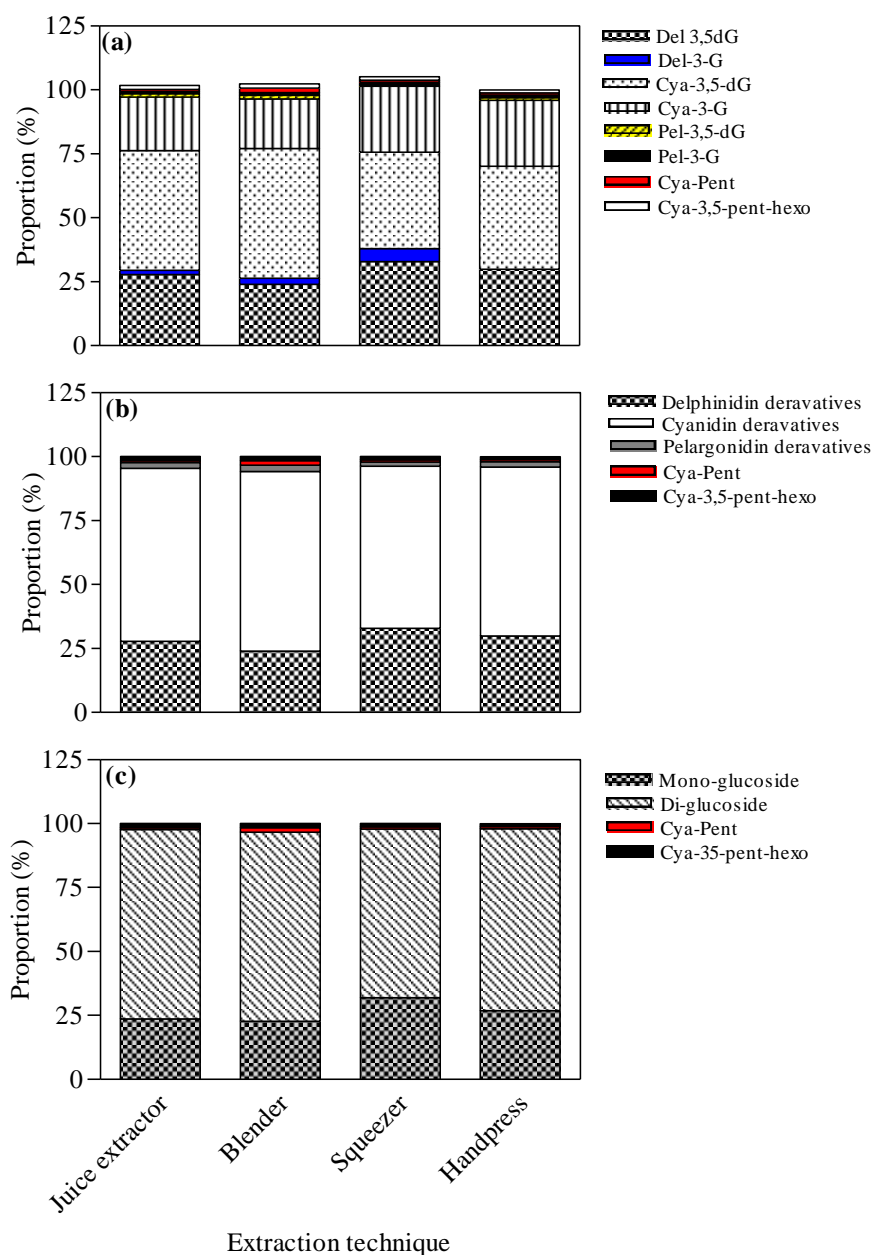


Fig. 4. Individual anthocyanin concentration of pomegranate juice extracted using different extraction technique. Individual anthocyanin compounds (a); anthocyanin derivatives (b); mono and di-glycosylated groups, cyanidin pentoside, cyanidin-3,5-pentoside-hexoside (c); Del-3,5-dG, delphinidin-3,5-diglucoside; Del-3-gluc, delphinidin-3-glucoside; Cya-3,5-dG, cyanidin-3,5-diglucoside; Cya-3-G, Cyanidin-3-glucoside; Pel-3,5-dG, pelargonidin-3,5-diglucoside; Pel-3-G, pelargonidin-3-diglucoside; Cya-pent, cyanidin pentoside; Cya-3,5-pent-hexo, cyanidin-3,5-pentoside-hexoside.

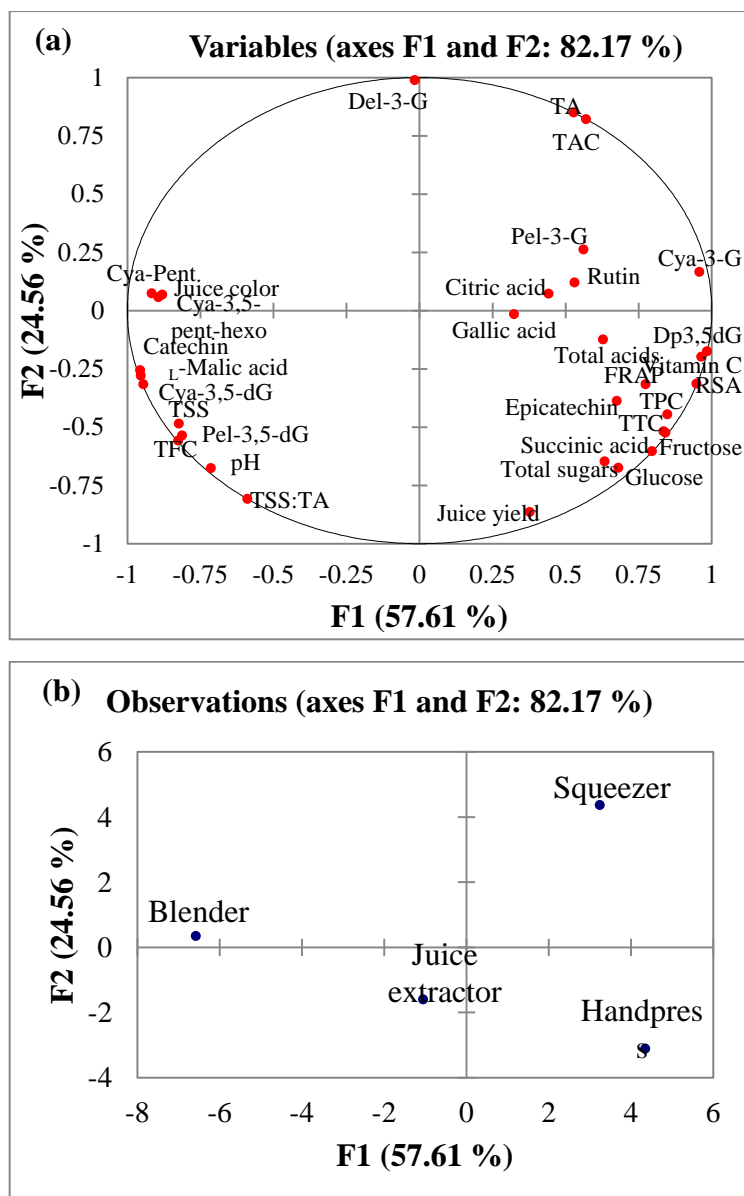


Fig. 5. Principal component analysis of the first two factors (F1 and F2) based on biochemical and bioactive compounds pomegranate juice cv. Wonderful using different extraction technique. Variable plot (a): TSS = total soluble solids, TA = titratable acidity, TPC= total phenolics, TTC= total tannin concentration, RSA = radical scavenging activity, FRAP = ferric reducing antioxidant power, TAC = total anthocyanin concentration, TFC = total flavonoid, Del-3,5-dG= delphinidin-3,5-diglucoside, Cya-3,5-dG = cyanidin-3,5-diglucoside, Pel-3,5-dG= pelargonidin-3,5-diglucoside, Del-3-G = delphinidin-3-glucoside, Cya-3-G= cyanidin-3-glucoside, pel-3-G = pelargonidin-3-glucoside, Cya-pent = cyanidin-pentoside, Cya-3,5-pent-hexo = cyanidin-3,5-pentoside-hexoside.

PAPER 5

Influence of packaging system and long term storage on pomegranate fruit. Part 1: Physiological attributes of whole fruit, biochemical quality, volatile composition and antioxidant properties of juice

Abstract

Commercially ripe pomegranate fruit were packed in ventilated carton with polyliner (referred to as passive modified atmosphere packaging, MAP), individual shrink wrap and open top carton (control) and stored under $7\pm 0.5^{\circ}\text{C}$ and $92\pm 2\%$ RH for 4 months. Incidence of physiological disorders and changes in biochemical properties, phenolic compounds, total phenolics, total flavonoids, total tannins, total anthocyanins, antioxidant activity and vitamin C were analysed monthly. The results showed that fruit stored under polyliner and individual shrink wrapped significantly minimized weight loss compared to control. Significantly higher fruit decay incidence was observed after 3 months, irrespective of package type. TSS content, TSS:TA, citric acid, and L-malic concentrations decreased considerably in all packaging systems with increasing storage time. Fructose and glucose concentrations fluctuated during storage with the lowest value observed at the end of storage in fruit packed under polyliner and shrink wrapped packaging. Amongst phenolic compounds identified, catechin and rutin increased by 65.43% and 139.39%, respectively, in fruit packed inside polyliners and individual shrink wrap after 4 months days of cold storage. Total phenolic and total tannin concentrations declined by 23.86 and 65.89% in fruit stored under polyliner and individual shrink wrap packaging after 3 months of storage, respectively. Furthermore, total anthocyanin concentration was significantly higher in fruit packed in MAP (10.35 mg C3gE/ 100 mL) than individual shrink wrap (8.47 mg C3gE/ 100 mL) after 4 months of storage. Volatiles organic compounds including ethanol, alpha-pinene and beta-pine accumulation increased significantly with prolonged storage regardless of packaging material used.

Keywords: Fruit quality, Individual shrink wrap, Modified atmosphere packaging, Polyphenols, Pomegranate, Storage

1. Introduction

Pomegranate fruit (*Punica granatum* L) is one of the oldest known fruit belonging to the Punicaceae family. Pomegranate is highly appreciated for its unique organoleptic properties and hence its wide production across the world and most recently in South Africa (Al-Said et al., 2009; Holland et al., 2009; Fawole and Opara, 2013a,b). The fruit contains substantial amount of

polyphenols of high biological value including flavonoids (anthocyanins, flavonols), hydrolysable tannins (ellagitannins, gallotannins, condensed tannins (proanthocyanidins) (Hernandez et al., 1999; Gil et al., 2000; Li et al., 2006). These polyphenols have been reported to have a broad range of potentially therapeutic uses, including treatment and prevention of cancer, cardiovascular diseases, Alzheimer's disease and inflammatory diseases (Fuhrman et al., 2005; Hong et al., 2008). These effects have been attributed to the exceptionally high amount of antioxidant capacity often attributed to the high concentration of polyphenols in the juice (Gil et al., 2000; Fischer et al., 2011).

Incidence of postharvest losses and poor keeping quality of pomegranate are largely attributed to high sensitivity of the fruit to temperatures below 4°C and above 10°C (Arendse et al., 2014; Fawole and Opara, 2014). The storage temperature recommended for pomegranates varies from 5 to 7.5°C, with shelf life ranging from 8 to 16 weeks depending on cultivar (Fawole and Opara, 2013a; Arendse et al., 2014; Opara et al., 2008). Pomegranate fruit is also highly susceptible to moisture loss due to the presence of micro-cracks that allow free movement of water from its surface (Elyatem and Kader, 1984; Opara et al., 2010). To reduce postharvest losses and maintain fruit quality, modified atmosphere packaging (MAP) in combination with postharvest treatments has been introduced (Caleb et al., 2013a; Opara et al., 2016). Modification of the atmosphere inside the package preserves quality of produce by retaining moisture and reducing pathological deterioration and metabolic activities (Mir and Beaudry, 2004; Caleb et al., 2012). Nevertheless, extending the shelf-life of pomegranate has been made possible using modified atmosphere packaging (MAP) (Caleb et al., 2013a).

For instance, Nanda et al. (2001) reported significant reduction in weight loss with an increased loss in vitamin C concentration after 12 weeks storage at 8°C in individually shrink wrapped fruit treated with sucrose polyester (SPE) Semprefresh™. Furthermore, D'Aquino et al. (2010) found that film wrapping in combination with fludioxonil completely inhibited weight loss, husk scald and overall improvement of fruit freshness stored at 8°C for 6 or 12 weeks. The authors also found significant reduction in total phenolic concentration whereas antioxidant activity remained relatively stable till the end of the storage. Furthermore, Selcuk and Erkan (2014) observed that prolonged storage up to 4 months at 6°C resulted in decreased total anthocyanin concentration in fruit treated with Prochloraz under modified atmosphere packaging. On the other hand, none of the above studies investigated the volatile evolution of pomegranate fruit with prolonged storage. Volatile organic compounds play a major role in determining the flavour life and the quality of pomegranate fruit during cold storage (Caleb et al., 2013a). It is only recently that Mayuoni-Kirshinbaum et al. (2013) investigated sensory quality and aroma profile during prolonged storage of

‘Wonderful’ pomegranate fruit stored in MAP. The authors found that the sensory quality of pomegranate arils decreased considerably after 16 and 20 weeks of cold storage at 7°C. Additionally, none of the study reported on individual flavonoids and phenolic compounds.

Despite the significant improvement in the fruit quality, effect of packaging on the bioactive compounds and volatile composition is often overlooked. In addition, cultivars may vary in sensitivity to modified atmospheres. Consumer acceptance of this crop requires that fruit be in excellent condition and exceptionally be rich in nutritional and sensory quality. Pomegranate ‘Wonderful’ is the most widely grown and consumed pomegranate cultivar globally (Holland et al., 2009) and during the past ten years, South Africa has seen tremendous increase in commercial production, accounting for over 1000 ha of total planted area and 56% of total production (Hortgro, 2014). Moreover, there has been vast research on pre- and postharvest handling of pomegranate fruit, however less information has been reported on modified atmosphere packages and their influence on concentrations of individual phenolic compounds, volatile composition and antioxidant activity of pomegranate fruit during prolonged storage conditions. The aim of the study was to determine the effect of modified atmosphere packaging and individual shrink wrap film on the biochemical, physiological attributes polyphenols, volatile composition and antioxidant activity of pomegranate fruit cv. Wonderful during long term storage.

2. Materials and methods

2.1. Fruit source

Pomegranate fruit (cv. Wonderful) were sourced during commercial harvest in 2015 from Sonlia packhouse in Western Cape (33°34'851"S, 19°00'360"E), South Africa. Fruit were picked and immediately transported inside air-conditioned car to the Postharvest Technology Laboratory at Stellenbosch University, where healthy with no defect were sorted based on uniform size shape and colour.

2.2. Fruit packaging

A batch of 600 fruit were randomly separated into three lots and each lot comprising 200 fruit was assigned the following three treatments: (1) control, with fruit packed in open top cartons without liner bag (dimensions: width 0.3 m, length 0.4 m, height 0.133 m and a total of 21 perforations (70.9%)); (2) passive MAP, with fruit packed in open top cartons with polyliner bag (ZOEpac, South Africa); (3) shrink film wrap, with each fruit shrink-wrapped using a double-layered

co-extruded polyolefin film (BDF-2001, Mipaq, South Africa), thickness of 25 micron, oxygen transmission rate 4500 cc/m²/day). Fruit were individually wrapped using a portable I-bar sealer (model: ME450IP-450SP) followed by heat-shrinking of the film using a portable heat gun (model: ME-1200-HG) with the operating temperature range of 315- 537°C. Dry cup technique (ASTM, 2005) method E96-95 was used with slight modification to determine water vapour transpiration rate (WVTR) gravimetrically (Hussein et al., 2015; Opara et al., 2015) at 7.5± 0.5 °C and 90±2 % RH over a period of 4 months. In triplicate, aluminium test cups (diameter 5.6 cm and depth 1.5 cm) with open top-screw lid (Comar International, Cape Town, South Africa) were filled with 8.0 ± 0.5 g of anhydrous calcium chloride salt (CaCl₂). Film was placed on top of each test cup and firmly closed exposing film surface area of 25 cm². Each cup was first sealed using an O-ring rubber and lubricated to ensure airtight and moisture proof condition. The WVTR (g/m²/day) of films was calculated on basis of mass gain in water by CaCl₂ salt in the test cup over time 4 months (equation 1):

$$WVTR = \frac{W_i - W_t}{\Delta t} - \frac{1}{\Delta P} \quad (1)$$

W_i represents the initial weight of the test cup; W_t is the weight (g) of the test cup at time Δt (daily); ΔP is the differential water vapour pressure (kPa). However, during each test, the cup was kept in a constant environment (°C and % RH) and therefore differential water vapour was not considered during calculations. Water vapour transmission rates of shrink wrap film obtained was 10.90 g/m²/day.

2.4. Gas composition analysis

Six cartons each containing 12 fruit were used to monitor MAP gas composition for the entire storage duration. Internal atmospheres created by the polyliner bag (MAP) were assessed daily during cold storage using a gas analyser with accuracy of 0.5% (Checkmate 3, PBI Dansensor, Ringstead, Denmark). Gas analysis was done by inserting a needle attached to the gas analyser through a rubber septum on the packaging film.

2.5. Fruit storage and sampling procedure

After applying the packaging treatments, fruit were stored at 7.5 ± 0.5°C and 90 ± 5% RH for 4 months and sampling was carried out at monthly intervals. Cold store temperature (°C) and RH (%) were monitored at hourly interval using Tiny Tag TV-4500 data loggers (Gemini Data Logger,

Sussex, UK). On each sampling date, 24 fruit per treatment were evaluated for physiological attributes (weight loss and physiological disorders).

2.6. *Weight loss and decay incidence*

Fruit weight was measured using an electronic weighing balance (ML3002.E, Mettler Toledo, Switzerland). After every month, samples of 24 fruit of known weight before storage were reweighed and weight loss was calculated using the equation 2:

$$WL = \left[\frac{W_0 - W_f}{W_0} \right] \times 100 \quad (2)$$

where WL is the weight loss (%), W_0 is the initial weight (g) and W_f is the final weight (g) at the time of sampling during storage. Visual appearance (shrivelling) was assessed based on a 5 point hedonic scale: 5 = excellent, 4 = good, 3 = poor, 2 = limit marketability, 1 = very poor. Fruit decay incidence was expressed as percentage using the following scoring system: 0= without decay; 1= 1-25%; 2 = 25-50%; 3 = 50-75%; 4 = 100%. An index of fruit decay was calculated by multiplying the scores of severity by the number of fruit affected and dividing by the total number of fruit (Artés et al., 1998).

2.7. *Biochemical properties*

2.7.1. *Total soluble solids (TSS), titratable acidity (TA)*

Total soluble solid in (°Brix) of pomegranate juice was measured using a digital refractometer (Atago, Tokyo, Japan, calibrated with distilled water) at 20°C. A metrohemn 862 compact titrosampler (Herisau, Switzerland) was used to determine titratable acidity (g citric acid (CA) /100 mL).

2.8. *Determination of individual compounds*

2.8.1. *Organic acids and sugars (refer to Chapter 1)*

A Thermo Scientific Arena 20XT random access chemistry analyser was used for enzyme robot assays. The concentrations of organic acids including L-malic, succinic and citric and sugars (D-glucose, D-fructose and sucrose) were determined using enzymatic test kits (R-Biopharm AG, Germany) by measuring the formation of NADPH at 340 nm.

2.9. Determination of phenolic acid, flavonoids and individual anthocyanin concentration

LC-MS and LC-MS^E analyses were conducted on a Waters Synapt G2 quadrupole time-of-flight mass spectrometer system (Milford, MA, USA). The instrument was connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC) and Acquity photo diode array (PDA) detector. The gradient for the analysis of phenolic compounds started with 100% using 0.1% (v/v) formic acid (solvent A) and kept at 100% for 0.5 min, followed by a linear gradient to 22% acetonitrile (solvent B) over 2.5 min, 44% solvent B over 4 min and finally to 100% solvent B over 5 min. The column was subjected to 100% solvent B for an extra 2 min. The column was then re-equilibrated over 1 min to yield a total run time of 15 min. Reference standards (Sigma-Aldrich, South Africa) of flavonoids and phenolic acids were used for the quantification of individual compounds in pomegranate juice (PJ). For anthocyanin, solvents that constituted a mobile phase were A (7.5% (v/v) formic in water) and B (7.5% (v/v) formic acid in acetonitrile). The gradient started with 1% B isocratically for 0.5 min followed by a linear increase to 15% at 15 min, 23% at 20 min and 28% at 25 min. Column precondition at 100% B subsequently followed for 1 min followed by re-equilibration for 4 min (total run-time of 30 min). Injection volume of 3 μ L at a flow rate of 0.1 mL/min was used. Anthocyanin was identified by comparison with mass spectra with those in the literature (Sentandreu et al., 2013). Proportion of individual anthocyanin was calculated and presented from the peak areas.

2.10. Determination of total phenolic concentration

Total phenolic concentration (TPC) was measured using the Folin-Ciocalteu (Folin-C) method as described by Makkar (2000) with slight modification (Fawole et al., 2012). Diluted pomegranate juice extract (50 μ L) was mixed with 450 μ L of 50% methanol followed by the addition of 500 μ L Folin-C and then sodium carbonate (2%) solution after 2 min. The mixture was vortexed and absorbance read at 725 nm using a UV-visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin) after incubation for 30 min at room temperature. Gallic acid standard curve (0.02– 0.10 mg/mL) was used and TPC was expressed as milligram gallic acid equivalent per 100 mL PJ (mg GAE /100 mL PJ).

2.11. Determination of total tannin concentration

Total tannin analysis was carried out using Folin-C method described by Makkar (2000). Polyvinylpyrrolidone (PVPP) was used to separate tannin from non-tannin compound in

pomegranate juice by adding 100 mg of PVPP to 1.0 mL of distilled water and 1.0 mL PJ in a test tube. The mixture was vortexed and kept at 4°C for 15 min followed by centrifugation at 4000 × g for 10 min. After the extraction, 50 µL of supernatant was mixed with 450 µL of 50% methanol followed by the addition of 500 µL Folin–C and then sodium carbonate (2%) solution after 2 min. The absorbance was recorded at 725 nm using UV–visible spectrophotometer after incubation for 40 min at room temperature. Separate juice extract not treated with polyvinylpyrrolidone (PVPP) was measured for total tannin concentration. Total tannin concentration was calculated using equation 3:

$$\text{Total tannin concentration (TTC)} = \text{TPC}_{(\text{in juice without PVPP})} - \text{TPC}_{(\text{in juice treated with PVPP})} \quad (\text{eqn. 3})$$

where TPC is the total phenolic concentration (mg GAE /100 mL PJ). Results were expressed as milligram gallic acid equivalent per 100 mL PJ (mg GAE /100 mL PJ).

2.12. Determination of total flavonoid concentration

Total flavonoid concentration was measured spectrophotometrically as described by Yang et al. (2009). PJ (1 mL) was extracted with 50% methanol (10 mL) and vortexed for 30 s. The mixture was sonicated in an ultrasonic bath for 10 min and centrifuged at 4000 × g for 12 min at 4°C. Distilled water (1.2 mL) was added to 250 µL of extracted PJ and then followed by 75 µL of 5% sodium nitrite. After 5 min, freshly prepared 10% aluminium chloride (150 µL) was added to the mixture, followed by the addition of 500 µL sodium hydroxide after a another 5 min, and 775 µL distilled water bringing the final volume to 3 mL. The mixture was vortexed and absorbance was immediately read using spectrophotometer at 510 nm. Catechin (0.025–0.100 mg/mL) was used to obtain the standard curve. The results were expressed as catechin equivalent per 100 mL PJ (mg CE/100 mL PJ).

2.13. Determination of total monomeric anthocyanin concentration

The pH differential method described by Giusti and Wrolstad (2001) was used to determine total monomeric anthocyanin concentration. PJ (1 mL) was extracted with of 50% methanol (14 mL) by sonication for 5 min and followed by centrifugation at 4000 g for 12 min. Juice supernatant (1 mL) was taken into vials and diluted with 7 mL of potassium chloride buffer (pH 1.0) and sodium acetate buffer (pH 4.5), separately. After 10 min absorbance values of each buffer mixture was measured at 510 nm and 700 nm in a UV-Visible spectrophotometer. Results were expressed as

milligram cyanidin-3-glucoside equivalent per 100 mL pomegranate juice (mg C₃g E /100 mL PJ) according to equations 3 & 4.

$$A = (A_{510} - A_{700})_{pH1.0} - (A_{510} - A_{700})_{pH4.5} \quad (\text{eqn. 4})$$

$$\text{Monomeric Anthocyanin Concentration (MAC)} = \frac{(A \times MW \times DF)}{\epsilon \times L} \quad (\text{eqn. 5})$$

where A = Absorbance values at 510 nm and 700 nm, ϵ = Cyanidin-3-glucoside molar absorbance (26,900), MW = Cyanidin-3-glucoside molecular weight (449.2 g/mol), DF = Dilution factor, L = Cell path length (1cm).

2.14. Determination of ascorbic acid concentration

Ascorbic acid was determined according to Klein and Perry (1982) with slight modifications (Barros et al., 2007). Pomegranate juice (1.0 mL) was mixed with 14 mL of 1% metaphosphoric acid followed by sonication on ice for 4 min and centrifugation at 4000 × g for 12 min. Supernatant (1.0 mL) was pipetted into a tube and mixed with 9 mL of 2,6 dichlorophenolindophenol dye (0.0025%). The mixture was incubated in the dark for 10 min before absorbance was measured at 515 nm. Calibration curve of authentic L-ascorbic acid (0.01 – 0.1 µg/mL) was used to calculate ascorbic acid concentration. Results were expressed as ascorbic acid equivalents per millilitre crude juice (µg AAE/mL PJ).

2.15. Radical scavenging activity (RSA)

The ability of PJ to scavenge the 2,2-diphenyl-1-picryl hydrazyl (DPPH) radical was measured following the procedure described by Karioti et al. (2004) with slight modifications (Fawole et al., 2012). Pomegranate juice extract (15 µL) was mixed with 735 µL methanol and 0.1 mM solution of DPPH (750 µL) dissolved in methanol. The mixture was incubated for 30 min in the dark at room temperature before measuring the absorbance at 517 nm using a UV–visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). The RSA was determined by ascorbic acid standard curve (0–2000 µM). The results were presented as micro gram ascorbic acid (AA) equivalent per millilitre of crude pomegranate juice (µM AAE/mL PJ).

2.16. Ferric reducing antioxidant power (FRAP)

Ferric reducing antioxidant power assay was performed according to the method of Benzie and Strain (1996). FRAP solutions contained 25 mL acetate buffer (300 mM acetate buffer, pH 3.6),

2.5 mL (10 mM of TPTZ solution), 2.5 mL (20 mM of FeCl₃ solution). Ten millilitre of aqueous methanol (50%) was added to PJ (1 mL), sonicated for 10 min in cold water and centrifuged for 5 min at 4°C. PJ (150 µL) was mixed with 2850 µL FRAP and the absorbance was read at 593 nm after 30 min incubation using a UV–visible spectrophotometer. Trolox (100–1000 µM) was used for calibration curve, and results were expressed as trolox (µM) equivalents per millilitre pomegranate juice (µM TE/mL PJ).

2.17. Extraction and gas chromatographic analyses of volatile compounds

Volatile compounds were trapped and extracted from the vial headspace using headspace solid-phase micro-extraction (HS-SPME) method described by Melgarejo et al. (2011). Ten millilitre aliquot of fresh pomegranate juice was in a 20 mL SPME vial. Sodium chloride (30% mass/volume) was added to the juice to facilitate evolution of volatiles into the headspace and inhibit enzymatic degradation and 10 µL of 3-octanol (at 1 ppm) was added as an internal standard. The SPME vials were equilibrated for 10 min at 50°C in the CTC autosampler incubator at 250 rpm. Subsequently, a 50/30 m divinylbenzene/-carboxen/-polydimethylsiloxane (DVB/CAR/PDMS) coated fibre was exposed to the sample headspace for 20 min at 50°C. The desorption of the volatile compounds from the fiber coating was made in the injection port of CTC at 250°C during 5 min in splitless mode. Separation, identification and quantification of the volatile compounds were performed on a gas chromatograph using Agilent 6890 N (Agilent, Palo Alto, CA), coupled with an Agilent mass spectrometer detector Agilent 5975 MS (Agilent, Palo Alto, CA). The GC–MS system was equipped with a polar Agilent Technologies DB-FFAP capillary column (model J&W 122-3263) with dimensions 60 m × 250 mm i.d. and 0.50 µm film thickness. Analyses were carried out using helium as carrier gas with a flow of 1.9 mL/min with nominal initial pressure of 216.3 kPa and average velocity of 36 cm sec⁻¹. The injector temperature was maintained at 250°C. The oven temperature was as follows: 70°C for 1 min; and then ramped up to 142°C at 3°C min⁻¹ and finally ramped up to 240°C at 5°C min⁻¹ and held for 3 min. Compounds were tentatively identified by comparison of the retention times (RI); Kovats retention indices (KI); and, by comparison with mass spectral libraries (NIST, version 2.0). For quantification, the calculated relative percentages were used.

2.18. Statistical analysis

The data was subjected to one-way anova using Statistica software versions (12.0, StatSoft Inc., USA). Data for non-destructive measurements (weight loss and decay incidence) were analysed

for repeated measures over time using general linear model (GLM) procedure and where appropriate, two-factorial analysis (factor A= package; factor B= storage duration) was conducted. Means were separated by least significant difference (LSD; $P = 0.05$) according to Duncan's multiple range test. GraphPad Prism software version 4.03 (GraphPad Software, Inc., San Diego, USA) was used for graphical presentations. Values are presented as mean \pm standard error.

3. Results and discussion

3.1. CO_2 and O_2 concentrations inside the modified atmosphere packaging

A concomitant decrease in O_2 and increase in CO_2 concentrations inside the modified atmosphere packaging (polyliner bag) during storage is shown in Fig. 1. The O_2 concentration decreased significantly ($P < 0.05$) during the first month of storage from 20.90 to 14.45% with corresponding increase in CO_2 level (from 0.04 to 2.40%). The CO_2 concentration continued to a small increase (from 2.49% to 3.05%) while O_2 concentration remained relatively stable until the end of storage period (from 14.45% to 15.10%). However, a steady decrease in O_2 and increase in CO_2 concentrations inside MAP of different pomegranate cultivars ('Mollar de Elche' and 'Hicrannar') during prolonged cold storage ($5^\circ C$ and $6^\circ C$) has been reported by previous researchers (Laribi et al., 2012; Selcuk and Erkan, 2014). In case of cold storage of shrink wrapped fruit, no head space gas analysis was done due to limited air space.

3.2. Weight loss and visual quality

Fruit weight loss (%) of pomegranate stored at $7 \pm 0.5^\circ C$ up to 4 months is shown in Fig. 2A. Pomegranate fruit has been reported to be highly sensitive to moisture loss due to the high porosity of peel which enables free vapour movement (Elyatem and Kader, 1984). Weight loss was significantly ($P < 0.001$) affected by packaging, storage duration and their interaction. In this study, weight loss was significantly minimised when fruit were packed in polyliner bags (modified atmosphere) and shrink wraps and there was no significant difference observed after 3 months of storage. In general, weight loss remained below 2% for fruit stored in MAP and shrink wrap throughout the storage duration whereas in control fruit weight loss was about 16.29% by after three months when the trial was terminated due to excessive shrivelling (Fig. 2A). According to Dhall et al. (2012), the reduction in weight loss of individually shrink wrapped fruit could be due to alleviation of water stress around each fruit which in turn minimises respiration rate. It is known that MAP of fresh fruit limits water vapour diffusion thereby generating higher water vapour pressure

and relative humidity within the package (Serrano et al., 2006). Similar trend in fruit weight loss was reported for pomegranate cvs. Hicrannar (Selcuk and Erkan, 2014) and Primosole (D'Aquino et al., 2010) under polyliner (MAP) and shrink wrap packaging, respectively.

Fruit visual appearance was highly maintained in MAP and individual shrink wrap packages compared to control fruit and this was consistent during the storage period (Fig. 2B). In particular, visual appearance remained relatively unchanged in MAP and shrink wrap packed fruit for the first month of storage, whereas visual appearance of control fruit decline by one score point (from 'excellent' to 'good' during the same period). Changes in fruit visual appearance became apparent after 3 months of storage in all treatments, with control fruit terminated due to excessive shrivelling graded as limit of marketability. Owing to significant interaction ($P*S = <0.0001$), storage duration affected different packaging options differently. The visual appearance of MAP and individual shrink wrap fruit were graded as acceptable until the end of storage.

3.3. Decay incidence

One of the advantages of using individual wrapping of fruit is that it prevents cross contamination. Prolonged storage duration significantly ($P<0.001$) influenced decay incidence (Fig. 2C). During the first month, decay started occurring in shrink wrapped fruit and control fruit with 4.17% decay incidences. Decay incidence increased significantly in all treatments during storage, with the least decay incidence observed in fruit packed inside open ventilated packaging (control, 16.68%) after 3 months of storage. At the end of the storage period, fruit stored inside polyliner and shrink wrapped had 33.93 and 29.17% decay, respectively with no significant ($P>0.05$) differences observed between the packaging methods. Similar to our findings, D'Aquino et al. (2010) observed no significant variation in decay incidence between control fruit (stored in plastic boxes) and shrink wrapped pomegranate fruit (cv. Primosole) between 1.5 and 2.5 months of cold storage. Selcuk and Erkan (2014) found that 'Hicrannar' pomegranate fruit stored in passive MAP at 6°C started to show decay after 2 months. Similarly, Laribi et al. (2012) found that decay percentage of pomegranate (cv. Mollar de Elche') increased with storage (5°C, 20 weeks) with high percentages observed on fruit packaged in passive or MA than control fruit.

3.4. Biochemical properties

There was a significant effect of packaging, storage duration and their interaction on TSS concentration ($P < 0.0001$) (Table 1). The general trend observed during storage was an initial increase in TSS followed by a decrease. TSS concentration decreased by 6.69% in fruit packed inside polyliner bag (MAP) during the first month of storage with no significant change observed in shrink wrap and control fruit. However, a significant decline was observed after the second month of storage in all the treatments, followed by further increase after the 3rd month of storage with control and MAP stored fruit having the highest TSS than individually shrink wrapped fruit. The increase in TSS concentration of control fruit might be as a result of concentrations of sugars due to moisture loss. At the end of storage, TSS concentration declined by approximately 10.00 and 5.89% in MAP and individual shrink wrapped fruit, respectively. Contrary to our findings, Selcuk and Erkan (2014) observed an increase in TSS concentration after 4 months of cold storage at 6°C when fruit were packed in passive modified atmosphere (polyliner) bags. The findings in the present study are in accordance with Mayouni-Kirshinbaum et al. (2013), D'Aquino et al. (2010) and Nanda et al. (2001) who observed decreases in TSS concentrations in different pomegranate cultivars during prolonged storage under various modified atmosphere packaging. The possible explanation for the observed decrease in TSS concentration in this study could be as a result of the degradation of sugars with prolonged storage period (Fawole and Opara, 2013a).

TA concentration was significantly affected by storage duration ($P < 0.0001$). TA concentrations (g /100 mL) decreased significantly with prolonged storage across all treatments. Decrease in TA could be linked to metabolic activities of pomegranate during storage (Selcuk and Erkan, 2014). Decrease in TA during storage is consistent with the result by other authors on different pomegranate cultivars under modified atmosphere package with increasing storage duration (Laribi et al., 2012; Selcuk and Erkan, 2014). These findings are inconsistent with those observed by Nanda et al. (2001), who reported higher retention of TA in individual shrink wrapped fruit when compared with control. Generally, a decrease in TA concentration during prolonged storage results in an increased TSS:TA ratio and increases sweetness perception of the juice. There were significant effects of packaging, storage duration and their interaction on the TSS:TA ($P < 0.0001$). TSS:TA showed an increasing trend with highest concentration observed after three months in all treatments. However, non-significant differences were observed between MAP and shrink wrap packages at the end of storage (4 months, appendix 1, Table 1). Flavour characteristics of pomegranate juice and indeed other fruit juices are influenced by the TSS:TA ratio.

Organic acids are important flavour components and can contribute to the formation of off flavour. Also, the perception of sweetness is strongly linked to the acidity (Magwaza and Opara, 2015). Citric acid concentration fluctuated with prolonged storage with the lowest concentration observed in MAP (13.31 g/L) and shrink wrapped (13.08 g/L) fruit at the end of 4 months in storage. Generally, a decline in citric acid during storage occurs as a result of on-going metabolism, as observed in pomegranate cultivars 'Wonderful' (Kader et al., 1984) and 'Mollar' (Artés et al., 2000). The L -malic was found to be significantly affected by packaging, storage duration and their interaction ($P < 0.0001$). A gradual decline in L -malic acid (0.34 g/L, harvest) concentration was observed till the end of storage (4 month, appendix 1, Table 1) with the lowest concentration observed for individual shrink wrapped fruit (0.12 g/L) than MAP (0.17 g/L). At harvest, succinic acid averaged 0.02 g/L, however, the concentration remained relatively stable in fruit from all packaging treatments till the end of storage with the exception of a negligible increase observed in individual shrink wrap fruit (0.03 g/L). In this study, citric acid was found to be the most predominant organic acid followed by L -malic acid and succinic acid as reported recently in other pomegranate cultivars (Mena et al., 2011; Fawole and Opara, 2013a,b). The initial total acids was 16.94 g/L, however, the concentrations fluctuated during storage. At the end of storage, MAP and individual shrink wrap stored fruit declined by 20.18% (to 13.52 g/L) and 21.84% (to 13.24 g/L), respectively. The result reveals that total acids declined significantly with storage duration regardless of package treatment. The observed fluctuations in total acid is linked to decline in citric and malic which highlight that total acid are not considerably influence by gas composition (Hess-Pierce and Kader, 2003). Our result is in agreement with Melgarejo et al. (2000) and Carbonell-Barrachina et al. (2012), who reported higher level of fructose than glucose in pomegranate cultivars grown in Spain.

Fructose concentration increased by approximately 1.48 fold when compare to the concentration at harvest (51.63 g/L) during the first months in all treatments and the concentration significantly declined till the end of storage (Table 1). Fructose concentration was higher in MAP fruit (64.66 g/L) compared to individual shrink wrap (57.56 g/L), but higher than the concentration observed at harvest. Similarly, significantly higher glucose concentration was observed in MAP stored fruit (51.48 g/L) than shrink wrap stored fruit (46.64 g/L) at the end of storage. In terms of total sugars, MAP had higher total sugars (116.15 g/L) whereas individual shrink wrap (104.20 g/L) remained relatively similar to that observed at harvest. Fructose was found at higher concentrations than glucose in this study. However, several studies reported relatively higher glucose than fructose concentration (Cam et al., 2009; Ozgen et al., 2008).

3.5. Total phenolics, total tannins, total anthocyanins and total flavonoids concentration

Total phenolic concentration was 492.56 mg GAE/100 mL during the first month and declined significantly with advancement in storage duration in all treatments (Fig. 3A). Control fruit (packed inside open top ventilated cartons) had the highest total phenolic concentration (413.61 mg GAE/100 mL) followed by fruit stored inside MAP (375.01 mg GAE/100 mL) and individual shrink wrap (168.00 mg GAE/100 mL) packages after 3 months of storage. This could be due to concentration effect arising from higher moisture loss of fruit packed in ventilated open top carton packaging. At the end of storage duration, a greater degradation of total phenolic concentration was observed for MAP (152.91 mg GAE/100 mL) and individual shrink wrapped fruit (145.54 mg GAE/100 mL). Degradation of total phenolic is related to enzymatic oxidation (polyphenol oxidase and peroxidase) during storage (Fawole and Opara, 2013c). These results are inconsistent with those reported by Selcuk and Erkan (2014), Fawole and Opara (2013a) who observed an increase in total phenolic concentration in various pomegranate cultivars stored at 6°C and 5°C, respectively. In this present study, it was observed that both MAP and individual shrink wrap packaging did not prevent degradation of total phenolics. In agreement with our findings, Arendse et al. (2014) observed a decrease in total phenolic concentration in pomegranate fruit cv. Wonderful stored at 7.5°C for 5 months using open carton boxes. In the case of total tannins, a similar pattern was observed with the highest tannin concentration found in control fruit after 3 months of storage (Fig. 3B), but similar to shrink wrap at months.

Total anthocyanin showed a progressive increase in all treatments during storage (Fig. 3C). For the MAP stored fruit, anthocyanin concentration increased by 2-folds whereas shrink wrapped fruit increased by 1-fold at the end of the storage, thus, suggesting continuous anthocyanin biosynthesis during cold storage. Increase in anthocyanin with prolonged cold storage has been reported in 'Ruby' pomegranates stored at 10°C and 7°C after 12 weeks (Fawole and Opara, 2013a). Selcuk and Erkan (2014) observed significant increase in total anthocyanin concentration until 3.5 months at 6°C under modified atmosphere packaging. It has been reported that an increase in anthocyanin is related with the activity of enzymes responsible for the biosynthetic pathways: phenylalanine ammonia lyase and uridine diphosphate glucose flavonoid-3-O-glucosyltransferase (Selcuk and Erkan, 2014). The result found in the present study is contrary to the recent study by Arendse et al. (2014) who reported a decline in total anthocyanin concentration of 'Wonderful' after 3 months of storage at 10°C, 7.5°C and 5°C. Several studies have also revealed that anthocyanin concentration in pomegranate fruit increased during cold storage (Artés et al., 2000; Miguel et al.,

2004). Total flavonoids increased steadily after 3 months in all packaging treatments with the highest concentration found in control fruit (108.35 mg CE/100 mL) from an initial concentration of 60.81 mg CE/100 mL PJ (Fig. 3D). However, a decrease by 19% (900.73 to 723.09 mg/L) was observed in MAP while shrink wrapped fruit remained relatively stable during the same storage period. At the end of storage, total flavonoid concentration was reduced by 45% and 43% in MAP and shrink wrapped fruit, respectively (Appendix 2, Table 3).

3.6. Individual bioactive compounds

The predominant bioactive compounds of pomegranate juice identified were flavonoids (catechin, epicatechin, and rutin) and phenolic acid (gallic acid) (Fig. 4). Catechin, epicatechin and rutin concentrations fluctuated throughout the storage period. After 4 months of storage, a gradual increase (> 49.38%) in catechin concentration was observed for both MAP and individual shrink wrapped fruit (Appendix 3, Table 4). There were observed significant effects of packaging and storage duration as well as their interaction ($P < 0.001$) on epicatechin (Fig. 4B). The initial value for epicatechin concentration was 0.60 mg/L. Higher epicatechin concentration was maintained in fruit stored under MAP fruit (0.97 mg/L) than control (0.79 mg/L) and individual shrink wrapped fruit (0.69 mg/L) after 3 months (Fig. 4B). At the end of storage, epicatechin concentration remained relatively stable in MAP (0.97 mg/L) than individual shrink wrapped fruit (0.85 mg/L) when compared to the value at harvest (0.60 mg/L).

Rutin compound showed a progressive and significant increase ($P < 0.05$) in all treatments (Fig. 4C). Moreover, a considerable increase was observed at the end of storage for both MAP (0.24 mg/L) and individual shrink wrapped fruit (0.26 mg/L). Gallic acid concentration declined by almost 89% from the initial concentration of 8.24 mg/L during storage in all the treatments with no significant change observed at the end of storage (Fig. 4D). From the study, it was observed that flavonoids investigated were stable during cold storage compared to the phenolic acid (gallic acid), irrespective of the package type.

3.7. Individual anthocyanin concentrations

Eight individual anthocyanins were detected and identified during cold storage of pomegranate (cv. Wonderful) including 3,5-diglucoside of delphinidin, cyanidin and pelargonidin, 3-diglucoside of delphinidin, cyanidin, pelargonidin and other minor anthocyanins including cyanidin-pentoside and cyanidin-3, 5-pentoside-hexoside (Fig. 5). In agreement to these findings, a similar profile was observed in a Peruvian pomegranate cultivar (Fischer et al., 2011). As can be observed

(Fig. 5a), the proportion (%) of individual anthocyanin during storage are in the order: cyanadin 3, 5-diglucoside > delphinidin-3,5-diglucoside > cyanidin-3-glucoside > pelargonidin-3,5-diglucoside > pelargonidin-3-glucoside > cyanidin-3, 5-pentoside-hexoside > cyanidin pentoside. Pelargonidin 3-diglucoside was greatly reduced after storage with a concurrent increase in cyanadin glucoside in all treatments. Likewise, cyanidin-3, 5-diglucoside seems to increase gradually in all treatments which indicate metabolic activity occurring during storage. Delphinidin-3-glucoside increased after 1 month of storage in all treatments, but the proportion declined afterwards.

3.8. Radical scavenging activity (RSA), ferric reducing antioxidant power (FRAP) and ascorbic acid concentration

Radical scavenging activity in pomegranate juice fluctuated with advancement in storage duration in all treatments (Fig. 6A). There were approximately 1.3 and 1.6-fold decreases in radical scavenging activity level for fruit stored in MAP and individual shrink wrap, respectively, while control fruit remained relatively unchanged after first month of storage. A 2-fold increase in radical scavenging activity was observed in fruit stored under MAP and individual shrink wrap packages at the end of storage (after four month). Lòpez-Rubira et al. (2005) and D'Aquino et al. (2010) did not observe significant change in antioxidant activity of pomegranate (cv. Primrose) during cold storage under modified atmosphere and shrink wrap packaging. Contrary to our findings, Arendse et al. (2014) observed a sharp decline in radical scavenging activity of 'Wonderful' after 5 months of storage. The present study reveals that even though the total phenolic concentration decreased at the end of storage, the radical scavenging activity was still maintained, thus highlighting the possible role of other phenolic compounds in contributing towards radical scavenging ability. The high level of radical scavenging activity of pomegranate juice is often linked to higher polyphenol concentration found in the juice (Viuda-Martos et al., 2010).

The FRAP method for quantifying antioxidant activity is based on the reduction of Fe (III)–tripyriddytriazine complex to Fe(II)–tripyriddytriazine at low pH by electron-donating antioxidants, resulting in an absorbance increase (Apak et al., 2004; Miguel, 2010). Ferric reducing antioxidant power remained relatively stable till month 3 in all treatments (Fig. 6B). At the end of storage, negligible decline was observed in fruit stored in both MAP and individual shrink wrap packages.

Ascorbic acid was found to be significantly influenced by packaging, storage duration and their interaction ($P < 0.0001$). Ascorbic acid concentration in fruit juice declined significantly with advancement in prolonged storage duration for all the treatments (Fig. 6C). Significantly higher concentration was found in individually shrink wrapped fruit (172.02 $\mu\text{g AAE/ mL}$) followed by

MAP (147.91 µg AAE/ mL) after 4 months of storage. The reduction in ascorbic acid during storage is largely due to conversion of ascorbic acid to dehydroascorbic acid due to the action of ascorbic acid oxidase (Singh et al., 2005). Significantly higher concentration of ascorbic acid was found in individually shrink wrapped fruit (172.02 µg AAE/ mL) than fruit packed inside polyliner bags (MAP) (147.91 µg AAE/ mL) after 4 months of storage. The findings are in agreement with the report by Arendse et al. (2014) who observed decrease of 16.94 and 22.94% in ascorbic acid at 5°C and 7.5°C respectively, after 5 months of storage. However, Miguel et al. (2006) found a significant increase in ascorbic concentration ‘Mollar de Elche’ and ‘Assaria’ fruit stored in the dark at 5°C for 4 months. It is worth noting that individually shrink wrapped fruit retained the same amount of ascorbic acid after 1 month of storage. According to Nath et al. (2012), variation in ascorbic acid retention might be due to different level of oxidation as affected by film permeability to the atmospheric oxygen. Therefore, it is possible that retention of ascorbic acid in individual shrink wrapped fruit in our study might be due to low O₂ permeability (4500 cc/m²/day) as observed in pears (Nath et al., 2012), resulting in limitation of atmospheric oxygen for oxidation. Further, the oxidizing enzymes might be reduced (Nath et al., 2012) in individual shrink wrap stored fruit that resulted in higher retention of ascorbic acid.

3.9. Volatile composition

A total of 13 volatile organic compounds (VOCs) from six chemical families were detected in the headspace of pomegranate juice, comprising: alcohols (ethanol; 1-hexanol), esters (ethyl acetate, isoamyl acetate), monoterpenes (limonene, α -terpineol, β -pinene, α -pinene, myrcene, γ -terpinene), sesquiterpenes (α -bergamontene), aldehyde (n-hexanal) and ketone (3-octanone) (Table 2). Seven volatile organic compounds were detected and identified at harvest using HS-SPME and were lower than those reported by other authors in various pomegranate cultivars (Caleb et al., 2013b; Mayouni-Kirshinbaum et al., 2013; Fawole and Opara, 2014). The most dominant volatile compounds observed at harvest were ethyl acetate and 3-octanone whereas monoterpenes including alpha pinene, beta pinene and limonene were detected in lower concentrations during the same period. From this study, alcohol (ethanol), monoterpenes (α -terpineol, myrcene, γ -terpineol) and sesquiterpenes (α -bergamontene) were detected as storage duration advanced in all treatments. However, ethyl acetate compound was only detected up to 3 months in all treatments. Ethanol accumulation during storage under MAP conditions was observed in ‘Wonderful’ pomegranates (Mayouni-Kirshinbaum et al., 2013) and other types of fruit such as strawberries, mandarins and apples (Ke et al., 1994; Rudell et al., 2002; Tietel et al., 2011). Several authors have reported that the accumulation of ethanol and

ethyl acetate compounds was primarily responsible for off-flavours in citrus fruits (Cohen et al., 1990; Shi et al., 2007; Obenland et al., 2011).

As observed in the present study, ethanol concentration (%) was detected after 2 months averaging 18.24% in control fruit while it was undetected under MAP and individual shrink wrapped fruit, thus indicating that the use of both packages resulted in increased ethanol concentration due to CO₂ build-up (Mattheis and Fellman, 2000). Mayouni-kirshinbaum et al. (2013) observed decreased flavour preference of MAP-stored 'Wonderful' pomegranate after 4 weeks at 7°C due to increased ethanol level much above its odour threshold. Therefore, it could be suggested that ethanol build up in control fruit as early as 2 months could exhibit the onset of off-flavour. However, further studies on odour threshold of ethanol in pomegranates in combination with sensory evaluation are needed. This observation that ethanol build-up as early as 2 months of cold storage confirms the principle that the flavour-life of fruit is shorter than the overall storage life as determined by external visual quality of the produce (Baldwin et al., 2007; Kader, 2008; Caleb et al., 2013a). As observed in this present study, the external appearance of fruit packed inside open top ventilated cartons (control) remained relatively appreciable after 2 months of cold storage. After 3 months storage, ethanol compounds was detected in MAP (30.75%) and individual shrink wrapped fruit (11.27%) with substantial increase observed in control fruit (23.92%). Moreover, a sharp increase ethanol compound was observed at the end of storage for fruit stored in MAP (46.67%) and individual shrink wrapped (58.50%). These results indicate that prolonged storage contributes to increase in ethanol accumulation in 'Wonderful' pomegranate, regardless of packaging material used.

Monoterpenes, including alpha pinene, beta pinene and limonene, were detected in lower concentrations (%), but increased slightly with prolonged storage for all the packaging treatments. Several other volatile compounds including α -terpineol and γ -terpinene, and myrcene, which were not detected at harvest accumulated with increasing storage duration. For instance, γ -terpinene was detected in fruit after 3 months storage inside open top ventilated cartons (control) and polyliner bags (MAP). It is therefore hypothesised that the accumulation of terpenes (γ -terpinene and myrcene) was likely as a response to exposure to chilling stress as observed in 'Wonderful' pomegranates during 4-5 months storage at 7°C (Mayuoni-Kirshinbaum et al., 2013) given that pomegranates are chilling sensitive (Kader, 2006).

4. Conclusions

The result of this study showed that packaging fruit inside polyliners (passive MAP) and individual shrink wrapping maintained the visual appearance of pomegranates up to 4 months of cold

storage; however, other quality attributes and phenolic compounds were severely affected. Storage of pomegranate in both passive MAP and individual shrink wrap significantly reduced fruit weight loss. Incidence of fruit decay was more pronounced after 3 months of storage, irrespective of packaging system. However, quality attributes including TSS, citric, L-malic acid and glucose concentration declined significantly during storage, also irrespective of packaging method. Although MAP and individual shrink wrapping kept the fruit until the fourth month, total phenolic, total tannin concentration and antioxidant activity measured by ferric reducing power as well as gallic acid concentration were severely affected. Total anthocyanin increased significantly after 4 months of storage. The study revealed that packaging fruit using MAP or shrink wrapping delayed alcohol accumulation up to 3 months during storage. The results of the study show that fruit could be stored up to 4 months using MAP or shrink wrapping; however, changes in sensory attributes and decay incidence must be taken into consideration in assessing quality of pomegranates stored for such a period. The study suggest that storing pomegranate fruit for up to fourth months under MAP and individual shrink wrap package might not be ideal and should be limited to less than 4 months.

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Table 1
Biochemical properties of ‘Wonderful’ pomegranate after storage for four months at $7 \pm 0.5^\circ\text{C}$ under different types of packaging.

Treatments	Storage duration (Months)	TSS ($^\circ\text{Brix}$)	TA g citric acid (CA) / 100 mL)	TSS:TA	Citric acid (g/L)	L-malic acid (g/L)	Succinic acid (g/L)	Total acids (g/L)	Fructose (g/L)	Glucose (g/L)	Total sugars (g/L)
	1										
Control		16.53 \pm 0.08a	1.86 \pm 0.09a	9.07 \pm 0.44d	22.35 \pm 0.43a	0.36 \pm 0.002b	0.02 \pm 0.002b	22.73 \pm 0.43a	76.54 \pm 0.32ab	71.85 \pm 0.47a	148.40 \pm 0.78ab
MAP		15.33 \pm 0.06cd	1.60 \pm 0.03bc	9.60 \pm 0.16de	22.01 \pm 0.69a	0.40 \pm 0.007a	0.03 \pm 0.001ab	22.44 \pm 0.70a	79.59 \pm 0.98a	74.34 \pm 1.25a	153.94 \pm 2.24a
Shrink wrap		16.16 \pm 0.07ab	1.67 \pm 0.08b	9.85 \pm 0.45e	21.63 \pm 1.01a	0.34 \pm 0.008b	0.03 \pm 0.002ab	22.01 \pm 1.00a	74.57 \pm 0.55b	70.28 \pm 0.08a	144.85 \pm 0.64bc
	2										
Control		15.30 \pm 0.17cd	1.41 \pm 0.08 de	11.20 \pm 0.28d	15.48 \pm 0.41c	0.16 \pm 0.007d	0.03 \pm 0.001ab	15.67 \pm 0.40c	62.41 \pm 2.14c	57.86 \pm 2.07c	120.28 \pm 4.22d
MAP		14.53 \pm 0.34e	1.43 \pm 0.02cd	10.15 \pm 0.78cd	15.07 \pm 0.07c	0.20 \pm 0.002c	0.03 \pm 0.001a	15.30 \pm 0.07c	59.95 \pm 0.49c	54.80 \pm 0.61c	114.76 \pm 1.04d
Shrink wrap		14.93 \pm 0.21de	1.49 \pm 0.02cd	9.99 \pm 0.09d	15.45 \pm 0.54c	0.19 \pm 0.018c	0.03 \pm 0.001ab	15.67 \pm 0.54c	58.91 \pm 3.19c	53.71 \pm 2.93c	112.63 \pm 6.12d
	3										
Control		16.15 \pm 0.05ab	1.23 \pm 0.02ef	13.08 \pm 0.27a	14.63 \pm 0.16c	0.16 \pm 0.003d	0.03 \pm 0.001ab	14.82 \pm 0.16c	62.87 \pm 1.52c	55.49 \pm 1.26c	118.36 \pm 2.78d
MAP		16.50 \pm 0.08a	1.42 \pm 0.04 d	11.72 \pm 0.42bc	15.00 \pm 0.36c	0.16 \pm 0.005d	0.03 \pm 0.001ab	15.19 \pm 0.36c	62.62 \pm 0.49c	56.40 \pm 0.32c	119.02 \pm 0.81d
Shrink wrap		15.78 \pm 0.14bc	1.23 \pm 0.02f	12.78 \pm 0.22ab	17.81 \pm 0.14b	0.20 \pm 0.002c	0.03 \pm 0.003ab	18.04 \pm 0.13b	73.63 \pm 0.33b	65.01 \pm 0.42b	138.64 \pm 0.75c
	<i>P-value</i>										
Packaging (P)		<0.0005	<0.3768	<0.0079	<0.0583	<0.0003	0.6246	0.0572	0.2596	0.4656	0.3478
Storage (S)		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0591	<0.0001	<0.0001	<0.0001	<0.0001
P x S		<0.0003	<0.0062	<0.0006	<0.0022	<0.0001	0.1599	<0.0017	<0.0001	<0.0001	<0.0001

Each value in the table is presented as a mean \pm standard error. Mean values followed by different letter (s) within same column are significantly different ($P < 0.05$) according to Duncan’s multiple range test. In other to determine interaction effects between packaging and storage duration, data for month 1, 2, and 3 for different packaging were used. The storage trial for the control was discontinued after 3 month due to excessive fruit shrivelling and data for month 4 is reported on the appendix 1 (Table 1). TSS, total soluble solids; TA, titratable acidity; P, Packaging, S, storage duration. MAP, modified atmosphere packaging. Harvest value, TSS = 16.43; TA = 2.89; TSS:TA = 5.68; Citric acid = 16.57; L-malic acid = 0.34; Succinic acid = 0.02; Total acids = 16.94; Fructose = 51.63; glucose = 48.62; total sugars = 100.25.

Table 2Volatile composition (%) in juice from 'Wonderful' pomegranate after storage at $7 \pm 0.5^\circ\text{C}$ for four months under different types of packaging.

Compound	Harvest	1			2			3			4	
		Control	MAP	Shrink wrap	Control	MAP	Shrink wrap	Control	MAP	Shrink wrap	MAP	Shrink wrap
Alcohols												
Ethanol	nd	nd	nd	nd	18.24±0.02e	nd	nd	23.92±0.01d	30.92±0.04c	11.27±0.02e	46.67±0.01b	58.50±0.02a
1-hexanol	nd	8.00±0.01a	5.84±1.14b	10.68±0.02a	3.35±0.06bc	nd	13.31±0.07a	2.75±0.04c	nd	2.03±0.06d	2.89±0.01cd	nd
Esters												
Ethyl acetate	64.31±0.07a-d	22.43±0.01e	44.40±0.06ab	50.25±0.05bcd	69.97±0.02a	68.89±0.01a	32.74±0.01e	45.23±0.001cd	41.41±0.07de	51.89±0.04bcd	nd	nd
Isoamyl acetate	0.60±0.01d	nd	nd	nd	1.38±0.01c	nd	nd	1.89±0.06b	nd	nd	5.77±0.06a	nd
Monoterpenes												
Limonene	0.41±0.02d	0.53±0.01c	0.72±0.05cd	1.05±0.04c	0.37±0.03c	0.80±0.01c	1.05±0.01c	2.24±0.01b	2.45±0.01b	1.02±0.04c	nd	3.72±0.03a
α -terpineol	0.35±0.04b	nd	nd	nd	nd	nd	0.52±0.05b	nd	2.45±0.01a	0.13±0.01c	nd	0.26±0.01b
γ -terpinene	nd	nd	nd	nd	nd	nd	nd	0.27	0.33	-	0.42	0.44
β -pinene	1.21±0.09d	1.56±0.02bc	4.56±0.16ac	4.66±0.07ab	nd	1.61±0.45cd	2.85±0.45a	1.87±0.04cd	1.48±0.01bcd	nd	4.83±0.11ab	4.82±0.02a
α -pinene	5.78±0.12cde	nd	4.65±0.13de	7.00±0.06b	2.91±0.10e	2.29±0.05e	2.69±0.03e	7.35±0.05bd	7.60±0.02abc	4.83±0.10cde	13.30±0.01a	10.11±0.07ab
myrcene	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.65	0.89
Sesquiterpenes												
α -bergamontene	nd	nd	nd	nd	nd	0.55	nd	0.83	nd	1.22	1.60	1.33
Aldehydes												
Hexanal	nd	nd	nd	2.83	nd	1.81	5.94	nd	nd	nd	nd	nd
Ketones												
3-Octanone	25.94±0.10cd	31.28±0.01a	31.81±0.18bcd	21.83±0.02abc	nd	17.35±0.01b	39.20±0.04a	11.64±0.02g	11.59±0.07def	21.19±0.08ab	22.90±0.03cd	18.61±0.02def

Mean values with different letter(s) in the same row are significantly differences ($P < 0.05$) according to Duncan's multiple range test; n.d= not detected. Storage trial for control was discontinued after 3 month due to excessive fruit shrivelling. MAP, modified atmosphere packaging.

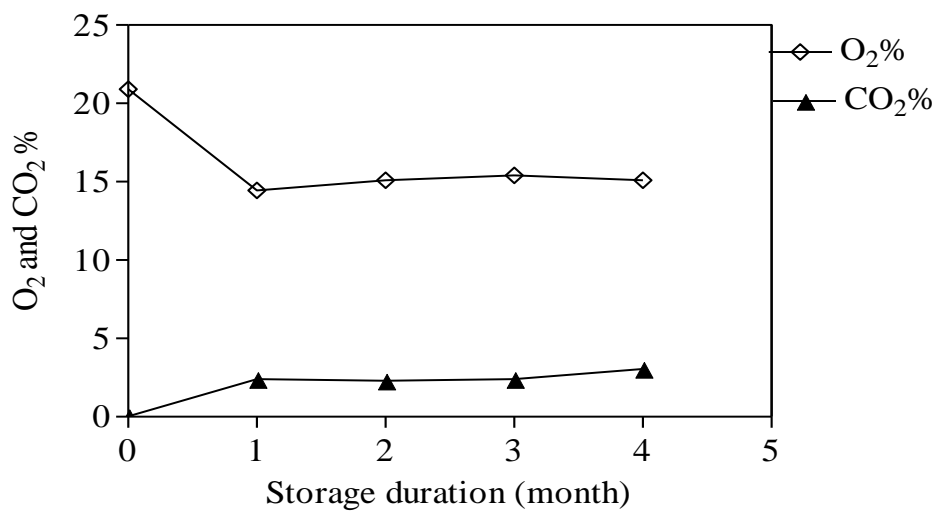


Fig. 1. Changes in CO₂ and O₂ concentrations inside MAP of pomegranate cv. 'Wonderful' during storage at $7 \pm 0.5^\circ\text{C}$ for four months. MAP, Modified atmosphere packaging.

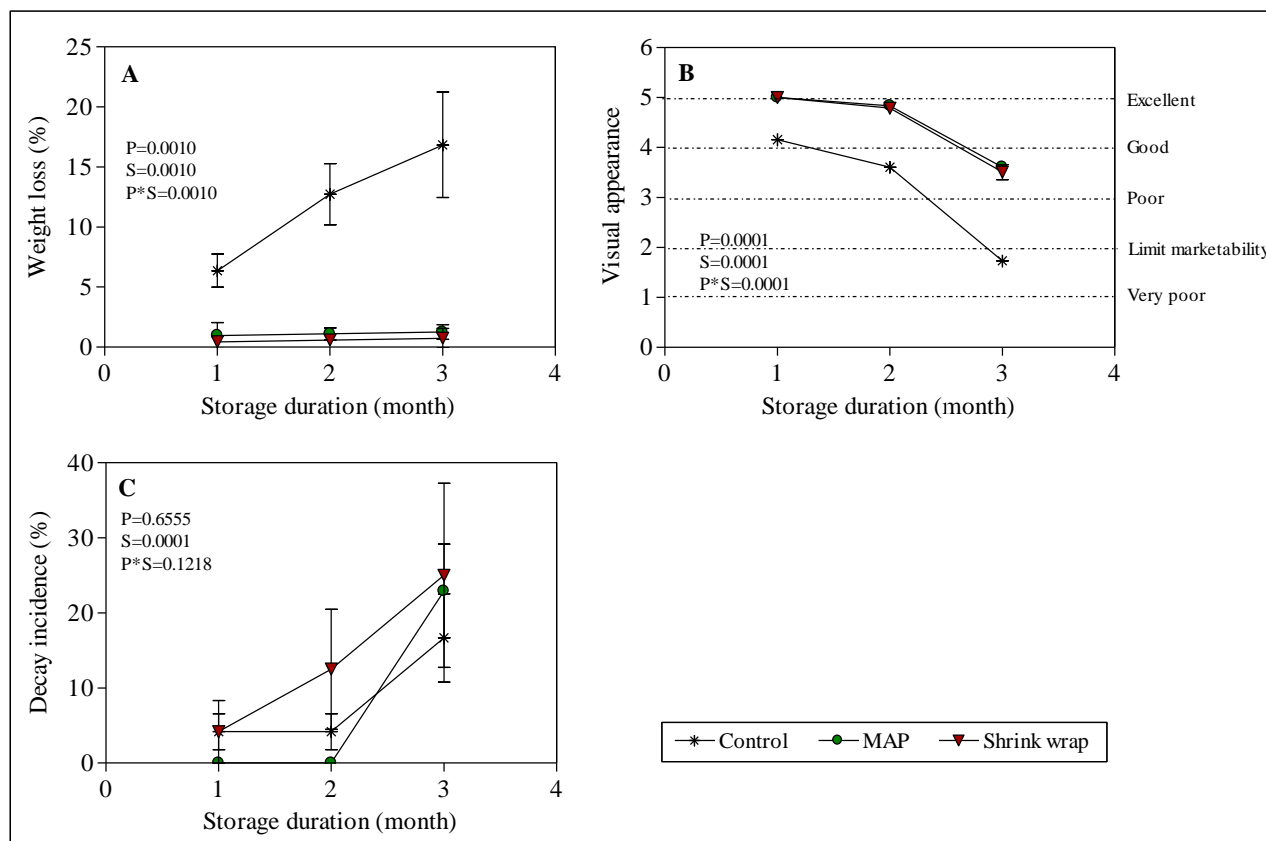


Fig. 2. Changes in the quality attributes of pomegranate fruit cv. Wonderful during storage over a four month period at $7 \pm 0.5^\circ\text{C}$. Each value in the graph is represented as a mean \pm SE. Decay index was assessed on a scale of 1-5, explaining the severity of fungal decay (0 = no decay, 1 = 1-25%, 2 = 25-50%, 3 = 50-75%, 4 = 100%) and the data was presented as decay incidence percentage. Visual appearance was conducted on the base of a 5 point hedonic scale as follows: 5 = excellent, 4 = good, 3 = poor, 2 = limit marketability, 1 = very poor. In other to determine interaction effects between packaging and storage duration, data for month 1, 2, and 3 for the different packaging treatment was presented. Storage trial for control was discontinued after 3 months due to excessive fruit shrivelling. MAP and shrink wrap data for month 4 is reported on the appendix 2 (Table 2). P, Packaging; S, Storage duration. MAP, Modified atmosphere packaging.

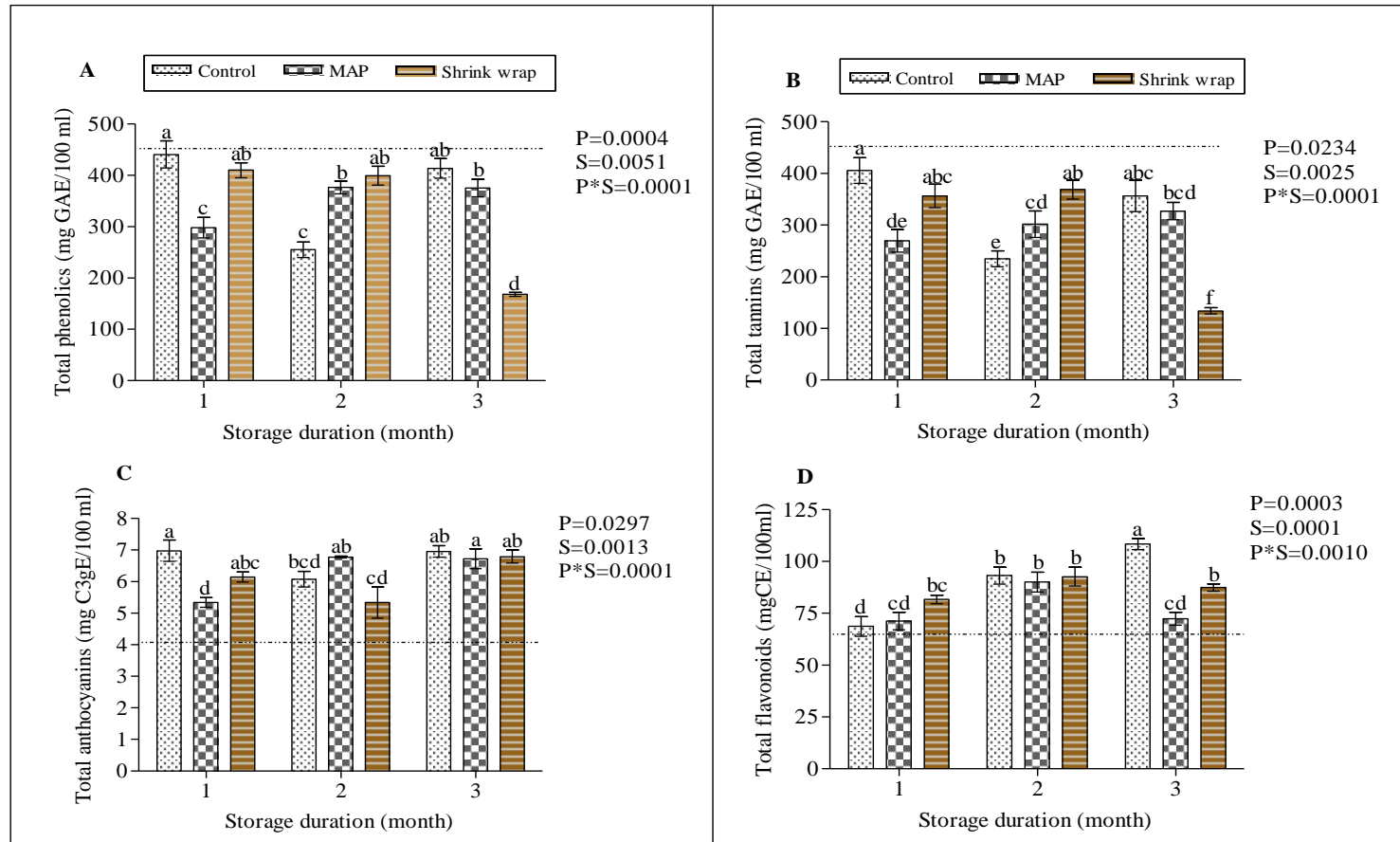


Fig. 3. Changes in the total phenolic (A), total tannins (B), total anthocyanins (C) and total flavonoids (D) concentrations of pomegranate fruit cv. Wonderful during storage over a four month period at $7 \pm 0.5^{\circ}\text{C}$. Each value in the graph is represented as a mean \pm SE. Different letters on bars represent statistical differences ($P < 0.05$) using Duncan's multiple range test. In order to determine interaction effects between packaging treatment and storage duration, data for month 1, 2, and 3 for different packaging were used. The storage trial for control was discontinued after months 3 due to excessive fruit shrivelling. MAP and shrink wrap data for month 4 is reported in appendix 3 (Table 3). P, Packaging, S, Storage duration. MAP, Modified atmosphere packaging. ---- measurements at harvest.

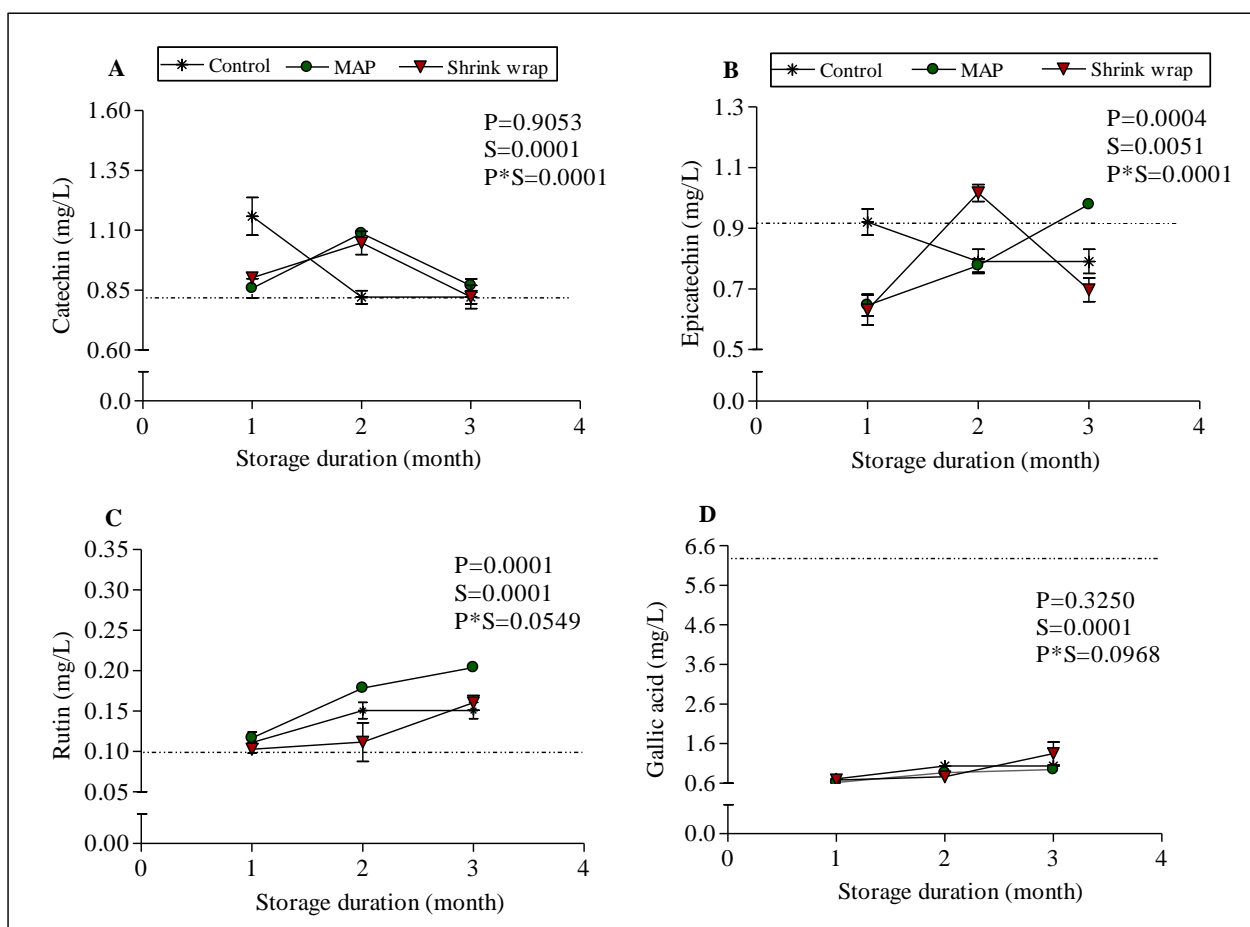


Fig. 4. Changes in the catechin (A), epicatechin (B), gallic acid (C) and rutin (D) of pomegranate fruit cv. Wonderful during storage over a four month period at $7 \pm 0.5^\circ\text{C}$. Each value in the graph is represented as a mean \pm SE. In order to determine interaction effects between packaging and storage duration, data for month 1, 2, and 3 for different packaging treatment was used. The storage trial for the control was discontinued after 3 months due to excessive fruit shrivelling. The data for month 4 is reported on the appendix 4 (Table 4).

---- measurements at harvest. P, Packaging, S, Storage duration. MAP, Modified atmosphere packaging.

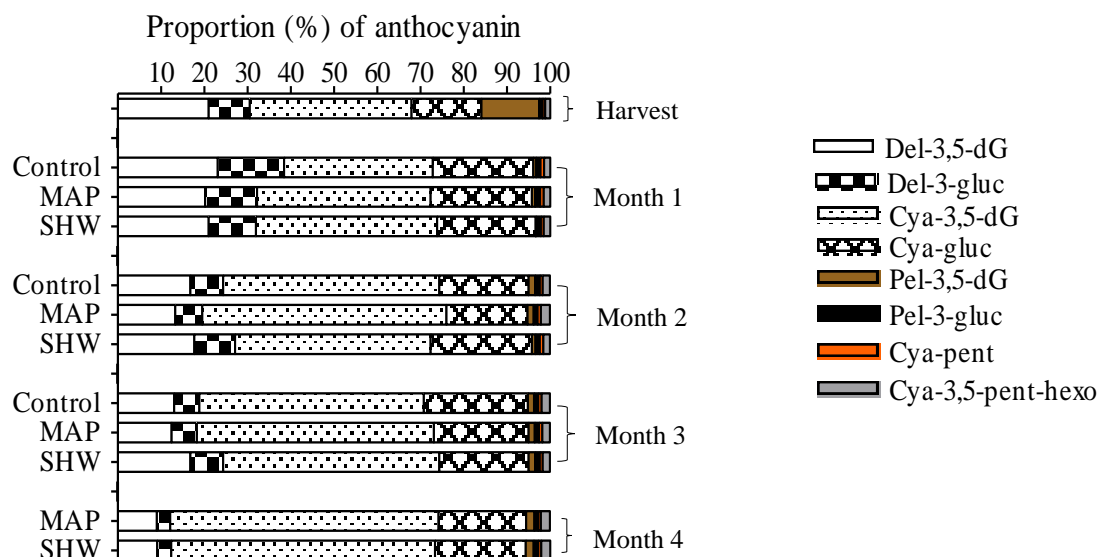


Fig. 5. Individual anthocyanin presented as proportion (%) during storage over a four month period at $7 \pm 0.5^\circ\text{C}$. Del-3,5-dG, delphinidin-3,5-diglucoside; Del-3-gluc, delphinidin 3-glucoside; Cya-3,5-dG, cyanidin-3,5-diglucoside; Cya-3-G, Cyanidin 3-glucoside; Pel-3,5-dG, pelargonidin 3,5-diglucoside; Pel-3-G, pelargonidin 3-diglucoside; Cya-pent, cyanidin pentoside; Cya-3,5-pent-hexo, cyanidin-3,5-pentoside-hexoside. Storage trial for control was discontinued after 3 month due to excessive fruit shrivelling. MAP, modified atmosphere packaging; SHW, shrink wrap.

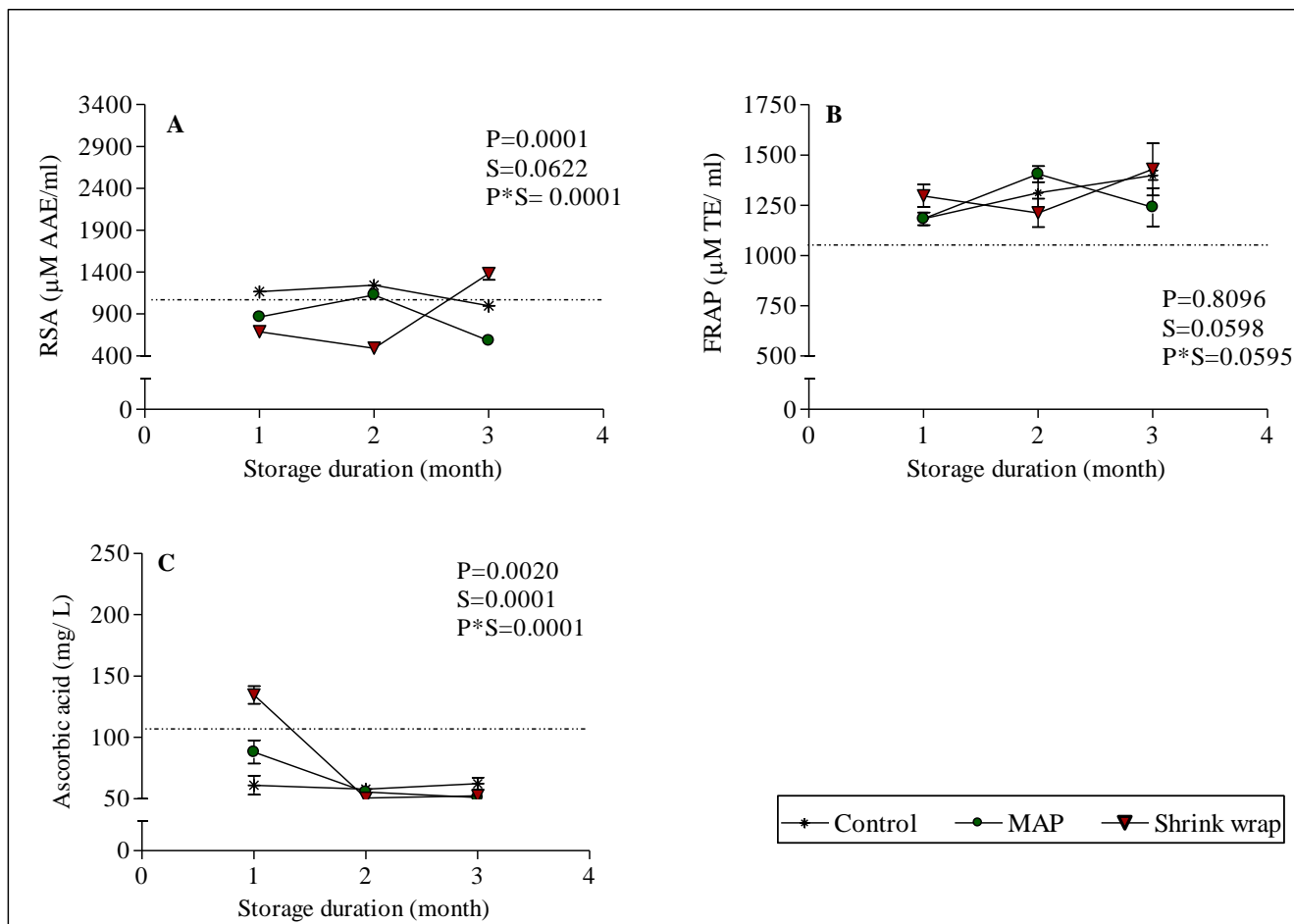


Fig. 6. Changes in ascorbic acid and antioxidant activities of pomegranate juice of cv. Wonderful during storage over a four month period at $7 \pm 0.5^\circ\text{C}$. In order to determine interaction effects between packaging and storage duration, data for month 1, 2, and 3 for different packaging was used. Storage trial for control was discontinued after 3 month due to excessive fruit shrivelling. The data for month 4 is reported on the appendix 5 (Table 5). MAP, Modified atmosphere packaging; P, Packaging, S, Storage duration; RSA, Radical scavenging activity; AAE, Ascorbic acid equivalent, TE, Trolox equivalent; FRAP, Ferric reducing antioxidant power. --- measurements at harvest.

PAPER 6

Influence of packaging systems and long term storage on pomegranate fruit. Part 2: Bioactive compounds and functional properties of fruit by-products (peel and seed oil)

Abstract

In Part 1 of this study on the effects of packaging and long term cold storage on pomegranate fruit quality, it was reported that packing ‘Wonderful’ pomegranates inside polyliner bags (modified atmosphere packaging, MAP) and individual shrink wrap satisfactorily reduced fruit weight loss, maintained visual appearance and delayed excessive accumulation alcohols responsible for off-flavours by up to three months. In the present complementary study (Part 2), changes in concentration of polyphenols, phenolic acids, vitamin C of fruit peel and fatty acids composition of seed oil were investigated as well as changes in functional properties. Total phenolic concentration of fruit peel declined ($P < 0.05$) progressively with prolonged storage whereas radical scavenging activity remained relatively stable. The results showed that rutin was the predominant flavonoid (3446.24 mg/kg dry matter) in peel, which declined by 65% in fruit packed inside polyliner bag (MAP) fruit at the end of four months storage period. Punicic acid was the highest fatty acids in the seed oil with the average percentage of 68.09% and the concentration remained relatively high at the end of storage for fruit packed inside polyliner bag or individually shrink wrapped fruit. Furthermore, there was a decline in the inhibitory activity (MIC, 1.56 mg/mL) of the extracts from the peel of shrink wrapped fruit against *Klebsiella pneumonia* at the end of storage. Peel extracts from fruit packed inside polyliner bag (MAP) during storage had the highest activity (29.70 µg/mL) against monophenolase after four month of storage. Seed oil of fruit stored under polyliner and shrink wrap had poor inhibitory activity against Gram positive bacteria, with MIC value of 1.56 µg/mL during storage for all types of packaging. With regard to anti-tyrosinase activity, seed oil extracted from shrink wrapped fruit had better activity against diphenolase (0.49 µg/mL) than those obtained from MAP stored fruit (3.78 µg/mL) at the end of storage. This information would be of interest for off-season processing of pomegranate by-products into value-added ingredients in the food, nutraceutical and pharmaceutical industries

Keywords: Diphenolase, Fatty acids, Gram positive, MIC, Rutin, Total phenolic, EC₅₀, Pomegranate by-products, Seed oil, Fruit Peel

1. Introduction

Several human chronic disease conditions including cancer have been associated with oxidative stress produced through either increased free radical generation or decreased antioxidant level in biological tissue (Graf et al., 2005). Consequently, consumption of polyphenol rich diet has been shown to have a protective role (Kirakosyan et al., 2003). Pomegranate fruit (*Punica granatum* L. Punicaceae) has been used to treat many diseases since time immemorial due to the high level of antioxidants and numerous bioactive compounds (Opara et al., 2009; Fawole et al., 2012). In fact, various fruit fractions have been extensively used as traditional remedy against acidosis, dysentery, microbial infections, diarrhoea, helminth infection, haemorrhage and respiratory pathologies (Reddy et al., 2007; Kim and Choi, 2009).

These therapeutic properties are associated with the remarkable amount of various biologically active compounds such as vitamin C and phenolic compounds such as gallic acid, punicalagin, gallotannins and anthocyanins (Noda et al., 2002; Cerdà et al., 2003), which are known to act as natural antioxidants. Pomegranate fruit is comprised of peels and arils (which contain juice and seeds/kernels). Inside the fruit, arils are clustered in sacs which are attached to the peel and covered with membrane (Aindongo et al., 2014). During juice processing, the peel is a major by-product and accounts for about 50% of whole fruit mass (Al-Said et al., 2009; Opara et al., 2009; Fawole et al., 2015). The peel is rich in polyphenols including flavonoids, phenolic acids and tannins (Opara et al., 2009; Fawole et al., 2012; Fawole et al., 2015). There has been an exponential growth in studies regarding the pharmacological properties of this fruit over the past few years owing to its high concentration of bioactive compounds contained therein. From the previous studies on the pharmacological properties of pomegranate fruit, the peel has antibacterial, antioxidant properties (Al-Zoreky, 2009; Fawole et al., 2012; Opara et al., 2009) and anti-tyrosinase activity (Fawole et al., 2012). Pomegranate seeds have also been shown to contain the estrogenic compounds estrone and estradiol (Kim and Choi, 2009) which have been reported to exhibit anti-inflammatory properties. In recent studies, seed oil has also shown to have antibacterial (Karaman et al., 2015), anti-inflammatory (Boussetta et al., 2009) and anti-cancer effects (Kim et al., 2002).

Although pharmacological properties of pomegranate peel and seed oil have been investigated by different researchers, little attention has been focused on the effects of packaging systems used to handle fruit during prolonged cold storage (such as the use of plastic bags and shrink films) on the bioactive compounds and functional properties. Pomegranate fruit is highly susceptible to moisture loss, decay development and husk scald during cold storage (Elyatem and Kader, 1984;

Koksal, 1989). Thus, the use of modified atmosphere packaging (MAP) in combination with postharvest treatments has been introduced in industry (Caleb et al., 2013a; Opara et al., 2016). MAP has been extensively used to prolong cold storage of whole pomegranate cultivars including ‘Ganesh’ (Nanda et al., 2001), ‘Primosole’ (D’Aquino et al., 2010), ‘Shlefy’ (Ghafir et al., 2010), ‘Mollar de Elche’ (Laribi et al., 2012), ‘Hicrannar’ (Selcuk and Erkan, 2014) and ‘Hicaznar’ (Selcuk and Erkan, 2015). In addition, MAP has been successfully used to prolong shelflife of minimally processed arils of pomegranate fruit (Banda et al., 2015; Caleb et al., 2013b).

Previous studies suggest that several preharvest and postharvest factors affect the chemical composition of pomegranate fruit and these play major roles on its pharmacological properties (Mphahlele et al., 2014a; Caleb et al., 2013a). It was reported in the first paper of this series that cold storage of ‘Wonderful’ pomegranates in polyliner bags (passive MAP) or shrink wrapping fruit individual reduced fruit weight loss, maintained visual appearance and delayed alcohol accumulation in the fruit. The aim of this work was to establish whether the packaging systems affect the bioactive compounds and functional properties of pomegranate peel and seed oil during long term cold storage. The study could provide valuable information about the suitability of pomegranate fruit waste (peel and seed) as potential biomaterials for nutraceutical applications.

2. Materials and methods

2.1. Fruit samples, packaging and storage condition

‘Wonderful’ pomegranate fruit were procured in 2015 from Sonlia packhouse (33°34’851”S, 19°00’360”E) in the Western Cape, South Africa. Fruit were sorted immediately after commercial harvest for uniformity of size and colour, and those with visual injuries or cracks were discarded. Detailed description of fruit source and the packaging systems and storage conditions investigated were presented in the previous paper/chapter. Briefly, three types of packaging were studied: (1) packing fruit inside open top ventilated cartons (control), (2) packing fruit inside ventilated cartons with polyliner bag (referred to passive modified atmosphere packaging, MAP), and (3) shrink-wrapping each fruit before packing inside ventilated cartons (shrink wrap). Internal atmosphere created by the polyliner (MAP) was determined. Water vapour transmission rate of shrink-wrap film was tested over 4-months period. All fruit were stored at $7\pm 0.5^{\circ}\text{C}$ and $92\pm 2\%$ RH (relative humidity) for 4 months.

2.2. Fruit sampling and preparation

On each sampling day, 15 fruit from each of type of packaging treatments were manually peeled. Seeds were separated by juicing the arils using juice extractor (LiquaFresh, Mellerware, South Africa) without breaking the seeds at low speed (6500 rpm). Fruit peels and seeds, regarded as by-products, were stored immediately in separate plastic bags at -80°C before freeze drying (VirTis, BenchTop “K”, USA) for 92 h. Each type of dried by-product was pulverized using a miller (IKA, A11B, Germany) and the powder was kept in air tight plastic containers and stored at -20°C until used for biochemical extraction.

2.2.1. Seed oil extraction

Approximately 10 g of powered seeds were extracted in 70 mL of hexane for 3 h using soxhlet apparatus (SER 148, Velp Scientifica, Europe). The oil yield was weighed and calculated as percentage of dry matter followed by storage at -20°C until analysis.

2.2.2. Extraction of pomegranate peel

Dried pomegranate peel (2 g) from each packaging type was extracted separately with 10 mL of 80% methanol using sonication for approximately 1 h (Al-Zoreky, 2009). The extracts were separately filtered with Whatman No.1 filter paper and residues were re-extracted following the same procedure. The extracts were pooled before drying under a stream of air.

2.3. Phytochemical analysis

2.3.1. Total phenolic, tannin, flavonoid and ascorbic concentrations

The same methods reported in the first paper of this series (preceding chapter) were adopted for quantification of polyphenolic concentrations. Total phenolic (TPC) and tannin concentrations were expressed as milligram gallic acid equivalent per kilogram peel extracts (mg GAE /kg DM), and total flavonoids expressed as catechin equivalent per kilograms peel extracts (mg CE /kg DM).

2.3.2. Individual phenolic acid and flavonoid concentration

The profiles of phenolic acids and flavonoids of peel extracts were determined following the procedure reported earlier established (Mphahlele et al., 2014b). The LC-MS and LC-MS^E systems comprised of a Waters Synapt G2 quadrupole time-of-flight mass spectrometer (Milford, MA, USA), equipped with a Waters Acquity ultra-performance liquid chromatograph (UPLC) and Acquity photo

diode array (PDA) detector. Extracts (3 μL) were injected into a waters UPLC BEH C18 column 2.1 x 50 mm diameter, 1.7 μm particle size. The mobile phase consisted of 0.1% (v/v) formic acid (solvent A) and 22% acetonitrile (solvent B) over 2.5 min, following a linear gradient of increasing polarity of 44% solvent B over 4 min and finally to 100% solvent B over 5 min. The column was subjected to 100% solvent B for an extra 2 min. The flow rate was 650 L/h and desolvation temperature of 275°C. The concentrations of individual flavonoids and phenolic acids were estimated from the calibration curve of reference standards (Sigma-Aldrich, South Africa).

2.4. Fatty acids methyl esters and chromatographic analyses

Approximately 0.5 g of seed oil was weighed and dissolved in 10 mL of hexane. In a glass vial with a PTFE lining, 1 mL of 2.5% (v/v) sulphuric acid in methanol was added into 1 mL of the sample. 100 μL of heptadecanoic acid (C17) was at a concentration of 1000 mg/L was added as internal standard. Subsequently, the mixture was vortexed for 30 seconds before incubating for one hour in an oven maintained at a temperature of 80°C. Hexane (500 μL) was added into cooled mixture followed with 1.5 mL of 1% (w/v) NaCl solution to extract fatty acids methyl esters (FAMES). The samples were shaken and the centrifuged to facilitate phase separation. The upper hexane phase was used for analysis of fatty acid. Separation was performed on a gas chromatograph (6890N, Agilent technologies network) coupled to an Agilent technologies inert XL EI/CI Mass Selective Detector (MSD) (5975B, Agilent technologies Inc., Palo Alto, CA). The GC-MS system was coupled to a CTC Analytics PAL autosampler. Separation of fatty acids was performed on a non-polar ZB-5MS GUARDIAN (30 m, 0.25 mm ID, 0.25 μm film thickness) ZB 7HG-G010-11 capillary column. Helium was used as the carrier gas at a flow rate of 1 mL/min. The injector temperature was maintained at 280°C. 1 μL of the sample was injected in a 10:1 split ratio. The oven temperature was programmed as follows: 100°C for 1 min; and then ramped up to 180°C at a rate of 25°C/min and held for 3min, ramped up to 200°C at 4°C/min for 5 min again up to 280°C at 8°C/min held for 0min and finally ramped up to 310°C at a rate of 10°C/min and held for 5 min. The MSD was operated in a full scan mode and the source and quad temperatures were maintained at 230°C and 150°C, respectively. The transfer line temperature was maintained at 280°C. The mass spectrometer was operated under electron impact mode at ionization energy of 70eV, scanning from 35 to 500m/z. The esterified fatty acids were identified by comparing their spectra to that of the Supelco 37 fame mix from Sigma-Aldrich to that of the NIST library. Sterols were tentatively identified by comparing their m/z spectra to that of the library due to lack of standards.

2.5. Functional properties

2.5.1. Antioxidant capacity

Radical scavenging activity (RSA) and Ferric reducing antioxidant power (FRAP) were determined. The methods described in the first paper of this series were adopted. Briefly, the ability of peel extract to scavenge 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical was measured using the DPPH assay (Fawole et al., 2012), and results presented as millimolar ascorbic acid equivalent per gram of peel extracts (mM AAE/g DM). Ferric reducing antioxidant power assay was performed according to the method of Benzie and Strain (1996), and results expressed as trolox (μM) equivalents per millilitre pomegranate juice ($\mu\text{M TE /g DM}$).

2.5.2. Microdilution assay for antibacterial activity

Antibacterial activity of pomegranate peel was determined following microdilution assay for the minimum inhibitory concentration values (Fawole et al., 2012). Four bacterial strains used included two Gram-negative bacteria (*Escherichia coli* ATCC 11775 and *Klebsiella pneumonia* ATCC 13883) and two Gram-positive bacteria (*Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 12600). All the bacteria were grown in sterile Mueller Hinton broth. The stock solutions of the peel extracts were dissolved in methanol to make 50.0 mg/mL. Under aseptic conditions, 100 μL of sterile water were added in a 96-well micro plate followed by 100 μL peel extracts as well as bacterial culture and serially diluted (two-fold). Similarly, two fold serial dilution of streptomycin (0.1 mg/mL) was used as positive control against each bacterium. Bacteria-free broth, methanol solvent (100%) and sterile water were included as negative controls. The final concentration of peel extract ranged from 0.097 – 12.5 mg/mL, whereas streptomycin was between (0.097- 12.5 mg/mL). Plates were incubated for 18 h at 37°C. After incubation, bacterial growth in the plate was indicated by adding 40 μL of *p*-iodonitrotetrazolium chloride (Sigma-Aldrich, Germany) after incubation. Bacterial growth was indicated by pink colour, while clear wells indicated inhibition. The results were recorded in terms of the minimal inhibitory concentration which is regarded as the lowest concentration of the extract without bacterial growth. The assay was measured in triplicate.

2.5.3. Mushroom tyrosinase inhibition assay

Tyrosinase inhibitory activity was determined using calorimetric method as described by Momtaz et al. (2008) with slight modification (Fawole et al., 2012). L-tyrosine and L-3,4-

dihydroxyphenylalanine (L-DOPA, Sigma) were used as substrates. Assays were carried out in a 96-well micro-titre plate and a Multiskan FC plate reader (Thermo scientific technologies, China) was used. Peel extracts and seed oil were dissolved in methanol and DMSO, respect to concentration of 50 mg/mL and further diluted in potassium phosphate buffer (50 mM, pH 6.5) to 1000 ug/mL. Each prepared sample (70 µL) was mixed with 30 µL of tyrosinase (333 Units/mL in phosphate buffer, pH 6.5). After 5 min incubation, 110 µL of substrate (2 mM L-tyrosine or 12 mM L-DOPA) was added to the reaction mixtures and incubated for 30 min. The final concentration of the extracts were between 2.6 - 333.3 µg/mL. Arbutin (1.04 – 133.33 µg/mL) was used as a positive control while a blank test was used as each sample that had all the components except L-tyrosine or L-DOPA. The final concentrations of the seed oil were between 0.17-5 mg/mL whereas the positive control (arbutin) was between 4.10-400 µg/L. All the steps in the assay were conducted at room temperature. Results were compared with a control consisting of DMSO instead of the test sample. After adding mushroom tyrosinase solution, the reaction mixture was incubated at room temperature (37°C) for 30 minutes. The absorbance of the reaction mixture was measured at 475 nm. The percentage mushroom tyrosinase inhibitory activity was calculated using the following equation:

$$\% \text{ inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

where A_{control} is the absorbance of Methanol and A_{extract} is the absorbance of the test reaction mixture containing extract or arbutin. The inhibition concentration of 50% (IC_{50}) values of extracts and arbutin were calculated. The assay was measured in triplicate.

3. Statistical analysis

Statistical analyses were carried out using statistical software (STATISTICA, Vers. 12.0, StatSoft Inc., USA). Data was subjected to one-way analysis of variance (ANOVA) and were appropriate, two-factorial (factor A= package; factor B= storage duration) was conducted. Means were separated by least significant difference (LSD; $P = 0.05$) according to Duncan's multiple range test. GraphPad Prism software version 4.03 (GraphPad Software, Inc., San Diego, USA) was used for graphical presentations. Values are presented as mean \pm standard error.

4. Results and Discussion

4.1. Oil yield

At harvest, pomegranate seed oil content was 9.95%. Changes in oil content fluctuated considerably during long term storage under different package types are shown in Fig. 1. However, small, but insignificant differences were observed between the packaging systems with the lowest content observed in shrink wrap (11.75%) compared to MAP (13.00%) and control fruit (12.65%) after 3 months of storage.

4.2. Total phenolic, tannin and flavonoid concentration of peels

The changes in total phenolic, total tannin and flavonoid concentration are shown in Fig. 2. Relative to the initial concentration, the total phenolic concentration of the peel increased about 10% from 2386.18 to 2626.31 g GAE/kg DM in control fruit whereas 7% loss was observed for peel of the fruit stored in both polyliner (MAP) and shrink wrap (to ~2556.14 g GAE/kg DM) during the first month of storage (Fig. 2A). Further decline was observed at the end of storage in MAP and individual shrink wrapped fruit peel. Moreover, TPC in peel of individually shrink wrapped fruit declined by approximately 26% (1743.64 g GAE/kg DM) and 34% (1537.50 g GAE/kg DM) in fruit peel stored under polyliner (MAP). Degradation of total phenolic is related to enzymatic oxidation (polyphenol oxidase and peroxidase) during storage (Fawole and Opara, 2013). Polyphenols have shown to be unstable during cold storage as reported by several researchers in various pomegranate cultivars (Fawole and Opara, 2013; Selcuk and Erkan, 2014; Palma et al., 2015). A study by Tarrozi et al. (2004) reported lower phenol values in the peel of apple fruit after cold storage for three months with no further effect after six months.

Total tannin concentration showed a declining trend for all treatments (Fig. 3B). At the end of storage, peel of the fruit stored in individual shrink wrap had the least degradation (34% loss) compared to MAP stored fruit peel (40% loss). It is possible that retention of total tannin concentration in individual shrink wrapped fruit peel in this study may be due to low O₂ permeability resulting in limitation of atmospheric oxygen for oxidation.

Total flavonoid concentration was 94.48 g CE/kg DM at harvest and fluctuated with increasing storage duration (Fig. 3C). After 3 months in cold storage, total flavonoid concentration of the peel of fruit stored inside open top ventilated cartons (control) and polyliner bags (MAP) increased by 1% (95.69 g CE/kg DM) and 2% (96.69 g CE/kg DM) respectively, compared to those from shrink wrapped fruit (10% loss, 84.54 g CE/kg DM). It has been reported that flavonoids are

more likely to reduce with increasing storage time, temperature and oxygen concentration (Raisi and Aroujalian, 2007). The decrease in total flavonoid concentration in the peel of shrink wrapped fruit during storage is not well understood, therefore, there is need for further research. At the end of 4 months storage, total flavonoid concentration in the peel of fruit stored in MAP and individually shrink wrapped decreased by 5% (89.24 g CE/kg DM) and 6% (88.84% g CE/kg DM), respectively. The result showed that total flavonoid of the pomegranate peel was highly retained regardless of the package type. Similar results were reported by Awad and De Jager (2000) in fruit peel of various apple cultivars stored for 8 months during and after regular ultra-low oxygen storage (Awad and De Jager, 2000).

4.3. Determination of phenolic acid and flavonoids concentration of peels

The concentration of bioactive compounds in pomegranate peel extracts during the investigated storage period using different types of packaging are presented in Fig. 3. The phenolic compounds quantified included -catechin, +epicatechin, hesperidin, rutin and punicalin. To the best of our knowledge, this is the first study showing the changes of individual flavonoid in pomegranate peel over a prolonged storage period. Total bioactive compound concentrations declined significantly during storage regardless of package type, until the end of storage (Fig. 3A). However, the peel of fruit stored in individual shrink wrap had higher total bioactive compounds than MAP stored fruit after four month of storage (Appendix 2, Table 4). High total bioactive compounds concentration in the peel of fruit stored in individual shrink wrap packaging was possibly due to reduced polyphenol oxidase enzymes in response to changes in fruit external atmosphere.

Rutin was identified as the most abundant flavonol in pomegranate peel extracts in this study. After 3 months of storage, there was a significant decline in rutin concentration in the peel of fruit packed in all types of packaging (Fig. 3B). These results suggest that significant decline in rutin concentration may be explained by high instability of this compound during prolonged cold storage. In the ventilated open top carton packaging (control), rutin in peel of fruit decreased by 80.27% (from an initial amount of 3446.24 to 680.02 mg/kg DM), with smaller reductions in concentration in the peel of fruit packed in shrink wrap (50.54%) and MAP (65.42 %) after 3 months of storage.

The effects of packaging, storage duration and their interaction on catechin and epicatechin were significant ($P < 0.001$). There was a negligible but consistent increase in catechin and epicatechin concentration in all treatments (Fig. 3C and D). At the end of storage, catechin concentration did not vary between the peel of MAP and shrink wrapped fruit (supplementary Table 4). Similarly, epicatechin concentration was higher in peel of fruit stored in MAP (55.05 mg/kg DM)

than shrink wrap package (45.93 mg/kg DM) (Appendix 2, Table 4). The significant reduction in catechin and epicatechin concentration in pomegranate peel extract could be as a result of oxygen levels inside the shrink wrap package. Several studies reported that many factors may affect catechins stability including temperature and oxygen level (Chen et al., 2001; Sang et al., 2005; Wang et al., 2006). The punicalin compound which forms the integral part of pomegranate peel was significantly ($P < 0.001$) higher in peel of fruit stored in individual shrink wrap packaging (987.76 mg CE/kg DM) compared to the peel of fruit stored in conventional open top cartons (711.55 mg/kg DM) and polyliner (705.27 mg/kg DM) after first months of storage (Fig 3C). After 2 months of storage, the punicalin concentration in peel of fruit stored in shrink wrap packaging declined by 22% (769.4 mg/kg DM) after 2 months of storage. During the same period, punicalin concentration in peel of fruit stored in conventional open top carton increased by 13% with negligible decrease observed in peel of fruit stored under polyliner packaging. However, a notable increase was observed for peel of fruit stored in shrink wrap than MAP at the end of storage (4 months) (Appendix, Table 4).

Hesperidin in the peel was significantly affected by both packaging and storage duration ($P < 0.001$). Hesperidin concentration showed increasing trend over storage duration (Fig 3F). Peel of the fruit stored under polyliner packaging had the highest concentration (14.39 mg/kg DM) at the end storage than individual shrink wrap fruit peel (10.94 mg/kg DM) compared to fruit peel at harvest (0.23 mg/kg DM) (Fig. 3F). The continued increase in hesperidin concentration during storage may be related to the reported stability and activity of manonyl transferase which is responsible for synthesis of hesperidin compound (Samir et al., 1999). The finding in the present study on the changes in hesperidin concentration is in agreement with Rapisdra et al. (2008) who reported significant increase in hesperidin concentration in blood oranges stored at 6° C for 65 days.

4.4. Fatty acids composition of seed oil

The fatty acid composition of pomegranate has recently received increasing attention, with emphasis on the health potential of polyunsaturated (n-3) fatty acids (Fernades et al., 2015). Overall, fatty acid composition was 6.77%, 3.85% and 72.80% for saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA) and poly-unsaturated fatty acids (PUFA) at harvest, respectively; however, significant fluctuation was observed during prolonged storage (Table 1). Furthermore, no significant change was observed between total MUFA and PUFA of total fatty acids of seed oil at the end of 3 months of cold storage of fruit packed in polyliner and shrink wrap compared to concentration at harvest, thus indicating high stability of these compounds with prolonged storage irrespective of package type. Similar trend was observed by Rastrelli et al. (2002) who showed that

the concentration of polyunsaturated fatty acids in olive oil remained nearly constant following 8 months of different storage conditions. Ayton et al. (2012) observed a similar result after 36-months storage of extra virgin olive oil at 15, 22 and 37°C.

A 27% increase in total SFA was observed in peel of pomegranate fruit stored in MAP whereas the seed oil from shrink wrapped fruit had 19% less saturated fatty acids at the end of storage. In addition, seed oil from conventional open top cartons fruit also had higher SFA concentration after the third month. The MUFA/PUFA and SFA/UFA ratio of seed oil were 0.05% and 0.09% at harvest, respectively. The MUFA/PUFA (0.05%) and SFA/UFA (0.09%) ratio found in the present study were within the range reported by Verardo et al., (2014) in pomegranate fruit cvs Hershkovitz (MUFA/PUFA (0.08%), SFA/UFA, (0.05%) and Mollar 1 (MUFA/PUFA (0.07%), SFA/UFA (0.06%). The authors reported that the MUFA/PUFA and SFA/UFA ratio of seed oil in the range of 0.06 to 0.07% and 0.10 to 0.12%, respectively had lower degree of unsaturation. The findings in the present study on the MUFA/PUFA and SFA/UFA ratio are lower than those observed by Verardo et al. (2014); thus it could be suggested that seed oil had higher degree of unsaturation. The MUFA/PUFA and SFA/UFA ratio of seed oil from fruit stored under polyliner and individual shrink wrap packaging did not significantly change at the end of storage (4 months). The study revealed that prolonged storage coupled with packaging treatment could maintain properties of pomegranate seed oil for later usage.

Results of the fatty acid composition of pomegranate seed oil showed that 6 fatty acids, 1 tocol, 1 phytosterol, and squalene were the major components identified (Table 1). Two major saturated fatty acid concentrations, stearic and palmitic acid, were 3.08 and 3.69%, respectively, while arachidic acid was not detected at harvest. At the end of storage, seed oil from fruit stored under polyliner had significantly ($P<0.05$) higher palmitic acid (19% increase from 3.69% to 4.40%) while seed oil obtained from shrink wrapped fruit had 47% less palmitic acid (3.08% to 1.95%) during the same period.

Stearic acid concentration fluctuated during storage. In seed oil from fruit stored in ventilated open top carton, the concentration declined to 1.32% compared to harvest (3.08%) after 2 months storage. During the 3rd month, stearic acid concentrations significantly increased in seed oil from fruit stored in ventilated open top carton (3.68%) and shrink wrap packaging (3.51%) with the lowest concentration found in seed oil of fruit stored under polyliner (1.89%). At the end of storage, seed oil from fruit stored under polyliner had the highest concentration (25% increases) than individually shrink wrapped fruit seed oil (1% increase). Similarly, arachidic acid concentration fluctuated significantly during prolonged storage. Polyliner stored fruit seed oil had higher arachidic acid

concentration (0.50%) than control (0.40%) and individually shrink wrapped fruit (0.40%) during the first month of storage. Despite fluctuations during storage, individually shrink wrapped fruit seed oil retained higher arachidic acid concentration (0.69%) than fruit packed in MAP (0.36%) at the end of 4 months storage duration. In general, there are no studies on changes in fatty acids concentration in pomegranate seed oil during storage.

The concentrations of major unsaturated fatty acids of seed oil at harvest were punicic acid (68.09%), linoleic (4.70%) and oleic (3.85%) concentration (Table 1). Linoleic acid decreased by 56% after 2 months of storage in control fruit seed oil but increased by 2 fold after 3 months. At the end of storage, seed oil from individually shrink wrapped fruit maintained higher linoleic acid concentration (5% increase) while polyliner stored fruit seed oil declined by 6%. Punicic acid was identified as major fatty acid in pomegranate cultivars (Fadavi et al., 2006; Verardo et al., 2014; Fernandes et al., 2015). Its presence is of high importance as it promotes potent biological and therapeutic effect of the pomegranate seed oil (Özgül-Yücel, 2005). Punicic acid showed a slight but significant decline in polyliner stored fruit seed oil after the first and second month of storage. However, no significant change was observed in seed oil from ventilated open top cartons fruit after the first month of storage. Additionally, punicic acid concentration remained relatively stable until the end of storage with the higher concentration observed for polyliner and individual shrink wrapped fruit seed oil. From the study, it could therefore be deduced that MAP and individual shrink wrapped stored fruit seed oil did not influence the punicic acid concentration during storage.

The oleic acid concentration at harvest was 3.85% and remained relatively stable throughout the storage for seed oil of fruit stored in polyliner and individual shrink wrap (Table 1). With prolonged storage, oleic acid concentration declined by almost 57% in seed oil from ventilated open top carton fruit after 2 months but an increase by 2 fold was observed after 3 months. The γ -tocopherol is often the most prevalent form of vitamin E in plant seeds and in products derived from them (McLaughlin and Weihrauch, 1979). The γ -tocopherol (vitamin E) generally known as a major antioxidant was the only tocol identified in this study and its concentration was 1.15% at harvest. During the first month of storage, a significant increase in γ -tocopherol was observed in seed oil from ventilated open top carton (65%) and polyliner (115%) stored fruit. However, γ -tocopherol remained relatively constant in shrink wrapped fruit seed oil during the same period. With prolonged storage, the γ -tocopherol concentration declined significantly until the end of storage irrespective of package type. According to Pellegrini et al. (2001) the degradation of tocopherol in extra virgin oil was suggested to be partly due to polyphenols.

The presence of γ -sitosterol was detected only at harvest, thus indicating the possibility of high degradation with prolonged storage (Table 1). The concentration of squalene significantly decreased by 89% in seed oil of fruit stored in ventilated open top carton fruit, 38% (individual shrink wrapped fruit seed oil) and 7% (polyliner) after 3 months of storage when compared to percentage value (1.77%) at harvest (Table 1). Nevertheless, squalene concentration increased significantly in seed oil of fruit stored in individual shrink wrap (57%) whereas 34% decline was observed for polyliner stored fruit seed oil at the end of storage.

4.5. Radical scavenging activity, ferric reducing antioxidant power and vitamin C of peel

Radical scavenging activity of pomegranate peel was significantly ($P < 0.05$) affected by type of packaging (Fig. 4A). For instance, a notable difference was observed during the first month of storage with the lowest activity observed in fruit peel packed inside shrink wrap while no significant change was observed for control and fruit packed under MAP. However, the antioxidant activity fruit peel was highly maintained, with about 3% lost after 3 months of cold storage in all package types. At the end of storage, the activity remained relatively stable for peel of fruit stored in polyliner and individual shrink wrap. The antioxidant activity of pomegranate peel has been widely reported to positively correlate with total phenolic concentration (Gil et al., 2000). The high level of radical scavenging activity of pomegranate juice is often associated with higher polyphenol concentrations found in the peel (Fawole et al., 2012).

The antioxidant activity measured by ferric antioxidant reducing power is presented in Fig. 4B. There was no significant effect of packaging and storage, as well as interaction effect on the antioxidant activity measured by ferric reducing antioxidant power ($P > 0.05$). This study indicates that ferric reducing antioxidant power was highly maintained irrespective of the package type. Chaovanalikit and Wrolstad (2004) reported that the possibility of higher antioxidant activity concentration might be that polyphenolic degradation products retain antioxidant activities.

There was a decline in vitamin C concentration during the first month of storage in the peel of control fruit (25% loss) and MAP (11% loss) while the concentration of peels of fruit packed in shrink wrap did not change (Fig. 4C). However, a slight increase was observed in the peel of fruit stored in ventilated open top carton fruit after 3 months of storage. At the end of storage (4 months), a significant decline in vitamin C concentration by up to 34% and 36% was observed in peel of fruit packed in MAP and shrink wrap, respectively. The loss in vitamin C with storage time duration could be explained by the indirect degradation through polyphenol oxidase, cytochrome oxidase and peroxide activity (Lee and Kader, 2000).

4.6. Antibacterial activity of pomegranate peel extracts

Changes in the antibacterial activity (MIC) of pomegranate peel extracts during prolonged storage under different types of packaging are presented in Table 2. Generally, MIC values less than 1.0 mg/mL are considered active for peel extracts (van Vuuren, 2008). Peel extracts showed the best MIC against Gram-negative (0.39 mg/mL) compared to Gram positive bacteria (1.562 mg/mL) at harvest (Table 2). Contrary to the study by Fawole et al. (2012) on the peel of 'Wonderful' cultivar, the extracts showed the best MIC against Gram positive bacteria (*Klebsiella pneumonia*, 0.33 mg/mL; *Staphylococcus aureus*, 0.39 mg/mL). With prolonged storage, the MIC values were within the highest level of potency against all the bacteria, irrespective of package type. However, less activity was observed for individual shrink wrapped fruit peel at the end of storage with the MIC value of 1.562 mg/mL against *K. pneumonia*. Low MIC value may be due to the high accumulation of CO₂ composition inside wrapped fruit and might have led to a decrease in compound responsible for the inhibitory activity. Comparatively, the peel extracts in all the treatments showed a good inhibitory activity against Gram negative (MIC value range between 0.195-0.781 mg/mL) than Gram positive bacteria (MIC value range between 0.781 and 1.562 mg/mL) during storage. As reported earlier, several bioactive compounds such as tannins have been implicated to the antibacterial activity of pomegranate peel (Opara et al., 2009; Miguel et al., 2010; Fawole et al., 2012) and their tendency to exhibit antibacterial activity is governed by their chemical structures (Heim et al., 2002). The study revealed that antibacterial activity of pomegranate peel extracts were not negatively impacted by the prolonged storage and package type used, and therefore, the peel extract could still be considered an active antibacterial agent.

4.7. Antibacterial activity of pomegranate seed oil

Results obtained on the antibacterial activity of pomegranate seed oil are presented in Table 2. The MIC values ranged from 0.39 and 0.781 mg/mL for Gram positive and Gram negative bacteria, respectively, at harvest. The inhibitory activity of seed oil against both Gram positive and Gram positive bacteria fluctuated during storage in all package types. Nonetheless, the best MIC values were observed against Gram negative than Gram positive bacteria regardless of the package type. As can be observed, the MIC values were in the range of 0.39 to 1.56 mg/mL for Gram negative as compared to Gram positive bacteria (in the range of 0.39-3.13 mg/mL). However, the MIC values against both Gram negative and Gram positive were similar at the end of storage for seed oil of fruit stored in polyliner and individual shrink wrap. The findings in the present study showed

that the seed oil of fruit showed lower inhibitory activity against Gram-positive bacteria during prolonged cold storage with lower MIC values than Gram-negative during storage in all package types. The antibacterial activity of pomegranate seed oil against Gram-negative *E. coli* O157:H7 has been reported by Karaman et al. (2015). Previously, it was reported that linoleic and oleic acids which are important constituents of oil have potential antibacterial properties and are attributable to long-chain unsaturation (McGaw et al., 2002, Agoramoorthy et al., 2007). Therefore, it could be suggested that the antibacterial activity observed in the present study may be associated with the presence of various free fatty acids in the pomegranate seed oil.

4.8. *Mushroom tyrosinase inhibition activity of peel extracts*

Tyrosinase is known to be a key enzyme in melanin production. Tyrosinase inhibitor has been used as a whitening agent or antihyperpigment agent because of its ability to suppress dermal-melanin production (Piao et al., 2002) along with food browning (Friedman, 1996). Among the whitening agent, pomegranate peel extracts have shown to be competitively effective against tyrosinase activity relative to arbutin (Fawole et al., 2012). Changes in the IC₅₀ of pomegranate peel extracts during prolonged storage under different packaging treatments are presented in Table 3. There was a significant effect of packaging and storage duration against monophenolase and diphenolase (P<0.05). The most active peel extracts were observed in control and MAP fruit peel extract against monophenolase with IC₅₀ values of 60.79 and 71.49 µg/mL, respectively, during the first month of storage as compared to the peel of individually shrink wrapped fruit (157.62 µg/mL). Moreover, the control fruit peel showed a better inhibition activity after 2 months of storage with IC₅₀ value of 99.9 µg/mL compared to MAP and individual shrink wrapped fruit peel having IC₅₀ values of 154.70 and 131.65 µg/mL, respectively. At the end of storage however, MAP stored fruit peel exhibited potent inhibitory activity with the IC₅₀ value of 29.70 µg/mL but lower than positive control (arbutin, 14.71 µg/mL). Potent inhibitory activity against monophenolase may be attributed to the higher antioxidant activity. In general, shrink wrapped fruit peel exhibited lesser degree of inhibition against monophenolase. Generally, bioactive compounds such as flavonoids, are well known to form complexes with metal ions and exhibit antioxidative action and were proved to be effective inhibitors of tyrosinase activity (Kubo et al., 2003; Momtaz et al., 2008). It has also been reported that compounds such as tannins have the capabilities to precipitate mainly proteins and flavonoid structure is analogous with the role of both substrates and inhibitors of tyrosinase (Fawole et al., 2012). Variation in inhibitory activity may be related to the fluctuation of bioactive compounds with prolonged storage and package treatments.

Diphenolase inhibition activity fluctuated during storage. The results showed that the best inhibition against diphenolase was observed after the first month of storage in peel of shrink wrapped fruit (27.80 µg/mL) compared to control (38.46 µg/mL) and MAP (82.04 µg/mL). After 3 months of storage, MAP stored fruit peel showed higher inhibition activity against diphenolase with the IC₅₀ value of 30.17 µg/mL, better than the positive control (arbutin, 44.00 µg/mL). However, peel activity was reduced to IC₅₀ values between 92.30 µg/mL and 137.39 µg/mL for individual shrink wrap and MAP stored fruit peel, respectively at the end of storage. During storage, the decline in bioactive compounds was observed which may have resulted in reduced inhibitory activity. Moreover, the IC₅₀ values observed at the end of storage were higher than that of positive control (arbutin, 44.00 µg/mL). Thus, it could be evidently concluded that the prolonged storage coupled with package type reduces the bioactive compounds responsible for inhibitory activity of pomegranate peel.

4.9. Anti-tyrosinase activity of pomegranate seed oil

Changes in IC₅₀ value of pomegranate seed oil during prolonged storage under different packaging treatments are shown in (Table 3). There was a significant interaction effect of packaging and storage duration on monophenolase and diphenolase. Pomegranate seed oil exhibited lower IC₅₀ value against monophenolase with prolonged storage in all package type. However, the concentration did not vary significantly in all treatments with prolonged storage. Therefore, the results were discussed based on the lowest IC₅₀ value observed. The IC₅₀ value against monophenolase was 0.37 µg/mL at harvest. The inhibition activity stayed within the best IC₅₀ values in all treatments during storage. However, a negligible decrease in IC₅₀ value was observed at the end of fruit storage for seed oil individual shrink wrapped fruit (2.84 µg/mL). Seed oil showed inhibitory activity against diphenolase with the IC₅₀ value of 1.40 µg/mL observed at harvest. Moreover, seed oil from fruit stored under MAP had the best inhibitory activity against diphenolase compared to seed oil from fruit stored in control packaging and shrink wrap (Table 3). However, inhibitory activity continued to fluctuate with storage duration. At the end of storage, seed oil from fruit stored in individual shrink wrap maintained higher IC₅₀ with value of 0.49 µg/mL compared to seed oil from fruit stored in MAP (3.78 µg/mL).

5. Conclusions

The findings from this study highlight the prospects of pomegranate peel and seed as sources of natural bioactive compounds and functional ingredients with potential applications in food, pharmaceutical and other bioprocess industries. In particular, fruit stored in shrink wrap packaging

maintained higher total phenolic, tannins and flavonoid concentration compared to MAP; however, vitamin C concentration was poorly preserved in both types of packaging at the end of 4 months in cold storage. Furthermore, rutin concentration in the peel of fruit stored was significantly reduced irrespective of type of packaging at the end of storage. Fatty acid composition (%) of seed oil including saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were best maintained at the end of storage regardless of the type of packaging used to store fruit. Peel extracts exhibited good inhibitory activity against Gram negative and Gram positive bacteria regardless of type of packaging after prolonged cold storage (4 months). Similarly, peel extract of fruit stored under MAP had good IC₅₀ against monophenolase and diphenolase than shrink wrapped fruit peel after 4 months of storage. Seed oil extracted from pomegranate fruit expressed significant antibacterial and anti-tyrosinase activity against selected strains of pathogenic microorganisms but not as efficient as peel extracts regardless of the package treatment. Overall, this study has demonstrated that pomegranate fruit by-products (peel and seed) can be considered as valuable waste material for value addition even after longer cold storage owing to their concentration of useful bioactive compounds and good antioxidant activity.

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Table 1

Fatty acid composition, tocol, phytosterol and triterpene (%) of pomegranate seed oil during storage at $7 \pm 0.5^\circ\text{C}$ for 4 months under different types of package.

Treatments	Storage duration (month)	Saturated fatty acids			Unsaturated fatty acids			Tocol/ vitamin E	Phyto- sterol	Triterpene
		Palmitic acid (C16:0)	Stearic acid (C18:0)	Arachidic acid (C20:0)	Linoleic acid (C18:2)	Punicic acid (9c, 11t, 13t, C18:3.n6)	Oleic acid (C18:1)	γ -tocopherol	γ -sitosterol	Squalene
Harvest	0	3.69±0.11ed	3.08±0.05bd	nd	4.70±0.08ef	68.09±0.86ac	3.85±0.06bc	1.15±0.16c	1.54	1.77±0.02cd
Control		3.58±0.01e	3.05±0.08bd	0.40±0.08de	4.70±0.11ef	69.98±15.64a	3.62±0.11bcd	1.90±0.24b	nd	2.38±0.15ab
MAP	1	3.74±0.05ed	2.99±0.05cd	0.50±0.001c	5.34±0.07bc	61.94±2.31d	3.44±0.05de	2.51±0.35a	nd	2.68±0.16a
Shrink wrap		2.05±0.92bc	3.15±0.01bd	0.40±0.09de	4.97±0.01ed	nd	3.86±0.01bc	0.80±0.02cd	nd	1.69±0.08cd
Control		nd	1.32±0.01d	nd	2.03±0.91g	nd	1.62±0.01e	nd	nd	2.33±0.01ab
MAP	2	4.19±0.12bc	3.73±0.02ab	0.65±0.01b	5.22±0.03cd	60.48±2.18d	3.90±0.12b	0.50±0.04de	nd	2.48±0.11ab
Shrink wrap		4.04±0.26c	3.51±0.12abc	0.42±0.07de	4.66±0.21ef	65.37±14.61c	3.56±0.02b	0.55±0.06de	nd	2.02±0.09bc
Control		4.73±0.05a	3.68±0.19abc	0.46±0.09cd	4.68±0.16ef	67.34±15.14ac	3.55±0.07ec	nd	nd	0.37±0.01f
MAP	3	4.12±0.05bc	1.89±0.65e	0.76±0.10a	5.66±0.05a	67.64±15.12ac	4.51±0.08a	0.20±0.03ef	nd	1.43±0.07de
Shrink wrap		4.10±0.04bc	3.51±0.01abc	0.68±0.17b	5.54±0.001ab	66.39±14.84ac	4.29±0.01a	nd	nd	1.08±0.36e

Table 1 (Continue)

Fatty acid composition, tocol, phytosterol and triterpene (%) of pomegranate seed oil during storage at $7 \pm 0.5^\circ\text{C}$ for 4 months under different types of package.

Treatments	Storage duration (month)	Saturated fatty acids			Unsaturated fatty acids			Tocol/vitamin E	Phytosterol	Triterpene
		Palmitic acid (C16:0)	Stearic acid (C18:0)	Arachidic acid (C20:0)	Linoleic acid (C18:2)	Punicic acid (9c, 11t, 13t, C18:3..n6)	Oleic acid (C18:1)	γ -tocopherol	γ -sitosterol	Squalene
Control		-	-	-	-	-	-	-	-	-
MAP	4	4.40±0.07b	3.88±0.18 a	0.36±0.001e	4.40±0.07f	69.76±0.19a	3.85±0.22bc	nd	nd	1.16±0.36e
Shrink wrap		1.95±0.87c de	3.13±0.24 bcd	0.69±0.15ab	4.98±0.14de	69.29±0.03ab	3.77±0.18bcd	0.17±0.07f	nd	2.78±0.04a

Each value in the table is presented as a mean± standard error. Mean values followed by different letter (s) within same column are significantly different ($P < 0.05$) according to Duncan's multiple range test. nd= not detected. Storage trial for control was discontinued after 3 month due to excessive fruit shrivelling.

Table 1 (Continue)

Total and ratio of fatty acid composition (%) of pomegranate seed oil during storage at $7 \pm 0.5^\circ\text{C}$ for 4 months under different types of package.

Treatments	Storage duration (month)	Total			Ratio	
		SFA	MUFA	PUFA	MUFA/PUFA	SFA/UFA
Harvest	0	6.77±0.17def	3.85±0.06bc	72.80±0.95ab	0.05±0.01d	0.09±0.001c
Control		6.84±0.07cef	3.62±0.11bcd	74.68±0.11b	0.04±0.01d	0.09±0.001c
MAP	1	7.24±0.01be	3.44±0.05de	67.29±2.38ab	0.05±0.01d	0.10±0.003c
Shrink wrap		5.42±0.92f	3.86±0.01bc	4.99±0.01c	0.77±0.01b	1.12±0.183a
Control		1.3±0.01g	1.62±0.01e	4.09±0.1c	0.80±0.01a	0.65±0.001b
MAP	2	8.59±0.07abc	3.90±0.12b	65.70±2.15ab	0.06±0.01c	0.13±0.003c
Shrink wrap		7.76±0.38abcd	3.56±0.02be	70.03±0.21b	0.05±0.01d	0.11±0.005c
Control		8.65±0.25a	3.55±0.07ec	72.03±0.48b	0.04±0.01d	0.12±0.002c
MAP	3	6.40±0.70def	4.5±0.08a	73.30±0.05b	0.06±0.01c	0.09±0.009c
Shrink wrap		8.30±0.01abcd	4.29±0.01a	71.94±0.01b	0.06±0.01c	0.11±0.001c

SFA, Saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; UFA, unsaturated fatty acids.

Table 1 (Continue)

Total and ratio of fatty acid composition (%) of pomegranate seed oil during storage at $7 \pm 0.5^\circ\text{C}$ for 4 months under different types of package.

Treatments	Storage duration (month)	Total			Ratio	
		SFA	MUFA	PUFA	MUFA/PUFA	SFA/UFA
Control		-	-	-	-	-
MAP	4	8.65±0.26ab	3.85±0.22c	74.16±0.11a	0.05±0.01b	0.11±0.003c
Shrink wrap		5.43±1.12ef	3.77±0.18bcd	74.27±0.18a	0.05±0.01b	0.07±0.014c

Table 2

Antibacterial activity (MIC, mg/mL) of pomegranate peel extracts and seed oil during storage at $7 \pm 0.5^\circ\text{C}$ for 4 months under different types of package.

Treatment	Storage duration (month)	Peel extracts				Seed oil			
		E.c	K.p	S.a	B.s	E.c	K.p	S.a	B.s
Harvest	0	0.39	0.39	1.56	1.56	0.78	0.78	0.39	0.39
Control	1	0.20	0.20	1.56	1.56	0.78	0.78	0.39	0.39
MAP		0.20	0.20	0.78	0.78	0.39	0.39	1.56	1.56
Shrink wrap		0.39	0.39	0.78	0.78	0.39	0.39	1.56	1.56
Control	2	0.20	0.39	0.78	0.78	0.39	0.39	1.56	1.56
MAP		0.39	0.39	0.78	0.78	1.56	1.56	3.13	3.13
Shrink wrap		0.39	0.39	0.78	0.78	1.56	1.56	3.13	3.13
Control	3	0.39	0.39	0.78	0.78	1.56	1.56	1.56	1.56
MAP		0.39	0.39	0.78	0.78	0.78	0.78	1.56	1.56
Shrink wrap		0.39	0.39	0.78	0.78	0.78	0.78	1.56	1.56
Control	4	-	-	-	-	-	-	-	-
MAP		0.78	0.78	0.78	0.78	1.56	1.56	1.56	1.56
Shrink wrap		0.78	1.56	0.78	0.78	1.56	1.56	1.56	1.56
Streptomycin (mg/mL)		0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Values less 1.0 mg/mL are considered very active. Storage trial for control was discontinued after 3 month due to excessive fruit shrivelling. E.c, *Escherichia coli*; K.p, *Klebsiella pneumonia*; S.a, *Staphylococcus aureus*; B.s, *Bacillus subtilis*.

Table 3

Effective inhibition concentration (EC₅₀) of pomegranate peel extracts during storage at 7 ± 0.5°C for 4 months under different types of package.

Treatment	Storage duration (month)	Peel extracts		Seed oil	
		EC ₅₀ Monophenolase (µg/mL)	EC ₅₀ Diphenolase (µg/mL)	EC ₅₀ Monophenolase (µg/mL)	EC ₅₀ Diphenolase (µg/mL)
Control		60.79±4.22d	38.46±4.03d	2.37±0.41a	1.43±0.02cd
MAP	1	71.49±2.64dc	82.04±2.20b	0.34±0.03b	0.88±0.06cd
Shrink wrap		157.62±21.46a	27.80±2.57e	0.14±0.03b	1.41±0.16cd
Control		99.9±19.11bc	80.73±5.32b	0.41±0.06b	3.84±0.17a
MAP	2	154.70±0.01a	65.04±3.43b	0.29±0.01b	2.59±0.47b
Shrink wrap		131.65±7.30ab	136.43±5.28a	0.45±0.02b	0.50±0.04d
Control		108.79±5.60b	83.48±4.40b	0.16±0.04b	0.82±0.08c
MAP	3	116.75±10.53b	30.17±2.34e	0.35±0.05b	1.63±0.12cd
Shrink wrap		110.61±12.40b	63.21±2.38c	0.59±0.08b	3.49±0.72ab
	<i>P-value</i>				
Packaging (P)		<0.0001	<0.0003	<0.0001	0.4182
Storage duration (S)		<0.0001	<0.0001	<0.0001	<0.0014

P*S

<0.0001

<0.0001

<0.0001

<0.0001

Data with the same letter in the same column indicate significant differences ($P < 0.05$) according to Duncan multiple range test. Each value in the table is represented as a mean \pm standard error. In order to determine interaction effects between packaging and storage duration, data for month 1, 2, and 3 for different packaging treatment was used. Storage trial for control was discontinued after 3 month due to excessive fruit shrivelling. Data for harvest and month 4 is reported on the appendix 2 (Table 2). P, Packaging; S, Storage duration; MAP, Modified atmosphere packaging. Harvest value: peel extracts (monophenolase = 107.00, arbutin=14.71; diphenolase = 115.58, arbutin = 44.00); seed oil (monophenolase = 0.37, arbutin= 42.37; diphenolase =1.40, arbutin= 42.36).

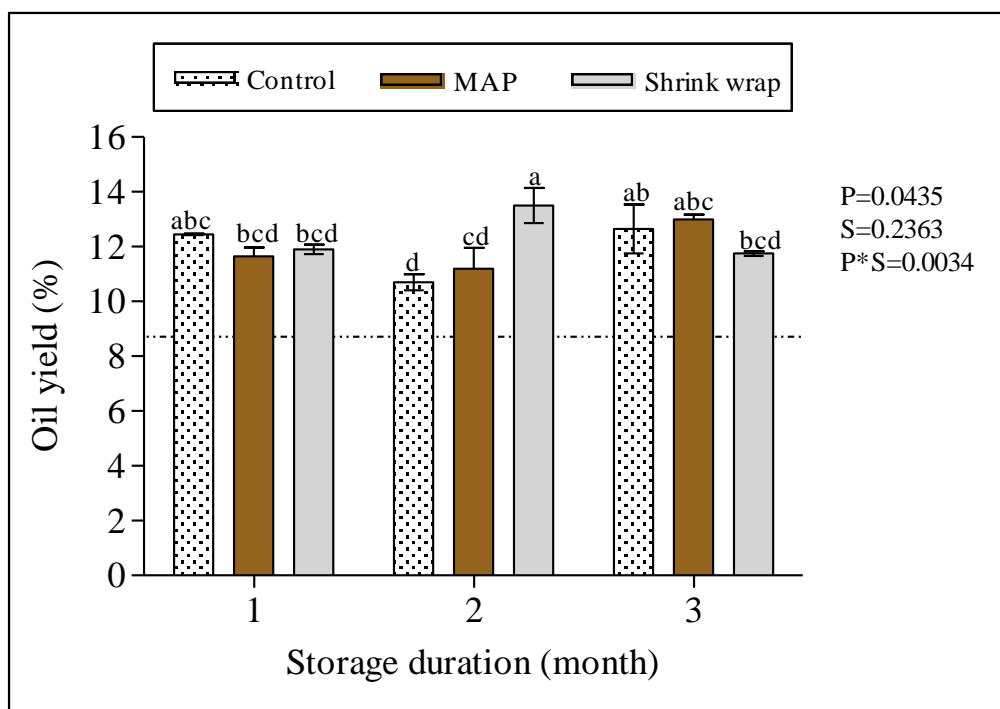


Fig. 1. Oil content of pomegranate seeds (% mean \pm SE, n=3). Bars followed by the same letters are not significantly different at $P < 0.05$ according to Duncan multiple range test. In order to determine interaction effects between packaging and storage duration, data for month 1, 2, and 3 for different packaging treatment was used. Storage trial for control was discontinued after 3 month due to excessive fruit shrivelling. The data for month 4 is reported on the appendix 2 (Table 1). P, Packaging; S, Storage duration; MAP, Modified atmosphere packaging. ---- measurements at harvest.

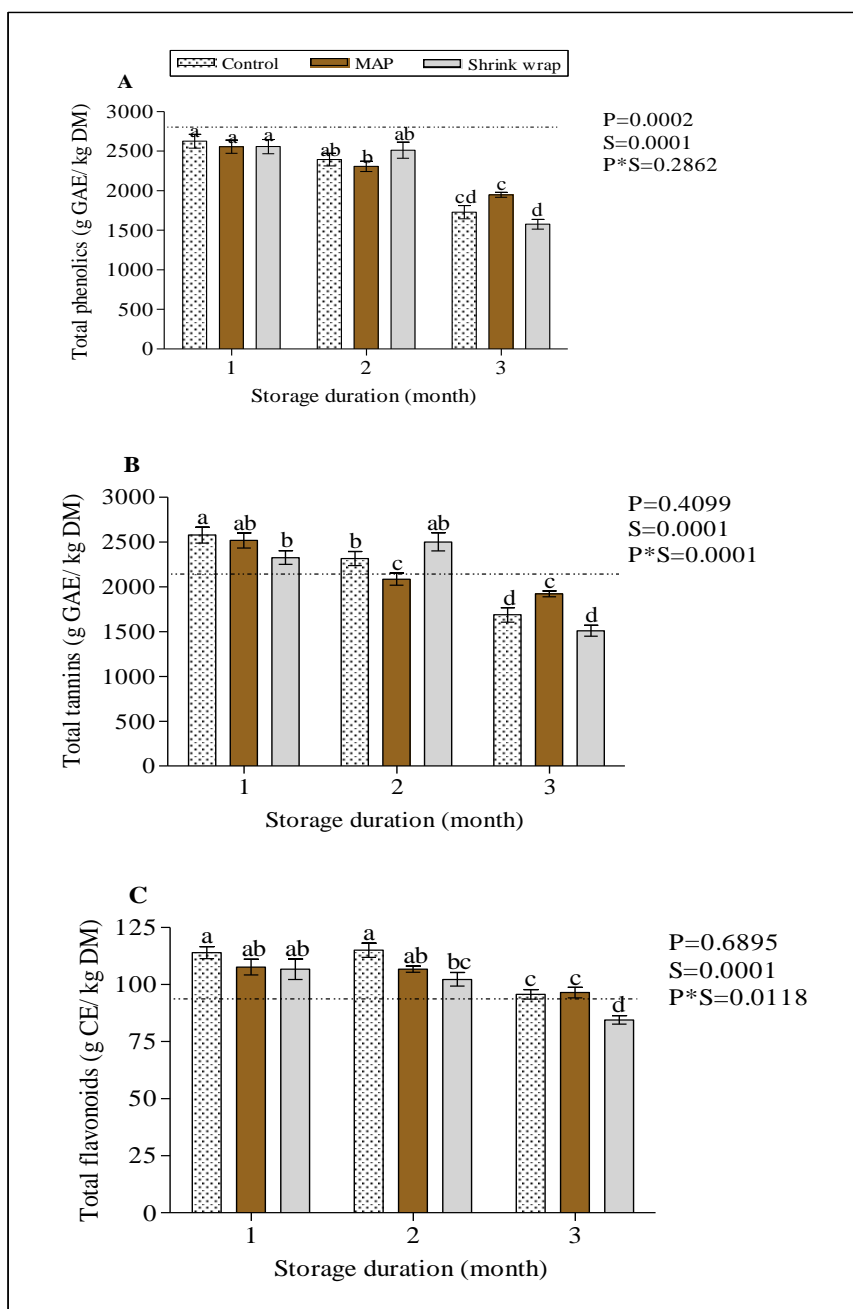


Fig. 2. Changes in the total phenolics, total tannin and total flavonoid concentration of pomegranate peel cv. Wonderful during storage at $7 \pm 0.5^\circ\text{C}$ for four months under different package types. Bars followed by the same letters are not significantly different at $P < 0.05$ according to Duncan multiple range test. Mean \pm SE presented ($n=3$). In order to determine interaction effects between packaging and storage duration, data for month 1, 2, and 3 for different packaging treatment was used. Storage trial was discontinued after 3 months for control fruit due to excessive fruit shrivelling. The data for month 4 is reported in the appendix 2 (Table 3).

---- Measurements at harvest. P, Packaging; S, Storage duration; MAP, Modified atmosphere packaging.

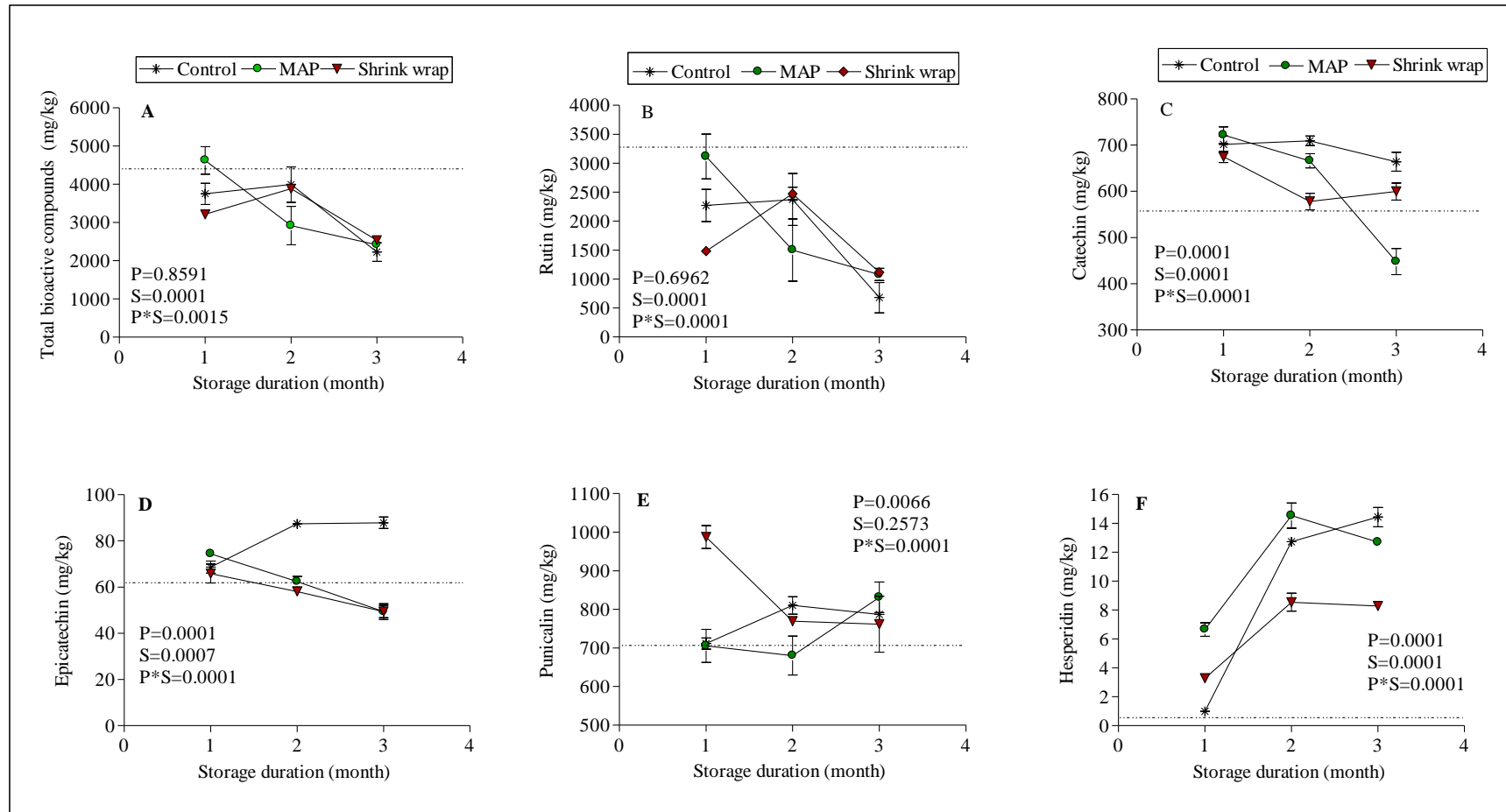


Fig. 3. Individual phenolic concentrations of cv. Wonderful pomegranate peel during storage at $7 \pm 0.5^\circ\text{C}$ for four months under different package types. In other to determine interaction effects between packaging and storage duration, data for month 1, 2, and 3 for different packaging treatment was used. Storage trial was discontinued after 3 months for control fruit due to excessive fruit shrivelling. The data for month 4 is reported on the appendix 3 (Table 3). ---- Measurements at harvest. P, Packaging; S, Storage duration; MAP, Modified atmosphere packaging.

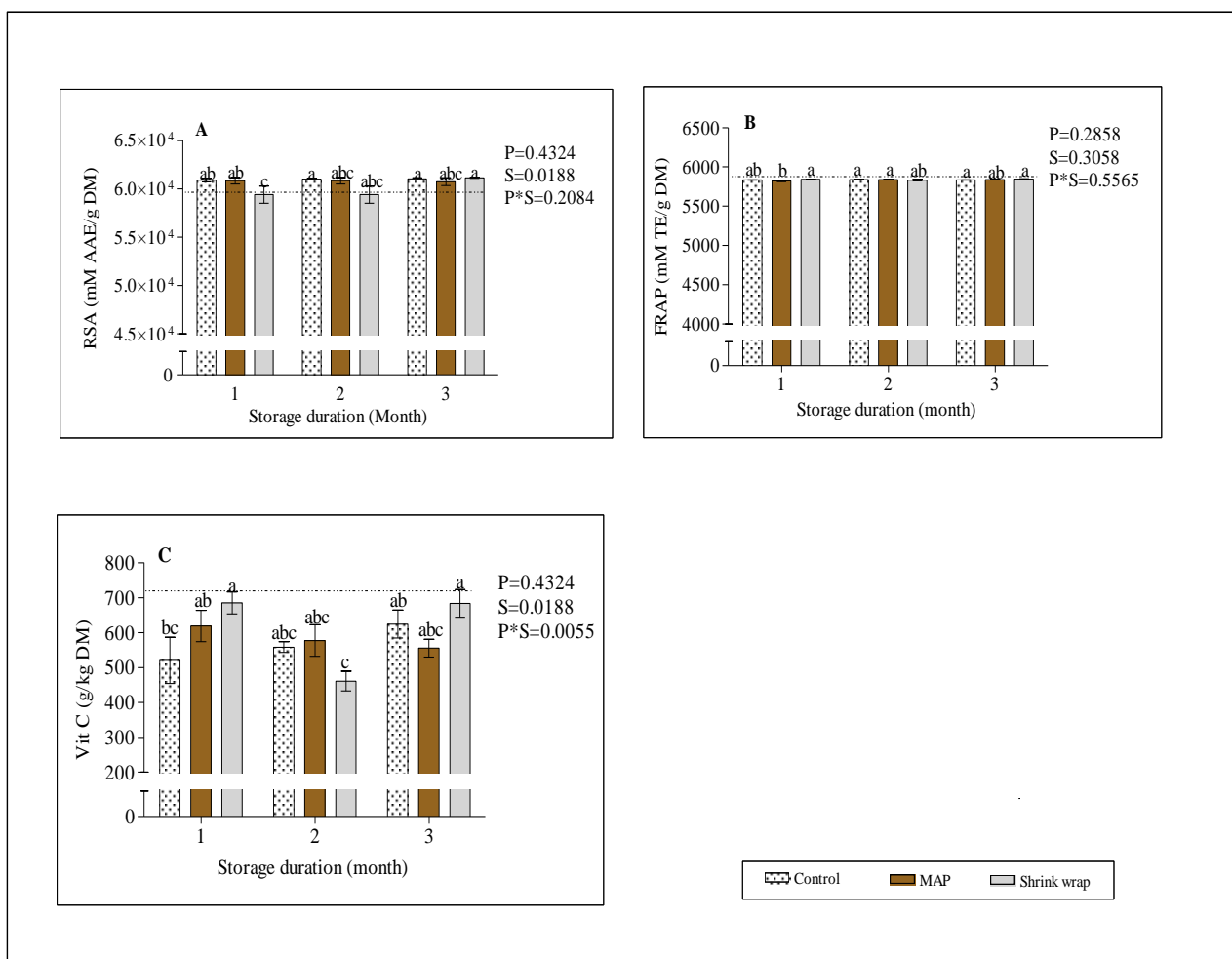


Fig. 4. Changes in the antioxidant activity and vit C of pomegranate peel cv. Wonderful during storage at $7 \pm 0.5^\circ\text{C}$ for four months under different package types. RSA, radical scavenging activity; FRAP, ferric reducing antioxidant power; vit C, vitamin C. Mean \pm SE presented (n=3). Bars followed by the same letters are not significantly different at $P < 0.05$ according to Duncan multiple range test. Storage trial was discontinued after 3 months for control fruit due to excessive fruit shrivelling. The data for month 4 is reported on the appendix 4 (Table 4). --- Measurements at harvest; P, Packaging, S, storage duration. MAP, modified atmosphere packaging.

PAPER 7

Effect of drying on the bioactive compounds, antioxidant, antibacterial and antityrosinase activities of pomegranate peel

Abstract

The use of pomegranate peel is highly associated with its rich phenolic concentration. Series of drying methods are recommended since bioactive compounds are highly sensitive to thermal degradation. The study was conducted to evaluate the effects of drying on the bioactive compounds, antioxidant as well as antibacterial and antityrosinase activities of pomegranate peel. Dried pomegranate peels with the initial moisture content of 70.30% wet basis were prepared by freeze and oven drying at 40°C, 50°C and 60°C. Difference in CIE-LAB, chroma (C*) and hue angle (h°) were determined using colorimeter. Individual polyphenol retention was determined using LC-MS and LC-MS^E while total phenolics concentration (TPC), total flavonoid concentration (TFC), total tannins concentration (TTC) and vitamin C concentration were measured using colorimetric methods. The antioxidant activity was measured by radical scavenging activity (RSA) and ferric reducing antioxidant power (FRAP). Furthermore, the antibacterial activity of methanolic peel extracts were tested on Gram negative (*Escherichia coli* and *Klebsiella pneumonia*) and Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilis*) using the *in vitro* microdilution assays. Tyrosinase enzyme inhibition was investigated against monophenolase (tyrosine) and diphenolase (DOPA), with arbutin as a positive control. Oven drying at 60°C resulted in high punicalin concentration (888.04 ± 141.03 mg CE/kg dried matter) along with poor red coloration (high hue angle). Freeze dried peel contained higher catechin concentration (674.51 mg/kg drying matter) +catechin and –epicatechin (70.56 mg/kg drying matter) compared to oven dried peel. Furthermore, freeze dried peel had the highest total phenolic, tannin and flavonoid concentrations compared to oven dried peel over the temperature range studied. High concentration of vitamin C (31.19 µg AAE/g dried matter) was observed in the oven dried (40°C) pomegranate peel. Drying at 50°C showed the highest inhibitory activity with the MIC values of 0.10 mg/mL against Gram positive (*S. aureus* and *B. subtilis*). Likewise, the extracts dried at 50°C showed potent inhibitory activity concentration (22.95 mg/mL) against monophenolase. Principal component analysis showed that the peel colour characteristics and bioactive compounds isolated the investigated drying method. The freeze and oven dried peel extracts exhibited a significant antibacterial and antioxidant activities. The freeze drying method had higher total phenolic, tannin and flavonoid concentration therefore can be explored as a

feasible method for processing pomegranate peel retaining the maximum amount of their naturally occurring bioactive compounds.

Keywords: Freeze drying; Oven drying; Rutin; Total phenolics; Vitamin C

1. Introduction

Pomegranate (*Punica granatum* L.) fruit is an important commercial crop cultivated in different parts of the world. The adaptability and health benefits are some of the characteristics responsible for its wide scale cultivation. About 50% of the total fruit weight corresponds to the peel, which is an important source of bioactive compounds (Opara et al., 2009). Meanwhile the edible part of pomegranate fruit consists of 40% arils and 10% seeds (Sreerkumar et al., 2014). Pomegranate peel is a waste from juice processing. Several studies have confirmed that pomegranate peel is a rich source of bioactive compounds including ellagitannins, catechin, rutin and epicatechin among others (Opara et al., 2009; Glazer et al., 2012; Fawole et al., 2012; Fawole et al., 2015). These bioactive compounds possess different biological activities such as scavenging reactive oxygen species (ROS), inhibiting oxidation and microbial growth and reducing the risk of chronic disease such as cancers and cardiovascular disorders (Opara et al., 2009; Viuda-Martos et al., 2010; Fawole et al., 2012).

However, the concentrations of bioactive compounds widely fluctuate among cultivars, environmental conditions, fruit maturity status, storage and postharvest treatments which may affect fruit quality and health beneficial compounds (Fawole and Opara, 2013a,b; Mphahlele et al., 2014a,b; Arendse et al., 2014). In the past, pomegranates was commonly used in conventional medicine for eliminating parasites and vermifuge, and to treat and cure apthae, ulcers, diarrhoea, acidosis, dysentery, haemorrhage, microbial infections and respiratory pathologies (Viuda-Martos et al., 2010). According to Gil et al. (2000), pomegranate peel has the higher antioxidant activity than the pith and juice.

Drying is an ancient process used to preserve and prolong shelflife of various food products (Ratti, 2001). The main aim of drying food products is to remove water in the solid to a level at which microbial spoilage and deterioration resulting from chemical reactions is significantly reduced (Krokida et al., 2003; Sablani, 2006; Tang et al., 2013; Chiewchan et al., 2015). This enables the product to be stored for longer periods since the activity of microorganisms and enzymes is inhibited through drying (Alibas et al., 2001; Jayaraman and Gupta, 1992). Generally, drying involves the application of thermal energy which causes water to evaporate into the vapour phase. However, drying results in structural, chemical and

phytochemical changes that can affect quality properties such as texture, colour and nutritional values (Maskan, 2000; Attanasio et al., 2004; Di Scala and Crapiste, 2008). Several drying techniques used for various products include air, oven and freeze drying. Generally, air-drying and oven drying are favoured due to processing cost and efficiency (Vega-Galvez et al., 2009). However, air drying has disadvantages of both long drying time required and poor quality (Soysal et al., 2006; Therdtai and Zhou, 2009). By far, freeze drying is regarded as the better method for moisture removal, with final products of the highest quality compared with air-drying (Ratti, 2001; Korus, 2011).

'Wonderful' is the most widely grown and consumed pomegranate cultivar globally (Holland et al., 2009) and during the past ten years, South Africa has seen tremendous increase in commercial production, accounting for over 1000ha of total planted area and 56% of total production (Hortgro, 2014). Pomegranate peel has been known for many years for its health benefit, including antibacterial activity. More recently, research indicated that pomegranate peel extracts also inhibit tyrosinase activity (Fawole et al., 2012), an enzyme that induces the production of melanin which leads to hyperpigmentation of the skin.

The high level of bioactive compounds in the peel as well as the reported health benefits to date make these desirable by-products as functional ingredients in food, nutraceuticals and pharmaceuticals (Espín et al., 2007; Fawole et al., 2012; Fawole et al., 2015). Previous researches have been limited to the characterization of phenolic compounds of the pomegranate peel extracts and the evaluation of its biological activities. However, the information on the effect of drying on the pharmacological properties is limited. Therefore, the aim of this study was to investigate the concentrations of polyphenols compounds, antioxidant activity and the in vitro pharmacological properties of pomegranate peel using freeze and oven drying (within a temperature range).

2. Material and methods

2.1. Plant material

Pomegranate fruit (cv. Wonderful) were sourced in 2015 during commercial harvest from a Sonlia packhouse (33°34'851"S, 19°00'360"E) in Western Cape, South Africa. Fruit were transported to the Postharvest Technology Laboratory at Stellenbosch University. Fruit of the same size shape, colour and without any physical defects were randomly selected. Fresh pomegranate peel was cut in the dimension of 20 ± 0.5 mm (length), 20 ± 0.5 mm (width) and 5 ± 0.5 mm (thickness) were used. Before drying, the peels were stored at -80°C until use. Moisture

content was measured using a modified AOAC method 925.45 (AOAC, 2005) with slight modifications by drying the peel using the oven at $105 \pm 0.5^\circ\text{C}$ for 24 h. The oven was kept functional for an hour to equilibrate the oven temperature before drying. The accuracy of the oven temperature was monitored using a thermometer (Thermco®, Germany). All the drying tests were run twice in triplicates at each temperature and averages were reported.

2.2. Drying procedure

Oven drying: Three different temperature levels (40°C , 50°C and 60°C) were used. The oven dryer (Model nr. 072160, Prolab Instruments, Sep Sci., South Africa) was operated at an air velocity of 1.0 m/s, parallel to the drying surface of the sample. Weight change was recorded by a digital balance (ML3002.E, Mettler Toledo, Switzerland) at an hourly interval during drying. Peels were dried until equilibrium (no weight change) was reached.

Freeze drying: Prior to drying, peels were frozen at -80°C for 2 days. Frozen pomegranate peels were freeze dried using a freeze dryer (VirTis Co., Gardiner, NY, USA) at a vacuum pressure of 7 milliTorr and the condenser temperature of -88.7°C . Similar procedure for monitoring weight loss as explained above was employed. Weight loss was recorded at 2 h intervals. The drying time needed to reach equilibrium weight and the residual moisture content in all drying methods is presented in Table 1.

2.3. Colour

Peel colour change was measured before and after drying using the CIE L^* , a^* , b^* coordinates with a calibrated Minolta Chroma Meter (Model CR-400/410, Minolta Corp, Osaka, Japan). The hue angle (h°) and colour intensity (C^*) were calculated (Pathare et al., 2013). The values provided for each sample were the average of three replicates. The total colour difference (ΔE^*) were calculated using the following formula:

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (1)$$

where ΔL^* , Δa^* and Δb^* are the differences between the colour of the fresh and dried sample.

2.4. Peel preparation

Dried peel was ground to a fine powder using a miller (Model A11, IKA, Germany) and screened through a plastic mesh sieve, with a mesh size of 1.4 mm particle size (Vanguard,

India). Dried pomegranate peel (2g) from each drying methods were extracted separately with 10 mL of 80% (w/v) methanol using sonication for approximately 1h (Al-Zoreky, 2009). The extracts were separately filtered with Whatman no.1 filter paper and residues were re-extracted following the same procedure. The extracts were pooled before drying under stream of air.

2.5. Determination of individual phenolic acids and flavonoids concentration

LC-MS and LC-MS^E analyses were conducted on a Waters Synapt G2 quadrupole time-of-flight mass spectrometer system (Milford, MA, USA). The instrument was connected to a Waters Acquity ultra-performance liquid chromatograph (UPLC) and Acquity photo diode array (PDA) detector. Ionisation was achieved with an electrospray source using a cone voltage of 15 V and capillary voltage of 2.5 kV using negative mode for analysis of phenolic compounds. Nitrogen was used as the desolvation gas, at a flow rate of 650 L/h and desolvation temperature of 275°C. The separations were carried on a waters UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm particle size), with injection volume of 3 µL at flow rate of 0.4 ml/min. The gradient for the analysis of phenolic compounds started with 100% using 0.1% (v/v) formic acid (solvent A) and kept at 100% for 0.5 min, followed by a linear gradient to 22% acetonitrile (solvent B) over 2.5 min, 44% solvent B over 4 min and finally to 100% solvent B over 5 min. The column was subjected to 100% solvent B for an extra 2 min. The column was then re-equilibrated over 1 min to yield a total run time of 15 min. Reference standards (Sigma-Aldrich, South Africa) of phenolic acids and flavonoids were used for the quantification of individual compounds in pomegranate peel extracts.

2.5.1. Determination of total phenolic concentration

Total phenolic concentration (TPC) was measured using the Folin-Ciocalteu (Folin-C) method as described by Makkar et al. (2000) with slight modification (Fawole et al., 2012). Diluted peel extracts (50 µL) was mixed with 450 µL of 50% methanol followed by the addition of 500 µL Folin-C and then sodium carbonate (2%) solution after 2 min. The mixture was vortexed and absorbance read at 725 nm using a UV-visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). Gallic acid standard curve (0.08– 0.32 mg/mL) was used and TPC was expressed as milligram gallic acid equivalent per kilogram peel extracts (mg GAE/kg DM).

2.5.2 Determination of total tannin concentration

Total tannin analysis was carried out using Folin-C method described by Makkar et al. (2000). Polyvinylpyrrolidone (PVPP) was used to separate tannin from non-tannin compound in peel extracts by adding 100 mg of PVPP to 1.0 mL of distilled water and 1.0 mL peel extracts in a test tube. The mixture was vortexed and kept at 4°C for 15 min followed by centrifugation at 4000g for 10 min. After the extraction, 50 µL of supernatant was mixed with 450 µL of 50% methanol followed by the addition of 500 µL Folin-C and then sodium carbonate (2%) solution after 2 min. The absorbance was recorded at 725 nm using UV-Visible spectrophotometer after incubation for 40 min at room temperature. Separate peel extracts not treated with PVPP was measured for total phenolic concentration. Total tannin concentration was calculated as:

$$\text{Total tannin concentrations (TTC)} = \text{TPC}_{(\text{in peel extract without PVPP})} - \text{TPC}_{(\text{in peel extract treated with PVPP})} \quad (1)$$

where TPC referred to total phenolic concentration (mg GAE /kg DM)

Results were expressed as milligram gallic acid equivalent per kilogram peel extracts (mg GAE /kg DM).

2.5.3 Determination of total flavonoid concentration

Total flavonoid concentration was measured spectrophotometrically as described by Yang et al. (2009). PJ (1.0 g) was extracted with 50% methanol (49 mL) and vortexed for 30 s. The mixture was sonicated in an ultrasonic bath for 10 min and centrifuged at 4000 g for 12 min at 4°C. Distilled water (1.2 mL) was added to 250 µL of extracted peel extracts and then followed by 75 µL of 5% sodium nitrite. After 5 min, freshly prepared 10% aluminium chloride (150 µL) was added to the mixture, followed by the addition of 500 µL sodium hydroxide after a another 5 min, and 775 µL distilled water bringing the final volume to 3 mL. The mixture was vortexed and absorbance was immediately read using spectrophotometer at 510 nm. Catechin (0.01–0.5 mg/mL) was used for the standard curve. The results were expressed as catechin equivalent per kilograms peel extracts (mg CE /kg DM).

2.5.4 Radical scavenging activity (RSA)

The ability of peel extract to scavenge 2, 2-diphenyl-1-picryl hydrazyl (DPPH) radical was measured following the procedure described by Karioti et al. (2004) with slight modifications (Fawole et al., 2012). Peel extract (15 µL) was mixed with 735 µL methanol and

0.1 mM solution of DPPH (750 μ L) dissolved in methanol. The mixture was incubated for 30 min in the dark at room temperature before measuring the absorbance at 517 nm using a UV-visible spectrophotometer (Thermo Scientific Technologies, Madison, Wisconsin). The RSA was determined by ascorbic acid standard curve (0–1500 μ M). The results were presented as millimolar ascorbic acid (AA) equivalent per gram of peel extracts (mM AAE/g DM).

2.5.5 Ferric reducing antioxidant power

Ferric reducing antioxidant power assay was performed according to the method of Benzie and Strain (1996). FRAP solutions contained 25 mL acetate buffer (300 mM acetate buffer, pH 3.6), 2.5 mL (10 mM of TPTZ solution), 2.5 mL (20 mM of FeCl_3 solution). Ten millilitre of aqueous methanol (50%) was added to peel extract (1 mL), sonicated for 10 min in cold water and centrifuged for 5 min at 4°C. PJ (150 μ L) was mixed with 2850 μ L FRAP and the absorbance was read at 593 nm after 30 min incubation using a UV-visible spectrophotometer. Trolox (0–1.5 mM) was used for calibration curve, and results were expressed as trolox (μ M) equivalents per millilitre pomegranate juice (μ M TE /g DW).

2.5.6 Determination of ascorbic acid concentration

Ascorbic acid was determined according to Klein and Perry (1982) with slight modifications (Barros et al., 2007). Briefly, peel extract (1.0 g) was mixed with 50 mL of 1% metaphosphoric acid followed by sonication on ice for 4 min and centrifugation at 4000 g for 12 min. Supernatant (1.0 mL) was pipetted into a tube and mixed with 9 mL of 2, 6 dichlorophenolindophenol dye (0.0025%). The mixture was incubated in the dark for 10 min before absorbance was measured at 515 nm. Calibration curve of authentic L-ascorbic acid (0.01–0.1 μ g/mL) was used to calculate ascorbic acid concentration. Results were expressed as ascorbic acid equivalents per millilitre crude juice (μ g AAE /g DM).

2.5.7 Microdilution assay for antibacterial activity

Antibacterial activity of pomegranate peel was determined following microdilution assay for the minimum inhibitory concentration values (Fawole et al., 2012). Four bacterial strains used included two Gram-negative bacteria (*Escherichia coli* ATCC 11775 and *Klebsiella pneumonia* ATCC 13883) and two Gram-positive bacteria (*Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 12600). All the bacteria were grown in sterile MH broth. The stock solutions of the peel extracts were dissolved in methanol to make 50.0 mg/mL. Under

aseptic conditions, 100 μ L of sterile water were added in a 96-well micro plate followed by 100 μ L peel extracts as well as bacterial culture and serially diluted (two-fold). Similarly, two fold serial dilution of streptomycin (0.1 mg/mL) was used as positive control against each bacterium. Bacteria-free broth, methanol solvent (100%) and sterile water were included as negative controls. The final concentration of peel extract ranged from 0.097 – 12.5 mg/mL, whereas streptomycin was between (0.097- 12.5 mg/mL). Plates were incubated for 18 h at 37°C. After incubation, bacterial growth in the plate was indicated by adding 40 μ L of *p*-iodonitrotetrazolium chloride (Sigma-Aldrich, Germany) after incubation. Bacterial growth was indicated by pink colour, while clear wells indicated inhibition. The results were recorded in terms of the minimal inhibitory concentration which is regarded as the lowest concentration of the extract without bacterial growth. The assay was measured in triplicate.

2.5.8 Mushroom tyrosinase inhibition assay

Tyrosinase inhibitory activity was determined using calorimetric method as described by Momtaz et al. (2008) with slight modification (Fawole et al., 2012). L-tyrosine and L-3,4-dihydroxyphenylalanine (L-DOPA, Sigma) were used as substrates. Assays were carried out in a 96-well micro-titre plate and a Multiskan FC plate reader (Thermo scientific technologies, China) was used. Peel extracts and seed oil were dissolved in methanol and DMSO, respect to concentration of 50 mg/mL and further diluted in potassium phosphate buffer (50 mM, pH 6.5) to 1000 μ g/mL. Each prepared sample (70 μ L) was mixed with 30 μ L of tyrosinase (333 Units/mL in phosphate buffer, pH 6.5). After 5 min incubation, 110 μ L of substrate (2 mM L-tyrosine or 12 mM L-DOPA) was added to the reaction mixtures and incubated for 30 min. The final concentration of the extracts were between 2.6 - 333.3 μ g/mL. Arbutin (1.04 – 133.33 μ g/mL) was used as a positive control while a blank test was used as each sample that had all the components except L-tyrosine or L-DOPA. The final concentrations of the seed oil were between 0.17-5 mg/mL whereas the positive control (arbutin) were between 4.10 – 400 μ g/L. All the steps in the assay were conducted at room temperature. Results were compared with a control consisting of DMSO instead of the test sample. After adding mushroom tyrosinase solution, the reaction mixture was incubated at room temperature (37°C) for 30 minutes. The absorbance of the reaction mixture was measured at 475 nm. The percentage mushroom tyrosinase inhibitory activity was calculated using the following equation:

$$\% \text{ inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

where A_{control} is the absorbance of Methanol and A_{extract} is the absorbance of the test reaction mixture containing extract or arbutin. The IC_{50} values of extracts and arbutin were calculated. The assay was measured in triplicate.

3. Data analysis

Statistical analyses were carried out using statistical software (STATISTICA, Vers. 12.0, StatSoft Inc., USA). Data was subjected to analysis of variance (ANOVA) and means were separated by least significant difference (LSD; $P = 0.05$) according to Duncan's multiple range test. GraphPad Prism software version 4.03 (GraphPad Software, Inc., San Diego, USA) was used for graphical presentations. Principal component analysis (PCA) was carried out using XLSTAT software version 2012.04.1 (Addinsoft, France). Triplicate measurements were carried out and the values are reported as mean \pm standard error.

4. Results and discussion

4.1. Drying time and residual moisture concentration

The drying time required to achieve a final moisture concentration of the peel were, 22, 17, 16 and 12 hours for 40°C, 50°C, freeze drier and 60°C, respectively (Table 1). Drying time resulted in the residual moisture concentration which varied from 0.087 to 0.096 kg water/ kg dry matter. Overall, freeze dried peel had the lowest residual moisture whereas oven dried peels did not vary within the temperature range.

4.2. Peel colour

The freeze dried peel showed a considerably higher L^* (lightness/ brightness) with no significant differences in colour lightness of peel dried in the oven at 40°C, 50°C and 60°C, indicating a darker coloration than that of freeze dried peel (Table 2). High decline in lightness (L^*) was observed by Toor and Savage (2006) and Ashebir et al. (2009) in different tomatoes cultivars dried at various temperature. Moreover, a^* (peel redness) value is in the order of 60°C > 50°C > 40°C > freeze dried. The chroma value indicates the degree of saturation of colour and is proportional to the strength of the colour (Maskan, 2001). In this study, the freeze dried peel had the lowest colour intensity compared to oven dried peel at the investigated temperatures. The results indicate that freeze dried peel were slightly bleached (lower chroma value) which was also confirmed by lower a^* value compared to the oven dried peel. Moreover, there was a

negligible increase in hue angle (darkness) and followed the order: 60°C > 40°C > 50°C > freeze dried. According to Bahloul et al. (2009) the increase in hue angle is indicative of a browning reaction as a result of activity of polyphenolic oxidase. Likewise, formation of brown compounds may be as a result of the Maillard reaction which occurs upon the reduction of sugar and amino acids (Carabasa-Giribet and Ibarz-Ribas, 2000). Comparable results were also reported by other authors. For instance, Wojdylo et al. (2014) reported high L^* (lightness) but less a^* (red colour) value in freeze dried sour cherries. However, Vega-Galv ez et al. (2008) reported an increased L^* and a^* value in pre-treated red bell pepper dried at an air temperature in the range of 50 and 80°C. The change in total colour difference (ΔE) is an important part for dried product, which express human eye's ability to differentiate between colours of various samples (Wojdylo et al., 2014). Slight but notable variation was observed in the total colour difference between the drying methods, with oven drying at 60°C having the lowest total color difference (TCD) (16.82) while the highest was observed in peel dried at 50°C (23.10) (Table 2).

4.3. Individual phenolic acid and flavonoid compound

The phenols identified in dried pomegranate peel include phenolic acid (p-coumaric), flavan-3-ols (+catechin, -epicatechin), flavanone (hesperidin), flavonol (rutin), ellagitannin (punicalin) (Table 3). Punicalin is hydrolysable tannin which is known to account for high antioxidant activity in pomegranate peel (Lin et al., 2001; Tzulker et al., 2007). Punicalin values ranged from 559.60 to 888.40 mg/kg DW. As can be observed, drying at 60°C resulted in relatively higher punicalin concentration, which was 32.98, 25.41, 15.66% higher than oven dried (40°C), (50°C) and freeze dried peel, respectively. Higher retention of punicalin compound at 60°C may be as a result of less exposure to oxygen as the drying time was shorter (12 h). The highest concentration of rutin was found in freeze dried peel (4666.03 mg/kg DW) followed by drying at 60°C (3401.36 mg/kg DW) and 40°C (2135.00 mg/kg DW). On the other hand, p-coumaric was only detected in the oven dried peel at 50°C and 60°C. Generally, it was observed that the concentrations of rutin, +catechin, -epicatechin, and hesperidin were significantly higher in freeze dried compared to oven dried for the whole temperature range (40, 50 and 60°C). It has been highlighted that high porosity of dehydrated food promotes greater contact of the material with oxygen and facilitate oxidation of compounds (N obrega et al., 2015), whereas drying treatments release bound phytochemicals from the matrix to make them more accessible in extraction (Wojdylo et al., 2014). Comparable results were reported by Katsube et al. (2009),

who observed high phenolic concentration of freeze dried mulberry leaves. Freeze dried peel induced an increase in bioactive compounds compared to oven drying as observed in this study.

4.4. Total phenolic (TPC), total tannins (TTC) and flavonoid concentration (TFC)

Concentrations of total phenolic, total tannin and flavonoid after drying in pomegranate peel are shown in Figure 1. On the dry basis, TPC, TTC and TFC were between 3 to 5 folds more in freeze dried peel than the oven dried peel at all temperatures. The results were consistent with those reported by Karaman et al. (2014) who observed the highest total phenolic in freeze dried persimmon powder because of limited thermal and chemical degradation, as it was performed at low temperatures. Asami et al. (2003) reported that hot-air drying promoted the oxidation and condensation of phenolic compounds compared to freeze-drying. Similar results were reported by Calín-Sánchez et al. (2013) who reported higher total phenolic concentration in pomegranate rind after freeze drying. Generally, thermal treatment has significant effect on the depletion of polyphenols in food products (Kaur and Kapoor, 2001). Vega-Gálvez et al. (2009) also reported loss of polyphenol compounds in air dried red pepper in which polyphenol concentration decreased with drying temperature. In this study, total phenolic concentration did not vary across all the temperatures for oven dried method. This may be due to the fact that some phenolic compounds are destroyed by the elevated temperature during the drying process. Our results were similar to the results of Wolfe and Liu (2003) who did not observe any significant difference in total phenolics and flavonoids in apple peels dried under oven conditions (40°C, 60°C, or 80°C). According to Wojdyło et al. (2014) this behaviour may be due to the fact that a large percentage of phenolic compounds are bound to cellular structures, and dehydration treatments release bound phytochemicals from the matrix to make them more accessible in extraction. Freeze drying is often considered to be the most effective technique for preserving temperature sensitive compounds since the ice crystals formed within the plant matrix can rupture the cell structure, which provides the release of cellular components (Nicoli et al., 1999). With regard to the present study, freeze drying would be a better drying method for preserving total phenolic, tannins and flavonoid concentration of pomegranate peel than oven drying process.

4.5. Radical scavenging activity (RSA) and ferric reducing antioxidant power (FRAP) and vitamin C concentration

Peel dried at 60°C significantly ($P < 0.05$) had higher radical scavenging activity compared to 40°C, 50°C and freeze drier (Fig. 2A). As can be observed, increased radical scavenging activities in peel dried at 60°C coincide with higher punicalin, +catechin, -epicatechin and rutin compound concentration. It has been reported that the antioxidant activity may be related with amount of compounds since they act as scavengers of free radicals produced during oxidation reaction (López et al., 2013). Moreover, recent studies showed that the radical scavenging activity was elevated at higher drying temperatures using oven drying treatments (Lee Mei Ling et al., 2013; Rodriguzer et al., 2014).

The reducing power of pomegranate peel was determined using ferric reducing antioxidant power method which measures reduction of Fe^{+3} to Fe^{+2} . In this study, antioxidant activity (FRAP) of pomegranate peel did not vary significantly ($P > 0.05$) between the drying methods. The reducing power was in the order of 40°C > 50°C, 60°C > freeze-dried (Fig. 2B). It can be observed that freeze and oven drying at various temperatures affected vitamin C ($P < 0.05$), thus a considerable higher vitamin C in samples dried at 40°C was observed (Fig. 2C). However, no significant difference ($P < 0.05$) was observed between freeze and oven-dried (at 50°C and 60°C) peel. According to Margues and Freire (2006), the small losses in vitamin C in freeze dried product are attributed to low temperature and to the use of vacuum in the process. Researchers observed loss of vitamin C during air-drying of pomegranate peel (Opara et al., 2009). Our findings are in agreement with those of Vega-Gálvez et al. (2009) who reported loss of vitamin C during oven drying at temperatures between 50 and 90°C in red pepper. Miranda et al. (2009) reported the loss of 70% of vitamin C after drying in *Aloe vera* gel and the authors concluded that this may be the result of irreversible oxidation during drying with hot air. Therefore, lower vitamin C concentration observed in this study may be as a result of irreversible oxidation during drying. Vitamin C is a thermo-sensitive compound, therefore lower concentration was likely due to elevated processing temperature (Sigge et al., 2001; Hawlader et al., 2006) and period of exposure required to dry the sample at 50°C and 60°C.

4.6. Antibacterial activity

The antibacterial activity of dried pomegranate peel extracts are presented in Table 4. As can be observed, all the extracts showed the broad-spectrum activity against the bacterial strains used. The minimum inhibitory activity values observed against the tested bacteria ranged from

0.10 to 0.39 mg/mL. Moreover, drying at 50°C showed the highest inhibitory activity with the MIC values of 0.10 mg/mL against gram positive bacteria in particular *Staphylococcus aureus* and *Bacillus subtilis* compared with the rest of the treatments. Results from this study indicated that the peel extracts were effective against the tested bacteria, irrespective of the drying methods employed. Possible explanation could be as a result of higher retention of antioxidant activity after drying. Likewise, the activity against all the test bacteria (Gram-positive and Gram-negative bacteria) indicates that extracts contain broad spectrum metabolic toxins. Wojdylo et al. (2014) indicated that polyphenols in an intermediates state of oxidation may exhibit higher radical scavenging efficiency than the non-oxidized ones, although a subsequent loss in the antioxidant properties has been found for advanced enzymatic oxidation steps (Nicoli et al., 1999). To some extent, this is consistent with previous studies on antibacterial activity pomegranate peel extracts (Negi and Jayaprakasha, 2003; Opara et al., 2009; Fawole et al., 2012). It has been reported that the antibacterial activity of pomegranate peel extracts can be attributed to the presence of high molecular weight compounds such as tannins. In addition, the tannin rich ellagitannins have antibacterial and antifungal and antiprotozoal activity (Supayang et al., 2005; Vasconcelos et al., 2003; Prashanth et al., 2001). The results of the study suggest that drying either by freeze or oven showed the best MIC which indicates high stability of compounds contained in the pomegranate peel.

4.7. Tyrosinase inhibitory activity

Tyrosinase plays a key role in biosynthesis of melanin which is responsible for pigments of the skin, eyes and hair in mammals as well as browning of the fruits (Friedman, 1996). There are two distinct reactions of melanin biosynthesis; the hydroxylation of L-tyrosine (monophenolase activity) and the conversion of L-DOPA (diphenolase activity) to the corresponding monophenolase and diphenolase which are the key substrate facilitating the O-quinones (Seo et al., 2003). These quinones are highly reactive, and tend to polymerize spontaneously to form brown pigments, namely melanin. The inhibitory effect of dried peel extracts on the activity of tyrosinase is presented in Table 5. The inhibitory activity (IC₅₀) against monophenolase was in the range of 22.95-107.73 mg/mL. The peel extract dried at 50°C notably showed better inhibition on monophenolase activity. The highest inhibition activity against monophenolase was found to be 22.95 mg/mL of peel extracts dried at 50°C concentration compared to the rest of treatment. Moreover, the extracts of peel dried at 50°C showed potent inhibitory activity than the arbutin. In general, peel extracts showed weaker diphenolase

inhibition in all treatments compared to arbutin (control). However, better inhibitory activity against diphenolase was observed in the peel extracts dried at 60°C with MIC value of 62.09 mg/mL. Nevertheless, pomegranate peel contains a mixture of many kinds of secondary metabolism products, including phenolics, which vary greatly in their antioxidant capacity and phenolic compounds composition.

4.8. *Multivariate analysis*

4.8.1. *Principal component analysis*

The results show the average of individual phenolics, total phenolics, antioxidant activity and color coordinates of pomegranate dried peel by oven and freeze drying. The two principal components (PC1 and PC2) explain 88.70% of the total data variance (Fig. 3). As observed, PC1 explained 70.98% of the total variance whilst PC2 explained only 17.71% of the total variability which showed that the disparity among pomegranate peel dried using different methods was described by the F1 (Fig. 3). The observations (Fig. 3) indicated that freeze dried peel could be associated with catechin, rutin, epicatechin hesperidin, total flavonoid, total phenolic, total tannin, ferric reducing antioxidant power and lightness which had higher positive scores along F1 (Table 6). Moreover, the higher negative scores (Table 6) along F1 (Fig. 3) correspond to chroma (C*), p-coumaric, radical scavenging activity, hue angle, punicalin and redness (a*), moisture content (wb %) and residual moisture content (db %) of the peel dried at 60°C. Along F1 (Fig. 3), lower positive scores correspond to total colour difference, of oven dried peel at 50°C. Likewise, high positive scores (Table 6) along F2 is associated with total colour difference, rutin, radical scavenging activity and punicalin of the oven dried (60°C) (Fig. 3). Along F2, high negative scores (as shown in Fig. 3 and Table 6) for oven dried (40°C) could characterize the peel for having high vitamin C concentration. However, lower positive scores along F2 were from freeze dried peel (associated with total phenolic concentration, total flavonoid concentration, ferric reducing antioxidant power, total tannin concentration, hesperidin, Chroma and hue angle). The lower negative scores (Fig. 3) along F2 (Table 6) were from oven dried (50°C) (associated with residual moisture content). The results demonstrated that PCA showed that freeze drying and oven drying at all temperature range have significantly different properties.

5. Conclusions

The results of the study showed that drying processes have an impact on the bioactive compounds of pomegranate peel. Freeze drying peels had a positive effect on the total phenolic, tannins and flavonoid than oven drying at all temperature ranges. Moreover, freeze drying had a positive impact on the +catechin, -epicatechin, hesperidin and rutin concentrations of fruit peel. Pomegranate fruit obtained from all the drying methods investigated were less effective against tyrosinase activity; however, they exhibited the best MIC against all the test bacteria. In addition, drying peels at 50°C had a positive influence on the inhibitory activity of peel extracts against monophenolase. The results of the present study reveal that freeze-drying can be explored as a viable method for processing pomegranate peel to retain the maximum amount of their naturally occurring bioactive compounds.

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Table 1 Residual moisture of pomegranate peel using freeze and oven drying methods.

Drying method and/or drying temperature	Drying time (h)	Residual moisture (kg water/ kg dry matter)
Freeze dried	16	0.087±0.002b
40°C	22	0.093±0.002a
50°C	17	0.094±0.002a
60°C	12	0.096±0.004a

Means in the same column with different letter(s) differ significantly ($P < 0.05$) according to Duncan's multiple range tests.

Table 2 Pomegranate peel colour attributes after drying.

Drying method and/or drying temperature	Colour attributes				
	L^*	a^*	C	h°	ΔE
Fresh peel	51.01±1.67b	28.85±1.70a	35.14±1.60a	34.61±2.05b	-
Freeze dried	61.46±1.59a	23.33±1.28b	29.99±0.86b	38.85±2.18ab	17.77±1.07b
40°C	41.51±1.09c	24.33±1.01b	33.13±0.77a	42.32±1.70a	18.72±1.14ab
50°C	39.29±1.37c	25.24±0.88 b	33.25±0.78a	39.82±1.76ab	23.10±2.42a
60°C	42.04±0.92c	25.24±1.16b	35.08±0.66a	43.78±1.90a	16.82±2.00b

Means in the same column with different letter(s) differ significantly ($P < 0.05$) according to Duncan's multiple range tests. L^* =lightness/darkness; a^* = redness/greenness; C = chroma; h° = hue angle; ΔE = total colour difference (TCD).

Table 3 Individual phenolic and flavonoid concentration in fresh and dried pomegranate peel.

Drying method and/ temperature	Punicalin	Rutin	p-Coumaric	+Catechin	-Epicatechin	Hesperidin
	(mg CE/ kg DM)			(mg/kg DM)		
Freeze dried	708.38±48.86b	4666.03±311.70a	nd	674.51±21.30a	70.56±0.22a	16.45±1.65a
40°C	768.11±1.67b	2135.00±0.00c	nd	377.26±22.05c	28.93±1.55c	5.07±0.02b
50°C	672.98±26.93b	nd	0.45±0.02	340.64 ±21.06c	31.95±3.37bc	4.59±0.54b
60°C	888.04±57.57 a	3401.36±0.00b	0.57±0.52	443.41±0.30b	34.74±0.11b	1.77±0.54c

Mean in column with different letter (s) differ significantly ($P < 0.05$) according to Duncan's multiple range test. Means \pm SE presented (n=3). nd, not detected.

Table 4 Antibacterial activity (MIC, mg/mL) of dried pomegranate peel extracts using two different drying methods.

Drying method and/ or temperature	Gram negative		Gram positive	
	<i>Escherichia coli</i>	<i>Klebsiella pneumonia</i>	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>
Freeze dried	0.39	0.39	0.20	0.20
40°C	0.20	0.20	0.20	0.20
50°C	0.20	0.20	0.10	0.10
60°C	0.39	0.39	0.39	0.39
Streptomycin (mg/mL)	0.02	0.02	0.02	0.02

Table 5 Inhibition concentration at 50% (IC₅₀) of fresh and dried fruit peel extracts against tyrosinase.

Drying method and/ or temperature	Monophenolase (IC ₅₀ mg/mL)	Diphenolase
Freeze dried	107.73±10.08a	86.93±15.23ab
40°C	45.07±6.05b	119.79±20.23a
50°C	22.95±1.53c	74.05±10.27ab
60°C	64.27±10.35b	62.09±2.98b
Arbutin (mg/mL)	44.00±5.56b	14.99±2.52c

Different letter within the same column are significantly different (P<0.05) according to Duncan multiple test range. IC₅₀ (mg/mL), inhibition concentration at 50%. Data represent the Mean ± SE (n=3). Arbutin, positive control.

Table 6 Factor loadings, eigenvalue, cumulative variance (%) and score for the first two principal (F1–F2) components based on pomegranate peel from two different drying methods.

Loadings	F1	F2
Catechin	0.864	0.487
Epicatechin	0.930	0.363
Punicalin	-0.543	0.653
Hesperedin	0.999	0.033
RSA	-0.649	0.760
Vit C	-0.300	-0.511
TF	0.983	0.182
TP	0.964	0.264
TT	1.000	0.014
FRAP	0.875	0.165
Rutin	0.564	0.652
p-coumaric	-0.715	0.457
Lightness (L^*)	0.945	0.315
Chroma (C^*)	-0.984	0.174
Hue angle (h°)	-0.791	0.303
Redness (a^*)	-0.930	0.053
TCD	-0.551	0.821
MC _{wb}	-0.969	0.236
RMC	-0.994	-0.114
Scores		
Freeze dried	6.147	0.816
40°C	-1.067	-2.055
50°C	-1.511	-1.356
60°C	-3.569	2.595

RSA, radical scavenging activity; Vit C, Vitamin C; TF, total flavonoid; TP, total phenolic; TT, total tannin; FRAP, ferric reducing antioxidant power, TCD, total color difference; MC_{wb}, moisture content (wet basis) RMC, residual moisture content.

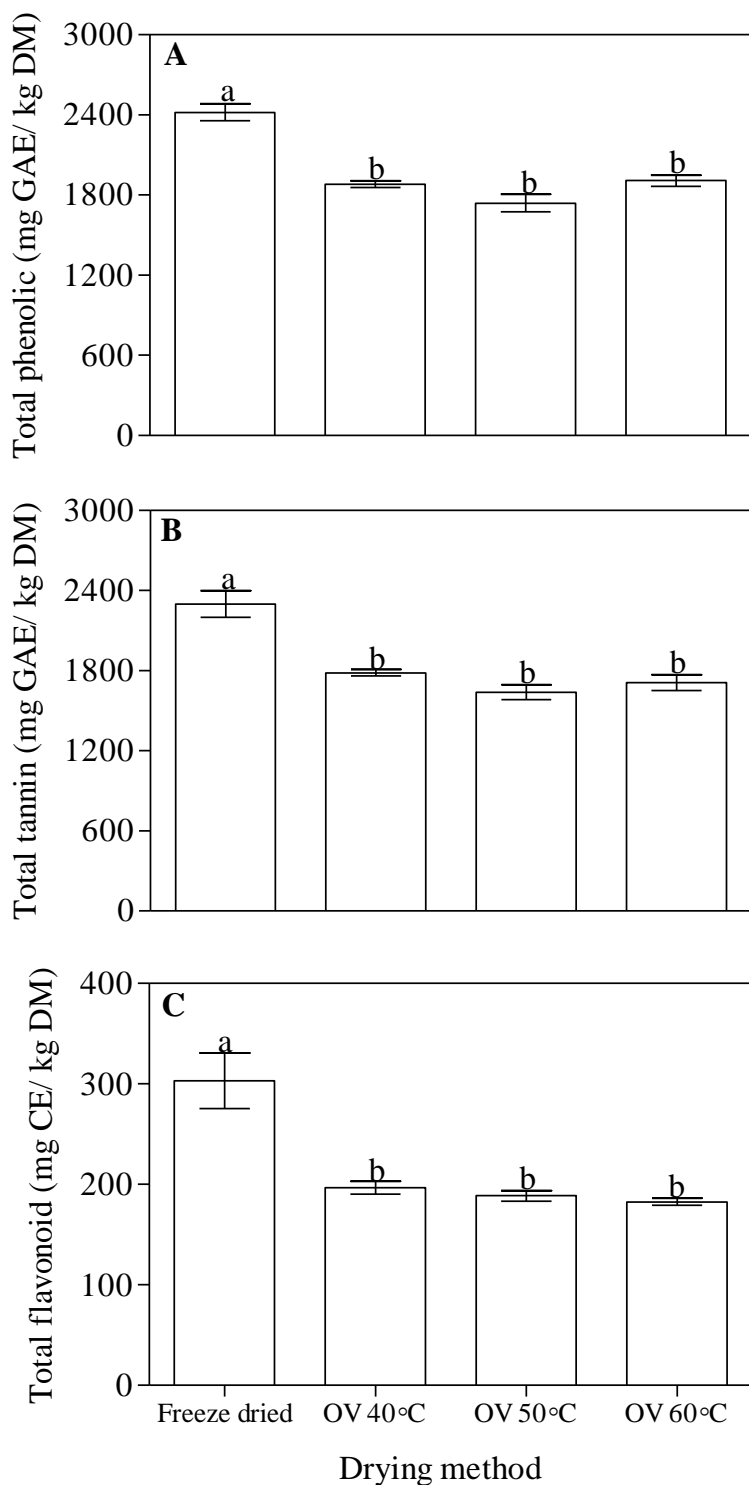


Fig. 1. Effects of drying methods on the total phenolics, total tannins and total flavonoid concentrations of pomegranate peel. Bars with same letter are not significantly different ($P < 0.05$; Duncan's multiple range test). Data represent the Mean \pm SE ($n=3$). OV, Oven drying.

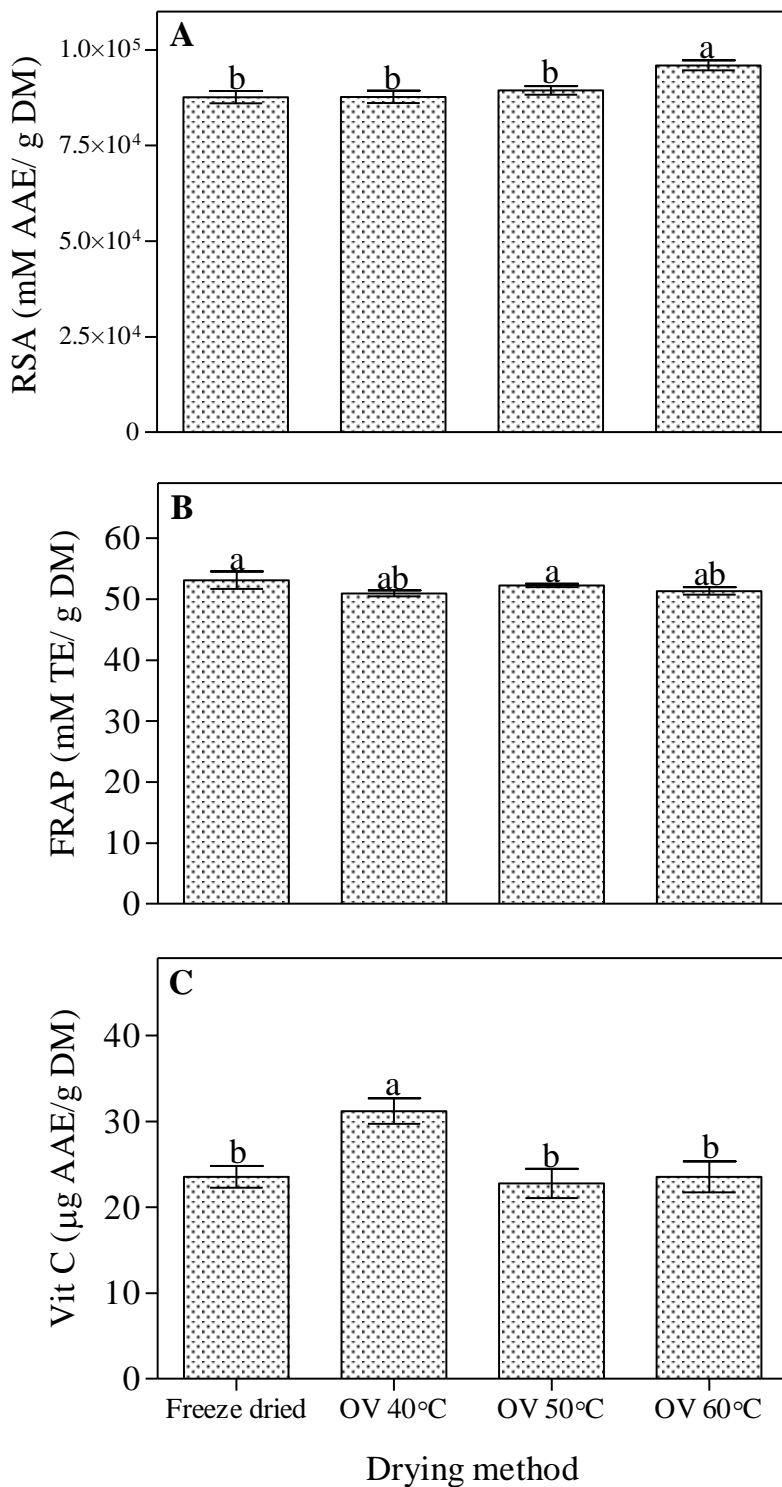


Fig. 2. Effects of drying methods on RSA, FRAP and vitamin C concentration of pomegranate peel. Bars with same letter are not significantly different ($P < 0.05$; Duncan's multiple range test). Data represent the Mean \pm SE ($n=3$). RSA, radical scavenging activity; FRAP, ferric reducing antioxidant power; Vit C, Vitamin C. OV, Oven drying.

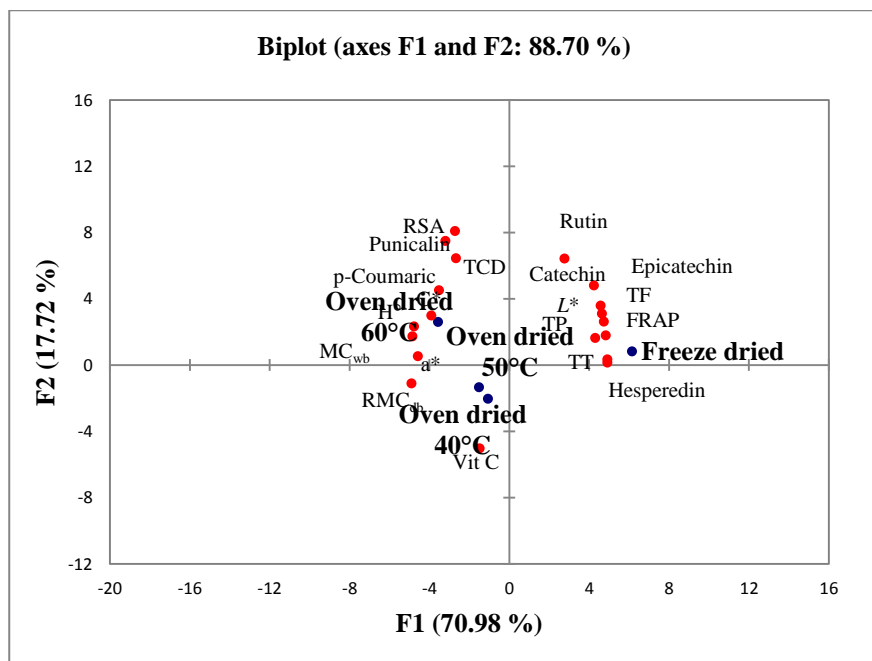


Fig. 3. Principal component analysis of the first two factors (F1 and F2) based on colour attributes and bioactive compounds of pomegranate peel cv. Wonderful obtained from different drying methods. TCD, total colour difference; TF, total flavonoid, TP, total phenolic; TT, total tannin; RSA, radical scavenging activity; Vit C, Vitamin C; FRAP, ferric reducing antioxidant power. L^* , lightness; C^* , chroma; H^o , hue angle; a^* , redness; RMC, residual moisture content (dry basis %); MC, moisture content (wet basis %).

PAPER 8

Drying kinetics of pomegranate peel (cv. Wonderful)

Abstract

Pomegranate juice processing produces large amount of peel as by-product or waste which is highly susceptible to microbial decomposition due to high moisture content. Drying the peel offers opportunities for value addition into novel products, thus reducing waste from the fruit processing operations. This study presents the mathematical models describing the thin layer drying behaviour of pomegranate peels (initial thickness 5.00 ± 0.05 mm and moisture content 70.30% wet basis) using three air temperatures (40°C, 50°C and 60°C) at a constant air velocity of 1.0 m/s. The results obtained showed that drying time decreased as the oven drying temperature increased. The drying process took place mainly in the falling rate period. Ten thin layer drying models were evaluated based on coefficient of determination (r^2) and standard error (e_s). Among the tested drying models, the Midilli et al. (2002) mathematical model was found to be the best fit for establishing the drying kinetics of pomegranate peel. Furthermore, the effective moisture diffusivity of pomegranate peel ranged from 4.05×10^{-10} to 8.10×10^{-10} m²/s over the temperature range investigated, with mean activation energy (E_a) of 22.25 kJ/ mol.

Keywords: Pomegranate peel; Drying; Diffusivity; Temperature; Activation energy

1. Introduction

Pomegranate fruit (*Punica granatum* L.) belongs to the Punicaceae family. It is relatively distributed around the world, including Asia, USA, Russia, North Africa, Spain and most recently South Africa (Al-Said et al., 2009; Holland et al., 2009; Fawole et al., 2012a). Pomegranate fruit is popularly consumed as juice and is used in food industry in the manufacture of jellies, concentrates, and flavouring and colouring agent (Opara et al., 2009). Pomegranate fruit consumption has continued to gain global interest among consumers due their wealth of nutritional properties and high content of polyphenols (Caleb et al., 2012; Fawole and Opara, 2013). The fruit is comprised of peels and arils (which contain juice and seeds/kernels), with the arils arranged in sacs. During juice processing, the peel is a major by-product and accounts for about 50% of whole fruit mass (Opara et al., 2009; Al-Said et al., 2009; Fawole et al., 2015) The peel is rich in polyphenols including flavonoids, phenolic acids and tannins (Opara et al., 2009; Fawole et al., 2012b; Fawole et al., 2015). These bioactive compounds possess different

biological activities such as scavenging reactive oxygen species (ROS), inhibiting oxidation and microbial growth and reducing the risk of chronic disease such as cancers and cardiovascular disorders (Opara et al., 2009; Viuda-Martos et al., 2010; Fawole et al., 2012b).

Since the pomegranate fruit peel is highly susceptible to microbial contamination and rapid spoilage in its wet state, drying could serve as an alternative method of preservation. Drying is an ancient process used to preserve and prolong shelf life of various food products (Ratti, 2001; Kim et al., 2002; Tang et al., 2013). The main aim in drying food products is the removal of water in the solid to a level at which microbial spoilage and deterioration resulting from chemical reactions is significantly reduced (Krokida et al., 2003; Sablani, 2006; Tang et al., 2013; Chiewchan et al., 2015). This enables the product to be stored for longer periods since the activity of microorganisms and enzymes is inhibited through drying (Alibas et al., 2001). One of the mostly widely used drying techniques in the agriculture and food industries involves the application of thermal energy.

Studies on drying characteristics and kinetics of by-products of a wide range of agricultural commodities have been reported such as carrot pomace (Kumar et al., 2012), olive pomace (Goula et al., 2015; Meziane, 2011; Vega-Gálvez et al., 2010), grape marc and pulp (Doymaz and Akgün, 2009), apple pomace (Sun, 2007), grape seeds (Roberts et al., 2008), vegetable baggase (Vijayaraj, 2007) and waste (Lopez et al., 2000). Studies on the drying characteristics and kinetics pomegranate peels are limited. Only recently, several papers have been published on the drying kinetics of pomegranate by-products (from juice processing) using a cabinet dryer (Kara and Doymaz, 2015), pomegranate peels cv. Hicaznar using cabinet dryer (Doymaz, 2011) and pomegranate seed cv. Hicaznar (from juice processing) using infrared radiation (Doymaz, 2012).

'Wonderful' is the most widely grown and consumed pomegranate cultivar globally (Holland et al., 2009) and during the past ten years, South Africa has seen tremendous increase in commercial production, accounting for over 1000 ha of the total 4500 ha planted area and 56% of total production (Hortgro, 2014). The high level of bioactive compounds in the peel as well as the reported health benefits highlight the potential of these by-products as functional ingredients in food, nutraceuticals and pharmaceuticals (Espín et al., 2007; Fawole et al., 2012b; Fawole et al., 2015). The aim of the study was to determine the drying characteristics and establish a suitable thin-layer drying model for pomegranate peel (cv. Wonderful) over a wide temperature range. Additionally, effective moisture diffusivity and activation energy are calculated.

2. Materials and methods

2.1. Fruit Material

Pomegranate fruit (cv. Wonderful) were sourced in 2015 during commercial harvest from Sonlia packhouse in Western Cape (33°34'851"S, 19°00'360"E), South Africa. Fruit were then transported to the Postharvest Technology Laboratory at Stellenbosch University and immediately, healthy fruit were sorted for uniformity in size, shape and colour. Fresh pomegranate peel was cut in the dimension of 20 ± 0.5 mm (length), 20 ± 0.5 mm (width) and $5 \text{ mm} \pm 0.5$ thicknesses were used. Moisture content was measured using a modified AOAC method 925.45 (AOAC, 2005) with slight modifications by drying the peel using the oven at $105 \pm 0.5^\circ\text{C}$ for 24 h. The oven was kept functional for an hour to equilibrate the inner temperature before drying. The accuracy of the inner temperature was monitored using thermometer (Thermco®, Germany).

2.2. Oven Drying Procedure

Three different temperature levels (40, 50 and 60°C) were used and the oven dryer was operated at an air velocity of $1.0 \text{ m}^2/\text{s}$, parallel to the drying surface of the sample. Moisture loss was recorded by a digital balance (ML3002.E, Mettler Toledo, Switzerland) at an hourly interval during drying for determination of drying curves. Peels were dried until equilibrium (no weight change) was reached. Drying tests were run four times at each temperature.

2.3. Modelling of the Drying Characteristics

Moisture ratio (MR) of pomegranate peels during drying was calculated using equation (1) (Jain and Pathare, 2007; Ngcobo et al., 2013).

$$MR = \frac{M_t - M_e}{M_o - M_e} \quad (1)$$

where M_t represents moisture content at time t (kg water/kg dry matter), M_o initial moisture content of the sample (kg water/kg dry matter), and M_e equilibrium moisture content (kg water/kg dry matter). However, MR was simplified to M_t/M_o instead of $(M_t - M_e)/(M_o - M_e)$ since the value of equilibrium moisture content (M_e) is negligible compared to M_t and M_o (Kingsly and Singh, 2007; Wang et al., 2007):

The drying rate of pomegranate peel was calculated using equation 2:

$$DR = \frac{M_{t_1} - M_{t_2}}{t_2 - t_1} \quad (2)$$

where t_1 and t_2 are drying times (h); M_{t_1} and M_{t_2} are moisture content of the samples at (g water/g dry matter) at time 1 and time 2, respectively (Doymaz, 2011).

3. Data Analysis

Ten thin-layer drying models were selected for fitting the data as detailed in Tables 1, 2 and 3. The r^2 (coefficient of determination) is one of main criteria for selecting the best model to describe drying curves (Chapra and Canale, 1989). The best fit model describing the drying characteristics was chosen based on the highest r^2 value and the lowest standard error.

3.1. Effective Moisture Diffusivity Determination

According to Pathare and Sharma (2006), moisture diffusivity is used to indicate the flow of moisture within a material and is primarily influenced by moisture content and temperature of the material. The moisture diffusivity of infinite slab is described by Eq. 3 (Crank, 1975). By assuming that there is uniform moisture distribution, equilibrium between the product surface and the drying air constant diffusivity, and negligible shrinkage of the test sample (García-Perez et al., 2006), we obtain Eq. 3 and 4:

$$\frac{\partial M}{\partial t} = \nabla [D_{eff}(\nabla M)] \quad (3)$$

$$MR = \frac{8}{\pi^2} \sum_{n=0}^{\infty} \frac{1}{(2n+1)^2} \exp\left(-\frac{(2n+1)^2 \pi^2 D_{eff} t}{4L^2}\right) \quad (4)$$

D_{eff} is the effective moisture diffusivity (m^2/s), t is the time (min), L denotes half-thickness of samples (m), and n is a positive integer. In the case of longer drying periods, the above equation can be simplified to the only first term of series, without much affecting the accuracy of the prediction (Movagharnejad and Nikzad, 2007; Lopez et al., 2000):

$$\ln MR = \ln\left(\frac{8}{\pi^2}\right) - \left(\frac{\pi^2 D_{eff} t}{4L^2}\right) \quad (5)$$

From Eq. (5), a plot of $\ln MR$ versus drying time give a straight line with a slope (K) of

$$K = \frac{\pi^2 D_{eff}}{4L^2} \quad (6)$$

3.2. Determination of Activation Energy

According to Aghbashlo et al. (2010), the dependence of effective moisture diffusivity on temperature is described by the Arrhenius equation:

$$D_{eff} = D_0 \exp\left(\frac{-E_a}{R(T+273.15)}\right) \quad (7)$$

where D_0 is the pre-exponential factor of the Arrhenius equation (m^2/s), E_a is activation energy (kJ/mol), R is the universal gas constant (kJ/mol K^{-1}), and T is temperature ($^{\circ}C$).

4. Results and discussion

The changes in pomegranate peel moisture content versus drying time for different drying temperatures are presented in Fig. 1. The result show that the moisture content of pomegranate peel decreased exponentially as the drying time increased resulting in 0.093, 0.094, 0.096 kg water/ kg DM for 40 $^{\circ}C$, 50 $^{\circ}C$ and 60 $^{\circ}C$ drying temperature, respectively. The drying time required to achieve a final constant moisture content of the peel were, 22, 17 and 12 hours at the oven temperature of 40 $^{\circ}C$, 50 $^{\circ}C$ and 60 $^{\circ}C$, respectively. It can be observed that increasing drying air temperature substantially reduced drying time. Rapid moisture ratio decrease is due to increased air heat supply rate to the peels which results in accelerated moisture migration out of the peel. Similar results were also obtained by several researchers on drying various agricultural by-products such as olive cake (Vega-Gálvez et al., 2010); pomegranate by-product after juice processing (Kara and Doymaz, 2015) and prickly pear seed (Motri et al., 2013).

The drying rate (kg water/ kg dry matter/hr) versus moisture content is presented in Fig. 2. The average drying rate of the pomegranate peel at the oven temperature of 40 $^{\circ}C$, 50 $^{\circ}C$ and 60 $^{\circ}C$ were 0.0010, 0.0031 and 0.0085 kg water/ kg dry matter/hr, respectively. As can be observed, higher temperature resulted in higher drying rate. Average drying rate was greater at the beginning of the drying process possibly due to evaporation and moisture from peel surface which later declined with decreasing moisture content for all the drying temperature range. In addition, constant rate drying was not well noticeable as the drying took place at the normal falling rate for all the temperature range indicating that internal mass transfer occurred by diffusion. According to Pathare and Sharma (2006) the accelerated drying rate was presumed to be attributed to internal heat generation. The results are in agreement with those findings reported by Doymaz (2011) using pomegranate peel and Kara and Doymaz (2015) using pomegranate by-product (after juice processing) and vegetable such as carrot (Kumar et al., 2012).

Fitting of the Drying Curves

Fig. 3 presents the variation of experimental and predicted moisture ratio using the best model with drying time for dried pomegranate peel. The best model selected was based on the highest coefficient of determination (r^2) and the lowest standard error (e_s) values. Midilli et al. (2002) model was identified as the best descriptive model for all the drying temperatures with the highest r^2 and the lowest e_s value compared to other layer drying models. The values for coefficient of determination and e_s were in the range of 0.9988 – 0.9999 and 0.0028 – 0.0112, respectively indicating that the thin layer drying of pomegranate peels occurs in the falling rate period. The results are similar to those found by (Doymaz, 2011), who dried pomegranate peel cv. Hicaznar in thin layer with drying temperature in the range 50 - 70°C. The author observed that Midilli model obtained the best fit. Likewise, Kara and Doymaz (2015) found that Midilli et al. (2002) model best represented the thin layer drying characteristics of pomegranate by-products (from juice processing) with drying air temperature in the range of 50 to 80°C.

Effective Moisture Diffusivity (D_{eff})

The values of D_{eff} were obtained using Eq. (7) and are presented in Table 4. In the present study, the calculated D_{eff} showed an increasing trend with the increasing drying temperature. The effective moisture diffusivity of the pomegranate peels at the drying temperature was 4.05×10^{-10} , 5.06×10^{-10} and 8.10×10^{-10} m²/s at 40°C, 50°C and 60°C, respectively. The D_{eff} observed in the study was within general range observed by several researchers such as pomegranate by-product from juice processing ($1.22 - 4.29 \times 10^{-10}$ m²/s), (Kara and Doymaz, 2015), pomegranate peel cv. Hicaznar ($4.02 - 5.31 \times 10^{-9}$ m²/s) (Doymaz, 2011), and grape seed ($1.57 - 8.03 \times 10^{-10}$ m²/s) (Roberts et al., 2008) using various air temperatures in the range of 40 – 80°C.

Activation Energy (E_a)

Activation energy is a measure of the temperature sensitivity of D_{eff} and is the energy needed to initiate moisture diffusion within the peels (Afolabi and Tunde-Akintunde, 2014). In the present study, the activation energy of pomegranate peels was found to be 21.98 kJ/mol. According to Zogzas et al. (1996), the value of E_a is within the general range of 12.7-110 kJ/mol for various food materials. Our results are in agreement with those reported by several researchers for various agricultural crops and by-products. For instance, the activation energy was found to be 23.05 kJ/mol in carrot (Kumar et al., 2012), 39.66 kJ/mol for pomegranate peel

cv. Hicaznar (Kara and Doymaz, 2015), 25.41 kJ/mol for grape marc (Doymaz, 2009), 13.47 kJ/mol for grape pulp (Doymaz, 2009) and 52.10 kJ/mol for tomato pomace (Al-muhtaseb et al., 2010).

Conclusions

This study established that the drying behaviour of pomegranate peels occurs in the falling rate period. Increasing air drying temperature increased the drying potential and therefore decreased drying time. Higher drying rate was observed for higher drying air temperature. Among the ten empirical drying models investigated, the model proposed by Midilli et al. (2002) best explained the drying characteristics of pomegranate peels, and therefore represents a good approximation for estimating the drying time of this by-product. The values of effective moisture diffusivity under different air temperature were in the range of $4.05 - 8.10 \times 10^{-10} \text{ m}^2/\text{s}$, with average activation energy of 21.98 kJ/mol.

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Nomenclature

A	Positive integer
$a_0, a,$	Coefficient of drying models
D_0	Pre-exponential factor of Arrhenius Eq.
D_{eff}	Effective moisture diffusivity, $m^2 s^{-1}$
DR	Drying rate, $kg (water) kg^{-1} (dry matter) h^{-1}$
E_a	Activation energy
e_s	Standard error
K	Drying coefficient
L	Half thickness
M_0	Initial moisture content, $kg (water) kg^{-1} (dry matter)$
M	Moisture content, $kg (water) kg^{-1} (dry matter)$
M_e	Equilibrium moisture content, $kg (water) kg^{-1} (dry matter)$
MR	Moisture ratio
M_t	Moisture content at any time
N	Number of observations
N	Exponential coefficient of Page's Eq.
N	Positive interger
r^2	Coefficient of determination
T	Time, s

Table 1 Statistical output of the thin layer drying models for the drying of the pomegranate peels cv. Wonderful at 40°C.

Model	Parameter	Value	Coefficient of determination (r^2)	Standard error (e_s)
Newton ($M_R = \exp(-kt)$)	$k(h^{-1})$	0.2223	0.9862	0.0183
Page ($M_R = \exp(-kt^n)$)	$k(h^{-1})$	0.3041	0.9979	0.0112
	n	0.8178		
Henderson and Pabis ($MR = a \exp(-kt)$)	$k(h^{-1})$	0.2098	0.9892	0.0228
	a	0.9485		
Asymptotic (logarithmic) ($MR = a_0 + a \exp(-kt)$)	$k(h^{-1})$	0.2519	0.9986	0.0098
	a	0.9343		
	a_1	0.0460		
Two term ($MR = a \exp(-k_0t) + b \exp(-k_1t)$)	k_0	0.3217	0.9995	0.0059
	a	0.7448		
	k_1	0.2491		
	b	0.0898		
Two-term exponential ($MR = a \exp(-kt) + (1-a) \exp(-kat)$)	k	0.5447	0.9975	0.0102
	n	0.3068		
Midilli et al ($MR = a \exp(-kt^n) + bt$)	$k(h^{-1})$	0.2868	0.9997	0.0046
	n	0.8789		
	a	1.0021		
	b	0.0014		
Modified page ($MR = \exp(-kt^n)$)	k	0.2969	0.9862	0.0168
	n	0.7486		
Approximation of diffusion ($MR = a \exp(-kt) + (1-a) \exp(-kbt)$)	k	0.0943	0.9994	0.0060
	a	0.2708		
	b	3.5250		
Verma et al. ($MR = a \exp(-kt) + (1-a) \exp(-gt)$)	k	0.3323	0.9994	0.0060
	a	0.7291		
	g	0.0943		

Table 2 Statistical output of the thin layer drying models for the drying of the pomegranate peels cv. Wonderful at 50°C.

	Parameter	Value	Coefficient of determination (r^2)	Standard error (e_s)
Newton ($MR = \exp(-kt)$)	$k(h^{-1})$	0.3252	0.9914	0.0155
Page ($MR = \exp(-kt^n)$)	$k(h^{-1})$	0.3911	0.9961	0.0158
	n	0.8609		
Henderson and Pabis ($MR = a \exp(-kt)$)	$k(h^{-1})$	0.3169	0.9920	0.0183
	a	0.9760		
Asymptotic (logarithmic) ($MR = a_0 + a \exp(-kt)$)	$k(h^{-1})$	0.3659	0.9998	0.0030
	a	0.9587		
	a0	0.0385		
Two term ($MR = a \exp(-k_0t) + b \exp(-k_1t)$)	k0	0.3668	0.9999	0.0030
	a	0.9573		
	k1	0.0401		
	b	0.0029		
Two-term exponential ($MR = a \exp(-kt) + (1-a) \exp(-kat)$)	k	0.5972	0.9968	0.0133
	a	0.3960		
Midilli et al. ($MR = a \exp(-kt^n) + bt$)	$k(h^{-1})$	0.3647	0.9999	0.0028
	n	0.9547		
	a	1.0006		
	b	0.0023		
Modified page ($MR = \exp(-(kt)^n)$)	$k(h^{-1})$	0.3591	0.9914	0.0146
	n	0.9054		
Approximation of diffusion ($MR = a \exp(-kt) + (1-a) \exp(-kbt)$)	$k(h^{-1})$	0.0182	0.9998	0.0032
	a	0.0497		
	b	20.5488		
Verma et al. ($MR = a \exp(-kt) + (1-a) \exp(-gt)$)	k	0.3689	0.9998	0.0031
	a	0.9580		
	g	0.0059		

Table 3 Statistical output of the thin layer drying models for the drying of the pomegranate peels cv. Wonderful at 60°C.

	Parameter	Value	Coefficient of determination (r^2)	Standard error (e_s)
Verma et al (MR=aexp(-kt) + (1 - a)exp(-gt))	k(h ⁻¹)	0.3323	0.9994	0.0060
	a	0.7291		
	g	0.0943		
Newton (MR=exp(-kt))	k(h ⁻¹)	0.4196	0.9923	0.0262
Page (MR=exp(-kt ⁿ))	k(h ⁻¹)	0.3952	0.9928	0.0237
	n	1.0577		
Henderson and Pabis (MR=aexp(-kt))	k(h ⁻¹)	0.4245	0.9925	0.0258
	a	1.0123		
Asymptotic (logarithmic) (MR=a ₀ + aexp(-kt))	k(h ⁻¹)	0.4565	0.9946	0.0232
	a	0.9974		
	a1	0.0230		
	n	1.0285		
Two term (MR=aexp(-k ₀ t) + bexp(-k ₁ t))	k ₀	0.4565	0.9946	0.0233
	n	0.9974		
	k ₁	0.0230		
	b	0.0000		
Two-term exponential (MR=aexp(-kt) + (1-a)exp(-kat))	k	0.4630	0.9923	0.0265
	a	0.7165		
Midilli et al (MR=aexp(-kt ⁿ) + bt)	k	0.3657	0.9988	0.0112
	n	1.1939		
	a	0.9970		
	b	0.0040		

Table 3 (Continued) Statistical output of the thin layer drying models for the drying of the pomegranate peels cv. Wonderful at 60°C.

	Parameter	Value	Coefficient of determination (r^2)	Standard error (e_s)
Modified page ($MR = \exp(-(kt)^n)$)	$k(h^{-1})$	0.4080	0.9923	0.0261
	n	1.0285		
Approximation of diffusion ($MR = a \exp(-kt) + (1 - a) \exp(-kbt)$)	k	0.4529	0.9933	0.0220
	a	1.0875		
	b	5.5494		
Verma et al. ($MR = a \exp(-kt) + (1 - a) \exp(-gt)$)	k	0.4460	0.9941	0.0235
	a	0.9787		
	g	0.0000		

Table 4 Effective moisture diffusivity at various drying oven temperatures.

Temperature (°C)	D_{eff} (m ² /s)
40	4.05×10^{-10}
50	5.06×10^{-10}
60	8.10×10^{-10}

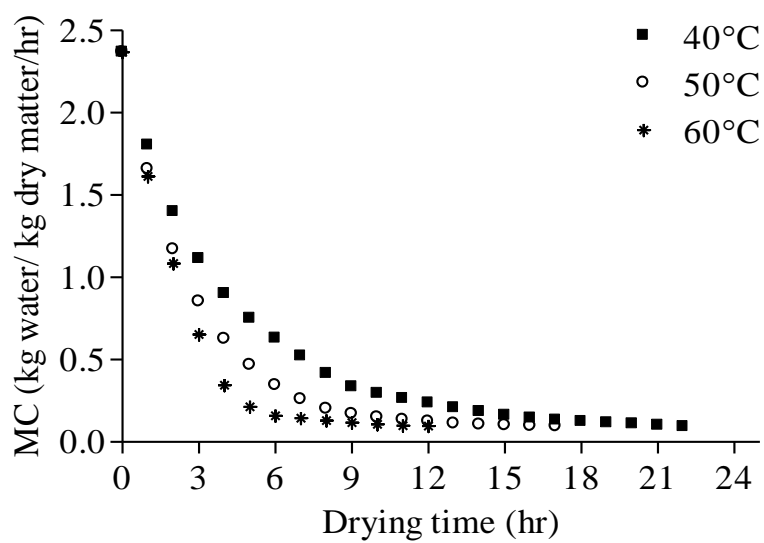


Fig. 1. Drying curves of pomegranate peel at different temperatures. MC, moisture.

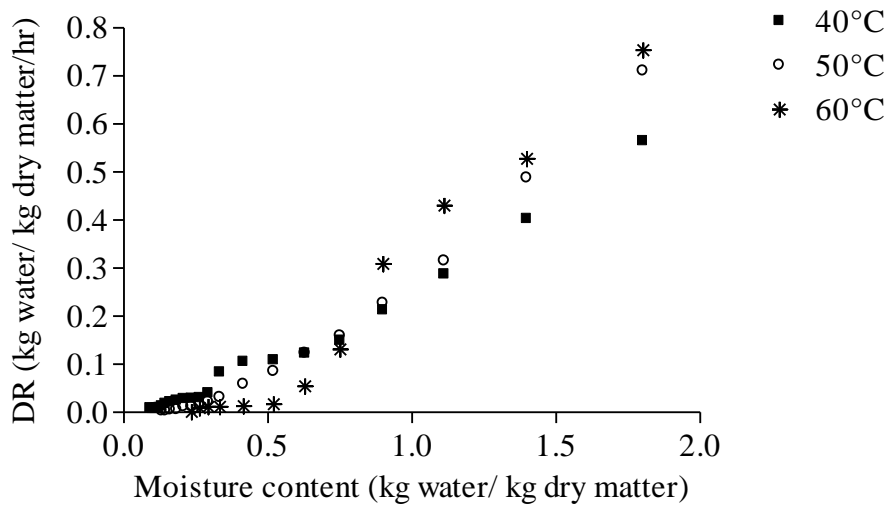


Fig. 2. Variation of drying rate as a function of moisture content of pomegranate peel. DR, drying rate.

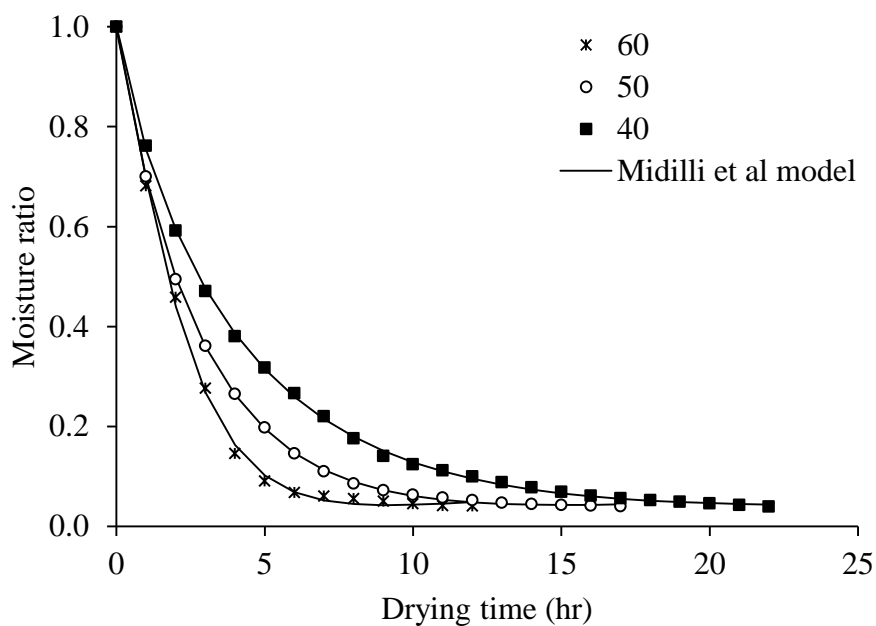


Fig. 3. Comparison between the experimental moisture ratios of pomegranate peels and those predicted by Midilli et al. (2002) model.

General Discussion and Conclusions

1. Introduction

Functional foods derived from fresh horticultural produce are becoming more popular as consumers recognize that a healthy diet is important to control and prevent diseases. Pomegranate is among the highly rated types of fruit (often referred to as ‘super fruits’) due to its high concentration of beneficial health compounds including high antioxidant activity (Opara et al., 2009; Fawole et al., 2012). The high concentration of health-promoting compounds have been reported to confer various functional properties such as anti-oxidant, anti-inflammatory, anti-tyrosinase and anti-microbial activities (Stover and Mercure, 2007; Jurenka, 2008; Fawole et al., 2012). Researches have shown that several preharvest and postharvest factors affect the composition and concentration of health-promoting compounds and the biochemical attributes of pomegranate fruit (Caleb et al., 2012; Fawole et al., 2013a, b; Mditshwa et al., 2013; Mphahlele et al., 2014). Juice extraction from arils is the most common method of processing pomegranate fruit, but this generates huge waste as by-products (peel, pulp and seeds) which are rich in bioactive compounds (Opara et al 2009; Fawole et al., 2012; Siano et al., 2015) and lipids (Pande and Akoh, 2009; Fernandes et al., 2015). The peel is a major by-product and accounts for about 50% of whole fruit mass (Opara et al., 2009; Al-Said et al., 2009; Fawole et al., 2015) and is rich in polyphenols including flavonoids, phenolic acids and tannins (Opara et al., 2009; Fawole et al., 2012).

Studies on drying characteristics and kinetics of by-products of a wide range of agricultural and horticultural commodities have been reported (Kumar et al., 2012; Goula et al., 2015). However, very limited research has been reported on drying kinetics of pomegranate peel, especially the globally most important commercial cultivars such as ‘Wonderful’. Drying pomegranate peel and indeed other by-products offers opportunities for value addition into novel products, thus reducing waste from fruit processing operations. Existing information on phytochemical concentration of pomegranate fruit fractions is generally based on determining the total phenolic concentration (Fawole and Opara, 2013a, b; Mditshwa et al., 2013) with less focus on individual bioactive compounds.

The increasing desire of consumers to maintain a diet which promotes better health has spurred increased global demand and consumption of pomegranate fruit. Therefore, improved postharvest handling, processing and drying procedures to maintain quality and

maximise potential nutritive value are necessary. The overall aim of this research was to develop science-based tools for proper handling of pomegranate fruit to minimize losses and waste through improved understanding of the preharvest and postharvest factors affecting fruit quality attributes and bioactive compounds in pomegranate by-products. To achieve this aim, this dissertation was structured into the following eight papers, namely;

1. **Paper 1:** Preharvest and postharvest factors influencing bioactive compounds in pomegranate (*Punica granatum* L.) - review
2. **Paper 2:** Effects of different maturity stages and growing locations on changes in biochemical and aroma volatile composition of 'Wonderful' pomegranate juice
3. **Paper 3:** Effect of fruit maturity and growing location on the postharvest concentrations of flavonoids, phenolic acids, vitamin C and antioxidant activity of pomegranate juice (cv. Wonderful)
4. **Paper 4:** Effect of extraction method on biochemical, volatile composition and antioxidant properties of pomegranate fruit juice
5. **Paper 5:** Influence of packaging system and long term storage on pomegranate fruit. Part 1: Physiological attributes of whole fruit, biochemical quality, volatile composition and antioxidant properties of juice
6. **Paper 6:** Influence of packaging system and long term storage on pomegranate fruit. Part 2: Bioactive compounds, antibacterial, anti-tyrosinase and antioxidant properties of pomegranate by-products (peel and seed oil)
7. **Paper 7:** Effect of drying on concentration of bioactive compounds and the antioxidant, antibacterial and anti-tyrosinase activities of pomegranate fruit peel
8. **Paper 8:** Drying kinetics of pomegranate peel (cv. Wonderful)

2. General discussion

Preharvest and postharvest factors influencing bioactive compounds of pomegranate – a review

The objective of **Paper 1** was to discuss recent knowledge on preharvest and postharvest factors influencing bioactive compounds of pomegranate fruit. Pomegranates have been the most studied fruit in the last decade and is reported to contain polyphenols in different fruit parts including the peel, juice and seed/kernel (juice and seeds are contained in arils). The arils inside a fruit are commonly packed in three to four compartments commonly

referred to as arils sacs separated by membranes (Aindongo et al., 2014 a,b). The major polyphenols in pomegranate fruit are flavonoids, condensed tannins and hydrolysable tannins (Gil et al., 2000; Seeram et al., 2008). Flavonoids, including flavonols, anthocyanins and phenolic acids are mainly found in the peel and juice of pomegranate while hydrolysable tannins including gallotannins and ellagitannins are found in the peel and membrane. Like other types of fruit, pomegranate fruit quality attributes are affected by a range of preharvest and postharvest conditions. Available evidence has shown that preharvest and postharvest factors influence the bioactive compounds of pomegranate fruit. However, these findings are limited to the general screening of the total phenolic concentration and very few studies have reported the influence of preharvest and postharvest factors on the individual bioactive compounds in both arils and peel. Several reports have, however, shown that the biochemical properties of pomegranates are highly dependent on the stage of fruit development and ripening, irrigation and fertilization (Borochoy-Neori and Shomer, 2001; Khattab et al., 2010). Moreover, the concentration of bioactive compounds was found to be higher in the peel than aril which also varies significantly in fruit from different growing locations (Schwartz et al., 2009).

Pomegranate is a non-climacteric fruit and it has been demonstrated that various postharvest physiological and biochemical changes can be retarded by applying diverse postharvest treatments and hurdle technologies (Opara et al., 2016; Lee and Kader, 2000). The storage life of pomegranate fruit has been extended as result of application of postharvest treatments such as heat treatment, modified atmosphere packaging, shrink wrapping, coating and controlled atmosphere storage (Caleb et al., 2013; Opara et al., 2016). The application of these treatments has been reported to affect the nutritional quality as well as bioactive compounds of pomegranates (Artés et al., 2000; Sayyari et al., 2010).

Most postharvest studies on pomegranate fruit quality did not examine the functional properties (Villaescusa et al., 2000) while others have reported opposing results such as the effects of packaging on loss of anthocyanin (Gil et al., 1996; López-Rubira et al., 2005). The effects of different packaging systems, long-term storage, methods of juice extraction and drying on bioactive components and functional properties of pomegranate have not been adequately investigated. This information is needed to optimize postharvest handling and processing protocols to support value addition of pomegranates as a functional ingredient in food, nutraceuticals and pharmaceuticals.

Effects of different fruit maturity and growing location on changes in biochemical and aroma volatile composition of 'Wonderful' pomegranate juice

Altitude of the growing location is a factor that is rarely assessed in relation to its influence on fruit quality and phytochemicals composition as well as antioxidant activity. Moreover, the concentration of phenolic compounds and antioxidant levels vary considerably among fruit maturity stages and cultivars. Previous studies focused on the phenolic compounds of 'Bhagwa' and 'Ruby' cultivars (Fawole and Opara, 2013a,b; Mditshwa et al., 2013). The aim of this study was to investigate the effect of maturity stages and growing locations on fruit quality, phytochemical concentration and antioxidant activity of pomegranate juice (cv. Wonderful). This study is important to the beverage industry and consumers looking for fruit juice with high functional qualities. The study reported in **Paper 2** focused on the effects of different maturity stages and growing locations on changes in biochemical and aroma volatile composition of 'Wonderful' pomegranate juice. Fruit were harvested from different altitudes (low, 222 m; medium, 662 m; high, 898 m) and maturity stages (unripe, midripe and full ripe). The study revealed fruit quality and volatile composition are highly driven by maturity status and altitude of the growing location. This was confirmed by significant ($P < 0.05$) interaction between fruit maturity and different altitudes. Total soluble solids, glucose, fructose and citric acid concentration were significantly ($P < 0.01$) influenced by fruit maturity and different altitudes. At commercial harvest, fruit from low altitude (222 m) had higher TSS, glucose and fructose concentrations than fruit from medium (662 m) and high altitudes (898 m).

Volatile compositions play a key role in determining sensory quality and acceptability of pomegranate fruit and thus influence consumer preference (Kader, 2008; Belitz et al., 2009). However, pomegranate fruit is characterized by a low concentration of volatile organic compounds (VOCs) when compared to other fruit. In this study, a total of 13 volatile compounds belonging to the chemical classes of aldehydes, alcohols and monoterpenes were detected during fruit maturation, indicating lower intensities of VOCs of the pomegranate fruit juice (Carbonell-Barrachina et al., 2012). Most of the identified VOCs were below 1% during fruit maturation across all the investigated altitudes. Fawole and Opara (2013c) reported only 10 volatile compounds of pomegranate cv. Bhagwa at different maturity stage. Limonene was detected across all maturity stages and agro-climatic locations in relative abundances between 0.004 and 0.01% which revealed that is the key aroma volatiles in the

investigated cultivar. Similarly, Andreu-Sevilla et al. (2013) found that limonene was the main compound in three pomegranate juices (obtained from halved fruit), representing about 55% of the total concentration of volatiles in the headspace of cultivars ‘Wonderful’ and ‘Mollar de Elche’. In the present study, pomegranate juice was obtained from arils without crushing the seeds. The observed variation in the concentration and characterization of VOCs might be as a result of sample preparation and method of determination. Thus, there is a need for further research focused on standardization of extraction methods to permit the traceability and inter-laboratory comparability of aroma profile data. In general, fruit from lower altitude (222 m) consisted mainly of alcohols, ketones and monoterpenes at full ripe stage, whereas fruit from medium (662 m) and high (898 m) altitudes contained only two chemical families (alcohols and monoterpenes).

Effect of fruit maturity and growing location on flavonoids, phenolic acids, vitamin C and antioxidant activity of pomegranate juice

Paper 3 is a continuation of **Paper 2** and investigated the influence of fruit maturity and growing location on flavonoids, phenolic acids, vitamin C and antioxidant activity of pomegranate fruit juice. The results from this study indicated that compounds including (+)-catechin, (–)-epicatechin, naringin, gallic acid were high irrespective of maturity stages and growing locations. However, there were significant ($P < 0.05$) interaction between fruit maturity and different altitude on the individual phenolic compound. Phenolic compounds are secondary plant metabolites that protect plants from various biotic and abiotic stresses (Hernandez et al., 1999; Tomas-Barberan and Espin, 2001). Considering quantitative changes in concentrations of total phenolic compound, unripe apricot fruit have been reported to have the highest levels of bioactive compounds, which decreased at the semi-mature stage and remained relatively unchanged at commercial harvest maturity (Dragovic-uzelac et al., 2007). Bioactive compounds in pomegranate fruit may vary as a function of several factors. Likewise, the fruit developmental stage (unripe, midripe and full ripe) can also drive the synthesis and accumulation of phenolic bioactive in pomegranate fruit. From this present study, a different trend was observed in several bioactive compounds identified and there were significant effects of the altitudes (low, medium and high) investigated. The flavonoids including epicatechin, eriodictyol 7-O- β -glucoside, kaempferol-3- β -D-glucoside, rutin and hesperidin as well as phenolic acids (gallic and protocatechuic acid) increased significantly at commercial harvest (full ripe) for fruit harvested from low altitude location. The findings

suggested that the photosynthetic active radiation available at different agro-climatic locations appears to be responsible in determining the final phenolic concentration in harvested fruit. For instance, the locations had different maximum temperatures during fruit ripening which possibly could have influenced the concentration of individual flavonoids given that the rate of developmental events in fruit is dependent on temperature (Hurd and Graves, 1985). Therefore, it can be concluded that flavonoids concentration was affected by fruit maturity and growing location.

The concentrations of total phenolics and total tannins as well as radical scavenging activity (RSA) in DPPH assay declined as fruit maturity advanced. It has been highlighted that higher antioxidant activity is attributed to higher total phenolic compound present in pomegranate cultivars and other fruit such as fig (Solomon et al., 2006; Tzulker et al., 2007). However, an opposite trend was observed in this study on 'Wonderful' pomegranate, which showed that RSA of juice of full ripe fruit decreased with advancing fruit maturity while total phenolic concentration increased, in particular for fruit harvested at medium altitude (662 m). These findings show, in part, that the antioxidant activity might be as a result of phenolic constituent.

The concentration of total anthocyanins, total flavonoids and vitamin C increased significantly ($P < 0.01$) as fruit maturity advanced regardless of altitude. Increase in anthocyanin pigment during fruit maturity was also observed for 'Bhagwa', 'Mollar' and 'Ganesh' pomegranate cultivars (Kulkarni and Aradhya, 2005; Fawole and Opara, 2013a). Fruit harvested at a medium altitude (662 m) had significantly ($P < 0.01$) higher vitamin C and lower total anthocyanin concentration when compared to higher (898 m) and lower (222 m) altitudes. High light intensity has been reported to accelerate biosynthesis of vitamin C since the compound serves a potent antioxidant activity against harmful substances as in the case with fruit harvested from area with high light intensity (Lee and Kader, 2000). An opposite trend was observed for total anthocyanin concentration. These findings also suggest that high light intensity (23.14 MJ/m^2) and temperature above 30°C associated with 662 m altitude did not favour anthocyanin synthesis. Haselgrove et al. (2000) reported that enzymes involved in anthocyanin biosynthesis pathways function at an ideal temperature between 17 and 26°C , beyond which anthocyanin synthesis is inhibited. Higher levels of total anthocyanin and total flavonoid concentration were observed in fruit from lower and higher altitudes with maximum temperature between 24°C and 26°C , respectively. The findings from the present study suggest that postharvest quality of pomegranate may therefore vary from region to

region depending on geographical position of the farm. The higher vitamin C concentration in pomegranate fruit harvested at medium altitude (662 m) found in the present study corroborates the results reported recently on ‘Bhagwa’ pomegranates grown under different micro-climatic conditions in South Africa (Mditshwa et al., 2013).

Principal component analysis (PCA) showed that fruit grown in area at low altitude were associated with higher bioactive compounds at full ripe stage. Furthermore, PCA plot also revealed that fruit growing location had a significant and prominent impact on the concentration of bioactive compounds than maturity status. From this study, significant variation in phenolic concentration could be associated with the maturity status of fruit at harvest since ripening is driven principally by temperature which in turn varied significantly across the altitudes. The study provides evidence on the effects of agro-climatic locations, altitude and maturity stages on bioactive compounds accumulation in ‘Wonderful’ pomegranate fruit. These suggest that production (orchard) site location and geographical features can influence fruit quality attributes and functional properties. However, the study did not look into seasonal changes as well as multiple farms with approximately similar agro-climatic conditions, and cultural practices. Therefore, the proposed variation as a result of temperature may not be generalized for all the growing locations and further studies are warranted to validate these before generalised conclusions can be made.

Effect of extraction method on biochemical, volatile composition and antioxidant properties of pomegranate juice

Pomegranate fruit has been found to be a rich source of phenolic compounds which are reported to be mainly located in the fruit peel and mesocarp than arils (Gil et al., 2000; Fischer et al., 2011). Current methods of juice extraction are largely limited to juice processing from the whole fruit by crushing and separated arils (Miguel et al., 2004a; Muhacir-Güzel et al., 2014). In this study (**Paper 4**), pomegranate juice was extracted from different fruit fractions including arils without damaging the seeds, arils plus seeds, whole fruit and halved fruit. Among the biochemical properties investigated, the study revealed that extraction method significantly influenced juice yield and colour absorbance, pH concentration, TA and TSS:TA. Halved fruit produced considerably higher percentage juice yield (96.58%) whereas the lowest yield percentage was observed in juice extracted from the whole fruit (28.01%) as a result of incomplete disruption of arils. Colour absorbance for juice sample obtained from crushed arils and seeds were significantly higher compared to rest of

the extraction. The underlying mechanism for the increased colour saturation of juice obtained from crushing of the aril and seed is not well understood in this study, however, oil from the seed/kernel might have contributed to the degree of saturation.

Juice obtained from whole fruit had significantly the highest TA (1.78 g (CA)/ 100 mL) compared to the rest of the extraction methods. Possible high TA concentration in juice obtained from whole fruit could be as a result of fruit peel (Al-Rawahi et al., 2014). The TA concentration of 1.78 g (CA)/ 100 mL found in this study is inconsistent with those reported by (Rinaldi et al., 2013) who found lower TA concentration (0.47 g (CA)/ 100 mL) in juice from the whole fruit (cv. Wonderful) at commercial harvest. The variation in TA concentration could be attributed to fruit maturity and agro-climatic conditions, thus highlighting the importance of differences in agro-climatic locations on postharvest quality and nutritional value of pomegranate fruit.

It is worth noting that fructose and glucose concentration were the predominant sugars and citric acid was the predominant acid found in pomegranate juice irrespective of the extraction method. Similar results were also obtained in the earlier study on the effects of fruit maturity status and growing location (**Paper 2**), where fructose and glucose as well as citric acid were identified to be prominent in pomegranate juice. In the present study, juice obtained from arils plus seeds showed significantly lower fructose concentration and citric acid compared to juice obtained from whole, halved and arils. Possible dilution effect on juice from arils plus seeds could have been as a result of the inclusion of seed concentrations such as the oil.

Flavonoid compounds including catechin, epicatechin and rutin were identified in pomegranate juice whereas gallic acid was the only phenolic acid found in all pomegranate juice investigated. The number of phenolic compounds identified and quantified in the present study was less than those observed in **Paper 3** where eight phenolic compounds were identified and quantified in pomegranate fruit juice. The amount phenolic compounds observed in the present study were higher than those observed by Fawole and Opara, (2013a) with only 6 phenolic compounds identified in pomegranate fruit juice cv. Bhagwa. Fischer et al. (2011) found array of phenolic compounds in Peruvian pomegranate much higher than those observed in this study thus demonstrating that the polyphenols profile of pomegranate fruit might be more complex. In this present study, catechin was the highest amongst flavonoid compounds detected irrespective of the fruit fraction used for juice extraction. In the case of epicatechin, halved fruit had the highest concentration than the arils, arils plus

seed and whole fruit juice. High concentration of catechin and gallic acids pomegranate juice were consistent in **Paper 3** but the concentration was much higher than those observed in the present study (**Paper 4**). He et al. (2011) showed that pomegranate seed residue is a rich source of catechin compound. de-Pascual-Teresa et al. (2000) found the presence of epicatechin in pomegranate peel. These findings suggest the possibility that the extraction methods of halved fruit and arils plus seed could facilitate in the increasing of epicatechin and catechin in pomegranate juice, respectively. Likewise, the results suggest that even though the classes of phenolic compounds found were similar among the methods of extraction, specific compounds may be more abundant as a result of different fruit fractions.

The study showed that juice obtained from whole fruit and halved fruit had significantly high concentration of total phenolic compounds and antioxidant activity. It is not surprising since pomegranate peel is a rich source of phenolic compounds (Fawole et al., 2012; Opara et al., 2009) which is responsible high level of antioxidant ability. Whole fruit juice had 23.72, 21.40, and 19.22% more abundant total monomeric anthocyanin concentration than juice from arils plus seed, arils, and halved fruit, respectively. Similar findings were also observed by Türkyilmaz et al. (2013), who reported higher concentration of anthocyanins in juice extracted from the arils (pomegranate quarters, cv. Hicaznar) at pressing pressure (1.2 bars, 5 min). Therefore, juice extraction from the whole fruit could serve as a method for increasing anthocyanin concentration in pomegranate juice.

A total of 10 VOCs belonging to classes of esters, ketones, alcohols, terpenes and monoterpenes were identified in pomegranate juice (cv. Wonderful) extracted from arils without damaging the seed, arils plus seed, whole fruit and halved fruit. The chemical classes identified in this study were more than those observed in **Paper 2** (aldehydes, alcohols and monoterpenes) in which juice was only extracted from the arils without crushing seed which highlight that the juice extraction method has a significant influence on the volatile composition of pomegranate fruit juice. On the contrary, differences in fruit maturity status could have also contributed to the observed differences in the composition of volatiles (Fawole and Opara, 2013e) owing to its differences in TSS:TA concentration in the range from 15.7-17.5°Brix and 16.03- 16.33°Brix observed in **Paper 2** and in the present study, respectively. Juice obtained from arils plus seeds had the highest total relative percentage volatile organic compounds (VOCs) (70.37%) followed by whole fruit (63.76%), halved fruit (55.93%) while arils (49.52%) had the least. These findings suggest that the inclusion of pomegranate seeds and peels during juice processing increases the concentration of VOCs in

the juice. According to Koppel et al. (2014), decreased volatile concentration could be linked to less intense flavour attributes of pomegranate fruit juice. Based on these findings, it could be hypothesized that pomegranate juice obtained from arils plus seed is characterized by higher total relative percentage of VOCs which imparts higher aroma and flavour compared to juice obtained from whole fruit or other fruit fractions. Thus, there is a need for further research focused on standardization of juice extraction methods thereby to maximise juice yield and also permit the traceability and inter-laboratory comparability of aroma profile data.

Influence of packaging system and long term storage on pomegranate fruit. Part 1: Physiological attributes of whole fruit, biochemical quality, volatile composition and antioxidant properties of juice

Packaging systems such as polyliners (referred to as passive modified atmosphere packaging (MAP) influence quality preservation of produce by altering gas composition around produce, retaining moisture and reducing pathological deterioration and metabolic activities (Mir and Beaudry, 2004; Caleb et al., 2012; Mditshwa and Opara, 2013). Despite significant progress in maintaining fruit quality over the years due the application of MAP alone or in combination with a wide range of postharvest technologies, very few studies have reported their effects on the bioactive compounds, antioxidant activity and volatile composition. Continuing consumer acceptance and demand for pomegranates requires that fruit be in excellent condition and exceptionally be rich in nutritional and sensory quality and the use of packaging materials such as polyliners which modify the atmosphere around fruit and shrink-wrapping of individual fruit are expected to continue to play a crucial role. The aim of the study was to investigate the effects of different packaging systems on the physiological attributes, biochemical properties, volatile composition and antioxidant activity of pomegranate fruit during long term storage (**Paper 5**). The study showed that fruit stored under polyliner and individual shrink wrap packages significantly had less weight loss, with fruit losing not more than 1.2% of initial weights at the end of storage compared to fruit packaged in conventional open top cartons which had about 16% weight loss after 3 months cold storage ($7\pm 0.5^{\circ}\text{C}$ and $92\pm 2\%$ RH). Similar trend on the weight loss was also reported for pomegranate cvs. Hicranar (Selcuk and Erkan, 2014) and Primosole (D'Aquino et al., 2010) in modified atmosphere and shrink wrap packaging during cold storage, respectively. Fruit stored in conventional open top did not exceed 3 months due to excessive shrivelling of fruit. The levels of CO_2 and O_2 concentration achieved with MAP had a noticeable effect in

maintaining fruit visual appearance until the end of 4 month storage. Shrink wrap packaging was equally effective in maintaining the visual appearance of pomegranate fruit than conventional open top cartons (control) after 3 months of storage with control fruit terminated due to excessive shrivelling. On the contrary, fruit decay was more pronounced after 3 months regardless of the package treatment, indicating that fruit decay was highly influenced by storage duration. Laribi et al. (2012) found that decay percentage of pomegranate cv. Mollar de Elche increased with storage duration (5°C, 20 weeks) with high percentages observed on fruit packaged in modified atmosphere than control fruit. With prolonged storage, fruit biochemical attributes including citric acid, L-malic acid and glucose concentration significantly ($P < 0.01$) decreased as storage duration progressed regardless of packaging systems, which indicated high rate of metabolic activity of the fruit during cold storage, even under modified atmospheres. TSS fluctuated but generally increased during 3 months of storage in all package types. After 4 months of storage, TSS declined in polyliner and individual shrink wrapped fruit.

Although polyliner and individual shrink wrapping kept the fruit until the end of 4-month storage, total phenolic and total tannin concentration and antioxidant activity (measured by ferric reducing power) as well as gallic acid concentration were severely affected. Degradation of total phenolic concentration is related to enzymatic oxidation (polyphenol oxidase and peroxidase) during storage (Fawole and Opara, 2013d). The results of the study highlight that none of the packaged treatments slowed down the polyphenol oxidase activity during cold storage. On the contrary, total anthocyanin concentration showed a progressive increase in all treatments during storage. Similar trend was reported by several studies that the anthocyanin concentration can increase after harvest, during cold storage in pomegranate fruit (Artés et al., 2000; Miguel et al., 2004b). Flavonoid compounds including catechin, epicatechin, and rutin remained relatively stable in all treatments during storage. Although pomegranate is a rich source of bioactive compounds, the stability of flavonoids compounds observed in the present study offers some new information about their behaviour during prolonged cold storage.

With regards to volatile composition, a total of 13 volatile organic compounds (VOCs) from six chemical families were detected in the headspace of pomegranate juice, comprised of alcohols (ethanol; 1-hexanol), esters (ethyl acetate, isoamyl acetate), monoterpenes (limonene, α -terpineol, β -pinene, α -pinene, myrcene, γ -terpinene), sesquiterpenes (α -bergamontene); aldehyde (n-hexanal) and ketone (3-octanone). The

concentration (%) of alcohol (ethanol), monoterpenes (α -terpineol, myrcene, γ -terpineol) and sesquiterpenes (α -bergamontene) were detected as storage duration advanced in all treatments. The result of the study revealed that ethanol concentration (%) was detected after 2 months which averaged about 18.24% in control fruit (conventional open top cartons) whereas it was not detected in MAP and individual shrink wrapped fruit. Ethanol concentration was detected after 3 months in pomegranate fruit stored under polyliner and individual shrink wrap packaging and increased significantly after 4 months. Therefore, it could be suggested that ethanol build up in fruit stored in conventional open top cartons as early as 2 months could exhibit the onset of off-flavour. This observation in the present study confirms the principle that the flavour-life of fruit is shorter than their overall storage life as determined by external visual quality of the produce (Baldwin et al., 2007; Kader, 2008). Previous research have shown that the accumulation of ethanol and ethyl acetate compounds were primarily responsible for off-flavours in citrus fruits (Cohen et al., 1990; Shi et al., 2007; Obenland et al., 2011). Ethanol can be an enhancer of flavour if present in low amounts (Nisperos-Carriedo and Shaw, 1990). Mayouni-kirshinbaum et al. (2013) linked decreased flavour preference of MAP-stored 'Wonderful' pomegranate after 4 weeks at 7°C with increased ethanol level much above its odour threshold. The result from the present study shows that flavour life appears to be shorter than postharvest life thus sensory attributes and ethanol threshold level, biochemical properties, as well as decay incidences must be taken into consideration for assessing quality of pomegranates stored for such a period. Therefore, the study suggest that storing pomegranate fruit for up to 4 months under polyliner and individual shrink wrap package might not be ideal and should be stored for 3 months.

Influence of packaging systems and long term storage on pomegranate fruit. Part 2: Bioactive compounds and functional properties of fruit by-products (peel and seed oil)

Paper 6 is the continuation from **Paper 5**, however, only by-products were assessed in this present study. Although pharmacological properties of pomegranate peel and seed oil have been studied by different researchers (Fawole and Opara, 2012; Karaman et al., 2015) little attention has been given to the effects of prolonged cold storage under MAP, in form of plastic bags or shrink film, on the bioactive compounds and functional properties. It was therefore crucial to explore the effects of packaging systems on the bioactive compounds and functional properties of pomegranate peel as well as fatty acid composition of seed oil during long term cold storage. In this study, only phenolic concentrations of pomegranate peel were

measured. The study showed that pomegranate fruit peel stored in shrink wrap packaging had significantly higher total phenolic, tannins and flavonoid concentration compared to peel of fruit stored under polyliner. Polyphenols have been reported to be unstable during cold storage as reported by several researchers in various pomegranate cultivars (Fawole and Opara, 2013d; Selcuk and Erkan, 2014; Palma et al., 2015). Total flavonoids in peel of fruit stored in individual shrink wrap package did not significantly vary after 4 months of storage.

The flavonoids identified and quantified in pomegranate fruit peel included catechin, epicatechin, hesperidin, rutin and punicalin. Several compounds characterized by this study were in agreement with studies done by other researchers in pomegranate peel (Fischer et al., 2011; Zahin et al., 2010; Tzulker et al., 2007). To the best of our knowledge, this is the first study showing the changes in individual flavonoids in pomegranate peel over a prolonged storage period and these results would be of interest for off-season processing of pomegranate co-products and by-products into value-added ingredients in the food, nutraceutical and pharmaceutical industries. The results showed that the investigated individual flavonoid compounds of the fruit peel were best preserved by the package systems at the end of storage (4 months). The antioxidant activity of peel of the fruit measured by radical scavenging activity and ferric reducing antioxidant power remained relatively stable regardless of the package type after 4 months of storage. Possible reasons for high antioxidant activity stability might be as a result of the presence of other compounds (Mahattanatawee et al., 2006) not detected in this study.

The fatty acid composition (%) of pomegranate seed oil including saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) remained relatively stable at the end of storage regardless of package treatment. From this study, punicalic acid was identified as the prominent saturated fatty acid in pomegranate seed oil with the average of 68.09% at harvest and the concentration remained relatively stable at the end of the storage irrespective of the package type. Previously, punicalic acid was identified as major fatty acid in various pomegranate cultivars (Fadavi et al., 2006; Verardo et al., 2014; Fernandes et al., 2015). Peel extracts exhibited the best inhibitory activity against Gram negative (*Escherichia coli* and *Klebsiella pneumonia*) and Gram positive (*Staphylococcus aureus* and *Bacillus subtilis*) regardless of package type after 4 months of prolonged cold storage. However, peel of fruit stored under polyliner showed good inhibition concentration at 50% (IC₅₀) against monophenolase and diphenolase than individually shrink wrapped fruit peel after 4 months of storage.

Seed oil extracted from pomegranate exhibited significant antibacterial activity against Gram negative (*E. coli* and *K. pneumonia*) and Gram positive (*S. aureus* and *B. subtilis*) but not as efficient as peel extracts regardless of the package type. With regard to anti-tyrosinase activity, seed oil extracted from shrink wrapped fruit had better activity against diphenolase (IC₅₀, 0.49 µg/mL) than those obtained from polyliner stored fruit (IC₅₀, 3.78 µg/mL) at the end of 4 months storage. This study highlights that the pomegranate peel can be considered as valuable waste material even after long cold storage fruit owing to its high antioxidant activity.

Effect of drying on the bioactive compounds, antioxidant, antibacterial and anti-tyrosinase activities of pomegranate peel

Drying of pomegranate peel is an important processing operation as it enables the product to be stored for longer periods since the activities of microorganisms and enzymes are inhibited after drying to lower moisture levels (Alibas et al., 2001; Jayaraman and Gupta, 1992). It was therefore crucial to explore several drying methods which could be applied for the purpose of preserving phytochemical concentrations and antioxidant activity of the peel. The aim of the study was determine the effect of drying on the bioactive compounds, antioxidant, antibacterial and anti-tyrosinase activities of pomegranate peel (**Paper 7**). In this study, two types of drying method namely, freeze drying and oven drying at 40, 50 and 60°C, were investigated. The study showed that rutin and catechin were identified as major flavonoids in the dried peel. The highest concentration of rutin was found in freeze dried peel (4666.03 mg/kg DW) followed by drying at 60°C (3401.36 mg/kg DW) and 40°C (2135.00 mg/kg DW). Punicalin concentration of the peel dried at 60°C was 32.98, 25.41, 15.66% higher than oven dried at 40°C, 50°C and freeze dried peel, respectively. Higher retention of punicalin compound at 60°C may be as a result of less exposure to oxygen as the drying time was shorter (12 h).

Pomegranate peel dried using freeze dryer had high +catechin, -epicatechin, hesperidin and rutin concentrations. Likewise, freeze dried peel had significantly higher concentration of total phenolic, total tannin and flavonoid concentration better than oven dried peel at all temperature range. The results were consistent with those reported by Karaman et al. (2015) who observed the highest total phenolic in freeze dried persimmon powder because of limited thermal and chemical degradation, as it was performed at low temperatures. The results of the present study revealed that freeze-drying can be explored as a

viable method for processing pomegranate peel retaining the maximum amount of their naturally occurring bioactive compounds.

More recently, research has indicated that pomegranate peel extracts inhibit tyrosinase activity (Fawole et al., 2012), an enzyme that induces the production of melanin which leads to hyperpigmentation of the skin. This relationship was attributed to the exceptionally high amount of antioxidant capacity often linked with high phenolic composition found in the peel (Fawole et al., 2012). Previous research has been limited to the characterization of phenolic compounds of the pomegranate peel extracts and the evaluation of its biological activities. Therefore, the present study went further to assess the influence of drying on the antibacterial, anti-tyrosinase and antioxidant properties of pomegranate peel. All the drying methods were less effective against tyrosinase activity (monophenolase and diphenolase); however, they exhibited good minimum inhibitory concentration (MIC) against all the test bacteria (Gram positive (*Escherichia coli* and *Klebsiella pneumonia*) and Gram negative (*Staphylococcus aureus* and *Bacillus subtilis*) in the range of 0.10-0.39 mg/mL. In particular, peel dried at 50°C had the lowest MIC values of 0.10 mg/mL against gram positive bacteria in particular *Staphylococcus aureus* and *Bacillus subtilis* compared with the rest of the treatments. This is consistent with previous studies on antibacterial activity pomegranate peel extracts (Negi and Jayaprakasha, 2003; Opara et al., 2009; Fawole et al., 2012). These findings showed that pomegranate peels dried using freeze drier or oven showed good MIC, indicating high stability of compounds contained in the pomegranate peel. With regards to tyrosinase activity, the highest inhibition activity against monophenolase was observed in peel dried at 50°C with the IC₅₀ value of 22.95 mg/mL compared to the rest of treatments. On the contrary, better inhibitory activity against diphenolase was observed in peel extracts dried at 60°C with IC₅₀ value of 62.09 mg/mL. Reasons for the observed responses in the inhibitory activity of pomegranate peel dried at different temperatures are unclear. Therefore, further research in this area is warranted.

Drying kinetics of pomegranate peel

Pomegranate juice processing produces huge quantities of peel as by-product or waste which is highly susceptible to microbial decomposition due to high moisture concentration, thus drying could serve as an alternative method of preservation. The study was conducted to establish the drying kinetics of pomegranate peel (**Paper 8**) as a tool for future prediction of drying performance. Ten thin layer drying models were evaluated based on coefficient of

determination (r^2) and standard error (e_s). The outcome of the study showed that the moisture concentration of pomegranate peel decreased exponentially as the drying time increased resulting in 0.093, 0.094, 0.096 kg water/ kg DM for 40°C, 50°C and 60°C drying temperature, respectively. The time required to reach the final moisture concentration (%) of pomegranate peel at 40, 50 and 60°C drying temperatures were 22, 17 and 12 h, respectively, indicating that higher temperature increased drying rate. This result is consistent with previous studies in the literature on drying kinetics of various agricultural by-products (Vega-Gálvez et al., 2010; Motri et al., 2013; Kara and Doymaz, 2015).

The model proposed by Midilli et al. (2002) was identified as the best descriptive model for all the drying temperatures with the highest r^2 and the lowest e_s , and which showed that drying occurred at a falling rate. The results are similar to those found by (Doymaz, 2011, Kara and Doymaz, 2015) for pomegranate by-products (cv. Hicaznar) in the temperature range of 50 – 80°C. From the observed results, we can conclude that Midilli et al. (2002) model best explained the drying characteristics of pomegranate peels therefore, represents a good approximation for estimating the drying time of pomegranate peel (cv. Wonderful). The effective moisture diffusivity of the pomegranate peels was 4.05×10^{-10} , 5.06×10^{-10} and 8.10×10^{-10} m²/s at 40°C, 50°C and 60°C, respectively, with average activation energy of 21.98 kJ/mol. The effective moisture diffusivity observed in the study was within general range observed by several researchers such as pomegranate by-product from juice processing ($1.22 - 4.29 \times 10^{-10}$ m²/s), (Kara and Doymaz, 2015), pomegranate peel cv. Hicaznar ($4.02 - 5.31 \times 10^{-9}$ m²/s) (Doymaz, 2011), and grape seed ($1.57 - 8.03 \times 10^{-10}$ m²/s) (Roberts et al., 2008) using various air temperatures in the range of 40 – 80°C. Zogzas et al. (1996) reported that the value of activation is within the general range of 12.7-110 kJ/mol for numerous food materials. The result from this study is in agreement with those reported by agricultural crops and by-products. For instance, the activation energy was found to be 39.66 kJ/mol for pomegranate peel cv. Hicaznar (Kara and Doymaz, 2015), 25.41 kJ/mol for grape marc (Doymaz, 2009) and 52.10 kJ/mol for tomato pomace (Al-muhtaseb et al., 2010). The findings allow the successful modelling of pomegranate peel drying between 40 and 60°C. Knowledge of drying characteristics is important in the design, simulation and optimization of drying process.

General conclusions

The studies reported in this thesis provide detailed information on the quantitative changes in health-promoting compounds and functional properties of pomegranates during postharvest handling and processing. This baseline information will assist in evaluating potential value-addition of fruit fractions and by-products for possible applications in food and other bioprocess industries. In addition, the information will also assist in optimizing postharvest and processing practices to minimize loss of functional properties.

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APPENDIX 1: Paper 5

Table 1

Biochemical properties of 'Wonderful' pomegranate after 4 months storage at $7 \pm 0.5^\circ\text{C}$ under different types of package.

Treatments	TSS ($^\circ\text{Brix}$)	TA g citric acid (CA) / 100 mL)	TSS:TA	Citric acid (g/L)	L-malic acid (g/L)	Succinic acid (g/L)	Total acids	Fructose (g/L)	Glucose (g/L)	Total sugars
MAP	14.85 \pm 0.18b	0.93 \pm 0.02b	13.63 \pm 0.58a	13.31 \pm 1.39b	0.17 \pm 0.01a	0.02 \pm 0.01b	13.52 \pm 0.43b	64.66 \pm 1.27a	51.48 \pm 1.02a	116.15 \pm 2.27a
Shrink wrap	14.85 \pm 0.18b	1.12 \pm b0.01b	13.63 \pm 0.54a	13.08 \pm 1.11b	0.12 \pm 0.01a	0.03 \pm 0.01b	13.24 \pm 0.39b	57.56 \pm 0.73b	46.64 \pm 0.72b	104.20 \pm 1.43b

Each value in the table is represented as a mean \pm standard error. Unpaired t-test, $P < 0.05$. TSS, total soluble solids; TA, titratable acidity; MAP, modified atmosphere packaging.

Table 2

Changes in the quality attributes in pomegranate cv. Wonderful after 4 months storage at $7 \pm 0.5^\circ\text{C}$ under different types of package.

Treatments	Weight loss (%)	Decay incidence (%)	Visual appearance
MAP	$1.08 \pm 0.75\text{a}$	$33.34 \pm 5.88\text{a}$	$3.60 \pm 0.13\text{a}$
Shrink wrap	$1.09 \pm 0.55\text{a}$	$29.17 \pm 16.32\text{a}$	$3.50 \pm 0.07\text{a}$

Each value in the table is represented as a mean \pm standard error. Unpaired t-test, $P < 0.05$. MAP, modified atmosphere packaging.

Table 3

Changes in the phenolic concentration of pomegranate fruit cv. Wonderful after 4 months storage at $7 \pm 0.5^\circ\text{C}$ under different types of package.

Treatments	Total phenolics (mg GAE/100mL PJ)	Total tannins (mg GAE/100mL PJ)	Total anthocyanins (mg C3gE/100 mL PJ)	Total flavonoids (mg CE/100 mL PJ)
MAP	152.91±2.03a	101.05±7.64a	10.35±0.43a	33.12±0.78a
Shrink wrap	145.54±10.98a	112.63±10.04a	8.47±0.62b	34.69±0.89a

Unpaired t-test, $P < 0.05$. Each value in the table is represented as a mean. MAP, modified atmosphere packaging.

Table 4

Individual flavonoids and phenolic acids of 'Wonderful' pomegranate after 4 months storage at $7 \pm 0.5^\circ\text{C}$ under different types of package.

Treatments	Catechin (mg/L)	Epicatechin (mg/L)	Rutin (mg/L)	Gallic acid (mg/L)
MAP	1.34±0.08a	0.97±0.09a	0.24±0.02a	1.01±0.06a
Shrink wrap	1.21±0.21a	0.85±0.12b	0.26±0.07a	0.86±0.05a

Each value in the table is represented as a mean. Unpaired t-test, $P < 0.05$. MAP, modified atmosphere packaging.

Table 5

Antioxidant activity of 'Wonderful' pomegranate after 4 months storage at $7 \pm 0.5^\circ\text{C}$ under different types of package.

Treatments	RSA (μM AAE/mL PJ)	FRAP (μM TE/mL PJ)	Ascorbic acid (μg AAE/mL PJ)
MAP	2674.91 \pm 50.73b	1061.68 \pm 50.75a	147.91 \pm 9.13a
Shrink wrap	3082.38 \pm 115.47a	1122.96 \pm 147.75a	172.02 \pm 21.04a

Each value in the table is represented as a mean. Unpaired t-test, $P < 0.05$. MAP, modified atmosphere packaging. RSA, radical scavenging activity; AAE, ascorbic acid equivalent, TE, trolox equivalent; FRAP, ferric reducing antioxidant power.

APPENDIX 2: Paper 6

Table 1

Oil content (%) of pomegranate seeds after 4 months storage at $7 \pm 0.5^{\circ}\text{C}$ under different types of package.

Treatments	Oil yield (%)
MAP	$8.85 \pm 0.49\text{b}$
Shrink wrap	$10.25 \pm 0.37\text{a}$

Unpaired t-test, $P < 0.05$. Each value in the table present as a mean \pm standard error. MAP, modified atmosphere packaging.

Table 2

Effective inhibition concentration (IC_{50}) of pomegranate peel extracts after 4 months storage at $7 \pm 0.5^\circ C$ under different types of package.

Treatment	Peel extracts		Seed oil	
	IC_{50} Monophenolase ($\mu g/mL$)	IC_{50} Diphenolase ($\mu g/mL$)	IC_{50} Monophenolase ($\mu g/mL$)	IC_{50} Diphenolase ($\mu g/mL$)
MAP	29.70 \pm 2.22b	92.30 \pm 3.33b	1.26 \pm 0.09b	3.78 \pm 0.45a
Shrink wrap	158.81 \pm 4.99a	137.39 \pm 5.27a	2.84 \pm 0.35a	0.49 \pm 0.10b

Unpaired t-test, $P < 0.05$. Each value in the table present as a mean \pm standard error. MAP, modified atmosphere packaging.

Table 3

Changes in the total phenolic, total tannin and total flavonoid concentration of pomegranate peel (cv. Wonderful) after 4 months storage at $7 \pm 0.5^\circ\text{C}$ under different types of package.

Type of package	Total phenolics (g GAE/kg DM)	Total tannins (g GAE/kg DM)	Total flavonoids (g CE/kg DM)
MAP	1537.50 \pm 29.16b	1482.456 \pm 31.02b	89.24 \pm 1.68a
Shrink wrap	1743.64 \pm 68.65a	1729.167 \pm 68.27a	88.84 \pm 0.86a

Unpaired t-test, $P < 0.05$. Each value in the table present as a mean \pm standard error. MAP, modified atmosphere packaging.

Table 4

Individual phenolic concentration of pomegranate peel (cv. Wonderful) after 4 months storage at $7 \pm 0.5^\circ\text{C}$ under different types of package.

Treatments	Rutin (mg/ kg DM)	Catechin (mg/ kg DM)	Epicatechin (mg/ kg DM)	Punicalin (mg CE/ kg DM)	Hesperidin (mg/ kg DM)	Total
MAP	1191.39±492b	530.27±7.01a	55.05±2.89a	548.99±5.00b	12.64±0.63a	2338.35±476.64b
Shrink wrap	1704.31±310a	526.60±16.01a	45.93±0.07b	801.14±12.17a	11.72±0.05a	3089.71±282.51a

Unpaired t-test, $P < 0.05$. Each value in the table present as a mean± standard error. MAP, modified atmosphere packaging.

Table 5

Changes in the antioxidant activity of pomegranate peel (cv. Wonderful) after 4 months storage at $7 \pm 0.5^\circ\text{C}$ under different types of package.

Treatments	RSA (mM AAE/g DM)	FRAP (mM TE/g DM)	Vit C (mg AAE/kg DM)
MAP	60824.07 \pm 213.41a	5836.56 \pm 2.52a	456.44 \pm 14.87a
Shrink wrap	60870.37 \pm 115.74a	5796.95 \pm 6.85b	442.68 \pm 19.22a

Unpaired t-test, $P < 0.05$. Each value in the table present as a mean \pm standard error. MAP, modified atmosphere packaging.