
**PHENOLIC COMPOSITION AND *IN VITRO* ANTIOXIDANT
CAPACITY OF SOUTH AFRICAN PLUMS
(*PRUNUS SALICINA* LINDL.)**

by
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DECLARATION

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

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ABSTRACT

Phenolic compounds of the types present in plums have been found to exhibit health-promoting properties associated with their antioxidant capacity. Fruits with red peel and/or flesh are thus sought-after for their high antioxidant levels. In the current study South African plum (*Prunus salicina* Lindl.) cultivars and selections, harvested during two consecutive fruit seasons, were compared in terms of general fruit attributes (colour, firmness, °Brix, pH, titratable acidity), phenolic composition and antioxidant capacity. The effect of season and a commercial cold storage and ripening regime was also investigated.

A reversed-phase high-performance liquid chromatography-diode-array-fluorescence detection (HPLC-DAD-FLD) method suitable for use with mass spectrometry (MS) detection, was optimised for separation and identification of phenolic compounds from four phenolic groups (phenolic acids, anthocyanins, flavan-3-ols and flavonols) in six South African plum cultivars and five selections. Parameters that were optimised include the mobile phases, analysis temperature and gradient program. Good stability, linearity and inter- and intra-day precision were obtained. Identification of compounds was based on comparison of retention times, UV-Vis spectra and mass fragments with available authentic phenolic standards and/or literature data. The optimised method allowed identification or tentative identification of twenty-four phenolic compounds, including cyanidin-3-*O*-glycosides, quercetin glycosides, monomeric, dimeric and trimeric flavan-3-ols, and hydroxycinnamic acids. An on-line ABTS^{•+} (2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)) antioxidant assay, performed for qualitative evaluation of the antioxidant response of individual phenolic compounds, indicated the flavan-3-ols as major antioxidants in plums.

Eighteen phenolic compounds were quantified, including anthocyanins and flavonol glycosides, flavan-3-ols (monomers and dimers) and hydroxycinnamic acids. Phenolic composition differed greatly between cultivars and selections. Cyanidin-3-*O*-glucoside was the predominant anthocyanin in plums with red peel and/or flesh, followed by cyanidin-3-*O*-rutinoside. Cyanidin-3-*O*-galactoside was present only in the cultivar Laetitia (red peel, yellow flesh). The ripe fruit of Ruby Red and PR04-19, both with red peel and flesh, had the highest anthocyanin content for the first and second harvest season, respectively. Neochlorogenic acid and quercetin-3-*O*-glucoside were the major phenolic acid and flavonol, respectively. Chlorogenic acid, 3-*O*-*p*-coumaroylquinic acid and several quercetin-glycosides and -diglycosides were also present in some cultivars and selections. Procyanidin B1 was the flavan-3-ol present in the highest concentration in the majority

of cultivars and selections and its content correlated with the (+)-catechin content, while the same was observed for procyanidin B2 and (-)-epicatechin.

The effect of cold storage and ripening on fruit attributes differed greatly between cultivars and selections. The increase and decrease in pH and titratable acidity, respectively, were as expected for ripe fruit as opposed to unripe fruit. Ripe fruit had higher a^* -values and lower L^* -values. The cold storage and ripening regime had no significant effect on total polyphenol and total flavan-3-ol content of the cultivars and selections, but the anthocyanin content increased in some cases.

In terms of *in vitro* antioxidant capacity, the selections PR04-32 and PR04-35, both with red peel and flesh, had the highest antioxidant capacity, irrespective of assay. Sapphire (red peel, yellow flesh), with the lowest total polyphenol content, also had the lowest antioxidant capacity in the ORAC and FRAP assays. Both the total polyphenol and flavan-3-ol contents correlated significantly to antioxidant capacity, irrespective of assay.

UITTREKSEL

Fenoliese verbindings, soos teenwoordig in pruime, is bekend vir hul gesondheidsbevorderende eienskappe wat geassosieer word met antioksidant kapasiteit. Vrugte met 'n rooi skil en/of vleis is veral gesog as gevolg van hul hoë antioksidant aktiwiteit. Met hierdie studie is Suid-Afrikaanse pruim (*Prunus salicina* Lindl.) kultivars en seleksies, geoes tydens twee opeenvolgende seisoene, vergelyk in terme van algemene vrug eienskappe (kleur, fermheid, °Brix, pH en titreerbare suurheid), fenoliese samestelling en antioksidant kapasiteit. Die effek van 'n kommersiële koelopberging en rypwording prosedure is ook ondersoek.

'n Omgekeerde-fase hoë-druk vloeistof chromatografie (HPLC) metode met diode-opstelling en fluoressensie deteksie, maar wat ook geskik is vir massa spektrometrie (MS), is geoptimeer om fenoliese verbindings te skei en te identifiseer in Suid-Afrikaanse pruime. Verbindings van vier fenoliese groepe (fenoliese sure, antosianiene, flavan-3-ole en flavonole) wat in ses kultivars en vyf seleksies voorgekom het, is ondersoek. Die vloeistof fases, skeidingstemperatuur en gradiënt van die metode is geoptimeer. Goeie resultate vir stabiliteit, lineariteit en inter- en intra-dag akkuraatheid is verkry. Verbindings is geïdentifiseer deur vergelyking van retensie tye, UV-Vis spektra en massa fragmente met dié van egte fenoliese standaarde en/of met literatuur data. Vier-en-twintig fenoliese verbindings is geïdentifiseer of voorlopig geïdentifiseer, insluitende sianidien- en kwersetien glikosiede, flavan-3-ol monomere, dimere en trimere, en hidrosikaneelsure. 'n Aanlyn ABTS^{•+} (2,2'-azino-di-(3-etielbensotialosien-sulfoon suur) radikaal kation blussingstoets is gebruik om die antioksidant reaksie van individuele polifenole op 'n kwalitatiewe wyse te evalueer en flavan-3-ole is as hoof antioksidante in pruime aangetoon. Kwantifisering van agtien verbindings, insluitende antosianiene, flavonol glikosiede, flavan-3-ole (monomere en dimere) en hidrosikaneelsure, was moontlik met hierdie geoptimeerde metode. Die fenoliese samestelling het aansienlik verskil tussen kultivars en seleksies. Sianidien-3-O-glukosied was die hoof antosianiene in pruime met 'n rooi skil en/of vleis, gevolg deur sianidien-3-O-rutinosied. Sianidien-3-O-galaktosied het slegs in Laetitia (rooi skil en geel vleis) voorgekom. Ryp vrugte van Ruby Red en PR04-19, beide met rooi skil en vleis, het onderskeidelik die hoogste antosianieneinhoud gehad met die eerste en tweede seisoen se oeste. Neochlorogeniese suur en kwersetien-3-O-glukosied was die hoof fenoliese suur en flavon-3-ol, onderskeidelik. Chlorogeniese suur, 3-O-p-kumarienkwiniesuur en verskeie kwersetien glikosiede en diglikosiede was teenwoordig in sekere kultivars/seleksies. Die flavan-3-ol, prosianidien B1, was

teenwoordig in die hoogste konsentrasie in die meerderheid kultivars/seleksies. Die prosianidien B1 inhoud het met die (+)-katekien inhoud gekorreleer, terwyl dieselfde gevind is vir prosianidien B2 en (-)-epikatekien.

Die effek van koelopberging en rypwording op die algemene vrug einskappe het tussen kultivars en seleksies verskil. Die pH en titreerbare suurheid het onderskeidelik toegeneem en afgeneem, soos verwag is vir ryp vrugte teenoor onryp vrugte. Ryp vrugte het hoër a^* -waardes en laer L^* -waardes getoon. Koelopberging en rypwording het geen beduidende effek op die totale polifenol- en totale flavan-3-ol inhoud gehad nie, maar die antosianieninhoud het vir sommige kultivars en seleksies toegeneem.

In terme van *in vitro* antioksidant kapasiteit het die seleksies PR04-32 en PR04-35, beide met rooi skil en vleis, die hoogste antioksidant kapasiteit getoon, ongeag die antioksidant toets wat gebruik is. Sapphire (rooi skil en geel vleis) het die laagste totale polifenolinhoud gehad, asook die laagste antioksidant kapasiteit soos bepaal deur die ORAC en FRAP toetse. Beide die totale polifenol- en flavan-3-ol inhoud het beduidend met die antioksidant kapasiteit korreleer, ongeag van die toets wat gebruik is.

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CHAPTER 1

INTRODUCTION

Plums are stone fruit and are classified as climacteric fruit, indicating that the ripening process is continued after the fruit have been harvested (Burg & Burg, 1965). Of all stone fruit, plums are the fruit produced in highest quantities, with approximately 49 million kg produced in South Africa during the 2010/2011 season (Ntombela, 2011). The country producing the largest quantity of plums per annum is China (Anon., 2011a), and during 2008 China produced approximately 5 223 001 metric tons of plums, while South Africa produced ca 62 043 metric tons (Anon., 2012). In South Africa the Western Cape is the main producer of plums in South Africa (Anon., 2011c) and the cultivar Laetitia (red skin and yellow flesh) is produced in the largest quantities compared to other cultivars (Anon., 2011b). Plums are also produced in various regions of the world, such as California, Spain, etc. and the two main species are known as Japanese plums (*Prunus salicina* Lindl.) and European plums (*Prunus domestica* Lindl.). European plums are the species generally cultivated specifically for drying of the fruit (Okie & Ramming, 1999).

Fruit and vegetables generally have a high phenolic content, with the composition varying between different foods (Imeh & Khokhar, 2002). Phenolic compounds are secondary metabolites abundant in fruit and vegetables and have various functional attributes (Kim & Lee, 2005). The major phenolic compounds present in plums are flavonoids and hydroxycinnamic acids (Chun *et al.*, 2003; Kim *et al.*, 2003). In addition, anthocyanins are responsible for the red colour of fruit peel and flesh (Macheix *et al.*, 1990a).

The interest in phenolic compounds has increased over recent years due to their potential health benefits (Gil *et al.*, 2002; Byrne *et al.*, 2009). Some reported beneficial functions include anticarcinogenic activity and a reduction in the risk of heart disease (Frankel *et al.*, 1993; Cai *et al.*, 2004; Noratto *et al.*, 2009). One of the functions that is of great interest is that of antioxidant, as they may play an important role in reducing the risk of certain pathological conditions. Antioxidants neutralize free radicals and other reactive species that cause degenerative reactions in the body associated with the above-mentioned medical conditions (Kim *et al.*, 2003; Cevallos-Casals *et al.*, 2006). More information is emerging regarding the *in vitro* antioxidant capacity of phenolic compounds and their bioavailability properties. In order to obtain accurate information regarding the possible health benefits of food products it is necessary to identify and quantify the applicable compounds. The preferred method of analysis for phenolic compounds in fruit is high-performance liquid chromatography (HPLC) to separate applicable phenolic compounds and allow subsequent identification and quantification (Tsao & Yang, 2003).

Phenolic compound research may directly benefit the food industry and could also be applied in the compilation of polyphenol databases. Several of these databases have already been

established and the amount of information is continually increasing. Some examples include Phenol-Explorer, the Brazilian flavonoid database, the USDA phenol database and the EuroFIR-BASIS database (Gry *et al.*, 2007; Neveu *et al.*, 2010; De Menezes *et al.*, 2011). In Phenol-Explorer there is currently only data for *Prunus domestica*. Phenolic compound information could also be used for the determination of dietary intake levels of certain food products. In addition, Carlsen *et al.* (2010) compiled *The Antioxidant Food Table* in which the antioxidant content of more than 3 000 foods is listed. However, only plums produced in Norway and the USA were included.

As plums are considered to have a high phenolic content (Gil *et al.*, 2002), it would be useful to fully evaluate the phenolic composition, quantify individual compounds and assess their contribution to the antioxidant capacity of plums. The information may interest consumers as well as allow the utilization of the functional phenolic properties found in plums. Phenolic compounds are of interest for the fruit industry as the effective extraction thereof may allow its use in the manufacturing of various food products (as colorant or as a functional ingredient). The possibility of applications in nutraceutical products also exist (Macheix *et al.*, 1990b).

At present there is limited published research regarding Japanese plums produced in South Africa and their general fruit attributes, phenolic composition and antioxidant activity. At present there is only one publication in which the phenolic composition of South African plums were analysed and only one cultivar was evaluated (De Beer *et al.*, 2012). Thus far the published research on plums has primarily been done on Japanese and European plums grown in Europe and America (Chun *et al.*, 2003; Rupasinghe *et al.*, 2006; Slimestad *et al.*, 2009). Currently the Cultivar Development division of the Agricultural Research Council (Stellenbosch) is breeding new South African plum cultivars (*P. salicina*) with red peel and flesh. In terms of South African plums there is a need for an improved HPLC method for identification and quantification of major and minor phenolic compounds in plums. It is also necessary that this method be suitable for the analysis of different South African plum cultivars and selections. In addition, it is not known which of the individual phenolic compounds in plums exhibit a high antioxidant activity. Cold storage and/or ripening procedures may also affect the phenolic composition and antioxidant activity.

In South Africa the phenolic composition, antioxidant activities and the possible effect of cold storage on these attributes in plums have not been adequately addressed. In this study the results on general fruit attributes, phenolic composition and antioxidant activity will be compared between the different cultivars and selections. This will give an indication about the quality and characteristics of new plum selections, compared to cultivars currently on the market. It is believed that data gathered by the project should be of value to other scientists in the food

industry, and provide valuable information regarding South African plums. One potential application would be in the evaluation of new plum selections for enhanced health benefit. The knowledge of phenolic compounds can also be used as markers in future breeding programmes (Wu & Prior, 2005).

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CHAPTER 2

LITERATURE REVIEW

BACKGROUND INFORMATION

Fruits and vegetables play an important part in our daily diets, and the concept of a healthier and more “natural” lifestyle has become a consumer trend (Anon., 2009). It has been reported that fruit and vegetables assist in reducing the occurrence of certain diseases, including cancer, diabetes and heart disease (Utsunomiya *et al.*, 2005; Belkaid *et al.*, 2006; Noratto *et al.*, 2009). The protective function of these foods is partly attributed to the presence of phenolic compounds (Crozier *et al.*, 2009). Phenolic compounds are important constituents of fruits and vegetables and contribute to the character (sensory and nutritional) of the food by influencing their colour, taste, astringency and health properties (Kim & Lee, 2005). Many of these compounds exhibit high *in vitro* antioxidant activity, which has been linked to several potential health benefits (Ignat *et al.*, 2011). Fruits are generally considered a good source of phenolic compounds, with the total phenol content varying between different fruit types. Plums have a fairly high phenolic content in comparison to some other fruits such as apple, grapes and kiwifruit (Table 1) (Imeh & Khokhar, 2002; Chun *et al.*, 2005; Fu *et al.*, 2011).

Table 1 Total phenol content for a range of fruits (Chun *et al.*, 2005)

Fruits	Total phenolic content (mg GAE.100 g ⁻¹ FW)
Apple	118.3
Grape	83.6
Orange	11.8
Banana	112.8
Plum	368.7
Cherries	55.8
Strawberries	225.0

*GAE = gallic acid equivalents; FW = fresh weight.

In South Africa plums are generally harvested between December and March. In addition to fresh consumption, plums are used in various processed products such as jam and juice, or sold as dried fruit (prunes) (Mazza & Miniati, 1993a). Japanese plums (*Prunus salicina* L.) are normally consumed fresh, while European plums (*Prunus domestica* L.) are often used for the production of prunes (Okie & Ramming, 1999).

In a study performed by Hurtado-Fernandez *et al.* (2010) the authors shared their view on the importance of gathering information on phenolic compounds and the combination of polyphenols in different fruit. If extracts from plant material are to be used in further applications (e.g. in processed food products or nutraceuticals) it is essential that the properties of the extract be understood. Knowledge regarding phenolic composition is important since the minor components could possibly have as large an effect as the major components. Therefore appropriate methods of analysis are necessary for acquiring sample information. South African plum cultivars have been used previously in research studies, but only limited published research regarding the phenolic composition of plums harvested in South Africa is available (Steyn, 2010; De Beer *et al.*, 2012).

Information from published studies on phenolic compounds, their antioxidant activity, health benefits, and methods of analysis will be discussed in this literature review. The bioavailability of antioxidants, which is also an important factor to consider when evaluating the health benefits of phenolic compounds, will be discussed briefly. As phenolic compounds could also prove valuable for application in the food and pharmaceutical industries their potential industrial applications will be considered. These topics are reviewed since they are directly applicable to this study, in which the antioxidant activity and phenolic compounds were compared between plum cultivars and an HPLC method was developed and used to identify and quantify phenolic compounds. The results will be compared to literature and this research chapter will serve as background to following research chapters.

PHENOLIC COMPOUNDS

Phenolic compounds are secondary metabolites that occur ubiquitously in plants and food products of plant origin. Polyphenols are aromatic compounds and can be divided into several groups according to their basic structure, which consists of an aromatic ring with one or more hydroxyl groups and/or sugar moieties (Kim *et al.*, 2004). These compounds normally originate from reactions via the shikimate pathway or phenylpropanoid metabolism of plants (Ryan *et al.*, 1999). Phenolic compounds can be found substituted with various functional groups and these, along with the number of constitutive carbon atoms, allow classification of phenolic compounds into several groups (Hollman *et al.*, 1996; Ryan *et al.*, 1999). Some of these groups are the tannins, flavonoids, stilbenes and phenolic acids (Shahidi & Naczk, 2004a) (Fig. 1).

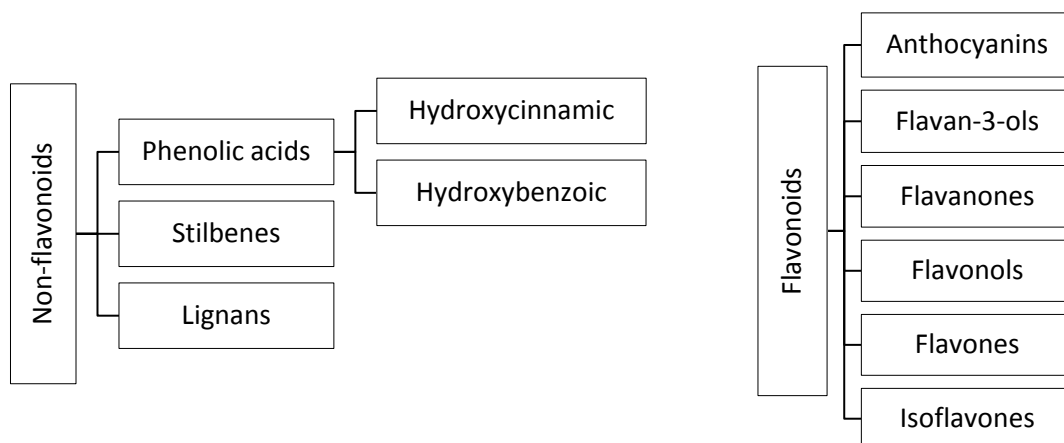


Figure 1 Schematic representation of the main phenolic groups and sub-groups.

Research regarding phenolic compounds has received widespread attention over the last decade, as evidence suggests these compounds exhibit certain health benefits, including antioxidant and anticarcinogenic activity. Many review and research papers report on the fact that phenolic compounds may also reduce the risk of cardiovascular disease, cancer, stroke, and diabetes (López-Vélez *et al.*, 2003; Cai *et al.*, 2004; Moyers & Kumar, 2004; Yao *et al.*, 2004; Del Rio *et al.*, 2010).

In addition to phenolic antioxidant activity, certain health benefits are portrayed through the role of phenolic compounds in cell signalling pathways (Rahman *et al.*, 2006). Research has shown that polyphenols may control cellular signalling through different mechanisms and consequently prevent the development of several diseases, especially those associated with or initiated by inflammation. Mechanisms for the modulation of signalling pathways have been reviewed (Williams *et al.*, 2004; Fresco *et al.*, 2006; Rahman *et al.*, 2006) and the effect of factors such as mitogen activated protein kinase (MAPK), nuclear factor-kappaB (NF-κB) and nuclear redox factor (Nrf2) have been investigated. The activation of mediators in inflammatory pathways, such as NF-κB and Nrf2, are often as a result of oxidative stress or the action of reactive oxygen species. The role of phenolic compounds in controlling the activation of NF-κB and other transcription factors may thus prevent inflammation (Fresco *et al.*, 2006).

Phenolic groups that have been reported to be common in plums include phenolic acids and three subclasses of the flavonoids, namely anthocyanins, flavonols and flavan-3-ols (Tomás-Barberán *et al.*, 2001; Fang *et al.*, 2002; Kim *et al.*, 2003). Some characteristic aspects regarding these groups are discussed.

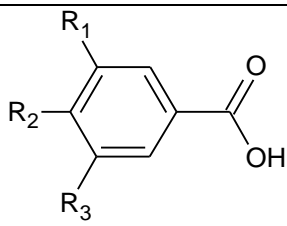
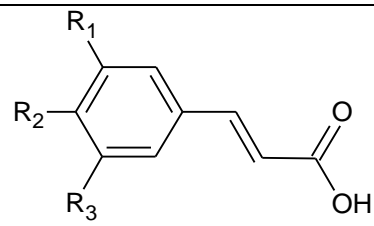
Non-flavonoids

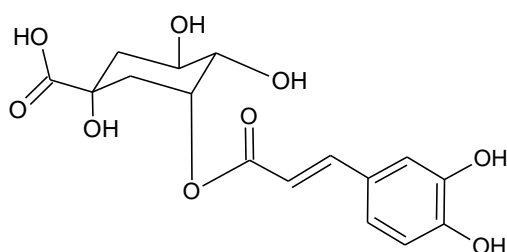
Phenolic acids

Phenolic acids can be divided into two subgroups: hydroxycinnamic acids and hydroxybenzoic acids (Ignat *et al.*, 2011). Examples of hydroxybenzoic acids are gallic acid, vanillic acid and *p*-hydroxybenzoic acid. The basic structure of a hydroxybenzoic acid is a C6-C1 (Table 2) carbon skeleton, and they are found in plant material as esters, glycosides or in their free form (Pérez-Jiménez *et al.*, 2010).

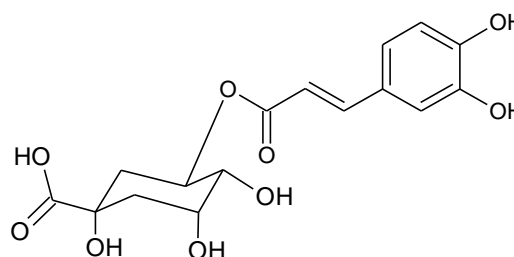
Hydroxycinnamic acids are often present in a bound state and are the predominant phenolic group in many foods, including apples, blueberries and plums (as reviewed by Lule & Xia, 2005). In addition, they generally form part of compounds with a complex structure, such as lignin. Hydroxycinnamic acids differ from hydroxybenzoic acids by having a C6-C3 basic structure. These phenolic compounds are abundant in fruit, and their concentration depends on the degree of ripeness and is affected by geographical and seasonal factors (D'Archivio *et al.*, 2007). They are also found in high concentrations in coffee (Pérez-Jiménez *et al.*, 2010). According to several studies (Chun *et al.*, 2003; Kim *et al.*, 2003; Matilla *et al.*, 2006) chlorogenic acid isomers were found to be the major polyphenols in several plum cultivars, with neochlorogenic acid (5-*O*-caffeoylquinic acid) generally present in higher concentrations than chlorogenic acid (3-*O*-caffeoylquinic acid) (Fig. 2). Both are formed by esterification of caffeic acid with quinic acid (Olthof *et al.*, 2001).

Table 2 Basic structures of hydroxybenzoic and hydroxycinnamic acids with structural variations for common phenolic acids

Hydroxybenzoic acid				Hydroxycinnamic acid			
							
Name of acid	R ₁	R ₂	R ₃	Name of acid	R ₁	R ₂	R ₃
Vanillic	OCH ₃	OH	H	Caffeic	OH	OH	H
Gallic	OH	OH	OH	Ferulic	OCH ₃	OH	H
<i>p</i> -Hydroxybenzoic	H	OH	H	<i>p</i> -Coumaric	H	OH	H



Neochlorogenic acid
(5-*O*-Caffeoylquinic acid)



Chlorogenic acid
(3-*O*-Caffeoylquinic acid)

Figure 2 Structures of the two most common hydroxycinnamic acids found in plums.

Flavonoids

Compounds in the flavonoid group have a diphenylpropane (C₆-C₃-C₆) basic structure (Shahidi & Naczki, 2004a). This structure contains a number of hydroxyl groups attached at various positions. Methylation and glycosylation are also common for flavonoids (Hollman *et al.*, 1996). Flavonoids can be classified into several subgroups including anthocyanins, isoflavones, flavan-3-ols, flavonols, flavones, and flavanones (D'Archivio *et al.*, 2007). Their classification is based on the oxidation state of the central pyran ring and on the substitution patterns (Shahidi & Naczki, 2004a). Three of these groups are discussed in this section as they are commonly found in plums (Chun *et al.*, 2003).

Anthocyanins

The anthocyanins are one of the most well-known phenolic groups and are primarily responsible for the colour of fruit and vegetables with a significant colour in the red to purple/blue range (Andersen & Jordheim, 2006). Anthocyanins are mainly found in nature as glycosides of their respective anthocyanidin aglycones. There are six basic anthocyanidins found ubiquitously in nature, namely cyanidin, peonidin, pelargonidin, petunidin, malvidin and delphinidin (Table 3) (Andersen & Jordheim, 2006). Some common sugars can be bound to the anthocyanidins include glucose, rhamnose and galactose (Mazza & Miniati, 1993b). Generally the sugar moiety of the anthocyanin compound is bound at the 3-position on the anthocyanidin C-ring (Fig. 3). Other possibilities are attachment to the 5- or 7-positions on the A ring, or the 3'- or 5'- positions on the B ring (Mazza & Miniati, 1993b). At a low pH anthocyanins also have a positive charge on the central ring of its structure (flavylium cation form) (Jaganath & Crozier, 2010).

Table 3 Substitution patterns of different anthocyanidin aglycones (with reference to Fig. 3)

Compound	Position					
	3	5	7	3'	4'	5'
Cyanidin	OH	OH	OH	OH	OH	H
Peonidin	OH	OH	OH	OCH ₃	OH	H
Pelargonidin	OH	OH	OH	H	OH	H
Petunidin	OH	OH	OH	OCH ₃	OH	OH
Malvidin	OH	OH	OH	OCH ₃	OH	OCH ₃
Delphinidin	H	OH	OH	OH	H	OH

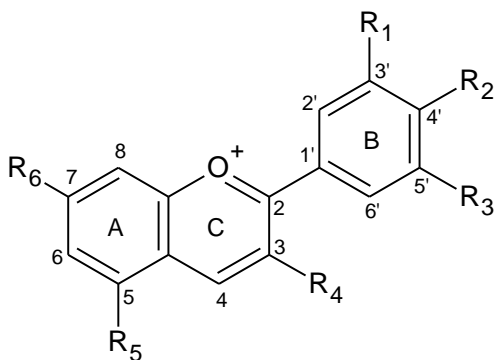


Figure 3 General structure of the anthocyanin flavylium cation.

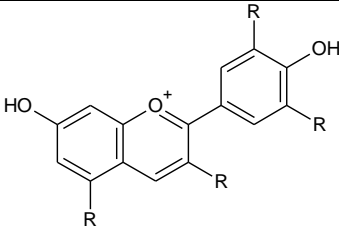
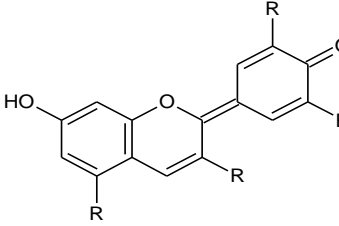
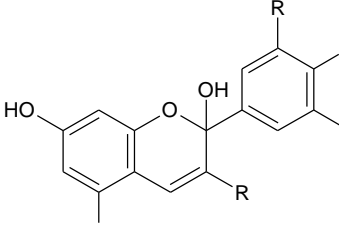
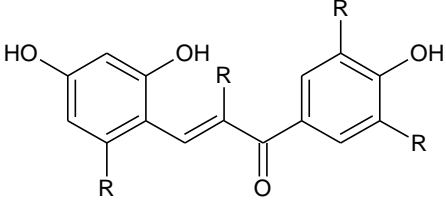
Anthocyanins are very sensitive to changes in factors such as pH, temperature, light, oxygen, and interferences from other compounds (Mazza & Miniati, 1993a; Rein, 2005). A significant change in the mentioned factors will influence the colour displayed (Brouillard, 1983). For example, a change in pH will cause alteration in the structure of the compound resulting in a colour change (Stintzing *et al.*, 2002). At a pH below two, the anthocyanins are in the flavylium cation state (Fig. 3) and display a red colour (Mazza & Miniati, 1993b). Table 4 lists the colour hues exhibited by the anthocyanin compounds at different pH values. From Fig. 4 it is clear that the flavylium form predominates at a pH below 2. As the pH increases all four forms are present in equilibrium and because of the slightly different retention times for these forms this causes very broad peaks on HPLC chromatograms. The different structural forms are in equilibrium at pH values between pH 4 and 6. The chalcone may also display a pale yellow colour, and the quinoidal base is known to be anhydrous (Cooper-Driver, 2001). Deprotonation of the quinoidal base takes place between pH 6 and 7, causing a subsequent change in colour in the purple-blue range. Mechanisms for the transformation of compounds during equilibrium that result in colour change have been described (Brouillard & Dubois, 1977; Cheminat & Brouillard, 1986; Fossen *et al.*, 1998).

Copigmentation may also occur between anthocyanins and other compounds, affecting the colour intensity displayed (Asen *et al.*, 1972; Boulton, 2001). Certain plant species (especially flowers) may exhibit a characteristic colour at pH values where anthocyanins are normally colourless. This phenomenon is caused by the copigmentation of anthocyanins with colourless phenolic compounds, such as flavonols and phenolic acids, or stabilisation due to complexation with metal (Davies & Mazza, 1993; Mazza & Miniati, 1993b).

The glycosidic forms of anthocyanidins such as cyanidin and petunidin have been known to form stable complexes with metals. Anthocyanins may also form association with themselves in certain aqueous solutions. Other factors that also may indirectly influence the colour include: individual anthocyanins present in the sample; influence from chlorophyll or carotenoids; and the influence of macromolecules (Asen *et al.*, 1972).

Fig. 5 displays some common anthocyanins reported to be present in plum cultivars with red peel or flesh. Wu and Prior (2005) found that cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-glucoside and cyanidin-3-*O*-galactoside are anthocyanins abundant in plums. Various others, including peonidin-3-*O*-glucoside, have also been reported. Tomás-Barberán *et al.* (2001) found that the anthocyanin content for a variety of plum samples was predominantly concentrated in the peel of the fruit. The phenolic composition will differ between cultivars.

Table 4 Colour changes caused by pH adjustment (Mazza & Miniati, 1993b)

pH values	Colour displayed	Structural form	Structure
pH 1	Red	Flavylium cation	
pH 2-4	Blue	Quinoidal base	
pH 5	Colourless	Carbinol pseudobase	
pH 6	Colourless/ pale yellow	Chalcone	

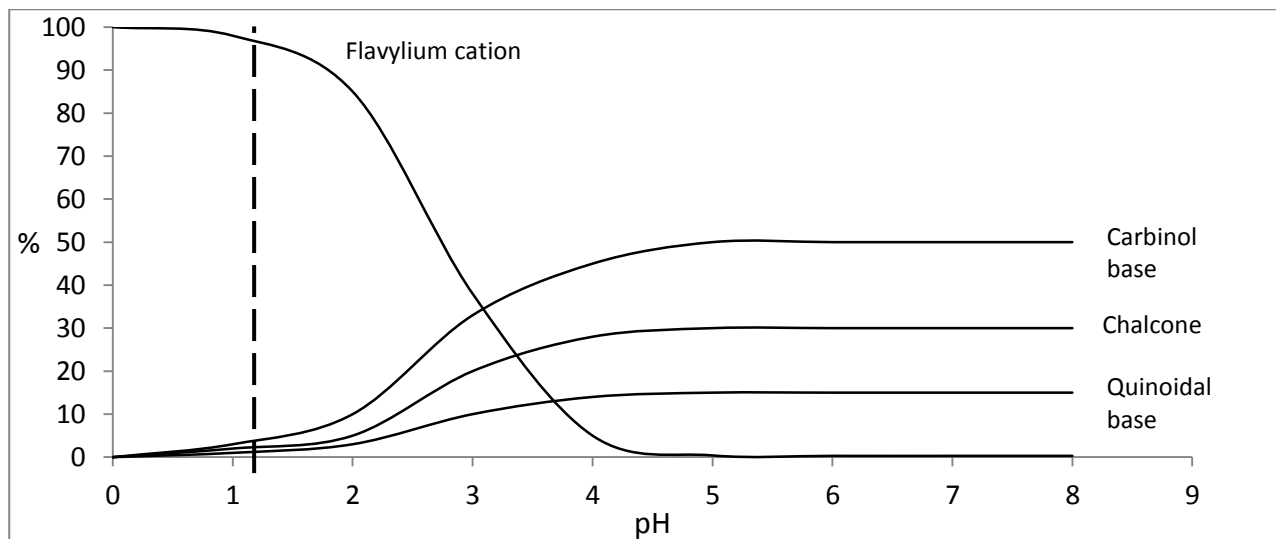


Figure 4 Graphical representation of anthocyanin equilibrium (Glories, 1984).

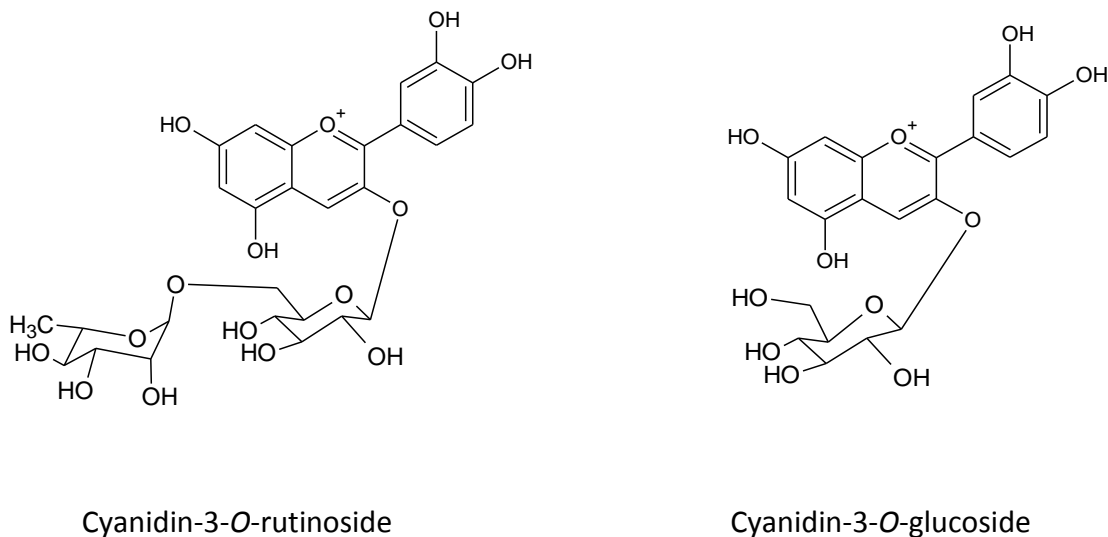


Figure 5 Molecular structures of anthocyanins reported in plums.

Flavan-3-ols

Typical examples of flavan-3-ols found in food are catechin, epicatechin and the proanthocyanidins (Fig. 6). Polymeric proanthocyanidins are also known as condensed tannins and is the term used to describe flavan-3-ols that are polymerized to form oligomers (3-10 subunits) and polymers (more than 10 subunits) (Souquet *et al.*, 1996; Santos-Buelga & Scalbert, 2000). These compounds are colourless, but exposure to an acidic medium at high temperature will cause the formation of the corresponding anthocyanidin (Santos-Buelga & Scalbert, 2000).

Flavan-3-ols possess chiral centres at the 2- and 3-positions on the C-ring (Fig. 6). Catechin monomers can be converted to gallo catechins through hydroxylation, or to procyanidins by esterification (dimers). Flavan-3-ols have a hydroxyl group in the third carbon position on the centre ring (C) and are the only flavonoid subgroup that does not normally appear in food products as a glycosylated compound (Arts *et al.*, 2000a). It is reported that the astringent character of certain fruit is caused by proanthocyanidins (Santos-Buelga & Scalbert, 2000). Plums are rich in flavan-3-ols (García-Alonso *et al.*, 2004) and studies have established (+)-catechin as the main flavan-3-ol in plums (Tomás-Barberán *et al.*, 2001). Procyanidin dimers B1, B2, B3, B4, B5 and B7 have been identified in plums (De Pascual-Teresa *et al.*, 2000). Other flavan-3-ols include (-)-epigallocatechin (EGC) and (-)-epicatechin gallate (ECg) as found in tea, and prodelpinidins which are present in pomegranates (Arts *et al.*, 2000b; Plumb *et al.*, 2002).

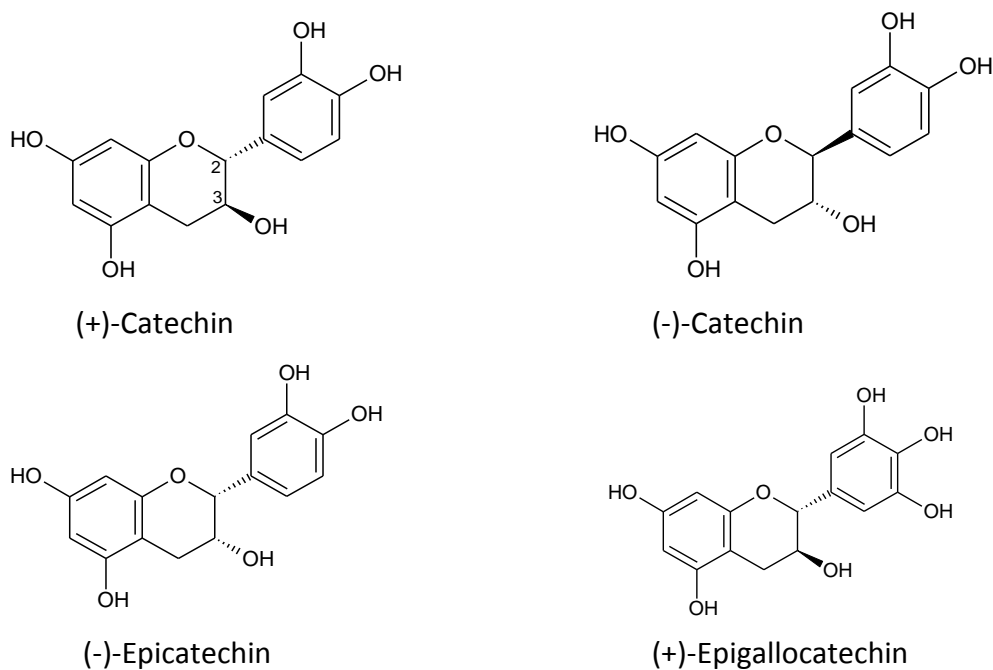
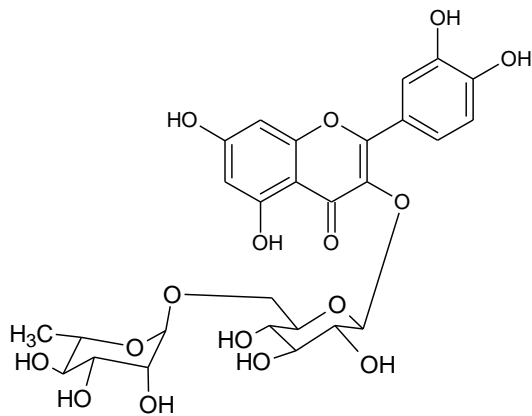


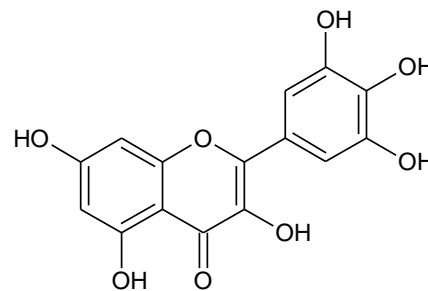
Figure 6 Chemical structures of flavan-3-ols.

Flavonols

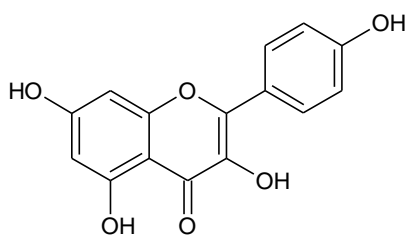
The flavonol most often found in plant material is a compound known as quercetin, with kaempferol and myricetin also present in this group (Hertog *et al.*, 1992) (Fig. 7). Flavonols are generally present in their glycosidic form, with flavonol-glucosides and -glucuronides being the most common (Mullen *et al.*, 2004). The synthesis of quercetin in biological systems is promoted by exposure to light, and thus flavonols are mainly found in the peel or leaves of plants (Awad *et al.*, 2000). From studies where plums were analysed, quercetin-3-*O*-rutinoside (Fig. 7) was often determined as the primary flavonol (Kim *et al.*, 2003). Various quercetin glycosides may be present in plums.



Quercetin-3-O-rutinoside (rutin)



Kaempferol



Myricetin

Figure 7 Chemical structures of flavonol compounds.

ANTIOXIDANTS: FUNCTION AND ACTIVITY

Free radicals and antioxidants in the body

Free radicals are compounds that have one or more unpaired electrons in their outer orbital and are capable of existing independently (Gutteridge, 1995). Reactive species (which include free radicals) are found in biological systems portraying physiological roles *in vivo*. However, in excess they may be harmful to the living system (Guo *et al.*, 2003).

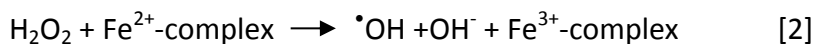
Antioxidants are believed to provide control over the levels of reactive species in a system and when an imbalance between the antioxidant levels and the production of free radicals exist, it is known as oxidative stress (Halliwell & Gutteridge, 2007). Oxidative stress is associated with the development of degenerative diseases. Halliwell and Gutteridge (2007) define an antioxidant as “any substance that, when present at low concentrations compared to those of an oxidizable

substrate, significantly delays or inhibits oxidation of that substrate". In addition, an antioxidant must produce a stable radical compound after scavenging (Halliwell, 1990; Shahidi & Wanasundara, 1992). In general the concentration of phenolic compounds present, as well as characteristic structural properties, may influence the *in vitro* antioxidant activity displayed (Stintzing *et al.*, 2002).

The mechanism by which an antioxidant reacts is influenced by the structure of the compound. Most antioxidants have a multifunctional nature, meaning that antioxidants may react through more than one type of mechanism in a particular food system (Prior *et al.*, 2005; Rice-Evans *et al.*, 1996; Singh & Singh, 2008). The different mechanisms thus require that antioxidant activity be evaluated by several assays that are based on different reaction principles (Prior *et al.*, 2005). Phenolic compounds may display antioxidant activity by acting as reducing agents, singlet oxygen quenchers and hydrogen donors (Gutteridge, 1995; Rice-Evans *et al.*, 1995). The ability to act as a metal chelator is a secondary mechanism of action. Metal chelation is especially important as it may reduce oxygen toxicity (Khokhar & Apenten, 2003). When iron is involved in metal chelation, the iron ions are extracted by the chelating agents and reactions that may lead to the formation of radical compounds are prevented (e.g. Fenton reaction, explained below). Metal chelation may also prevent the initiation of lipid peroxidation (Afanas'ev *et al.*, 1989; Ferrali *et al.*, 1997).

Metal ions are necessary for biological processes, but may also promote the development of oxidative stress (Morel *et al.*, 1999) due to the fact that transition metals (such as divalent copper, Cu^{2+}) contribute to the formation of reactive oxygen species. It has been found that certain phenolic compounds may act as pro-oxidants by generating radicals in environments where large concentrations are found in the presence of metal ions (Laughton *et al.*, 1989; Cao *et al.*, 1997). These active radical species may cause damage to DNA, protein, lipids, and other molecules.

The formation of hydrogen peroxide (H_2O_2) is the initial step in the formation of hydroxyl radicals [1]. Subsequently the Fenton reaction [2] may take place which is a key route to the formation of oxygen radicals (Afanas'ev *et al.*, 1989). Fenton-type reactions are dependent on the type of metal-ligand complex and the metal-ligand ratio, and radical formation is promoted in the presence of ascorbic acid and under conditions of oxidative stress (Laughton *et al.*, 1989; Engelmann *et al.*, 2003).



Thus the pro-oxidant activity displayed will be greatly affected by the ability of the compound to chelate metals or reduce O_2 , as this may inhibit the formation of hydrogen peroxide and active hydroxyl radicals, respectively (Sakihama *et al.*, 2002). In addition to their ability to reduce metals and act as chelators, the antioxidant/pro-oxidant behaviour of phenolic compounds will also depend on pH and solubility (Moran *et al.*, 1997). At a lower pH the antioxidants have a greater iron-reducing capacity. Pro-oxidative forms of reactive iron can also cause lipid peroxidation. Lipid peroxidation occurs when free radicals cause the oxidation of polyunsaturated fatty acids (Gutteridge, 1995). The molecules formed by this process (known as lipid peroxides) are stable compounds, but the presence of transition metals may cause their decomposition. The reaction between metal complexes and lipid peroxides results in the formation of reactive radicals (Gutteridge, 1995). This once again confirms that the metal chelation properties of antioxidants are important in preventing oxidative stress.

Structure-activity relationships

The structure of the phenolic compound has a great effect on the antioxidant activity displayed. For example, the amount and positioning of hydroxyl groups, functional characteristics of substitutions, and the interaction between these functional groups influences the activity of an antioxidant (Rice-Evans *et al.*, 1996).

The position of the hydroxyl groups on the respective rings of a flavonoid compound has a significant effect on the antioxidant activity and several structural arrangements have been confirmed to exhibit greater antioxidant activity (Fig. 8):

- 1) an *ortho*-dihydroxy structure on the B ring (Sichel *et al.*, 1991)
- 2) a 2,3 double bond in conjugation with the 4-oxo group of the C ring (Bors *et al.*, 1990)
- 3) the 5- and 3-OH groups on A and C rings, respectively, in combination with the 4-keto on the C-ring (Bors *et al.*, 1990; Khokhar & Apenten, 2003)

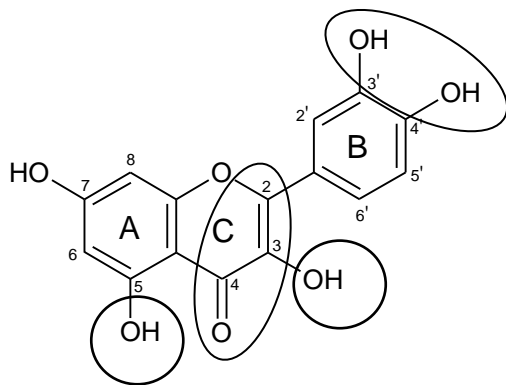


Figure 8 Structural features found to provide optimum antioxidant activity.

Salah *et al.* (1995) confirmed that the *o*-dihydroxy structure is an essential structural attribute in the stabilisation of the radical formed. This is due to the delocalisation of electrons allowed through the arrangement (Bors *et al.*, 1990). It also assists in the ability of the compound to form metal chelates. The second factor (a 2,3 double bond) relates to the electron delocalisation from the B ring and leads to formation of a stable free radical (Rice-Evans *et al.*, 1996). The conjugation effect that occurs between the A ring and B ring favours the formation of a stable flavonoid radical due to resonance (Bors *et al.*, 1990). The hydroxyl groups have been found to be a structural attribute that is essential for display of optimum radical scavenging potential. An increase in -OH substitutions have been found to result in an increase in *in vitro* antioxidant activity (Cao *et al.*, 1997). For example, from Figs. 6 and 7 it is clear that basic structure for catechin and quercetin possess the same number of hydroxyl groups. However the flavonol, quercetin, has a higher antioxidant activity compared to catechin due to the 2,3 double bond and the 4-keto group in the C ring. It was confirmed by Salah *et al.* (1995) that the *in vitro* antioxidant capacity (using the TEAC assay) was higher for quercetin.

The structural features regarded significant in metal chelation (Khokhar & Apenten, 2003) are similar to those for radical scavenging. The authors also found that a catechol group (dihydroxy group) on positions 7 and 8 on the A ring is also correlated with a higher iron binding capacity. The catechol is known as the chelating unit. The presence of the ketone group at position C4 is also an important structural factor (Soczynska-Kordala *et al.*, 2001). In the flavan-3-ol group catechin displayed the highest metal chelating capacity in comparison to its derivatives (Khokhar & Apenten, 2003). In a study by Soczynska-Kordala *et al.* (2001) both quercetin and quercetin-3-*O*-rutinoside were able to perform as metal chelators. The phenol group in its bound state can no longer bind metals and metal chelation then takes place at another metal-binding site (Hider *et al.*, 2001).

Cao *et al.* (1997) determined that the pro-oxidant activity would increase linearly with the number of hydroxyl groups. Interestingly, the modification of a hydroxyl group by the addition of a methyl group diminished the display of both antioxidant and pro-oxidant activity. In the case where there is no double bond at the 2,3 position on the C-ring in conjugation with the 4-keto group, the formation of reactive species may be initiated. This process is advanced in the presence of oxygen and Cu^{2+} (Cao *et al.*, 1997).

The type of sugar moiety attached to the basic structure also plays a role. Based on the ORAC assay anthocyanidins were shown to have a higher *in vitro* antioxidant activity than anthocyanins (Wang *et al.*, 1997). In another study by Seeram *et al.* (2001) it was found that a decrease in the number of glycosides attached to an anthocyanin compound tends to result in an increase in the biological activity of the anthocyanin. The attachment of glycoside units to flavonol compounds also resulted in a decrease in antioxidant activity. Antioxidant activity of both quercetin and kaempferol aglycones is lowered with the addition of a glycoside (Plumb *et al.*, 1999). Flavonoids with a glycoside attached at the C7 position on the A ring causes greater decrease in antioxidant properties than attachment at the C3 position (Mora *et al.*, 1990). Evaluation of glycoside subunits of quercetin confirmed that the type of sugar does affect the antioxidant activity, as quercetin-3-*O*-rutinoside displayed a higher antioxidant activity than quercetin-3-*O*-rhamnoside (chemiluminescence assay) (Limasset *et al.*, 1993).

In terms of phenolic acids, the introduction of a hydroxyl group *ortho* or *para* to the first hydroxyl results in an increase in antioxidant activity. The activity of ferulic acid was also found higher than that of vanillic and *p*-hydroxybenzoic acid due to methoxylation in a position *ortho* to the hydroxyl group (Pokorny, 1987). Derivatives of cinnamic acids display a greater antioxidant activity than benzoic acid derivatives as radical stabilization is favoured by the double bond on the cinnamic acid side-chain (Natella *et al.*, 1999).

Antioxidant assays

Different methods are available for analysing the antioxidant capacity of a sample, each based on different principles and with their own benefits (Prior *et al.*, 2005). Several review papers have been published discussing the different methods and their principles and application (Prior *et al.*, 2005; Magalhães *et al.*, 2008; Singh & Singh, 2008). One way to classify antioxidant assays is by the physiological relevance of the assay. Two groups can be identified, namely assays that involve the scavenging of synthetic radicals, and assays which measure free radical scavenging of

physiologically relevant radicals (Prior *et al.*, 2005). Synthetic radicals are those that are not found in the body (Prior *et al.*, 2005). These include the ABTS [2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)] cation radical and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical. Other assays such as ORAC (Oxygen Radical Absorbance Capacity), nitric oxide and superoxide scavenging assays utilize biological radicals which may closely reflect *in vivo* antioxidant action (Prior *et al.*, 2005). The ORAC and TRAP (total peroxy radical-trapping potential) assays are known to best portray biological relevance. In the following section assays that have been applied in the analysis of fruits and other plant material are discussed.

In the ABTS radical cation scavenging method, the radical cation (ABTS^{•+}) is generated prior to the addition of the sample by oxidation of ABTS with potassium persulfate leading to the formation of a blue-green colour with maximum absorbance at 734 nm (Re *et al.*, 1999). As ABTS^{•+} is subsequently reduced by the antioxidant, a decrease in colour occurs, which is measured spectrophotometrically at 734 nm (Prior *et al.*, 2005). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analogue of Vitamin E, is used as an antioxidant standard, and antioxidant activity is expressed as Trolox equivalents (Prior *et al.*, 2005). The ABTS^{•+} scavenging assay is referred to by some as the TEAC (Trolox Equivalent Antioxidant Capacity) assay. Benefits involving the use of the ABTS^{•+} scavenging assay include the fact that the radical is generated without the need for an intermediary radical and proved popular due to its efficiency in samples of an aqueous or lipid nature (Re *et al.*, 1999; MacDonald-Wicks *et al.*, 2006).

In the DPPH radical scavenging assay, the decrease of absorbance at 515 nm (due to scavenging of DPPH[•]) is measured. This is an indication of the ability of the antioxidant to reduce the DPPH[•] (deep purple colour) to DPPH-H (yellow) (Prior *et al.*, 2005). DPPH[•] is also a synthetic organic nitrogen radical. The results are expressed as Trolox equivalent units (Moon & Shobamoto, 2009). The DPPH[•] scavenging assay is effective for use in antioxidant screening due to its simplicity and ease of execution (Prior *et al.*, 2005).

The FRAP (Ferric Reducing Ability of Plasma/Ferric Reducing Antioxidant Power) assay is used to measure the ability of a sample to reduce iron. A ferric tripyridyltriazine complex (Fe³⁺-TPTZ) is reduced to its ferrous form (Fe²⁺-TPTZ) by the antioxidant (Benzie & Strain, 1996). This assay takes place at a low pH (pH 3.6) and the antioxidant activity is measured through the change in absorbance at 592 nm, i.e. development of a deep blue colour. Trolox or Fe²⁺ can be used as the reference antioxidant (Moon & Shibamoto, 2009). In a review by Singh and Singh (2008) it was reported that some of the benefits of this assay include its sensitivity, reproducibility

and ease of use. On the other hand, the FRAP assay does not display any information regarding antioxidant properties such as its chain breaking ability or preventative capacity (Singh & Singh, 2008).

The ORAC assay measures the total antioxidant activity against a peroxy radical generated by thermal decomposition of an azo-compound (Cao & Prior, 1999). A reaction takes place between the peroxy radical and fluorescein (a fluorescent probe), which results in the formation of a non-fluorescent product. Initially β -phycoerythrin was used as fluorescent probe, but it was found that the fluorescence signal of this probe declines over time (Ou *et al.*, 2001). Using a microplate reader, the decrease in fluorescence caused by the scavenging of the peroxy radical by an antioxidant is determined (Prior *et al.*, 2005). The difference in the AUC (area under the curve) between a control and the sample is used to determine the ORAC value in terms of Trolox equivalents. AAPH [2,2'-azobis (2-amidinopropane) dihydrochloride] is used as a peroxy radical generator (Cao & Prior, 1999). The decomposition of AAPH is temperature-dependent and occurs spontaneously during the assay to releases peroxy radicals. These radicals are scavenged by antioxidants and the ability of the antioxidant to quench the radicals is measured (Cao & Prior, 1999).

Huang *et al.* (2002a) were of the first to identify the need for full automation of the original ORAC method. This allowed the test to be done for multiple samples at the same time, and eliminates experimental fault caused by manual execution. Subsequently, the original ORAC assay (Cao & Prior, 1999) was adapted for a microplate fluorescence reader (96-well) using automated dispensing of AAPH. The ORAC method was reported to be more sensitive than the FRAP and original ABTS^{•+} assays (Cao & Prior, 1999). Originally the ORAC assay was used to measure hydrophilic antioxidants, and was adapted by Huang *et al.* (2002b) to allow analysis of antioxidants of a lipophilic nature.

On-line antioxidant assays

While antioxidant assays are effective in the determination of total antioxidant activity displayed, they do not give any indication of the contribution of individual compounds to the antioxidant capacity (Kusznierewicz *et al.*, 2011). This limitation can be partly overcome through the use of on-line antioxidant activity determinations. In on-line antioxidant assays, phenolic compounds are separated by HPLC and reacted post-column with the applicable reagents (for the chosen antioxidant assay). The reaction will result in the formation of coloured or non-coloured

compounds (depending on the assay) which is detected as an increase or decrease in absorbance, respectively (Kusznierewicz *et al.*, 2011). On-line methods are used for screening complex mixtures with the aim of identifying radical scavenging compounds and have also been used to determine the total antioxidant activity (Koleva *et al.*, 2000; Kusznierewicz *et al.*, 2011). On-line methods for ABTS^{•+} and DPPH[•] have been successfully applied to plant material by several researchers (e.g Dapkevicius *et al.*, 2001; Koleva *et al.*, 2000). On-line antioxidant assays can be used for quantitative and qualitative analysis of phenolic compounds.

Antioxidant activity of plums

In a study by Wang *et al.* (1996) the antioxidant capacity of several fruits was determined using the ORAC assay, whereby plums displayed the second highest value. The antioxidant activity of plums was analysed with the FRAP and ORAC assays (Imeh & Khokhar, 2002), and the results indicated that plums exhibit very high activity compared to a range of other fruit, including kiwi, apple and pear. Red plums specifically were proposed by García-Alonso *et al.* (2004) to contain a high antioxidant activity, possibly due to the high anthocyanin content. Plums also have a higher activity compared to other stone fruit such as nectarines and peaches (Gil *et al.*, 2002). The same authors found that the total phenolic content of plums strongly correlated with the antioxidant activity. When evaluating the correlation between individual phenolic groups it was found that the flavan-3-ols were correlated best to the antioxidant capacity. Hydroxycinnamic acids, however, did not correlate well with the *in vitro* antioxidant activity (Gil *et al.*, 2002). A similar study by Chun *et al.* (2003) confirmed these findings. These findings may be due to higher flavan-3-ol contents in plums compared to hydroxycinnamic acids.

The phenolic content of plums increases as the fruit matures (Puerta-Gomez & Cisneros-Zevallos, 2011), and it may be expected that the antioxidant activity will show the same trend. Phenolic composition and content will vary between cultivar and is also affected by environmental factors such as light and climate. In a study on grapes it was determined that increased exposure to natural light led to an increase in flavonol content (Price *et al.*, 1995). In addition, it has been found that grapes cultivated in warmer geographical areas tend to have a higher total concentration of flavonols than those in cooler areas (McDonald *et al.*, 1998). Therefore it is necessary to evaluate different plums cultivars from South Africa, as the majority of previous studies were performed on Japanese and European plums from different climates (generally grown in the USA or Japan).

Table 5 summarizes the antioxidant capacity of various fruit. In comparison to other fruit plums may be classified as having a relatively high *in vitro* antioxidant activity. Poor correlation of results from different assays can be attributed to the multifunctional nature of antioxidants. Certain phenolic compounds may act as reducing agents while others act by scavenging free radicals (Fu *et al.*, 2011).

Table 5 Typical antioxidant activities of several fruit as measured by FRAP, TEAC and ORAC assays

Fruit	FRAP ^a	TEAC ^a	ORAC ^b
	($\mu\text{mol Fe(II).g}^{-1}$ FW)	($\mu\text{mol Trolox.g}^{-1}$ FW)	($\mu\text{mol Trolox.g}^{-1}$ FW)
Pomegranate	25.6	40.6	-
Cherries	14.7	5.5	-
Plum (black)	9.8	6.5	9.5
Kiwifruit	10.8	4.6	6.0
Grape (red)	6.7	4.0	7.4
Apple (red)	5.9	4.6	2.2
Blueberry	10.1	1.3	-
Grape (green)	5.0	1.3	-
Olive	2.7	80.7	-
Strawberry	-	-	15.4

^aFu *et al.*, 2011; ^bWang *et al.*, 1996.

REPORTED HEALTH BENEFITS OF PHENOLIC COMPOUNDS

Reactive oxygen species may cause oxidative stress which cause damage to molecules such as DNA and protein, and lead to a variety of diseases or biological damage in the body (Ames *et al.*, 1993). These include diabetes, cancer, arthritis, arteriosclerosis, inflammation or infections (Shahidi & Naczki, 2004b). Many reports have been published on the contribution of phenolic compounds to the health benefits of fruits and vegetables (Yochum *et al.*, 1999; Park *et al.*, 2004, etc.). The majority of these are *in vitro* or animal studies, with studies on *in vivo* effects being less frequent.

While the ability of phenolic compounds to act as antioxidants play a large part in the health benefits displayed, phenolic compounds may also contribute to disease prevention through other mechanisms, such as activation of the P-glycoprotein or through the modulation of liver enzyme systems, which may both prevent activation of certain carcinogens (as reviewed by Duthie

et al., 2000). Mechanisms by which phenolic compounds act in the prevention of diseases have been reviewed previously (Ishige *et al.*, 2001) and will not be discussed. Selected studies focussing on the health benefits of phenolic compounds will be examined.

The oxidation of low-density lipoprotein (LDL) has been proposed to contribute to the development of heart disease by causing blockages in major arteries. Phenolic compounds may counteract the onset of heart disease due to their ability to inhibit LDL oxidation (Frankel *et al.*, 1993). Flavonoids are potentially beneficial against arteriosclerosis due to the prevention of LDL oxidation in atherosclerotic lesions. Several flavonoid aglycones including flavone, quercetin, gossypetin and morin were compared, of which flavone showed the most effective inhibition and gossypetin the lowest (De Whalley *et al.*, 1990).

A high intake of saturated fats is generally associated with the development of coronary heart disease. However, there are some exceptions. In certain areas in France it has been found that in addition to high intake of products high in saturated fats, individuals are also regular consumers of red wine. These areas also show a lower incidence of mortality from heart diseases. This occurrence is known as the "French paradox" and is attributed partly to the flavonoids in red wine (Frankel *et al.*, 1993). Epidemiological studies support the concept that phenolic compounds exhibit certain health benefits. Studies performed on older women and men agree that the intake of flavonoids (including quercetin and kaempferol) is related to a reduced risk of cardiovascular disease (Hertog *et al.*, 1993; Yochum *et al.*, 1999).

Furthermore, phenolic compounds may play an important role in prevention of cancer. Chlorogenic acid and its derivatives in plums were found to possess certain chemopreventative properties and may inhibit the growth of breast cancer (Noratto *et al.*, 2009). Belkaid *et al.* (2006) found that chlorogenic acid may also display anticancer properties by contributing to the prevention of brain tumour development. Flavones have been inversely associated with the occurrence of breast cancer (Peterson *et al.*, 2003). Beneficial effects of several flavonoids have also been reported regarding other types of cancer including thyroid (Yin *et al.*, 1999), skin (Wei *et al.*, 1990) and colon cancer (Wenzel *et al.*, 2004).

In addition there is experimental proof that phenolic compounds are capable of exhibiting anti-inflammatory action. It was found that cyanidin and certain cyanidin glycosides effectively inhibit enzymes associated with inflammation and thus contribute to relieving pain associated with conditions such as arthritis (Seeram *et al.*, 2001). A study by Park *et al.* (2004) confirmed certain phenolic compounds (e.g. honokiol) to be effective in the treatment of skin conditions such as acne through anti-bacterial and anti-inflammatory action.

Oxidation and inflammation are believed to be promoters of tumour development and thus the counteracting effect of phenolic compounds on inflammation could be chemopreventative (Surh, 2002). The anti-inflammatory effect of certain polyphenols can be ascribed to the modulation of cell signalling pathways. Several mechanisms of action are suggested and one possible explanation is the prevention of the activation of nuclear factor-kappa B (NF- κ B). This transcription factor in its activated form could initiate inflammatory diseases, cancer and allergies (as reviewed by Kumar *et al.*, 2004). Compounds such as resveratrol and curcumin (present in grapes and turmeric, respectively) have been found to prevent activation of NF- κ B and the influence of polyphenols in cell signalling could therefore assist in the prevention of various diseases (Singh & Aggarwal, 1995; Surh *et al.*, 2001).

However, many studies on the health benefits of phenolic compounds are contradictory to one another. In comparing results from different studies it is important to take into consideration the methods used, the specific phenolic compound under evaluation, the subjects used (age, gender, health, etc.), dosage ingested and the method of application (via food, supplements, etc.). It would be valuable to continue research on the absorption and bioavailability regarding health benefits of phenolic compounds, especially with *in vivo* studies, as phenolic compounds could be beneficial in the prevention of the treatment of various diseases (Wang *et al.*, 1997; Fu *et al.*, 2011).

BIOAVAILABILITY OF PHENOLIC COMPOUNDS

In order to assess the health benefits of phenolic compounds it is necessary to not only consider the amount of phenolic compounds present in foods, but also whether these compounds are bioavailable (Srinivasan, 2001). It is well known that for a compound to exert a beneficial effect in the body it must be efficiently digested, absorbed and possibly metabolised. It should also be examined whether the compounds will reach a physiologically relevant concentration in the body. The knowledge of absorption of phenolic compounds is thus essential in order to determine the health implications thereof (Olthof *et al.*, 2001). In addition, compounds may be hydrolysed, metabolised or degraded in the gastrointestinal tract or during blood circulation (Kühnau, 1976). In certain cases it is thus the antioxidant activity and possible health implications of these metabolites that should be studied. If not absorbed into the blood stream it may exert a biological effect elsewhere in the body (e.g. in the colon) (Olthof *et al.*, 2001). It is also necessary to establish

whether the compound will stay in the body for an adequate period in order to display the necessary action (Karakaya, 2004).

The majority of hydroxycinnamic acids are linked to the cell wall of plant material by means of an ester bond. This bond must be cleaved in order for the compounds to be metabolised or taken up in the gut, which is accomplished by microbial esterase (Andreasen *et al.*, 2001). High esterase activity has been detected in colonic microflora, indicating that these acids may be effectively cleaved from the food material (cereal bran was used in the study) and subsequently absorbed. It was concluded by the authors of the mentioned study that diferulic acids are indeed bioavailable. The absorption of chlorogenic acid and caffeic acid in humans was determined by Olthof *et al.* (2001) and it was established that 95% of the ingested caffeic acid and 33% of chlorogenic acid were effectively absorbed in the small intestine. Healthy ileostomy subjects were used for this study since the phenolic compounds of interest are degraded in the colon.

The type of glycoside attached to a flavonol plays an important role in the rate of absorption and bioavailability. Substitutions with sugar determined that quercetin-3-*O*-glucoside was absorbed more rapidly than quercetin-3-*O*-rutinoside and other derivatives (Hollman *et al.*, 1997). A possible mechanism of action can also be derived from the amount of time measured until these compounds are found in plasma (Hollman *et al.*, 1997). Microorganisms necessary for rutinoside hydrolysis are found only in the colon, and the increased period of time until rutin metabolites are detected is an indication that it is likely absorbed after hydrolysis in the colon. However, in another study it was suggested that the quercetin aglycone is effectively absorbed in the small intestine (Hollman *et al.*, 1995). In contrast to other studies, Sesink *et al.* (2001) showed that quercetin-glycosides are not absorbed in their intact form. Rather, this study showed that the flavonols were either hydrolysed and metabolites separately absorbed, or found in the body in a conjugated form.

The bioavailability of the flavan-3-ol, (+)-catechin, has been studied by various authors with similar results. Two such studies (Lee *et al.*, 2002; Henning *et al.*, 2004) are in agreement that catechins from tea display a low bioavailability. Although a very low percentage of each catechin derivative was absorbed it was confirmed that (-)-epigallocatechin and (-)-epicatechin display better absorption than (-)-epigallocatechin-3-gallate (Henning *et al.*, 2004).

Published data indicates that anthocyanins tend to display a low bioavailability. Müllleder *et al.* (2002) found that anthocyanins from elderberry concentrate were not effectively absorbed. In a study by Murkovic *et al.* (2000) the authors also found very low bioavailability of anthocyanins based on the analysis of human plasma. Only 10 µg was found in the plasma after ingestion of

200 mg anthocyanins. Wu *et al.* (2005a) confirmed the low bioavailability of anthocyanins, and also found that the flavonoid aglycone and the different sugar moieties attached affect their bioavailability.

Confirmation of the bioavailability of anthocyanins is particularly challenging due to the complex nature of this compound. For example, many forms of anthocyanins (aglycones and glycosides) are found in food material and may all display different bioavailability properties (Wu *et al.*, 2005b). In addition, structural changes occur as the pH of the matrix is altered. During analysis of anthocyanins the occurrence of the flavylum compound is measured. However, this form is not likely to be found *in vivo* (McGhie & Walton, 2007).

ANALYSIS OF PHENOLIC COMPOUNDS

Various methods have been developed and applied to assess the phenolic composition of samples from plant origin. The choice of method is dependent on the purpose of analysis (Escarpa & González, 2001). Some techniques include liquid chromatography (LC), gas chromatography (GC), thin-layer chromatography (TLC) and capillary zone electrophoresis (as reviewed by Stalikas, 2007). Of these, high-performance liquid chromatography (HPLC) is predominantly used for separation and quantification of phenolic compounds (Tsao & Yang, 2003). HPLC is the preferred method as it is reported to be a more versatile and precise technique (Escarpa & González, 2001; Tsao & Yang, 2003).

In addition, mass spectrometry (MS) is a technique frequently used to identify phenolic compounds, especially those in trace amounts. The application of mass spectrometry may also provide information on the structural arrangement of the compound (Ryan *et al.*, 1999). Furthermore, additional fragmentation can be accomplished through MS/MS (tandem MS) which assists in the identification of compounds (Häkkinen & Auriola, 1998).

Alternatively, the total phenol content can be determined by methods such as the Folin-Ciocalteu method (a spectrophotometric method) to evaluate overall phenolic content, but not individual compounds (Parejo *et al.*, 2004).

Sample preparation

Before a sample is analysed using one of the abovementioned techniques, the sample must be correctly prepared for analysis. The main objective of sample preparation is ensuring the sample is in a form that is suitable for a specific analytical technique. For samples in solid form, an extraction process is first required to obtain a liquid sample. Depending on the nature of the sample and the analysis to be performed, extraction might include steps such as sonication, centrifugation, filtration and evaporation (as reviewed by Stalikas, 2007). Thus fruit samples such as plums are first extracted with a suitable solvent and then centrifuged and filtered to remove any remaining particles. Sonication is also often used to enhance the extraction procedure (Smith, 2003).

One should keep in mind that sample preparation starts from the collection of the sample (Snyder *et al.*, 1997). Thus the effect of each step until the point of analysis should be considered. For example, samples should be collected, transported and stored in a way which ensures that products are not damaged and compounds of interest are not degraded (Snyder *et al.*, 1997). One such example is the addition of sodium fluoride during homogenization. This prevents oxidation during homogenization and freezing by inhibition of the polyphenol oxidases present in the fruit (Tomás-Barberán *et al.*, 2001).

For certain sample matrices, filtration alone will be sufficient, while other samples require a more extensive process. It is important to use a method that is most effective in terms of subsequent analysis and that is also time efficient. Filtration is an important part of sample preparation when using liquid chromatography. Any small, insoluble particles will negatively affect the column by causing blockage (Smith, 2003).

In certain circumstances it is useful to separate different phenolic groups into fractions in order to simplify complex mixtures and thus improve identification of individual compounds. Different techniques are available for fractionation of phenolic compounds. These include liquid-liquid extraction (LLE), solid-phase extraction (SPE) and supercritical fluid extraction (SFE), to name a few (Snyder *et al.*, 1997). Depending on the nature of the sample, LLE and SPE are the procedures most commonly used.

During LLE the sample is suspended in a mixture of solvents that form two phases, generally an aqueous and an organic phase. After allowing the phases to separate, the compounds which are more hydrophobic will be suspended in the organic phase, while the remaining compounds will be found in the aqueous phase (Snyder *et al.*, 1997).

SPE utilizes a disposable cartridge that is pre-packaged with an appropriate solid phase. The cartridges are preconditioned with a solvent of appropriate polarity and pH, allowing retention of applicable compounds from the sample solution (Smith, 2003). By altering the polarity or pH of the sample matrix, the trapped compounds can be eluted by the applicable solvent. Effective preconditioning and a constant flow rate are important aspects in the procedure (Smith, 2003). If the flow rate is too fast there may not be adequate contact between the sample and the stationary phase (Snyder *et al.*, 1997). When analysing a large number of samples at a time, a multicartridge vacuum manifold system can be used. The process can be performed under vacuum or using gravity. The solvent selected for elution allows either strong retention of the compound in the cartridge, or causes the sample to be weakly retained and washed from the cartridge (Snyder *et al.*, 1997). Through the use of solvents of different polarities or pH, phenolic compounds are separated from one another (Da Costa *et al.*, 2000). SPE thus also allows the removal of compounds that are undesirable in the analysis of certain samples (such as sugar, acids and proteins) and allows separation of different phenolic compounds in a sample (Da Costa *et al.*, 2000). The separated fractions are used in subsequent analyses.

SPE provides several benefits over LLE, including more efficient extraction and separation and most likely a reduction in the amount of solvent used (Smith, 2003). Another positive aspect is that a variety of SPE phases are commercially available. Therefore there is a wider choice as to which type of trapping mechanism is to be utilized. Compounds can be separated based on their ionisation, polarity or hydrophobic/hydrophilic properties (Smith, 2003). The SPE process can also be automated together with HPLC or GC. However, the reproducibility of SPE is often questioned due to the possible variation between cartridges (Snyder *et al.*, 2010).

High-performance liquid chromatography (HPLC)

High-performance liquid chromatography is a popular technique used to separate, identify and quantify phenolic compounds in food (Tsao & Yang, 2003). The separation of compounds is illustrated by means of a chromatogram. The choice of method parameters such as the stationary phase, mobile phases and temperature is dependent on the sample characteristics and compounds which must be separated.

Different detection systems can be coupled to the HPLC system depending on the task to be performed. Chromatograms of a sample can be collected at different wavelengths at the same time through the use of an ultraviolet-visible (UV-Vis) diode-array detector (DAD). Information

about the composition of a sample can be obtained in one HPLC run, eliminating the need for several single-wavelength runs (Snyder *et al.*, 1997). Alternatively, the use of mass spectrometry (MS) as detection method allows tentative identification of individual compounds. HPLC-MS is also used to evaluate peak purity, which is necessary in order to validate methods and to regulate the purity of the analyte assessed (Bryant *et al.*, 1996). From previous HPLC-MS studies it was found that co-eluting compounds which appear as a single peak may be distinguished from one another using MS data (Wu & Prior, 2005).

An MS detector is only able to detect sample species that are in a charged form and thus the analyte to be detected is firstly ionized by the source of the mass spectrometer (Snyder *et al.*, 1997). Different MS ionization techniques are possible, but the one that is most frequently used for phenolic compounds is electrospray ionization (ESI) (Wu & Prior, 2005). ESI creates charged polyphenolic molecules from evaporating droplets of liquid and the results provide a mass-to-charge ratio (m/z) to be used in the identification process. The spectrum obtained will generally show the pseudomolecular ion ($[M + H]^+$ or $[M - H]^-$ for positive and negative mode, respectively) as the most intense ion which also displays the molecular weight of the compound. ESI is known as a soft ionization procedure, which allows application in studies with small metabolites such as phenolic compounds, polar molecules, ionic molecules and molecules with a high molecular mass (Baldi *et al.*, 1995; Careri *et al.*, 1998). Mass spectrometry data are rarely used to fully determine the structure of a compound. Rather, collected data allow determination of molecular weight and the substituents on the compound (Ryan *et al.*, 1999). The MS/MS fragmentation pattern can be used together with molecular weight for tentative compound identification (Ryan *et al.*, 1999). For identification the retention time, UV-Vis characteristics and MS characteristics of peaks are compared to those of authentic phenolic standards and with results found in literature (Wu & Prior, 2005).

With modern HPLC systems a gradient elution program is commonly used to allow the separation of different compounds by gradually changing the composition of the mobile phase. Gradient elution is preferred over isocratic conditions if compounds in a sample will be eluted over a wide range of retention times (Snyder *et al.*, 1997). Commonly, acetonitrile or methanol serves as organic modifier in the system, while an organic acid (e.g. formic acid) is used as acid modifier in the aqueous phase.

When developing a new HPLC method or improving a previous method, different aspects can be evaluated in order to optimize efficiency. Parameters of HPLC analysis to experiment with include temperature, flow rate, injection volume, column dimensions and packing material, mobile

phase composition, and gradient program (Parejo *et al.*, 2004; Schellinger & Carr, 2006; Francisco & Resurreccion, 2009). Table 6 summarizes different method parameters that have been applied in analysis of phenolic compounds.

The final step of a method development process should be the validation of the method. A well-designed HPLC method should be both accurate and precise in its intended application. In addition to accuracy and precision, other components of method validation may include limit of detection, specificity and sample stability (Snyder *et al.*, 1997). The accuracy of the method refers to the closeness of results to a reference standard, while through determination of the precision of a method one would evaluate the reproducibility and/or repeatability of the method (Snyder *et al.*, 1997). A possible way to evaluate specificity is to determine peak purity, normally using a technique such as HPLC-MS. The sample stability may be determined simply by injecting the sample over a period of time and observing whether degradation occurred (Snyder *et al.*, 1997). Statistical analysis also proves to be a necessary step regarding the validation process. Tests such as analysis of variance (ANOVA) and the *t*-test are commonly applied (Snyder *et al.*, 1997).

Many studies have been performed in which the phenolic compounds of fruit are analysed using HPLC, LC-MS or both (Chandra *et al.*, 2001; Määttä *et al.*, 2003; Wu & Prior, 2005). However, the methods used in these studies must be adjusted to obtain the best results for the specific samples (e.g. South African vs. European plums). The method development process could be expensive and time-consuming. The fact that there are no pure standards available for many of the compounds may prove challenging in terms of identification (Wu & Prior, 2005).

In past studies it was found that the coupling of HPLC with MS detection poses some challenges in terms of finding the correct interface between the two methods. These challenges include the amount of mobile phase to be introduced into the column, the characteristics of the solvent used, application of heat and influence from the compound of interest (as reviewed by Welch *et al.*, 2008). The HPLC-MS system is generally not compatible with strong acids such as trifluoroacetic acid (TFA) or high concentrations of weaker acids (e.g. formic acid). TFA causes ion suppression which diminishes the signal displayed (Emmert & Rueck, 2006). In addition, the use of non-volatile solvents may suppress ionization and ultimately influence how much of the charged ion reaches the detector (Annesley, 2003).

Table 6 Examples of HPLC method parameters used in the analysis of phenolic compounds

Sample analysed	Column parameters	Mobile phases	Gradient programme	Temperature (°C)	Flow rate (mL.min ⁻¹)	Injection volume (µL)	Reference
Plums	Eclipse XDB-C8 Diameter: 4.6 x 150 mm Particle size: 5 µm	A = 0.05% TFA B = 0.05% TFA in acetonitrile	5-10% B in 5 min, 10-25% B in 5 min, 25 – 85% B in 6 min, 85 to 5% B in 2min, 5% B for 2 min	30	0.8	10	Slimestad <i>et al.</i> , 2009
Plums: anthocyanins, flavonols & phenolic acids	Gemini-NX C18 Diameter: 4.6 x 150 mm Particle size: 3 µm	A = 7.5% FA B = 7.5% FA in acetonitrile	0–1 min = 3% B, 1–4 min = 3–7% B, 4–7 min = 7% B, 7–27 min = 7–35% B, 27–29 min = 35–80% B, 29–31 min = 80% B, 31–35 min = 80–3% B, 35–45 min = 3% B	30	1.0	50	De Beer <i>et al.</i> , 2012
Plums	Alltech C18 Diameter: 4.6 x 250 mm Particle size: 5 µm	A = 2.0% FA B = acetonitrile	0-15 min = 10% B, 15-35 min = 10-50% B, 35-38 min = 50-80% B, 38-42 min = 80-100% B, 42-43 = 100-10% B, 43-61 min = 10% B	Not mentioned	1.0	20	Mubarak <i>et al.</i> , 2012
Pears & apples	Nucleosil 120 C18 Diameter: 4.6 x 250 mm Particle size: 5 µm	A = 0.01 M phosphoric acid B = acetonitrile	0 min = 2% B, 10 min = 15% B, 35 min = 35% B	Ambient	2.0	20	Escarpa & González, 1999
Berry juice concentrates	Polymer Labs PLRP-S Diameter: 4.6 x 250 mm Particle size: 5 µm	A = 4% phosphoric acid B = acetonitrile	0-10 min = 6% B, 10-55 min = 6-20% B, 50-60 min = 20% B.	Ambient	1.0	20	Hong & Wrolstad, 1990
Berries	Zorbax SB-C18 Diameter: 4.6 x 250 mm Particle size: 5 µm	A = 5% FA B = methanol	0-2 min = 5% B, 2-10 min = 5-20% B, 10-15 min = 20% B, 15-30 min = 20-30% B, 30-35 min = 30% B, 35-50 min = 30-45% B, 50-55 min = 45% B, 55-65 min = 45-5% B, 65-68 min = 5% B	Not mentioned	1.0	Not mentioned	Wu <i>et al.</i> , 2005a

*TFA = trifluoroacetic acid; FA = formic acid.

Total polyphenol assay

In terms of spectrophotometric methods used to determine the total phenolic content of a product, the Folin-Ciocalteu (FC) reagent is most often used (Soong & Barlow, 2004; Herken & Guzel, 2010). The total phenol assay gives an indication of the total amount of total phenolic compounds in a sample, taking different phenolic groups into account. The mechanism by which the FC method functions is an oxidation/reduction reaction. The molybdotungstate compounds in the FC reagent cause oxidation of phenolic compounds in an alkaline environment, resulting in the formation of a coloured product with detection at 765 nm (Prior *et al.*, 2005). Due to its mechanism of action this method could in some cases be applied in the determination of antioxidant activity.

It is important to keep in mind that certain interfering compounds may influence the accuracy of the FC assay. Interferences can cause the under- or overestimation of total polyphenols by reacting with the assay reagents (Singleton *et al.*, 1999). One common interfering substance in fruits is ascorbic acid (Brat *et al.*, 2007). Methods are available for elimination of substances before the assay, or to subtract from the final quantification results (Brat *et al.*, 2007). Singleton and co-authors (1999) found that the relative colour formation during the FC method is dependent on the molar absorptivity and reactive groups of different phenolic compounds. The authors stated that comparison to gallic acid under the same assay conditions to evaluate the contribution of specific compounds to colour formation would be beneficial.

Although it is of interest to determine the total phenol content of a material, it might not relate directly to certain important properties, such as the colour of the product. In cases where the colour is of importance, it is advisable to evaluate the phenolic compound of interest (e.g. anthocyanins) directly (De Beer *et al.*, 2004).

INDUSTRIAL IMPORTANCE OF PHENOLIC COMPOSITION

Information gathered regarding the phenolic composition and content of food products can have great impact on future research concerning industrial applications. This knowledge may be beneficial for future breeding programmes, for use of waste products, or for the incorporation of phenolic compounds in products as antioxidants.

Information is continually being compiled to summarize which food products contain a high phenolic content or antioxidant capacity, and the correlation between these have been

extensively researched (Fu *et al.*, 2011; Paixão *et al.*, 2007; Soong & Barlow, 2004). There is an interest in this subject due to an increase in breeding programmes aimed at producing fruit with an enhanced functional value (Jaganath & Crozier, 2010). Possibilities exist for plant products with a higher antioxidant capacity or phenolic content to be further utilized in the nutraceutical industry (antioxidants), in food products (functional ingredient and/or colorant), or provide benefit by consumption of fresh produce.

The colour of a product is very important in terms of consumer acceptability, as this is the first perception a consumer has of a product. The use of colorants in the food industry serve as enhancement of colour, to restore colour lost due to processing or as complement to certain flavours (Bridle & Timberlake, 1997). During recent years there has been a decrease in the amount of synthetic colorants that are permitted by legislation due to safety concerns and the demand from consumers for natural colorants (Bridle & Timberlake, 1997; Downham & Collins, 2000).

Due to the variety of functional properties phenolic compounds exhibit, it may be possible to utilize these functions in certain products in the food and/or nutraceutical industries (Fossen *et al.*, 1998). The interest in the use of anthocyanins as antioxidants, for example, is partly due to the potential health benefits it may impart to a food product (Giusti & Wrolstad, 2003).

Thus far, anthocyanins have been successfully used in acidic solutions as a natural pigment to impart red colour, particularly in products such as jams, soft drinks and baked products (Konczak-Islam *et al.*, 2003). The choice of which anthocyanidin derivative to be used will depend on the colour desired, stability of the compound under applicable conditions, and the food product in which it is to be incorporated (Giusti & Wrolstad, 2003). According to Cevallos-Casals *et al.* (2006), it is indeed possible to utilize anthocyanins as natural colorant sources, as they exhibit an attractive colour that is similar to that of synthetic food colorants. In a study by Camire *et al.* (2002) it was found that anthocyanins extracted from grapes and blueberries were effectively incorporated as colorants.

As anthocyanins are water-soluble pigments, their success as colorants may be due to the ease of solubility in a variety of solvents (e.g. water, alcohol, etc.). The incorporation of anthocyanins as colorant is highly dependent on the pH of the food matrix. At a pH below 2.0, the anthocyanins predominantly exist as a flavylium cation, which provides a desirable red colour (Giusti & Wrolstad, 2003). There are, however, some restrictions to the use of phenolic compounds such as anthocyanins in food products. Natural colorants are not compatible with certain processing procedures or storage conditions and may reduce the colour stability of the product (Giusti & Wrolstad, 2003). One should also consider the effect of functional ingredients on

the taste of a product. A recent study investigated the use of plum peel extract to increase the phenolic content and antioxidant activity of plum nectar (De Beer *et al.*, 2012). It was found that peel extract could be effectively incorporated into plum nectar at different concentrations. A difference in sensory character was, however, perceived with different concentrations. At higher concentrations negative attributes such as astringency and acidity were more noticeable.

It may also be possible to extract phenolic compounds from production waste, such as the pulp, seed and peel of fruit that remains from juice or wine production (Lee & Wrolstad, 2004; Shrikhande, 1999). It has been reported that there are bioprocesses that may improve recovery from waste products (Correia *et al.*, 2004). Other published studies confirm that by-products from industrial production processes may be adequate sources of phenolic compounds, and thus also possible sources of antioxidants (Parejo *et al.*, 2004; Balasundram *et al.*, 2006). Puerta-Gomez and Cisneros-Zevallos (2011) took an interesting approach by monitoring phenolic content in peaches and plums beyond their eating ripe stage. They found that the anthocyanin content of the fruit continued to increase during the ripening period after the fruit has reached the ripeness stage for fresh consumption. There is thus potential to use fruit, unfit for sale as fresh fruit, for production of functional ingredients. The phenolic content of plum waste from processing is currently not known and allows room for investigation.

However, only certain phenolic compounds are acceptable for use in other food products. Extensive research regarding their activity, toxicity and effect of products formed from degradation of the phenolic compounds is still required (Shahidi & Naczki, 2004b). The efficiency of incorporation into pharmaceutical or food products must also be considered. The utilisation of waste products for extraction of phenolic compounds needs further attention concerning practical application. The extraction process might not be practically feasible if enough raw material is not available, or if the extraction process is not efficient.

CONCLUSIONS

Currently limited information is available regarding the phenolic composition and antioxidant activities of plums produced in South Africa. HPLC is the preferred method of analysis of phenolic compounds for identification and quantification, and a specific method is necessary for evaluation of South African cultivars and selections since phenolic content is influenced by the geographical environment, cultivars, etc. The analysis of phenolic compounds is of interest due to their antioxidant activity and the potential health benefits they may exhibit.

Adequate knowledge of plum phenolic compounds and their antioxidant activity is of interest for possible future breeding programs and the incorporation of data in phenolic databases or the compilation of phenolic intake data.

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CHAPTER 3

CHARACTERISATION OF PHENOLIC COMPOUNDS IN PLUMS USING HPLC-DAD-FLD, HPLC-DAD-MS AND AN ON-LINE ANTIOXIDANT ASSAY

ABSTRACT

Phenolic compounds are abundant secondary metabolites in plums, with potential health benefits believed to be largely due to their antioxidant activity. It is of interest to identify antioxidant compounds in plums to fully evaluate their health implications. A standard method of analysis is necessary to evaluate phenolic composition in different plum cultivars and selections. This method should also be suitable for use with mass spectrometry to confirm known compound identities and identify previously unidentified phenolic compounds.

A high-performance liquid chromatography (HPLC) method was optimised and used to characterise the phenolic composition of different South African plum cultivars and selections including red-fleshed plums. Twenty-four phenolic compounds were identified by comparison of their UV-Vis and mass spectrometric characteristics to that of authentic reference standards or to compounds in literature. Eighteen phenolic compounds could be quantified from four phenolic groups, namely phenolic acids (neochlorogenic acid, chlorogenic acid, 3-*O*-*p*-coumaroylquinic acid, anthocyanins (cyanidin-3-*O*-glucoside and -rutinoside), flavan-3-ols ((+)-catechin, (-)-epicatechin, procyanidin B1 and B2), and flavonols quercetin-3-*O*-rutinoside, -glucoside, -arabinoside, -xyloside, -rhamnoside, quercetin pentosyl-pentoside, quercetin pentosyl-hexoside and acetylated quercetin-hexoside). Neochlorogenic acid and cyanidin-3-*O*-glucoside were the major compounds in the majority of red-fleshed plums. Additional compounds identified included flavan-3-ol A-type procyanidins (dimers and trimers) and B-type procyanidins (trimers). Method validation proved acceptable in terms of linearity, stability and intra-day precision. The inter-day precision for not as good, especially for flavan-3-ols in the standard calibration mixtures where percentage relative standard deviation ranged between 5 and 13.2%. This may be due to small peak areas or co-elution of unknown compounds.

On-line coupling of the ABTS^{•+} scavenging assay with HPLC enabled qualitative evaluation of the antioxidant activity of individual phenolic compounds. The flavan-3-ols displayed the greatest antioxidant response peaks, followed by the anthocyanins.

The optimised method of analysis was successfully applied to characterise eleven different South African plum cultivars and selections and to assess their antioxidant activity. Future research on the phenolic fractions in plums will benefit from these methods of analyses, especially in breeding programs aimed at increasing the antioxidant activity of plums.

INTRODUCTION

Phenolic compounds have various functional attributes, of which their potential health benefits are regarded with the highest interest. For this reason research on the application of phenolic compounds and antioxidants in processed food products (as colorants, functional ingredients) or as nutraceuticals is on the increase. However, to fully utilise these functions of phenolic compounds it is necessary to gain more information regarding their identity, amount present and their antioxidant activity. To obtain accurate results it is vital to use suitable techniques and optimised methods.

One of these techniques commonly used for the analysis of phenolic compounds is high-performance liquid chromatography (HPLC). HPLC is a reliable method for the analysis of phenolic compounds in fruit matrices and has been successfully applied to plums and other fruit (Merken & Beecher, 2000; Welch *et al.*, 2008; Castañeda-Ovando *et al.*, 2009; Moon & Shibamoto, 2009; Ignat, *et al.*, 2011). HPLC is reported to be more acceptable than certain other methods (such as gas chromatography and spectrophotometric methods) in terms of accuracy and technical operation. Different detection systems are available depending on the aim of the analysis (quantification, nature of compounds, etc.). An ultraviolet (UV) diode array detector (DAD) allows detection at a range of wavelengths simultaneously (Snyder *et al.*, 1997). Compounds can be classified by the shape of their UV-Vis spectra and the wavelengths at which maximum absorption occur. In addition, fluorescence detection (FLD) can also be used for compounds with fluorescent properties that are present in lower concentrations, as fluorescence detectors are highly sensitive (Meyer, 2010). When coupled to a mass spectrometer (HPLC-MS) the technique can be used to identify unknown or trace compounds through their molecular weights and fragmentation patterns.

Diverse samples require different HPLC methods due to the variation in phenolic composition. The method development process is thus essential for ensuring optimum separation to allow identification and quantification of compounds in a specific matrix. There are several aspects of the HPLC process that can be adapted to provide optimum separation. These are the gradient program, mobile phases, stationary phase (column), column temperature, flow rate and injection volume (Parejo *et al.*, 2004; Schellinger & Carr, 2006; Francisco & Resurreccion, 2009). In addition it may be necessary to adjust parameters for HPLC-DAD-MS analysis such as the injection volume and flow rate (Papadoyannis & Theodoridis, 2005).

Coupling of HPLC analysis with antioxidant assays (known as on-line antioxidant assays) can be achieved through the post-column reaction of the antioxidant reagents with the HPLC effluent. The second chromatogram indicating antioxidant activity can be matched to specific compounds in the DAD chromatogram. In this manner individual phenolic compounds exhibiting antioxidant activity can be identified (Pellegrini *et al.*, 2003).

Several papers have been published on the quantification and/or identification of phenolic compounds in plums of both *Prunus salicina* (Gil *et al.*, 2002; Kim *et al.*, 2003; Noratto *et al.*, 2009, etc.) and *Prunus domestica* (Fang *et al.*, 2002). According to compiled data on the Phenol-Explorer database (gathered from a variety of sources), neochlorogenic acid and cyanidin-3-*O*-rutinoside are the main phenolic compounds in plums (*P. domestica*) (Neveu *et al.*, 2010). Of the anthocyanins, cyanidin and its glycosides are the most common in red plums (Anon., 2007). No anthocyanins are present in cultivars with yellow peel and flesh (Lozano *et al.*, 2009). Authors have also found that there is a difference in phenolic composition between different cultivars of *P. salicina* (Kim *et al.*, 2003) and *P. domestica* (Kristl *et al.*, 2012).

The abovementioned studies were, however, primarily performed on Japanese and European plums grown in Europe and America. Currently very little information is available regarding the phenolic composition and antioxidant activity of South African plums, with only one paper making mention of the phenolic content in one South African plum cultivar (De Beer *et al.*, 2013). In order to comprehensively characterise the different cultivars and selections there is a need for a HPLC-DAD-FLD method that is applicable specifically to South African plums. One of the aims of this study was to develop and validate a method to effectively separate and quantify individual phenolic compounds present in South African plums. The method was subsequently used to identify phenolic compounds in different plum cultivars and selections using HPLC-MS, and to identify individual compounds with high antioxidant activity using an on-line antioxidant assay.

MATERIALS AND METHODS

Chemicals

Authentic reference standards for quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnoside and chlorogenic acid were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Quercetin-3-*O*-arabinoside, neochlorogenic acid and procyanidins B1 and B2 were obtained from PhytoLab (Vestenbergsgreuth, Germany). Quercetin-3-*O*-rutinoside, (+)-catechin and (-)-epicatechin were

from Sigma-Aldrich (St. Louis, MO, USA) and cyanidin-3-*O*-rutinoside and quercetin-3-*O*-galactoside from Extrasynthese (Genay, France). Cyanidin-3-*O*-glucoside and -galactoside were purchased from Polyphenols Laboratories (Sandnes, Norway). Trifluoroacetic acid (TFA) and acetonitrile (gradient grade for liquid chromatography) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid and methanol (UniVar) was purchased from Merck (Darmstadt, Germany). The ABTS (2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)) reagent was from Roche diagnostics GmbH (Indianapolis, IN, USA). Deionised water was prepared using an Elix (Merck Millipore, Darmstadt, Germany) water purification system. Deionised water was subject to an additional purification step to obtain HPLC-grade water using a Milli-Q academic (Millipore) water purification system.

Sample preparation

Plums were harvested from Bien Donn  (Groot Drakenstein, South Africa; S 33.84, E 18.98) between December and February during the 2010/2011 and 2011/2012 harvest seasons (Table 1). Twelve fruit were randomly selected from three different trees for each cultivar/selection. Harvest dates were determined by evaluation of firmness and total soluble solids parameters, as set out by the Agricultural Product Standards Act (Anon., 1991) for different cultivars. Five fruits per tree were analysed on the day of harvest, while the remaining fruits underwent a commercial cold storage regime for approximately 35 days (10 days at -0.5 C, 9 days at 7.5 C, 16 days at -0.5 C) followed by 7 days of ripening at 10 C (Anon., 2006). For this research chapter only fruit after cold storage and ripening were analysed. Fruit (5 plums per tree) were homogenized with 4 g.L⁻¹ sodium fluoride (1 ml for every 4 g fruit) and samples frozen in 50 mL centrifuge tubes at ca -20 C until extraction. For extraction, the frozen plum pulp was defrosted at room temperature and ca 5.0 g weighed into a 50 mL screw-cap centrifuge tube and 10 mL methanol added. Thereafter the tubes were shaken, placed in a sonication bath (Branson 8510, Branson Ultrasonic Corporation, CT, USA) for 10 minutes, and centrifuged to separate the solids from the extract. Centrifugation was performed for 10 min at 8000 rpm (ca 6000 x g) using a Biofuge primo Centrifuge (Thermo Scientific, AEC-Amersham, Johannesburg, South Africa). The supernatant was filtered using a Millex-HV hydrophilic polyvinylidene difluoride (PVDF) 0.45  m syringe-driven filter (Millipore). Thereafter 300  L aliquots of the filtrate were diluted with 1 mL water and frozen at ca -20 C until analysis. For HPLC-DAD-MS and on-line antioxidant analyses, a more concentrated sample was required. For this purpose, the filtrate (without dilution) was concentrated using a Savant SPD

2010 SpeedVac Concentrator (Savant SPD 2010, Thermo Scientific, MA, USA). Different time, vacuum and ramp settings were tested to establish parameters suitable to reduce the sample volume from 12 mL to 3 mL (Table 2).

Table 1 Plum selections and cultivars evaluated

Cultivars/selections	Peel colour (ripe)	Flesh Colour (ripe)	Harvest date
Sun Breeze	Yellow	Yellow	14 February 2012
Laetitia	Red	Yellow	8 February 2012
African Delight	Red	Yellow	12 February 2012
Sapphire	Red	Yellow	13 December 2011
Ruby Red	Red	Red	3 January 2012
Ruby Crunch (PR02-62) ^a	Red	Red	31 January 2012
PR02-55	Red	Red	21 December 2010
PR03-34	Red	Red	20 December 2011
PR04-32	Red	Red	17 January 2012
PR04-19	Red	Red	13 December 2011
PR04-35	Red	Red	20 December 2011

^a Selection PR02-62 was released as a cultivar, Ruby Crunch, in May 2012.

Table 2 SpeedVac parameters for concentration of plum extract

Parameter	Setting
Temperature	45°C
Ramp	5
Vacuum	10.0 Torr
Heat time	CCC (continuous heating)
Run time	4.0 h

High-performance liquid chromatography with diode-array and fluorescence detection (HPLC-DAD-FLD)

Analyses were performed using an Agilent 1200 series HPLC (Waldbronn, Germany). The system consisted of an autosampler, quaternary pump, column thermostat, diode-array detector and fluorescence detector. Chemstation software for LC 3D systems (Agilent) was used for data acquisition and analysis. A Gemini-NX C18 column (3 μm particle size, 110 \AA pore size, 150 x 4.6 mm; Phenomenex, Santa Clara, CA, USA) was used for the analysis. The column was fitted with a guard column with the same packing material (4 x 3.0 mm; Phenomenex).

The method used by De Beer *et al.* (2012) was used as the initial method of analysis for South African plums and subsequently optimized. The final method was established by experimenting with different method parameters such as temperature, solvents and gradient program during the method development period. Method parameters are shown in Table 3 and the final gradient program in Table 4. Details regarding the method development process are discussed in the *Results and discussion* section.

A DAD was used to allow quantification at different wavelengths simultaneously. Hydroxycinnamic acids were quantified at 320 nm, flavonols at 350 nm and anthocyanins at 520 nm. Flavan-3-ols were quantified with a fluorescence detector (excitation = 275 nm; emission = 315 nm). Two injection volumes (100 μL and either 40 or 50 μL) were used for samples to ensure accurate quantification of compounds present in small concentrations and those present in large amounts. A calibration mixture containing the authentic reference standards was injected at 1, 5, 10, 20, 30, 40 and 50 μL to obtain calibration ranges as follows: neochlorogenic acid (0.03-1.6 μg injected); chlorogenic acid (0.03-1.5 μg); cyanidin-3-*O*-glucoside (0.07-3.2 μg); cyanidin-3-*O*-rutinoside (0.04-2.0 μg); quercetin-3-*O*-rutinoside and -galactoside (0.01-0.5 μg); quercetin-3-*O*-glucoside, -rhamnoside and -arabinoside (0.02-1.0 μg); (+)-catechin, (-)-epicatechin, procyanidin B1 and procyanidin B2 (0.02-1.0 μg). The calibration ranges were chosen to span the variation in the phenolic compound contents of the different cultivars and selections, as determined through trials during the development process.

Table 3 Method parameters for final HPLC method

Parameter	Details
Mobile phases	Solvent A = 0.05% TFA Solvent B = Acetonitrile
Temperature	40°C
First injection volume	100 µL
Second injection volume	40 or 50 µL
Flow rate	1 ml.min ⁻¹

Table 4 Final solvent gradient program for analysis of plum samples

Time (min.)	Acetonitrile (%)
0	3
1	3
30	35
31	50
33	50
35	3
45	3

HPLC method validation

Seven-point calibration curves were set up for all of the standards (as described in the previous section) in order to test the linearity of the DAD and FLD responses. Linear regression on the calibration curve data for each compound was performed using the least squares method (Microsoft Excel 2003, Microsoft Corporation, Redmond, WA, USA). The slope, y-intercept and correlation coefficients (R^2) were determined.

Two representative plum samples (Ruby Red and African Delight) and a standard calibration mixture were injected over a 28.2 hour period to evaluate sample stability. The standard calibration mixtures were prepared in the same manner as for HPLC analysis and injected at 1 µL and 30 µL and the plum samples injected at 100 µL. The relative standard deviation (RSD) over all injections and percentage change over the period were determined for each compound.

To determine the inter- and intra-day precision the samples and standard mixtures were injected six times each day for three consecutive days. The same injection volumes were used as for stability analysis. The RSD for each compound in the samples and standards was calculated for the replicate injections per day, as well as for the mean values of the three days.

Liquid chromatography-mass spectrometry (HPLC-DAD-MS)

HPLC-DAD-MS analyses were performed using a Waters Synapt G2 system (Waters, Milford, MA, USA) with an electrospray ionization (ESI) source. A Waters Acquity ultra-high-pressure liquid chromatography (UPLC) system equipped with autosampler, binary pump and DAD was used under HPLC conditions. Separation was performed using the same temperature and gradient program as for HPLC-DAD-FLD. An external column heating compartment was used. The settings for HPLC-DAD-MS are shown in Table 5. MassLynx software (Version 4.1, Waters) was used to analyse the mass spectra.

Table 5 Method parameters for HPLC-MS

Parameters	Settings
Ionisation	ESI positive
Capillary voltage	3 kV
Cone voltage	15 V
Mode	Resolution
MS/MS mode	MS ^E
Injection volume	10 µL
Split	60:40
Lock mass	Leucine enkaphalin
Collision energy	15-60 eV
Source temperature	120°C
Desolvation temperature	275°C
Cone gas	50 L.h ⁻¹
Desolvation gas	650 L.h ⁻¹

On-line antioxidant analysis

The method of Pellegrini *et al.* (2003) was modified for the on-line ABTS^{•+} antioxidant assay. The ABTS^{•+} stock solution was prepared according to Pellegrini *et al.* (1999): 440 μL 140 mM potassium-persulphate solution was added to 25 mL 7 mM ABTS. The ABTS^{•+} stock solution was stored at room temperature for 12-16 h before analysis to allow reaction time between ABTS and potassium persulfate. The final working solution (25 mL ABTS^{•+} stock added to 1 L 75 mM potassium phosphate buffer at pH 7.4) was filtered before analysis and placed in a cooling unit (4°C) to stabilise the radical for the duration of the analysis. The combination of solvents (TFA, acetonitrile and the phosphate buffer reagent) was evaluated beforehand to ensure that no precipitation would occur during analysis.

The Agilent HPLC system used for quantification of phenolic compounds was coupled to additional components installed for the on-line antioxidant activity assay. The on-line system consisted of a second pump (LKB Bromma 2150) which pumps the ABTS^{•+} reagent to a high pressure static mixing tee at 0.5 $\text{ml}\cdot\text{min}^{-1}$, allowing post-column addition of the reagent to the HPLC effluent flow. The combined reagents flowed through a reaction coil (15.24 m PEEK tubing) which allows time (36 s) for reaction between the separated compounds and the ABTS^{•+} reagent. The decrease in absorbance caused by the reaction was detected by a variable wavelength detector (VWD) (Agilent 1200 series HPLC, Waldbronn, Germany Agilent) at 600 nm. The absorbance was indicated as a second chromatogram with negative peaks.

Trolox (40 μL of a 1.25 $\text{mg}\cdot\mu\text{L}^{-1}$ solution in ethanol) was added to 1000 μL of concentrated plum extract as internal standard. The mixture was injected at 5 μL . Trolox served as reference to align the antioxidant peaks to correspond to the phenolic compounds on the DAD chromatogram.

RESULTS AND DISCUSSION

HPLC method development

Optimization of the HPLC method previously used for quantification of the major phenolic compounds (5 compounds only) in plums (De Beer *et al.*, 2012) was necessary. Improvement in the separation of a number of phenolic compounds present at lower concentrations was needed to enable their quantification. Using the optimised method a total of 18 compounds could be quantified and several others identified from subsequent HPLC-DAD-MS analysis. The HPLC

method as previously used in the analysis of South African plums (De Beer *et al.*, 2012) employed 7.5% formic acid in water (Solvent A) and 7.5% formic acid in acetonitrile (Solvent B) as mobile phases, and only quantified five compounds. These conditions were used as basis and the column temperature, gradient program, and mobile phases were adjusted systematically until optimum separation was achieved.

As the Gemini-NX C18 column provided satisfactory peak shapes, other stationary phases were not investigated. Firstly, different analysis temperatures were evaluated, namely 25°C, 35°C and 40°C (in addition to the original 30°C). Of these options the sample runs performed at 40°C displayed the best separation. Other gradient programs were also evaluated with different run times.

Temperature adjustments did not result in the desired separation for all major compounds and the use of a different mobile phase was explored, namely trifluoroacetic acid (TFA). TFA has been used in previous polyphenol analyses with HPLC (Ibern-Gómez *et al.*, 2002; Baranowski *et al.*, 2004; Kim *et al.*, 2008) to ensure the desired acidic conditions needed for the separation of anthocyanins. It is required that the pH should be below 2 to keep the anthocyanin compound in its flavylium cation form (red colour) for analysis (Mazza & Miniati, 1993). The solvent gradient program was adjusted numerous times in order to reach the desired separation. The use of the fluorescence detector was also added to the method protocol to allow quantification of flavan-3-ol compounds. In the final method 0.05% TFA (Solvent A) and 100% acetonitrile (Solvent B) were used as mobile phases. This method could be successfully applied for HPLC-DAD-MS analysis with minor changes.

TFA has been used in previous studies during HPLC analysis of phenolic compounds, but is normally advised against for HPLC-MS analysis due to suppression of ionisation (Emmert & Rueck, 2006). However, 0.05% TFA was tested and found suitable during mass spectrometry analysis for the identification of plum phenolic compounds. Chromatograms for cultivars and selections displaying separation of compounds will be discussed in the following sections along with their identification. Enlarged sections of chromatograms for each wavelength used for quantification are shown in Fig. 1 to show separation and identities of compounds of interest.

Identification of phenolic compounds using HPLC-DAD-MS

Several phenolic compounds from four phenolic groups were identified using HPLC-DAD-MS by comparing UV-Vis spectra, mass-to-charge ratio (m/z) values for the pseudo-molecular ion and

compound fragments to that of literature and/or authentic standards. During HPLC-DAD-FLD certain compounds were identified by comparing their UV-Vis spectra and retention times to that of authentic reference standards. The identities of these compounds were confirmed during HPLC-DAD-MS. Additional peaks were identified from their mass spectrometry data (MS and MS/MS fragments). All peaks and their corresponding UV-Vis and mass spectrometry data are listed in Table 6. Table 7 shows the occurrence of phenolic compounds not present in all plum cultivars and selections, while Table 8 displays the structures of identified phenolic compounds.

In the phenolic acid category neochlorogenic acid (**3**) and chlorogenic acid (**7**) (Table 6) were identified and confirmed using reference standards. Generally, neochlorogenic acid is the predominant compound in plums, whether Japanese or European (Möller & Herrmann, 1983; Nakatani *et al.*, 2000; Tomás-Barberán *et al.*, 2001). The same was found for South African plums in the current study, although this compound was absent in the cultivar Sapphire. Neochlorogenic acid and chlorogenic acid are isomers (3-*O*-caffeoylquinic acid and 5-*O*-caffeoylquinic acid, respectively) and thus have the same pseudo-molecular (m/z 355) and fragment (m/z 163) ions. Chlorogenic acid is also found often in plums, although in lesser amounts than neochlorogenic acid (Möller & Herrmann, 1983; Slimestad *et al.*, 2009). A similar trend was observed for several South African plum cultivars and selections. An unidentified hydroxycinnamic acid (**5**) was detected in almost every cultivar and selection (Fig. 2, Table 7). The compound was identified as a 3-*O-p*-coumaroylquinic acid from its mass fragments. The presence of a *p*-coumaroylquinic acid has previously been confirmed in plums (Donovan *et al.*, 1998; Fang *et al.*, 2002; Slimestad *et al.*, 2009). Möller and Herrmann (1993) and Fang *et al.* (2002) found the 3-, 4- and 5-isomers of *p*-coumaroylquinic acid present in stone fruit (including *P. domestica*). Based on the knowledge that the 3-isomers are the most abundant in stone fruit it is believed that the 3-*O-p*-coumaroylquinic acid was the derivative present in South African plums.

The anthocyanins, cyanidin-3-*O*-rutinoside and –glucoside, were present in every plum sample except the yellow cultivar, Sun Breeze (Fig. 2). Laetitia was the only cultivar containing three anthocyanin compounds. Cyanidin-3-*O*-galactoside (**8**) was identified in addition to cyanidin-3-*O*-glucoside (**10**) and –rutinoside (**11**) in this cultivar (Fig. 1, C). Cyanidin-3-*O*-galactoside (m/z 449) has the same pseudomolecular ion and MS/MS fragment (m/z 287) as cyanidin-3-*O*-glucoside and eluted just before cyanidin-3-*O*-glucoside. Authentic reference standards for the three anthocyanins were injected to confirm the identities of the compounds. The elution order of cyanidin compounds corresponds to that found by Abad-García *et al.* (2009) for the analysis of

fruit juice, and the three identified anthocyanins have been found previously in plums (Tomás-Barberán *et al.*, 2001; Wu & Prior, 2005).

Four quercetin glycosides were identified after comparing their UV-Vis spectra and retention times to authentic standards (Fig. 2). The compounds were quercetin-3-*O*-glucoside (**17**), -rhamnoside (**21**), -arabinoside (**20**) and -rutinoside (**15**). All of these have previously been detected in plums (Williams & Wender, 1953; Tomás-Barberán *et al.*, 2001; Kim *et al.*, 2003). Research has shown that rutin is the predominant quercetin glycoside found in plums (Kim *et al.*, 2003; Slimestad *et al.*, 2009). Four other flavonol compounds (**16**, **18**, **19** and **22**) were present in the majority of the cultivars. From the HPLC-DAD-MS data these compounds were confirmed to be quercetin derivatives due to the presence of a fragment for the quercetin aglycone (m/z 303). Peak **16** was tentatively identified as a quercetin pentosyl-hexoside and peak **18** as quercetin-3-*O*-xyloside. Quercetin-3-*O*-arabinoside and -xyloside have the same pseudomolecular ion (m/z 435) and MS/MS fragments (303, 229). The sugars xylose and arabinose are isomers resulting in the same molecular mass. Quercetin-3-*O*-arabinoside was distinguished from -xyloside during HPLC analysis by comparison of their retention times to that of an authentic standard of quercetin-3-*O*-arabinoside. Peak **22** was tentatively identified as an acetylated quercetin-hexoside, based on the loss of m/z 204, which matches the mass of a hexoside and acetyl residue (162 and 42 amu, respectively) (Barros *et al.*, 2012). Slimestad and Hostettmann (1996) classified an unknown phenolic compound in Norwegian spruce with ions with m/z 507 and 303 as quercetin 3-*O*-(6-acetyl) glucoside. Tomás-Barberán *et al.* (2001) also mention the presence of small amounts of this compound in plums. Peak **19** was identified as a quercetin derivative (m/z 303) based on data from a study by Tomás-Barberán *et al.* (2001). The compound was tentatively assigned the identity of quercetin pentosyl-pentoside. The authors found this compound with pseudomolecular ion m/z 567 and fragment m/z 229 in addition to 303 in plum peels.

In the flavan-3-ol category the presence of (+)-catechin (**6**), (-)-epicatechin (**12**) and procyanidin B1 and B2 (**4** and **9**) were confirmed by comparing their retention times to authentic reference standards (Table 6). Flavan-3-ol monomers (+)-catechin (C) and (-)-epicatechin (EC) ($[M+H]^+$ m/z 291) are the basic sub-units for flavan-3-ol dimers and trimers in fruit. The presence of A-type procyanidin dimers (m/z 577) was found by interpretation of MS data. A-type dimers were not visible on the chromatogram, and peak numbers were not assigned. Due to the inter-catechin bonds of this compound it has two mass units less than B-type procyanidin dimers (m/z 579) (Nunes *et al.*, 2008). Mass fragments also correspond to that of A-type procyanidins (m/z 425, 287) previously found in plums (Tomás-Barberán *et al.*, 2001; Nunes *et al.*, 2008). Gu *et*

al. (2003) reported that the m/z 577 ion of the A-type trimer represents an interflavan bond between the middle and the base unit. This compound would then be composed of (+)-catechin or (-)-epicatechin units in the sequence (E)C-(E)C-A-(E)C (Ou *et al.*, 2012). Other compounds were recognized as procyanidin B-type trimers (**2** and **14**) and an A-type trimer (**13**), with pseudomolecular ions corresponding to m/z 867 and 865, respectively. However, the specific dimers and trimers could not be distinguished due the lack of authentic standards. Possible flavan-3-ol B-type trimers may be procyanidin C2 (C-(4,8)-C-(4,8)-C) or EEC (EC-(4,8)-EC-(4,8)-C). Procyanidin B-type dimers could be procyanidin B3, B4, or B5 (De Pascual-Teresa *et al.*, 2000) and A-type dimers procyanidin A1 or A2 (Table 8).

Table 6 Phenolic compounds detected and identified using HPLC-DAD-MS

Peak nr.	t _R (min) ^a	λ _{max}	[M+H] ⁺ /M ⁺ ^b	Na-adduct ions	Fragment ions	Phenolic compound
1	5.5	275	353	-	177, 160*	Unknown compound 1
2	6.6	279	867	-	579*, 247	B-type procyanidin trimer 1
3	8.4	324	355	-	163	Neochlorogenic acid ^c
4	10.3	278	579	-	427, 409*, 291	Procyanidin B1 ^c
5	10.6	312	339	-	147	3- <i>O-p</i> -Coumaroylquinic acid
6	10.8	278	291	-	139	(+)-Catechin ^c
7	11.8	325	355	-	163	Chlorogenic acid ^c
8	12.4	276, 515	449	-	287	Cyanidin-3- <i>O</i> -galactoside ^c
9	12.7	278	579	-	-	Procyanidin B2 ^c
10	12.9	276, 515	449	-	287	Cyanidin-3- <i>O</i> -glucoside ^c
11	13.5	280, 515	595	-	287	Cyanidin-3- <i>O</i> -rutinoside ^c
12	13.3	278	291	-	139	(-)-Epicatechin ^c
13	14.8	278	865	-	577, 425*, 287	A-type procyanidin trimer
14	15.3	279	867	-	579*, 409, 291, 247	B-type procyanidin trimer 2
N.A. ^d	16.8	278	577	-	425, 287*	A-type procyanidin dimer 1
15	18.5	254, 352	611	633	465, 303*, 229	Quercetin-3- <i>O</i> -rutinoside ^c
16	18.9	254, 351	597	619	303*, 229	Quercetin pentosyl-hexoside
17	19.1	254, 353	465	-	303*	Quercetin-3- <i>O</i> -glucoside ^c
N.A. ^d	19.1	278	577	-	425, 287*	A-type procyanidin dimer 2
18	19.9	254, 351	435	891, 457	303*, 229	Quercetin-3- <i>O</i> -xyloside
19	20.1	254, 351	567	589	303*, 229	Quercetin pentosyl-pentoside
20	20.6	255, 352	435	891, 457	303*, 229	Quercetin-3- <i>O</i> -arabinoside ^c
21	21.1	255, 347	449	919, 471	303*, 229	Quercetin-3- <i>O</i> -rhamnoside ^c
22	21.6	256, 351	507	529	303*, 229	Acetylated quercetin-hexoside

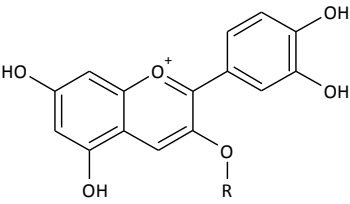
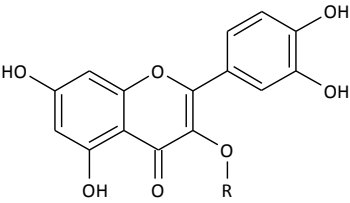
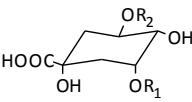
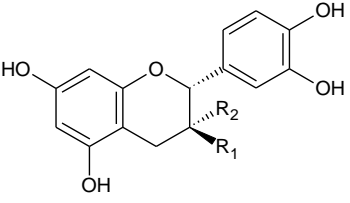
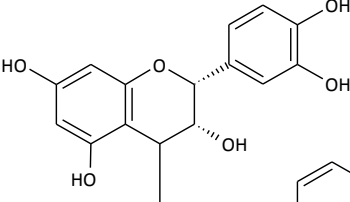
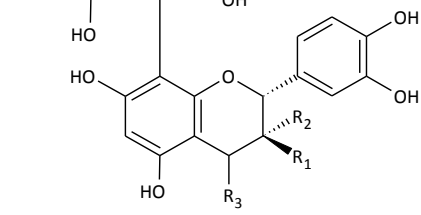
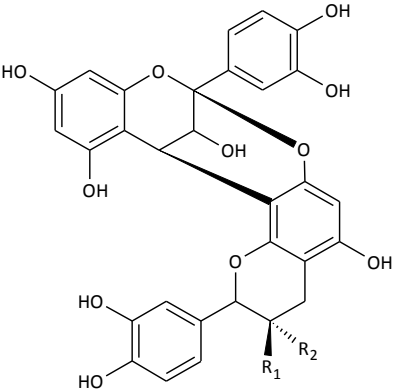
*most abundant fragment ion; ^a equivalent retention times for HPLC-DAD-FLD analysis using the Agilent 1200 HPLC to facilitate comparison with chromatograms; ^b M⁺ for anthocyanins and [M+H]⁺ for other compounds; ^c identified based on comparison to authentic reference standard; ^d no visible peaks on chromatograms.

Table 7 Occurrence of phenolic compounds not present in all plum cultivars and selections

Cultivar/ selection	Neochloro- genic acid	Chlorogenic acid	3- <i>O</i> - <i>p</i> - Coumaroyl- quinic acid	Cyanidin-3- <i>O</i> - glucoside	Cyanidin-3- <i>O</i> - rutinoside	Quercetin pentosyl- hexoside	A-type procyanidin dimer 2	B-type procyanidin trimer 2
Sun Breeze	+	N.D.	+	N.D.	N.D.	+	N.D.	+
Laetitia	+	N.D.	+	+	+	+	N.D.	N.D.
African Delight	+	+	N.D.	+	+	+	+	+
Sapphire	N.D.	N.D.	N.D.	+	+	+	N.D.	+
Ruby Red	+	N.D.	+	+	+	N.D.	+	+
Ruby Crunch	+	N.D.	N.D.	+	+	+	N.D.	+
PR02-55	+	N.D.	N.D.	+	+	N.D.	+	N.D.
PR03-34	+	+	+	+	+	+	N.D.	+
PR04-32	+	N.D.	N.D.	+	+	N.D.	+	+
PR04-19	+	N.D.	N.D.	+	+	+	N.D.	+
PR04-35	+	+	+	+	+	N.D.	+	+

+ = present; N.D. = not detected.

Table 8 Structures of phenolic compounds present in plums

General structure	Nr.	Phenolic compound	Substituents
	8	<i>Anthocyanins</i> Cyanidin-3- <i>O</i> -galactoside	R = galactosyl
	10	Cyanidin-3- <i>O</i> -glucoside	R = glucosyl
	11	Cyanidin-3- <i>O</i> -rutinoside	R = rutinosyl
	15	<i>Flavonols</i> Quercetin-3- <i>O</i> -rutinoside	R = rutinosyl
	17	Quercetin-3- <i>O</i> -glucoside	R = glucosyl
	18	Quercetin-3- <i>O</i> -xyloside	R = xylosyl
	20	Quercetin-3- <i>O</i> -arabinoside	R = arabinosyl
	21	Quercetin-3- <i>O</i> -rhamnoside	R = rhamnosyl
	3	<i>Hydroxycinnamic acids</i> Neochlorogenic acid	R ₁ = H; R ₂ = caffeoyl
	5	<i>p</i> -Coumaroylquinic acid	R ₁ = <i>p</i> -coumaroyl; R ₂ = H
	7	Chlorogenic acid	R ₁ = caffeoyl; R ₂ = H
	6	<i>Flavan-3-ols</i> (+)-Catechin	R ₁ = OH; R ₂ = H
	12	(-)-Epicatechin	R ₁ = H; R ₂ = OH
	4	<i>B-type procyanidin dimers</i> Procyanidin B1	R ₁ = OH; R ₂ = H
	9	Procyanidin B2	R ₁ = H; R ₂ = OH
		<i>B-type procyanidin trimers</i> Procyanidin C1	R ₁ = H; R ₂ = OH; R ₃ = (4,8)-EC
		<i>A-type procyanidin dimers</i> Procyanidin A1	R ₁ = H; R ₂ = OH
		Procyanidin A2	R ₁ = OH; R ₂ = H

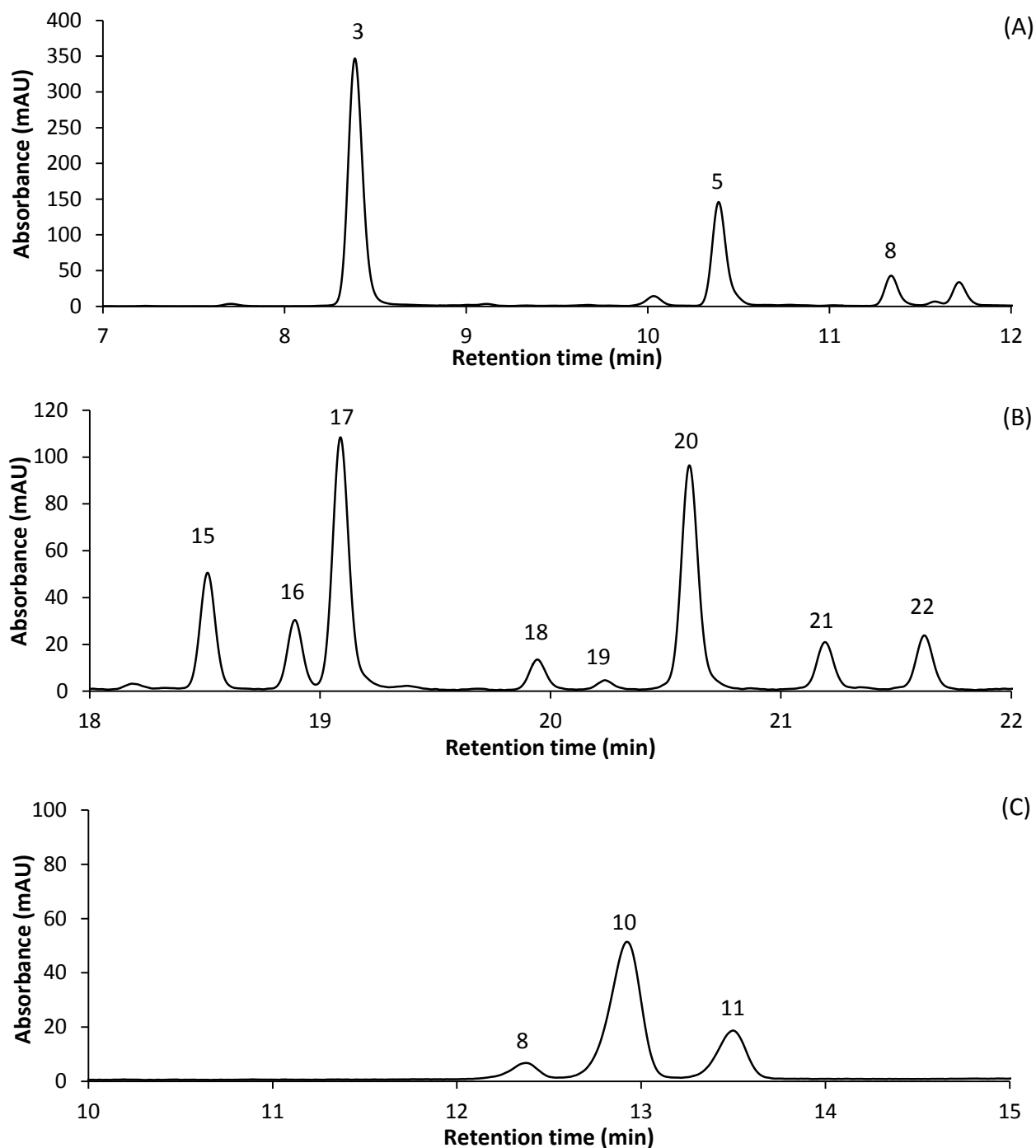


Figure 1 Enlarged sections of chromatograms for different phenolic groups in selected plum cultivars/selections (see Table 6 for peak numbers). (A) phenolic acids in PR04-35 at 320 nm (B) flavonols in PR04-19 at 350 nm (C) anthocyanins in Laetitia at 520 nm (D) flavan-3-ols in Ruby Crunch using fluorescence detection.

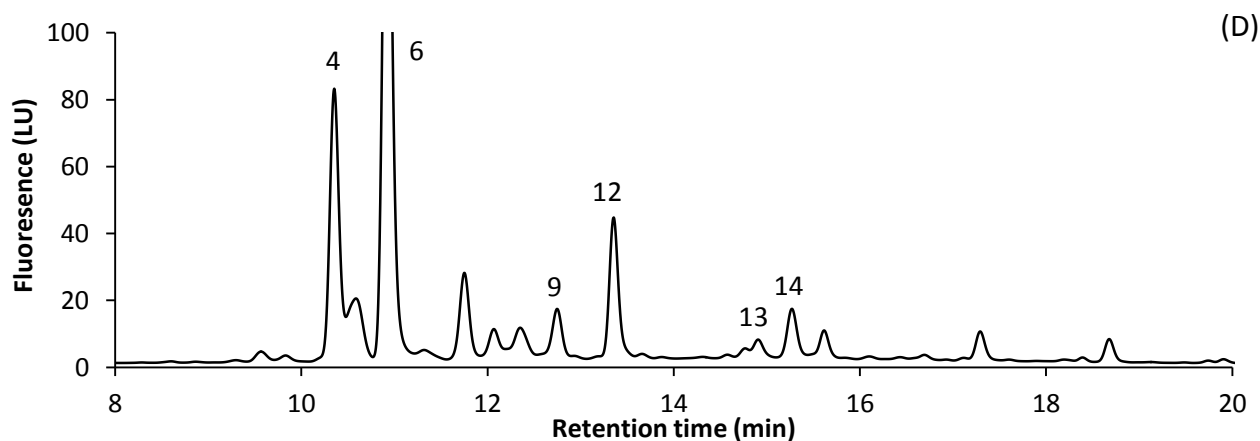


Figure 1 (continued) Enlarged sections of chromatograms for different phenolic groups in selected plum cultivars/selections (see Table 6 for peak numbers). (A) phenolic acids in PR04-35 at 320 nm (B) flavonols in PR04-19 at 350 nm (C) anthocyanins in Laetitia at 520 nm (D) flavan-3-ols in Ruby Crunch using fluorescence detection.

HPLC method validation

The linearity for authentic reference standards as shown in Table 9 was acceptable ($r > 0.999$). Pearson correlation values (r) were equal to 1 up to three decimal places, and y -intercepts were relatively low.

In terms of compound stability the % RSD for compounds in the standard mixtures were all below 5% (Table 10). Over the 28 h period the % change of the calibration mixture was between -5 and 5% in all cases, except for quercetin-3-*O*-rutinoside in the 1 μ L injection (6.8%) and cyanidin-3-*O*-rutinoside in the 30 μ L injection (-5.7%). The stability of compounds in the Ruby Red and African Delight reference samples also displayed a good stability. No compounds showed a % change lower than -5% or higher than 5% or % RSD higher than 5%. Due to the low % RSD and % change values of 28 h one could suggest the samples may be prepared up to 24 hrs in advance and degradation of compounds will not likely occur over this time period.

Excellent intra-day precision results (% RSD $\leq 4.1\%$) were obtained for all compounds in standard mixtures as well as reference samples (Table 11). The inter-day precision (% RSD) of the phenolic acid and anthocyanin compounds, as well as quercetin-3-*O*-arabinoside, were excellent (< 5%) in standard mixtures and samples. The inter-day precision for the other flavonol compounds in standard mixtures were not as good (% RSD between 5 and 10%), although values for samples were all below 5%. On the other hand, the inter-day precision of the flavan-3-ol

compounds was greater than 5% in most cases and between 10 and 13.2% in some cases. Poor precision can be attributed to either small peak areas or difficulty integrating peaks due to interference from unidentified co-eluting compounds. In the case of the flavan-3-ol compounds co-elution is a problem warranting future improvement of the method.

Table 9 Linear regression data for calibration curves

Compound	Calibration range (μg injected)	Slope	Y-Intercept	<i>r</i>
Neochlorogenic	0.03-1.60	2482.8	-9.1	1.000
Chlorogenic acid	0.03-1.52	2680.0	-16.6	1.000
Cyanidin-3- <i>O</i> -glucoside	0.07-3.57	1889.1	6.9	1.000
Cyanidin-3- <i>O</i> -rutinoside	0.04-2.00	1918.4	8.1	1.000
Quercetin-3- <i>O</i> -rutinoside	0.01-0.51	1622.9	-1.4	1.000
Quercetin-3- <i>O</i> -glucoside	0.02-1.02	1692.1	-4.1	1.000
Quercetin-3- <i>O</i> -arabinoside	0.2-0.98	1854.6	-11.9	1.000
Quercetin-3- <i>O</i> -rhamnoside	0.02-0.99	1911.1	-7.5	1.000
(+)-Catechin	0.2-0.99	1444.6	3.4	1.000
(-)-Epicatechin	0.02-1.0	1121.7	1.59	1.000
Procyanidin B1	0.02-0.99	415.6	0.40	1.000
Procyanidin B2	0.02-1.00	750.3	-0.11	1.000

Table 10 Stability of compounds (% RSD^a and % change) of calibration mixtures and two plum samples over a period of 28.2 h

Compound	Standard: 1 μ L		Standard: 30 μ L		Ruby Red		African Delight	
	% RSD	% change	% RSD	% change	% RSD	% change	% RSD	% change
Neochlorogenic	1.1	2.3	0.1	0.1	0.3	0.8	0.3	1.1
Chlorogenic acid	1.0	0	0.1	0.4	N.D.	N.D.	1.3	2.8
3- <i>O-p</i> -Coumaroylquinic acid	N.A.	N.A.	N.A.	N.A.	1.4	-1.6	N.D.	N.D.
Cyanidin-3- <i>O</i> -glucoside	1.5	-1.4	1.4	-4.4	0.3	-0.7	0.8	-0.8
Cyanidin-3- <i>O</i> -rutinoside	2.4	-4.3	1.9	-5.7	0.7	-1.6	2.2	-1.3
Quercetin-3- <i>O</i> -rutinoside	2.7	6.8	0.1	0.1	0.2	0.2	0.3	1.1
Quercetin pentosyl-hexoside	N.A.	N.A.	N.A.	N.A.	N.D.	N.D.	2.1	1.9
Quercetin-3- <i>O</i> -glucoside	1.0	0	0.2	0	0.4	1.1	0.4	1.0
Quercetin-3- <i>O</i> -arabinoside	0.8	2.3	0.6	0	0.4	-1.1	0.5	1.1
Quercetin-3- <i>O</i> -rhamnoside	0.9	1.3	0.6	-0.1	0.3	0.8	1.5	2.5
Quercetin-3- <i>O</i> -xyloside	N.A.	N.A.	N.A.	N.A.	0.4	1.2	1.4	2.3
Quercetin pentosyl-pentoside	N.A.	N.A.	N.A.	N.A.	0.5	1.5	2.0	-2.0
Acetylated quercetin-hexoside	N.A.	N.A.	N.A.	N.A.	0.3	0.7	0.3	0.6
(+)-Catechin	1.3	0.30	1.3	-0.6	1.1	2.6	1.1	2.1
(-)-Epicatechin	1.5	0.4	1.3	-0.7	1.5	2.0	2.0	3.0
Procyanidin B1	1.5	1.1	1.2	-0.9	1.4	2.8	1.2	3.4
Procyanidin B2	1.7	0.9	1.4	-0.9	2.3	0.9	2.0	3.1

^a RSD = Relative Standard Deviation; N.A. = not applicable; N.D. = not detected

Table 11 Intra- and inter-day precision (% RSD) for selected phenolic compounds

Sample	Compound	Day 1 (n=6)	Day 2 (n=6)	Day 3 (n=6)	Inter-day (n=3)
Standard: 1 μL ^a	Neochlorogenic acid	2.1	0.6	0.7	3.0
	Chlorogenic acid	1.6	0.8	0.6	2.4
	Cyanidin-3- <i>O</i> -glucoside	2.2	1.9	1.7	4.2
	Cyanidin-3- <i>O</i> -rutinoside	2.4	1.5	1.5	3.8
	Quercetin-3- <i>O</i> -rutinoside	2.0	1.2	2.3	5.7
	Quercetin-3- <i>O</i> -glucoside	1.2	1.0	3.0	2.7
	Quercetin-3- <i>O</i> -arabinoside	1.4	0.5	1.3	3.5
	Quercetin-3- <i>O</i> -rhamnoside	1.2	2.3	1.8	5.3
	(+)-Catechin	2.1	3.1	0.9	7.1
	(-)-Epicatechin	2.3	3.5	0.8	6.4
	Procyanidin B1	2.5	3.2	1.4	1.2
	Procyanidin B2	2.5	3.6	0.9	6.6
Standard: 30 μL ^a	Neochlorogenic acid	0.2	0.1	0.2	0.3
	Chlorogenic acid	0.2	0.1	0.2	2.3
	Cyanidin-3- <i>O</i> -glucoside	0.2	0.3	0.1	0.7
	Cyanidin-3- <i>O</i> -rutinoside	0.3	0.5	0.2	2.2
	Quercetin-3- <i>O</i> -rutinoside	0.1	0.1	0.1	7.5
	Quercetin-3- <i>O</i> -glucoside	0.2	0.2	0.2	6.1
	Quercetin-3- <i>O</i> -arabinoside	0.6	0.5	0.2	3.7
	Quercetin-3- <i>O</i> -rhamnoside	0.1	0.1	0.2	6.1
	(+)-Catechin	0.4	0.1	0.2	5.3
	(-)-Epicatechin	0.2	0.3	0.3	4.6
	Procyanidin B1	0.3	0.2	0.3	7.5
	Procyanidin B2	0.2	0.3	0.3	4.6
Ruby Red	Neochlorogenic acid	0.2	0.2	0.2	1.1
	Chlorogenic acid	N.D.	N.D.	N.D.	N.D.
	3- <i>O</i> - <i>p</i> -Coumaroylquinic acid	1.7	1.7	0.3	1.6
	Cyanidin-3- <i>O</i> -glucoside	0.1	0.0	0.1	2.2
	Cyanidin-3- <i>O</i> -rutinoside	0.3	0.1	0.2	1.4
	Quercetin-3- <i>O</i> -rutinoside	0.4	0.1	0.2	1.0
	Quercetin pentosyl-hexoside	N.D.	N.D.	N.D.	N.D.
	Quercetin-3- <i>O</i> -glucoside	0.1	0.1	0.1	1.5
	Quercetin-3- <i>O</i> -arabinoside	0.3	0.1	0.4	2.3
	Quercetin-3- <i>O</i> -rhamnoside	0.1	1.5	0.2	1.2
	Quercetin-3- <i>O</i> -xyloside	0.1	0.3	1.1	2.7
	Quercetin pentosyl-pentoside	0.5	0.4	3.1	3.2
	Acetylated quercetin-hexoside	0.2	0.4	0.3	1.6
	(+)-Catechin	0.5	0.2	0.1	6.4
	(-)-Epicatechin	0.6	1.0	1.2	7.4
	Procyanidin B1	1.4	4.1	3.2	4.2
Procyanidin B2	0.2	2.2	0.3	8.7	

^a Neochlorogenic acid, chlorogenic acid = 0.03 mg.mL⁻¹; Cyanidin-3-*O*-glucoside = 0.07 mg.mL⁻¹; Cyanidin-3-*O*-rutinoside = 0.04 mg.mL⁻¹; Quercetin-3-*O*-rutinoside = 0.01 mg.mL⁻¹; Quercetin-3-*O*-glucoside, -arabinoside, -rhamnoside, (+)-catechin, (-)-epicatechin, procyanidin B1, procyanidin B2 = 0.02 mg.mL⁻¹.

Table 11 (continued) Intra- and inter-day precision (% RSD) for selected phenolic compounds

Sample	Compound	Day 1 (n=6)	Day 2 (n=6)	Day 3 (n=6)	Inter-day (n=3)
African Delight	Neochlorogenic acid	0.9	0.1	0.1	0.8
	Chlorogenic acid	1.2	0.5	1.0	2.9
	3- <i>O-p</i> -Coumaroylquinic acid	N.D.	N.D.	N.D.	N.D.
	Cyanidin-3- <i>O</i> -glucoside	1.0	0.4	0.6	1.5
	Cyanidin-3- <i>O</i> -rutinoside	2.6	2.1	2.2	1.1
	Quercetin-3- <i>O</i> -rutinoside	0.1	0.1	0.2	0.5
	Quercetin pentosyl-hexoside	0.3	2.0	0.2	2.9
	Quercetin-3- <i>O</i> -glucoside	0	0.2	0.2	1.0
	Quercetin-3- <i>O</i> -arabinoside	0.5	0.6	0.1	2.2
	Quercetin-3- <i>O</i> -rhamnoside	1.3	0.5	0.7	1.0
	Quercetin-3- <i>O</i> -xyloside	0.9	1.2	1.0	2.3
	Quercetin-pentosyl-pentoside	3.4	3.4	1.7	0.8
	Acetylated quercetin-hexoside	0.2	0.4	0.3	1.6
	(+)-Catechin	0.9	0.4	0.7	7.4
	(-)-Epicatechin	0.7	0.7	1.2	11.6
	Procyanidin B1	1.4	0.9	1.3	9.6
	Procyanidin B2	0.7	0.6	0.8	13.2

^a Neochlorogenic acid, chlorogenic acid = 0.03 mg.mL⁻¹; Cyanidin-3-*O*-glucoside = 0.07 mg.mL⁻¹; Cyanidin-3-*O*-rutinoside = 0.04 mg.mL⁻¹; Quercetin-3-*O*-rutinoside = 0.01 mg.mL⁻¹; Quercetin-3-*O*-glucoside, -arabinoside, -rhamnoside, (+)-catechin, (-)-epicatechin, procyanidin B1, procyanidin B2 = 0.02 mg.mL⁻¹.

On-line antioxidant analysis

The ABTS^{•+} on-line antioxidant assay is a useful analysis tool with one of the advantages being the ability to evaluate the individual antioxidant contribution of each phenolic compound. Since the compounds in a complex mixture are separated before analysis, the antioxidant activities caused by the combined effect of phenolic compounds (as with microplate assays) are not taken into consideration (Raudonis *et al.*, 2012). The total antioxidant activity of a sample is often thought to be attributed to the combined effects of polyphenols or other compounds. Post-column detection can in some cases be used to quantitatively determine the antioxidant activity of individual compounds. During the current study this was not possible due to co-eluting peaks, resulting in broad co-eluted antioxidant response peaks, and oligomeric flavan-3-ols that were not separated, resulting in an irregular antioxidant response baseline (Rzeppa *et al.*, 2011). Phenolic groups could be quantified at different wavelengths, but were not distinguished in the antioxidant response. Compounds with similar retention times may result in enlarged or split antioxidant response peaks. The concentration of phenolic compounds in each cultivar should also be taken into consideration when examining its antioxidant activity, and was not discussed in this chapter.

All eleven cultivars and selections were analysed using the on-line antioxidant activity assay and for the majority a large antioxidant response peak was observed corresponding to neochlorogenic acid, indicating that this compound has a large contribution to the antioxidant activity of the sample. Generally the greatest antioxidant responses in each chromatogram corresponded to that of the flavan-3-ol compounds (especially that of procyanidin B1 and (+)-catechin) and in some cultivars the anthocyanins (Fig. 2). Anthocyanins display a high antioxidant activity corresponding to cyanidin-3-*O*-glucoside (**10**) and –rutinoside (**11**) in all samples except Laetitia (low anthocyanin content) and Sun Breeze (no anthocyanins). The antioxidant responses for the two anthocyanins were very similar despite the fact that cyanidin-3-*O*-rutinoside was present in higher concentrations than cyanidin-3-*O*-glucoside. In certain cultivars/selections (Sapphire, Ruby Red, PR02-55, PR04-19 and PR04-35) a slight split is visible in the antioxidant response peak of cyanidin-3-*O*-glucoside. Upon evaluation of the UV-Vis chromatogram (288 nm) it is seen that procyanidin B2 (**9**) elutes right before cyanidin-3-*O*-glucoside, resulting in a combined antioxidant response. Where three anthocyanins were identified (Laetitia), cyanidin-3-*O*-rutinoside displayed the greatest antioxidant response, followed by cyanidin-3-*O*-glucoside and cyanidin-3-*O*-galactoside. Regarding the different glycoside substitutions Wang *et al.* (1999) reported that the antioxidant activity of anthocyanins with fewer sugar substitutions display a higher antioxidant activity. In agreement Kähkönen and Heinonen (2003) found that the antioxidant activities of anthocyanin-glucosides (in the form of authentic reference standards) were higher than derivatives with arabinoside, galactoside and rutinoside substitution (as determined with the DPPH[•] assay). This was not always apparent for many South African plum samples since cyanidin-3-*O*-rutinoside (**11**) elutes with (-)-epicatechin (**12**), resulting in a greater antioxidant response peak. In red plums (and other red fruit) the anthocyanins provide the greatest contribution to the total antioxidant activity (as determined by the Vitamin C Equivalent Antioxidant Capacity assay) (Chun *et al.*, 2003).

Large antioxidant response peaks were generally observed for the flavan-3-ols. Procyanidin B1 and catechin showed a large antioxidant response for all cultivars and selections, with the exception of PR04-19, which had a low content of these compounds. In some cultivars the antioxidant response of catechin combined with that of 3-*O*-*p*-coumaroylquinic acid (**5**), due to close retention times. Tentatively identified B-type procyanidin trimers (peaks **2** and **14**) were also observed to display a relatively large antioxidant response compared to their UV-Vis peak areas. The UV-Vis peaks for these compounds at 288 nm are very small, and still a large antioxidant peak can be seen. One should keep in mind that the UV-Vis peaks are not an accurate representation of

the flavan-3-ol concentration, as these compounds generally display low extinction coefficients in UV-Vis compared to the flavonols. Antioxidant peaks were matched with flavan-3-ol peaks at 288 nm, and not in the fluorescence chromatogram, since a fluorescence detector was not coupled to the HPLC on-line antioxidant system.

Flavan-3-ols generally display a higher *in vitro* antioxidant activity than other phenolic groups such as flavonols. Teissedre *et al.* (1996) found that flavan-3-ols also exhibit a higher activity compared to anthocyanins when evaluating their ability to inhibit LDL oxidation, while Tsao *et al.* (2005) found anthocyanins have a higher antioxidant activity in the FRAP assay. Flavan-3-ol dimers and trimers generally display a greater antioxidant activity than their monomer constituents (+)-catechin and (-)-epicatechin (Teissedre *et al.*, 1996; Lotito *et al.*, 2000; Counet & Collin, 2003). It is suggested that the antioxidant activity of the procyanidin increase with an increase in the chain length of the compound (Zhao *et al.*, 1999). Using the FRAP assay Tsao *et al.* (2005) found that procyanidin dimers (B1 and B2) were more effective than catechin and epicatechin in its ability to reduce iron to its ferrous form. In the chromatogram at 288 nm a slight but visible baseline drift is observed, which is likely due to the presence of oligomeric procyanidins (Rzeppa *et al.*, 2011). This results in an uneven baseline in the antioxidant chromatogram caused by the combined antioxidant response of these compounds.

The uneven baseline prevented the separate evaluation of the antioxidant activity of the flavonol compounds especially. Where flavonol antioxidant peaks could be distinguished, quercetin-3-*O*-glucoside displayed the largest antioxidant activity between all the quercetin derivatives (as observed in Laetitia, African Delight, Ruby Red and Ruby Crunch), followed by either quercetin-3-*O*-rutinoside or -arabinoside. The different glycosides attached to the aglycone of a phenolic compound affect the antioxidant activity of the compound. For example Limasset *et al.* (1993) found that quercetin-3-*O*-rutinoside had a higher antioxidant activity than quercetin-3-*O*-rhamnoside. Matching trends were seen for the plum cultivars and selections. On the other hand, Tsao *et al.* (2005) reported that quercetin-3-*O*-rhamnoside had a higher antioxidant activity than quercetin-3-*O*-glucoside, -galactoside and -xyloside. The tests were performed using authentic phenolic standards and measured with the FRAP assay.

The antioxidant activity of flavan-3-ols and flavonols agree with literature in that the flavan-3-ols generally display a greater antioxidant activity than flavonol glycosides (e.g Teissedre *et al.*, 1996, Tsao *et al.*, 2005). It is known that certain structural features are beneficial for maximum antioxidant activity, such as the *o*-dihydroxy group in the B ring, the 2,3 double bond and 4-keto group on the C ring. The 4-keto group is also beneficial in combination with hydroxyl

groups in positions C3 and C5 on the C and A ring respectively (Bors *et al.*, 1990). Thus the aglycone quercetin should theoretically display a higher in vitro antioxidant activity than catechin (Sichel *et al.*, 1991; Rice-Evans *et al.*, 1996). The unsaturation of the B ring is believed to be a promising site for electron delocalisation and subsequent stabilisation of the radical formed (as reviewed by Rice-Evans *et al.*, 1996). However, Fukumoto and Mazza (2000) reported that the antioxidant activity of a compound increased as the amount of hydroxyl groups increase, and a lower antioxidant activity was observed with the presence of more glycoside groups. This may explain the greater antioxidant activity for the flavan-3-ol compounds, since no sugar moieties are attached and more hydroxyl groups are available for antioxidant function. The attachment of more sugar moieties result in steric hindrance by blocking antioxidant sites necessary for radical scavenging and iron chelation (Fukumoto & Mazza, 2000). One must also keep in mind that the antioxidant activity of a compound may depend on its environment (e.g. lipid vs. aqueous matrixes).

In terms of the hydroxycinnamic acids literature states that chlorogenic acid may have a slightly higher antioxidant activity than neochlorogenic acid (Meyer *et al.*, 1998; Nakatani *et al.*, 2000). For the South African plum samples analysed neochlorogenic acid (**3**) display the higher antioxidant activity of the phenolic acids as it was generally present in higher amounts. In the selections PR03-34 and PR04-35 the antioxidant activity for 3-*O-p*-coumarolyquinic acid (**5**) and chlorogenic acid (**7**) was noticeably greater than the other compounds. From the chromatograms it is seen that these two selections contained the greatest amount of 3-*O-p*-coumarolyquinic acid. In the selection PR03-34 and the cultivar Ruby Crunch, 3-*O-p*-coumaroylquinic acid co-eluted with procyanidin B1 and could not be independently evaluated. A large amount of this hydroxycinnamic acid is present and a large combined antioxidant peak was observed.

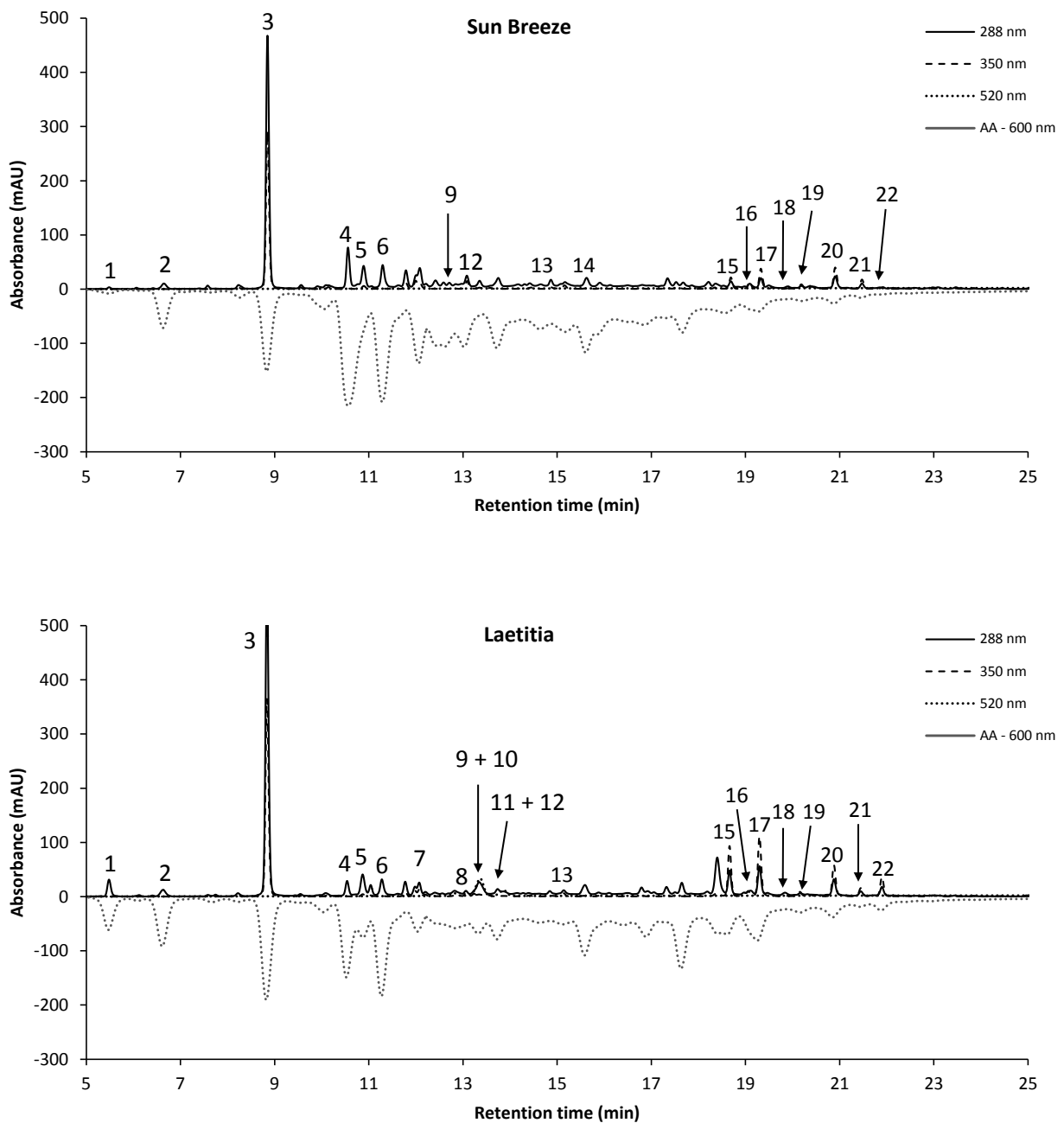


Figure 2 Chromatograms (absorbance vs. retention time) of the on-line antioxidant assays for all cultivars and selections. Peak numbers correspond to those in Table 8.

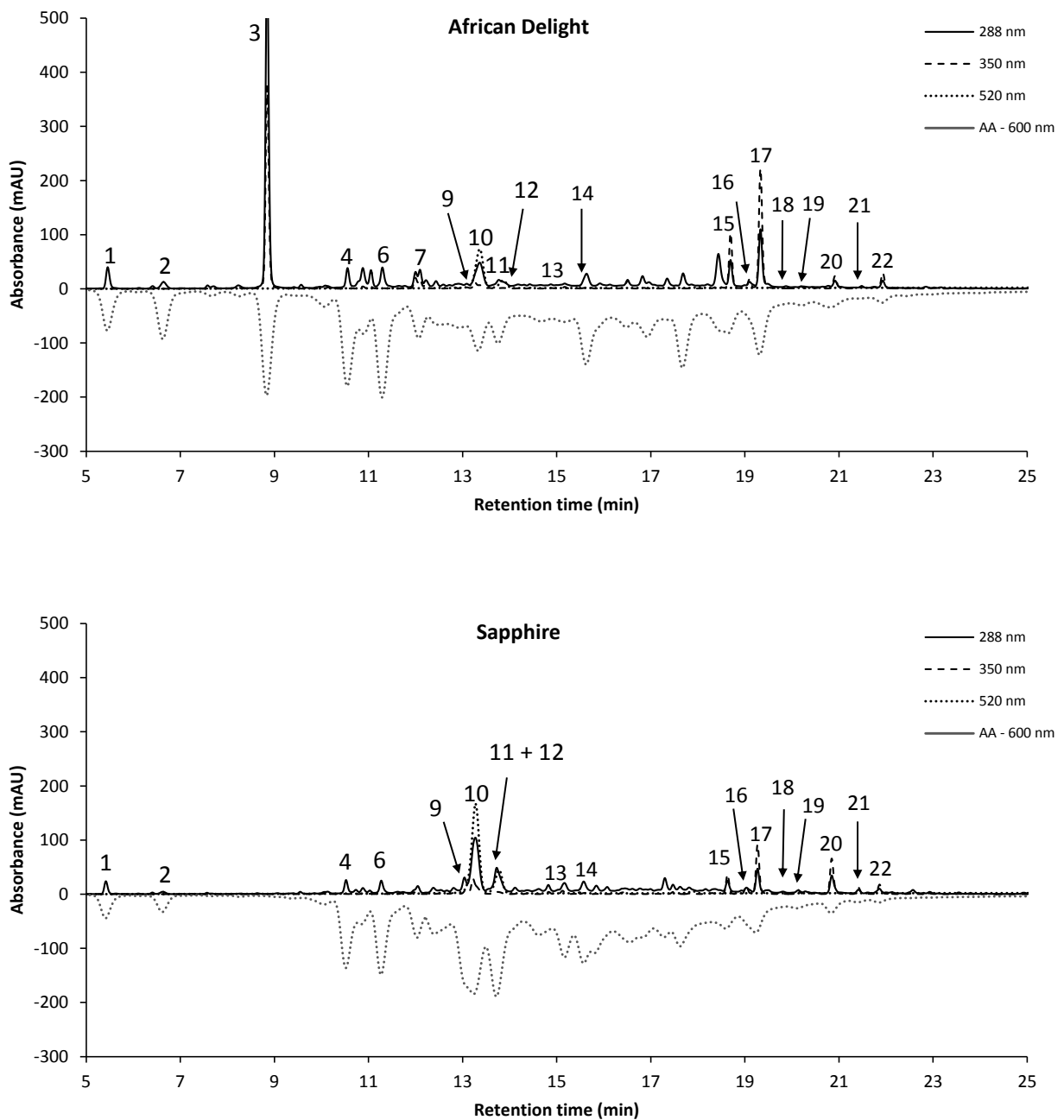


Figure 2 (continued) Chromatograms (absorbance vs. retention time) of the on-line antioxidant assays for all cultivars and selections. Peak numbers correspond to those in Table 8.

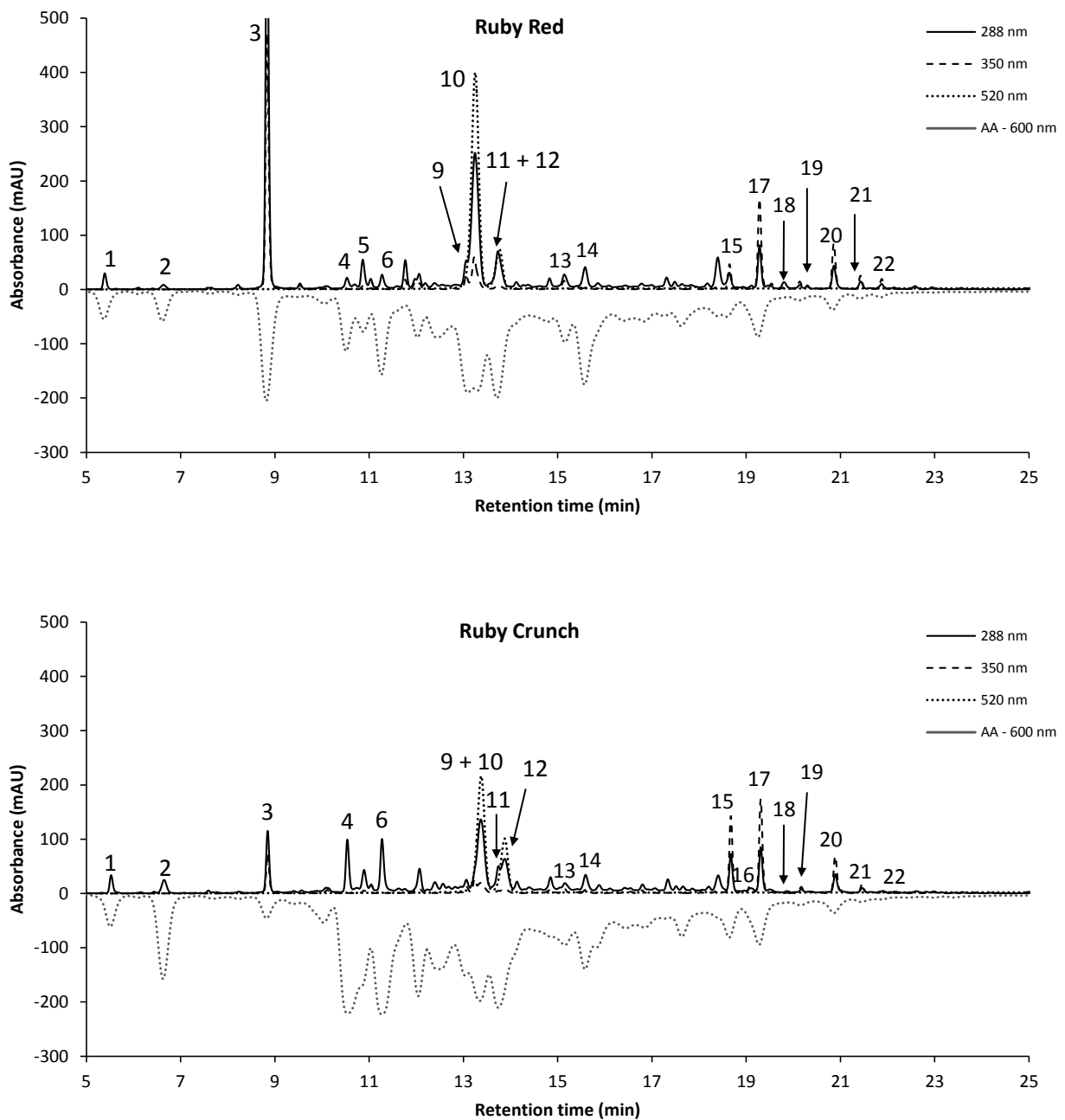


Figure 2 (continued) Chromatograms (absorbance vs. retention time) of the on-line antioxidant assays for all cultivars and selections. Peak numbers correspond to those in Table 8.

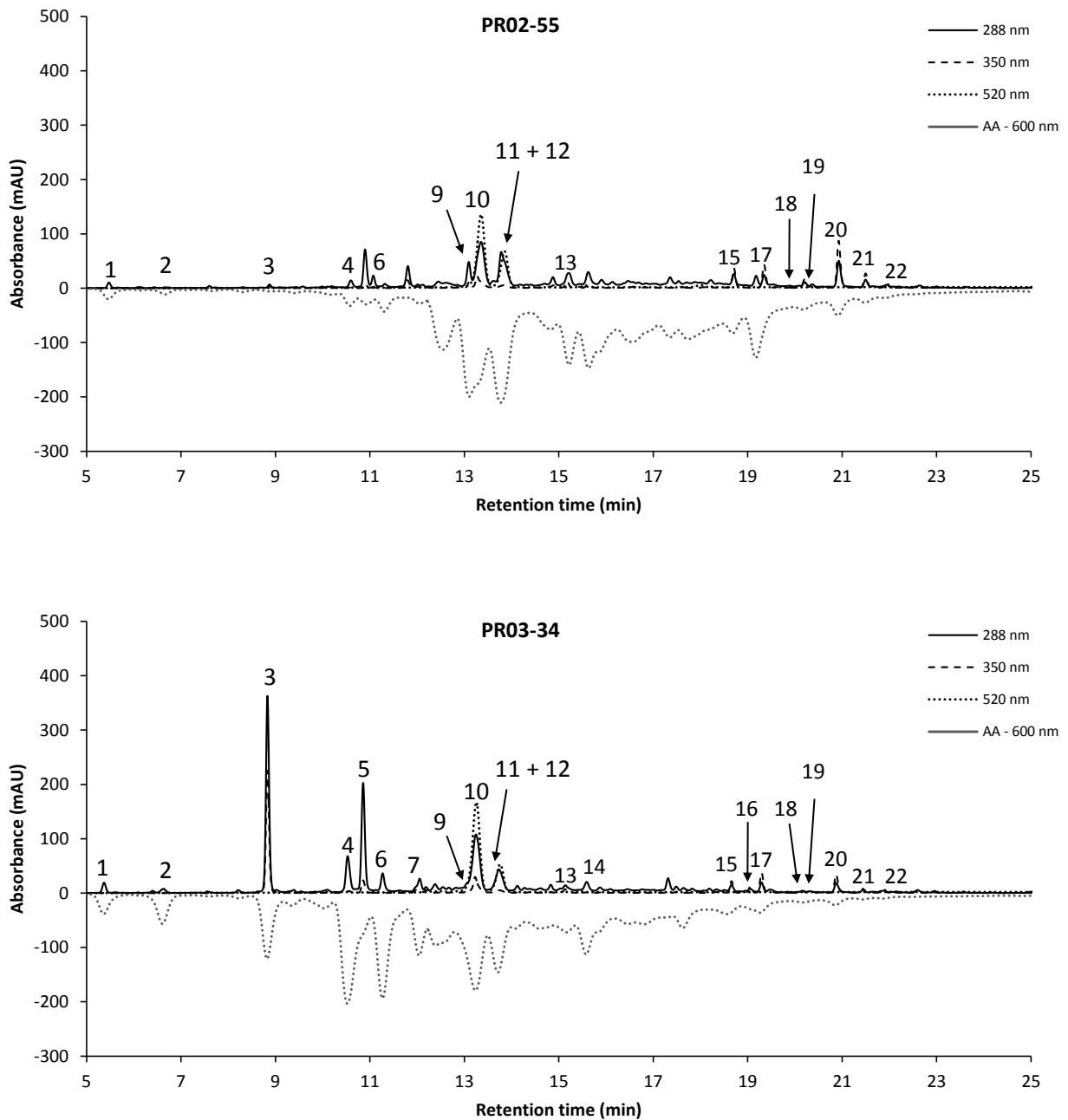


Figure 2 (continued) Chromatograms (absorbance vs. retention time) of the on-line antioxidant assays for all cultivars and selections. Peak numbers correspond to those in Table 8.

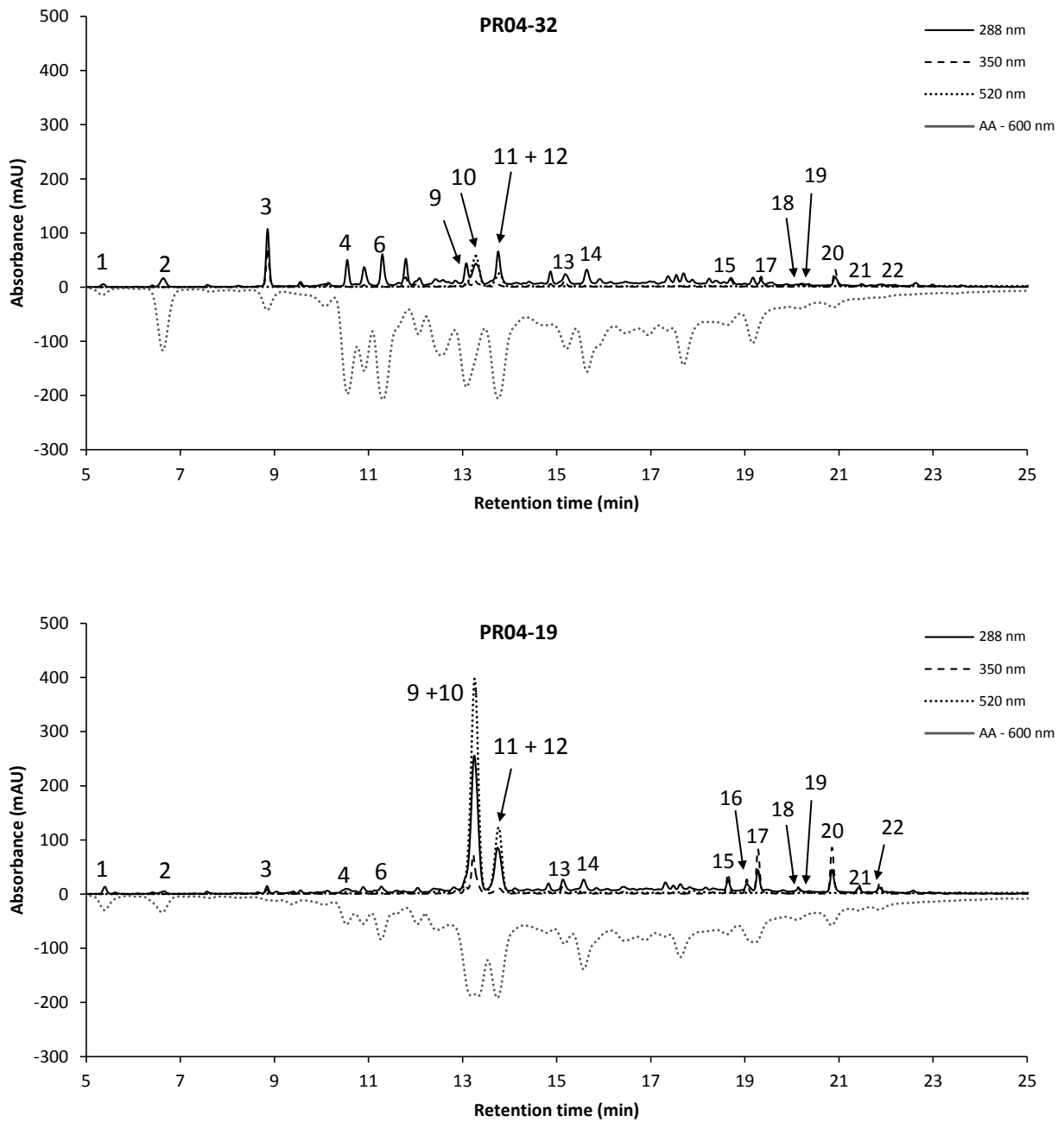


Figure 2 (continued) Chromatograms (absorbance vs. retention time) of the on-line antioxidant assays for all cultivars and selections. Peak numbers correspond to those in Table 8.

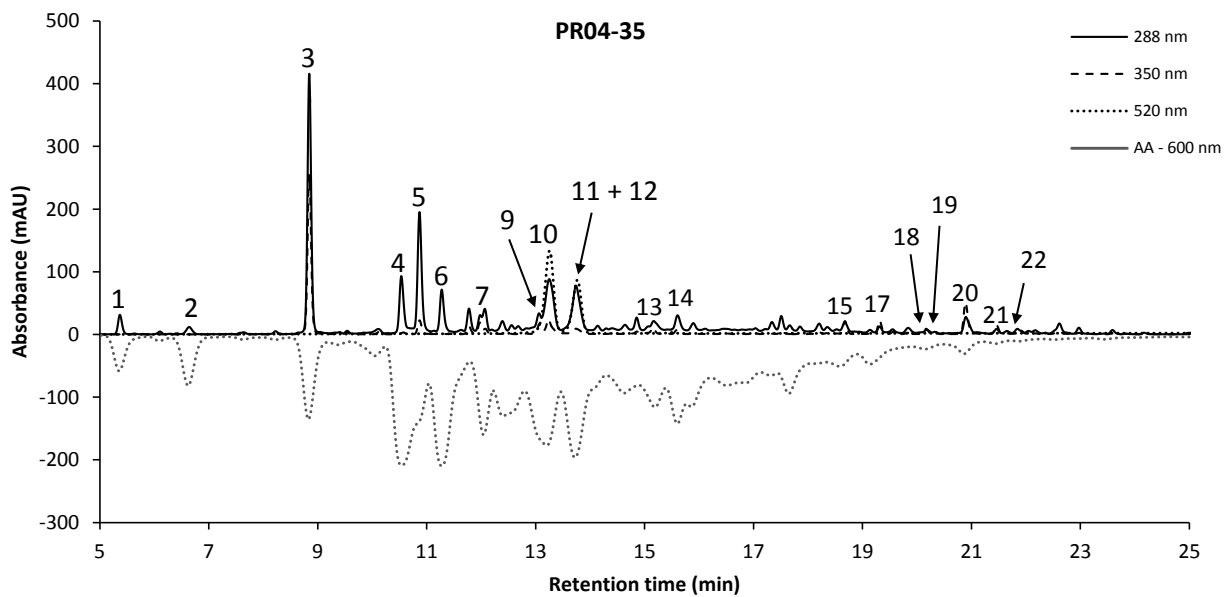


Figure 2 (continued) Chromatograms (absorbance vs. retention time) of the on-line antioxidant assays for all cultivars and selections. Peak numbers correspond to those in Table 8.

CONCLUSIONS

An optimised HPLC method was successfully developed and applied to separate a variety of phenolic compounds from four phenolic groups. A total of 24 compounds were identified or tentatively identified of which 18 were quantified. The optimised HPLC method was compatible with eleven South African plum cultivars and was applied with mass spectrometry analyses and the on-line antioxidant assays. Validation results indicated that the compounds are stable during the analysis and that the method has an acceptable precision for most compounds. Precision for the flavan-3-ols was less good (%RSD > 10% in some cases). In this respect the method should still be further improved or a method specifically focussed on the flavan-3-ol compounds should be developed in future.

Thirteen of the compounds were confirmed by comparison to authentic reference standards (retention times and UV-Vis spectra), while nine peaks were identified using comparison of UV-Vis and MS characteristics with literature. Neochlorogenic was the predominant compound in the majority of samples, with cyanidin-3-*O*-glucoside and –rutinoside also generally present in large amounts. Four flavonols which have not previously been identified in South African plums were identified as quercetin derivatives, namely quercetin-3-*O*-xyloside, quercetin pentosyl-pentoside, quercetin pentosyl-hexoside and an acetylated quercetin-hexoside. A- and B-type procyanidin dimers and trimers were present in most samples. One peak found in the majority of

samples remains unknown. According to literature all compounds have previously been identified in plums.

From the on-line antioxidant response peaks it was found that the flavan-3-ol group displayed a great antioxidant response, as well as the anthocyanins. However antioxidant activity could not be quantitatively evaluated due to combined response peaks and an uneven baseline. From literature anthocyanins are reported to have great antioxidant activity in red fruit.

It is believed that the optimised HPLC method and knowledge about the specific phenolic compounds present will be a helpful tool for future research on South African plums, especially in breeding programs and the evaluation of new plum selections. The fact that this can be successfully coupled to different detection systems and is compatible for analysis of a variety of cultivars is a great benefit. The application of this method, with minor adjustments, in the analysis of phenolic compounds of other stone fruit is also a possibility.

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CHAPTER 4

FRUIT ATTRIBUTES, PHENOLIC COMPOSITION AND ANTIOXIDANT CAPACITY OF SOUTH AFRICAN PLUMS: COMPARISON OF CULTIVARS AND SELECTIONS AND THE ROLE OF SEASON AND A COMMERCIAL COLD STORAGE AND RIPENING REGIME

ABSTRACT

Plums are climacteric stone fruit known to have a high phenolic content compared to fruit such as apples, grapes and oranges. Phenolic compounds in plums contribute to various sensory and nutritional functions, such as colour and antioxidant capacity. These attributes are affected by maturity, climacteric conditions, etc.

Eleven South African plum cultivars and selections from two harvest seasons were comparatively evaluated in terms of fruit attributes such as colour of the peel and flesh, firmness, °Brix, pH and, titratable acidity (TA), as well as phenolic composition and antioxidant capacity. Plums were analysed at harvest (unripe) and after a commercial cold storage and ripening regime. For quantification of phenolic compounds (phenolic acids, anthocyanins, flavan-3-ols and flavonols) an optimised HPLC-DAD-FLD (high-performance liquid chromatography with diode array and fluorescence detector) method was used. The total polyphenol content was determined using the Folin-Ciocalteu method and *in vitro* antioxidant capacity was determined using the Ferric Reducing Antioxidant Power (FRAP), Oxygen Radical Absorbance Capacity (ORAC), 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical cation scavenging and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays. The fruit attributes, colour, firmness, °Brix, pH and titratable acidity (TA), were measured using instrumental techniques.

The phenolic compositions of South African plums differ greatly between cultivars and selections. Neochlorogenic acid was the predominant hydroxycinnamic acid, although absent in the cultivar Sapphire. Cyanidin-3-*O*-glucoside and quercetin-3-*O*-glucoside were the predominant anthocyanin and flavonol, respectively, although not present in all cultivars/selections. The flavan-3-ols, (+)-catechin, (-)-epicatechin and procyanidins B1 and B2, were present in all cultivars and selections in varying amounts, with selection PR04-32 having the highest flavan-3-ol concentration.

The plum selection PR04-32, displayed the highest antioxidant capacity, irrespective of the assay in the first harvest season, and PR04-35 in the second season. The antioxidant capacity of Ruby Red and PR04-19, harvested during the 2011/2012 season, increased significantly ($P \leq 0.05$) during cold storage and ripening, as measured with the DPPH[•], ABTS^{•+} and FRAP assays. Principal component analysis (PCA) biplots revealed that the total antioxidant capacity was positively associated with PR04-32 (first season) and negatively associated with Sapphire and Sun Breeze. The latter were the cultivars with the lowest total polyphenol content, indicating that the phenolic content may contribute greatly to the total antioxidant capacity.

The firmness of fruit generally showed a decrease during cold storage and ripening. This was accompanied by a decrease in TA and an increase in pH.

Ultimately results from the current study compared well to literature on plums in the following aspects: phenolic composition differs between cultivars and selections and is affected by stage of maturity; *in vitro* antioxidant capacity of red-fleshed plums are generally higher than plums with yellow flesh; and all phenolic compounds identified in the South African plum varieties have previously been found in plums.

INTRODUCTION

Plums grown in South Africa are classified as Japanese plums (*Prunus salicina* Lindl.) and differ from European plums (*Prunus domestica* L.) in various aspects such as size and phenolic composition. European plums are generally used in the production of prunes, while Japanese plums are consumed as fresh fruit (Okie & Ramming, 1999). Plums are one of the fruit known to generally contain a high concentration of phenolic compounds (Vinson *et al.*, 2001; Fu *et al.*, 2011). Phenolic compounds are secondary plant metabolites known to exhibit several health benefits, including prevention of cancer, diabetes and cardiovascular disease (Utsunomiya *et al.*, 2005; Belkaid *et al.*, 2006; Noratto *et al.*, 2009). Antioxidant activity of phenolic compounds is believed to be the main function pertaining to their health benefits (Crozier *et al.*, 2009). Due to the nutritional value of plums and health benefits of phenolic compounds, plums have been suggested as an important fruit to incorporate in the diet (Vinson *et al.*, 2001). Phenolic compounds can be classified into different groups according to their basic structure and common groups found in plums include hydroxycinnamic acids, anthocyanins, flavan-3-ols and flavonols (Ryan *et al.*, 1999; Crozier *et al.*, 2009).

Attributes such as phenolic composition, antioxidant capacity, nutrient content and physical aspects (colour, firmness, etc.) differ between plum cultivars and possibly at different stages of maturity (Kim *et al.*, 2003; Lombardi-Boccia *et al.*, 2004). Certain compounds only develop during the ripening process, while the concentration of others may increase or decrease over time. These factors are also influenced by pre- and post-harvest elements such as the environment, cold storage parameters, etc. An increase in the anthocyanin content during cold storage and ripening is visible in certain red cultivars where the peel or flesh colour changes from yellow to predominantly red during cold storage (Abdi *et al.*, 1998). It is thus expected that the

phenolic composition will change during cold storage and ripening, and that this may also affect the *in vitro* antioxidant capacity. However, there are varying results on this subject (Puerta-Gomez & Cisneros-Zevallos, 2001) and currently very little is known about the effect of cold storage and ripening on the phenolic composition and antioxidant capacity of South African plums.

The aim of this research chapter was to compare the fruit attributes (firmness, colour, etc.), phenolic composition and antioxidant capacity of different plum cultivars and selections from South Africa, and evaluate the effect of cold storage and ripening on these factors. Fruit of two harvest seasons were compared.

MATERIALS AND METHODS

Chemicals

Gallic acid, pH buffers, DPPH (2,2-diphenyl-1-picrylhydrazyl) reagent, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), fluorescein sodium reagent and AAPH (2,2'-azobis-2-methyl-propanimidamide dihydrochloride) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ferric chloride (FeCl_3), KH_2PO_4 solution, Folin-Ciocalteu reagent, potassium hydroxide and sodium fluoride were purchased from Merck (Darmstadt, Germany). Roche Diagnostics GmbH (Indianapolis, IN, USA) and B & M Scientific (Cape Town, South Africa) supplied the ABTS reagent and sodium hydroxide, respectively. The standards, solvents, acid modifiers and purified water for HPLC analysis were the same as described in Chapter 3.

Analysis of whole fruit

Sample collection

On the day of harvest, 12 randomly selected plums from each of three trees of the different plum cultivars and selections (Table 1) were collected (Bien Donné, Groot Drakenstein, South Africa; S 33.84, E 18.98). Five fruit were sampled from each tree for analysis on the day of harvest. The remaining fruit were subjected to a commonly used commercial cold storage and ripening regime, advancing the fruit to eating ripeness. This involved storage for 10 days at -0.5°C , followed by 9 days at 7.5°C , 16 days at -0.5°C , and ripening at 10°C for 7 days (Anon., 2006). After the appropriate time had passed (± 45 days), five fruits from each tree were sampled for analysis.

Table 1 Cultivars and selections evaluated over two harvest seasons

Cultivar/selection	Peel colour (ripe)	Flesh Colour (ripe)	Season 1 harvest date	Season 2 harvest date
Sun Breeze	Yellow	Yellow	1 February 2011	14 February 2012
Laetitia	Red	Yellow	8 February 2011	8 February 2012
African Delight	Red	Yellow	22 February 2011	12 February 2012
Sapphire	Red	Yellow	14 December 2010	13 December 2011
Ruby Red	Red	Red	4 January 2011	3 January 2012
Ruby Crunch (PR02-62) ^a	Red	Red	- ^b	31 January 2012
PR02-55	Red	Red	21 December 2010	-
PR03-34	Red	Red	21 December 2010	20 December 2011
PR04-32	Red	Red	25 January 2011	17 January 2012
PR04-19	Red	Red	-	13 December 2011
PR04-35	Red	Red	-	20 December 2011

^a Selection PR02-62 was released as a cultivar, Ruby Crunch, in May 2012; ^b Cultivar/selection not harvested.

Analyses on whole fruit (colour, firmness and soluble solids) were performed on each of the plum selections/cultivars on the day of harvest, as well as after the cold storage and ripening period. After the analyses for the specific day were completed, the depitted fruit of each tree were homogenized together with sodium fluoride (added as 1 ml for every 4 g fruit at a concentration of 4 g.L⁻¹) (Tomás-Barberán *et al.*, 2001) using a food blender. Aliquots were frozen until required for further analysis (total solids, titratable acidity and extraction).

Colour analysis

The peel and flesh colour was measured with a CR-400 Konica Minolta Colorimeter (Tokyo, Japan). Four measurements were taken in random positions on the peel for peel colour. For flesh colour three measurements were taken on opposite sides of the fruit immediately after removal of a piece of peel. Flesh was exposed by removing a slice of peel and flesh with a knife to provide a flat surface for colour measurement. The colorimeter measured the colour of the sample on the CIE Lab scale using the 2° observer and C-illuminant. The values measured indicate the lightness (L*), red and green colour (a*), and the yellow or blue colour (b*) (Anon., 2008).

Firmness

The firmness of the fruit flesh, expressed in kg, was measured using a penetrometer with an 11 mm probe on opposite sides of the plum after removal of a piece of peel.

Soluble solids content (°Brix)

The soluble solids content of the individual fruits (°Brix) was measured in duplicate using an Atago PAL-1 refractometer (Scientific Gear, Fairfax, VA, USA). Average °Brix values for each tree were calculated.

Analysis of fruit pulp

Total solids

The total solids content of the plum pulp samples (5.0 g) was determined gravimetrically (in triplicate). The plum pulp was spread out in a nickel moisture dish to allow a large drying surface. The moisture dishes were placed in a laboratory oven at 70°C for 4 h to remove most of the moisture, whereafter it was placed in a vacuum oven at 70°C overnight (16 h).

pH-measurement

The pH of the plum pulp was measured in duplicate using a standard pH meter. The pH meter was calibrated using buffers at pH 7.0 and 4.0 at the start of each series of measurements.

Titrateable acidity

The titrateable acidity (TA) of the plum pulp was determined using an automatic titrator (Crison compact titrator, Version D, Alella, Spain). Analysis was done using a standard 13.33% sodium hydroxide solution. The pulp samples were allowed to thaw whereafter the pulp was placed in a glass beaker and the mass noted (between 13 and 30 g). Distilled water was added until a mass of approximately 65 g was reached. The pulp-water mixture was then divided into two 50 mL

centrifuge tubes and centrifuged at 8000 rpm (ca 6000 x g) for 10 min (Biofuge Primo Centrifuge, Thermo Scientific, AEC-Amersham, Johannesburg, South Africa). The supernatant was filtered using a 0.45 µm Millex-HV hydrophilic polyvinylidene difluoride (PVDF) (Merck Millipore) syringe-driven filter and ca 50 ml filtrate collected. The results were expressed as g malic acid⁻¹ FW (fresh weight).

Extraction of pulp

Plum pulp was extracted using the optimized protocol as described in Chapter 3 for HPLC analysis. The same dilution ratio was used for the antioxidant assays and Folin-Ciocalteu assay as for HPLC analysis (300 µL extract and 1 mL distilled water).

Analysis of extract

All antioxidant assays were performed using a BioTek SynergyHT microplate reader with Gen5 software (Jericho, VT, USA) for the collection of data. Clear polystyrene 96-well microplates (Greiner Bio-One, Frickenhausen, Germany) were used for the absorbance assays, while a black plate with a clear bottom (Greiner Bio-One, Frickenhausen, Germany) was used for the fluorescence assay (ORAC). Triplicate measurements were obtained for each sample in all spectrophotometric assays, and in duplicate for HPLC analysis.

Folin-Ciocalteu assay for total polyphenols

The determination of total polyphenols was done using a modified version of the Folin-Ciocalteu method described by Singleton *et al.* (1999). A standard series of gallic acid was prepared in a concentration range from 10-100 mg.L⁻¹. Pulp extracts were diluted appropriately to fall in the range of the standard curve. Samples, standards and blanks (20 µL) were added to assigned wells on the microplate, followed by addition of 100 µL Folin-Ciocalteu reagent (10x diluted) using a multi-channel pipette. Immediately thereafter 80 µL of 7.5% Na₂CO₃ (w.v⁻¹) was added to each well. After mixing (0.3 min, 1000 rpm), using an Eppendorf MixMate (Merck, Darmstadt, Germany), the plate was incubated for two hours at 30°C. The plate was read at 765 nm and results were calculated as gallic acid equivalents (GAE) 100 g⁻¹ FW.

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The determination of antioxidant activity with the DPPH[•] scavenging assay was done using a modified version of the method by Rangkadilok *et al.* (2007). The DPPH[•] reagent (5 mg DPPH[•] in 50 ml methanol) was prepared fresh on the day of analysis. The solution flask was sonicated in a foil-covered sonication bath, and the reagent was kept in the dark until analysis. The DPPH[•] concentration was adjusted by diluting with methanol to obtain an absorbance of ca 0.60 at 515 nm. The Trolox standard was prepared by dissolving 0.01251 g Trolox in 50 mL methanol. Sonication was used to ensure the Trolox was dissolved. Six Trolox solutions ranging from 50 to 400 μ M were prepared from the original stock solution.

The blanks (deionised water), standards and samples (30 μ L of each) were pipetted into the assigned wells on a deep-well plate. Using a multi-channel pipette, 270 μ L of the DPPH[•] reagent was added to each well and the plate sealed with a silicone sealing mat. After mixing (0.3 min, 1630 rpm, Eppendorf MixMate) the plate was incubated in a dark cupboard for two hours. After the appropriate time had passed, 200 μ L of the mixture in each well of the deep-welled plate was pipetted into the corresponding wells of the flat-bottom plate. Readings were done at 515 nm and results expressed as μ mol Trolox equivalents (TE).g⁻¹ FW.

2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical cation scavenging assay

The ABTS^{•+} reagent was prepared by dissolving 0.0192 g ABTS in 5 ml deionised water, whereafter K₂S₂O₈ (88 μ L) was added and the reagent allowed to stand in the dark for 12-16 h before use (Pellegrini *et al.*, 1999). The ABTS^{•+} concentration was adjusted by diluting with potassium-phosphate buffer (75 nM, pH 7.4) to obtain an absorbance of ca 0.70 at 734 nm. The correct dilution for the ABTS^{•+} reagent was made up to 100 mL. The reservoir with ABTS^{•+} reagent was kept on ice and the lid kept closed between the pipetting steps. Trolox was used as standard and a stock solution was prepared by dissolving 0.01251 g in 50 mL ethanol. A Trolox standard series range of 50 to 300 μ M was prepared by appropriate dilution of the stock solution with ethanol.

Measurements for the ABTS^{•+} assay were performed row by row of the plate. Thus a volume of 20 μ L of the blank (deionised water), standards and samples were placed in wells at the

allocated positions in the first row of the microplate and 180 μL ABTS^{•+} reagent added to reach a total volume of 200 μL in each well. The method was programmed to shake the plate, incubate it for 4 min at 30°C and take a reading at 734 nm. Results were expressed as $\mu\text{mol TE.g}^{-1}\text{FW}$.

Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC assay as described by Huang *et al.* (2002) was modified. In contrast to the other antioxidant methods, ORAC makes use of fluorescent measurements. Distilled water (300 μL) was placed in the outer wells of the plate and served as a thermal barrier. The Trolox standard series ranged from 5 to 30 μM . In the remaining wells the blanks, standards and samples were pipetted into their allocated positions in volumes of 25 μL each. To prepare the fluorescein solution 0.0031 g fluorescein disodium was dissolved in 100 mL 15 mM K-phosphate buffer (10.2 g KH_2PO_4 in 1 L deionised water, pH 7.4). The working solution was prepared by diluting 100 μL of the stock solution in 100 mL K-phosphate buffer, of which 150 μL was added to the wells (samples and blanks). The plate was then incubated for 10 min at 37°C. AAPH (153 mM) was prepared by dissolving 0.414 g AAPH in 10 mL K-phosphate buffer. The AAPH solution (25 μL) was automatically added to the applicable wells, using the dispenser of the microplate reader, the plate was shaken and the reading started. A reading was taken at one-min intervals for a total of 35 min. The area under the curve (AUC) was used for calculation. Results were expressed as $\mu\text{mol TE.g}^{-1}\text{FW}$.

Ferric Reducing Antioxidant Power (FRAP) assay

A modified method of Benzie and Strain (1999) was followed for the FRAP assay. It is important to note that the FRAP reagent was covered with foil and kept in a dark cupboard during the assay to avoid light degradation. A Trolox standard series range of 50 to 500 μM was prepared by appropriate dilution of the stock solution with ethanol. Blanks (deionised water), standards and samples (20 μL of each) were placed in the allocated wells on the plate and 180 μL of FRAP reagent added. Two readings were taken, the second after 4 minutes. Readings were done at 592 nm and a temperature of 37°C (pre-set microplate reader temperature). Results were expressed as $\mu\text{mol TE.g}^{-1}\text{FW}$.

High-performance liquid chromatography (HPLC)

Phenolic compounds were analysed using the optimised HPLC method as described in Chapter 3. All samples were injected at 100 μL , as well as at an additional lower volume, depending on the cultivar or selection (Table 2). Samples were injected in duplicate. Individual phenolic compounds were quantified using the peak areas from chromatograms of each cultivar. Compounds were identified by comparing the retention times and UV-Vis spectra of compounds to authentic standards and subsequently quantified. Authentic standards were not available for all compounds and thus quercetin-3-xyloside, quercetin pentosyl-pentoside and acetylated quercetin-hexoside were quantified using the reference standard for quercetin-3-*O*-glucoside. The hydroxycinnamic acid, 3-*O*-*p*-coumaroylquinic acid, was quantified using neochlorogenic acid. Data were expressed in terms of the fresh weight of the sample ($\text{mg compound.kg}^{-1}$ FW).

Table 2 Additional injection volumes per sample

Cultivar/selection	Injection volume (μL)
Sun Breeze	50
Laetitia	50
African Delight	50
Sapphire	50
Ruby Red	40
Ruby Crunch	50
PR02-55	-
PR03-34	50
PR04-32	40
PR04-19	40
PR04-35	50

Statistical analysis

The experimental design for statistical analysis was treated as a split plot. Plum cultivars were regarded as main plot treatments, while the different storage factors (at harvest and after cold storage and ripening) were regarded as split plot factors. For the main plot three trees of each of the 11 cultivars and selections were used as replicates, forming a randomised main plot design.

Half of the fruit collected were analysed on the day of harvest and the remaining fruit after cold storage and ripening.

SAS statistical software (SAS®, Version 9.2; SAS Institute Inc., Cary, NC, USA) was used for the univariate analysis of variance (ANOVA) tests. This was done on all variables assessed during the study and the General Linear Models (GLM) procedure was applied. In order to compare the sample treatment means Student's t-test was used, with the least significant difference calculated at 5% (Ott, 1998). The Shapiro-Wilk test was used to test for normality (Shapiro & Wilk, 1965) and a 5% probability level was regarded as significant for all tests.

Multivariate statistical analysis was performed using XLSTAT software (Version 7.5.2, Addinsoft, New York, USA). Principal Component Analysis (PCA) was used to evaluate relationships between sample attributes and cultivars/selections.

RESULTS AND DISCUSSION

First harvest season

Principal Component Analysis (PCA) biplots were compiled to evaluate the association between samples, fruit attributes, antioxidant capacity and phenolic composition. The correlations between variables were also statistically evaluated using Pearson's correlation matrix and coefficients of determination (R^2 values). ANOVA was performed on the average values of fruit from three trees (15 fruit). These results will be used to discuss the main observations and comparisons.

Fig. 1 represents all data collected during the first harvest season, before and after cold storage and ripening. The biplot explains 51.8% of the variation for plum cultivars and selections. Some cultivars and selections such as African Delight, PR04-32 and PR03-34 formed distinct clusters without any overlap with others. Furthermore, observations before and after cold storage and ripening were clearly separated for Ruby Red, Sapphire, Laetitia and PR04-32.

In terms of physical attributes, pH and °Brix were negatively associated with TA on the plot. This was to be expected as a lower pH would represent greater titratable acid (TA) concentration. The pH increased slightly during cold storage and ripening, while TA decreased significantly for all cultivars and selections, attributed to the decrease in malic acid, one of the major organic acids in plums (Lombardi-Boccia *et al.*, 2004). In addition, sugar content increases while acidity decreases with ripening (Guerra & Casquero, 2008). However, during this study the °Brix showed no significant increase ($P>0.05$). For the cultivars African Delight, Ruby Red and Sapphire a significant

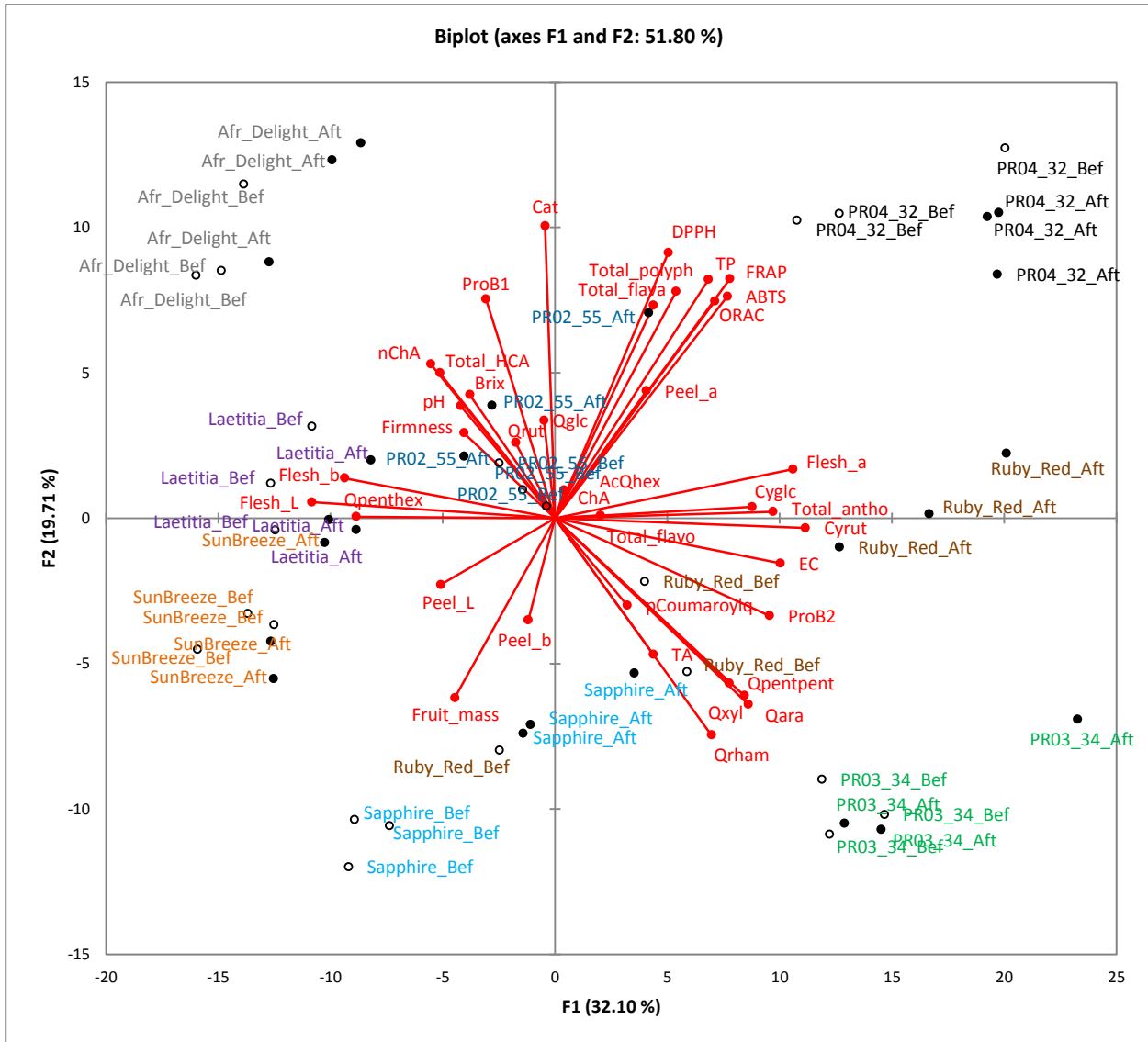


Figure 1 PCA biplot of associations between phenolic compounds, antioxidant capacity, fruit attributes and cultivars and selections of the first harvest season.

*Key: Bef = before cold storage and ripening (open circles); Aft = after cold storage and ripening (closed circles); HCA = hydroxycinnamic acids; Flavo = flavonol; Flava = flavan-3-ol; Antho = anthocyanin; Cyrut = cyanidin-3-*O*-rutinoside; Cyglc = cyanidin-3-*O*-glucoside; NeoChA = neochlorogenic acid; ChA = chlorogenic acid; pCoumaroylq = 3-*O*-*p*-coumaroylquinic acid; Cat = (+)-catechin; EC = (-)-epicatechin; ProB1 = procyanidin B1; ProB2 = procyanidin B2; Qrut = quercetin-3-*O*-rutinoside; Qglc = quercetin-3-*O*-glucoside; Qxyl = quercetin-3-*O*-xyloside; Qara = quercetin-3-*O*-arabinoside; Qrham = quercetin-3-*O*-rhamnoside; Qpentpent = quercetin pentosyl-pentoside; Qpenthex = quercetin pentosyl-hexoside; AcQhex = acetylated quercetin-hexoside.

decrease in °Brix during cold storage and ripening occurred. A decrease in °Brix during cold storage was seen previously in plums (Taylor *et al.*, 1995), and may be related to the formation of pectin-sugar gels during storage. In term terms of colour, peel and flesh a*-values corresponded to Ruby

Red and PR04-32. A positive a^* -value represents a red colour (Anon., 2008). In addition, Ruby Red after cold storage and ripening associated more closely with the a^* -value of the flesh than the unripe fruit (Fig. 1). A significant increase ($P \leq 0.05$) in the flesh a^* -reading occurred as a result of ripening (Addendum Table A1).

At harvest, firmness was positively associated with African Delight and Laetitia (Fig. 1). African Delight had the highest average firmness (8.7 kg) at harvest, while PR02-55 had the highest firmness after storage and ripening (8.3 kg) (Table A1). According to the Agricultural Product Standards Act (Anon., 1991) the firmness for African Delight at harvest should be between 5.5 and 9.0 kg. The required firmness at harvest differs between cultivars and selections, and official requirements are not yet available for all plum cultivars or selections. Unexpectedly, the plum selection PR02-55 showed an increase in firmness during cold storage, although it was not significant ($P > 0.05$) (Table A1). Firmness is expected to decrease during ripening partly due to the breakdown of pectin during ripening (as reviewed by Brownleader *et al.*, 2012). Although specific firmness values could not be directly compared to literature (due to different measurement units and methods), similar trends were seen (i.e. decreased firmness after ripening).

Tomás-Barberán *et al.* (2001) found the firmness of Japanese plums to range from 24.5 to 52.5 N at harvest and from 7.6 to 19.6 N after storage (5 days at 20°C). Ozturk *et al.* (2012) found that the firmness of plums decreased significantly over a 21 day cold storage period at 0°C. Argenta *et al.* (2003) also found that the firmness of the cultivar Laetitia (South African cultivar grown in Brazil) decreased from harvest to a ripe stage (16 days at 23°C). Negative correlations (R^2) ranging from -0.391 to -0.597 were observed between the fruit mass and antioxidant assays. A possible reason for the greater antioxidant capacity of smaller fruit is the higher peel-to-flesh ratio. Phenolic compounds are more concentrated in the peel of fruit (Tomás-Barberán *et al.*, 2001; Gil *et al.*, 2002) and would thus provide a greater contribution to the total antioxidant capacity of smaller fruit.

The total polyphenol content of the fruit associated greatly with selection PR04-32 and partly with Ruby Red (ripe). PR04-32 selection had a significantly higher ($P \leq 0.05$) total polyphenol content than other cultivars (Table A2). Sapphire had the lowest total polyphenol content and was negatively associated with total polyphenol content (Fig. 1). The current study is in agreement with Kim and co-authors (2012) in finding that plums with red peel (Ruby Red, PR02-55, PR04-32) are higher in total polyphenols than fruit with yellow peel and/or flesh (Sun Breeze, Laetitia, Sapphire). African Delight, a cultivar with red peel and yellow flesh, was the only exception as its total polyphenol content was higher than that of Sun Breeze, Laetitia, Sapphire and Ruby Red

(Table A2). The total polyphenol content varied between cultivars and selections and differed significantly ($P \leq 0.05$) between the lowest (1.9 mg GAE.g⁻¹ FW), obtained for Sapphire, and the highest (3.5 mg GAE.g⁻¹ FW) content, obtained for PR04-32. Literature has shown that plum cultivars differ from one another in terms of total polyphenol content (Kim *et al.*, 2004; Kristl *et al.*, 2012; Mubarak *et al.*, 2012). A study by Chun and co-authors (2003) found the total polyphenol content of plum cultivars *P. domestica* to range from 0.14 to 0.69 mg GAE.g⁻¹ (FW), varying greatly from one cultivar to the next. Mubarak *et al.* (2012) compared 29 plum selections in terms of total polyphenol content and found great variation between selections (0.02-0.17 mg GAE.g⁻¹ FW). In the present study the total polyphenol content generally was higher for ripe fruits, although significantly higher values ($P \leq 0.05$) after cold storage and ripening were only observed for Sapphire and Ruby Red (Table A2). Similar results have been found in previous studies (Karaman *et al.*, 2012; Ozturk *et al.*, 2012). For example, Karaman *et al.* (2012) found the total polyphenol content of a specific Japanese plum cultivar to increase from 0.45 to 1.27 mg GAE.g⁻¹ FW during cold storage (28 d). Usenik *et al.* (2008) reported that no significant changes occurred in total polyphenol content during ripening of *P. domestica*.

Total polyphenol content is a useful parameter to show whether the phenolic content changed as a result of cold storage and/or ripening, but it provides no insight into the changes of specific types of compounds or individual compounds.

Fig. 1 shows that cyanidin-3-*O*-glucoside and -rutinoside are closely associated with Ruby Red (ripe), with cyanidin-3-*O*-glucoside identified as the predominant anthocyanin in South African plums (Table A3). The cyanidin-3-*O*-glucoside content of Ruby Red increased significantly ($P \leq 0.05$) during cold storage and ripening from 80.4 to 447.0 mg.kg⁻¹ FW (data not shown). The individual and total anthocyanins were positively associated with Ruby Red after cold storage and ripening, and negatively associated with Sun Breeze (no anthocyanins) and Laetitia (low anthocyanin content) (Fig. 1). Cyanidin-3-*O*-glucoside and -rutinoside are some of the major polyphenols in plums with red peel and/or flesh (Kim *et al.*, 2003; Wu & Prior, 2005; Usenik *et al.*, 2008). Both ripe Ruby Red and Sapphire were more closely associated with the anthocyanins than their unripe (before cold storage and ripening) counterparts (Fig. 1) and the a^* -value for both cultivars increased significantly during ripening (Table A1). This can be explained by the accumulation of anthocyanins during ripening, resulting in a change in colour (Usenik *et al.*, 2009). Miletić and co-authors (2012) found the total anthocyanin content of a European plum cultivar increased significantly as a result of ripening. Research done on blueberries and cherries found that there was an increase in anthocyanin content during storage at low temperatures (Connor *et al.*, 2002;

Conçalves *et al.*, 2004). In contrast, Cordenunsi *et al.* (2005) found a greater anthocyanin accumulation at higher storage temperatures for strawberries.

A factor to consider regarding anthocyanins is the phenomenon of co-pigmentation. It has been found that anthocyanins form complexes with colourless phenolic compounds, such as flavonols, resulting in a more intense colour (Asen *et al.*, 1972). Red colour would thus not only depend on anthocyanin content but the flavonol content would also be important.

Quercetin-3-*O*-xyloside, -arabinoside and -rhamnoside were positively associated with PR03-34 (unripe and ripe) and Ruby Red (unripe). The greatest quantity of quercetin-3-*O*-arabinoside was observed in PR03-34 after cold storage and ripening (61.2 mg.kg⁻¹ FW) and the lowest quantity in African Delight (negative association in Fig. 1) (Table A3). In the flavonol group quercetin-3-*O*-glucoside was the predominant flavonol and increased significantly ($P \leq 0.05$) after cold storage and ripening in Ruby Red, African Delight and Sapphire. However, cold storage and ripening had no effect on the other flavonol compounds. Ozturk *et al.* (2012) found the quercetin-3-*O*-rutinoside content of a certain plum cultivar increased after 28 d at 0°C storage. However, flavonol aglycones (quercetin and kaempferol) decreased during the same storage period. Olsson *et al.* (2004) found that the quercetin content of strawberries increased during storage and ripening.

At harvest and after ripening the total hydroxycinnamic acid and neochlorogenic acid content associated positively with Laetitia and African Delight. Neochlorogenic acid is also one of the predominant polyphenols in plums (Kim *et al.*, 2003) with the highest quantity in the current study present in the cultivar Laetitia (395.8 mg.kg⁻¹ FW; unripe). Neochlorogenic was not present in the cultivar Sapphire and selection PR02-55. Chlorogenic acid was present only in African Delight and PR03-34, and in much lower amounts than neochlorogenic acid. Total flavan-3-ol content (sum of individual flavan-3-ols) associated positively with PR04-32 and negatively with Sapphire at harvest and after cold storage and ripening (Fig. 1). This selection and cultivar (ripe) also had the highest and lowest total flavan-3-ol content, respectively. Fig. 2 shows the average values for flavan-3-ol compounds present in the cultivars after cold storage and ripening. The PCA biplot (Fig. 1) shows that procyanidin B2 and (-)-epicatechin are closely associated with one another, while procyanidin B1 and (+)-catechin are closely associated. The same trend is seen in Fig. 2. From this figure it is also obvious that the flavan-3-ol composition of different cultivars and selections are very different. In most cases procyanidin B1 was the major flavan-3-ol.

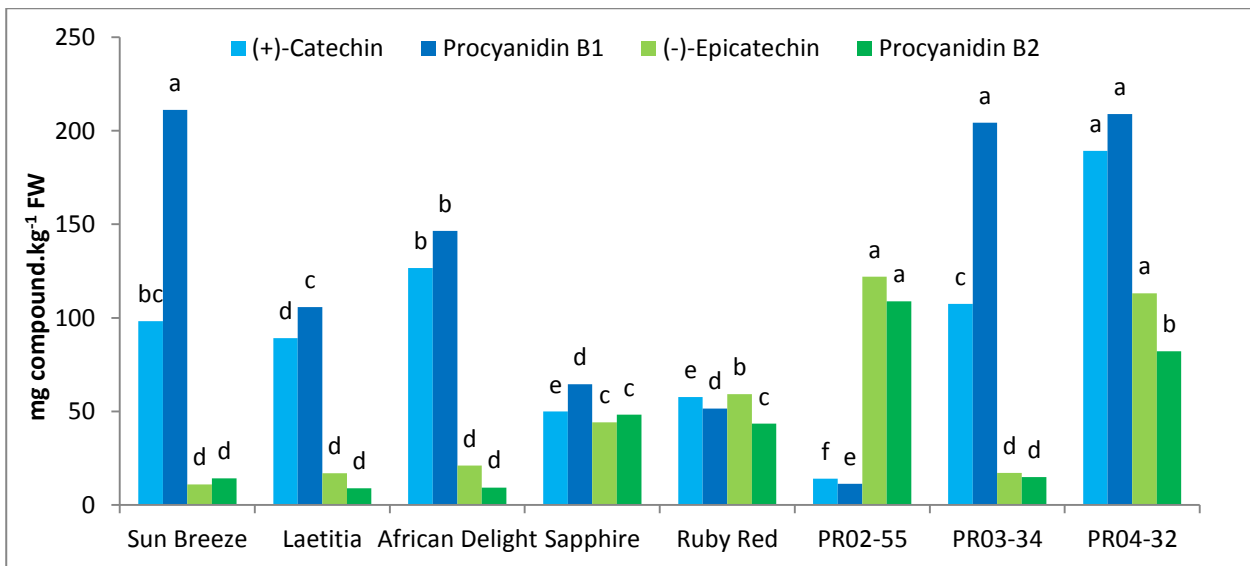


Figure 2 Flavan-3-ol compounds present in the different cultivars and selections after cold storage and ripening for the 2010/2011 harvest season.

*Different letters over columns for a specific compound represent significant differences at $P \leq 0.05$.

The antioxidant capacity of various Japanese and European plum cultivars has been studied previously *in vitro* (Chun *et al.*, 2003; Kim *et al.*, 2004). The ability of phenolic compounds to act as antioxidants is a topic that has received widespread attention. Fig. 1 shows that selection PR04-32 is closely associated with antioxidant capacity. PR04-32 had the highest *in vitro* antioxidant capacity in all the assays, except for the DPPH[•] assay in which PR04-32 did not differ significantly ($P > 0.05$) from African Delight (ripe) (Table A4). Sun Breeze was negatively associated with antioxidant capacity on the PCA biplot and had among the lowest antioxidant capacities in all the assays (Table A4). Overall Sapphire displayed the lowest antioxidant capacity (Table A4).

The Pearson's correlation matrix shows that the a^* -value of the peel and flesh is positively correlated with antioxidant capacity (Table 3), while a negative correlation is observed between the L^* -values of the peel and the antioxidant assays. Therefore, plums with a darker peel (lower L^* -value) show a closer correlation to antioxidant capacity. Plums with a lower L^* -value are generally the red plums containing a greater concentration of anthocyanins. The association of the a^* - and L^* -values with antioxidant capacity is a confirmation that colour plays an important role in *in vitro* antioxidant capacity of plums.

Table 3 Correlations between antioxidant assays, L*-value of the plum peel, and a*-values of plum peel and flesh for the first harvest season (Pearson correlation matrix)

Variables	Peel a*	Flesh a*	Peel L*
ABTS ^{•+}	0.333*	0.700***	-0.232
DPPH [•]	0.353*	0.479**	-0.161
FRAP	0.388**	0.697***	-0.334*
ORAC	0.353**	0.574***	-0.246

*P<0.05; **P<0.01; ***P<0.001; no superscript indicates no significant correlation.

Generally, large variation in the phenolic composition of the different South African plum cultivars and selections was observed. This was to be expected as a previous study by Mubarak *et al.* (2012), comparing 29 plum selections, found great variation regarding the presence of neochlorogenic acid, chlorogenic acid, quercetin-3-*O*-rutinoside and (-)-epicatechin between selections.

The antioxidant capacity of the individual cultivars/selections rarely showed a significant increase as a result of cold storage and ripening (Table A4). The antioxidant capacity increased significantly ($P \leq 0.05$) only for Sapphire and Ruby Red as measure with the ABTS^{•+}, FRAP and DPPH[•] assays. African Delight showed a significant increase ($P \leq 0.05$) only when using the DPPH[•] assay. Even though cold storage and ripening had little effect on the antioxidant capacity, a general increase was observed for the majority of cultivars and selections, except for Laetitia and PR04-32, showing a slight decrease ($P > 0.05$) (Table A4). According to literature the antioxidant capacity of plums tend to increase during cold storage and/or ripening. Karaman *et al.* (2012) found the antioxidant capacity of plums (*P. salicina*) to increase from 19.5 to 32.0 $\mu\text{mol TE}\cdot\text{g}^{-1}$ FW during storage (28 d) according to the ABTS^{•+} assay, and 10.8 to 14.0 $\mu\text{mol TE}\cdot\text{g}^{-1}$ FW according to the FRAP assay. Kevers *et al.* (2007) also found that the antioxidant capacity (ORAC and DPPH[•] assays) of plums increased during the initial 15 days of storage, whereafter a decrease was observed. The same trend was observed for the total polyphenol content. With the latter study one must keep in mind that fruit were obtained from a retail outlet and were not analysed at harvest (Kevers *et al.*, 2007).

The total polyphenol content as determined by the Folin-Ciocalteu (FC) assay, as well as the sum of total phenol compounds, was closely correlated with antioxidant capacity. Table 4 shows the positive correlations between total polyphenols and the different antioxidant assays, indicating that phenolic compounds may contribute greatly to the total antioxidant capacity. The

same conclusion has been made by several authors regarding Japanese and European plums (Gil *et al.*, 2002; Chun *et al.*, 2003; Kim *et al.*, 2003, etc.).

It was expected that Sun Breeze, a yellow peeled and fleshed cultivar, would display an antioxidant capacity relatively lower than other cultivars due to the absence of anthocyanins, which make up a large part of the total polyphenols. Sun Breeze had among the lowest antioxidant capacity (Table A6), but it was Sapphire (red peel and yellow flesh) that consistently displayed the lowest antioxidant capacity in all assays. Previous studies on plums (*P. domestica*) have found that yellow plums display a lower antioxidant capacity than red plums (Kim *et al.*, 2004). It was interesting to note that Sun Breeze displayed an average antioxidant capacity, despite a low concentration of the majority of phenolic compounds and the lack of anthocyanins.

The two predominant compounds in Sun Breeze were procyanidin B1 and neochlorogenic acid (Table A7), suggesting that these compounds may contribute greatly to the total antioxidant capacity. The average content of procyanidin B1 and neochlorogenic acid in Sun Breeze (ripe) harvested during the 2010/2011 harvest season was 211.1 and 216.4 mg.kg⁻¹ FW, respectively. The Pearson's correlation matrix shows that procyanidin B1, but not neochlorogenic acid, was positively correlated with all antioxidant assays (Table 4). The mechanism by which different phenolic compounds exhibit antioxidant activity may explain its correlation to different antioxidant assays. The structure of phenolic compounds has a great effect on their ability to act as antioxidants, and has been discussed in previous chapters. For example, the flavonoids are known to be effective radical scavengers, due to presence of the catechol group (Silva *et al.*, 2002), while hydroxycinnamic acids act as radical scavengers through hydrogen donation from the carboxyl side-chain (as reviewed by El-Seedi *et al.*, 2012).

Flavonol compounds did not show good correlation to antioxidant capacity (data not shown). No positive significant correlations were seen with Pearson's correlation matrix, and some compounds (quercetin-3-*O*-rhamnoside, quercetin pentosyl-hexoside and acetylated quercetin-hexoside) were negatively correlated with all antioxidant assays.

Tsao *et al.* (2005) used the FRAP assay to compare antioxidant activity of various phenolic standards and found that procyanidin B1, B2 and (-)-epicatechin exhibits a higher antioxidant capacity than quercetin-3-*O*-glycosides and chlorogenic acid. Tabart *et al.* (2009) found that phenolic acids exhibits a lower antioxidant capacity than flavonols, flavan-3-ols and anthocyanins as determined with the ORAC assay, and lower than flavan-3-ols with the DPPH[•] assay.

Table 4 Pearson correlation matrix values for phenolic compounds and antioxidant assays concerning cultivars and selections evaluated in the first season

Variables	ABTS ^{•+}	DPPH [•]	FRAP	ORAC	TP
(+)-Catechin	0.623***	0.599***	0.598***	0.676***	0.617***
(-)-Epicatechin	0.543***	0.338**	0.493***	0.498***	0.473**
Procyanidin B1	0.403**	0.382**	0.338*	0.372**	0.417**
Procyanidin B2	0.433**	0.220	0.364*	0.366*	0.347*
Cyanidin-3-O-glucoside	0.377**	0.327*	0.496***	0.412**	0.327*
Cyanidin-3-O-rutinoside	0.563***	0.393**	0.598***	0.535***	0.504***
Total anthocyanins	0.438**	0.356*	0.541***	0.459**	0.384**
Neochlorogenic acid	-0.127	0.059	0.018	-0.011	0.022
Total flavan-3-ols	0.835***	0.676***	0.746***	0.803***	0.790***
Total polyphenols (FC)	0.962***	0.886***	0.962***	0.839***	1.000***

*P<0.05; **P<0.01; ***P<0.001; no superscript indicates no significant correlation.

The antioxidant assays associate closely with one another (Fig. 1) and comparing R²-values of the antioxidant assays revealed a positive correlation to one another on fresh weight basis (Table 5). The fact that the different assays correlate well with one another indicates that the different assays should represent the same trend in results. The weakest correlation was observed between the DPPH[•] and ORAC assays. A low correlation between different assays can be attributed to the fact that antioxidants have a multifunctional nature and the assays are based on different mechanisms. The DPPH[•] and ABTS^{•+} assays are based on the ability of the antioxidant to scavenge a synthetic radical (Prior *et al.*, 2005), and the ORAC assay measures the ability of an antioxidant to act against peroxy radicals (Benzie & Strain, 1996; Prior *et al.*, 2005). Kevers *et al.* (2007) observed opposite trends in antioxidant capacity for certain fruit and vegetables analysed with the ORAC and DPPH[•] assays, due to the different radical sources. Although the DPPH[•] and ABTS^{•+} assays are both based on radical scavenging they displayed moderate correlation (Table 5). This may be explained by the fact that the DPPH[•] assay is based on electron transfer, while ABTS^{•+} acts by hydrogen donation (Salah *et al.*, 1995; Prior *et al.*, 2005).

Table 5 Correlation matrix (R^2) for antioxidant capacity for the first harvest season

Variables	ABTS ^{•+}	DPPH [•]	FRAP	ORAC
ABTS ^{•+}	1.000***	0.764***	0.921***	0.735***
DPPH [•]	0.764***	1.000***	0.784***	0.536***
FRAP	0.921***	0.784***	1.000***	0.780***
ORAC	0.735***	0.536***	0.780***	1.000***

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; no superscript indicates no significant correlation.

Second harvest season

Ten South African plum cultivars and selections were evaluated during the second harvest season. To simplify interpretation and discussion the data were separated into two PCA biplots: associations between fruit attributes and cultivars/selections; and associations between antioxidant capacity, phenolic compounds and cultivars and selections).

Fig. 3 shows that the cultivars with yellow peel and/or flesh are located on the left-hand side of the plot, closely associated with the b^* - and L^* -values of the peel and flesh. The red-fleshed plums are closely associated with the a^* -values of the peel and flesh (Fig. 3). As expected, the red-fleshed plums had significantly higher a^* -values than plums with yellow flesh (Fig. 4; Table A5). The difference in flesh colour between yellow-fleshed plums (Sun Breeze, Laetitia and African Delight) and the other cultivars and selections is the main reason for the clear separation on the PCA biplot (Fig. 3).

Associations based on phenolic composition, antioxidant capacity and cultivars/selections (Fig. 5) shows that the observations for selections PR04-35 and PR04-32 form an overlapping cluster on the PCA biplot, while replicates for the other cultivars/selections generally cluster together. This was not the case for the cultivar Ruby Red, for which the replicates did not form a cluster. In contrast to the first harvest season, the antioxidant assays for the second season were not clustered as closely, since the data of FRAP and ABTS^{•+} assays closely associated with one another, while that of the ORAC and DPPH[•] assays closely associated (Fig. 5). PR04-35, having the highest antioxidant capacity according to the FRAP and ABTS^{•+} assays, and PR04-32 the highest antioxidant capacity according to the ORAC and DPPH[•] assays (Table A6), caused the antioxidant assays to separate into different quadrants in the PCA biplot (Fig. 5). Sapphire and PR04-19 had among the lowest antioxidant capacities and were negatively associated with antioxidant capacity on the PCA biplot (Fig. 5, Table A6). However, all assays correlated significantly with one another

($R^2 > 0.440$) on a significance level of $P < 0.0001$ (data not shown), although not as strongly as during the first harvest season.

Similar to the first harvest season, negative correlations were seen between fruit mass and antioxidant capacity, as well as negative correlation between antioxidant capacity and L^* -values of the peel (data not shown).

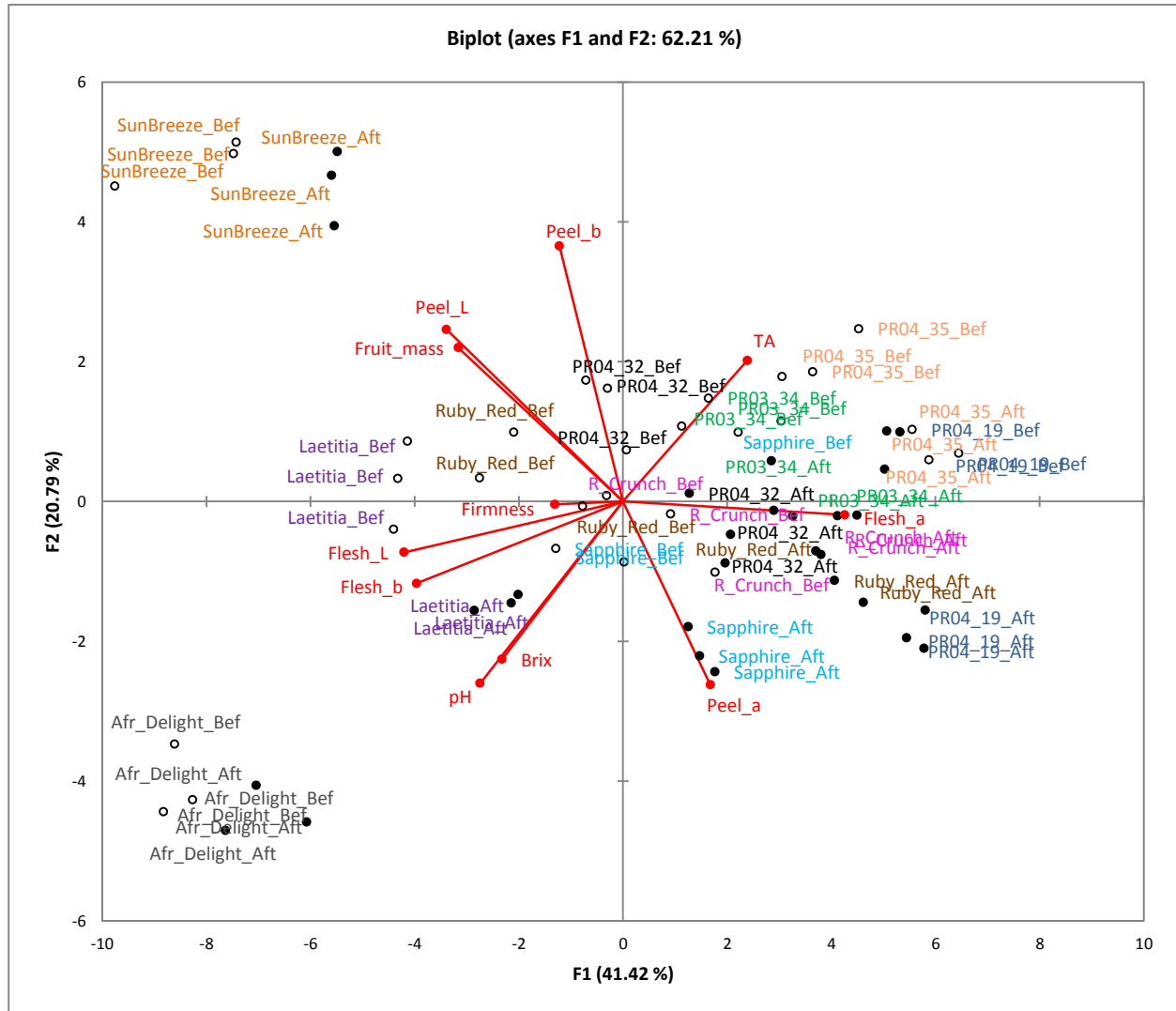


Figure 3 PCA biplot of associations between fruit attributes and cultivars and selections of the second harvest season.

*Refer to Fig. 1 for abbreviations key.

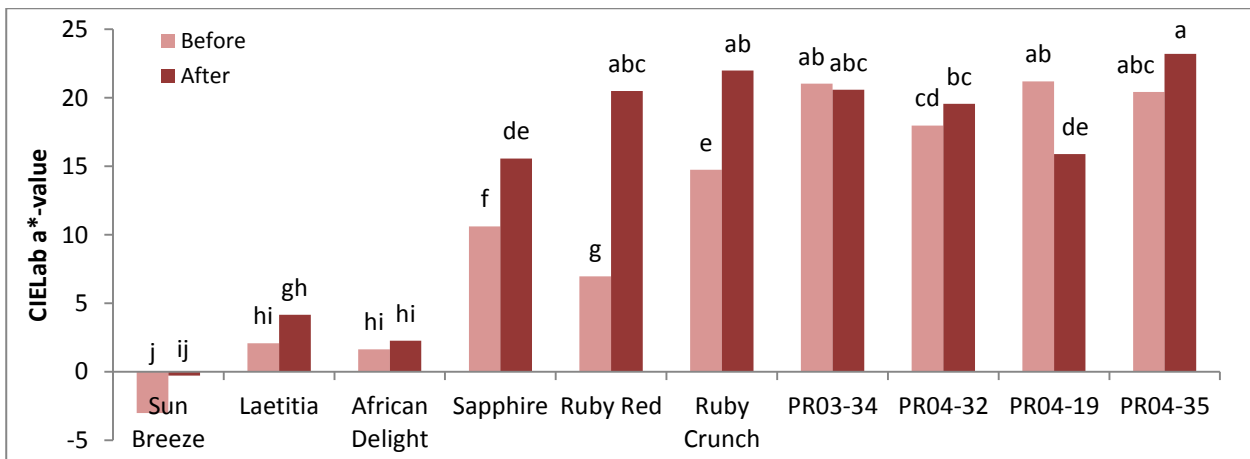


Figure 4 Flesh colour a^* -measurement (CIELab) for cultivars and selections of the second harvest season (2011/2012). Different letters above the column indicate statistically significant differences ($P < 0.05$).

The total polyphenol content was positively associated with selections PR04-35 and PR04-32 (Fig. 5). The unripe fruit of these two selections had the highest total polyphenol content of all cultivars/selections, while when ripe PR04-35 had the highest total polyphenol content ($P \leq 0.05$) (Table A2). The total polyphenol content negatively associated with Sapphire and PR04-19, the cultivar and selection, respectively, with the lowest total polyphenol content (Table A2). However, the trend for total polyphenol content was less noticeable than for the first harvest season, i.e. the total polyphenol content of the red-fleshed plums Ruby Red, Ruby Crunch and PR03-34, was not significantly higher ($P > 0.05$) than that of the yellow-fleshed plums (Sun Breeze, Laetitia and African Delight).

Ruby Red (ripe) and PR04-19 (ripe) were positively associated with the individual and total anthocyanin content (Fig. 5). PR04-19 (ripe) had a significantly higher cyanidin-3-*O*-rutinoside content than other cultivars with $161.5 \text{ mg} \cdot \text{kg}^{-1} \text{ FW}$ (Table A7). The cyanidin-3-*O*-glucoside content did not differ significantly ($P > 0.05$) between PR04-19 and Ruby Red after cold storage and ripening (Table A7). Their cyanidin-3-*O*-glucoside contents were substantially higher than that of the other cultivars and selections.

Similar to the first harvest season, procyanidin B2 and (-)-epicatechin were closely associated on the PCA biplot, while (+)-catechin and procyanidin B1 were correlated (Fig. 5). A trend was seen between flavan-3-ol compounds as procyanidin B2 and (-)-epicatechin were present in the highest concentration in PR04-35, and procyanidin B1 and (+)-catechin in selection PR04-32 (Table A7). The total flavan-3-ol content was closely associated with both PR04-32 and

PR04-35 (Fig. 5), having the highest total flavan-3-ol content of all the cultivars and selections (Table A10).

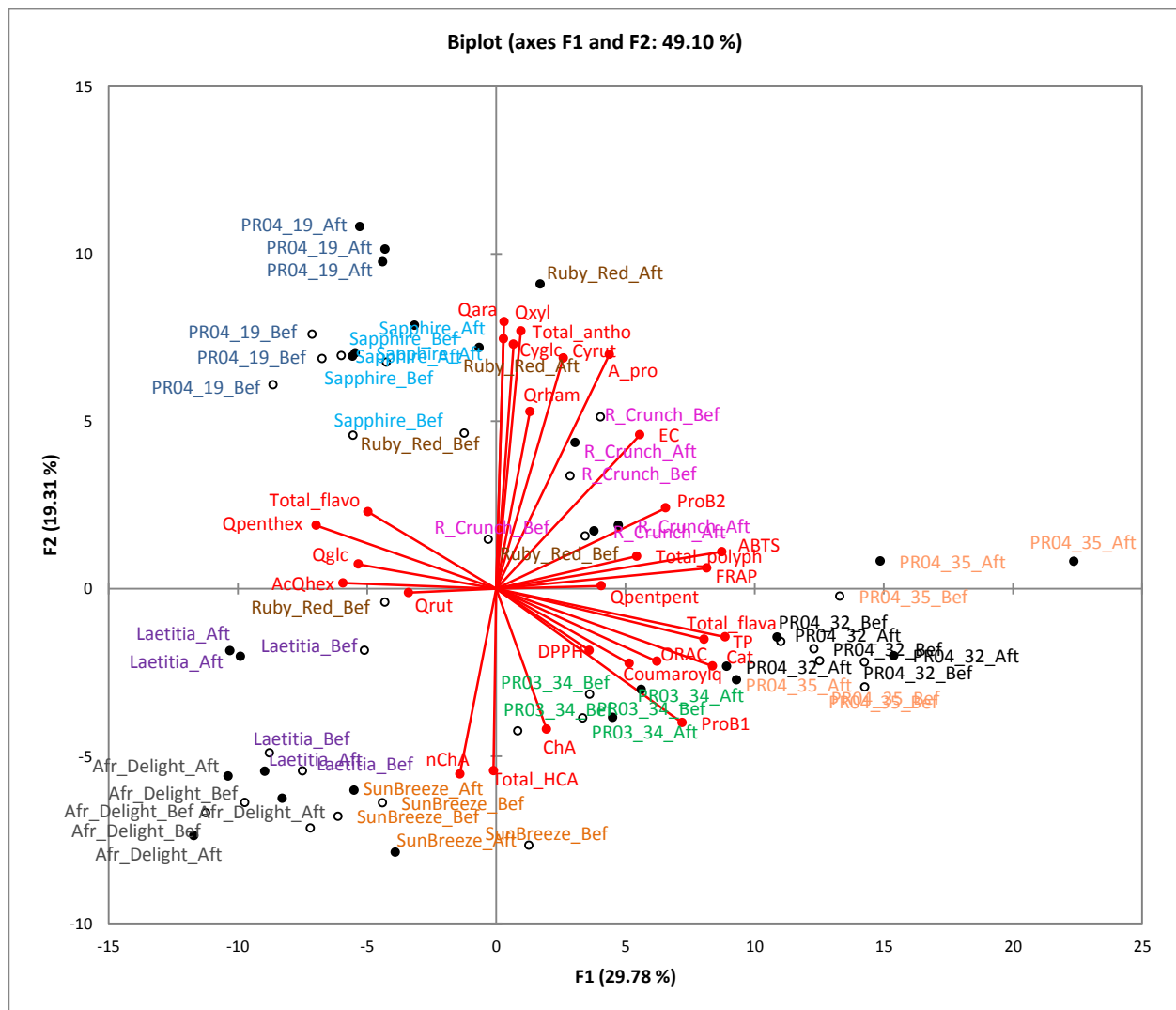


Figure 5 PCA biplot of associations between phenolic compounds, antioxidant capacity, and cultivars and selections of the second harvest season.

*Refer to Fig. 1 for abbreviations key.

From the flavonol group quercetin-3-*O*-rhamnoside, -arabinoside and -xyloside were closely associated with Ruby Red (Fig. 5). Ruby Red (ripe) possessed a significantly higher ($P \leq 0.05$) content of these compounds than other cultivars and selections (Table A7). Acetylated quercetin-hexoside was closely associated with Laetitia (ripe) which contained the highest concentration of this compound ($21.8 \text{ mg.kg}^{-1} \text{ FW}$) (Table A7). Quercetin pentosyl-hexoside, quercetin-3-*O*-glucoside and quercetin-3-*O*-rutinoside were negatively associated with PR04-35 and PR04-32 (Fig. 5). The latter two quercetin derivatives were present in the lowest concentrations in the mentioned selections, while quercetin pentosyl-hexoside was absent in PR04-35 and PR04-32

(Table A7). A trend also observed for the first harvest season was the close association between quercetin-3-*O*-arabinoside, -xyloside and rhamnoside.

The phenolic acids, neochlorogenic acid and chlorogenic acid, as well as the total hydroxycinnamic acids were associated with Sun Breeze, African Delight and Laetitia (Fig. 5). African Delight and PR04-35 contained the highest amount of chlorogenic acid (Table A7), while selections PR04-35 and PR04-34 possessed the highest amount of 3-*O*-*p*-coumaroylquinic acid (Table A7).

Phenolic compounds showing a close association with antioxidant capacity were the flavan-3-ols, 3-*O*-*p*-coumaroylquinic acid, total flavan-3-ols and total polyphenols (Fig. 5). Pearson's correlation matrix shows positive significant correlations between flavan-3-ol content and antioxidant capacity measured with all antioxidant assays, which was also observed for the 2010/2011 harvest season. In the second harvest season 3-*O*-*p*-coumaroylquinic acid was significantly correlated antioxidant capacity according to the ABTS^{•+} and FRAP assays (data not shown), while no significant correlations were observed for the first harvest season. In terms of flavonols, quercetin pentosyl-pentoside was the only compound with a positive significant correlation to antioxidant capacity (0.355 with the ORAC assay on a $P < 0.0001$ significance level). Similar to the first harvest season, quercetin pentosyl-hexoside and acetylated quercetin-hexoside correlated negatively (Pearson's correlation matrix) to all antioxidant assays. These flavonols, as well as quercetin-3-*O*-glucoside and the total flavonols associate negatively with antioxidant capacity on the PCA biplot (Fig. 5).

Comparison of two harvest seasons

Only data after cold storage and ripening will be discussed in this section for the comparison of cultivars and selections harvested during the two consecutive seasons. From the PCA biplot for fruit attributes (Fig. 6) it is clear that separate clusters formed for the respective seasons, indicating that the two seasons differed from one another in terms of fruit attributes. Overlap between seasons was only observed for African Delight. Larger variation was visible between cultivars/selections for associations between antioxidant capacity, phenolic compounds and cultivars/selections (Fig. 7). Clear separation between seasons was seen for African Delight and PR04-32, although clusters for a specific cultivar remain in the same general space on the biplot, indicating that seasonal effects did not overwhelm cultivar differences.

Using the DPPH[•] assay PR04-32 and African Delight had the highest antioxidant capacity during the first harvest season. Based on the DPPH[•] assay, a significant decrease occurred in antioxidant capacity of all cultivars and selections from the first to the second harvest season (Table A8). A different trend was observed using the ABTS^{•+} assay to assess antioxidant capacity. PR03-34 and Sapphire increased significantly ($P \leq 0.05$) from the first to the second harvest season, but no significant differences were observed between the two years for the other cultivars/selections. The FRAP assay also indicated a higher antioxidant capacity for PR04-32 ($P \leq 0.05$) in the first harvest season, while none of the other cultivars/selections showed a significant change. Interestingly, PR04-32 showed a significant decrease ($P \leq 0.05$) in antioxidant capacity from the first to the second season according to the ORAC assay, similar to the results obtained with the DPPH[•] and FRAP assays. For the other cultivars/selections, the ORAC assay showed a significant increase in antioxidant capacity for Ruby Red, while no significant change ($P > 0.05$) occurred between two seasons for the other cultivars/selections. The PCA biplot (Fig. 7) indicated that observations for the 2010/2011 harvest season for PR04-32 shows closer association to antioxidant capacity than the second harvest season, while the opposite was seen for PR03-34.

Generally little significant changes were observed for the anthocyanin content between harvest seasons. The only exception was PR03-34, for which both anthocyanins (cyanidin-3-*O*-glucoside and –rutinoside) showed a significant increase ($P \leq 0.05$) from the first to the second season, and PR04-32 for which the content of these anthocyanins decreased significantly ($P \leq 0.05$) (Table A9). The content of individual flavan-3-ols was not greatly affected by harvest season (Table A9). Only the flavan-3-ol content of African Delight and PR03-34 was affected, showing a decrease in two or more compounds from the first to the second season. This was particularly the case for the monomeric catechin content of African Delight and PR04-32 and the procyanidin B1 content of African Delight.

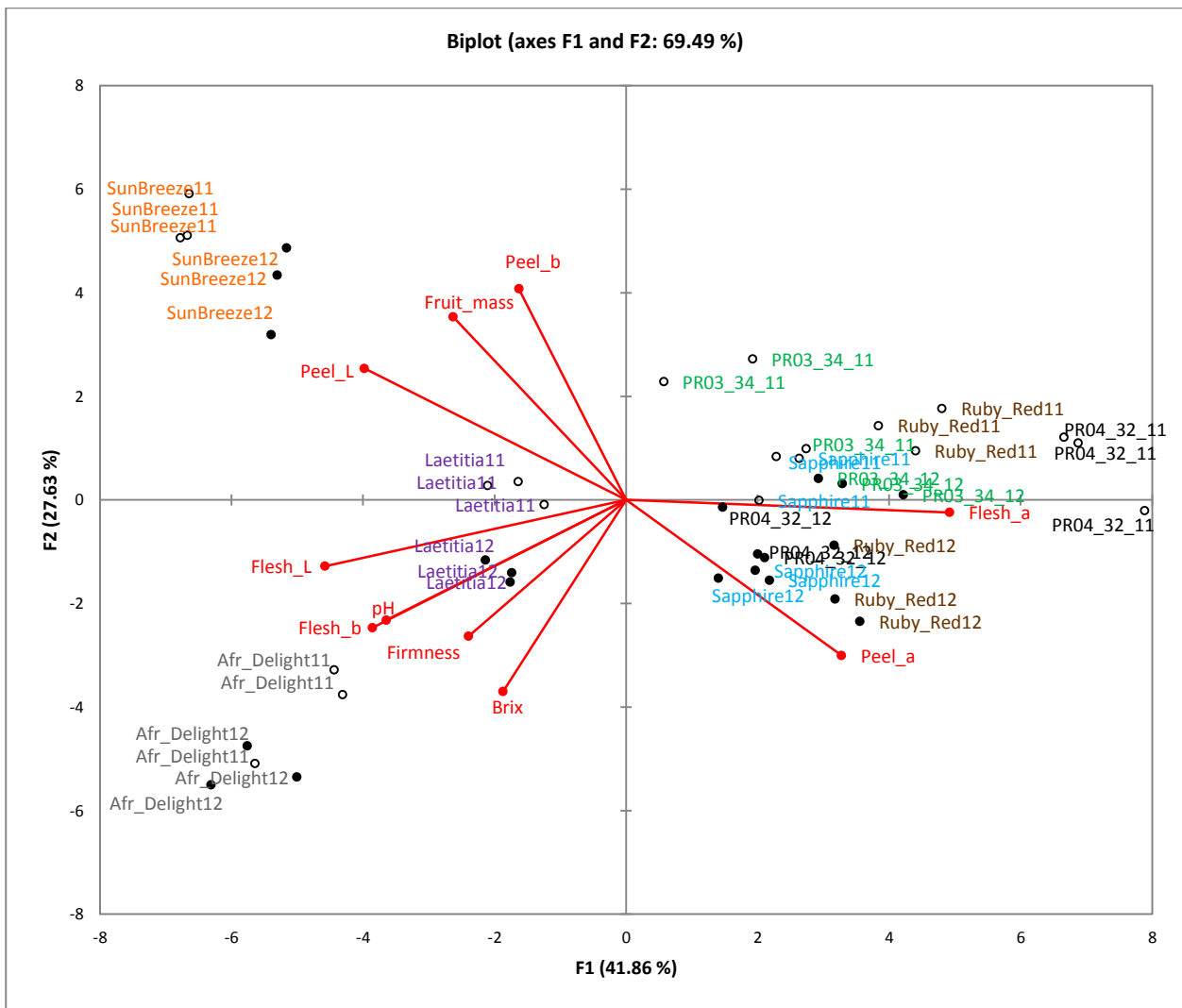


Figure 6 PCA biplot reflecting associations between cultivars and selections and different fruit attributes (colour, °Brix, pH, mass and solids) of two consecutive harvest seasons (ripe).

* 11 = 2010/2011 harvest season (open circles); 12 = 2011/2012 harvest season (closed circles). Refer to Fig. 1 for rest of abbreviations key.

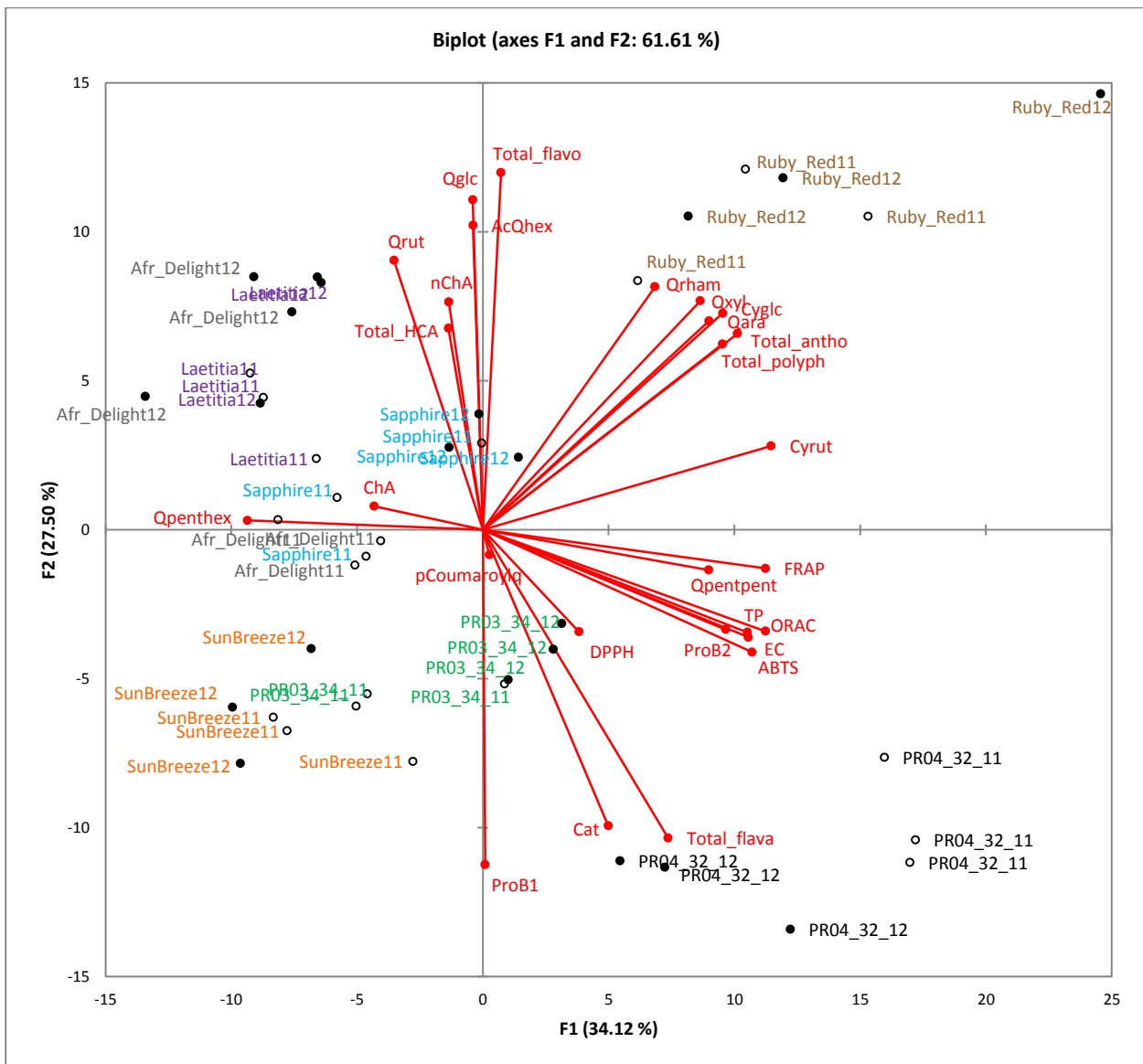


Figure 7 PCA biplot displaying associations between phenolic compounds, antioxidant capacity and cultivars and selections of two consecutive harvest seasons (ripe fruit).

*11 = 2010/2011 harvest season (open circles); 12 = 2011/2012 harvest season (closed circles). Refer to Fig. 1 for rest of abbreviations key.

Overall, the individual flavonol content of cultivars/selections was either unaffected by season, or increased from the first to the second harvest season ($P \leq 0.05$), except in the case of Ruby Red and PR04-32. For both, the quercetin-3-*O*-rutinoside content decreased from the first to the second harvest season, while the acetylated quercetin-hexoside content of PR04-32 also decreased (Table A9).

By comparing two harvest seasons it is evident that season affects the composition of fruit, but that it does not obscure cultivar differences. Due to the diversity of phenolic content, fruit attributes and antioxidant activity a specific trend could not be assigned for the change from one

season to the next. In a study by Kim *et al.* (2004) plum cultivars (*P. domestica*) grown in New York were compared in terms of phenolic composition over two harvest seasons. This study shows that the content of various phenolic compounds does differ between cultivars as well as from one harvest season to a following.

CONCLUSIONS

Fruit attributes, phenolic content and antioxidant capacity was successfully evaluated for eight South African plum cultivars and selections during the first harvest season, and ten during the second harvest season. The phenolic composition varied greatly between different cultivars and selections. Plums with red peel and flesh contain a greater amount of phenolic compounds than plums with yellow flesh, which can be attributed to the presence of anthocyanins. Anthocyanin content increased during cold storage and ripening, while flavan-3-ol, flavonol and hydroxycinnamic acid content was not affected.

Red-fleshed plums generally displayed a greater antioxidant capacity. The total polyphenol content is highly correlated with antioxidant capacity, which suggests that phenolic compounds are largely responsible for the antioxidant capacity of fruit. Flavan-3-ols and anthocyanins showed positive significant correlations to antioxidant capacity. During both seasons the antioxidant capacity of several cultivars and selections increased significantly with the ABTS^{•+}, FRAP and DPPH[•] assays as a result of cold storage and ripening. Cold storage and ripening had no significant effect on antioxidant capacity measured with the ORAC assay. A slight difference in phenolic content and composition is observed between harvest seasons.

The current study provided insight into the phenolic composition and antioxidant activity of South African plum cultivars and selections and the effect of a commercial cold storage and ripening regime, a topic on which there is limited knowledge. It is believed these findings could benefit future studies on South African plums and possibly other stone fruit, especially in breeding programs. Possible incorporation of data on the content of individual phenolic compounds in phenolic databases (e.g. Phenol-Explorer) would benefit other researchers. The current data may also inspire further evaluation into the antioxidant activity of phenolic compounds in plums, and their corresponding health benefits.

CHAPTER 4

ADDENDUM TABLES

Table A1 Fruit attributes for the 2010/2011 harvest season before and after cold storage and ripening ^a

Cultivar/ selection	Stage of maturity	Firmness (kg)	pH	TA (g malic acid.kg ⁻¹ fruit)	°Brix	Peel a*	Flesh a*	Peel b*	Flesh b*	Peel L*	Flesh L*
Sun Breeze	Unripe	4.8 bcd	3.9 b	25.1 d	13.6 efgh	-9.9 i	-5.0 j	32.4 a	23.7 c	57.0 a	49.4 bc
	Ripe	3.8 cd	3.9 bcd	22.0 e	12.6 hi	-3.4 h	-1.1 i	3.5 a	19.7 e	59.3 a	48.0 c
Laetitia	Unripe	5.2 bc	3.9 cd	22.7 c	14.0 def	23.3 bc	2.0 gh	6.1 d	28.1 a	46.4 b	53.8 a
	Ripe	0.2 f	4.0 b	19.1 f	13.2 fghi	20.6 bcd	4.7 f	0.8 efg	22.1 d	41.7 c	48.3 c
African Delight	Unripe	8.7 a	4.2 a	12.6 g	19.7 a	23.5 b	1.2 ghi	4.2 de	28.2 a	48.4 b	51.2 b
	Ripe	5.4 bc	4.2 a	9.9 h	18.6 b	18.0 de	3.5 gh	-0.5 fg	25.7 b	42.7 c	49.0 bc
Sapphire	Unripe	5.0 bcd	3.4 h	29.5 c	14.2 de	15.2 ef	-0.1 hi	5.7 d	25.9 b	36.5 d	47.2 c
	Ripe	0.0 f	3.5 g	24.7 d	13.0 fghi	19.3 d	8.9 e	2.9 de	20.1 e	30.0 f	41.3 b
Ruby Red	Unripe	3.8 cd	3.7 f	43.8 a	16.5 c	17.7 ed	12.3 d	3.3 de	20.0 e	37.6 d	40.5 de
	Ripe	0.2 f	3.8 e	33.8 b	15.0 d	13.8 fg	18.8 bc	-2.0 g	10.0 i	29.8 fg	27.6 i
PR02-55	Unripe	6.3 ab	3.6 gh	34.9 b	13.8 ef	14.1 fg	17.7 c	2.9 de	17.7 f	27.5 gh	38.0 ef
	Ripe	8.2 a	3.8 de	24.1 de	13.2 fghi	13.2 fg	18.2 c	1.6 ef	16.1 fg	25.4 h	40.3 de
PR03-34	Unripe	5.1 bcd	3.9 d	29.5 c	13.7 efg	11.3 g	23.9 a	16.9 b	17.4 f	47.5 b	34.9 gh
	Ripe	2.8 de	3.9 bc	23.3 de	12.7 ghi	20.1 cd	21.3 b	16.4 b	14.0 h	46.2 b	30.2 i
PR04-32	Unripe	6.7 ab	3.8 de	28.8 c	13.3 efghi	23.8 b	25.8 a	11.9 c	19.1 e	35.7 d	37.0 fg
	Ripe	0.9 ef	3.4 h	24.6 d	12.5 i	28.9 a	25.8 a	10.8 c	15.1 gh	32.6 e	30.2 i

^a Different letters in the same column indicate a statistically significant difference (P<0.05).

Table A2 Total polyphenol content for South African plum cultivars and selections (Folin-Ciocalteu method)

Cultivar/ selection	2010/2011 season ^a		2011/2012 season ^b	
	(mg GAE.g ⁻¹ FW) ^c		(mg GAE.g ⁻¹ FW) ^c	
	Unripe	Ripe	Unripe	Ripe
Sun Breeze	2.4 ed	2.6 bcd	2.6 ghij	2.3 ij
Laetitia	2.6 cd	2.4 de	2.8 defgh	2.6 fghij
African Delight	2.7 bcd	2.9 b	2.6 efghij	2.7 efghij
Sapphire	1.9 f	2.2 e	2.3 j	2.3 ij
Ruby Red	2.4 ed	2.8 bc	2.4 hij	3.0 cdefg
Ruby Crunch	-	-	2.7 efghi	3.0 cde
PR02-55	2.7 bcd	2.8 bc	-	-
PR03-34	2.8 bc	2.8 bc	2.7 efgh	3.0 defg
PR04-32	3.5 a	3.4 a	3.2 bc	3.2 bcd
PR04-19	-	-	2.3 ij	2.6 efghij
PR04-35	-	-	3.5 ab	3.8 a

^a Values represent averages of total polyphenol contents of plums from three trees; different letters between two columns indicate a statistically significant difference ($P \leq 0.05$); ^b Values represent averages of total polyphenol contents of plums from three trees; different letters between two columns indicate a statistically significant difference ($P \leq 0.05$); ^c GAE = gallic acid equivalents.

Table A3 Concentration of individual phenolic compounds after cold storage and ripening for the first harvest season

Cultivar/selection	Compound (mg.kg ⁻¹ FW) ^a								
	Neochlorogenic acid	Chlorogenic acid	3-O- <i>p</i> -coumaroyl-quinic acid	Cyanidin-3-O-glucoside	Cyanidin-3-O-rutinoside	(-)-Epicatechin	(+)-Catechin	Procyanidin B1	Procyanidin B2
Sun Breeze	216.3 d	N.D. c	14.6 c	N.D. g	N.D. h	11.0 f	98.2 de	211.1 a	14.1 e
Laetitia	334.2 c	N.D. c	16.3 c	51.5 defg	15.6 fgh	19.9 ef	89.2 e	105.7 e	8.9 e
African Delight	388.9 ab	19.2 a	N.D. d	72.33 def	10.0 gh	21.0 e	126.5 c	146.5 c	9.3 e
Sapphire	N.D. f	N.D. c	N.D. d	208.9 b	43.7 de	44.1 d	49.9 g	64.6 f	48.2 e
Ruby Red	353.7 abc	N.D. c	20.8 b	447.0 a	91.4 ab	59.3 c	57.7 g	51.6 f	43.5 d
PR02-55	3.7 f	N.D. c	N.D. d	207.2 b	95.9 a	122.1 a	14.1 h	11.4 g	108.8 b
PR03-34	218.1 d	8.8 b	99.6 a	92.2 cd	37.4 def	17.0 ef	107.5 d	204.3 ab	14.9 e
PR04-32	65.8 e	N.D. c	N.D. d	188.7 b	100.8 a	113.1 b	189.3 b	209.0 a	82.2 c

^a Different letters in a column indicate a statistically significant difference at P<0.05; N.D. = none detected.

Table A3 (continued) Concentration of individual phenolic compounds after cold storage and ripening for the first harvest season

Cultivar/selection	Compound (mg.kg ⁻¹ FW) ^a							
	Quercetin-3-O-rutinoside	Quercetin-3-O-glucoside	Quercetin-3-O-arabinoside	Quercetin-3-O-rhamnoside	Quercetin-3-O-xyloside	Quercetin pentosyl-hexoside	Quercetin pentosyl-pentoside	Acetylated Quercetin-hexoside
Sun Breeze	6.8 h	11.7 gh	18.0 ghi	5.7 cd	3.6 de	6.0 c	3.3 bc	N.D. g
Laetitia	53.6 a	63.5 b	20.1 fghi	4.6 cde	3.3 de	2.8 d	N.D. g	23.6 a
African Delight	44.7 ab	91.9 a	10.2 i	2.7 de	2.0 ef	6.7 abc	N.D. g	10.2 cde
Sapphire	23.2 de	49.8 c	30.5 cdef	6.3 c	4.1 d	7.0 ab	2.7 ed	10.5 cd
Ruby Red	39.1 bc	95.9 a	40.6 bc	10.6 b	6.9 b	N.D. e	2.9 cd	16.7 b
PR02-55	22.8 de	27.6 ef	53.3 ab	14.5 a	9.1 a	N.D. e	4.6 a	6.7 ef
PR03-34	8.5 fgh	10.1 h	20.5 fghi	5.1 cd	2.8 de	5.9 c	2.3 ef	5.6 f
PR04-32	20.8 defg	24.9 fg	29.3 cdef	4.7 cde	4.6 cd	N.D. e	3.4 b	8.6 cdef

^a Different letters in a column indicate a statistically significant difference at P<0.05; N.D. = none detected.

Table A4 Antioxidant capacity of cultivars of the first harvest season before and after cold storage and ripening

Cultivar/ selection	Stage of maturity	Antioxidant capacity ($\mu\text{mol TE}\cdot\text{g}^{-1}\text{FW}$) ^{a b}			
		ABTS ^{•+}	DPPH [•]	FRAP	ORAC
Sun Breeze	Unripe	23.0 ed	19.8 defg	9.2 g	38.5 def
	Ripe	23.9 cde	20.5 efg	9.6 efg	38.9 cdef
Laetitia	Unripe	23.0 ed	19.6 efg	9.9 efg	37.1 ef
	Ripe	22.3 e	19.1 fg	9.5 efg	37.1 ef
African Delight	Unripe	24.9 bcde	24.7 b	10.9 cde	41.5 cde
	Ripe	27.5 b	27.9 a	11.8 bc	46.8 bcd
Sapphire	Unripe	18.0 f	16.2 h	7.6 fg	31.1 f
	Ripe	21.8 e	20.5 efg	9.3 h	33.9 ef
Ruby Red	Unripe	22.2 e	18.5 gh	10.1 defg	47.6 b
	Ripe	27.5 b	23.3 bc	12.7 b	51.2 b
PR02-55	Unripe	26.0 bcd	21.1 cdef	10.9 cde	39.0 cdef
	Ripe	27.0 bc	23.2 bc	11.4 bcd	40.4 cde
PR03-34	Unripe	26.7 bc	21.8 cde	10.9 cde	38.8 cdef
	Ripe	26.8 bc	22.7 bcd	10.8 cdef	40.7 cde
PR04-32	Unripe	36.3 a	27.5 a	14.7 a	69.0 a
	Ripe	35.4 a	27.8 a	14.4 a	67.1 a

^a TE = Trolox equivalents; ^b different letters in the same column indicate a statistically significant difference ($P < 0.05$).

Table A5 Fruit attributes for the 2011/2012 harvest season before and after cold storage and ripening ^a

Cultivar/ selection	Stage of maturity	Firmness (kg)	pH	TA (g malic acid.kg ⁻¹ fruit)	°Brix	Peel a*	Flesh a*	Peel b*	Flesh b*	Peel L*	Flesh L*
Sun Breeze	Unripe	10.0 ab	3.9 cd	23.9 efg	15.0 ef	-8.7 l	-3.0 j	24.5 a	21.3 d	58.7 a	47.5 c
	Ripe	3.0 hi	4.0 bc	24.5 defg	14.3 gh	-0.5 k	-0.3 ij	27.3 a	17.6 ef	58.4 a	41.7 e
Laetitia	Unripe	8.0 cd	3.9 bcd	30.8 c	14.2 gh	22.1 c	2.1 hi	10.4 e	26.5 b	49.7 b	50.5 b
	Ripe	2.7 i	4.0 bc	22.9 fg	13.7 h	21.8 cd	4.2 gh	1.3 hi	23.0 c	41.4 ef	48.3 c
African Delight	Unripe	11.1 a	4.2 a	14.9 h	21.0 a	21.9 c	1.6 hi	4.0 g	29.1 a	48.9 b	53.9 a
	Ripe	7.9 cde	4.2 a	12.5 h	19.3 b	18.8 e	2.3 hi	-0.04 ij	27.4 b	42.8 de	51.5 b
Sapphire	Unripe	3.4 hi	3.8 ef	29.6 cd	15.2 ef	13.6 ji	10.6 f	1.7 ghi	21.4 d	31.7 jk	43.5 d
	Ripe	2.4 i	4.0 b	20.4 g	14.1 gh	19.6 de	15.6 de	3.2 gh	18.5 e	20.0 lm	38.1 f
Ruby Red	Unripe	7.9 cd	3.8 def	37.8 b	17.2 c	19.3 e	6.7 g	11.9 de	22.9 c	42.0 ef	44.8 d
	Ripe	5.4 fg	3.9 de	30.4 c	16.4 d	15.0 hi	20.5 abc	-1.7 jk	12.2 hi	29.7 kl	30.5 ij
Ruby Crunch	Unripe	9.0 bc	3.9 de	29.8 c	15.1 ef	12.4 j	14.8 e	-1.5 jk	17.6 ef	27.2 h	37.7 f
	Ripe	4.4 gh	3.9 cd	30.7 c	14.0 gh	12.0 j	22.0 ab	-2.6 k	13.1 h	33.3 ij	32.5 gh
PR03-34	Unripe	3.6 hi	3.8 def	30.1 c	16.1 d	16.2 fgh	21.0 ab	14.3 cd	14.6 g	44.2 cd	33.8 g
	Ripe	2.9 hi	3.9 bcd	22.5 fg	14.6 fg	22.5 b	20.6 abc	14.5 c	11.6 i	42.3 de	29.1 j
PR04-32	Unripe	8.5 bc	3.9 bcd	27.6 cdef	12.8 i	18.4 ef	18.0 cd	20.2 b	21.4 d	44.8	40.5 e
	Ripe	6.3 ef	4.0 bc	20.7 g	12.3 i	29.2 a	19.6 bc	15.1 c	16.6 f	40.2 fg	36.6 f
PR04-19	Unripe	7.4 cde	3.8 f	47.1 a	15.4 e	15.8 ghi	21.2 ab	6.9 f	12.1 hi	34.5 i	31.9 hi
	Ripe	6.6 def	4.0 bc	24.4 defg	14.5 fg	15.2 hi	15.9 de	2.8 gh	7.2 j	27.5 m	25.9 k
PR04-35	Unripe	8.8 bc	3.8 ef	36.7 b	12.8 i	18.0 efg	20.4 abc	13.6 cd	12.7 hi	43.4 cde	33.9 g
	Ripe	8.9 bc	3.8 ef	28.8 cde	11.4 j	29.2 a	23.2 a	13.2 cd	12.0 hi	39.1 gh	34.6 hi

Table A6 Antioxidant capacity of cultivars of the second harvest season before and after cold storage and ripening

Cultivar/ selection	Stage of maturity	Antioxidant capacity ($\mu\text{mol TE}\cdot\text{g}^{-1}\text{FW}$) ^{a b}			
		ABTS ⁺⁺	DPPH [•]	FRAP	ORAC
Sun Breeze	Unripe	23.4 e	12.6 ghij	9.5 gh	40.2 defg
	Ripe	21.9 e	12.5 hij	9.0 h	41.4 cdef
Laetitia	Unripe	24.5 e	13.1 fghij	10.5 efg	36.7 defg
	Ripe	23.0 e	12.4 hij	9.8 gh	35.4 defg
African Delight	Unripe	24.8 e	16.4 abc	10.6 efg	37.5 defg
	Ripe	23.8 e	16.4 bc	10.5 efg	38.4 defg
Sapphire	Unripe	25.0 e	12.6 ghij	9.3 gh	32.0 g
	Ripe	25.0 e	13.3 fghi	9.7 gh	32.6 fg
Ruby Red	Unripe	23.2 e	11.4 i	9.5 gh	58.1 ab
	Ripe	29.7 cd	14.6 cdef	12.5 cd	55.4 ab
Ruby Crunch	Unripe	29.3 c	13.9 efgh	11.7 cde	42.5 cde
	Ripe	32.1 bcd	15.2 cde	12.6 cd	50.5 bc
PR03-34	Unripe	29.3 cd	13.8 efghi	11.3 def	44.2 cd
	Ripe	33.0 bc	15.5 cde	12.3 cd	42.1 cde
PR04-32	Unripe	33.6 b	18.2 a	13.0 bc	54.7 ab
	Ripe	32.5 bcd	17.7 ab	12.5 cd	62.2 a
PR04-19	Unripe	25.3 e	12.0 ij	10.0 fgh	31.4 g
	Ripe	29.1 d	14.4 defg	11.8 cde	33.7 efg
PR04-35	Unripe	37.8 a	15.8 cd	14.2 ab	39.1 defg
	Ripe	38.0 a	15.9 cd	15.2 a	33.7 cd

^a TE = Trolox equivalents; ^b different letters in a column indicate a statistically significant difference ($P < 0.05$).

Table A7 Concentration of individual phenolic compounds after cold storage and ripening of the second harvest season

Cultivar/ selection	Compound (mg.kg ⁻¹ FW) ^a									
	Neochlorogenic acid	Chlorogenic acid	3-O-p-coumaroyl- quinic acid	Cyanidin-3-O- glucoside	Cyanidin-3-O- rutinoside	(-)-Epicatechin	(+)-Catechin	Procyanidin B1	Procyanidin B2	A-type procyanidin trimer
Sun Breeze	212.7 g	N.D.	10.7 c	N.D. i	N.D. i	8.4 hi	75.5 ef	204.8 cd	17.0 gh	7.4 jk
Laetitia	376.3 bc	N.D.	15.1 bc	52.5 fg	15.3 h	10.5 hi	65.5 efg	99.8 efg	8.6 h	6.6 jk
African Delight	356.0 c	21.7 a	N.D. d	69.8 ef	13.1 hi	5.0 i	66.7 efg	104.4 efg	9.9 h	4.5 k
Sapphire	N.D. i	N.D.	N.D. d	231.6 b	56.6 d	51.5 c	60.9 efg	73.5 gh	47.6 cd	36.5 ab
Ruby Red	433.0 a	N.D.	23.4 b	474.9 a	87.4 c	57.6 b	56.4 efg	64.9 gh	52.6 c	32.2 bc
Ruby Crunch	57.5 h	N.D.	N.D. d	230.0 b	120.4 b	42.4 g	156.9 abc	243.9 bc	34.8 ef	17.7 gh
PR03-34	255.6 def	11.4 b	115.4 a	232.8 b	55.5 d	22.4 g	122.2 cd	216.6 cd	16.6 gh	13.2 hi
PR04-32	73.5 h	N.D.	N.D. d	70.4 ef	33.2 ef	97.1 a	154.7 abcd	194.0 d	90.3 a	27.4 cde
PR04-19	8.4 i	N.D.	N.D. d	484.7 a	161.5 a	43.4 de	34.1 g	22.3 i	17.1 gh	25.8 de
PR04-35	284.5 d	22.3 a	117.3 a	159.9 cd	95.4 c	38.7 ef	133.7 bcd	328.6 a	52.3 c	30.2 cd

^a Different letters in a column indicate a statistically significant difference at P<0.05.

Table A7 (continued) Concentration of phenolic compounds after cold storage and ripening of the second harvest season

Cultivar/selection	Compound (mg.kg ⁻¹ FW) ^a								
	Quercetin-3-O-rutinoside	Quercetin-3-O-glucoside	Quercetin-3-O-arabinoside	Quercetin-3-O-rhamnoside	Quercetin-3-O-xyloside	Quercetin-pentosyl-hexoside	Quercetin-pentosyl-pentoside	Acetylated Quercetin-hexoside	
Sun Breeze	7.6 ij	13.3 ij	12.7 j	5.9 defgh	3.0 ghij	4.8 e	2.4 cd	1.0 de	
Laetitia	64.2 c	69.8 d	26.3 fghij	8.1 bcde	4.3 defghi	4.5 e	1.4 e	21.8 a	
African Delight	70.2 c	12.94 a	11.7 j	3.3 h	2.6 ij	8.6 ab	1.4 e	18.3 ab	
Sapphire	23.3 ef	65.8 d	39.9 bcd	8.4 bcd	6.0 bcd	7.4 bcd	3.4 ab	11.9 c	
Ruby Red	28.8 e	93.0 c	52.7 a	17.0 a	9.2 a	N.D. g	4.3 a	14.1 bc	
Ruby Crunch	92.9 a	87.8 c	25.2 ghi	6.8 cdefg	4.4 defgh	4.5 d	N.D. g	3.0 de	
PR03-34	11.7 ghij	25.2 gh	20.3 hij	5.0 efgh	3.3 efghij	5.6 ed	2.8 bc	5.2 d	
PR04-32	9.8 hij	13.6 ij	19.3 hij	3.7 gh	3.0 ghij	N.D. g	3.3 bc	4.5 de	
PR04-19	22.4 ef	48.1 e	34.0 bcdefg	7.3 cdef	5.1 cde	9.3 ab	N.D. fg	10.1 c	
PR04-35	11.8 ghij	15.1 hij	32.1 cdefg	11.1 b	5.4 cd	N.D. g	3.1 bc	4.9 d	

^a Different letters in a column indicate a statistically significant difference at P<0.05.

Table A8 Comparison of the antioxidant capacity for cultivars and selections (ripe) harvested during two consecutive harvest seasons

Cultivar/ selection	Harvest season ^a	Antioxidant capacity ($\mu\text{mol TE}\cdot\text{g}^{-1}\text{FW}$) ^{b,c}			
		ABTS ⁺⁺	DPPH [•]	FRAP	ORAC
Sun Breeze	First	23.5 de	20.2 cd	9.4 fgh	38.7 def
	Second	22.8 ef	12.5 i	9.3 gh	40.8 de
Laetitia	First	22.7 ef	19.4 de	9.7 fg	37.1 def
	Second	23.7 cde	12.8 i	10.1 efg	36.0 ef
African Delight	First	26.2 cde	26.3 a	11.3 cd	44.2 cd
	Second	23.3 cde	16.4 g	10.5 def	37.9 def
Sapphire	First	19.9 f	18.3 ef	8.5 h	32.5 f
	Second	25.0 cde	13.0 i	9.5 fgh	32.2 f
Ruby Red	First	24.9 cde	20.9 bc	11.4 cd	49.4 c
	Second	26.6 c	13.0 i	11.0 cde	56.7 b
PR03-34	First	24.6 cde	22.2 b	10.8 cde	39.7 de
	Second	31.2 b	14.7 h	11.8 bc	43.1 cd
PR04-32	First	35.8 a	27.6 a	14.5 a	68.0 a
	Second	33.1 ab	18.0 f	12.7 b	58.4 b

^a First = 2010/2011 harvest season; Second = 2011/2012 harvest season; ^b TE = Trolox equivalents; ^c different letters in a column indicate a statistically significant difference ($P < 0.05$).

Table A9 Individual phenolic compounds (ripe) for cultivars and selections evaluated during two consecutive harvest seasons

Cultivar/selection	Harvest year	Compound (mg.kg ⁻¹ FW) ^a								
		Neochlorogenic acid	Chlorogenic acid	3-O- <i>p</i> -coumaroyl-quinic acid	Cyanidin-3-O-glucoside	Cyanidin-3-O-rutinoside	(-)-Epicatechin	(+)-Catechin	Procyanidin B1	Procyanidin B2
Sun Breeze	First	216.3 e	N.D. e	14.6 def	N.D. j	N.D. j	11.0 hij	98.2 fg	211.1 a	14.1 e
	Second	212.7 e	N.D. e	10.7 f	N.D. j	N.D. j	8.4 ij	75.5 hijkl	204.8 ab	17.0 e
Laetitia	First	334.2 d	N.D. e	16.3 cdef	51.4 fghi	15.6 hi	17.0 ghi	89.1 gh	105.7 de	8.9 e
	Second	376.3 bcd	N.D. e	15.1 def	52.5 fghi	15.3 hi	10.5 hij	66.5 ijklmn	99.8 defg	8.6 e
African Delight	First	388.9 abc	19.9 b	N.D. g	72.33 fgh	10.0 ij	20.8 gh	126.6 c	146.5 c	9.3 e
	Second	156.0 cd	21.7 a	N.D. g	69.8 fgh	13.1 ij	5.0 j	67.0 ijklm	104.4 def	9.9 e
Sapphire	First	N.D. g	N.D. e	N.D. g	208.9 bcd	43.7 cde	44.1 f	49.9 n	64.6 hi	48.2 d
	Second	N.D. g	N.D. e	N.D. g	231.6 bc	56.5 bc	51.5 def	60.9 lmn	73.5 fghi	47.6 d
Ruby Red	First	353.7 cd	N.D. e	20.8 cd	447.0 a	91.4 a	59.2 cde	57.7 mn	64.9 hi	43.5 d
	Second	433.0 a	N.D. e	23.4 c	474.9 a	87.4 a	57.5 cde	56.4 mn	64.9 hi	52.6 cd
PR03-34	First	218.1 e	8.8 d	99.6 b	92.2 f	38.0 de	17.0 ghi	107.5 def	204.3 ab	14.9 e
	Second	255.6 e	11.4 c	115.4 a	232.8 b	55.5 bc	22.4 g	122.2 cd	216.6 a	16.6 e
PR04-32	First	65.8 f	N.D. e	N.D. g	188.8 cde	100.8 a	113.1 a	189.3 a	209.0 ab	82.1 b
	Second	73.5 f	N.D. e	N.D. g	70.4 fgh	33.2 def	97.1 b	154.7 b	194.0 ab	90.3 ab

^a Different letters in a column indicate a statistically significant difference at P<0.05; N.D. = not detected.

Table A9 (continued) Individual phenolic compounds (ripe) for cultivars and selections evaluated during two consecutive harvest seasons

Cultivar/selection	Harvest year	Compound (mg.kg ⁻¹ FW) ^a							
		Quercetin-3-O-rutinoside	Quercetin-3-O-glucoside	Quercetin-3-O-arabinoside	Quercetin-3-O-rhamnoside	Quercetin-3-O-xyloside	Quercetin pentosyl-hexoside	Quercetin pentosyl-pentoside	Acetylated Quercetin-hexoside
Sun Breeze	First	6.8 l	11.7 jk	18.0 hijkl	5.7 efghij	3.6 fg	6.0 cdef	2.4 efgh	0.4 n
	Second	7.6 kl	13.3 jk	12.7 jkl	5.9 efghij	3.0 fg	4.8 ef	3.3 cd	1.0 mn
Laetitia	First	53.6 cd	63.5 de	20.1 fghijkl	4.6 hijk	3.3 fg	2.8 h	N.D. k	23.6 ab
	Second	64.2 ab	69.8 d	26.3 efghi	8.1 cdefg	4.3 defg	4.5 fg	1.4 ij	21.8 bc
African Delight	First	44.7 ef	90.9 c	10.2 kl	2.7 jk	2.0 ij	6.7 bcd	N.D. k	10.2 gh
	Second	70.2 a	129.3 a	11.8 kl	3.3 ijk	2.6 ghij	8.6 a	1.4 ij	18.3 cd
Sapphire	First	23.2 gh	49.8 ef	30.5 cdef	6.3 defghi	4.1 efgh	7.0 bcd	2.7 cdefg	10.5 fgh
	Second	23.3 gh	65.8 d	39.9 bcd	8.4 bcde	6.0 bcd	7.4 abc	3.4 c	11.9 fgh
Ruby Red	First	39.1 f	95.9 c	40.6 bc	10.6 bc	6.9 b	N.D. i	3.0 cdefg	16.7 de
	Second	28.8 g	93.0 c	52.7 a	17.0 a	9.2 a	N.D. i	4.2 a	14.1 ef
PR03-34	First	8.5 jkl	10.1 k	20.5 fghijkl	5.1 efghij	2.9 fg	5.9 cdef	2.3 fgh	5.6 ijk
	Second	11.7 jkl	25.2 hij	20.3 fghijkl	5.0 fghijk	3.3 fg	5.6 def	2.8 cdefg	5.2 ijk
PR04-32	First	20.8 hi	24.9 hij	19.3 ghijkl	4.7 hijk	4.6 cdef	N.D. i	3.4 bc	8.6 hi
	Second	9.8 jkl	13.6 jk	29.3 defg	3.7 hijk	2.9 fg	N.D. i	3.3 cd	4.5 klm

^a Different letters in a column indicate a statistically significant difference (P<0.05); N.D. = not detected.

Table A10 Total phenolic compounds for each phenolic group for the respective harvest seasons (ripe)

Cultivars/ selections	Compound (mg.kg ⁻¹ FW)									
	Season 1 (2010/2011) ^a					Season 2 (2011/2012) ^a				
	Total polyphenols	Total hydroxycinnamic acids	Total anthocyanins	Total flavonols	Total flavan-3-ols	Total polyphenols	Total hydroxycinnamic acids	Total anthocyanins	Total flavonols	Total flavan-3-ols
Sun Breeze	620.8 gh	230.9 f	N.D.	55.5 h	334.4 b	587.1 k	223.4 e	N.D.	50.7 hi	313.0 efg
Laetitia	809.7 def	350.5 cd	66.9 fgh	171.5 bc	220.7 ef	850.6 fghi	391.5 bcd	67.8 ijk	200.3 bc	191.1 ij
African Delight	961.9 bcd	408.8 ab	82.4 efg	167.4 bc	303.3 bc	897.3 fgh	377.8 d	82.9 hi	245.5 a	191.1 ij
Sapphire	493.5 h	N.D. h	252.6 bc	134.1 bcde	206.8 f	742.2 ij	N.D. g	28.1 d	166.2 cd	270.0 gh
Ruby Red	1337.7 a	374.4 abc	538.5 a	212.8 a	212.0 ef	1501.1 a	456.3 a	561.2 b	218.9 ab	263.6 gh
Ruby Crunch	N.A.	N.A.	N.A.	N.A.	N.A.	1127.8 cd	57.5 f	350.5 c	224.5 ab	495.3 c
PR02-55	701.5 fgh	3.7 h	303.0 b	138.5 bcd	256.2 ed	N.A.	N.A.	N.A.	N.A.	N.A.
PR03-34	861.2 cdef	326.5 de	130.2 def	60.7 gh	343.7 b	1140.8 cd	382.4 cd	288.4 d	79.0 h	391.0 d
PR04-32	1045.2 b	65.8 g	289.6 b	96.4 efg	593.5 a	797.7 hi	73.5 f	103.6 h	57.0 hi	563.5 ab
PR04-19	N.A.	N.A.	N.A.	N.A.	N.A.	943.2 efg	8.4 g	646.1 a	136.7 def	142.9 j
PR04-35	N.A.	N.A.	N.A.	N.A.	N.A.	1346.7 b	421.2 abc	255.4 de	83.6 gh	583.6 a

^a Different letters in a column indicate a statistically significant difference ($P < 0.05$); N.D. = not detected; N.A. = not applicable.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

During the current study eleven South African plum cultivars and selections were evaluated in terms of phenolic composition, antioxidant capacity and other fruit attributes (such as colour, pH, °Brix and firmness). The importance attached to phenolic compounds and antioxidant activity of fruit (especially red-fleshed fruits) due to their assorted health-promoting properties, prompted this investigation. Currently limited information is available on South African plums and their phenolic profile and antioxidant capacity. The preferred method of analysis of phenolic compounds in fruit is high-performance liquid chromatography (HPLC), and thus one of the aims of this study was the improvement of a previous HPLC method (De Beer *et al.*, 2012) to obtain better separation of phenolic acids, anthocyanins, flavonols and flavan-3-ols. Subsequently, quantification of a larger spectrum of compounds was possible compared to the initial method of analysis. This method was also suitable for use with subsequent mass spectrometry (MS) analysis for compound identification, and for an on-line antioxidant assay to evaluate the antioxidant activity of individual phenolic compounds without the need for prior isolation. It was also necessary that the method be applicable to all cultivars and selections.

Another objective of this study, and its main focus, was the quantification of the identified phenolic compounds, for evaluation of the phenolic profile of the cultivars and selections obtained during two consecutive harvest seasons. Phenolic compounds may display antioxidant activity through various mechanisms, such as hydrogen donation, electron transfer and metal chelation (Khokhar & Apenten, 2003; Prior *et al.*, 2005). Therefore four different *in vitro* antioxidant assays were used, namely the 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) radical cation scavenging, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorbance Capacity (ORAC) assays. The effect of a commercial cold storage and ripening regime on phenolic content and antioxidant capacity was also investigated. Fruit attributes such as the colour of the peel and flesh, pH, TA, °Brix and firmness were measured and compared between cultivars and selections at unripe and ripe stages.

In the current study a HPLC-DAD-FLD (HPLC with diode array and fluorescence detection) method was successfully optimised for analysis of phenolic compounds in eleven plum cultivars and selections grown in South Africa (Chapter 3). Fluorescence detection was used to quantify flavan-3-ols, as these compounds possess fluorescent properties and are generally present in lower concentrations. The optimised method allowed the separation of compounds from four phenolic groups, namely phenolic acids, flavonols, flavan-3-ols and anthocyanins. Twenty-four compounds were identified or tentatively identified of which 18 were quantitatively evaluated.

Parameters evaluated and optimised include the analysis temperature, gradient program, injection volumes and mobile phases. A reversed-phase C18 column was used (3 μm , 150 x 4.6 mm) and acetonitrile and 0.05% trifluoroacetic acid (TFA) established as mobile phases to ensure optimum separation. The analysis temperature for the final method was 40°C and the total run time was 45 min. In the original method (De Beer *et al.*, 2012) acetonitrile and 7.5% formic acid was used as mobile phases, but due to the high acid concentration was not acceptable for LC-MS. In addition the method was only used for quantification of five major phenolic compounds, and flavan-3-ols were not evaluated.

After establishment of the final method parameters method validation was performed. The stability, intra-day precision and linearity of the method showed acceptable results with the percentage relative standard deviation (% RSD) and percentage change ranging from -5% to 5%. Less acceptable results were obtained for the inter-day precision, especially concerning the flavan-3-ols in the standard calibration mixtures. The % RSD for this group was between 5.0 and 13.2% in some cases. Due to the acceptable sample stability over a 28 h period, sample preparation can be done up to 24 h in advance without affecting sample integrity and thus results. Method validation showed acceptable results, but it is recommended that the method be further improved if the flavan-3-ols are of primary interest. Alternatively, methods focussing specifically on the flavan-3-ols could be used.

HPLC-DAD-MS and -MS/MS were used to identify phenolic compounds by comparing mass fragments and UV-Vis spectra to authentic standards and literature. The main compounds present in the majority of red-fleshed plums were neochlorogenic acid and cyanidin-3-*O*-glucoside. By comparison to authentic reference standards the identities of thirteen compounds were confirmed. An additional five flavan-3-ols (dimers and trimers) and four flavonols (quercetin derivatives) were tentatively identified by comparing MS data to literature. All flavonols identified were quercetin-derivatives, some of which were diglycosides. The phenolic acids, chlorogenic acid and 3-*O*-*p*-coumaroylquinic acid, were present in some plum cultivars/selections. The main flavan-3-ols were (+)-catechin, (-)-epicatechin and procyanidin dimers and trimers. All compounds were not present in every cultivar/selection, but have been previously identified or tentatively identified in plums (Tomás-Barberán *et al.*, 2001; Kim *et al.*, 2003; Wu & Prior, 2005). One prominent compound present in all cultivars and selections remains unknown.

By coupling the HPLC-DAD to a second pump and variable wavelength detector (VWD) as previously described by Pellegrini *et al.* (2003) it was possible to evaluate the relative antioxidant capacity of individual phenolic compounds by means of an on-line antioxidant assay. The ABTS^{•+}

scavenging assay was successfully applied and the antioxidant response of phenolic compounds evaluated in a qualitative manner. Interpretation of the negative antioxidant response chromatograms revealed that the flavan-3-ols displayed the greatest antioxidant response peaks. Neochlorogenic acid also displayed a prominent antioxidant response peak due to its high concentration in most cultivars. Anthocyanins have also been found to exhibit antioxidant activity (Chun *et al.*, 2003), and corresponding antioxidant response peaks were seen, but could not be independently evaluated due to co-elution with flavan-3-ols.

Quantitative determination was not possible due to co-eluting compounds causing enlarged antioxidant response peaks, as well as an uneven baseline. Rzeppa *et al.* (2011) attributed the latter to the presence of oligomeric procyanidins. Improvement of the HPLC method for possible separation of co-eluting compounds (cyanidins and flavan-3-ols) may allow quantitative evaluation of their antioxidant capacity.

Results from the current study compare well to literature in that flavan-3-ols generally display a greater antioxidant capacity than phenolic acids and flavonols (Salah *et al.*, 1995; Tsao *et al.*, 2005). Procyanidin dimers and trimers displayed greater antioxidant response than (+)-catechin and (-)-epicatechin, which has also been found by other authors (Teissedre *et al.*, 1996; Counet & Collin, 2003).

Recommendations for future work include the improvement of the HPLC-DAD-FLD method for better separation, especially between anthocyanins and flavan-3-ols, and evaluation of additional peaks with HPLC-DAD-MS. Tentatively identified compounds should be compared with authentic reference standards to confirm their identities. In the case where standards are not available, methods such as acid hydrolysis may be used to confirm the identities of glycosidic substituents (Kennedy & Jones, 2001).

After identification of the phenolic compounds the major compounds were quantified and the cultivars and selections compared, along with antioxidant capacity and other fruit attributes (Chapter 4). Several trends concerning fruit attributes were seen during both harvest seasons. A decrease in firmness was observed during cold storage and ripening, due to the degradation of pectin (as reviewed by Brownleader *et al.*, 2012). The only exception was PR02-55 for which the firmness increased, although it was not significant. In terms of fruit colour, the a^* -value (red colour) of the flesh generally increased after cold storage and ripening, due to the increase in anthocyanin (cyanidin-3-*O*-rutinoside and -glucoside) concentration. Cyanidin-glycosides are partly responsible for the red colour in fruit, while co-pigmentation of anthocyanins with other phenolic compounds, such as flavonols, may affect colour intensity.

Compounds from the flavonol group showed very little significant difference between unripe and ripe fruit. No significant increases were observed for quercetin-3-*O*-xyloside, -arabinoside, -rhamnoside, quercetin pentosyl-pentoside, quercetin pentosyl-hexoside and acetylated quercetin-hexoside. Quercetin-3-*O*-glucoside was the predominant flavonol and increased significantly after cold storage and ripening in Sapphire of the first harvest season, in Laetitia and Ruby Crunch of the second harvest season, and in Ruby Red and African Delight of both harvest seasons.

In the flavan-3-ol group close association was seen between (+)-catechin and procyanidin B1, and similarly between (-)-epicatechin and procyanidin B2. This association was observed for both harvest seasons. For example, in the first season (+)-catechin and procyanidin B1 were positively associated with African Delight, while procyanidin B2 and (-)-epicatechin were associated with Ruby Red and PR03-34. Cold storage and ripening had little effect on the flavan-3-ol content. The total polyphenol content (Folin-Ciocalteu) associated positively with PR04-32 in the first harvest season, and PR04-32, PR04-35 and PR03-34 in the second season.

New selections, in particular PR04-32 and PR04-35, were shown to have higher antioxidant capacities than existing cultivars. Both have red peel and flesh. Selection PR04-32 showed the highest antioxidant capacity for all assays in the first harvest season, as well as the highest antioxidant capacity with ORAC and DPPH[•] assays in the second season. PR04-35 had the highest antioxidant capacity as measured with ABTS^{•+} and FRAP assays in the second season. Considering the correlation of antioxidant capacity with phenolic content, total polyphenol, as well as individual and total flavan-3-ol content correlated significantly with antioxidant capacity, irrespective of season and assay used. Total anthocyanins correlated significantly with all antioxidant assays during the first harvest season irrespective of the assay used, while for the second season it only correlated with results obtained with ABTS^{•+} and FRAP.

Comparison of specific results obtained in the current study for antioxidant capacity and phenolic concentration with that from literature was complicated due to differences in methods and samples, but similar trends were seen as reported in literature. Results from the current study are in agreement with literature in that fruit with red peel and/or flesh generally have a higher total polyphenol content than yellow fruit (Kim *et al.*, 2012), likely due to the contribution of anthocyanins. Similar results have also been found regarding the major phenolic compounds. Neochlorogenic acid have previously been found to be the major phenolic acid, quercetin-3-*O*-rutinoside the predominant flavonol and cyanidin-3-*O*-glucoside and -rutinoside the major anthocyanins (Tomás-Barberán *et al.*, 2001; Kim *et al.*, 2003).

The current study clearly demonstrates that phenolic composition differs between cultivars/selections, not only quantitatively, but also qualitatively. Harvest season also had an effect on the phenolic composition for some cultivars and selections (Sun Breeze, PR04-32, African Delight and PR03-34), while others were not significantly affected by season.

For future studies it is recommended that additional cultivars and selections, as well as more trees of the same cultivar/selection, be evaluated for comprehensive evaluation of phenolic profiles. One of the limitations during the current study was that all cultivars/selections could not be obtained for both harvest seasons. This was due to the fact that many selections are in the early phases of evaluation and not enough fruit were available for analysis, as fruit were also required for other experimental work as part of the evaluation of breeding programs. It is recommended that the same cultivars/selections be evaluated in consecutive harvest years where possible. The evaluation of fruit from more trees would also be beneficial, in order to assess larger variation in phenolic composition between cultivars and selections. This is however a practical limitation as during early phases of evaluation only a small number of trees are planted. In addition, the same cultivars/selections produced in other areas could be included in future studies, as it would be interesting to determine to what extent the geographical location and climate affects the phenolic composition and antioxidant capacity of plums. Analysis of tree-ripened fruit instead of unripe fruit, and evaluation of fruit at different stages of ripeness could also be considered. Analysis of temperature and rainfall data during specific harvest seasons would also provide insight into changes occurring between consecutive seasons.

One of the major conclusions drawn from this study was the high antioxidant capacity of the flavan-3-ols, which is in agreement with previous published studies (Teissedre *et al.*, 1996; Tsao *et al.*, 2005). This was seen both with *in vitro* antioxidant assays as well as the on-line antioxidant assays. To fully evaluate the antioxidant capacity of flavan-3-ols that are not available in pure form it would be beneficial to improve the HPLC-DAD-FLD method for better separation, or investigate alternative sample preparation techniques to simplify the complex mixture. For example solid-phase extraction (SPE) and liquid-liquid fractionation have previously been used for the separation of flavan-3-ols from other phenolic groups (Sánchez-Ilárduya *et al.*, 2012). This would facilitate the separation of these compounds by HPLC.

If particular plum cultivars and/or selections evaluated during the current study were to be selected for future research, the best candidates would be Ruby Red, PR04-32 and PR04-35. Ruby Red displays good antioxidant capacity, high total polyphenol content and a distinct phenolic profile. Selections PR04-32 and PR04-35 both have a high phenolic content and antioxidant

capacity, giving these fruits market potential in terms of nutritional character. Determination of consumer perception and preference for these plum selections may lead to marketability and cultivar status.

This study is the first to provide comprehensive information on the phenolic composition and antioxidant capacity of plum cultivars and selections produced in South Africa. The optimisation of HPLC-DAD-FLD and HPLC-DAD-MS methods allowed identification or tentative identification of twenty-four phenolic compounds and quantification of eighteen phenolic compounds from eleven cultivars/selections. The optimised method would be of benefit for future research on South African plums. Prior to this study no information was available regarding the effect of a commercial cold storage and ripening regime on the phenolic composition and antioxidant activity of South African plums. The ARC only investigates the effect of cold storage on internal defects and eating quality, and thus limited information is available regarding the effect of cold storage and ripening on fruit attributes. The information gathered may prompt further research regarding phenolic composition, antioxidant capacity and the potential health benefits of South African plums.

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