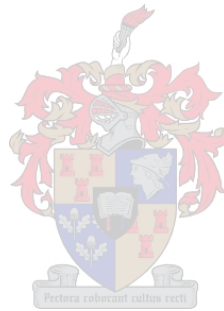# Antioxidant properties and effect of forced convection roasting on South African wheats

by

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Master of Science in Food Science

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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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March 2016

Abstract

The intake of whole wheat products has many health benefits, partly due to it containing considerable amounts of antioxidants. Although wheat cultivation started as early as the 17th century in South Africa (SA), no peer-reviewed publication was found regarding the antioxidant properties of South African wheat. This study investigated the antioxidant properties of 26 SA wheat cultivars, which were planted in a randomised complete block design in three regions. Samples were extracted using acidified methanol, and the antioxidant properties were determined using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and total phenolic content (TPC) assays.

Cooking of wheat is required for human consumption. The effect of thermal processing on the antioxidant properties of South African wheat was investigated using a forced convection continuous tumble roaster (FCCTR) according to a central composite design (CCD). FCCTR is an innovative processing method with several benefits (fast, energy efficient and easy to operate) and it has great potential for food manufacturing. PAN3161 and PAN3379 wheat cultivars were selected and roasted at different temperatures and speeds settings between 136 and 234°C and 20 and 90 Hz, respectively. The DPPH radical scavenging properties, TPC and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS$^{•+}$) radical scavenging capacity were determined to represent the antioxidant properties of thermally processed sample. The CCD was also used to determine the optimal roasting conditions by means of response surface methodology (RSM).

The DPPH radical scavenging capacity assay was modified to use acidified-methanol as extraction solvent. DPPH reagent is stable at pH 5.0-5.6, however the acidified-methanol extract had a pH<1. Modification using potassium phosphate buffer (75 mM) was tested. High similarity was found when comparing the stability of DPPH in the potassium phosphate buffer and 80% (v/v) methanol.

South African wheat was found to have moderate levels of antioxidants compared to published research done in Europe, USA and Canada. The antioxidant properties varied with locality. Additionally, samples collected from the irrigation region had higher antioxidant properties than the samples collected from the dry land regions. Significant correlation was found between wheat hardness and antioxidant capacity.

A prediction model generated by RSM, revealed antioxidant properties to be significantly affected linearly by roasting temperature, speed and their interaction. For PAN3161, minimum processing (136°C, 90Hz) was required to achieve the highest TPC, while roasting at 234°C, 20Hz achieved the highest DPPH radical scavenging. PAN3379 showed different behaviour, the RSM estimated roasting at 234°C, 90 Hz would result in the highest TPC, and 136°C at 90 Hz in the highest DPPH radical scavenging capacity. The difference in optimal processing conditions observed could have been due to the high temperatures destroying some of the free phenolic acids. Some antioxidant compounds may also have been produced through the Maillard reaction, not detected with the Folin-Ciocalteu method. The difference in free and bound phenolic acids content,

the natural composition of the amino acids, reducing sugar content of the wheat cultivars might also have contributed to the different results.

Uittreksel

Die inname van volgraan produkte het baie gesondheidsvoordele, gedeeltelik as gevolg van die aansienlike hoeveelhede antioksidante wat dit bevat. Al het koringverbouing reeds so vroeg as die 17de eeu in Suid-Afrika (SA) begin, kon geen eweknie-hersiende publikasie rakende die antioksidant eienskappe van Suid-Afrikaanse koring gevind word nie. Hierdie studie het die antioksidant eienskappe van 26 SA koringkultivars, wat in 'n ewekansige blok-ontwerp in drie streke geplant is, ondersoek. Monsters is onttrek deur aangesuurde metanol te gebruik, en die antioksidant eienskappe is bepaal deur 2,2-difeniel-1-pikrielhidrasiel (DPPH) radikale opruiming en totale fenoliese inhoud (TPC) toetse.

Koring moet gekook word vir menslike gebruik. Die effek van termiese prosessering op die antioksidant eienskappe van Suid-Afrikaanse koring is ondersoek deur 'n geforseerde-konveksie deurlopende tuimelrooster (FCCTR) volgens 'n sentrale saamgestelde ontwerp (CCD). FCCTR is 'n innoverende prosesseringsmetode met verskeie voordele (vinnig, energie-doeltreffend en maklik om te gebruik) en dit het groot potensiaal vir voedselvervaardiging. PAN2161 en PAN2279 koringkultivars is gekies en teen verskillende temperature en spoedstellings, tussen 136 en 234°C en 20 en 90 Hz, respektiewelik, gerooster. Die DPPH radikale opruimingseienskappe, TPC en 2,2'-azinobis-(3-etielbenzotiazolien-6-sulfoonsuur) (ABTS$^{Ÿ+}$) radikale opruimingskapasiteit is bepaal om die antioksidant eienskappe van die termies-geprosesseerde monster voor te stel. Die CCD is ook gebruik om die optimale roosterkondisies te bepaal deur middel van reaksie oppervlak metodologie (RSM).

Die DPPH radikale opruimingskapasiteitstoets is aangepas om aangesuurde metanol as onttrekkingsoplosmiddel te gebruik. Die DPPH reagens is stabiel by 'n pH 5.0-5.6, maar die aangesuurde metanol ekstrak het 'n pH<1. 'n Aanpassing met natriumfosfaatbuffer (75 mM) is getoets. 'n Hoë ooreenstemming is gevind wanneer die stabiliteit van DPPH in die fosfaatbuffer en 80% (v/v) metanol vergelyk is.

Daar is gevind dat Suid-Afrikaanse koring matige antioksidantvlakke bevat in vergelyking met gepubliseerde navorsing uit Europa, VSA en Kanada. Die antioksidant eienskappe varieër met betrekking tot ligging. Daarbenewens het monsters wat in die besproeiingsgebied ingesamel is, hoër antioksidant eienskappe as monsters wat in die droëland areas ingesamel is. Beduidende korrelasies is gevind tussen koringhardheid en antioksidant kapasiteit.

'n Voorspellingsmodel wat deur RSM gegenereer is, het getoon dat antioksidant eienskappe beduidend lineêr beïnvloed word deur roostertemperature, spoed en hul interaksie. Vir PAN3161 was minimum prosessering (136°C, 90Hz) nodig om die hoogste TPC te bereik, terwyl roostertoestande van 234°C en 20Hz die hoogste DPPH radikale opruiming meegebring het. Die verskille wat in die optimale prosesseringskondisies waargeneem is, kan moontlik toegeskryf word aan die vernietiging van vrye fenoliese sure deur die hoë temperature. Sommige antioksidant-verbindings mag ook deur die Maillard reaksie geproduseer word, en is nie deur die Folin-Ciocalteu metode opgespoor nie. Die verskil in vrye en gebinde fenoliese suurinhoud, die natuurlike

samestelling van die aminosure, en die reduserende suikerinhoud van die koringkultivars mag ook bydra tot die verskillende resultate.

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List of Abbreviations

ABTS: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)

AE: Antiradical efficiency

CCD: Central composite design

DNA: Deoxyribonucleic acid

DPPH: 2,2-diphenyl-1-picrylhydrazyl

FAE: Ferulic acid equivalent

FCCTR: Forced convection continuous tumble roaster

FCR: Forced convection roasting

FRAP: Ferric reducing ability of plasma

GAE: Gallic acid equivalent

HCl: Hydrochloric acid

HI: Hardness index

LSD: Least significant difference

MAE: Microwave-assisted extraction

NaOH: Sodium hydroxide

ORAC: Oxygen radical absorbance capacity

PBS: Phosphate buffer solution

RDSC: Relative DPPH$^\bullet$ scavenging capacity

RSE: Radical scavenging efficiency

RSM: Response surface methodology

SA: South Africa

SAE: Sonication assisted extraction

TCC: Total carotenoid contents

TE: Trolox equivalent

TFC: Total flavonoid content

TPC: Total phenolic contents

Trolox: (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid

Chapter 1

Introduction

A diet including whole grains could reduce the risk of chronic diseases, including cancer, cardiovascular diseases, atherosclerosis, hypertension and diabetes (Okarter & Liu, 2010). In spite of current controversy regarding whether cereal grains (carbohydrates) should be included in the daily diet or not, it is agreed that when cereal grains are consumed at least half, if not all, should be whole grain. Grains offer a wide range of nutrients and phytochemicals, i.e. antioxidants that may work synergistically to optimise human health (Liu, 2007). Consumers are aware of the potential health benefits of whole grains as a source of phytonutrients.

Wheat is a well known crop, which contains considerable amount of antioxidants (Manach *et al.*, 2004). Although its antioxidant content is not as high as some other cereals such as sorghum and rye (Ragaee *et al.*, 2006), it is one of the 'big three' cereal crops worldwide, and can be used as an ingredient for many food products (Shewry, 2009).

During thousands of years of wheat cultivation over 25,000 cultivars were developed throughout the world with various individual characteristics, able to adapt to different kinds of environments (Shewry, 2009). The antioxidants properties can differ depending on their genotype and their growing environment (Lv *et al.*, 2013; Lu *et al.*, 2015; Rascio *et al.*, 2015)

A wheat kernel generally consists 81-84% (w/w) of endosperm, 2-3% (w/w) of germ and 14-16% (w/w) of bran (Fig. 1.1) (MacMasters *et al.*, 1971). Wheat is commonly pearled and milled before further processing to achieve desirable flour qualities (Mousia *et al.*, 2004). Pearling removes around 30% of the wheat bran, while milling further separates the bran from endosperm, although the bran can never be completely separated (Mousia *et al.*, 2004). Earlier studies found that the majority of phytochemicals and dietary fibers are located in wheat bran. Thus products made from whole wheat grain are recommended for increasing health dietary benefits (Ragaee *et al.*, 2006; Liu, 2007).

Phenolic acids were found as the main antioxidant in many cereals, including wheat (Bunzel *et al.*, 2001), and ferulic acid is the predominant phenolic acid in wheat (Okarter *et al.*, 2010). Phenolic acids were found to be present as free and bound (Sosulski *et al.*, 1982). The bound form of phenolic acids are conjugated to the cell wall components of wheat through ether or ester-linkages (Sun *et al.*, 2002). Wheat was found to contain 80-95% of the bound form of phenolic acids (Adom *et al.*, 2003). Furthermore, Rufián-Henares and Delgado-Andrade (2009) found humans might have low bio-accessibility (can absorb very little amount) of the bound phenolic acids if wheat is uncooked. The extraction of phenolic compounds mimics the human's large and small intestine conditions.

Thermal processing was found to increase the antioxidant activity of wheat products. The increase of antioxidant activity has been attributed to the release of the bound form of antioxidants, as well as development of new compounds like Amadori products and hydroxymethylfurfural from

Maillard reaction and caramelisation (Rufián-Henares & Delgado-Andrade, 2009). Thermal processing can also cause degradation of thermal labile antioxidants (Araña *et al.*, 2007; Hidalgo *et al.*, 2010), however ferulic acid was found to have good thermal stability (Fiddler *et al.*, 1967).
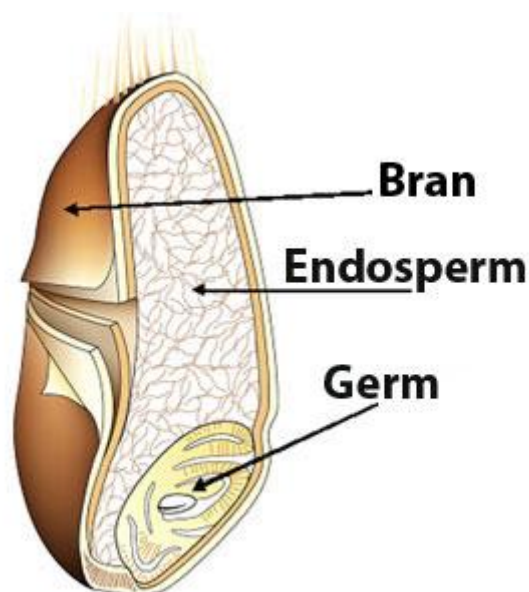
Figure 1.1. Model of a wheat kernel (Anonymous, 2014)

For determination of the antioxidant properties of wheat, it is essential to choose the correct extraction solvent and method depending on the objective. Organic solvents methanol, ethanol and acetone are commonly mixed with deionised water for extracting free phenolic compounds. Hydrolysis methods use high concentration of HCl or NaOH for determination of the bound phenolics (Sun *et al.*, 2002). A compromised method using 1% v/v to 2 M of acidified methanol was used to determine the total phenolic compounds (Okarter *et al.*, 2010). Using ultrasonic assisted extraction methods are known to accelerate the extraction rate, because the thermal treatment can disrupt cell wall structure, thus helping with the release of the bound phenolic compounds (Muñiz-Márquez *et al.*, 2013). An optimisation study, using ultrasonic assisted extraction recommended using 64% v/v ethanol, extracting at 60°C and sonication for 25 min for extracting antioxidants from wheat bran samples (Wang *et al.*, 2008). In genotype and environment effect study of antioxidant properties of Canadian wheats, Beta *et al.* (2005) used a mixture of methanol, deionised water and concentrated HCl (80:10:1 v/v) for extracting the total phenolic content (TPC) from wheats.

Although including acid as an ingredient of extraction solvent can increase the extraction efficiency, there is a drawback. That is the acid would lower the pH of the sample extract, with the antioxidant only able to be determined with a compound in the assay to stabilise the pH, such as sodium bicarbonate in TPC assay (Singleton *et al.*, 1999), and phosphate buffered saline, pH 7.4, in 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay (Re *et al.*, 1999a). Traditional DPPH assay does not include such a compound to stabilise the pH. The 2,2-diphenyl-1-

picrylhydrazyl (DPPH) reagent has a relative narrow stable pH range, and becomes unstable when working outside of the suggested working pH (Koleva *et al.*, 2000; Ozcelik *et al.*, 2003).

To our knowledge no information regarding the antioxidant properties of South African wheat cultivars is available. Therefore the aim of this study was to investigate the antioxidant properties of South African wheat cultivars. In addition the effect of thermal processing on South African wheat cultivars was determined using Forced Convection Continuous tumble Roaster (FCCTR). Acidified methanol (methanol/water/HCl, 80:10:1 v/v) was used as extraction solvent to determine the total antioxidant properties. Thus it was necessary to develop a workable and reliable method when using the potassium phosphate buffer in the DPPH assay, along with HCl-acidified methanol as an extraction solvent. Only extractable free antioxidants was determined for the roasted samples, 64% ethanol was thus used as extraction solvent.

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Chapter 2

Literature Review

Introduction

Wheat cultivation dates back to approximately 10 000 BC, where it was first planted in the Fertile Crescent in Egypt (Araus *et al.*, 2007). Presently, wheat is a well-known crop that is planted worldwide, and is predominantly used for human consumption. More so, wheat is one of the most important staple foods globally, as it provides macro and micro-nutrients such as carbohydrates, minerals, vitamins and phytochemicals. In South Africa, the cultivation of wheat started in the 17th century with the arrival of the Dutch settlers at the Cape of Good Hope and has since grown to be a substantial agricultural commodity (Guelke, 1976). As reported by the Department of Agriculture, Forestry and Fisheries (DAFF), the South African wheat production was 852 800 tons for the 2003/2004 season which increased to 1 924 000 tons for the 2012/2013 season (SAGL, 2014).

The antioxidant properties of wheat is mainly a genetic trait. More than 25,000 types were developed over the years, adapted for different environmental conditions (Feldman, 1976; Shewry, 2009). Environmental conditions such as rainfall, sunshine intensity, humidity, temperature and soil type also affect the antioxidant levels in wheat (Moore *et al.*, 2006).

Once wheat is harvested, it is transported to storage facilities before being processed. The processing steps basically include cleaning, milling, pre-mixing and thermal processing (Ragaee *et al.*, 2012). Each step could potentially influence its natural antioxidant properties (Ragaee *et al.*, 2012).

This literature review will focus on the differences among wheat cultivars and the effect of growing environment and thermal processing on the antioxidant properties in wheat. The following will be discussed: (1) the principles of antioxidant reduction-oxidation; (2) sources of antioxidants present in wheat; (3) antioxidants present in wheat fractions; (4) free and bound form of phenolic compounds in wheat; (5) genotype and environmental influences on wheat antioxidant properties; (6) processing effects of antioxidants in wheat and wheat products; and (7) methods for the determination of antioxidant properties.

Oxidation reactions

The only planet in the universe that supports respiration is our planet, i.e. earth due to its unique atmospheric composition. Oxygen, which forms 21% of the atmosphere, is the essential element that allows respiration in living organisms. Two percent oxygen is converted into free radicals through mitochondrial respiration and phagocytosis (Kunwar & Priyadarsini, 2011). Free radicals are important as they play a critical role in cell signaling and are utilised by immune cells in pathogen elimination (Kunwar & Priyadarsini, 2011). However, excess radicals need to be effectively eliminated as they may attack intra-cellular molecules like DNA, lipids, and proteins and may further

lead to chronic disease. Additionally, antioxidants are used to scavenge potentially harmful radicals (Kunwar & Priyadarsini, 2011). Antioxidants are thus the solution for living organisms in dealing with excessive free radicals.

The following factors explain the process of lipid oxidation: 1) initiation (1st and 2nd), 2) propagation and 3) termination (Fig. 2.1) (Rubbo *et al.*, 1995). Trace amounts of hydroperoxides are formed by the action of lipoxygenase when extracting oil from plant seeds (Leenhardt *et al.*, 2006). A hydroperoxide molecule can further break down into a hydroxyl radical, which is the compound that initiates lipid oxidation (McClements & Decker, 2000). At this stage (stage 1), a hydrogen atom is detached from a lipid molecule to become a lipid radical. Once the lipid radical is available in the system, the propagation stage becomes dominant in the oxidation process and the lipid radical reacts with oxygen to form a peroxy radical. Peroxy radicals extract hydrogen molecules from non-attacked lipid molecules and convert them into lipid radicals, thus constitutes the propagation stage (McClements & Decker, 2000). The reaction rate required to reach enthalpy is lower than initiation. The propagation stage is very rapid (Pokorný *et al.*, 2001). The last stage, namely the termination stage, binds two lipid radicals to form a complement of electrons molecule. For example, a lipid hydroxyl radical (ROO•) binds with an alkoxy radical (RO•) and forms a larger molecule with complement of electrons (ROOR). The second initiation normally cleaves the lipid hydroperoxide (ROOH) to an alkoxy radical (RO•) and a lipid hydroxyl radical (ROO•). Metal ions present in the system are the catalysts for this reaction (Pokorný *et al.*, 2001).
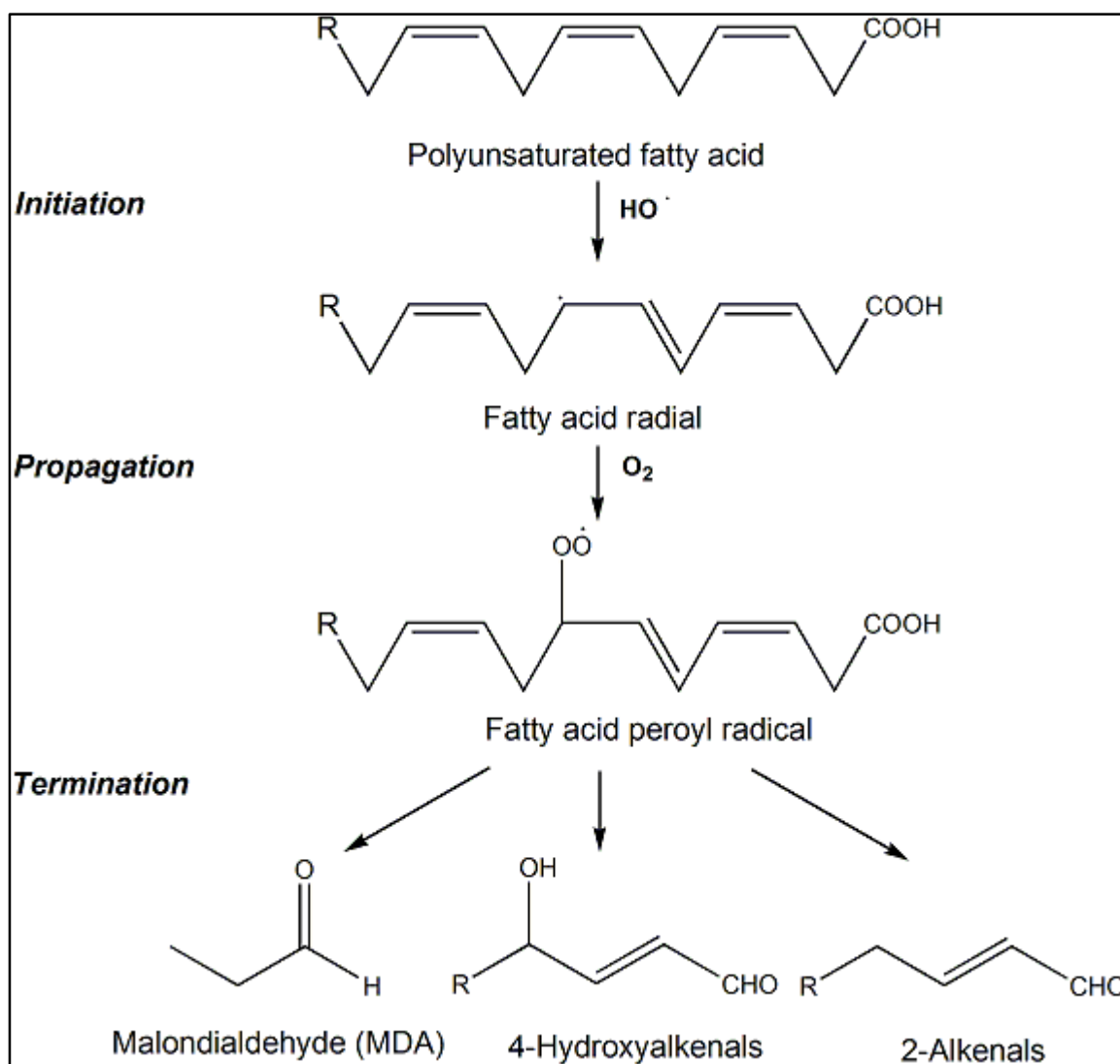
Figure 2.1. The three stages of lipid oxidation

The intake (ingestion) of natural antioxidants (as opposed to synthetic antioxidants) is one of the solutions of living organisms in dealing with the danger of excessive free radicals. The first line of defense is the preventive antioxidants that inhibit the formation of free radicals. Examples include antioxidants that have the catalase enzymes to decompose hydrogen peroxide, or they are antioxidants that have ions chelating agents, and quenchers of active oxygen molecules. The second defense line is radical scavenging mechanisms, which are compounds like vitamin C, vitamin E, carotenoids, and phenolic compounds (Lattanzio 2006). The third defense line is mainly provided by antioxidants with specific enzymes, which repair, replace and remove the oxidative damaged lipids, proteins and DNA. Apart from the latter antioxidants, even more complex mechanisms in the *in vivo* system are considered as the fourth line of defense, where appropriate amount of antioxidants can be produced and transferred to the right place according to their needs (Pokorný *et al.*, 2001).

Sources of antioxidants in wheat

One of the recognized definition of an antioxidant is 'any substance that, when present at low concentrations compared to those of an oxidisable substrate, significantly delays or prevents oxidation of that substrate' (Halliwell *et al.*, 1995). Plants produce a wide variety of antioxidants which can be divided into categories, including phenolic compounds, carotenoids, tocopherols and tocotrienols, certain amino acids and proteins (Pokorný *et al.*, 2001). By comparing the antioxidant content of 3 139 products which were collected from all over the world over a 9 year period, Carlsen *et al.* (2010) found that plant-based products contained significantly higher amounts of antioxidants than non-plant food products. The TPC differs significantly between several types of fruits, vegetables and cereals (Liu 2007). In general, a wide range in TPC was found between species. Total phenolic content in cranberries were found to be ten times more in comparison to grapefruit, whereas some fruits contained similar amounts of total phenolics to that of vegetables. Cereals contained relatively higher amounts of phenolics than vegetables and some fruit types, but mostly present as bound phenols (Liu, 2007).

Many cereals are classified as staple foods, as it is known that they contain a large portion of carbohydrates in the form of starch, compared to other nutrients. Maize, rice and wheat are the most important crops in the human diet and contribute towards two-thirds of our food consumption (Anonymous, 2014). Farmers and food producers usually focus on macronutrients (mainly carbohydrates and proteins) in cereals, consequently making use of processing methods, which include the removal of the germ and bran to produce desired texture product for their consumers (Gujral *et al.*, 2003). However, it has been shown that the consumption of whole grain has many health benefits, due to their more abundant dietary fiber, resistant starch, oligosaccharides, and phytochemicals contents (Slavin, 2000).

Esterified ferulic and caffeic acid of long chain mono and dialcohols are commonly found in grains (Daniels & Martin, 1967). Part of their function is to be a backup antioxidant for vitamin E to protect lipid membranes against oxidative stress in cereals (Miller *et al.*, 2000). These lipid soluble esters have similar antioxidant properties to that of tocopherols. In addition to soluble antioxidants, a significant amount of grain phenolics is covalently bound to cell wall polysaccharides and may be important to human health in this form through a digestive processes (Miller *et al.*, 2000).

Recent studies indicated wheat to contain several phytochemical compounds, such as phenolic acids, flavonoids, lignin, carotenoids, tocopherols and tocotrienols, and research in the last decade has shown that the consumption of food containing phytochemicals results in many health benefits (Liu, 2007).

Phenolic compounds

Phenolic compounds are known to be produced during the second metabolism in plants and are responsible as defense compounds against microbes, viruses, herbivores and competing plants, as well as protecting against oxidation and ultraviolet radiation (Kutchan, 2001; Lattanzio *et al.*, 2006;

Luthria *et al.*, 2015). The chemical structure of a phenolic compound is defined as a substance which possesses an aromatic ring, bearing one or more hydroxyl substituent, including functional derivatives (Harborne, 1989). Depending on their chemical nature, Lattanzio *et al.* (2006) summarised the phenolic compounds into several basic skeleton classes: C_6 (simple phenol, benzoquinones), C_6-C_1 (phenolic acids), C_6-C_2 (acetophenone, phenylacetic acid), C_6-C_3 (hydroxycinnamic acids, coumarins, phenylpropanes, chromones), C_6-C_4 (naphthoquinones), C_6-C_1-C_6 (xanthones), C_6-C_2-C_6 (stilbenes, anthraquinones), C_6-C_3-C_6 (flavonoids, isoflavonoids), $(C_6$-$C_3)_2$ (lignans, neolignans), $(C_6$-C_3-$C_6)_2$ (biflavonoids), $(C_6$-$C_3)_n$ (lignins), $(C_6)_n$ (catechol melanins) and $(C_6$-C_3-$C_6)_n$ (condensed tannins). Phenolic acids are the most abundant phenolic compound in wheat, whereas polyphenols, such as lignans and flavonoids are also commonly detected in wheat, but in smaller amounts (Verma *et al.*, 2009). Anthocyanins are only found in purple wheat varieties (Hosseinian *et al.*, 2008).

Phenolic acids

Phenolic acids have free radical scavenging and hydrogen donating properties (Liu, 2007). They can be classified into two primary groups: hydroxybenzoic acid and hydroxycinnamic acid derivatives (Liu, 2007). Ferulic acid, caffeic acid and *p*-coumaric acid are typical hydroxycinnamic acid derivatives, whereas gallic acid, protocatechuic acid and vanillic acid are hydroxybenzoic acid derivatives, commonly found in wheat (Fig. 2.2, Table 2.1). A research group compared the phytochemical content of six wheat varieties grown in the USA, and they found the ferulic acid content to be 301.8 to 496.1 µmol/100g (Okarter *et al.*, 2010). It revealed ferulic acid to be the predominant phenolic acid, which was about nine times more than the second main phenolic acid, *p*-coumaric acid, which ranged from 33.5 to 52.3 µmol/100g (Okarter *et al.*, 2010). Syringic acid, vanillic acid and caffeic acid were also detected in trace amounts (Okarter *et al.*, 2010). However, despite the phenolic acid content that may vary depending on cultivars and their growing environment, ferulic acid tended to be predominant (Kim *et al.*, 2006; Ragaee, Seetharaman*, et al.*, 2012). Similar results obtained by Mpofu *et al.* (2006) seemed to agree with the above statement, additionally their results included *o*-coumaric acid, the second most predominant phenolic acid found in their study (approximately half of the ferulic acid content). Both hydroxybenzoic acid and hydroxycinnamic acid derivatives are mainly present in conjugated form, which are covalently bound to cell wall structure compounds and mostly found in wheat bran fractions (Adom & Liu, 2002; Gallardo *et al.*, 2006).

Cinnamic acid derivatives	R$_1$	R$_2$	Benzoic acid derivatives	R$_1$	R$_2$
p-Coumaric acid	H	H	Protoctechuic acid	OH	H
Ferulic acid	H	OCH$_3$	Vanillic acid	H	OCH$_3$
Caffeic acid	OH	H	Gallic acid	OH	OH
Sinapic acid	OCH$_3$	OCH$_3$	Syringic acid	OCH$_3$	OCH$_3$
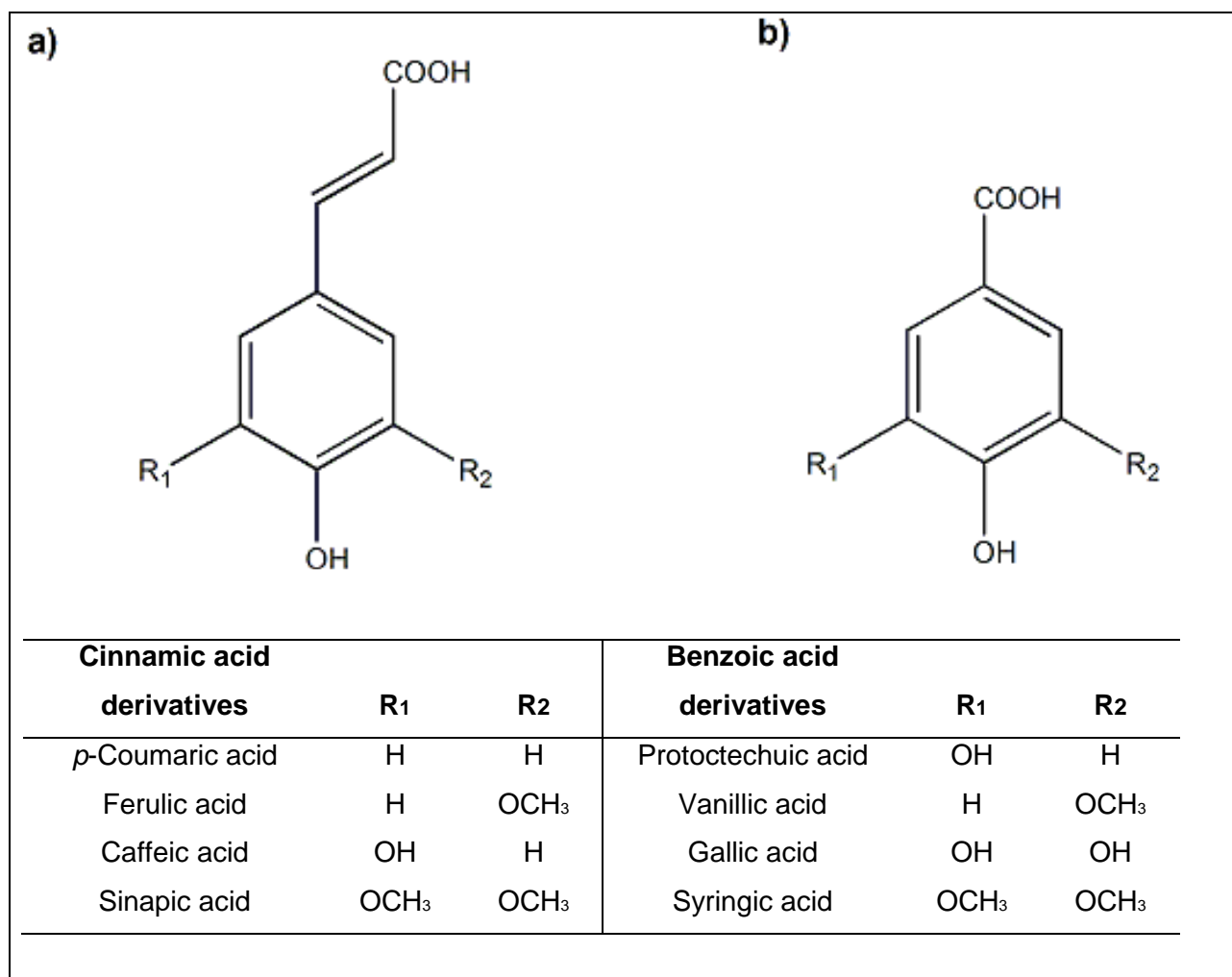
Figure 2.2 The chemical structure of (a) cinnamic acid derivatives and (b) benzoic acid derivatives, common phenolic acids found in wheat.

Lignans

Lignans belong to the phytochemical group which consist of two *p*-propylphenol moieties through β-β linkages (Ayres & Loike, 1990). Depending on the chemical structure of the lignan, their antioxidant capacity can be very different from one another. Some have more than twice the radical scavenging capacity compared to vitamin-C, while others have half the strength of vitamin-C (Eklund *et al.*, 2005). Lignans are found in the outer layers in many cereals (Nilsson *et al.*, 1997; Brouns *et al.*, 2012). Secoisolariciresinol and pinoresinol are the common lignans found in wheat (Aynun Nahar et al., 2004). However, the lignan syringaresinol is found in some wheat varieties and in these cases they are known to be the predominant lignan, whereas matiresinol is found in all varieties, but in small amounts (Aynun Nahar *et al.*, 2004; Dinelli *et al.*, 2007). The structure of the common lignans are shown in Fig. 2.3. Although lignans only account for a small portion of the total phenolic compounds (Dinelli *et al.*, 2007; Vaher *et al.*, 2010), their in vivo function should not be neglected. Although humans may not be able to absorb plant lignans directly, they can be converted to mammalian lignans, enterodiol and enterolactone by intestinal microflora (Aynun Nahar *et al.*, 2004; Liu, 2007). The converted compounds are found to be associated with cancer prevention and as antioxidants fight against oxidative stress (Eklund *et al.*, 2005; Thompson *et al.*, 2005).
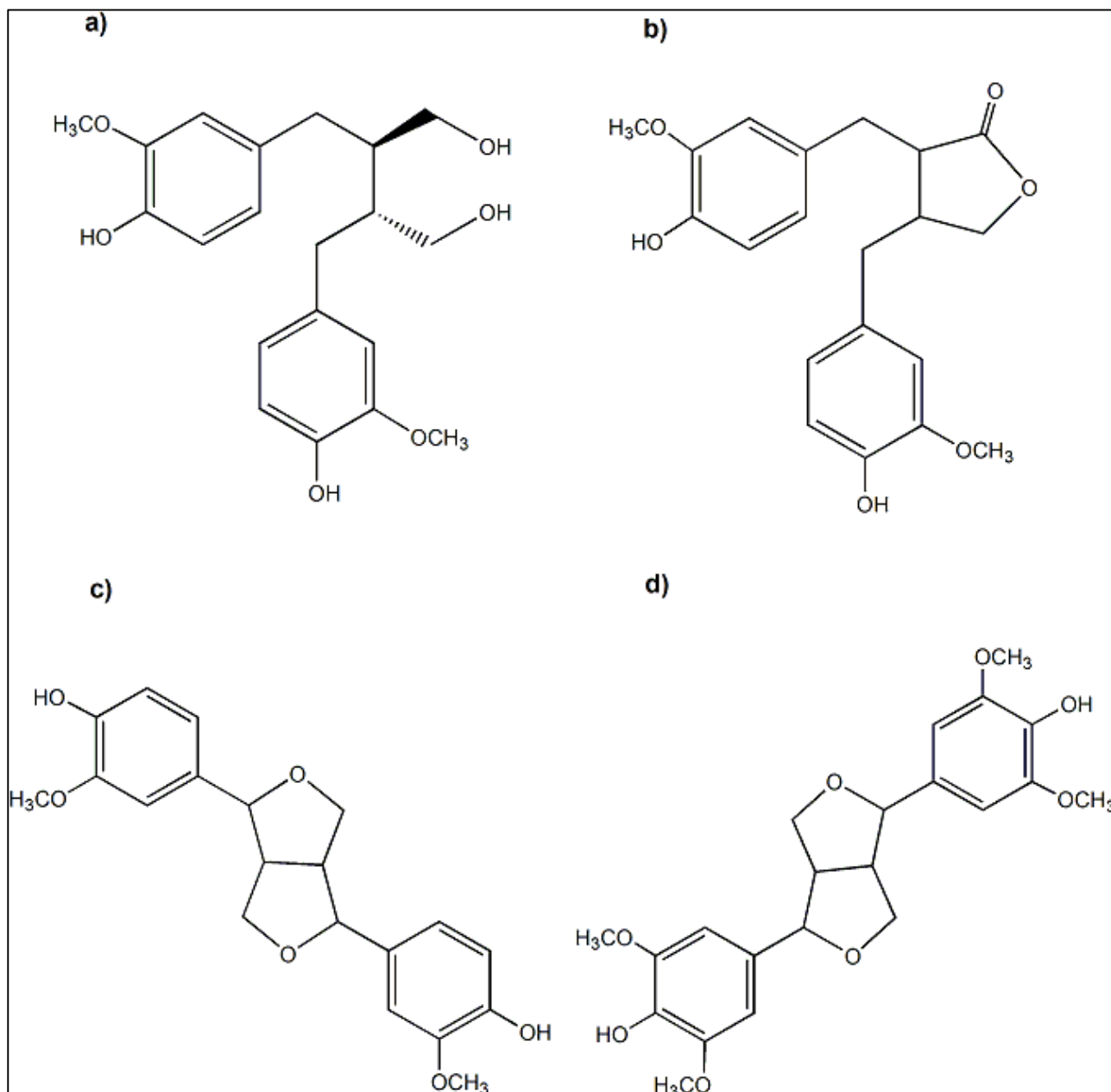
Figure 2.3. Chemical structure of common lignans in wheat: (a) secoisolariciresinol, (b) mataisoresinol, (c) pinoresinol, and (d) syringarsinol (Bryan & Fallon, 1976).

Flavonoids

The flavonoid content is relatively low compared to the phenolic acid content in wheat (Yu, 2008c). Their C_6-C_3-C_6 chemical structure (Fig. 2.4, Table 2.2) allows for complexity of antioxidant properties. Such mechanisms include hydrogen donating, free radical scavenging and metal ion chelating (Rafat Husain *et al.*, 1987; Afanas'ev *et al.*, 1989). Their antioxidant activity is highly dependent on the number of hydroxyl groups and the position thereof (Dziedzic & Hudson, 1983). Compounds include anthocyanidines, anthocyanides, flavonoles, iso-flavonoles, flavones, iso-flavones and flavanols, all belonging to the flavonoid family (Havsteen, 2002). Over 4000 flavonoid compounds were identified in 1986 (Middleton *et al.*, 2000). In 2004 the number increased to 8150, and it was more recently

estimated that about 10 000 flavonoids may exist in their natural form (Tahara, 2007). However, in comparison to the flavonoids pool, only small numbers of flavonoids may be found in wheat. A study by Asenstorfer *et al.* (2006) found flavonols and anthocyanins to be the two major flavonoids in wheat. Another study showed de-hulled whole grain wheat contained in total 124 *µ*mol of catechin/100g flavonoids, with 7% as free flavonoids and 93% as bound flavonoids (Adom & Liu, 2002). Additionally, it was found that the total flavonoids content was not much influenced by cultivar, including red and white, spring and winter, soft and hard wheat cultivars (Adom *et al.*, 2003).

Flavonols	R_1	R_2	Anthocyanins	R_1	R_2	R_3
A-6-C-Ara-8-C-Glc	arabinose	glucose	Cy-3-Gal	OH	H	galactose
A-6-C-Glc-8-C-Ara	glucose	arabinose	Cy-3-Glc	OH	H	glucose
A-6-C-Ara-8-C-Gal	arabinose	galactose	Pg-3-Glc	H	H	glucose
A-6-C-Gal-8-C-Ara	galactose	arabinose	Pn-3-Glc	OCH_3	H	glucose
			Mv-3-Glc	OCH_3	OCH_3	glucose
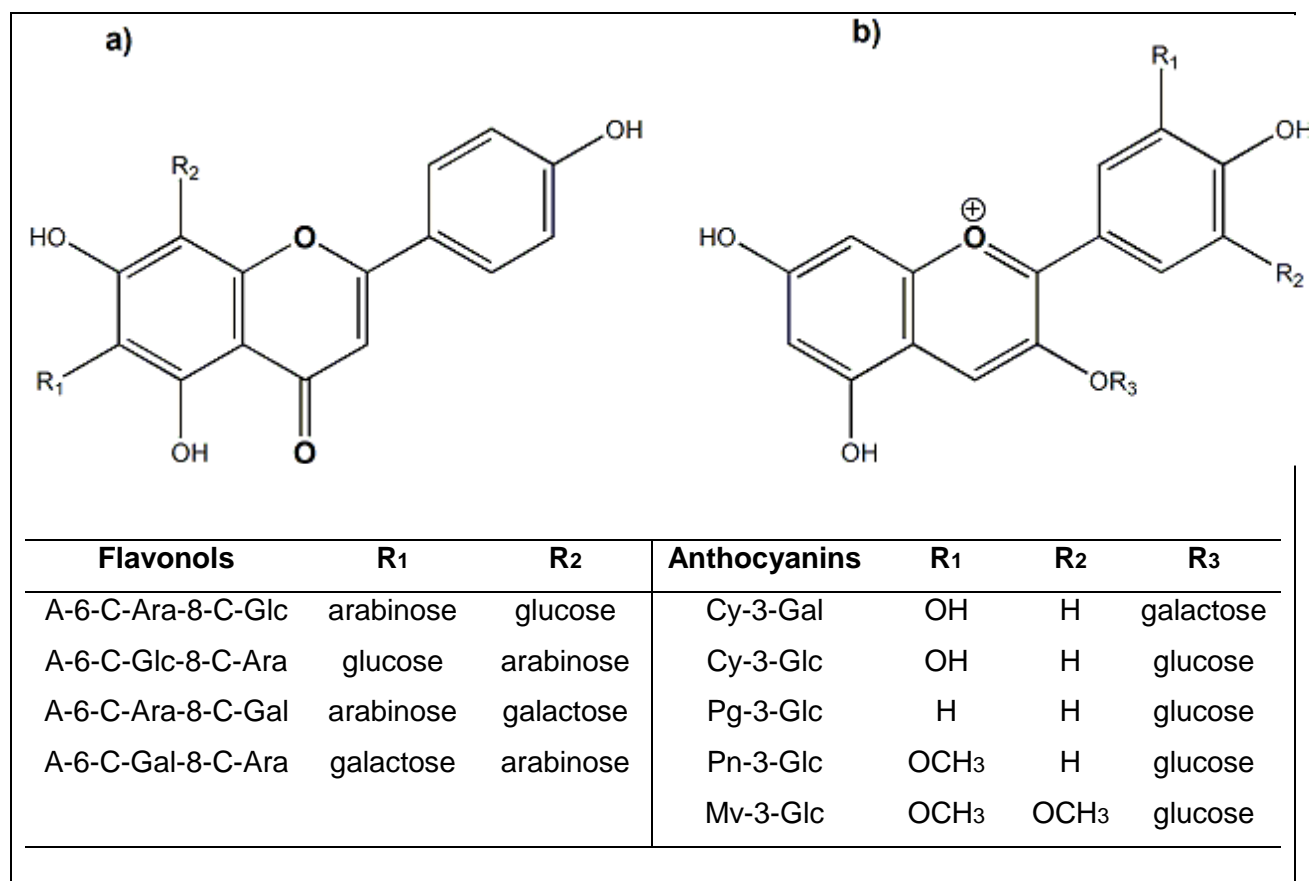
Figure 2.4. Common (a) flavonols and (b) anthocyanins found in wheat. A: apigenin, Cy: cyanidin, Pg: pelargonidin, Pn: peonidin, Ara: arabinoside, Glc: glucoside, Gal: galactoside. Adapted from Asenstorfer *et al.* (2006) and Hosseinian *et al.* (2008)

Carotenoids

Carotenoids are synthesised in plants and appear in yellow, orange or red colours in plants (Giuliano *et al.*, 1993). Likewise, carotenoids have free radical scavenging and oxygen quenching properties (Leenhardt *et al.*, 2006). Additionally, carotenoids can protect humans against oxidative stress and therefore play a role in preventing chronic diseases (Meydani, 2002). Ndolo and Beta (2013) quantified the total carotenoid content (TCC) of four wheat cultivars to be between 2.11 and 2.84 mg/kg, while Konopka *et al.* (2006) found spring wheat to have a higher carotenoid content than winter wheat. Although TCC does not always positively correlate to antioxidant scavenging activity, the Ndolo and Beta (2013) study proved that lutein and zeaxanthin have antioxidant property. The

two carotenoids most commonly found in wheat, are lutein and zeaxanthin, with lutein being the predominant carotenoid (Adom *et al.*, 2003) (Fig. 2.5). As shown by Abdel-Aal *et al.* (2007), lutein contributed 77 to 83% of carotenoids in einkorn, Khorasan, and durum wheat, while zeaxanthin contributed only 9 to13%.
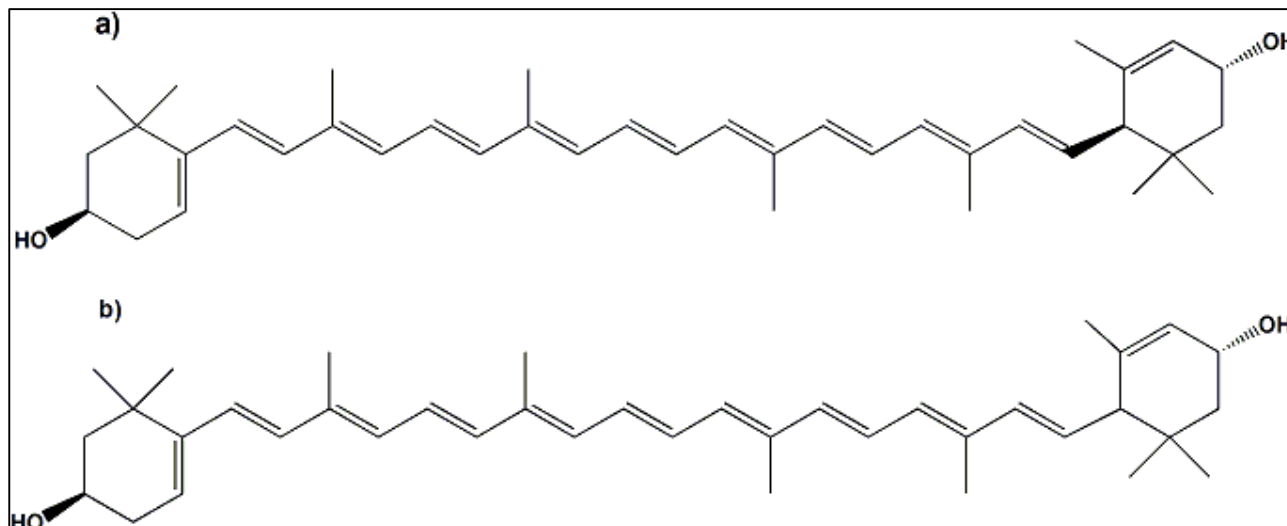
Figure 2.5. Common carotenoids found in wheat. (a) Lutein and (b) Zeaxanthin

Location of antioxidants in wheat

A wheat kernel consists mainly of three parts, i.e. the germ, endosperm and bran. Each part has its own biological function, which was described as early as 1919 (Osborne *et al.*), '*embryo, or germ, situated at one end of the kernel as a small, yellow mass, easily distinguished from the rest of the seed; the endosperm, which forms much the greater part of the entire kernel, and furnishes food for the embryonic plant when the seed germinates; the outer seed coats and underlying layer containing the protein cells which cover the entire seed, and protect the embryo and endosperm from damage during the resting period of the seed's existence.*'

The endosperm accounts for 83.5%, bran for 14.5%, whereas the germ accounts for 1.5%, respectively for a common wheat kernel (Osborne). A more recent study found the variation between soft white wheat and soft red wheat varieties to be in the range of 74.5 to 78.1% for the endosperm and 21.9 to 25.5% for the bran and germ fractions (Adom *et al.*, 2005).

Antioxidants are unevenly distributed between fractions, such as the bran/germ fraction that contains 1005 to 1130 µmol/100 g ferulic acid, while the endosperm fraction only contains 15 to 21 µmol/100 g (Adom *et al.*, 2005). Similar results were found by Vaher *et al.* (2010) using the Folin-Ciocalteu assay, in that the phenolic compounds in wheat bran were found to be 42 time higher than the endosperm. Their study also showed that the distribution of bound phenolic acids present in wheat bran were significantly higher than in wheat flour (Vaher *et al.*, 2010). Also evident from their

study was the fact that de-branned wheat flour were mainly in the endosperm fraction (Vaher *et al.*, 2010).

Wheat bran can be further separated into an outer pericarp, cross cells, testa or nucellar epidermis and aleurone cells (Parker *et al.*, 2005). *Trans*-ferulic acid and *cis*-ferulic acid contribute a total of 95% (w/w) of phenolic compounds in these four layers, with the aleurone layer having the highest phenolic content. The remaining 5% constitutes vanillin, vanillic acid, ρ-hydroxybenzoic acid and ρ-coumaric acid in the wheat bran (Parker *et al.*, 2005).

State of polyphenolics existing in wheat

Phenolic compounds can exist in three states in wheat, either in their free soluble state, their conjugated soluble state, or in their bound insoluble state. Traditional extraction methods, such as Soxhlet and maceration, were mainly used to extract unbound (free) phenolic compounds from cereals, whereas very little amounts of soluble conjugated and insoluble bound phenols can be separated from the wheat kernels (Krygier *et al.*, 1982; Adom & Liu, 2002). This led to the underestimation of the total antioxidant content of wheat until scientists realised the existence of 'non-extractable' phenolics. Perhaps, Geissmann and Neukom (1973) were the first group that discovered ferulic acid to be present in its insoluble form in wheat, which was extracted using alkaline saponification. Furthermore, their assumption that ferulic acid would bind to pentosans through esterification in the wheat kernel, was proven to be accurate by later research (Geissmann & Neukom, 1973). Thereafter, many methods, either physically, physiologically or chemically, had been developed to increase the extraction yield. An *in vitro physiological* procedure mimics the intestinal condition and serves as an alternative for the bio-accessibility of antioxidants when consuming foods. The results showed the antioxidant capacity using *in vitro physiological* procedure were significantly higher in comparison to chemical extraction (Serrano *et al.*, 2007).

Insoluble forms of phenolic compounds are covalently bound to cell wall structural components such as cellulose, hemicellulose (e.g. arabinoxylans), lignin, pectin and rod-shaped structural proteins (Wong, 2006) (Fig. 2.6). These phenolic compounds play an important role in a plant's cell wall, for example, to form a physical and a chemical barrier, to protect cells against autoxidation, their astringency taste repels insects and animals, and lastly as antibacterial and antifungal agents (Sancho *et al.*, 2001; Liu, 2007; Luthria *et al.*, 2015).

Phenolic acids, such as hydroxycinnamic and hydroxybenzoic acids, form ether linkages with lignin through their hydroxyl groups in the aromatic ring and ester linkages with structural carbohydrates and proteins through their carboxylic group (Liyana-Pathirana & Shahidi, 2006; Liu, 2007; Bhanja et al., 2009)
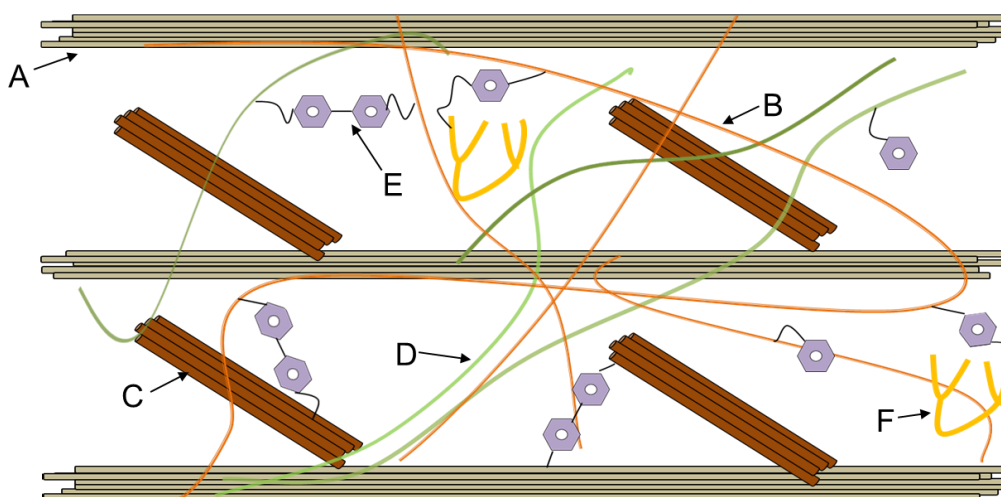
Figure 2.6. Representations of primary cell wall structure of plant material and cross-linking between structural components and phenolic compounds. A = Cellulose; B = Hemicellulose; C = Structural proteins; D = Pectin; E = Phenolic acids; F = Lignin (Acosta-Estrada et al., 2014).

A previous study on wheat revealed that major antioxidant compounds were present in their bound form (Gélinas & McKinnon, 2006). The latter studied the phytochemical profile of 11 wheat cultivars and found the bound phenolics to be 2.5 to 5.4 times higher than the free phenolics, and to contribute 72-84% of the TPC (Adom *et al.*, 2003). The flavonoid profile showed similar results with the bound flavonoids being 7 to 17 times more abundantly found compared to the free flavonoids, again with the bound form contributing 87-93% of the total flavonoids (Adom *et al.*, 2003).

Genotype and environment influence of antioxidant property in wheat

Numerous studies showed antioxidant properties could be effected by genotype, growing environment and a combination of the two (Moore *et al.*, 2006; Mpofu *et al.*, 2006; Menga *et al.*, 2010; Lv *et al.*, 2013; Rascio *et al.*, 2015). One of these studies used 10 winter wheat cultivars planted at four locations in Maryland, USA (Lv *et al.*, 2013). Their study revealed that the total carotenoids were preliminary influenced by the growing environment. When comparing the weather records during the plantation period it was clearly seen that the average air temperature and precipitation were the major environmental factors influencing the antioxidant properties of wheat (Lv *et al.*, 2013). The interaction of genotype and growing environment significantly affected the total tocopherol content. However, the growing environment was a more important factor determining antioxidant activity than genotype (Lv *et al.*, 2013). Their research also showed that wheat that was grown at cooler regions had higher 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS$^{\bullet+}$) scavenging capacity (Lv *et al.*, 2013). As this study only focused on comparing the environmental effects on soft red winter wheat cultivars grown in Maryland, there remain questions regarding the variation due to different cultivars and locations. Interestingly, Ragaee, Guzar*, et al.* (2012) studied 21 wheat varieties, which included hard and soft, white and red, as well as winter and spring wheat varieties, all planted in Ontario province, Canada. Their study showed that TPC in wheat was more correlated with genotype than environment.

Coincidently, a soft red winter wheat variety Branson, was used in the research of Lv *et al.* (2013). Lv *et al.* (2013) found the total tocopherols contents to range from 0.07 to 0.13 μmol/100 g, which was significantly different between location ($p < 0.05$), while Ragaee, Guzar*, et al.* (2012) found the phenolic compounds were not significantly affected by different locations ($p < 0.05$). Although these two studies did not agree on the effect of location on the phenolic compounds, they did however agree that the radical scavenging capacity of Branson were more stable to environmental conditions than the other wheat varieties. The results from the study by Gélinas and McKinnon (2006) using three wheat varieties and each variety collected from several locations, confirmed that location had more influence then genotype on the TPC in wheat.

It is not easy to make a universal statement when comparing the genotype and environment influence on antioxidant property in wheat. However, it is more likely that wheat varieties respond and adapt differently to their growing environment. Different regions have their unique environmental conditions such as temperature and precipitation, the soil type, sunlight intensity, atmosphere humidity, altitude, irrigation, insects and microbes, and many other factors.

Health benefits

For human beings, about 2% oxygen are converted into free radicals such as superoxide radicals (OO˙) and nitric oxide radicals (˙OH), through mitochondrial respiration and phagocytosis (Cadenas & Davies, 2000; Kunwar & Priyadarsini, 2011). These free radicals may attack cellular molecules like DNA, lipids, and proteins which may further lead to chronic disease (Kunwar & Priyadarsini, 2011). Our body prevents oxidative stress through the direct intake of antioxidants from our diet or by synthesising antioxidants from building blocks. A good example of the latter is glutathione, which is synthesised from L-cysteine, L-glutamic acid, and glycine in our bodies (Cadenas & Davies, 2000; Townsend *et al.*, 2003). On the other hand, glutathione can only be absorbed in its breaked-down format of smaller molecules, namely amino acids (Townsend *et al.*, 2003). Data obtained by Svilaas *et al.* (2004) showed that the total intake of antioxidants was significantly correlated with lutein, zeaxanthin and lycopene levels in human plasma and their result supported the hypothesis that dietary antioxidants contribute towards our oxidant defense ability.

Cereals are considered as a secondary source of antioxidants after fruits (Halvorsen *et al.*, 2002). Consumption of whole grain is recommended by dietary guidelines, since whole grains contain dietary fiber, resistant starch, oligosaccharides, minerals, phytoestrogens and antioxidants (Plaami, 1997). Phytic acid was found to play an important role in the treatment of cancer, hypercholesterolemia, hypercalcuria and kidney stones (Slavin, 2000). However, phenolic acids present in their bound forms which are ester-linked to the cell wall polymers, can not be absorbed through digestion (Menga *et al.*, 2010).

Thermal processing

Wheat flour is used as an important ingredient in many foods, for example bread, biscuit, pasta, pizza and cake, all requiring a cooking or baking step. During the cooking or baking step changes in flavour and textural properties occur, improving the product and making it more acceptable for human consumption. These flavour and textural properties changes result from the gelatinisation of starch and the denaturation of protein, which also makes the products more digestible for the consumers (Ezeogu *et al.*, 2005).

Pasteurisation, extrusion, steaming, baking and roasting are commonly used as thermal processing methods in the food industry. These methods change the natural antioxidant properties in wheat (Ragaee, Seetharaman*, et al.*, 2012) as they cause the release of bound phenolic acids from their cell wall structures (Dewanto *et al.*, 2002), also destroy thermal labile antioxidants (Sharma & Gujral, 2011), and lastly produce new products due to the Maillard reaction that have strong antioxidant properties (Rufián-Henares & Delgado-Andrade, 2009).

N'Dri *et al.* (2013) studied the effect of cooking on to the antioxidant properties of sorghum, fonio and millet. Their study found a decrease of total phenolic content and total antioxidant capacity on all the cooked samples under investigation. However, the soluble phenolic acids increased for sorghum and millet, while fonio showed a decrease in total phenolic contents. Their study revealed that thermal processing could lead to a decrease in antioxidant properties, while bound phenolic acid could be released as soluble phenolic acids by thermal processing (Dewanto *et al.*, 2002). Their study indicated that different types of cereal may respond differently regarding their antioxidant properties during thermal processing. Sharma *et al.* (2012) studied the influence that extrusion processing had on the antioxidant activity of barley. TPC, total flavonoid content (TFC), antioxidant activity (DPPH radical scavenging activity) and metal chelating activity, were compared amongst eight cultivars. Their study found that a higher temperature (180°C compared to 150°C) resulted in the most significant decrease of TPC, whereas a higher moisture (20%) caused a more extreme decrease in TFC than that of the lower moisture (15%). This could be explained by the phenolic compounds that start to degrade from 180°C. Contrary, phenolic and flavonoid compounds interact, or have the potential to interact with proteins, which gives these compounds a better stability against high temperature (Sharma *et al.*, 2012). Thus, the high level of destruction during moist heating explains why higher moisture in grains cause more degradation of TPC and TFC (Moreira, 2001). The increase of antioxidant activity and metal chelating activity had a conflicting effect on TPC and TFC, which seemed unexpected. Nonetheless, this could be explained by the products of the Maillard reaction that contributed towards the antioxidant properties as shown in many studies (Fogliano *et al.*, 1999; Rufián-Henares & Delgado-Andrade, 2009; Sharma *et al.*, 2012).

Another study that investigated the thermal effect of the antioxidant properties were done by Dewanto *et al.* (2002), on tomatoes. Their results indicated that the total antioxidant activity was enhanced by 27.93%, 33.88% and 62.09%, respectively, after heat treatments of 2, 15 and 30 min at 88°C. A slight increase in TPC and TFC was observed, but the difference was not significant (*P* >

0.05), which indicated that the majority of the phenolics content in tomatoes could tolerate the high temperature (Dewanto *et al.*, 2002). Lycopene (a carotenoid found in tomatoes and not in grains) content seemed to correspond with the total antioxidant activity at 2 to 15 min. It was found that the lycopene content decreased after 30 min, while the total antioxidants activity increased, which indicated the degradation of lycopene when exposed to long periods of thermal processing. Lycopenes contribute very little of the total antioxidant activity in tomatoes, while other components such as phenolic compounds are more accountable for antioxidant properties (Dewanto *et al.*, 2002). As the Maillard reaction took place at a very slow rate at all the relevant temperatures in this study, it did not influence the total antioxidant content. Additionally, the thermal processing might have aided with the release of the antioxidant compounds from cells and cell wall structures. The stability of the carotenoids were relatively low as carotenoids are sensitive to heat processing (Hidalgo *et al.*, 2010).

The effect of thermal processing (i.e. baking, toasting, cooking and microwaving) on the phytochemicals level in wheat was reviewed by Luthria *et al.* (2015). This review confirmed that phenolic compounds were stable during thermal processing. In addition, the free phenolic acids showed a slight increase, while the bound phenolic compounds showed a decrease. Evidently, this could prove that thermal processing released the bound form of phenolic compounds. The degradation of tocopherols and carotenoids were less during all forms of processing and started from 130°C for tocopherols and tocotrienols, caroteinoids, and other relevant phytochemicals (i.e. steryl ferulates), as indicated by the review.

The above review show that thermal treatment impacts on the TPC, TFC and total antioxidant activity in different types of samples, at temperatures as high as 180°C. The treatment time also plays a role that affecting the TPC, TFC and antioxidant activities.

Forced convection continuous tumble roaster

The FCCTR was designed and manufactured by a South African engineer at the beginning of the 21st century. This FCCTR with its unique process has been registered as a worldwide patent (PCT/IB 2008/001008). With the design of a semi-closed roasting chamber, the FCCTR re-circulates the heated air during roasting. With a rotating mixer in the center of the chamber, it continuously mixes and moves the products throughout the roasting process. This roaster has numerous industrial-wise advantages, such as efficient energy usage, precise control, even heat transfer and stable continuous roasting. During the roasting process, moisture that is released from the product stays in the roasting chamber, while the moisture vapour replaces part of the air gradually, and converts heated air into semi–superheated steam, which allows for more efficient and even heat transfer to the product. A review which compared hot air and superheated steam used for impingement drying of foods indicated that superheated steam processing reduces the oxidation rate during the process, thereby indicating the potential to better maintain the nutritional value of the products (Moreira, 2001). The latter review also mentioned that a higher degree of gelatinisation occurred when using superheated steam than when using dry air under the same condition. This was explained by the

19

superheated steam drying that has a high humidity at high temperatures in the drying chamber, which in turn causes moisture condensation on the product surface when the product first enters the drying chamber (when the initial product is much colder). As the temperature of the product increases externally as well as internally, faster moisture migration to the internal level of the product occurs, and interaction with starch granule at cellular level takes place (Moreira, 2001). Furthermore, higher temperature and higher heat transfer coefficients result in less gelatinisation in the product during superheated steam drying (Moreira, 2001). The temperature, steam-air ratio, heat transfer coefficient and nature of the product plays a roll with respect to how the texture, microstructure, nutritional value and pasting properties of the product (Moreira, 2001) is influenced.

Two factors can be controlled when roasting using FCCTR, i.e. the roasting temperature and the roasting speed (inversely related to roasting time). Although the FCCTR has been used to manufacture pet food as well as snacks and nuts for human consumption, limited research has been done on the optimisation of the roasting conditions.

A process similar to FCCTR might be that of convection oven roasting and will be discussed in the next section, i.e. thermal processing.
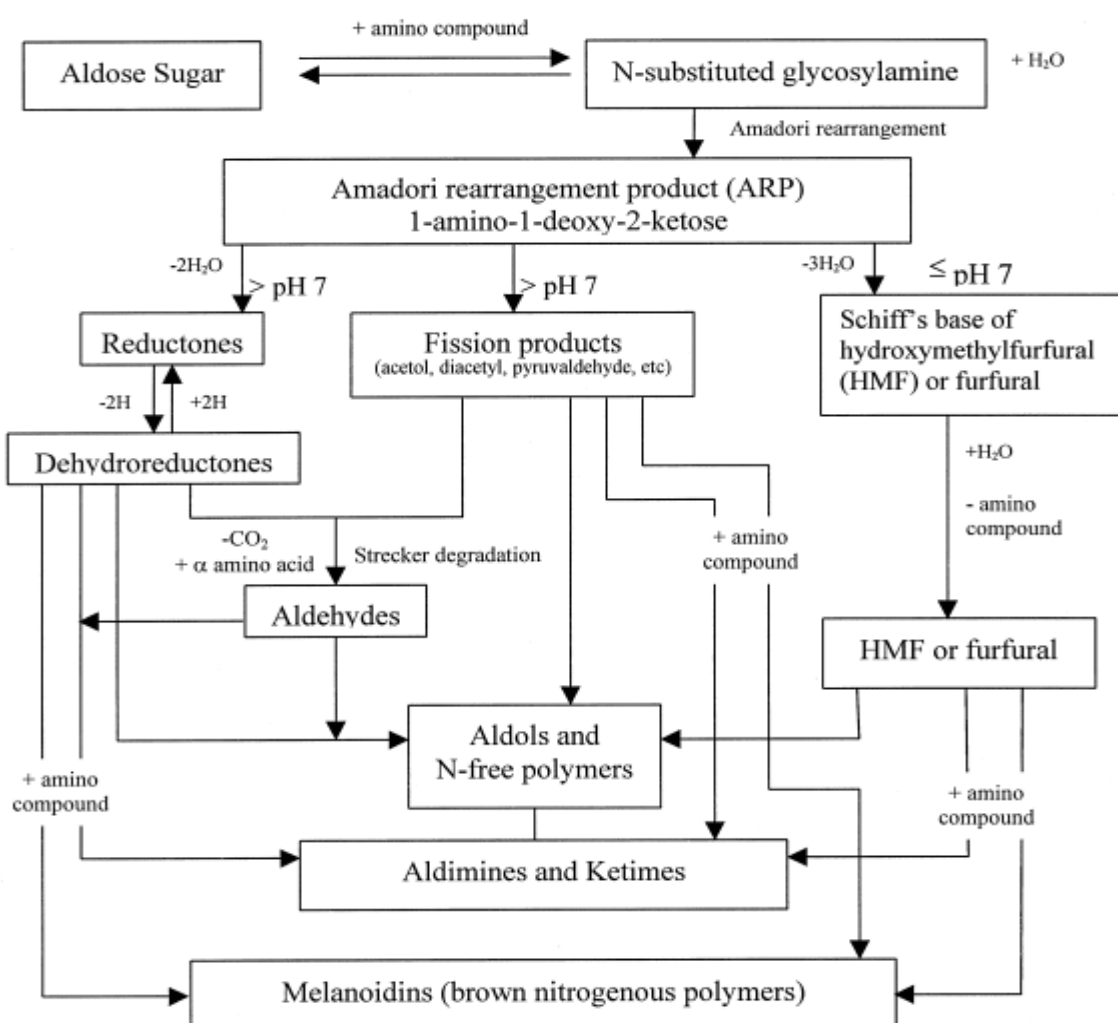
Figure 2.7. Compounds produced during Maillard reaction (Hodge, 1953; Martins et al., 2000)

Antioxidant analysis

Extraction

The first step for most cereal antioxidant analysis is the extraction process. Depending on the experimental requirements, the availability of equipment and the researchers and institutions' preference, the extraction methods may differ with respect to the solvents used, the sample-to-solvent ratio, assisted techniques, the separation technique, the extraction time, temperature amongst others. The extraction yield are highly dependent on the choice of the above mentioned factors (Shi *et al.*, 2005). The following steps explain the process and details of antioxidant measurements.

Milling

Milling is normally the first step when performing extraction. The key factor for the phytochemical extraction is the particle size. Smaller particles results in larger surface areas that interact with the extraction solvents, therefore achieving higher extraction efficiency (Rosa *et al.*, 2013).

Solvent

Commonly used solvents for antioxidant extraction of plant materials include acetone, ethanol, ethyl acetate, methanol and water. These can be used, either individually or combined, along with different water proportions (Dai & Mumper, 2010). Normally, pure water is not ideal for the extraction of polyphenols, due to the low solubility of antioxidants in water. However, the dissolution of proteins and poly-saccharides are not desired (Bey *et al.*, 2013) as can be explained by polarity differences between the solvent and extracts (Naczk & Shahidi, 2006).

The polarity can be adjusted by mixing the selected solvent with water in a certain ratio (the polarity of antioxidants differ depending on their molecular structure). In a review article based on a methodology for plant phenolic analysis, it was stated that the efficiency of solvents also differ depending on the molecular weight of the polyphenols (Dai & Mumper, 2010). For example, at a lower molecular weight of polyphenols, methanol is more efficient, while aqueous acetone has a better efficiency when extracting polyphenols with a higher molecular weight, such as flavonoid (Metivier *et al.*, 1980; Guyot *et al.*, 2001; Prior *et al.*, 2001).

Ethanol and methanol are the most popularly used solvents, they are commonly mixed with water to obtain a concentrations between 50% to 80% (Liazid *et al.*, 2007; Serpen *et al.*, 2008; Gallo *et al.*, 2010; Menga *et al.*, 2010; Laus *et al.*, 2012). A study that compared the use of absolute ethanol for the Soxhlet method and 50% acetone for maceration, indicated the best TPC and ABTS free radical extraction when using 50% acetone. The best solvent for oxygen radical absorbance capacity (ORAC) extraction was with 70% ethanol, the best DPPH free radical extraction with 70% methanol, and the best overall extraction solvent with 50% acetone (Zhou & Yu, 2004).

Wang *et al.* (2008) suggested that 64% ethanol should be used for the extraction of polyphenol compounds from wheat bran. It should be noted that Wang *et al.*'s method was combined with ultrasonic assisted technique, which will be discussed in detail in the following discussion on

ultrasonic assisted extraction. Also, Xu & Chang (2007) showed that 70% acetone is the most effective solvent for extracting polyphenols in legumes, whereas 80% acetone was the best solvent when excluding the acidic treatment . Some studies used acid or alkaline to extract bound phenolic acids from cereal samples. Those methods may extract more antioxidants from samples and more accurate to the actual antioxidant content from sample. However, those methods are more complicated, more expensive and more steps were involved, which were ideal to be used for small sample size (Kim *et al.*, 2006; Hosseinian *et al.*, 2008; Okarter *et al.*, 2010). Additionally, samples extracted using low concentration HCl and mixed with aqua-methanol can be used for Folin-Ciocalteu method, but not suitable for the DPPH radical scavenging assay (Mpofu *et al.*, 2006), as the DPPH reagent is stable at a pH range 4-8 (Okarter *et al.*, 2010).

Subsequently, there is no universal solvent that is suitable for extracting all types of antioxidants, although acetone, ethanol and methanol are the most commonly used solvents at concentrations between 50 to 80% v/v.

Temperature

Temperature is another important factor that affects the antioxidant extraction rate of cereals. The reason being that an increase in temperature leads to an increase in the solubility of solute and the diffusion coefficient (Spigno *et al.*, 2007), thereby increasing the extraction efficiency. On the other hand, high temperatures cause degradation of the thermal labile antioxidants (Spigno *et al.*, 2007). For example, anthocyanins degrade rapidly when temperatures of 70°C and higher are reached. Therefore, it is suggested that the extraction temperature of anthocyanins should not exceed 50°C (Aramwit *et al.*, 2010; Dai & Mumper, 2010). More so, higher extracting temperatures increase the solubility and mass transfer rate, as well as the reduction of the viscosity and surface tension of the solvents. These factors facilitate better interactions between the solvent and the sample, thus improve the extraction rate and yield at higher temperature (Dai & Mumper, 2010).

Some antioxidants are relatively heat stable, as Soong and Barlow (2004) observed in mango seeds with the total antioxidant capacity that remained stable at a high temperature of 160°C. Another research group reported most phenolic compounds to be stable at temperatures as high as 125°C when using microwave-assisted extraction (MAE). The same study also found the benzoic acid family to be even more stable at 175°C (Liazid *et al.*, 2007). Consequently, the selection of the correct extraction temperature is of critical importance for maintaining the stability of the phenolic compounds present (Dai & Mumper, 2010). Most of the phenolic compounds are stable below 180°C and can thus tolerate temperatures not exceeding 180°C.

Interaction time

If appropriate extraction conditions are provided (and the extraction temperatures do not cause degradation), the antioxidant yield correlates with the extraction time and the correlation curve takes on the shape of a logarithmic graph. The extraction rate starts with a steep increase, but eventually reaches an equilibrium state in the extraction eco-system (Spigno *et al.*, 2007; Wang *et al.*, 2008).

The rate may be influenced by several factors, such as temperature, types of solvent and the selection method. It is ideal to set the extraction time for the start of the equilibrium state.

Extraction methods

Many extraction techniques have been developed along with new technologies and equipment. Two examples are the development of sonication-assisted extraction (SAE) and MAE (Wang & Weller, 2006). No matter what method is being used, achieving the equilibrium state with higher mass transfer rate is the key element that determines the success of the extraction process (Shi *et al.*, 2005).

Soxhlet and maceration

The traditional extraction techniques such as maceration and Soxhlet extraction have been used for many decades; these methods are simple but normally require long extraction time and large quantity of extraction solvent (Reverchon & Senatore, 1992; Zhou & Yu, 2004). The Soxhlet method was invented in 1879, and this method is nowadays still widely used in many laboratories for lipid extraction (Sparr Eskilsson & Björklund, 2000). However, Soxhlet is not very popular for antioxidant extraction. Though Soxhlet showed better efficiency than maceration when extracting antioxidants at the same conditions (Zhou & Yu, 2004), maceration are more flexible to adapt with other techniques to achieve better extraction. Maceration can adapt with a wide range of solvent combination, therefore the polarity of the extracting solvent can be easily controlled by using either mono or multi-ingredient at selected concentrations (Zhou & Yu, 2004; Serpen *et al.*, 2008; Menga *et al.*, 2010). For example, 1% of HCl can be added with 80% of methanol to assist with the extraction of bound phenolic acids from cell walls for wheat samples (Beta *et al.*, 2005).

Shaking

Shaking is one of the simplest methods to improve the extraction yield. It provides the mechanical force that cause continuous movement of solvents and samples in the closed system, which increase the chance of contact. The movement also homogenises the mixture that keep the osmosis force pressure until equilibrium state is reached. With assistance of shaking, extracting time has reduced to 0.5-3 hours (Velioglu *et al.*, 1998; Ragaee *et al.*, 2006; Xu & Chang, 2007). Compared with 3-48 hours of Soxhlet extraction (Yu *et al.*, 2002), or up to 1 week maceration extraction at room temperature (Sun & Ho, 2005). The benefit of this method is obvious even though this is not efficient enough to satisfy the scientific research nowadays.

The shaking method is normally used with 80% methanol, while shaking in orbital movements. Some methods mentioned at 300 rpm, either at room temperature or heated up to 70°C, for anthocyanin extraction and for it to be done at lower temperatures (ambient). Separation can be done by centrifuge or vacuum filtration (Velioglu *et al.*, 1998; Ragaee *et al.*, 2006; Xu & Chang, 2007).

Sonication-assisted extraction

A good example is SAE, with frequencies above 20 kHz of mechanical vibrations, sound wave generate expansion and compression cycles to the sample and this can disrupt biological cell walls,

and lead to greater penetration of solvent into cellular materials (Wang & Weller, 2006). With ultrasound treatment during extraction, some compounds such as conjugated phenolic can be released from plant materials. A good example is the increase in yield of rutin from buckwheat by up to 15 times in contrast with the commonly used process (Fabjan *et al.*, 2003; Peričin *et al.*, 2009; Acosta-Estrada *et al.*, 2014). With 70% methanol as a solvent extracting wheat bran using SAE for 20 min obtained about 2.2 GAE (mg/g). Under similar extraction condition without sonication-assist only 0.11 GAE (mg/g) was recovered. From this data, the efficiency during the extraction has been multiplied 20 times using SAE compare to the normal maceration method (Gallardo *et al.*, 2006; Wang *et al.*, 2008). By optimising the ultrasonic-assisted extraction using wheat bran as a model, Wang *et al.* (2008) found that using 64% ethanol, at temperature of 60°C, for 25 min was the optimal condition for TPC determined by the Folin-Ciocalteu method.

Microwave-assisted extraction

Unlike sound-waves which are based on mechanical vibrations, microwaves are a form of electromagnetic energy, which alter electro-magnetic field at frequency between 0.3-300 GHz and interact with polar molecules of the material, and this causes the internal superheating of the sample (Pan *et al.*, 2003). As water is a polar substance and widely distributed in large portions of most plant and animal tissues, microwaves can be used to disrupt the cell in the tissue, improving the extraction yield (Wang & Weller, 2006). Gallo *et al.* (2010) compared the efficiency of MAE and SAE method when extracting phenolic compounds from spices, and indicated that the MAE method was four times more efficient than the SAE method with shorter time. In this experiment, the optimimum condition for extraction was found to be 64% ethanol at 60°C. Using different methods for separating the liquid and solid between SAE and MAE. Though, other studies also state that the MAE obtained better efficiency than SAE and maceration methods (Pan *et al.*, 2003; Kalia *et al.*, 2008).

Determination of antioxidant properties

Determination of the antioxidant properties involves determination of the antioxidant compounds and activities. These methods include quantifying the specific compounds and the total compounds within a specific group. A good example is that the individual phenolic acids can be determined using a chromatography and the TPC can be determined using Folin-Ciocalteu spectrophotometric technique. Many antioxidant activity determination methods have been developed based on determination of the free radical scavenging capacity and the metal ion chelating ability of antioxidants. As discussed in the previous section, the phenolic acids are the major source of antioxidant in wheat, and a strong significant correlation exists between total phenolics and antioxidant activity (Singleton & Rossi, 1965b; Brand-Williams *et al.*, 1995; Arnao *et al.*, 1996; Velioglu *et al.*, 1998; Zieliński & Kozłowska, 2000; Huang *et al.*, 2002; Beta *et al.*, 2005).

Table 2.1 Comparison of traditional and newer extraction techniques (Sparr Eskilsson & Björklund, 2000)

	Extraction technique		
	MAE	**Soxhlet**	**SAE**
Brief Description	Sample is immersed in a microwave-absorbing solvent in a closed vessel and irradiated with microwave energy.	Sample is placed in a glass fibre thimble and, by using a Soxhlet extractor, the sample is repeatedly percolated with condensed vapours of the solvent.	Sample is immersed in solvent in a vessel and placed in an ultrasonication bath.
Extraction time	3-30 min	3-48 h	10-60 min
Sample size	1-10 g	1-30 g	1-30 g
Solvent useage	10-40 mL	100-500 mL	30-200 mL
Advantages	- Fast and multiple extraction - Low solvent volumes - Elevated temperatures	- No filtration required - Low cost	- Multiple extractions - Low cost
Drawbacks	- Extraction solvent must be able to absorb microwaves - Clean-up step needed - Waiting time for the vessels to cool down	- Long extraction times - Large solvent volumes - Clean-up step needed	- Repeated extractions may be required - Clean-up step needed

Total phenolic contents determination with Folin-Ciocalteu reagent

The importance of understanding the total phenolic content (TPC) for antioxidant study in wheat was explained by Yu (2008a). One of the most popular methods used for the total phenolics content determination is Folin-Ciocalteu assay.

The method was initially designed for tyrosine and tryptophan determinations (Folin & Ciocalteu, 1927), and extended for total phenolic determination in wine by Singleton and Rossi (1965b) after many years. The Folin-Denis and Folin-Ciocalteu reagents were compared for estimation of plant phenols by colour yield, spectrum, time-temperature effects, and interferences. The Folin-Ciocalteu

formulation was found to have several advantages, including no precipitation observed, more intensity and consistency of colour formed, and better recovery. Although the formulation for Folin-Ciocalteau reagent is available to researchers, the exact chemical nature still remains a mystery to date (Huang *et al.*, 2005).

However, there is a debate about using Folin-Ciocalteu assay for total phenolic content measurement. As the method measures the reducing capacity based on electron transfer, the Folin-Ciocalteu reagent can be reduced by many nonphenolic compounds (Huang *et al.*, 2005). As TPC can only be determined when phenolics have been dissociated to phenolate anion under alkaline condition, a revisited method was suggested with a correction action, by simply subtracting the antioxidant activity value which was determined under acid condition from the total antioxidant value obtained under alkaline condition (Sanchez-Rangel *et al.*, 2013).

Preparation of several concentrations of gallic acid was recommended to be used to generate a standard curve, thus results should be expressed as gallic acid equivalent value (Singleton & Rossi, 1965a).

Ferric reducing ability of plasma (FRAP)

The ferric reducing ability of plasma assay is another method which is widely used to determine antioxidant properties. It measures the amount of ferric-tripyridyltriazine (Fe^{III}-TPTZ) complex to be reduced to the Fe^{II}-TPTZ (ferrous) form by the responsible antioxidants presented in the sample. The ferrous form complex results in an intense blue colour which gives a maximum absorption value at 593 nm. It should be noted this method measures the ability of antioxidants to delay or to prevent oxidation of substrates and to reduce or inactivate the oxidants (Benzie & Strain, 1996).

Fe^{III}-TPTZ was found to have a low reaction rate with the protein antioxidant – albumin (Roche *et al.*, 2008). It was thus proposed that the FRAP assay measures antioxidant power without interfering with protein-associated antioxidants (Benzie & Strain, 1996). This correspond to the previous study which revealed that glutathione and other thiols do not contribute to the results obtained with FRAP assay (Buettner, 1993). Since very little amount of glutathione can be absorbed through the digestion system and can hardly be utilised as a source of antioxidants, it becomes an advantage to use the FRAP assay for bioavailability studies of antioxidants (Stahl *et al.*, 2002).

DPPH

The DPPH free radical (2,2-diphenyl-1-picrylhydrazyl) scavenging assay is a rapid, simple and inexpensive method. This method has been extensively used for measuring overall antioxidant activity of foods and biological systems, including solid and liquid samples (Kedare & Singh, 2011). The chemical structure of a DPPH radical has an odd electron of nitrogen atom in the center with a deep violet colour. The DPPH radical becomes colourless when being reduced by receiving a hydrogen atom or an electron. Thus the antioxidant activity of samples can be monitored based on the colour reduction using a spectrophotometer, depending on their hydrogen or electron donating

ability (Brand-Williams *et al.*, 1995). It was found the DPPH radical has a strong absorbance band at 517 nm (Kedare & Singh, 2011).

The use of the DPPH radical for antioxidant studies first appeared in publication in 1958 (Blois, 1958; Kedare & Singh, 2011). The implementation of spectrophotometers associated with DPPH radical for antioxidant analysis was only published 37 years later (Brand-Williams *et al.*, 1995). Although the concept of this assay is not complicated, the methods to present the results had drawn many criticism. It was originally quantified as amount of antioxidant required to reduce the DPPH radical to 50% of its initial concentration at steady state (EC_{50}), and expressed in terms of antiradical power (ARP), which was calculated as $1/EC_{50}$ (Brand-Williams *et al.*, 1995). Soon after, some laboratories expressed their results as % DPPH radical remaining defined at random time, which is similar to the EC_{50} impression. The results obtained from both methods depend on the initial concentration of DPPH radical and the reaction time of measurement. This made it impossible to compare results between laboratories (Yu, 2008f). Other calculation methods such as 'antiradical efficiency' (AE) or 'radical scavenging efficiency' (RSE), take into account the kinetic properties by including measurement at more than one time point (Sánchez-Moreno *et al.*, 1998; De Beer *et al.*, 2003). These methods were reliant on EC_{50} calculation, therefore comparing results between laboratories were still impossible using the latter methods.

Correlating the DPPH radical scavenging capacity with a series of known concentration of antioxidant standards (e.g. trolox), Cheng *et al.* (2006) suggested the 'relative DPPH˙ scavenging capacity' (RDSC) estimation method, which addressed the criticisms of the other methods and made comparison of results possible between laboratories. It should be noted that the standard curve requires pre-adjustment for defining either the concentration range of antioxidant standard or the dilution factor of the sample extracts.

DPPH radical is relative stable and considered as a reliable reagent for antioxidant studies (Gil *et al.*, 2000). However, it still requires consideration of the operating conditions. It is advised that the experiment should be done at ambient temperature (Bondet *et al.*, 1997; Ozcelik *et al.*, 2003) and no significant colour change was found at 25°C in solvents such as methanol or acetone with appropriate control of other factors. It has also been suggested that experiments should be performed in a dark environment, pH should be in the range of 5.0-5.6, contact with oxygen and evaporation of solvent should be avoided and solvents should be carefully selected (Blois, 1958; Ozcelik *et al.*, 2003).

<u>2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) cation radical scavenging capacity assay</u>

The ABTS cation radical (ABTS˙+) scavenging capacity assay was used for the determination of antioxidant capacity. It was first published by Miller *et al.* in 1993. Similar to DPPH radical scavenging assay, ABTS˙+ scavenging capacity assay measures the overall antioxidant capacity of the sample against the ABTS cation radicals. The ABTS˙+ can be generated by oxidising ABTS with oxidants such as hydrogen peroxide (Miller *et al.*, 1993). The ABTS˙+ appears as a typical blue-greenish colour,

and become colourless when being reduced by antioxidants. The determination of antioxidant capacity is based on measuring the absorbance of the remaining ABTS$^{•+}$ using a spectrophotometer (Yu, 2008d) with a reference of antioxidant standard series, Trolox is the common standard.

Several factors may effect the reliability of the assay, mainly including selected wavelength, reagent used to generate ABTS$^{•+}$ and selected time point (Miller *et al.*, 1993; Arnao *et al.*, 1996; Miller *et al.*, 1996; Yu, 2008d). Miller *et al.* (1993) reported ABTS$^{•+}$ generated using ABTS/metmyoglobin peroxidase/H_2O_2 had the highest absorbance value at 734 nm. Arnao *et al.* (1996) suggested 414 nm should be used for the ABTS$^{•+}$ which was generated using ABTS/horseradish peroxidase/H_2O_2 system. However, the two methods were found to generate an intermediary radical, thus may have influenced the accuracy of the antioxidant activity (Miller *et al.*, 1993; Re *et al.*, 1999b). The improved method was suggested by Re *et al.* (1999) using potassium persulfate to generate ABTS$^{•+}$, with generation of monocation ABTS$^{•+}$ that avoided the generation of the intermediary radical. Re *et al.*'s method allowed enough time to pre-generate the ABTS$^{•+}$ before addition of antioxidant, that overcome the deficiency of uncertainty of the earlier methods by directly generate the radicals without generating of an intermediary radical., i.e. if the ABTS$^{•+}$ was completed. Later study confirmed that the ABTS$^{•+}$ is stable within a wide pH range of pH 1–8 (Ferri *et al.*, 2013).

Conclusion

The natural properties of antioxidants in wheat had been extensively studied in the past. Including the mechanisms, chemical structures, locations, effect of processing, environment and genotype effect, health benefit to human beings, determination methods, and as well as many other topics. This literature review was not able to discuss all the topics. However, the selected topics provided an overview of the past and recent research that had been achieved. Type of antioxidants in wheat, effect of genotype and environment and thermal processing effect on the antioxidant properties of wheat, was discussed in more detail as these topics are more relevant to this study. This literature review also confirmed there is no information available, to our knowledge, on the antioxidant properties of South African wheats, and the effect of thermal processing on wheat in South Africa, especially with the recently designed FCCTR.

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Chapter 3

DPPH radical stability under buffered conditions: survey and method development

Abstract

Sample extraction is a crucial step when studying antioxidants of cereal samples. About 80-90% of phenolic compounds are bound to cell wall components of wheat grains. Thus the appropriate extraction solvent and method should be carefully chosen depending on the objective.

Acidified methanol was used as extraction solution for the analysis of the antioxidant properties of South African wheat. The acid reduced the extract to pH<1, which was outside of the stability pH range of the DPPH reagent. This study found by mixing the DPPH reagent with both gallic acid and Trolox standards linear regression standard curves (R^2>0.99) could be obtained in 80% methanol, as well as HCl-acidified methanol (pH controlled with 75 mM potassium dihydrogen phosphate buffer). Identical DPPH stability curves were obtained for both 80% methanol and HCl-methanol/buffer conditions. This study found potassium dihydrogen phosphate buffer can be used to stabilise the pH for DPPH assay. A protocol was recommended to pre-mix the DPPH stock reagent with a buffer/aqueous-methanol mixture for spectrophotometry assay using the microplate reader.

Introduction

Wheat contains considerable amounts of antioxidants including phenolic acids, flavonoids, lignans, carotenoids, tocopherols and tocotrienols, with phenolics being the most predominant phytochemicals contributing towards the antioxidant activity (Yu, 2008a). Phenolics are presented in three forms, namely free soluble, conjugated soluble and conjugated insoluble (Manach *et al.*, 2004; Abdel-Aal *et al.*, 2012). Past studies indicated that the majority of phenolics are insoluble conjugated phenolics (Adom *et al.*, 2003; Gélinas & McKinnon, 2006). These are bound to the compounds in the cell wall structure through ester or ether linkages (Liu, 2007). Traditional extraction methods (such as Soxhlet) are only efficient for extracting free phenolics from cereals. These extraction methods, when used for determining phenolic content and antioxidant activity, do not result in the actual total antioxidant properties.

Several methods have been developed to overcome this shortcoming, for example enzymatic extraction, esterification and hydrolysis (Sancho *et al.*, 2001; Vaher *et al.*, 2010). These methods are time-consuming, tedious and often expensive. A hydrolytic method using HCl-acidified methanol (1% concentrated hydrochloric acid) was recommended by Beta *et al.* (2005) as an American Association of Cereal Chemists (AACC) method for wheat antioxidants determination. The wheat samples were extracted with HCl-acidified methanol (methanol/water/HCl, 80:10:1 v/v) to determine the total phenolic content and compared with DPPH scavenging activity where 80% methanol was used as an extraction solvent. This acidified method (80% methanol extract) cannot be used for the DPPH assay due to the DPPH free radical being only stable between the pH range of 5 to 5.6 (Blois, 1958). A DPPH radical can still maintain its function and be useful for some methods at lower pH (pH 3) (Koleva *et al.*, 2000), but the HCl-acidified methanol extract (1% HCl) resulted in a pH lower than 1. Using different extract solvents for different assays may be misinterpreted, especially when comparing two methods.

The DPPH radical was used for antioxidant measurements originally in the 1950s (Blois, 1958). However, the appropriate method using this reagent to evaluate the antioxidant scavenging capacity involving spectrophotometry was first published by Brand-Williams *et al.* (1995). This method involved ARP that was expressed as the amount of antioxidants required for reducing the initial DPPH free radical by 50% until it reached the steady state (EC_{50}). Since antioxidants differ in reaction speed to reduce the DPPH radical, the study by Sánchez-Moreno *et al.* (1998) further explored this phenomenon. They suggested that time should be taken into account when measuring antioxidant properties. Their assay was able to determine the ARE, expressed as $AE = 1/EC_{50}$ x T_{EC50}, where T_{EC50} is the time required to reach the steady state (50% of the initial DPPH) (Brand-Williams *et al.*, 1995).

DPPH free radicals are relatively stable, yet its stability can be influenced by different factors, for example the exposure to light, pH and type of solvents used (Ozcelik *et al.*, 2003). Buffers are commonly used for conditioning the pH in a solution. However, there is no literature describing the use of buffers to condition the pH of extract containing acidified organic solvents for DPPH assay.

Therefore, this study firstly aimed to determine the stability of DPPH using a potassium diphosphate buffer system to balance the HCl-acidified methanol solvent. The stability of the reagent under potassium phosphate buffer conditions must be verified first as being similar to that of the normal methanol extract, for further studies to continue.

A more recent study suggested that 25 to 70 µM DPPH is needed to ensure the spectrophotometric accuracy in order to achieve an absorbance of 0.220 to 0.698 (Sharma & Bhat, 2009). In practice, some laboratories would prepare approximately ten times more concentrated DPPH stock solution and then dilute it for further use. DPPH dissolves in high concentration of organic solvent (e.g. absolute methanol) because of its low polarity. This may lead to very little, but detectable amounts of precipitation when mixing the acidified extracts with the DPPH reagent during the assay. The products of the reactions are hydrochloric acid and potassium diphosphate buffer salts.

Although the current scientific tools available are reliable and accurate (such as pipettes and tips), the DPPH reagent may still differ with respect to their absorbance values as they are highly sensitive towards the respective pH values (Ozcelik *et al.*, 2003). Small differences are present due to buffers being transferred individually into each well of the 96-well plates, which may cause variation in pH values between the wells. It is thus suggested that a buffer/methanol (80% v/v) mixture should be used as a dilution solvent when preparing DPPH stock reagent.

Once the stability of the reagent under potassium phosphate buffer conditions has been verified, this study aimed to develop a workable and reliable method when using the potassium phosphate buffer in the DPPH assay, along with HCl-acidified methanol as an extraction solvent.

Materials and methods

Solvent reagents and standards preparation

Acidified methanol extract solvent

For the preparation of the HCl-acidified methanol extract solvent, absolute methanol (Sigma-Aldrich, Kempton Park, South Africa), distilled water and concentrate hydrochloric acid (Kimix Chemicals, Airport Industria, Cape Town) were mixed in the ratio of 80:10:1 v/v. The prepared solvent was stored in an airtight Schott bottle at room temperature for up to 10 days.

Potassium dihydrogen phosphate buffered methanol

A Cyberscan 1000 pH meter (Eutech Instruments, Stanger, South Africa) was used for pH measurements, and calibrated twice a day with calibration buffer pH 7 and pH 4 (Merck, Modderfontein, South Africa).

A 75 mM potassium dihydrogen phosphate solution (Scienceworld, Parow Industria, South Africa,) was prepared by dissolving its salt into distilled water.

a. For the stability survey, the solution was adjusted to pH 11.25 with 2 M potassium hydroxide (Scienceworld, Parow Industria, South Africa), followed by mixing with absolute methanol in a ratio of 52:48 v/v (achieved pH 11.74).

b. For the method development, the solution was adjusted to pH 7.57 with the 2 M potassium hydroxide, followed by mixing with absolute methanol (Sigma-Aldrich, Kempton Park, South Africa) in a ratio of 20:80 v/v (achieved pH 9.62).

<u>2,2-Diphenyl-1-picrylhydrazyl (DPPH) reagent</u>

A solution of 0.1 mg/mL of DPPH reagent (Sigma-Aldrich, Kempton Park, South Africa) was prepared by dissolving DPPH into absolute methanol using a volumetric flask.

a. For the stability survey, this reagent was further diluted with absolute methanol to reach an absorbance value of *ca.* 0.6 (DPPH reagent / absolute methanol, 37:63 v/v obtained absorbance of 0.62), when measured by the EON microplate spectrophotometer (BioTek, Winooski, USA).

b. For the method development, this stock solution was prepared by mixing 40 mL of DPPH stock with 60 mL of the buffered methanol (pH 9.62), this achieved a concentration of 101.4 µM, with average absorbance of 0.65.

<u>Preparation of Trolox and gallic acid standards</u>

Trolox

a. For the stability survey, Trolox ((±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (Sigma-Aldrich, Kempton Park, South Africa) was dissolved in 80% v/v methanol to obtain a concentration of 1 mM. From this solution two series of standards were prepared. For the first series, 80% v/v methanol was used to make the eight different concentrations of standard solution between 0 and 200 µM. The second series of standards were prepared, using the mixture of 80% methanol, HCl-acidified methanol and buffered methanol (Table 3.1).

b. For the method development, Trolox was dissolved in the HCl-acidified methanol solvent to a concentration of 1 mM. This stock solution was further diluted with HCl-acidified methanol to prepare the 0 to 200 µM Trolox standard.

Table 3.1. Trolox standards preparation in HCl-acidified methanol buffer solution

1 mM Trolox (µL)	80% v/v Methanol (µL)	Acidified methanol (µL)	Buffered methanol (µL)	Conc. Trolox (µM)
0	500	500	1000	0
25	475	500	1000	12.5
50	450	500	1000	25
100	400	500	1000	50
150	350	500	1000	75
200	300	500	1000	100
300	200	500	1000	150
400	100	500	1000	200

Gallic acid

A solution of 1 mM gallic acid (Merck, Modderfontein, South Africa) was prepared with de-ionised water and eight different concentrations of the standard solution between 0 and 200 µM (Table 3.2). Two series of standards were prepared from the 1 mM gallic stock solution. For the first series, 80% v/v methanol was used to make the eight different concentrations of the standard solution between 0 and 200 µM. The second series with the same standards concentration were prepared, using the mixture of 80% v/v methanol, HCl-acidified methanol and buffered methanol (Table 3.2).

Table 3.2. Gallic acid standards preparation in HCl-acidified methanol buffer solution

1 mM Gallic acid solution (µL)	80% v/v methanol (µL)	Acidified methanol (µL)	Buffer (µL)	Conc. Gallic acid (µM)
0	500	500	1000	0
25	475	500	1000	12.5
50	450	500	1000	25
100	400	500	1000	50
150	350	500	1000	75
200	300	500	1000	100
300	200	500	1000	150
400	100	500	1000	200

DPPH stability test

Trolox and gallic acid were used as standard antioxidants for testing the stability of DPPH reagent when measuring the antioxidants in the samples. For all the standards series prepared (gallic acid in 80% v/v methanol, trolox in 80% methanol, gallic acid in HCl-acidified methanol with buffer, and trolox in HCl-acidified methanol with buffer), 20 µL of the prepared standard series solution were mixed with 180 µL DPPH reagent in to a flat bottom 96 well microplate plate. Absorbance at 515 nm was measured in triplicate, every minute for the first 20 min, followed by every 5 min for the next 20 min, and ended by absorbance readings every 10 min for the last 30 min of the cycle. Readings were done using an EON microplate spectrophotometer.

DPPH assay development for the buffered HCl-acidified methanol extract

Wheat extract preparation

One soft wheat (PAN3161) and one hard wheat (PAN3379) cultivar were selected for this study. Approximately 30 g of wheat was milled using a Perten LM 3100 (Hägersten, Sweden) mill fitted with a sieve size 0.5 mm. One gram (± 0.005 g) milled samples was placed in 50 mL Corning tubes and mixed with 10 mL HCl-acidified methanol (methanol/distilled water/HCl, 80:10:1 v/v). This was followed by sonication at 40 kHz for 30 min at room temperature using a Scientech ultrasonic cleaner (Labotech, Pinelands, South Africa). The extracts obtained were centrifuged for 10 min at 3000 X G

(TJ-25 Centrifuge, Beckman Coulter, South Kraemer Boulevard, US), extraction was done in triplicate. The extracts were collected in 2 mL cryotubes and stored at -18°C.

<u>Absorbance repeatability</u>

Duplicate mixtures were prepared by mixing the wheat extract and buffered DPPH reagent in a glass beaker at a ratio of 1:9, the pH value of the mixture was measured with the Cyberscan 1000 pH meter. For each prepared Trolox standard, 30 µL was pipetted into 96 deep well plates, followed by 270 µL buffered DPPH reagent and sealed with a rubber mat to prevent evaporation. After 1 h of incubation at room temperature and shaking, 200 µL of the mixture was carefully transferred into a flat bottom 96 well microplate using a Gilson multichannel pipette (Lasec, Johannesburg, South Africa). Absorbance at 515 nm was measured in triplicate for the first two extracts, and in duplicate for the third extract, using an EON microplate spectrophotometer. This enabled simultaneous analysis of all samples in a 96 well plate which was more practical and expected to result in more reproducible measurements (Rascio et al., 2015).

Statistical analysis

Absorbance data was analysed using the Gen5 v2.05 software (BioTek, Winooski, US), as well as Microsoft Excel 2013 (Microsoft Corporation, Seattle, WA) to construct linear regression curves.

Results and discussion

Comparison between extraction solvents (80% methanol and potassium phosphate buffered HCl-acidified methanol)

Gallic acid is commonly used for the construction of standard curves when determining the total phenolic content of samples (Singleton *et al.*, 1999). It is, however, important to test the linearity of the standard curve created under the buffered HCl-acidified methanol conditions. Trolox is an antioxidant commonly used to create the standard curve in methods such as DPPH and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{•+}$) scavenging assays (Brand-Williams *et al.*, 1995; Re *et al.*, 1999b). It acts as an index for antioxidant activity.

Gallic acid is an antioxidant which is responsible for decolourising the DPPH radicals by reducing them to their non-radical form (Yu, 2008f). The remaining DPPH radicals which had not been reduced by gallic acid were measured at 515 nm and are reflected as absorbance values. The linear regression curve had an R^2 value of 0.92 (Fig 3.1a), which indicates a low predictability. Furthermore, it was observed that at high concentration, the absorbance value was close to zero, the large amount of gallic acid was sufficient to reduce the majority of the DPPH radicals that led DPPH to appear colourless. This led to a reduction in the confidence of accuracy when including this absorbance value in the standard curve. Thus the high concentration of gallic acid (100 – 200 µM) is not ideal for creating a standard prediction curve. However, by selecting the data points up to 100 µM gallic acid (Fig. 3.1b), the standard curve showed high predictability (R^2 = 0.995).

The linear regression standard curve formula, normally expressed as y = ax + b. DPPH was tested with gallic acid standards in buffered HCl-acidified methanol extract. Its standard curve

achieved a R^2 value of 0.997, with similar matrix (b_1 = 0.5271, b_2 = 0.5367) and vector (a_1 = -0.0033, a_2 = -0.0033) values for the standard curve formula compared to gallic acid in 80% methanol (Fig. 3.1b & Fig. 3.2). No considerable difference was observed in reliability between the two types of extract solutions used to prepare the standard curve.
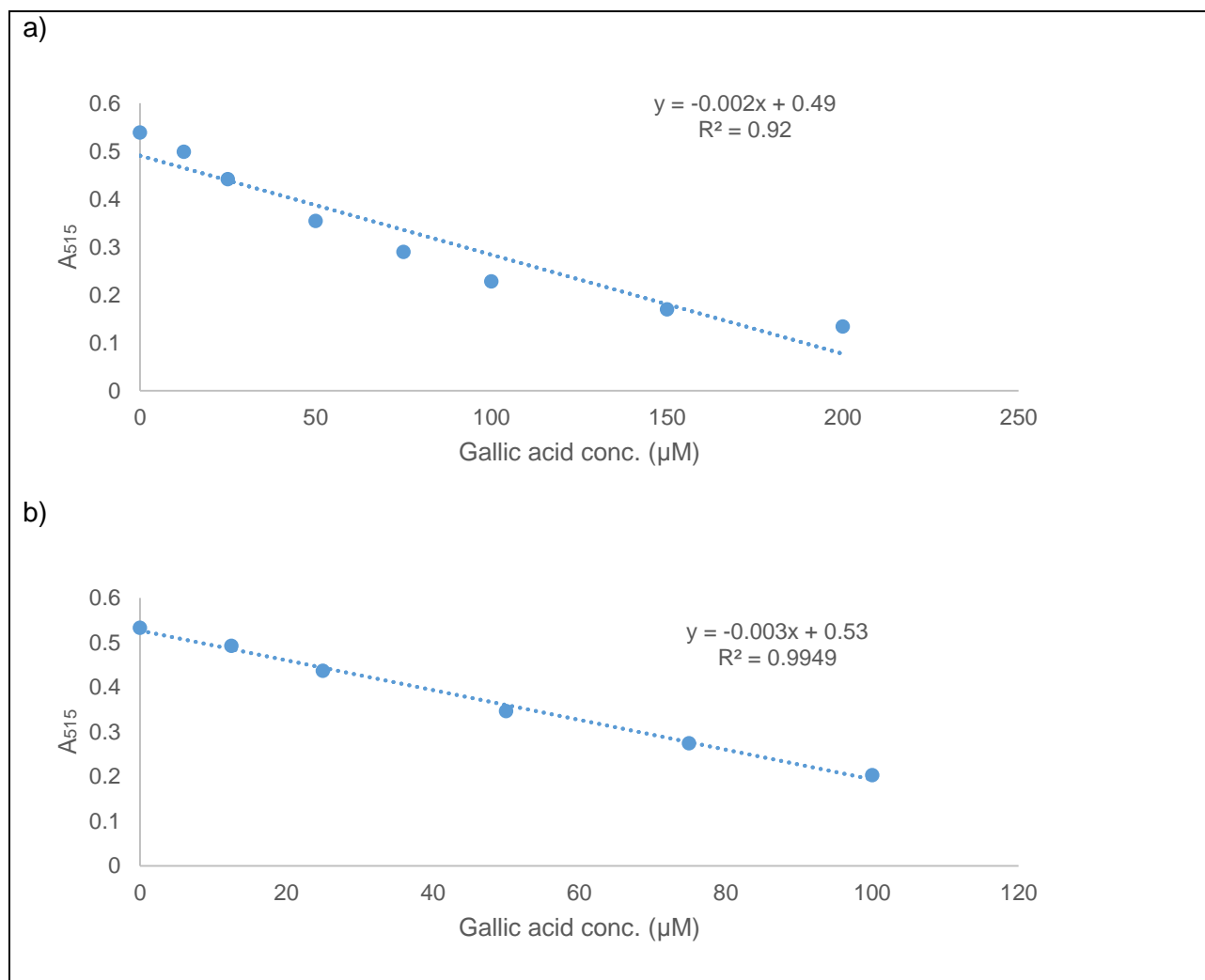
Figure 3.1. Absorbance values (515 nm) of remaining DPPH radical at various gallic acid concentrations in 80% methanol after 30 min incubation with (a) a full standard curve obtained from 0 to 200 μM gallic acid concentration, and (b) a standard curve obtained from a lower concentration of gallic acid vs DPPH reagent (0-100 μM).

The Trolox standard in 80% methanol reacted with DPPH and resulted in a great linear correlation between absorbance and Trolox concentration, and achieved R^2 = 0.996. The same Trolox concentration in buffered HCl-acidified methanol extract solution showed a good prediction in the standard curve with a slightly lower R^2 value (0.99). The vector and matrix values were quite similar between the two types of extraction solvents (Fig. 3.3).
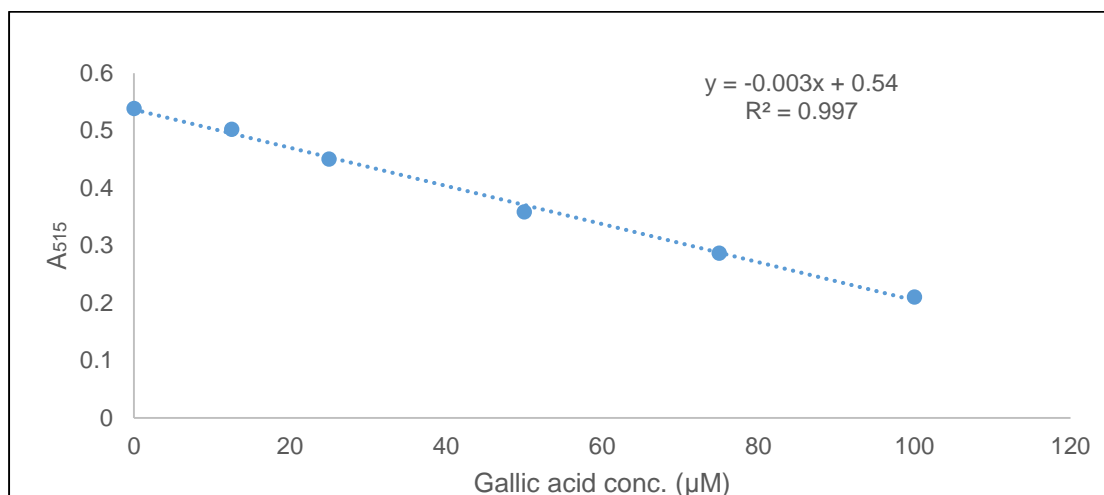
Figure 3.2. Absorbance values measured at 515 nm of the remaining DPPH radical at various gallic acid concentrations in buffered HCl-acidified methanol after 30 min incubation time. The standard curve obtained from the lower concentration of gallic acid (0-100 µM) was used.

The use of potassium phosphate for buffering the HCl-acidified methanol solvent showed high similarity compared to 80% methanol solvent for both gallic acid and Trolox as standard antioxidants to create the standard curve. Since gallic acid is one of the commonly found phenolic acids, this experiment confirmed that potassium phosphate buffer can be used to control the pH in the DPPH assay for cereals.
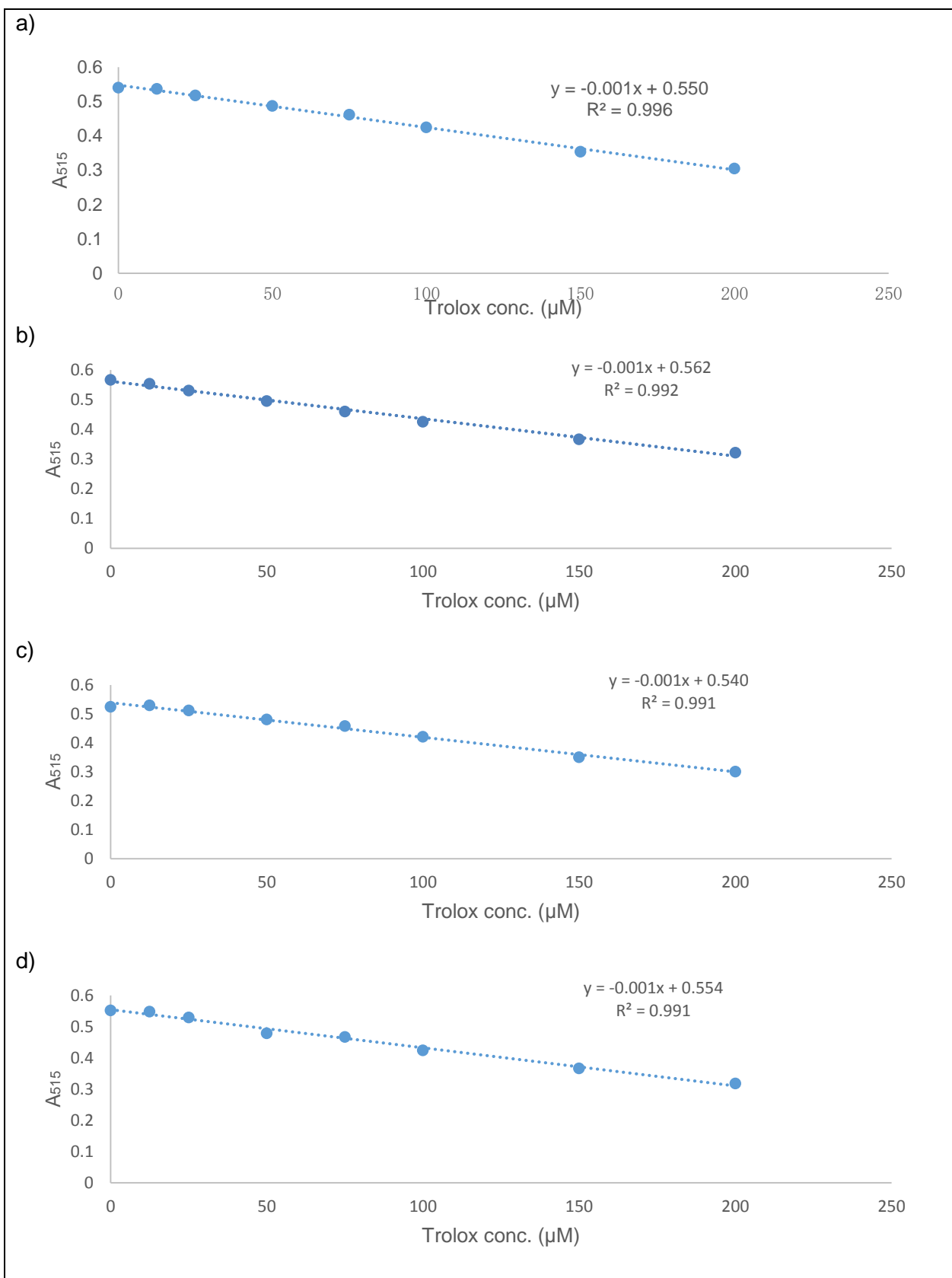
Figure 3.3. Absorbance at 515 nm of remaining DPPH react with Trolox standards after (a) 30 min incubation in 80% methanol, (b) 30 min in buffered HCl-acidified methanol, (c) 60 min in 80% methanol, and (d) 60 min in buffered HCl-acidified methanol.

DPPH stability under potassium phosphate buffered HCl-acidified methanol

The mixture of the potassium phosphate buffered methanol, gallic acid / Trolox in HCl-acidified methanol solution and DPPH reagent in absolute methanol resulted in a pH range of 5.25 to 6.28. This was within the stable pH range for DPPH (pH 5 to 6.5) (Koleva *et al.*, 2000). Eighty percent methanol is a commonly used solvent for antioxidant extraction from cereals (Zieliński & Kozłowska, 2000; Lahouar *et al.*, 2014). The kinetics of DPPH degradation was analysed by the reacting gallic acid in 80% methanol. The difference of the DPPH stability between the two could be measured by comparing the latter data obtained with gallic acid in buffered HCl-acidified methanol (Fig. 3.4). In the 80% methanol solvent, the absorbance of DPPH with a blank sample (0 µM gallic acid) reduced from 0.546 to 0.521 after 60 min incubation, whereas the absorbance at buffered HCl-acidified methanol showed a slight increase from 0.525 to 0.548. A rapid decrease in absorbance was observed after 1 min, with little change up to 60 min (Fig. 3.4). This indicated that the DPPH radical could be neutralised instantly by gallic acid, and that the residue of DPPH radicals remained stable for both types of solvents (as no considerable difference was observed between the two). Although Brand-Williams *et al.* (1995) classified gallic acid as a slow kinetic behaviour in reaction to DPPH, their study also found it was high in ARP. They explained that the high ARE was due to its triphenol structure. Generally, DPPH shows slightly better stability when reacting with gallic acid at buffered methanol extract solvent than 80% methanol.
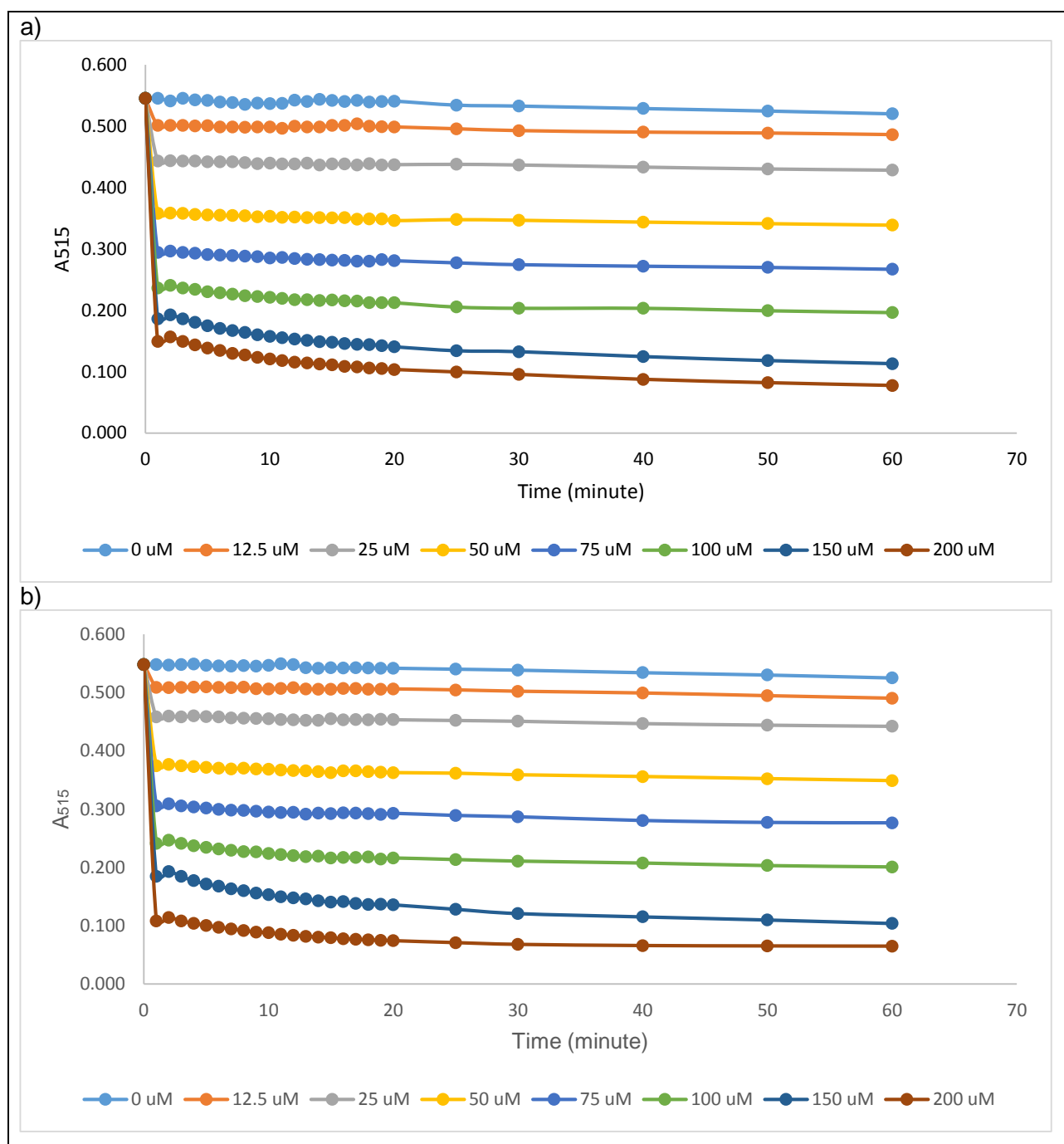
Figure 3.4. DPPH stability curves under various concentration of gallic acid in (a) 80% methanol and (b) buffered HCl-acidified methanol extract solvent. The absorbance values were taken at 515 nm, following the Lambert-Beer's law.

DPPH kinetic test with Trolox showed a similar kinetic trend as with gallic acid. The rapid decrease of the absorbance values at all the concentrations of Trolox standards indicated that the majority of the DPPH radicals could be rapidly reduced by Trolox. Trolox in the buffered HCl-acidified methanol solvent had shown a slight fluctuation compared to Trolox in 80% methanol. However, the high predictability ($R^2 > 0.99$) of the standard curves generated at all the time points presented strong evidence that the fluctuation was within the acceptable range. The stability curve (Fig. 3.4a) revealed Trolox in 80% methanol maintained the greatest stability throughout all the measured time points (1

to 60 min), whereas the Trolox in buffered HCl-acidified methanol solution maintained its absorbance stability until 50 min (Fig. 3.4b). Nevertheless, the Trolox in buffered HCl-acidified methanol extract solvent appeared to be better separated at low concentration (0–25 µM Trolox standard).
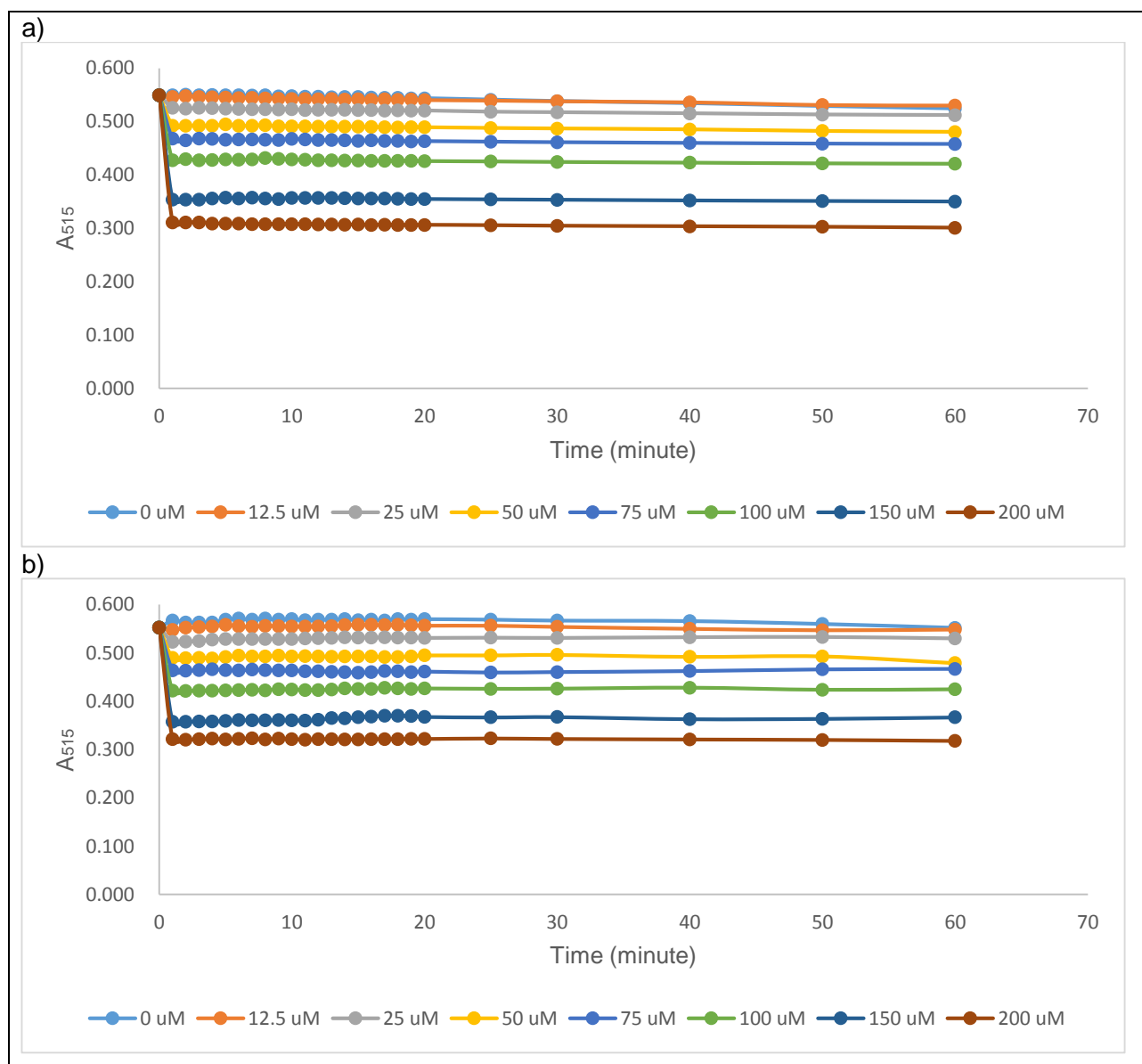
Figure 3.5. DPPH stability curves under various concentration of Trolox in (a) 80% methanol and (b) buffered HCl-acidified methanol extract solvent. Absorbance values were taken at 515 nm, following the Lambert-Beer's law.

In general, DPPH was less affected when reacting with gallic acid under buffered HCl-acidified methanol extract solvent than when reacting with Trolox. However, DPPH reagent in both antioxidant standards appeared to have good stability under the testing conditions.

DPPH assay development: reproducibility and reliability

Deep well plates were used in case of precipitation occurring during the reaction. The 1 h incubation allowed antioxidants in wheat to have enough time to react with DPPH reagent, although a preliminary study showed absorbance values changed slightly after 30 min incubation (results not

shown). The absorbance values obtained from 8 measurements of each sample had achieved the coefficient of variation of 2.896% and 2.776% (Table 3.3), which indicated good reproducibility under the buffered condition for HCl-acidified methanol extract (Mpofu *et al.*, 2006).

Table 3.3 Statistic analysis of absorbance of wheat extracts under buffered HCl-acidified methanol environment

Wheat	Absorbance range	Number of measurements	Mean & standard deviation	CV (%)
PAN3161 (soft wheat)	0.47-0.52	8	0.495 ±0.014	2.896
PAN3379 (hard wheat)	0.46-0.50	8	0.483 ±0.013	2.776

The Trolox standards were prepared in the same reagent as wheat extract solvent, which is commonly used as a reference to predict the antioxidant property in DPPH assay. However, the linear regression (R^2 value = 0.9943) (Fig. 3.6) also gave an indication of how DPPH reagent and the antioxidant behaved under the specific condition (potassium phosphate buffer for conditioning the HCl-acidified methanol extract).

$$y = -0.0009x + 0.5767$$
$$R^2 = 0.9943$$
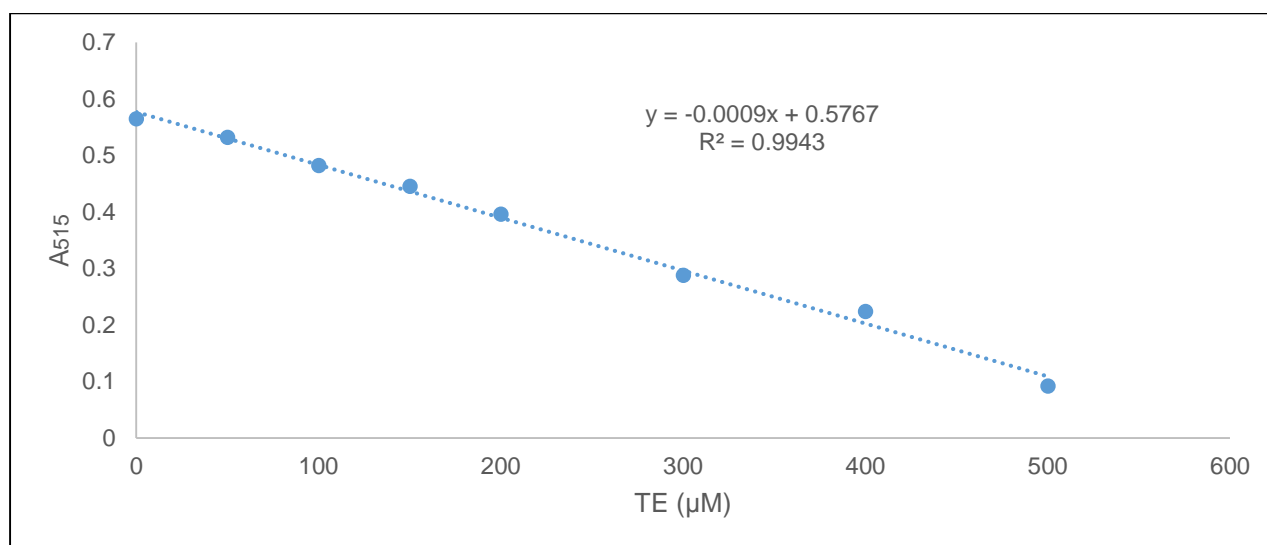
Figure 3.6. Trolox standard curve achieved at 515 nm under buffered condition for HCl-acidified methanol extract in DPPH assay.

Recommended protocol based on this study

Reagent preparation:

1) Prepare the HCl-acidified methanol as the extract solvent by mixing absolute methanol, water and hydrochloric acid (10.2 M) at a ratio of 80:10:1 v/v

2) Prepare a 75 mM potassium phosphate buffer adjusted to pH 7.4 - 7.6 with 2M potassium hydroxide solution

3) Prepare buffer-methanol solution by adding 200 mL prepared buffer (2) into 800 mL methanol

4) Prepare stock DPPH solution with absolute methanol at concentration of 0.1 mg/mL. Dilute this stock DPPH solution with prepared buffer-methanol solution (3) at ratio of 40:60 v/v

5) Prepare 1 mM Trolox stock standard by dissolving 0.01251 g Trolox directly into a 50 mL volumetric flask, fill up to the mark with HCl-acidified methanol (1). Prepare the standard series (0 - 500 µM) by diluting with HCl-acidified methanol (1)

Procedure:

1. Mix 1 g milled sample with extraction solvent (1), sonicate at room temperature at frequency of 40 kHz for 30 min, centrifuge at 3000 X G for 10 min

2. Transfer 30 µL of prepared standards (5) and sample (6) into 96 deep-well plate

3. Add 270 µL of DPPH reagent (4) into the same 96 deep-well plate, seal with rubber mat or parafilm, incubate for 60 min at room temperature in a dark chamber

4. After incubation, carefully transfer 200 µL into clear-bottom 96 well micro-plate

5. Shake for a few seconds, followed by measuring the absorbance values at 515 nm using a microplate spectrophotometer

Conclusion

This study confirmed the use of potassium dihydrogen phosphate buffer to control the pH of HCl-acidified methanol as an extraction solution. Standard curves generated from both Trolox and gallic acid standards showed high similarity between 80% methanol and HCl-acidified methanol with the buffer. Stability tests indicated that the DPPH assay can be used when the acidified extract were conditioned with potassium dihydrogen phosphate buffer for up to 50 min. The assay was also confirmed to be reliable for antioxidant activity determination for hard and soft wheat cultivars. Therefore, the same extract for total phenolic contents determination can be used in DPPH free radical scavenging assays, as a correlation between the two sets of data is now possible.

In practice, a buffer-methanol mixture is recommended to be used as a dilution solvent for adjusting the DPPH reagent concentration. This new method is highly reliability and recommended to be tested for determining the total antioxidant properties of other cereal products.

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Chapter 4

Antioxidant properties of South African wheat cultivars

Abstract

South Africa produce close to two million tons of wheat annually during the last 10 years. The antioxidant content of wheat had been extensively studied world wide, however the actual antioxidant properties of South African wheat has to date not been investigated.

This chapter studied the antioxidant properties of 26 South African wheat cultivars from three different regions. Additionally, the correlation between wheat kernel hardness and antioxidant properties were investigated. Acidified extraction solvent (HCl-methanol) along with assisted sonication extraction method was used to increase the efficiency of extracting the antioxidants from wheat samples. This required determination of the most efficient and practical extraction time. Wheat planted under irrigation were found to have higher antioxidant properties than climate dependent regions. Strong evidence was found that softer wheat had higher antioxidant properties. The study suggested samples from more seasons should be collected to statistically confirm the genotype and environment effect on antioxidant properties of South African wheat. It was also advised to be attentive of the effect of environmental stress, particularly water stress on antioxidant activity of wheat.

Introduction

Wheat is an important staple food globally and is being used as a main ingredient in various types of foods. In South Africa, a yearly average of 1.89 million tons of wheat has been produced over the past ten years (SAGL, 2015). In order to meet the demand, South Africa had to import approximately 1.3 million tons of wheat from Russia, and other countries in the 2014/2015 season (SAGL, 2015).

Wheat is a source of carbohydrates and protein (gluten), as well as minerals and several micro-nutrients (Lopez *et al.*, 2003). Whole wheat contains higher amounts of dietary fiber and phytochemicals than processed wheat, therefore the consumption of whole wheat is considered to provide many health benefits (Slavin, 2000). It is commonly agreed that wheat contains considerable amounts of antioxidants, with phenolic acids being the most predominant component contributing to the antioxidant properties of wheat (Kim *et al.*, 2006; Anson *et al.*, 2011). Other phytochemicals, such as flavonoids, carotenoids and anthocyanins were also found, but in lesser amounts (Okarter *et al.*, 2010). It is believed that the consumption of natural antioxidants reduce the risk of many chronic diseases, such as cardiovascular diseases, type II diabetes, as well as cancer (Okarter & Liu, 2010).

The antioxidant content of wheat varies between genotypes, as well as their growing environment (Mpofu *et al.*, 2006). Genotype and environmental effects have been extensively studied, particularly in Canada, USA, China and several European countries (Gélinas & McKinnon, 2006). Many studies found antioxidant properties to be more affected by the growing environment than that of genotype (Mpofu *et al.*, 2006; Lv *et al.*, 2013; Lu *et al.*, 2015). Another finding was that the antioxidant content amongst different cultivars were also region specific (Yu *et al.*, 2003). However, nothing regarding South African wheats' antioxidant properties were found in the literature. In this current study, the antioxidant properties of wheat cultivars planted in different regions in South Africa are presented. Before analysing the samples, the optimal extraction time using acidified methanol solution as extraction solvent, combined with sonication, was determined.

Materials and methods

Wheat samples

Twenty six cultivars of South African bread wheat, originating from 2012 harvest season's wheat evaluation trials, were kindly supplied by Sensako Pty (Ltd) (Bethlehem, South Africa). These cultivars came from three regions and different localities within the regions (Table 4.1). Samples were planted in a randomised complete block design, with three replicates each, although only one replicate was used in this study. Before milling, the samples were stored in sealed containers at ambient temperature.

Table 4.1 Wheat samples grown at different locations in the Western Cape, Free State and under irrigation

Region	Locality	Cultivars
Western Cape	Napier	Kariega
	Riversdal	PAN 3434
	Klipheuwel	Ratel
	Moorreesburg	Baviaans
		SST 015
		SST 096
		SST 056
		SST 087
		SST 088
Under irrigation	Hartsvallei	SST 806
	Lichtenburg	Duzi
	Marble Hall	Baviaans
	Winterton	Buffels
		PAN 3471
		SST 835
		Olifants
		PAN 3478
		SST 875
Free State	Bethlehem	Elands
	Bultfontein	PAN 3161
	Clocolan	PAN 3144
		Gariep
		SST 398
		PAN 3355
		SST 347
		SST 356
		SST3379

Methods

<u>Extraction: time determination</u>

To determine the appropriate extraction time for the sample set, one soft wheat (Kariega) and one hard wheat (SST 096) were selected. Approximately 30 g of each sample was milled, using a hammer type cyclone Laboratory Mill LM 3100 (Perten, Hägersten, Sweden) fitted with a 0.5 mm sieve. Milled wheat (1 ± 0.005 g) was placed in 50 mL Corning tubes and mixed with 10 mL acidified HCl–methanol (methanol/H_2O/HCl, 80:10:1 v/v) (HCl: Kimix Chemicals, Cape Town, South Africa;

methanol: Sigma–Aldrich, Kempton Park, South Africa). The samples were sonicated at room temperature using a Scientech ultrasonic cleaner (Labotech, Pinelands, South Africa). The sonication was done at 40 kHz for 30, 60, 90 and 120 min (respective extraction times). Thereafter, the samples were centrifuged for 10 min at 3000 RCF (TJ–25 Centrifuge, Beckman Coulter, South Kraemer Boulevard, USA). Extractions were done in triplicate and the extracts were collected into 2 mL cryotubes and stored at -18°C. The total phenolic content was determined at each extraction (sonication) time.

Extraction: antioxidant determination

For the determination of the antioxidant properties of the entire sample set, a modified extraction method (Mpofu *et al.*, 2006), which includes the use of sonication, was used. As before, 30 g of each sample were milled, using a hammer type cyclone Laboratory Mill LM 3100 fitted with a 0.5 mm sieve. Milled wheat (1 ± 0.005 g) was placed in 50 mL corning tubes and mixed with 10 mL acidified HCl–methanol (methanol/H_2O/HCl, 80:10:1 v/v). To facilitate extraction, samples were sonicated at room temperature using a Scientech ultrasonic cleaner at 40 kHz for 30 min (as determined in previous experiment) . Thereafter, the samples were centrifuged for 10 min at 3000 RCF. Extractions were done in triplicate and the extracts were collected into 2 mL cryotubes and stored at -18°C.

Total phenolic content

TPC was determined using the Folin–Ciocalteu method (Singleton & Rossi, 1965b; Arthur *et al.*, 2011) on a 200 µL microplate scale. Extracts were acclimated to room temperature before being analysed. For the construction of a standard curve, a gallic acid (Merck, Modderfontein, South Africa) standard range (0–150 mg/L), consisting of eight standard solutions, was prepared by diluting gallic acid with de–ionised water. For the total phenolic content determination, Folin–Ciocalteu reagent (Merck, Modderfontein, South Africa) was diluted with de–ionised water in a ratio of 1:10, where after 10 µL extract was mixed with 90 µL of the diluted Folin–Ciocalteu reagent in a flat bottom 96 well microplate plate. This was followed by adding 100 µL sodium carbonate (7.5% m/v) (Sigma–Aldrich, Kempton Park, South Africa), using a multi–channel pipette (Gilson, Germany). After gentle shaking, the mixtures were incubated at 30°C in a dark room for 2 h. The absorbance values were measured using an Eon microplate spectrophotometer (BioTek, Winooski, USA) at 765 nm. Each extract was measured in triplicate, and one extract was selected for the correction of variation observed between experiments that was done on different days. Absorbance values were calculated as the TPC based on the standard curve constructed from the gallic acid standards. Thus, the TPC were expressed as gallic acid equivalent (mg GAE/g).

DPPH radical scavenging capacity

The DPPH radical scavenging assay was used to determine the antioxidant activity of the wheat samples. The assay was adapted from Brand-Williams *et al.* (1995) and Arthur *et al.* (2011) and the modification involved the application of potassium phosphate buffer, as discussed in Chapter 3. The

absorbance of the samples were compared with Trolox standards, and expressed as Trolox equivalent (µmol TE/g).

The potassium phosphate solution preparation involved the adjustment of 75 mM potassium phosphate to pH 7.57 with 2 M potassium hydroxide. One portion of this buffer was mixed with four portions of absolute methanol using a volume scale, thereby 80% methanol buffer mixture was obtained with pH 9.97.

DPPH stock solution was prepared to a concentration of 0.1 mg/mL, where after it was mixed with the 80% methanol buffer mixture at ratio of 40:60 v/v (*solution A*).

A Trolox stock solution (1 mM) was prepared by dissolving 0.0125 g Trolox in 50 mL acidified methanol in a volumetric flask. A standard range of 8 Trolox solutions (0–500 µM) was thereafter prepared, diluting the stock solution with acidified methanol.

The DPPH radical scavenging assay was done in a dark environment where 30 µL standard and sample extracts were pipetted into corresponding wells of a 96 deep–well plate, followed by adding 270 µL DPPH reagent (*solution A*). The plates were sealed with silicon sealing mats, and incubated in a dark room for 60 min at room temperature (20°C). After the incubation period, the absorbance was measured using an Eon microplate spectrophotometer at 515 nm. Each extract was measured in triplicate.

Determination of wheat hardness

The hardness index of the wheat samples was determined using the Single–Kernel Characterization System (Perten SKCS 4100, AES Agri–Enviro Solutions, Gauteng, South Africa) according to the AACC International Method 55–31.01 (AACC, 1999). Approximately 12 to16 g of wheat kernels were added to the access hopper of the instrument. The instrument automatically counted 300 kernels on which hardness index was measured and reported. Results were interpreted according to Table 4.2.

Table 4.2 Guidelines for Kernel texture (hardness index, HI)

Category	HI
Extra Hard	90+
Very Hard	81-90
Hard	65-80
Medium Hard	45-64
Medium Soft	35-44
Soft	25-34
Very Soft	10-24
Extra Soft	up to 10

Statistical analysis

GAE and TE values were calculated using the Gen5 v2.05 software (BioTek, Winooski, US) and Microsoft Excel 2013 (Microsoft Corporation, Seattle, WA). Mean differences for the GAE and TE values were evaluated by one–way analysis of variance (ANOVA) using STATISTICA version 12 (StatSoft, Inc., Tulsa, USA). Significant results were analysed by Fisher's Least Significant

Difference (LSD) test at a level of *P*<0.05 for the determination of significant differences in TPC and DPPH scavenging activity between genotypes and localities of the tested samples.

Results and discussion

Extraction time determination

The solvent used for extraction is an important factor when extracting antioxidants. As phenolic acids are the most abundant antioxidants in wheat (Ragaee, Seetharaman*, et al.*, 2012), organic solvents such as methanol, acetone and ethanol are normally used as extraction solvents. These solvents are also commonly mixed with water to adjust the polarity thereof (Zhou & Yu, 2004). Kim *et al.* (2006) found that acidified extraction solvents achieved a higher total phenolic extraction. This was specifically found when methanol was acidified with hydrochloric acid. Additionally, it was found that ultrasonic assisted extraction increased the efficiency and accessibility when extracting antioxidant compounds from cereals (Wang *et al.*, 2008). For this current study, a combination of acidified methanol and sonication was used, with the aim of improving the efficiency of extracting antioxidants from wheat. This combination required the optimal extraction time needed to be established first.

The Folin–Ciocalteu method was used to monitor the extraction efficiency of two wheat samples, differing in hardness. TPC was calculated from absorbance values based on a standard curve of prepared gallic acid standards (Fig. 4.1). The standard curve achieved a good prediction value (R^2=0.999), indicating a good reliability of the assay.
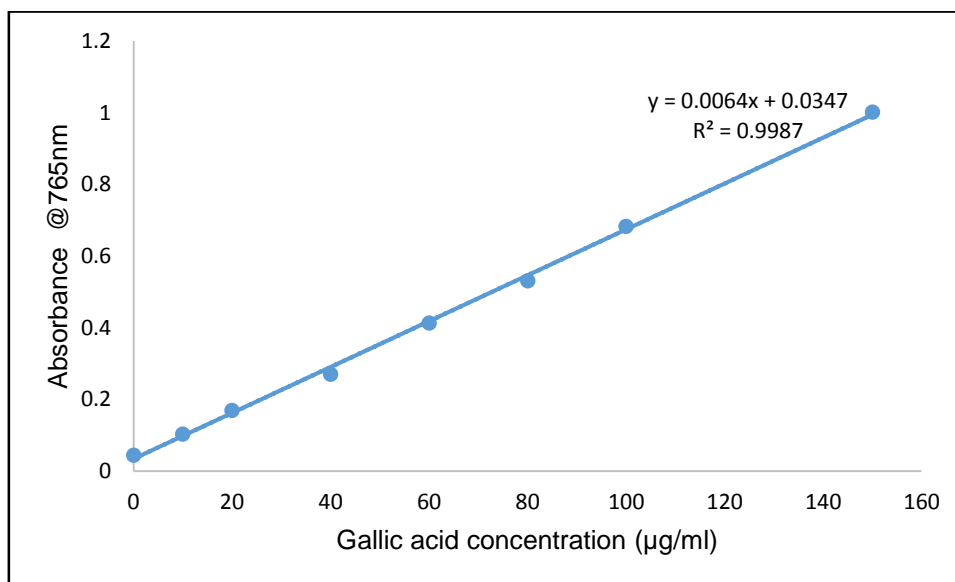
Figure 4.1. A standard curve constructed from gallic acid standard solutions.

No significant difference was observed between 30 and 60 min extraction times for both samples. At 30 min an extraction of 0.864 mg GAE/g was achieved for the soft wheat (Kariega) and 0.757 mg GAE/g for the hard wheat (SST096). A study on Canadian soft wheat by Moore *et al.* (2005) showed 0.4 to 0.8 mg GAE/g TPC using 50% acetone with a 24 h extraction period. Another study reported

TPC ranging from 0.109 to 0.145 mg/g extraction (based on ferulic acid equivalent), using 80% methanol when extracted for 30 min (Ragaee, Guzar, *et al.*, 2012). The shorter extraction time (30 min) used in this study seemed to have achieved better efficiency than the method used in the latter study. From 30 to 60 min extraction a gradual increase was observed as shown in Figure 4.2, however the difference was not significant (*P*<0.05). The TPC content significantly increased when the sample was sonicated for 90 min. At a sonication time above 100 min, more TPC was extracted from the hard than the soft wheat sample (Fig. 4.2).
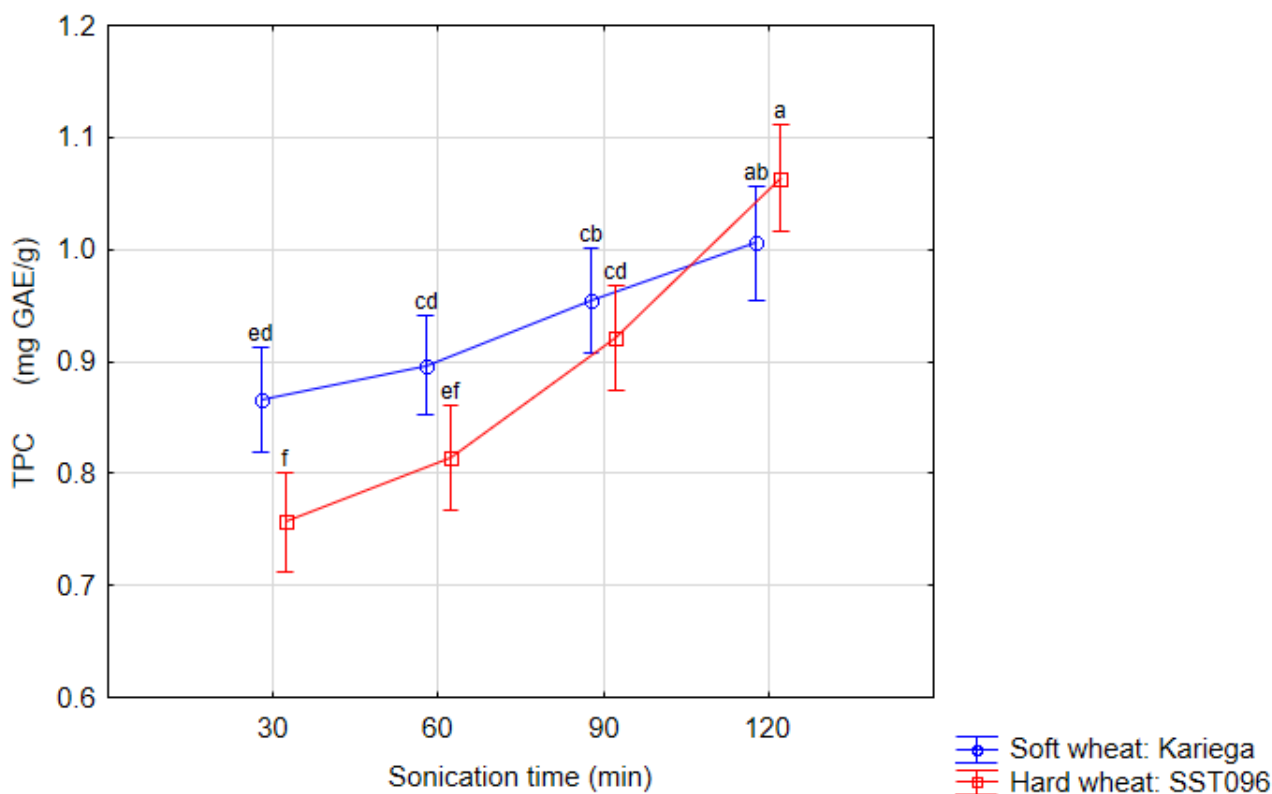
Figure 4.2. The extraction efficiency curve using ultrasonic assisted extraction method combined with acidified methanol (methanol/H_2O/HCl, 80:10:1 v/v). Vertical bars denote 95% confidence intervals. Different letter on top of the vertical bar indicate significant difference between the extraction times.

It should be noted that during the sonication, the ultrasonic energy is converted to thermal energy, whereby the temperature increased from 18°C to 48°C after 30 min of sonication. The temperature reached over 76°C after 60 min, and boiling temperature was reached after 120 min sonication. An earlier study (Sharma & Gujral, 2011) indicated some of the antioxidants, such as carotenoids and certain phenolic acids are thermal labile, and that some phenolic acids may lose their antioxidant properties more rapidly above 80°C. Furthermore literature suggested the extraction should be done below 70°C (Aramwit *et al.*, 2010; Dai & Mumper, 2010). The extraction time can thus influence the results significantly, depending on the temperature. Therefore, although longer sonication time showed significant increase in extraction efficiency of the phenolic compounds, the extraction temperature should be kept below the sensitive range to prevent the rapid decomposition of antioxidants. As extraction for 60 min did not result in significantly higher TPC, an extraction time of

30min was considered adequate and used in this study. An earlier study on optimization of ultrasound–assisted extraction of phenolic compounds from wheat bran, suggested 25 min extraction using sonication (Wang *et al.*, 2008)

Total phenolic content

As phenolic compounds are the major antioxidants in wheat, the TPC provides valuable information about the antioxidant properties of the samples. However, they can be either presented in wheat as free phenolics or bound phenolics with the bound form dominating (Vaher *et al.*, 2010). Gallic acid and ferulic acid are commonly used as standard phenolic acids for constructing a standard curve as reference. Results are thus often expressed as GAE or ferulic acid equivalent (FAE) (Laus *et al.*, 2012; Ragaee, Guzar*, et al.*, 2012).

All the standard curves achieved R^2–values above 0.984. The phenolic contents of all the cultivars ranged from 0.63–0.85 mg GAE/g across all regions (Appendix 4.1). Result obtained for Kariega (0.79 mg GAE/g), Olifants (0.83) and PAN3161 (0.76 mg GAE/g) showed the highest TPC in the Western Cape, irrigation and Free State regions, respectively. The lowest TPC of 0.604 (SST088), 0.581 (Duzi), and 0.654 (Gariep) mg GAE/g were observed in the Western Cape, irrigation and Free State, respectively. In comparison, Kim *et al.* (2006) determined free, acid hydrolysable and total phenolic content of Canadian wheat brans using 80% methanol, pH 2 HCl–methanol and 2M NaOH. They reported wheat bran contained 0.19–0.34 mg GAE/g free phenolics, 0.65–1.07 mg GAE/g acid–hydrolysable phenolics and 3.3–3.8 mg GAE/g of the TPC. On the other hand, Okarter *et al.* (2010) reported 1.43–1.89 mg GAE/g of the total phenolic acids content, which were extracted using 2M NaOH. It should be noted that the extraction method differed and that may be a reason for causing the difference in TPC. The present study obtained similar TPC to that of the acid–hydrolysable phenolic acids study on Canadian wheat by Kim *et al.* (2006).

There was no significant difference in TPC between cultivars for all the experimental regions (Fig. 4.3). The TPC was, however, found to vary significantly between planting localities (Fig. 4.4), which indicated the environment affects the TPC content. In the Western Cape, wheat grown in Riversdal contained higher TPC than wheat from the other three localities. Wheat that was grown in Hartsvallei and Marble Hall showed significantly higher TPC compared to that grown in Lichtenburg. For the Free State region, wheat that was grown in Bethlehem obtained significantly higher TPC than the other two localities.
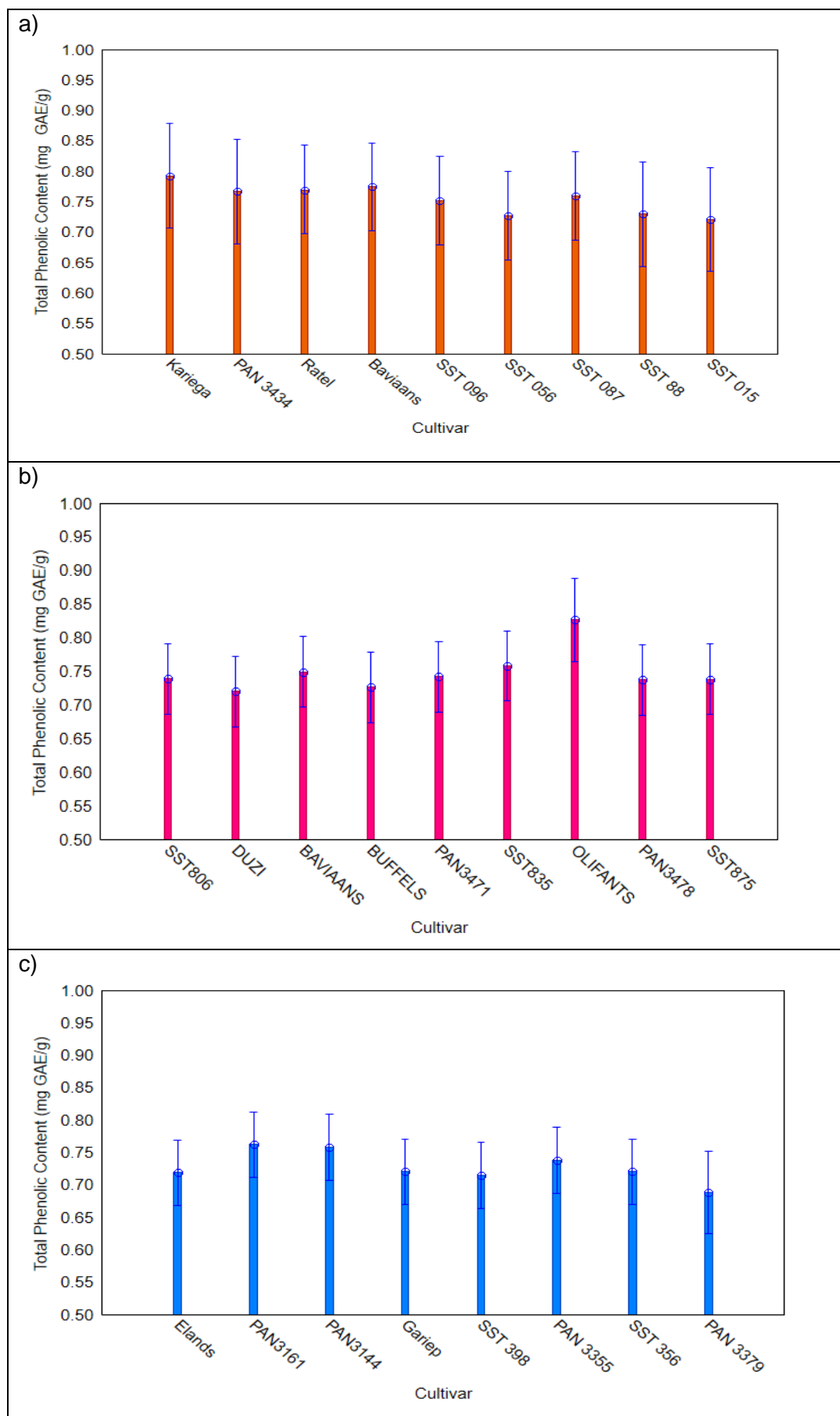
Figure 4.3. Fisher's LSD test of TPC between wheat cultivars on a). Western Cape region (*P*>0.05), b). Irrigation region (*P*>0.05), and c). Free State region (*P*>0.05). Vertical bars denote 95% confidence interval. No significant difference were observed between cultivars.

In an earlier study, Kim *et al.* (2006) found TPC to differ significantly between cultivars. In particular, bran from red wheat cultivars were found to contain higher amounts of TPC than that from white wheat cultivars (Kim *et al.*, 2006), and a highly significant difference was found among six western Canadian wheat genotypes (Mpofu *et al.*, 2006). However, the location had more influence on the TPC than genotypes. A similar phenomenon was observed by Mpofu *et al.* (2006), Yu *et al.* (2003) and Moore *et al.* (2005), although contradicting results were found by Ragaee, Guzar*, et al.* (2012).
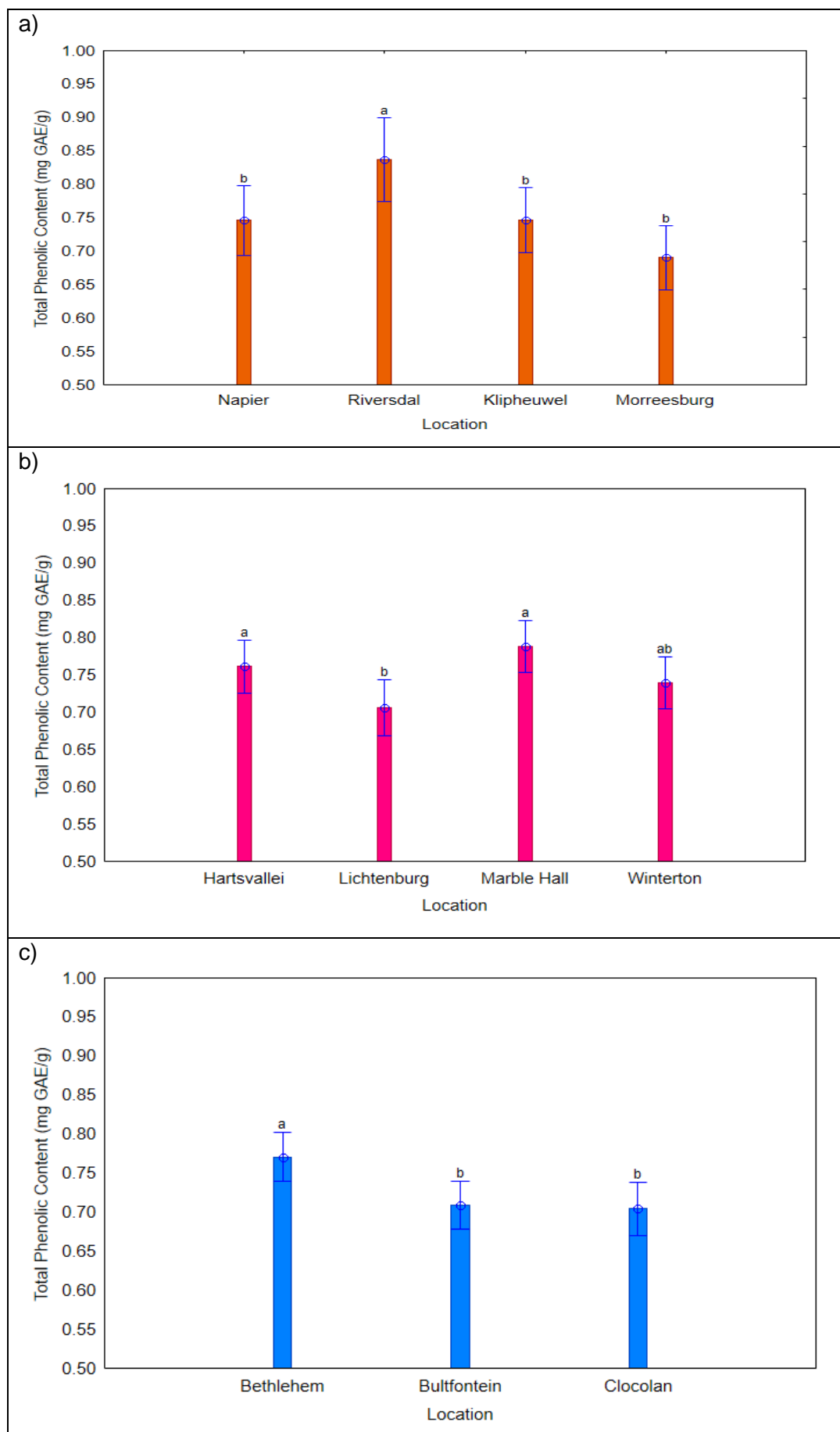
Figure 4.4. Fisher's LSD test of TPC between locations on a) Western Cape (*P*<0.05), b) Irrigation (*P*<0.05), and c) Free State regions (*P*<0.05). Vertical bars denote 95% confidence interval.

DPPH radical scavenging capacity

The DPPH radical scavenging capacity is an indication of the ability of a sample to donate an electron or hydrogen atom from antioxidants in order to reduce the DPPH radicals (Brand-Williams *et al.*, 1995). EC_{50} (amount of tested compound required to reduce the initial DPPH$^{\bullet}$ concentration by 50% at a steady–state) is a term that is used to express the DPPH scavenging activity. However, due to discrepancies found in literature regarding the EC_{50} method (Yu, 2008f), the results were expressed as Trolox antioxidant equivalent capacity (TEAC, μmol TE/g) in the current study.

The DPPH scavenging capacity ranged from 0.891 to 1.691 μmol TE/g. In the Western Cape region, SST88 had the highest DPPH scavenging capacity of 1.430 μmol TE/g, whereas SST056 had the lowest DPPH scavenging capacity of 1.037 μmol TE/g. Olifants had the highest DPPH scavenging capacity with 1.691 μmol TE/g and the cultivar PAN3478 the lowest TEAC value of 1.422 μmol TE/g in the irrigated region. In the Free State region, PAN3144 was the cultivar that had the highest DPPH radical scavenging capacity of 1.016 μmol TE/g, where as PAN3379 had the lowest DPPH scavenging capacity of 0.891 μmol TE/g.

The DPPH radical scavenging capacity of the wheat cultivars within each region was not significantly different (Figure 4.5). However, this study is not statistically adequate to compare the DPPH radical scavenging capacity of the different cultivars across regions.
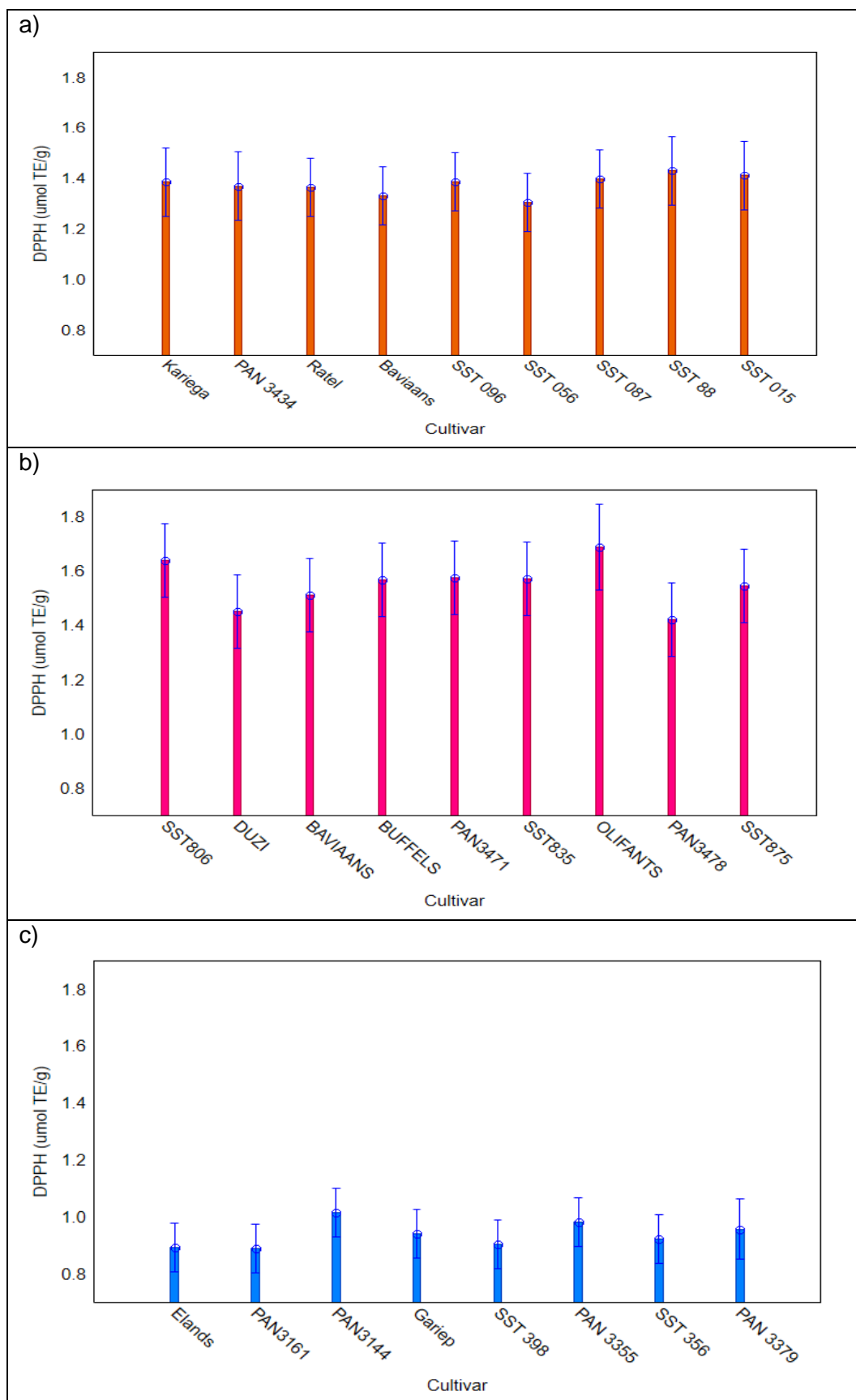
Figure 4.5. Fisher's LSD test of DPPH radical scavenging capacity between wheat cultivars on a). Western Cape region (*P*>0.05), b). Irrigation region (*P*>0.05), and c). Free State region (*P*>0.05). Vertical bars denote 95% confidence interval. No significant difference were observed between cultivars.

Wheat from Lichtenburg had a higher average DPPH radical scavenging capacity than wheat from Marble Hall (Fig. 4.6). However, there was not much of a difference in the DPPH radical scavenging capacity compared to wheat from Hartsvallei. Wheat from Hartsvallei had higher DPPH radical scavenging capacity, although not significantly higher than wheat from Marble Hall. In the Free State region, wheat that grew in Bultfontein had the highest DPPH ($P<0.05$) radical scavenging capacity of 0.93 µmol TE/g, followed by Bethlehem and Clocolan ($P<0.05$).
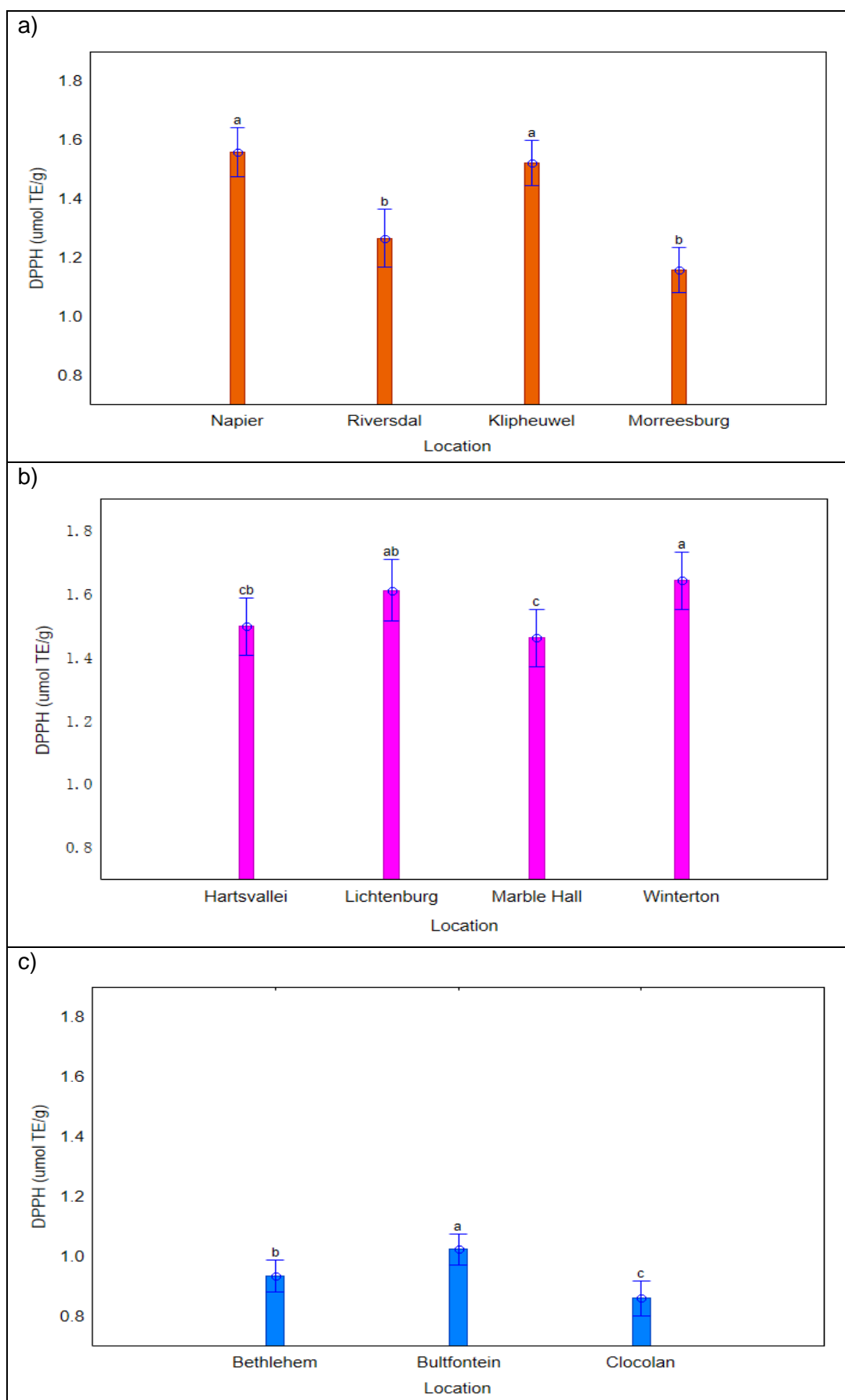
Figure 4.6. Fisher's LSD test of DPPH scavenging capacity between locations on a) Western Cape (*P*<0.05), b) Irrigation (*P*<0.05), and c) Free State regions (*P*<0.05). Vertical bars denote 95% confidence interval.

Hardness and antioxidant properties

Soft wheat showed higher antioxidant activity than the hard wheat of Canadian commercial wheat samples (Liyana-Pathirana & Shahidi, 2006). Although this study found no significant difference in both TPC and DPPH radical scavenging capacity between wheat cultivars, there could be a correlation between wheat hardness and antioxidant activities.

The correlations between hardness and TPC (Fig. 4.7) were analysed within the individual regions. TPC was inversely correlated to wheat hardness in the Western Cape region (r=-0.50, *P*<0.01). For wheat from the Free State region, a negative but insignificant correlation was recorded (r=-0.36, *P*=0.10). No correlation was observed between hardness and TPC of the wheat samples in the Irrigation region (r=0.016, *P*=0.93).
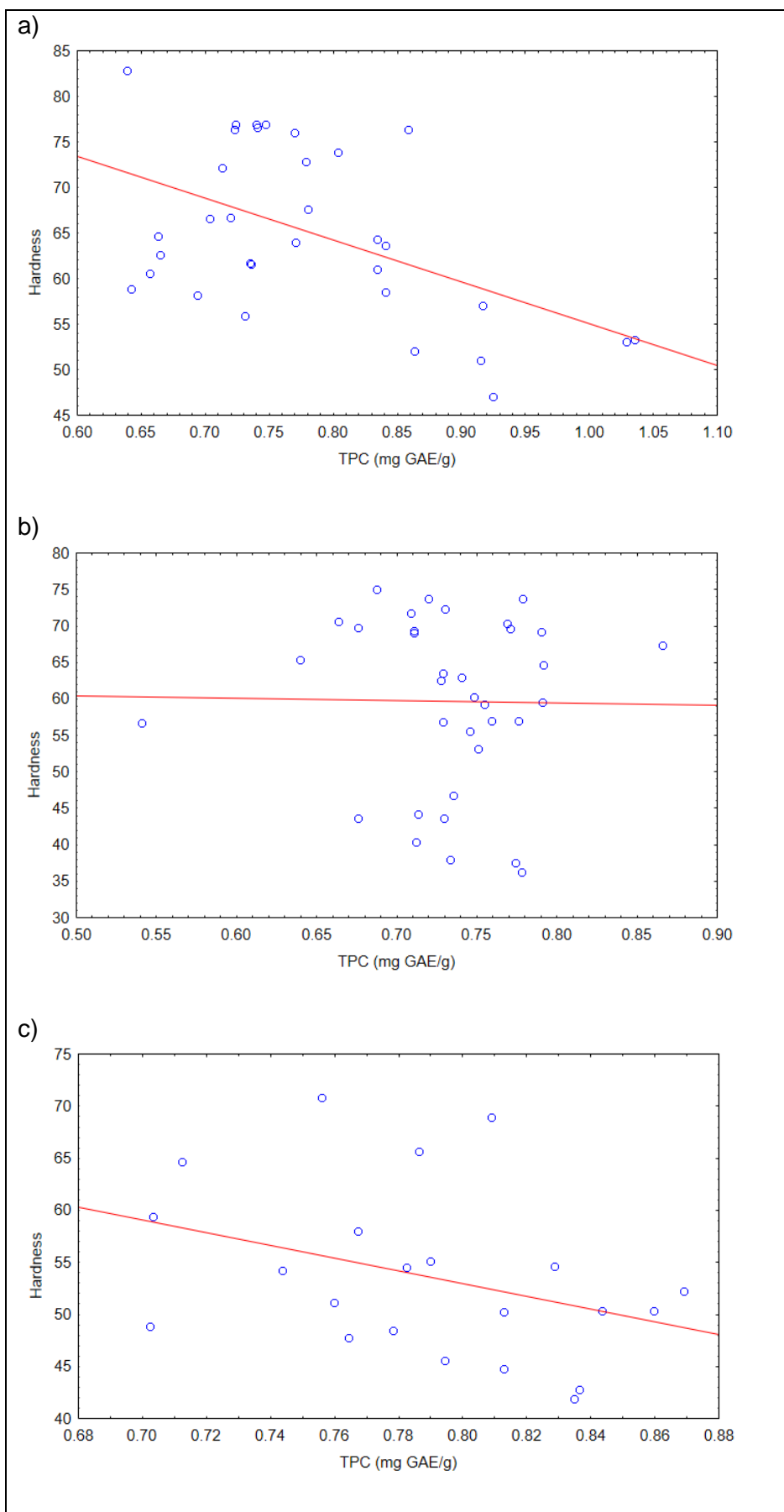
Figure 4.7. Descriptive statistic analysis between TPC and hardness of wheat sample: (a) Western Cape region (r=-0.502, *P*=0.003), (b) Irrigation region (r=-0.016, *P*=0.93), (c) Free State region (r=-0.363, *P*=0.097).

A good correlation was observed between hardness and DPPH radical scavenging capacity for the wheat planted under irrigation and in the Free State region (Fig. 4.8) with r=-0.649, *P*<0.001 and r=-0.467, *P*=0.029, respectively. Interestingly, the wheat planted in Western Cape showed a positive correlation between hardness and DPPH radical scavenging capacity.

Generally, hardness showed stronger correlation to DPPH radical scavenging capacity than TPC. The Folin–Ciocalteu method was designed for estimating the reducing capacity credited to the phenolic compounds (Singleton *et al.*, 1999), while DPPH assay was developed to predict the total antioxidant activity from any compound that could cause discolouration of the DPPH reagent (Liyana-Pathirana & Shahidi, 2006). The present study found that harder wheat had lower antioxidant activity, which agreed with a study by Chandrika & Shahidi (2006). It should be noted that samples planted in the Western Cape showed positive correlation between hardness and the DPPH radical scavenging capacity, while the TPC/hardness correlation was negative. According to Yu *et al.* (2003), the TPC may not relate to DPPH radical scavenging capacity, depending on the samples. The uniqueness about the Western Cape region is the cultivars that were planted in this region are adapted/requires winter rainfall, while the Free State and irrigation regions require summer rainfall. However, the data collected in this study is not sufficient to explain this exception, it is required to collect data for more seasons to be able to provide statistical evidence to explain the environment effect on the antioxidant properties of South African wheat.
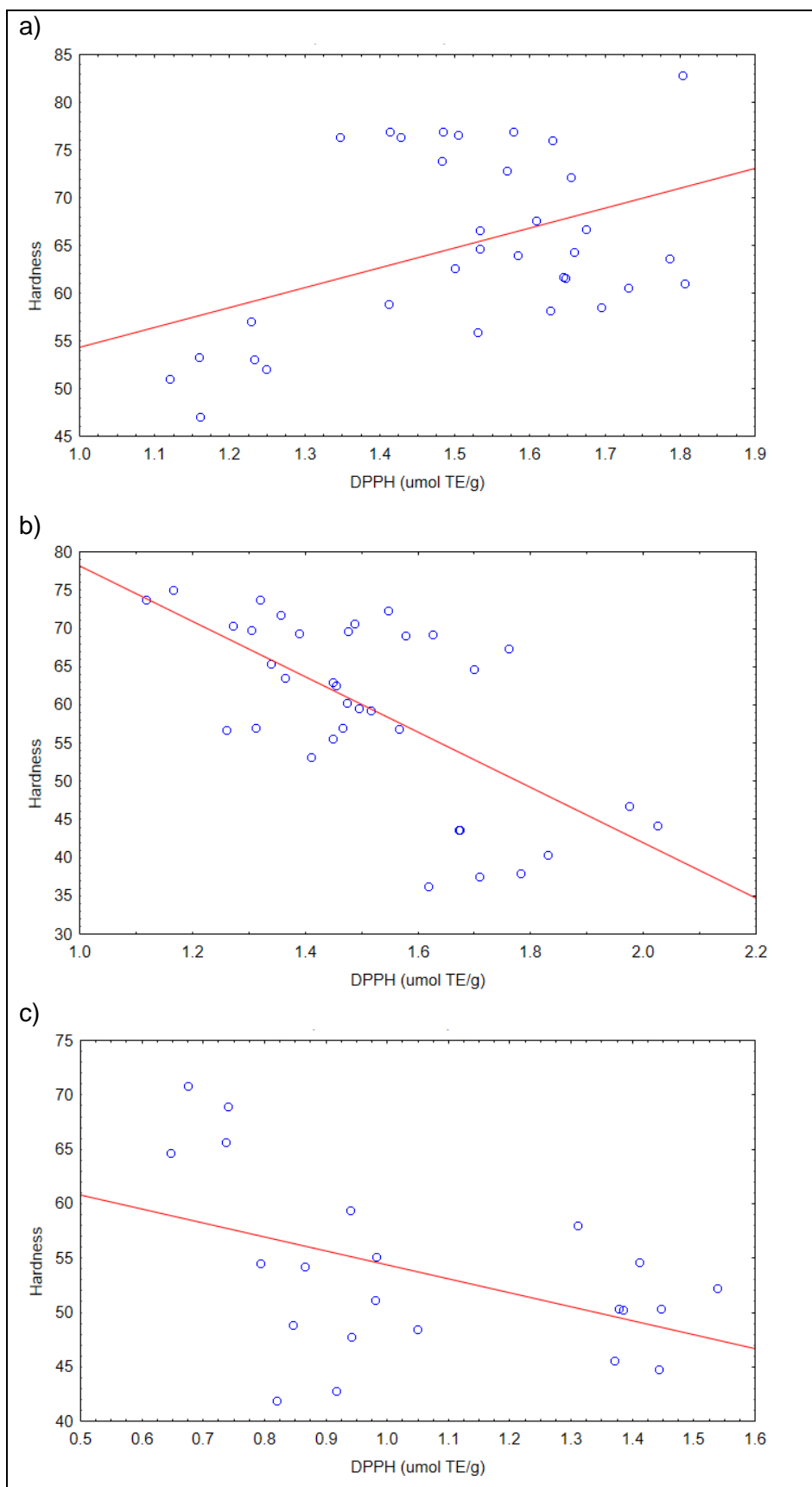
Figure 4.8. Descriptive statistic analysis between DPPH radical scavenging capacity and hardness of wheat sample: (a) Western Cape region (r=0.429, *P*=0.013), (b) Irrigation r (r=-0.649, *P*<0.001), (c) Free State region (r=-0.467, *P*=0.029)

Degree of effect

As the Western Cape and Free State regions were non-irrigated, the water quantity was entirely dependent on the climate. A comparison between irrigated and non–irrigated farming was thus possible. This allowed the comparison between irrigation versus none irrigation production. Irrigated farming provided better control and more consistent watering, thus the plants were normally not experiencing stress during drought periods. Table 4.1 presents the level of significance (*P*–value) of the relationship of the cultivar of the plant, or the location thereof and the antioxidant properties. The irrigation region showed higher *P*–values than both that of the other regions, indicating that wheat planted in this region had less variation between their antioxidant properties among the localities. This indicated environmental stress including water stress could be an important factor that affect the antioxidant properties in wheat. Water stress was found to lead to an increase in the lipo–oxidation in wheat during the growth period (Sairam & Saxena, 2000). It should be noted that the precipitation (rainfall) during the wheat growing season in this study was recorded as normal (Fig. 4.9).

Table 4.1 The significant value (*P*) between the cultivars and locality which was obtained in Fisher's LSD test

	TPC		DPPH	
Region	Cultivar	Locality	Cultivar	Locality
Western cape	0.908	0.009	0.879	<0.0001
Irrigation	0.344	0.022	0.206	0.021
Free State	0.529	0.012	0.343	0.002

The wheat planted under irrigation had higher DPPH radical scavenging capacity than the other two regions (Fig. 4.6). However, there were other factors that could also have influenced the DPPH radical scavenging capacity in wheat, for example soil type, intensity of sunlight and the fertiliser used. Even more so, with different cultivars planted between the regions, it was difficult to compare the regions with each other. Even though no significant difference were found between cultivars, Baviaans was the only cultivar that were planted in two regions (Western Cape region and irrigation region. It had high DPPH radical scavenging capacity in both the Western Cape region and under irrigation (Fig. 4.5). This showed a promising evidence that wheat that was grown with irrigation may result with a high DPPH radical scavenging capacity.

April 2012	May 2012

June 2012	July 2012

August 2012	September 2012

October 2012	November 2012

December 2012	Rainfall (mm)

Rainfall (mm)

- 0 - 10
- 10 - 25
- 25 - 50
- 50 - 100
- 100 - 200
- 200 - 500
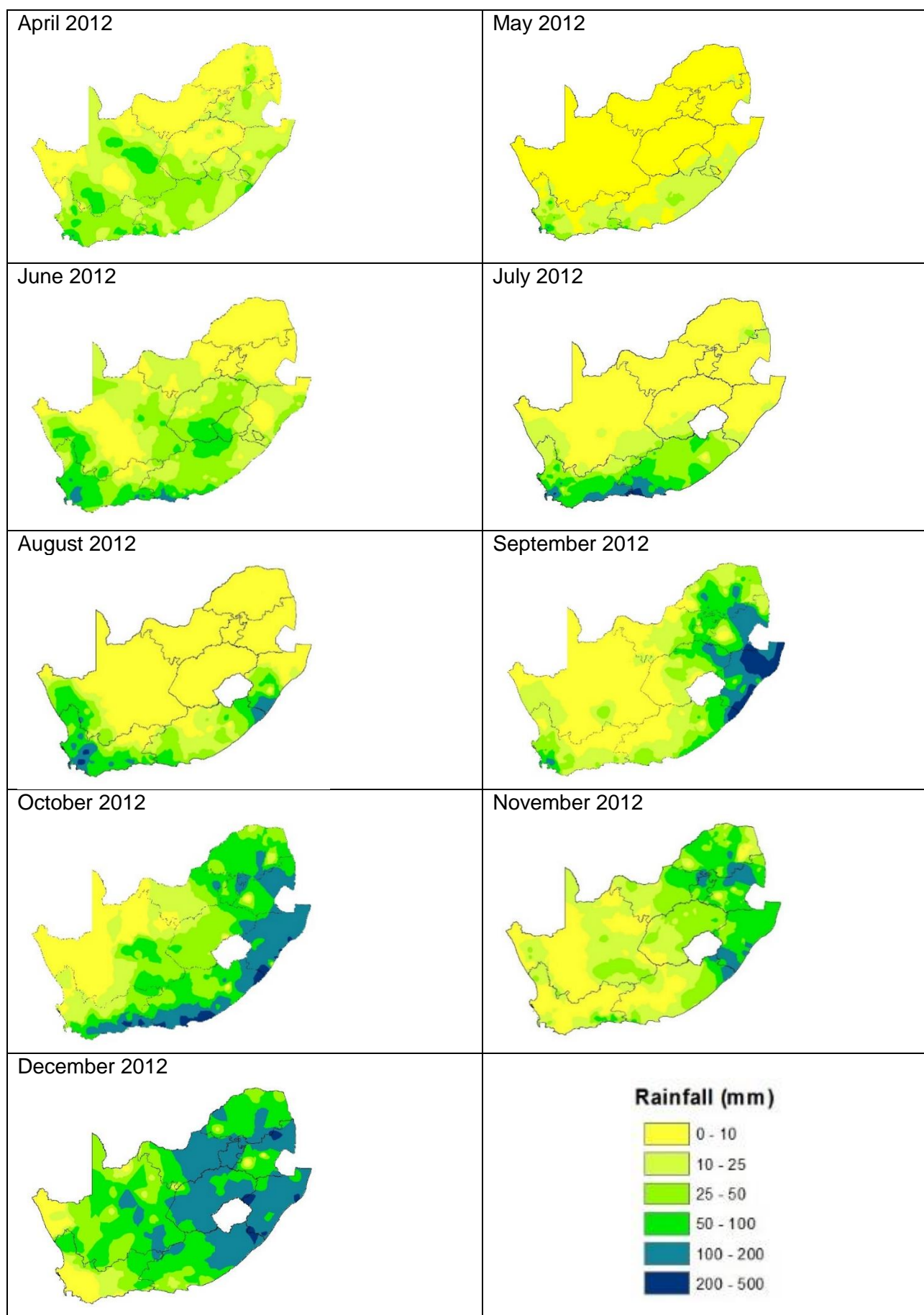
Figure 4.9. Record of rainfall in South Africa during growth period of wheat samples, for the period of April to December 2012 (SAWS, 2015).

Conclusion

This study provides a preliminary overview of the antioxidant properties (TPC and DPPH radical scavenging capacity) of some of the South African wheat cultivars. These wheat samples had similar levels of antioxidant content compared to wheat from other countries, such as Canadian wheat. The antioxidant properties of all the wheat cultivars used in this study did not differ significantly with respect to the production region. The locality of the wheat samples had a larger influence on the antioxidant properties than the difference caused by genotypes (wheat cultivars). This study showed the importance of monitoring the environmental effects with respect to the antioxidant properties of wheat. It is therefore advised to collect data for another year or two, in order to be able to statistically confirm that the variation was indeed caused by the locality. Furthermore, the irrigated farms showed higher DPPH radical scavenging capacity and was less influenced by respective localities. However, the cultivars planted in the specific region can not grow optimally in another region, thus impossible to compare the antioxidant properties of wheat across the regions. Further studies are advised to be attentive of the effect of environmental stress, particularly water stress on the DPPH radical scavenging capacity of wheat.

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Appendix 4.1 TPC of wheat samples

Region	Cultivars	Total Phenolic Contents (mg GAE/g)			
Western Cape		Napier	Riversdal	Klipheuwel	Moorreesburg
	Kariega	0.723	N.A	0.841	0.733
	PAN 3434	0.746	N.A	0.847	0.628
	Ratel	0.770	0.921	0.699	0.691
	Baviaans	0.740	0.914	0.841	0.604
	SST 015	N.A	0.811	0.664	0.699
	SST 096	0.804	0.803	0.719	0.683
	SST 056	0.741	0.802	0.669	0.696
	SST 087	0.841	0.749	0.710	0.741
	SST 088	0.639	N.A	0.730	0.740
Irrigation		Hartsvallei	Lichtenburg	Marble Hall	Winterton
	SST 806	0.754	0.678	0.756	0.770
	Duzi	0.751	0.740	0.809	0.581
	Baviaans	0.775	0.743	0.778	0.703
	Buffels	0.745	0.643	0.840	0.680
	PAN 3471	0.730	0.699	0.760	0.781
	SST 835	0.746	0.701	0.819	0.769
	Olifants	0.789	N.A	0.829	0.906
	PAN 3478	0.771	0.694	0.769	0.717
	SST 875	0.791	0.678	0.736	0.750
Free State		Bethlehem	Bultfontein	Clocolan	
	Elands	0.700	0.745	0.714	-
	PAN 3161	0.834	0.699	0.755	-
	PAN 3144	0.835	0.753	0.689	-
	Gariep	0.779	0.654	0.730	-
	SST 398	0.765	0.681	0.700	-
	PAN 3355	0.760	0.729	0.726	-
	SST 356	0.790	0.715	0.658	-
	SST 3379	0.704	0.699	N.A	-

Appendix 4.2 DPPH radical scavenging capacity of wheat samples

Region	Cultivars	DPPH˙ (µmol TE/g)			
Western Cape		Napier	Riversdal	Klipheuwel	Moorreesburg
	Kariega	1.427	N.A	1.671	1.169
	PAN 3434	1.485	N.A	1.651	1.085
	Ratel	1.629	1.225	1.493	1.116
	Baviaans	1.505	1.298	1.524	0.997
	SST 015	N.A	1.227	1.596	1.233
	SST 096	1.483	1.294	1.520	1.260
	SST 056	1.413	1.186	1.399	1.230
	SST 087	1.695	1.315	1.399	1.193
	SST 088	1.804	N.A	1.443	1.155
Irrigation		Hartsvallei	Lichtenburg	Marble Hall	Winterton
	SST 806	1.516	1.833	1.486	1.726
	Duzi	1.410	1.517	1.442	1.438
	Baviaans	1.466	1.428	1.493	1.666
	Buffels	1.449	1.482	1.830	1.519
	PAN 3471	1.567	1.591	1.518	1.627
	SST 835	1.475	1.784	1.401	1.633
	Olifants	1.626	N.A	1.449	1.940
	PAN 3478	1.477	1.480	1.247	1.484
	SST 875	1.496	1.638	1.295	1.756
Free State		Bethlehem	Bultfontein	Clocolan	
	Elands	0.847	0.991	0.848	-
	PAN 3161	0.819	0.997	0.856	-
	PAN 3144	0.917	1.151	0.980	-
	Gariep	1.049	0.924	0.852	-
	SST 398	0.942	0.984	0.790	-
	PAN 3355	0.980	1.060	0.908	-
	SST 356	0.982	1.025	0.762	-
	SST3379	0.940	1.056	N.A	-

Appendix 4.3 HI of wheat samples

Region	Cultivars	Hardness Index			
Western Cape		Napier	Riversdal	Klipheuwel	Moorreesburg
	Kariega	76.4	n.a.	61.0	64.0
	PAN3434	77.0	n.a.	63.6	62.6
	Ratel	76.0	53.3	58.2	55.9
	Baviaans	76.6	53.1	64.3	58.9
	SST 015	n.a.	47.1	60.6	61.6
	SST 096	73.9	57.1	72.2	66.7
	SST 056	76.9	51.0	64.7	61.7
	SST 087	58.5	52.1	66.6	67.6
	SST 088	82.9	n.a.	76.9	72.9
Irrigation		Hartsvallei	Lichtenburg	Marble Hall	Winterton
	SST 806	59.3	44.2	71.8	72.4
	Duzi	53.2	37.5	57.0	56.7
	Baviaans	57.0	36.2	63.5	70.7
	Buffels	55.6	43.7	64.7	65.4
	PAN3471	56.8	38.0	69.4	62.9
	SST 835	60.3	46.8	70.4	62.5
	Ollifants	69.2	n.a.	73.7	67.4
	PAN3478	69.6	43.6	73.8	69.8
	SST 875	59.6	40.4	75.1	69.1
Free State		Bethlehem	Bultfontein	Clocolan	
	Elands	48.9	50.4	n.a.	-
	PAN3161	41.9	50.2	68.9	-
	PAN3144	42.8	52.2	54.2	-
	Gariep	48.4	58.0	65.7	-
	SST 398	47.7	45.6	70.8	-
	PAN3355	51.1	50.4	54.5	-
	SST 356	55.1	54.6	64.7	-
	SST3379	59.4	44.8	n.a.	-

Chapter 5

Effect of forced convection roasting on antioxidant properties and the optimisation of roasting conditions of South African wheat cultivars for the production of whole grain flour

Abstract

An innovative processing method, i.e. forced convection continuous tumble roasting (FCCTR), which is fast, energy efficient, and easy to operate, has great potential for food manufacturing. This study assessed the application of FCCTR for pre-treatment of whole wheat grain, and more specifically the effect on antioxidant properties. Two wheat cultivars (PAN3161 and PAN3379) were roasted using a South African patented forced convection continuous tumble roaster. A central composite design (CCD) was used with temperature and rotating speed set in the range of 136 and 234°C and 20 and 90 Hz, respectively. The roasting time is inversely correlated to its rotating speed. Subsequently total phenolic content (TPC; Folin-Ciocalteu method) and antioxidant activity (DPPH and ABTS methods) have been determined. For PAN3161, minimum processing (136°C, 90Hz) was required to achieve the highest TPC, while roasting at 234°C, 20Hz achieved the highest DPPH radical scavenging. PAN3379 showed different behaviour, the RSM estimated roasting at 234°C, 90 Hz would result in the highest TPC, and 136°C at 90 Hz in the highest DPPH radical scavenging capacity. The difference in optimal processing conditions observed for the two cultivars could partly have been due to the high temperatures destroying some of the free phenolic acids. On the other hand, some antioxidant compounds may have been produced through the Maillard reaction, not detected by means of the Folin-Ciocalteu method. The difference in free and bound phenolic acids content, the natural composition of the amino acids, reducing sugar content of the wheat cultivars might also have been the reason for the different roasting conditions required for optimising the antioxidant properties.

Introduction

Many studies have found whole wheat grain to contain considerable amount of antioxidant properties (Kim *et al.*, 2006; Lu *et al.*, 2015). The consumption of whole grain wheat may therefore be beneficial for the prevention of chronical disease (Slavin, 2000).

Wheat has to be cooked to be acceptable as a food to be consumed by humans. As technology developed, many methods became available for the cooking of wheat based products. Examples of commonly found methods are steaming, boiling, baking, extruding and roasting. It is known that certain antioxidants such as phenolic acids, carotenoids and anthocyanin are heat labile, and these substances may lose their antioxidant properties during cooking (Ragaee, Seetharaman*, et al.*, 2012). Since phenolic acids are the most predominant substance responsible for antioxidant properties in wheat (Zieliński & Kozłowska, 2000), it is important to understand retention of phenolic acids and antioxidant properties in heat processed foods. Most phenolic compounds in wheat are present in bound form conjugated to the component in the cell wall structure (Wong, 2006). Bound forms of phenolic compounds have been reported to be more heat stable than the free phenolic compounds (Sharma *et al.*, 2012). The cooking step releases the conjugated phenolic compounds from the cell wall components, creating free phenolic compounds which are more accessible for utilisation through the human digestion system (Dewanto *et al.*, 2002). Other research found that certain products that were generated through the Maillard reaction had strong antioxidant activities (Rufián-Henares & Delgado-Andrade, 2009). The Maillard reaction could occur at room temperature at a very slow rate, but is accelerated with an increase in temperature (Martins *et al.*, 2000). However, the products of Maillard reaction can be dependent on various factors, such as the availability of the substrates and the reacting temperature (Martins *et al.*, 2000). Thus, by correctly controlling the cooking conditions, a product with good antioxidant properties could be achieved.

An innovative processing method called FCR, has many advantages such as ease of operating, efficient usage of energy, precise control of operating temperature, even heat transfer, limited temperature fluctuation, and continuous roasting design. During the roasting process, moisture is released from the product and stays in the roasting chamber, whereas the moisture vapour gradually replaces parts of the air, therefore converting heated air into semi–superheated steam. This enables more efficient and even heat transfer to the product (Moreira, 2001). An earlier review compared hot air with super-heated steam used for impingement drying of foods and indicated that super-heated steam processing reduced the oxidation rate, thus has potential to maintain more nutritional value in the products (Moreira, 2001).

Due to its simple heating principle and several advantages, the effect of FCR on the antioxidant properties of South African was studied. This study also aimed to provide a reference guide for optimising roasting conditions when producing wheat based products with high antioxidant benefits.

Materials and methods

Materials

One soft wheat cultivar (PAN3161) and one hard wheat cultivar (PAN3379) were kindly provided by PANNAR SEED (Pty) Ltd. The samples were stored in airtight plastic containers at room temperature. Samples were visually inspected for purity and foreign particles such as stems and stones were removed before use.

Determination of moisture content

The moisture content was determined for the untreated sample, moisture tempered samples, as well as the FCR samples. The moisture content was determined according to the AACCI approved method 44-19.01 (1999). The wheat was hulled into flour before moisture analysis. Approximate 2 g of wheat flour were weighed in an aluminium moisture dish, the weight was recorded in three decimals. With covers removed, the samples were dried in an EM10 oven (CHOPIN Technologies, Cedex, France) at 135°C for 2 h. After drying, the dishes were closed with cover and cooled to room temperature in a desiccator. The samples were then weighed and the moisture content was calculated according the Eq. 1.

$$\% \, Moisture = \frac{loss \ in \ moisture}{weight \ of \ sample} \times 100$$

Eq.1

Tempering of wheat grain

The FCCTR requires 18 to 20% of the moisture content of the sample during the roasting. The received samples had moisture contents in the range of 10.6 to 10.65 %. Thus, a tempering method was applied to increase the moisture content of the sample before roasting.

Wheat samples were tempered according to AACCI approved Method 26-95.01 (1999). Exactly 200 g (± 0.2 g) of wheat sample was placed in an air-tight plastic container. The amount of distilled water to be added to the sample was calculated based on Eq. 2. Following the water addition, the containers were sealed to prevent any water loss. The samples were placed in a dark environment at room temperature for 18 h. The samples were shaken for 5 min by hand every 2 h for the first 6 h, then shaken again 2 h before roasting, in order to ensure that each grain has an equal chance of being in contact with the added water.

$$weight \ of \ water \ to \ add \ (mL) = \left(\frac{100\% - initial \, moisture \, \%}{100\% - required \, moisture \, \%} - 1 \right) \times sample \ weight$$

Eq. 2

Experimental design

A central composite design (CCD) was used to study the roasting speed and temperature effect of the antioxidant properties of the samples roasted using FCCTR. The roasting speed of the FCCTR ranged from 20 to 90 Hz and the temperature ranged from 136 to 234°C. The roasting speed is inversely correlated to the roasting time. The range of the operating conditions used in this study was determined through a preliminary study. The lowest roasting temperature and the highest roasting speed limit was determined as the minimum degree of roasting that showed an obvious reduction of moisture content. The highest roasting temperature and the lowest roasting speed limit was determined as the highest degree of roasting without over-roasting the wheat samples. The over-roasted wheat grain appeared dark in colour with an undesirable carbon-smoky aroma. Based on the maximum and minimum temperatures (150–220°C) and speed (30–90 Hz) as determined in the preliminary study the CCD for the experiment was done.

The CCD consisted of 10 experimental runs which included 4 axial points, 4 factorial points and 2 central points that were employed to optimise the independent variables. It was a two-level factorial design with temperature (X_1) and speed (X_2) as the two variables. The roasting setting are listed in Table 5.1. The response surface methodology (RSM) was used for the investigation of the nature of the relationship between the antioxidant properties (response, Y) and the roasting settings (temperature and speed). From the RSM, the optimal operating setting to achieve the desirable antioxidant properties were determined. The RSM was obtained according to the Eq. 3.

$$Y = \beta_O + \beta_1 X_1 + \beta_2 X_2 + \beta_{12} X_1 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2$$

β_o is constant (intercept), β_1 and β_2 are linear coefficients, β_{11} and β_{22} are quadratic coefficients and β_{12} is the coefficient of interaction.

Eq. 3

Forced convection roasting

The tempered wheat samples were roasted using a FCCTR R100E (Roastech, Bloemfontein, South Africa). As the product being roasted can release its moisture and replace the air in the roasting chamber with the moisture, dry air is converted into super steam during roasting. Therefore, it was necessary to modify the atmosphere before roasting the experimental samples. Thus, 2 kg of tempered wheat kernels were gradually added into the roaster when the chamber temperature reached 150°C and the roasting speed was at 20 Hz. Wheat samples were roasted according to the CCD (Table 5.1). After roasting, the samples were air-cooled to room temperature, collected in air-tight plastic containers and stored at room temperature.

Table 5.1 Roasting temperature and speed of experimental runs generated using CCD

Experimental runs	Roasting temperature (°C)	Rotating speed (Hz)
1	150	30
2	150	80
3	220	30
4	220	80
5	136	55
6	234	55
7	185	20
8	185	90
9 (C)	185	55
10 (C)	185	55

Extraction of antioxidants

The sample extraction was done according to Wang *et al.* (2008). A portion (30 g) of sample was milled, using a hammer type cyclone Laboratory Mill LM 3100 fitted with a 0.5 mm sieve. Each milled wheat (2 ± 0.01 g) sample was extracted twice in a 50 mL Erlenmeyer flask and mixed with 10 mL of 64% aqueous ethanol. The samples were sonicated at 60°C using a Scientech ultrasonic cleaner at 40 kHz for 25 min to facilitate extraction. Thereafter, the samples were placed in 50 mL centrifuge tubes and centrifuged for 10 min at 3000 RCF. Extractions were done in triplicate and the supernatant (extracts) were collected into 50 mL centrifuge tubes and stored at -18°C.

Determination of total phenolics content

TPC of the FCR roasted wheat samples was determined using the Folin-Ciocalteau method (Singleton & Rossi, 1965b; Arthur *et al.*, 2011) on a 200 µL microplate scale. Extracts were acclimated to room temperature before being analysed. For the construction of a standard curve, a gallic acid (Merck, Modderfontein, South Africa) standard range (0–150 mg/L), consisting of eight standard solutions, was prepared by diluting gallic acid with de-ionised water. For the TPC determination, Folin-Ciocalteau reagent (Merck, Modderfontein, South Africa) was diluted with de-ionised water in a ratio of 1:10, where after 10 µL extract was mixed with 90 µL of the diluted Folin-Ciocalteau reagent in a flat bottom 96 well microplate plate. This was followed by adding 100 µL sodium carbonate (7.5% m/v) (Sigma-Aldrich, Kempton Park, South Africa), using a multi-channel pipette (Gilson, Germany). After gentle shaking, the mixtures were incubated at 30°C in a dark room for 2 hours. The absorbance values were measured using an Eon microplate spectrophotometer (BioTek, Winooski, USA) at 765 nm. Each extract was measured in triplicate, and one extract was selected for the correction of variation found between the experiments that was done on different days. Absorbance values were calculated as the TPC based on the standard curve constructed from the gallic acid standards. Thus, the TPC were expressed as gallic acid equivalent (mg GAE/g).

DPPH radical scavenging capacity

The DPPH radical scavenging assay was used to determine the antioxidant activity of the wheat samples. The assay was adapted from Brand-Williams *et al.* (1995) and (Arthur *et al.*, 2011). The absorbance of the samples were compared with Trolox standards, and expressed as Trolox equivalent (µmol TE/g).

DPPH stock solution was prepared to a concentration of 0.1 mg / mL, where after this stock DPPH solution was diluted with the 64% ethanol to achieve an absorbance value of 0.58–0.62.

A Trolox stock solution (1 mM) was prepared by dissolving 0.0125 g Trolox in 50 mL 64% ethanol in a volumetric flask. A standard range of 8 Trolox solutions, 0–500 µM was used.

The DPPH radical scavenging assay was done in a dark environment where 30 µL standard and sample extracts were pipetted into corresponding wells of a 96 deep-well plate, followed by adding 270 µL DPPH reagent (solution A). The plates were sealed with silicon sealing mats, and incubated for 60 min at room temperature (20°C) in a dark room. After the incubation period, the absorbance was measured, using an Eon microplate spectrophotometer at 515 nm. Each extract was measured in triplicate.

ABTS cation scavenging activity

The ABTS cation scavenging activity was determined according to methods suggested by Miller *et al.* (1993) and Re *et al.* (1999b). A 75 mM potassium phosphate buffer solution (PBS) was prepared by adjusting the potassium phosphate solution with 2 M KOH to pH of 7.4 in a beaker, followed by transfering the solution into a volumetric flask and filling the flask with distilled water. A potassium persulphate solution was prepared by dissolving potassium persulphate with de-ionized water to a concentration of 140 mM. Two steps were used for the preparation of the ABTS radical cation (ABTS$^{•+}$). The first step was to dissolve ABTS powder in de-ionised water to a concentration of 7 mM, whereas the second step was to allow the ABTS solution to be oxidised by adding prepared potassium persulphate (140 mM) to a final concentration of 2.45 mM. The prepared solution was incubated in a dark chamber at room temperature for 12–16 hours before use. After incubation, the prepared ABTS$^{•+}$ solution was further diluted with PBS to the absorbance of 0.7 at 734 nm. Trolox standards were prepared to the concentration ranged 0–450 µM, which was prepared in the same way as described in the DPPH assay.

The determination of the ABTS$^{•+}$ scavenging capacity was done using a EON microplate reader at 734 nm. The sample extracts were diluted in a ratio of 1/3 before measurement. Exactly 20 µL of each Trolox standard and sample were pipetted into corresponding wells in a flat bottom 96 well micro plate, followed by adding 180 µL of the ABTS$^{•+}$ reagent (diluted to absorbance of 0.7). The plate was incubated at 30°C for 2 h, whereafter the absorbance was measured at 734 nm in the microplate reader.

Statistical analysis

Absorbance data was analysed using the Gen5 v2.05 software (BioTek, Winooski, US), as well as Microsoft Excel 2013 (Microsoft Corporation, Seattle, WA) to construct linear regression curves. The Response Surface Methodology (RSM) including the analysis of variance (ANOVA) was analysed using Statistica version 12 (StatSoft Inc, Oklahoma, USA).

Results and discussion

ANOVA and RSM data analyses

The antioxidant properties examined included TPC, DPPH scavenging capacity and ABTS$^{\bullet+}$ scavenging capacity. The results of the roasted samples of CCD experiment (Table 5.2) were further analysed using RSM and ANOVA. The three-dimensional response surface plots and the two dimensional contour plots reflect the interaction between the roasting temperatures and roasting speeds that affected the antioxidant properties. The Pareto chart indicates the significance of the linear, quadratic and interaction effects of the temperature and speed on the antioxidant properties. The significance level was determined at P=0.05, which was shown as a vertical line in the Pareto chart. If the effect of its corresponding bar crossed the vertical line, it indicated the particular factor that affected the antioxidant properties significantly, whilst if the corresponding bar did not cross the vertical line that showed that the effect of the factor was not significant. The positive and negative values showed that the corresponding factors were positively or negatively influenced by the antioxidant properties. The regression coefficient (R^2-value) and lack-of-fit (*P*-value) as obtained in the ANOVA indicate the level of confidence that the effect of the variable had on the prediction model.

Table 5.2 Central Composite Design and the response values for TPC, DPPH scavenging capacity and ABTS[•+] scavenging capacity of the roasted wheat samples

Run Number	Cultivar	Temp. °C	Speed (cycles/min)	TPC (mg GAE/g)	DPPH (µmol TE/g)	ABTS (µmol TE/g)	Moisture content %
5	PAN3161	136	55	2.40	4.72	6.30	13.24
1	PAN3161	150	30	2.19	5.03	5.92	11.14
2	PAN3161	150	80	2.17	4.71	6.34	12.96
10 c	PAN3161	185	55	2.09	5.16	6.59	11.52
7	PAN3161	185	20	1.69	5.01	5.69	6.80
8	PAN3161	185	90	2.36	4.51	6.67	12.02
9 c	PAN3161	185	55	2.04	5.09	6.06	10.44
4	PAN3161	220	80	2.01	4.69	6.16	10.60
3	PAN3161	220	30	1.86	6.17	5.57	4.40
6	PAN3161	234	55	1.68	4.87	5.69	7.65
5	PAN3379	136	55	2.05	4.40	6.39	13.30
1	PAN3379	150	30	1.87	5.32	6.52	10.38
2	PAN3379	150	80	2.23	5.70	6.89	13.19
10 c	PAN3379	185	55	2.21	4.65	6.51	11.97
7	PAN3379	185	20	1.62	5.26	5.55	6.64
8	PAN3379	185	90	2.24	4.81	6.61	12.67
9 c	PAN3379	185	55	1.61	4.55	5.80	10.45
4	PAN3379	220	80	2.23	4.22	6.74	11.24
3	PAN3379	220	30	1.52	5.74	5.99	6.67
6	PAN3379	234	55	1.73	5.77	5.44	7.03

The RSM and ANOVA were based on the mean values of all the measurements of the individual samples. However, the RSM and ANOVA for median values was available, which showed similar results compared to the mean values. An exception was the Pareto chart on DPPH radical scavenging capacity that found none of the variables to show significant median values, whilst significant variables were found when analysis was done for mean values. Additionally, lower R^2-values with higher standard errors were obtained when analysis based on the median values were done, indicating that analysing median values did not achieve a better reliability and repeatability. Therefore, the analysis of median value will not be discussed in this chapter.

Effects of roasting temperature and speed

The regression coefficients of the second order polynomial equations are given in Table 5.3. The response surfaces for moisture content, TPC, DPPH radical scavenging capacity and ABTS[•+] scavenging activity from the roasted wheat samples are presented in Figures. 5.1, 5.2 and 5.3.

Table 5.3 Regression coefficients of the second-degree polynomial for the relationship between roasting conditions and antioxidant properties in the wheat sample

| Coefficient	TPC		DPPH		ABTS	
	PAN3161	PAN3379	PAN3161	PAN3379	PAN3161	PAN3379
β_0	3.036458	3.189980	-0.714103	8.467069	1.281495	8.942714
β_1	-0.006312	-0.010024	0.042975	-0.055381	0.049875	-0.019389
β_2	-0.001327	-0.012906	0.063981	0.044191	0.020410	-0.025266
β_{12}	0.000048	0.000098	-0.000335	-0.000543	0.000047	0.000108
β_{11}	-0.000005	0.000005	-0.000053	0.000239	-0.000155	0.000017
β_{22}	-0.000021	0.000041	-0.000134	0.000430	-0.000155	0.000167

Moisture content

Roasting greatly affected the moisture content of the tempered wheat samples. The tempered wheat samples had a moisture content of 15.2 to 15.6% for both soft and hard wheats. The moisture content of the roasted wheat ranged from 4.4–13.3% (Table 5.2). The percentage moisture lost was calculated according to Eq. 4, the calculated results can be found in Appendix 5.1, which was further analysed with RSM. The R^2-values were 0.99 and 0.96 for the PAN3161 and PAN3379, respectively.

$$\% \text{ Moisture lost} = \frac{\text{moisture content of tempered sample} - \text{moisture content of roasted sample}}{\text{moisture content of tempered sample}} \times 100$$

Eq. 4

The response surface plots and Pareto charts revealed the effect of FCR on moisture content of tempered wheat samples (Fig. 5.1). Both cultivars were significantly affected with respect to their moisture loss. The temperature showed a positive influence, whilst the roasting speed showed a negative influence. The linear effect showed a higher effect size on two wheat cultivars rather than their quadratic effect and interactions. However, it was noted that the slight difference of intensiveness of the temperature (linear) and speed (linear) affected the moisture loss in the two cultivars (Fig. 5.1 b & d). This could be attributed to the ability of wheat kernels to absorb and release moisture, as well as the difference between the starch and protein composition of the hard and soft wheat cultivars (Turhan & Gunasekaran, 2002). The grain size could be another factor that influenced the moisture absorption and release ability. It was found that PAN3379 grains were smaller than PAN3361 in size, which resulted in PAN3379 having had a larger surface area exposed to the media (mixture of superheated steam and air) during roasting.

Although this study was not focused on the FRC's effect on moisture migration, the moisture data provide indirect indication of reliability and repeatability of the roasting experiment. It should be taken into consideration that the moisture content could influence the antioxidant determination. However, the difference can either be achieved by a correction step when re-calculating, or the re-calculation may not be necessary if it was found that the difference did not interfere with the results.
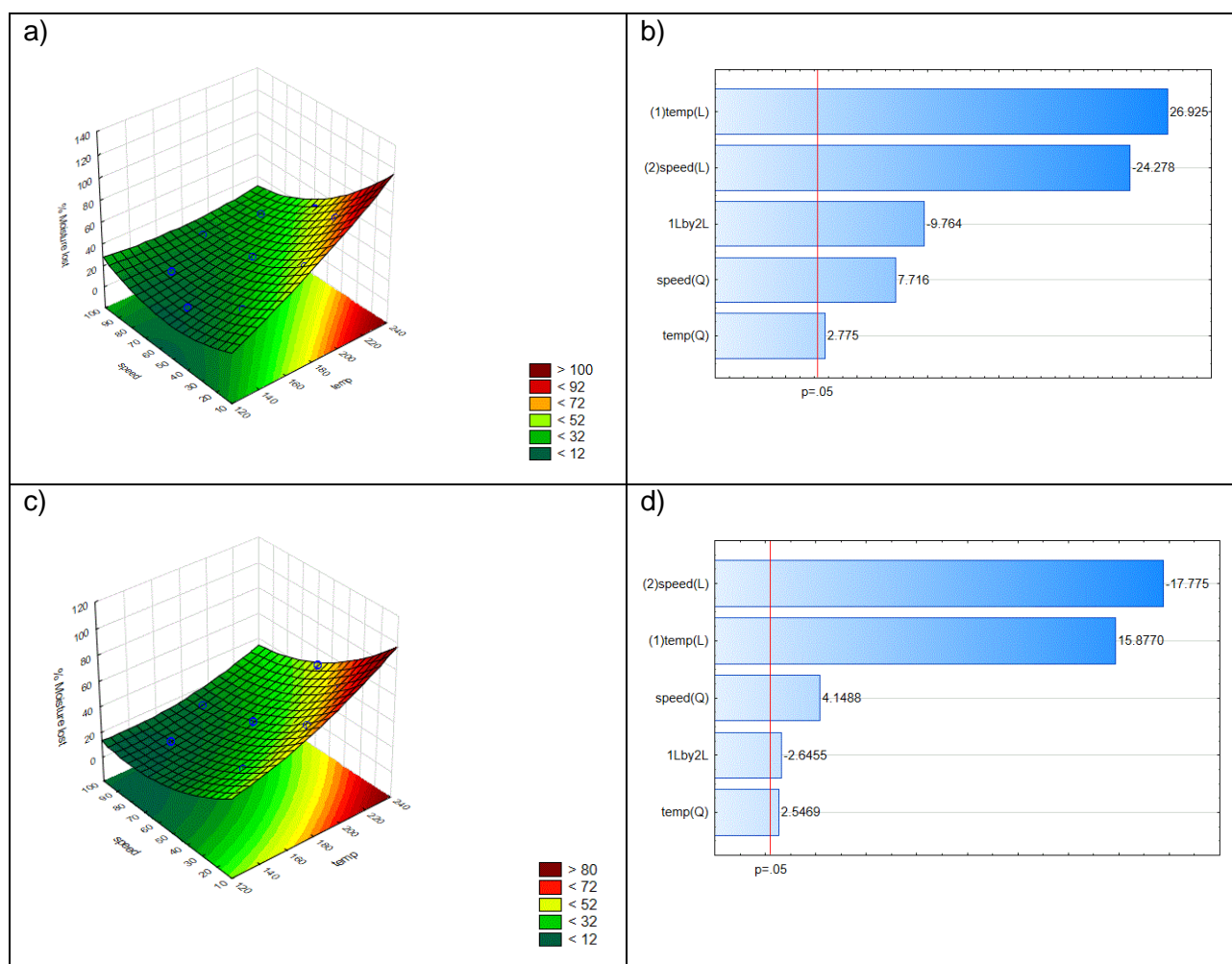
Figure 5.1. RSM analysis of moisture content for a) fitted response surface plots of PAN3161, b) Pareto Chart of PAN3161, c) fitted response surface plots of PAN3379, d) Pareto Chart of PAN3379.

Antioxidant properties

Roasting temperature and speed performed at different significance levels affected the TPC, DPPH radical scavenging capacity and ABTS$^{•+}$ scavenging capacity. Results from analysis performed on the entire sample set, showed that the antioxidant properties were linearly related to the roasting speed and temperature (Table 5.4).

The RSM analysis showed that the two wheat cultivars, PAN3161 and PAN3379 had different significance levels with respect to their TPC. FCR models were not significantly affected by the TPC of PAN3379, whilst roasting temperature negatively influenced the TPC of PAN3161, ($P<0.05$). The roasting speed ($P=0.06$), as well as the TPC (Table 5.4), did not indicate a significant effect. Furthermore, the response surface plot showed the TPC decreased with an increase in roasting temperature and a decrease in roasting speed (Fig. 5.2a). Since the roasting speed was inversely correlated to the roasting time, it could also be interpreted that the longer roasting time caused a lower TPC. For example, roasting at 90 Hz resulted in a roasting time of 2 min, at 55 Hz the roasting time was 3.25 min, whilst at a speed of 20 Hz the roasting time increased to 8.75 min. Even though

the roasting did not show to have a significant effect on the TPC for PAN3379, the response surface plot showed a similar trend as that of the TPC in PAN3161 (Fig. 5.2b).

Table 5.4 The effect of roasting temperature and speed on the antioxidant properties of wheat samples (PAN3161 and PAN3379) by Pareto chart (L: linear, Q: quadratic)

Assay	Factor	Soft (PAN3161)		Hard (PAN3379)	
		Effect size	P-Value	Effect size	P-Value
TPC	Temp. (L)	-16.32	0.04	-0.68	0.62
	Temp. (Q)	-0.38	0.77	0.03	0.98
	Speed (L)	11.53	0.06	1.62	0.35
	Speed (Q)	-0.83	0.56	0.13	0.92
	Interaction (L)	2.59	0.23	0.41	0.75
DPPH	Temp. (L)	9.29	0.07	4.58	0.14
	Temp. (Q)	-2.73	0.22	9.32	0.07
	Speed (L)	-17.60	0.04	-9.55	0.07
	Speed (Q)	-3.49	0.18	8.56	0.07
	Interaction (L)	-11.56	0.05	-14.34	0.04
ABTS	Temp. (L)	-1.33	0.41	-1.41	0.39
	Temp. (Q)	-1.08	0.47	0.08	0.95
	Speed (L)	2.28	0.26	1.82	0.32
	Speed (Q)	-0.55	0.68	0.43	0.74
	Interaction (L)	0.22	0.86	0.37	0.77

The DPPH radical scavenging capacity and ABTS$^{•+}$ scavenging capacity determined the ability of the antioxidant to reduce (scavenge) the free radicals present in the wheat samples. Roasting speed was revealed as a factor that significantly ($P<0.05$) affected the DPPH radical scavenging capacity for PAN3161. The interaction of the roasting speed and roasting temperature significantly affected the DPPH radical scavenging capacity of PAN3379 ($P=0.04$), the results following a linear model. The interaction of the temperature and speed showed to have a significant effect on both cultivars (Table 5.4). A similar trend was found with respect to the roasting temperature for both the cultivars. At a lower temperature and as the speed increased the DPPH scavenging capacity increased as well. Interestingly, the roasting speed inversely affected the DPPH radical scavenging capacity at a higher temperature. It should be noted that the roasting speed had a greater influence on PAN3379 than on PAN3161 at lower temperature. The response surface plot of PAN3161 showed a convex shape, whilst PAN3379 showed a concave shape (Fig. 5.2 c & d).

For both cultivars, no factors affected the ABTS$^{•+}$ scavenging capacity significantly (Table 5.4). The response surface plot showed that the roasting affected the ABTS$^{•+}$ scavenging capacity in a similar way as that of TPC. Additionally, PAN3161 scavenging performance was illustrated as a convex shaped graph, whereas PAN3379 showed to have a concave plot, which corresponded with the response surface plot obtained from DPPH radical scavenging capacity result.

The behaviour of antioxidant properties of the wheat samples during roasting could be attributed to several mechanisms. Firstly, FCR may cause the decomposition of several types of heat labile phenolic acids, explaining the decrease of TPC during roasting. Moreover, their linear correlation to the roasting temperature also indicated that as the temperature increased more phenolic acids decreased. Interestingly, the PAN3379 showed an increase in TPC at high speed that could be attributed to the liberation of bound phenolic acids during roasting (Anton *et al.*, 2008). The thermal labile phenolic acids might not be massively decomposed if the exposing time was not long enough to lead to decomposition (Martino & Savage, 1997). It should be noted that ferulic acid which is the most predominant phenolic acid in wheat (Adom & Liu, 2002), has been found to have high stability to high temperature (Fiddler *et al.*, 1967; Ragaee, Seetharaman*, et al.*, 2012). However, it can still be degraded into 4-vinylguaiacol and oxidised to vanillin, vanillic acid and acetovanillone (Fiddler *et al.*, 1967). Other factors such as air composition, grain size and initial moisture content of wheat grains may also influence the decomposition rate of phenolic acids besides exposing time and temperature. The roasting chamber captures the steam released from the product over a period of time, which converts the dry air roasting into a semi-supersteam roasting. This causes more invasive heat transfer to products with less oxygen present in the processing environment. Our results agreed with a thermal treatment study of buckwheat by Zhang *et al.* (2010). Their study found that thermal treatment decreased the phenolic acid content. Since buckwheat contains a majority of the free form of phenolic compounds (Şensoy *et al.*, 2006), the FCR may not have released large amount of bound form of heat stable phenolic acids from the cell wall structure of wheat. On the other hand, Gélinas and McKinnon (2006) found that baking increased the TPC of wholegrain bread, regardless of the baking time that ranged from 10 to 35 min at 177°C. They further explained this to be due to the compounds produced during Maillard reaction.
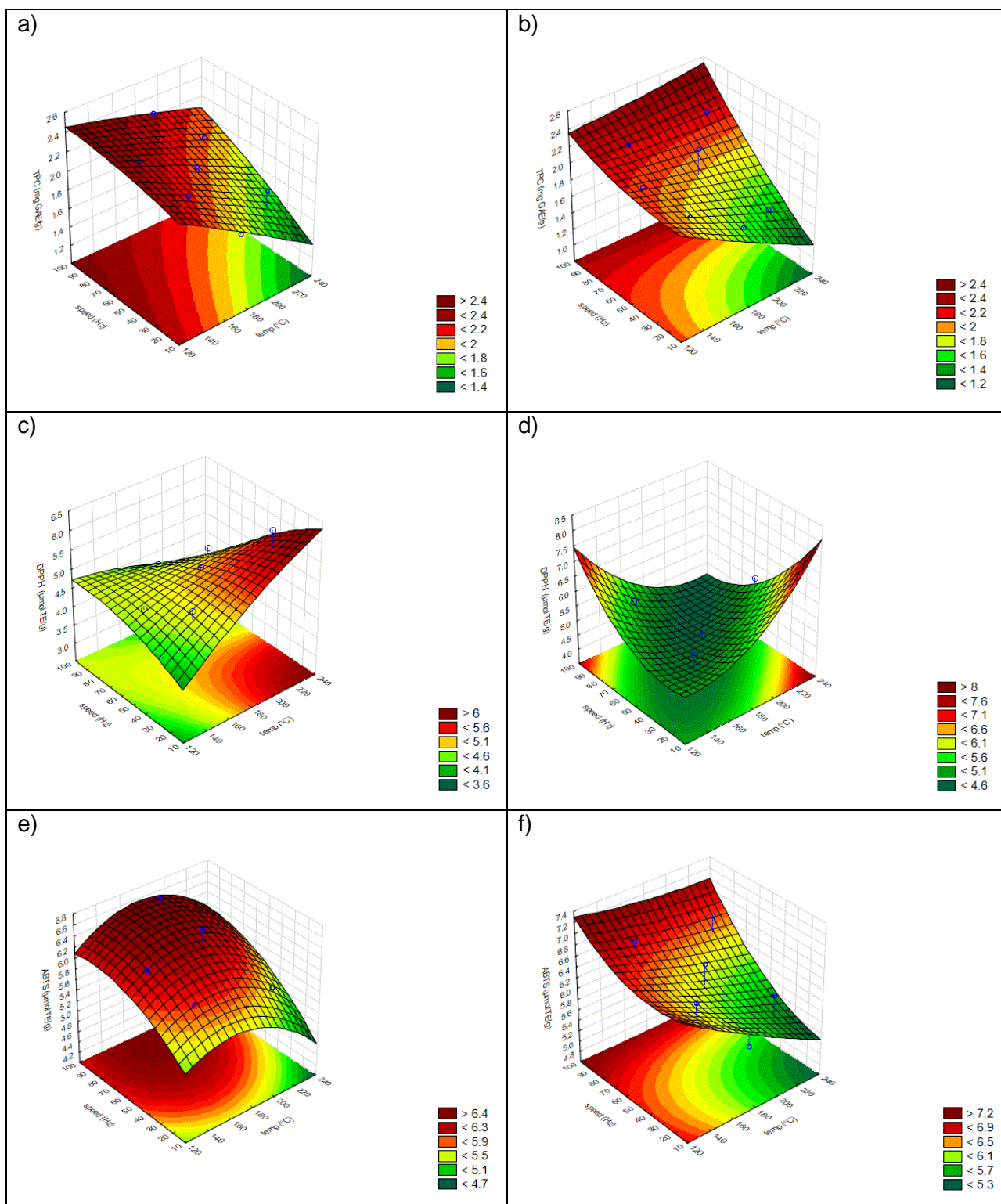
Figure 5.2. Response surfaces plot for (a) TPC of PAN3161, (b) TPC of PAN3379, (c) DPPH for PAN3161, (d) DPPH for PAN3379, (e) ABTS for PAN3161, (f) ABTS for PAN3379.

The Maillard reaction may produce various products depending on the temperature, available substrates, pH, and many other factors (Martins *et al.*, 2000). The increase of the DPPH radical scavenging capacity could be attributed to the formation of substances such as melanoidins through the Maillard reaction (Wang *et al.*, 2014; Przygodzka *et al.*, 2015). A similar result was found in a study of baking and fermentation effect on antioxidant properties of whole wheat pizza crusts (Moore

et al., 2009). They found that the DPPH radical scavenging capacity significantly increased when the baking time of the pizza dough increased from 7 to 14 min at 204°C; the DPPH radical scavenging capacity also increased when the baking temperature increased from 204 to 288°C for 7 min. Their ABTS$^{•+}$ scavenging capacity strongly correlates with the DPPH radical scavenging capacity. The ABTS$^{•+}$ scavenging capacity obtained in this study did not show a strong correlation to the DPPH radical scavenging capacity. Although its response surface plot was similar to the response surface plot obtained from TPC data, it was not significantly affected by any of the roasting settings used in this study.

It was reported that thermal processing, such as bread baking, could produce acrylamide, which was reported as a potential carcinogenic compound for humans (Mojska *et al.*, 2010; Przygodzka *et al.*, 2015). This study did not measure the presence of the acrylamide. It is, however, recommended to take this into consideration for future studies. With good control of optimal processing condition, it is possible to increase the antioxidant properties that benefit human health, although this needs to be weighed against the risk of producing a compound that could be associated with health problems.

Optimal roasting conditions determination

Desirability profile was determined with the RSM analysis, which was used to identify the optimal roasting temperature and speed to achieve highest antioxidant properties of roasted wheat.

As the FCR affected TPC, DPPH radical scavenging capacity and ABTS$^{•+}$ scavenging capacity differently on the two wheat cultivars, the optimal roasting conditions were also different. Therefore it is important to determine the objective based on the results and principium (arguments). The TPC is more focused on the determination of the presence of the phenolic compounds in the sample, whilst the emphasis of DPPH radical and ABTS$^{•+}$ scavenging capacity determine the antioxidant activity among all the compound able to scavenge the free radicals. Not all the detectable phenolic compounds have antioxidant properties, therefore the DPPH and ABTS$^{•+}$ scavenging capacity data should be taken into consideration. Table 5.4 revealed neither roasting temperature nor roasting speed resulted in a significant effect of ABTS$^{•+}$ scavenging capacity for both cultivars, thus it was not included for determining the optimal conditions.

The desirability profile revealed roasting PAN3161 at 136°C with 90 Hz of roasting speed would obtain the highest TPC based on the prediction model of RSM (Fig. 5.3 a). However, the roasting condition should be 234°C with 20 Hz to achieve the highest DPPH radical scavenging capacity (Fig. 5.3 b).

The highest TPC for PAN3379 was obtained by roasting at 234°C with speed of 90 Hz. It should be noted that roasting temperature did not significantly affect the TPC (Fig.5.4 a), and roasting at 136°C could still obtain the maximum desirability level of 1.0 (in a scale of 0–1). Roasting at 136°C with 90 Hz resulted in the maximum DPPH radical scavenging capacity for PAN3379 (Fig. 5.4 b).
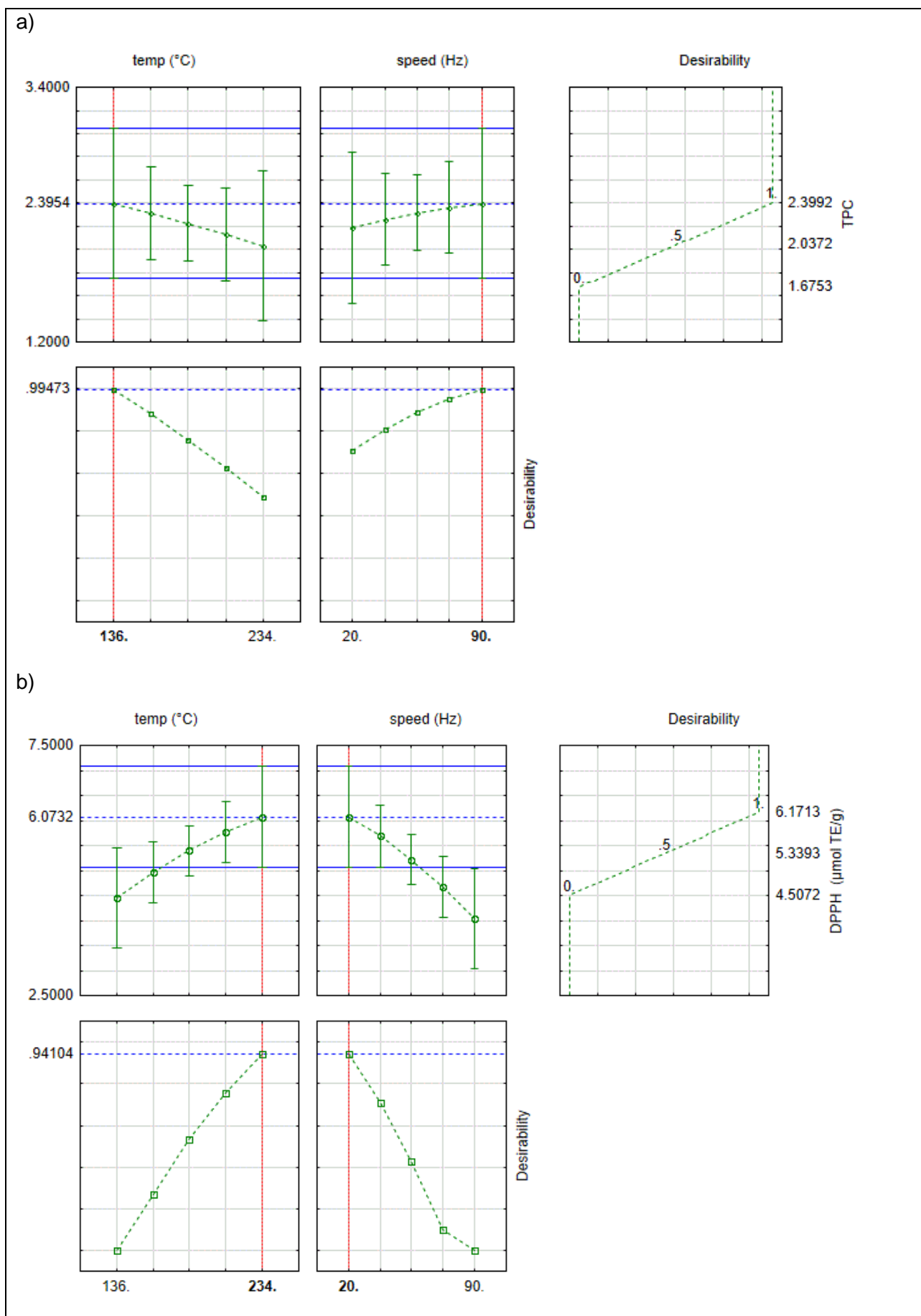
Figure 5.3. Individual prediction profile graph for optimum roasting conditions to obtain maximum of a) TPC of PAN3161, (R^2=0.78, *P*=0.12), b) DPPH radical scavenging capacity of PAN3161, (R^2=0.73, *P*=0.09).
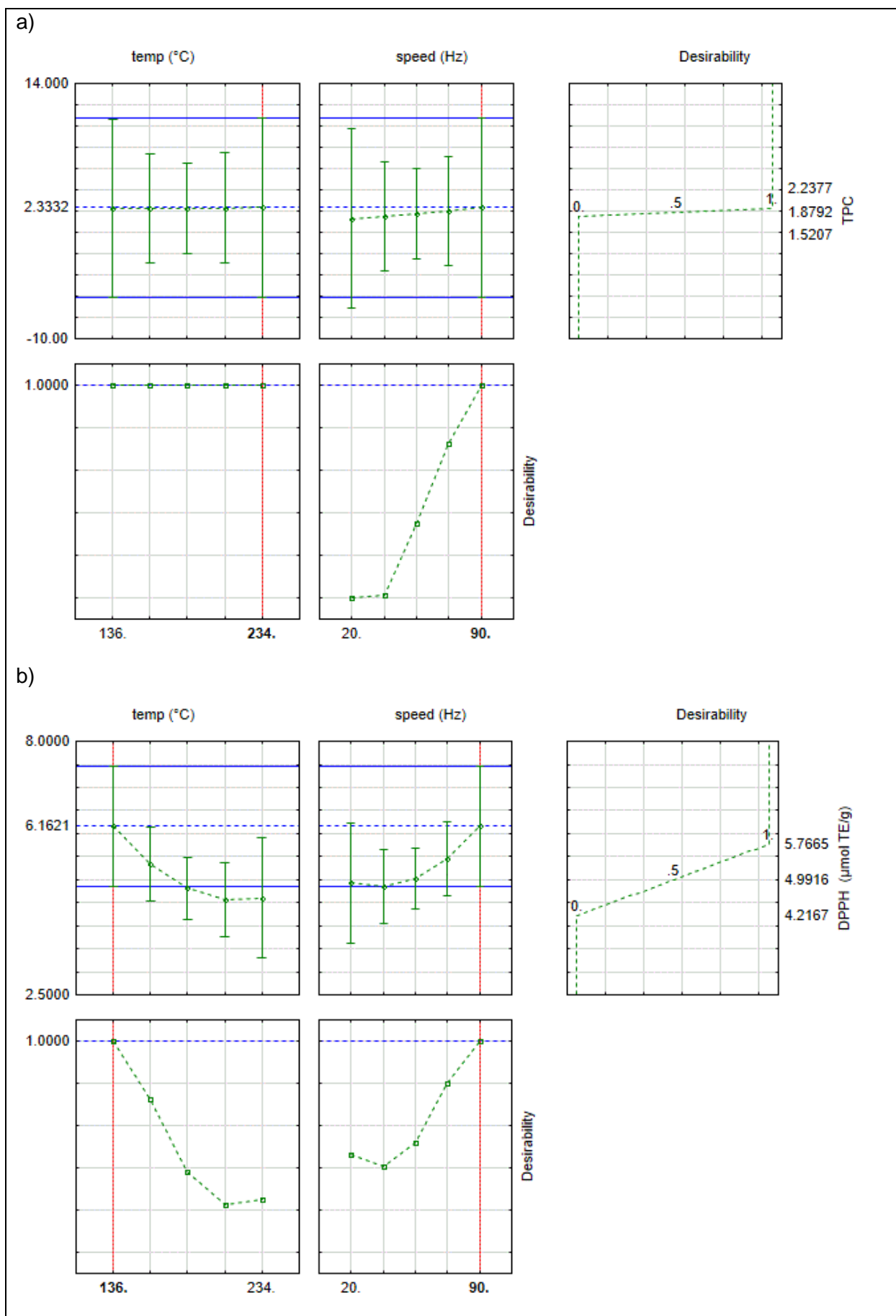
Figure 5.4. Individual prediction profile graph for optimum roasting conditions to maximise of a) TPC of PAN3379 (R^2=0.75, *P*=0.75), b) DPPH radical scavenging capacity of PAN3379, (R^2=0.61, *P*=0.08).

Conclusion

The antioxidant properties were affected during FCR for the wheat samples tested. Similarities and differences were found regarding the antioxidant properties of the two wheat cultivars studied. Significant linear effect were observed on roasting temperature, speed and the interaction among two wheat cultivars and different antioxidant assays. In contrast, no quadratic effects of the response variables were found on the antioxidant properties. This study observed the change of antioxidant properties caused by FCR, which revealed the complexity of the mechanisms of antioxidants release and degradation during thermal treatment. The study also showed that optimal processing conditions for releasing of phytochemicals in wheat varies between cultivars. In this study, PAN3161 exhibited maximum antioxidant activity when roasting at 234°C at a speed of 20 Hz, and roasting PAN3379 at 136°C at a speed of 90 Hz to maximize the antioxidant activity. The difference could be attributed to different cell wall structure that hold the bound phenolic compounds, as well as attributed to the available substrates to products new antioxidant compounds during Maillard reaction and caramelisation.

In future studies, it is advised to determine the acrylamide content to prevent the development of this potential carcinogen. Therefore the sample can be roasted at higher temperature for longer time that may produce more compounds have antioxidant properties without producing the acrylamide. However the implementation of the FCR and the use of its products still need to be assessed and perhaps may lead to a new innovation of food processing era.

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Appendix 5.1 Moisture loss of roasted wheat samples

Run number	Replicate	Cultivar	temp	speed	% moisture	% Moisture lost
5	a	PAN3161	136	55	13.28	14.06
	b	PAN3161	136	55	13.24	14.35
1	a	PAN3161	150	30	11.11	28.10
	b	PAN3161	150	30	11.14	27.93
2	a	PAN3161	150	80	12.92	16.40
	b	PAN3161	150	80	12.96	16.13
10 c	a	PAN3161	185	55	11.41	26.16
	b	PAN3161	185	55	11.52	25.48
7	a	PAN3161	185	20	6.80	56.00
	b	PAN3161	185	20	6.80	56.03
8	a	PAN3161	185	90	11.98	22.46
	b	PAN3161	185	90	12.02	22.21
9 c	a	PAN3161	185	55	10.41	32.65
	b	PAN3161	185	55	10.44	32.47
4	a	PAN3161	220	80	10.62	31.26
	b	PAN3161	220	80	10.60	31.39
3	a	PAN3161	220	30	4.46	71.15
	b	PAN3161	220	30	4.40	71.55
6	a	PAN3161	234	55	7.54	51.20
	b	PAN3161	234	55	7.65	50.52
5	a	PAN3379	136	55	13.31	13.85
	b	PAN3379	136	55	13.30	13.91
1	a	PAN3379	150	30	10.30	33.35
	b	PAN3379	150	30	10.38	32.82
2	a	PAN3379	150	80	13.37	13.48
	b	PAN3379	150	80	13.19	14.65
10 c	a	PAN3379	185	55	11.91	22.92
	b	PAN3379	185	55	11.97	22.56
7	a	PAN3379	185	20	6.66	56.90
	b	PAN3379	185	20	6.64	57.06
8	a	PAN3379	185	90	12.92	16.37
	b	PAN3379	185	90	12.67	17.99
9 c	a	PAN3379	185	55	10.44	32.45
	b	PAN3379	185	55	10.45	32.37
4	a	PAN3379	220	80	11.25	27.18
	b	PAN3379	220	80	11.24	27.28
3	a	PAN3379	220	30	6.52	57.82
	b	PAN3379	220	30	6.67	56.86
6	a	PAN3379	234	55	7.18	53.57
	b	PAN3379	234	55	7.03	54.52

Chapter 6

General discussion and conclusions

Wheat (*Triticum aestivum*) is cultivated in many countries as a staple crop, and is being used as a main ingredient in many foods. Intake of whole wheat grain is recommended to promote nutritional benefit for the human diet. Many studies have previously reported the antioxidant properties of whole wheat grains, their milling fractions and wheat based products. Some studies further showed the antioxidant properties can be effected by genotype, environment and interaction of the two. This study investigated the antioxidant properties of 26 South African wheat cultivars, and the effect of thermal processing, i.e. forced convection roasting (FCR) on the antioxidant properties of two selected wheat cultivars.

In this study, extraction of antioxidants was considered a crucial step. HCl-acidified methanol was used as the extraction solvent for determining the antioxidant properties of the 26 wheat samples. We aimed to investigate the total antioxidant properties of the South African wheats including free and bound form of phenolic compounds. Literature revealed the most reliable method would be to hydrolyse the samples with high concentration of NaOH and HCl, followed by extraction with organic solvents. However, this method is time-consuming and ideal for only a few samples. Our study included 288 samples extractions, which made the latter extraction method not practical. Thus the HCl-acidified methanol associated with ultra-sonication extraction was used. The HCl-acidified methanol had been used in several studies to determine the antioxidants in wheat (Beta *et al.*, 2005; Mpofu *et al.*, 2006). To determine the accessible antioxidant properties (e.g. free phenolic acids) of the wheat samples that was roasted using the forced convection continuous tumbling roaster (FCCTR), 64% ethanol was used as extraction solvent.

The phenolic compounds of South African wheats ranged from 0.69-0.83 mg GAE/g and DPPH scavenging capacity from 0.891 to 1.691 µmol TE/g across all regions. In comparison with earlier research, Mpofu *et al.* (2006) reported 1.71-1.99 mg GAE/g phenolic compounds in Canadian wheat samples. Zhou *et al.* (2004) reported Australian wheat bran contains on average 2.2 mg GAE/g and 0.79 µmol TE/g phenolic compounds. South African wheat had lower total phenolic content (TPC) than Canadian wheat and Australian wheat brans, however it had higher DPPH radical scavenging capacity. This indicate some antioxidant capacity might be attributed to other components present in South African wheats.

It was expected to observe lower TPC and DPPH scavenging capacity in thermal processed wheat samples, because the extraction solvent was designed to extract only the free antioxidants. On the other hand, degradation of thermal labile phenolic compounds may also have contributed to the decrease TPC of the roasted samples. Surprisingly, FCR samples had TPC ranging from 1.52-2.4 mg GAE/g, and 4.22-6.17µmol TE/g DPPH radical scavenging capacity. The unroasted samples of the same cultivars (PAN3161 and PAN3379) extracted by the HCl-acidified methanol had 0.70-

0.83 mg GAE/g TPC and 0.89-1.056 µmol TE/g DPPH radical scavenging capacity. The observed increase of TPC was magnificent, which should not only be attributed to the release of bound phenolics from cell wall components. It had to be the formation of new compounds during roasting. Literature mentioned Amadori products and hydroxymethylfurfural products of Maillard reaction and caramelisation have strong antioxidant properties (Rufián-Henares & Delgado-Andrade, 2009). It has to be mentioned, the central composite design (CCD) did not include the raw sample (unroasted sample). The effect of the roasting on wheat samples thus needs to be validated by comparing the unroasted sample with the roasted samples. This is recommended to be done in future studies.

Although it was observed that South African wheats have lower antioxidant properties than Canadian wheats, thermal processing (especially FCR) can be useful to elevate the antioxidant properties of wheat.

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