CHARACTERISATION OF CHEMICAL COMPONENTS IN MANUALLY ISOLATED ALEURONE AND ASSOCIATED LAYERS FROM MAIZE, WHEAT AND BARLEY KERNELS

By

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DEDICATION

To my husband Phillemon, children (Patrick, Abigail and Watipaso), brothers (John and Khumbo) and sister (Penjani) and to my parents who never lived to see the fruition of the hard working spirit they had cultivated in me.

THESIS FORMAT

This thesis has been prepared in the manuscript format. Referencing style used is for the journal Cereal Chemistry. The thesis has eight chapters of which Chapters 1 and 2 are overall introduction and literature Review. Chapters 3 to 7 are manuscripts at different stages of publication in peer-reviewed scientific journals. Chapter 8 provides the general discussion, conclusions and areas for further research.

Chapter 3 entitled "Ferulic acid fluorescence intensity profiles and concentration measured by HPLC in pigmented and non-pigmented cereals" by Ndolo, V. U., Beta, T. and Fulcher, R. G. 2013, was originally published in Food Research International 52:109-118.

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ABSTRACT

Health benefits related to consumption of whole grains have been attributed to their phytochemical components and micronutrient constituents. Understanding the composition, structure and distribution of these components in different cereal grains is of potential importance in aiding the selection of whole grains and their processed fractions for inclusion in the diet, and as ingredients in development of new food products. The aim of this study was to characterise and compare the composition and distribution of phenolic acids, carotenoids, amino acids and micronutrients in whole grain cereals and botanical fractions of yellow maize, barley, wheat and oat. Manual separation, a tedious and laborious technique that yields purified or concentrated fractions, suitable for compositional analysis, was used to separate whole grains into pericarp (inner+outer pericarp, testa, nucellus), aleurone layer, germ and endosperm fractions. Identification and quantification of tissue components was subsequently accomplished by several techniques including spectrophometry, HPLC, ICP-AES and LC-MS/MS for further confirmation of molecular structure. The study also explored the possibility of using spectral characteristics (primarily fluorescence intensity values) to provide rapid estimates of the concentrations and distribution of ferulic acid (FA), a major phenolic compound in cereal grains. While composition of phenolic acids and carotenoids was similar the distribution was significantly different ($P < 0.05$) among cereal types and grain fractions. Phenolic acids were concentrated in pericarp and aleurone fractions. The germ was intermediate while the endosperm had the lowest levels. Yellow maize exhibited the highest values. Carotenoids, lutein and zeaxanthin were concentrated in the germ and aleurone layer of wheat, barley and oat but zeaxanthin was not found in endosperm. Lutein and zeaxanthin were concentrated in the endosperm and aleurone layer of yellow maize. This is the first study to report on carotenoids

composition of aleurone fractions. Protein content in aleurone layers was highest in purple barley followed by yellow maize and MSUD wheat. Mineral elements, thiamine (vitamin B1), niacin (vitamin B3) and the essential amino acids (EAA) were more concentrated in the aleurone layer than endosperm fractions except for leucine in yellow maize. Macro- and micromineral elements, thiamine and niacin were generally higher in wheat aleurone than in purple barley and yellow maize. The findings suggest that yellow maize aleurone layers have potential as alternative food ingredients to the commercial wheat aleurone. A positive, significant correlation ($r= 0.421$, $p <0.0001$) was found between fluorescence intensity values and ferulic acid (FA) concentration. Therefore, fluorescence intensity profiles are a promising approach for rapid assessment of FA concentration in grain *in-situ*. Overall this work has provided information that would act as a database for selection of cereal fractions and guide the miller to obtain grain fractions with enriched levels of phytochemicals and micronutrients including phenolic acids, carotenoids, mineral elements, and amino acids.

CHAPTER 1

Introduction, Hypotheses and Objectives

1.1 Introduction

Cereals grains have been part of the human diet for centuries. Over the years, scientists seem to agree that inclusion of whole grains in the diet has protective effects against the development of diet-related disorders (Jacobs et al. 2000; Slavin 2004) hence contributing to promotion of human health (Dykes and Rooney 2007). These protective effects are attributed in part to fiber and bioactive compounds (phytochemicals) in the whole grains (Jacob et al. 2000; Jensen et al. 2004; Liu 2007). Phytochemicals of importance in whole grains include phenolic acids, carotenoids, flavonoids, β-glucan, vitamin E compounds and lignin, among others (Liu 2007; Sidhu et al. 2007). Therefore, understanding the composition and distribution of these phytochemicals in different cereal grains or grain fractions is important in the selection of appropriate grain fractions for inclusion in the diet and as ingredients in food product development.

The distribution of phenolic acids can be measured *in-situ* in grains using microspectrofluorometry (Pussayanawin et al. 1988; Saadi et al. 1998; Sen et al. 1994) and flourescence microscopy. Use of fluorescence microscope is underutilised despite its high resolution, sensitivity and potential chemical specificity (Autio and Salmenkallio-Marttila, 2001; Wood and Fulcher, 1983). Its use has been limited to localisation of FA, identification of pericarp or aleurone in milling fractions (Fulcher et al. 1972b; Fulcher and Wong 1980; Pussayanawin et al. 1988). However, fluorescence intensity (FI) is often directly proportional to the concentration of the fluorescing compounds (Guilbault 1989; Waters 2009). Therefore,

fluorescence microscope would not only be used to visualise the locations and distributions of FA within the grain, but also to estimate FA concentrations using fluorescence intensity values in grain fractions with potential as food ingredients.

Phenolic acids and carotenoid composition and distribution have been determined mainly in mechanically separated grain fractions from wheat (Abdel-Aal et al. 2002; Bellido and Beta 2009; Beta et al. 2005; Fratianni et al. 2005; Zielinski and Kozlowska 2000), barley (Nordkvist et al. 1984; Hernanz et al. 2001; Zielinski and Kozlowska 2000), maize (Kean et al. 2008; Panfili et al. 2004) and in a few hand dissected grain fractions (Barron et al. 2007; Hemery et al. 2007). However, the challenge with mechanical separation is that fractions are not homogenous, whereas manual separation of whole grains likely yields tissue samples of much higher purity which are consequently more suitable for compositional analysis (Brouns et al. 2012). Thus, the latter would provide a representation of original composition and distribution of these bioactive components within the grain. This knowledge is of importance in identification, separation and selection of fractions for use as food ingredients.

Additionally, data in the literature often show variation in the content of these bioactive compounds in wheat, barley and maize tissues from one author to another. This variation may be due to differences in the extraction and analytical methods, to genetic variation and variable environmental influences. Sample characteristics may also vary, ranging from hand-dissected tissues to the entire bran or different milling fractions. Furthermore, phenolic acids and carotenoid content in whole grains or grain fractions are commonly analysed in isolation or focussing on one grain type and its varieties (Beta et al. 2005; Fratianni et al. 2005; Hernanz et

al. 2001; Kean et al. 2008). Such variations pose a challenge when comparing the composition and concentration of bioactive compounds. Therefore, a systematic study which utilises samples with relatively similar characteristics and performed under the same experimental conditions would not only contribute to development of a more strictly comparable database but would also enhance our understanding of the variations in the distribution and composition of these health promoting components in different grains and grain fractions.

Cereal aleurone layers comprise most of the physiologically beneficial substances (Atwell 2010) including several micronutrients. Studies have shown that wheat aleurone layers, as commercialised food ingredients, offer a rich source of B-vitamins and minerals (Buri et al. 2004; Pomeranz 1973; 1988). However, not much is known about the nutritional composition of aleurone layers from other cereals. Therefore studies to evaluate the nutritional composition of aleurone from other cereals as alternative food ingredients, are essential. In addition, the structure, concentration and distribution of niacin, the most abundant B-vitamins in the aleurone layer has not been well characterised. Therefore part of this work will elucidate the structure of niacin using LC-MS/MS.

In summary, as consumers become more aware of benefits of health foods, focusing on cereal fractions with potential health benefits is therefore essential (Beta et al. 2005; Hemery et al. 2007; Jones et al. 2002). Use of grain fractions requires knowledge of both their chemical composition and distribution patterns within the grain. Therefore, understanding the variations in the distribution of these bioactive components and nutrients in the different grain fractions is an area of interest (Liu, 2007) if the food industry is to produce health foods that meet consumer

expectations as well as milling fractions with enhanced levels of these bioactive compounds. Therefore, the following hypotheses and objectives were used to guide the investigations:

1.2 Hypotheses

1) Fluorescence microscopic imaging can be used to determine the distribution and concentrations of fluorescent phenolic compounds in grains.

2) Manually separated botanical fractions may provide a representation of the original composition and distribution of phenolic acids and carotenoids within the grain

3) There are substantial differences in amino acid, mineral and B-vitamin composition and concentration in the aleurone layer and endosperm fraction of whole grains.

1.3 Objectives

The overall aim of this research was to manually separate the whole grain and examine the variations in the composition of selected phytochemicals and micronutrients in the aleurone layer and other grain fractions from wheat, barley, yellow maize and oat. Specifically the study;

1) Visualised the distribution ferulic acid in grains *in-situ* and explored the possibility of using florescence intensity grey level values to predict FA concentrations;
2) Investigated the distribution and composition of phenolic acids and carotenoids in manually separated grain fractions (pericarp, aleurone layer, germ and endosperm) from wheat, barley, yellow maize and oats in order to identify which fraction is the major contributor to health benefits of whole grains;
3) Compared the protein, amino acid, B-vitamins (thiamine, niacin, riboflavin) and mineral contents of wheat aleurone to that of samples of purple barley and yellow maize aleurone; and

4) Confirmed the structure of niacin in aleurone layers of yellow maize, normal barley and wheat kernels using LC-MS/MS

CHAPTER 2

Review of the Literature

This chapter briefly describes the structure of the grain, chemical composition, cereal grain utilisation and the association between whole grains and health. It also describes the distribution across the grain kernel of different types of grain components associated with health benefits. Lastly, it reviews the role of microscopy in cereal research.

2.1 Grain structure

Cereals are one-seeded fruits, produced by a grass family Gramineae (Hoseney 1986). Industrial processing of cereals, most commonly by milling or similar methods, typically produces three components (**Figure 2.1**). The largest fraction is the starchy endosperm (the bulk of the refined flour). The proportions of these anatomical parts vary widely in different types of grains, (**Table 2.1**) (Evers et al. 1999; Hoseney 1986). Two additional and typical fractions, the bran and germ are composed of outer kernel coverings (the bran), and the embryonic tissues that include a new plant and associated specialised nutrient-containing tissue (the germ). These industrial fractions are quite different in their organisation and biochemistry (Pascoe and Fulcher 2007) in turn providing, unique challenges to processors, scientists, and consumers.

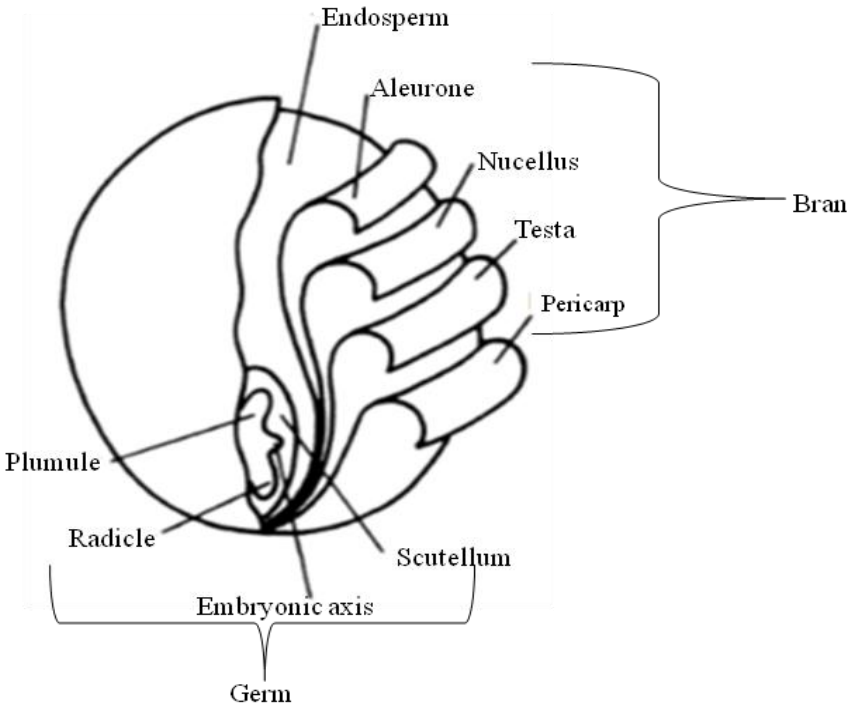

Figure 2.1 **Generalised grain showing main common characteristics adapted from Kent and Evers 1994 (**http://www.knovel.com.proxy1.lib.umanitoba.ca**)**

The pericarp is made up of multilayer peripheral tissues consisting of epicarp- mesocarp and endocarp (comprising cross cells and tube cells) (Evers et al. 1999; Serna-Saldivar 2010) (**Figure 2.1**). The pericarp surrounds the kernels and protects it against insects and pathogens attack and prevents grain dehydration (Serna-Saldivar 2010). The pericarp is a rich source of dietary fiber and cell-wall bound phenolic compounds (Slavin et al. 2001; Serna-Saldivar 2010). Below the tube cells is the testa (seed coat) followed by nucellar epidermis (hyaline layer) and the aleurone layer (**Figure 2.1**). The aleurone layer is a viable, enzyme-, mineral-, and vitamin-rich layer that is both protective and physiologically active in the seed, providing numerous nutrients, enzymes, and antioxidants (Fincher 1998; Fulcher et al. 1972a,1972b; Serna-Saldivar 2010). The aleurone layer, nucellus, and testa\pericarp complex collectively form the "bran" fraction of milled grains.

Each of these layers ultimately provide tough, antioxidant-rich fiber that resists fungal, bacterial, and insect attack (Bily et al. 2003; García-Lara et al. 2004; McKeehen et al. 1999), while providing many nutrients for a potentially new cereal. More importantly, each of these layers is quite distinctive in terms of biochemical composition (Pascoe and Fulcher 2007), hence providing equally distinctive nutritonal attributes to the human or livestock diet.

Table 2.1 Percentage contribution of different parts of cereal grain to whole grain

Cereal		Bran*	Pericarp	Aleurone	Endosperm	Germ
		(Pericarp + Testa + Aleurone)				
Maize	Dent		6.0	2.8	78	12
	Flint		6.5	2.2	79.6	11.7
Maize*		5.1-6.5			79.7-82.0	11.7-15.2
Wheat	Hard		8.2	6.7	82	3.6
	Durum		12	na	86.4	1.6
Wheat*		14.1-15.9		81.4-84.1		2.5-3.6
Barley	Hulled		2.9	4.8	76.2	3.0
	Hull-less		3.3	5.5	87.6	3.4
Barley*		8.8			87.6	3.4
Oat		12	12	na	84	3.7

Adapted from Serna-Saldivar (2010) and *Kent and Evers 1994

Each of these individual tissue are closely associated with each other and have different thickness. In wheat, the aleurone layer is the thickest measuring up to 65 μm, the outer pericarp is of intermediate thickness (15–30 μm), and the seed coat is the thinnest (5–8 μm) (Barron et al. 2007). In maize, the pericarp is thicker than the aleurone layer. Antoine et al. (2004) demonstrated that during manual separation, the outer layer strip corresponds to outer pericarp, the intermediate layer corresponds to several tissues consisting of the inner pericarp, testa and nucellus tissue and the inner strip corresponds to the aleurone layer.

Botanically, the aleurone is the outermost component of the starchy endosperm (Hoseney 1986). However, as an industrial product, it is also described as the innermost layer of the bran (Serna-Saldivar 2010). Aleurone cell size, cell wall thickness and shape vary among different types of grains. Wheat, oats, maize and sorghum have a single-celled aleurone layer while barley and rice have multiple two- to three-celled aleurone layers (Evers et al. 1999; Rooney et al. 1983). Such variations contribute to differences in percentage proportion of aleurone layer in the bran (**Table 2.1**). In addition, variation in number of aleurone layers has been implicated in differences in concentrations of some chemical components such as proteins (Wolf et al. 1972) and niacin (Teas 1952) in maize. Chemical components present in the aleurone layer include protein, B-vitamins, minerals, fat deposits, phytic acid and phenolic compounds (Evers et al. 1999; Hoseney 1986; Meziani et al. 2012; Serna-Saldivar 2010).

Several scholars have studied the structure and chemical components of wheat and barley aleurone layers, either in grain *in-situ* or in isolated fractions (Antoine et al. 2004; Bacic and Stone 1981; Evers and Millar 2002; Fulcher et al. 1972b; Jacobsen et al. 1971; Parker et al. 2005; Rhodes et al. 2002). Wheat aleurone is a rich source of both nutrients and phytochemicals as summarised in a review (Brouns et al. 2012). About 15% of the total protein and 80% of the total minerals in wheat are found in wheat aleurone (Pomeranz 1973; 1988). In addition, the wheat aleurone is a rich source of the B-vitamins (Buri et al. 2004). The wheat aleurone also has higher content of ferulic acid than the endosperm fraction (Adom et al. 2005; Antoine et al. 2003). In barley, studies focussed on understanding aleurone structure and chemical identification in-*situ* (Bacic and Stone 1981; Jacobsen et al. 1971). Mineral composition (Liu and Pomeranz 1975; Liu et al. 2007; Singh et al. 2014; Stewart et al. 1988) and protein content (Macewicz et al. 2006;

Sullivan et al. 2013; Yupsanis et al. 1990) of barley aleurone have been reported. Studies using maize, on the contrary, focussed on the development of aleurone layer and subaleurone layers (Kyle and Styles 1977) and its phytosterol content (Moreau et al. 2000). Some studies have also examined protein content and amino acid profiles of maize aleurone layer (Landry and Moureaux 1980; Wolf et al. 1972). Phenolic acid profiles in maize bran have been reported (Chiremba et al. 2012; Zhao et al. 2005). However, maize aleurone layer has not been targeted for analysis of phenolic acids and carotenoid profiles despite the high concentration of the former in the maize bran.

Endosperm: The endosperm is the largest tissue of the grain (Evers et al. 1999). The size of the endosperm is relatively the same in different types of grains, constituting greater than 80% of the grain weight (**Table 2.1**). The endosperm is packed with starch granules which are surrounded by a protein matrix. The major chemical component is carbohydrate. The endosperm also contains some vitamins and minerals. The starchy endosperm consists of larger cells compared to those in the aleurone layer and varies in size and shape within the grain and in different types of grains (Hoseney 1986). The endosperm is divided into the peripheral, also known as "subaleurone endosperm", the vitreous and floury endosperm (Serna-Saldivar 2010).

Germ: The germ is made up of the scutellum and embryo. The size of the germ varies among the different types of grain (**Table 2.1**). Maize has the largest germ. Unlike the wheat and maize germ, oat germ is longer and narrower taking up to a third of the length of the whole groat (Hoseney 1986). The germ contains high oil content, some proteins, B-vitamins and vitamin E

(α-tocopherol and α-tocotrienol). The germ also contains considerable amounts of phenolic acids (Sen et al. 1994).

2.2 General characteristics of cereal grains

Although all cereals have similar botanical structure, there are minor features that differentiate them and contribute to their uniqueness in size, shape and proportions of the different chemical components. The size and weight of grains (kernels) vary depending on the type of grain. The average length of a wheat grain is 8 mm; it weighs about 35 mg and has a crease on its ventral side (Hoseney 1986). Barley grain weighs about 35 mg on average. It may be hulled or hulless. The maize kernel is the largest among cereals weighing about 300-400 mg.

Another important characteristic of grains is the kernel colour. Cereal grains can either be pigmented or non-pigmented. The pigmented grains vary in colour from orange, yellow, red, blue to purple and black. Pigmented cereals are important as a source of natural colorants and antioxidants although they are currently being produced only in small amounts (Abdel-Aal et al. 2006). In New Zealand, purple wheat has been in use since 1980 as a colorant on the surface of bread (Bezar 1982) while blue and red maize are used in production of tortillas (Cortes-Gomez et al. 2005) as well as alote, gruels and tortilla chips. The colours are due to the presence of anthocyanins and carotenoids. Anthocyanins are a major group of flavonoids in cereals (Dykes and Rooney 2007). They are the primary pigments responsible for blue, red and purple colours of the grains (Abdel-Aal et al. 2006; Dykes and Rooney 2007; Siebenhandl et al. 2007). Anthocyanins are found either in the pericarp or aleurone layer of wheat and barley (Siebenhandl et al. 2007). Carotenoids are also natural pigments that are present in some cereals. They are

responsible for the orange and yellow colours. Carotenoids will be discussed in detail under phytochemicals in grains (Section 2.5.2).

2.3 Cereal grain utilisation

The major cereal grains in the human diet are wheat, maize and rice while minor contributors constitute barley, oat, rye, sorghum, millet and buckwheat (Liu 2007). Wheat contributes about 20 to 80% of the total food consumption of different regions of the world (Sidhu et al. 2007) mostly in the form of baked products. Although maize is used mainly as feed in the United States (US) and Canada, it is a major food crop in developing countries. However, the small percentage (10-11%) used for food in the US is converted to various maize products that are used as food ingredients (Rooney and Serna-Saldivar 2003). Two-thirds of barley is used for feed, one-third for malting and about 2% for food (Baik and Ullrich 2008). Recently there has been a renewed interest in use of barley or its fractions in cereal-based foods due to its nutritional value and health benefits (Baik and Ullrich 2008; Sullivan et al. 2013). Barley has low fat, fairly well balanced amino acids, minerals, vitamins, polyphenolic, and soluble and insoluble dietary fiber. In addition barley is a rich source of β-glucans associated with lowering blood cholesterol levels (Baik and Ullrich 2008).

Most cereals grains are processed before they are consumed or used as food ingredients. Wheat is milled to produce refined flour that is used in various baked products and semolina for production of pasta. Barley is abraded to produce pearled barley that is further processed into grits, flakes and flour (Baik and Ullrich 2008). Pearled barley has been used as an ingredient in different food products such as soy paste and sauce (Slavin et al. 2000). Barley flour has been

applied in wheat-based products including pasta (Marconi et al. 2000). Similarly, milling of maize produces maize meal, maize grit and maize flour. The latter is used mostly as an ingredient in making dry mixes such as pancakes, muffins and doughnuts, and ready-to-eat breakfast cereals (Serna-Saldivar 2010). Dry milling is commonly done to separate the outer layers (bran) and germ from the endosperm (Peyron et al. 2002). However, the bran and germ contain most of the micronutrients and phytochemicals that have been associated with whole grain consumption and health (Slavin et al. 2001).

2.4 Whole grains and health

Epidemiological studies have shown that a diet which contains whole grains and its products is associated with reduced risk for developing coronary heart disease (CHD), type 2 diabetes and certain cancers (Jensen et al. 2004; Liu et al. 1999; Slavin 1994). Jensen et al. (2004) studied the effects of consuming whole grains, bran, and germ and the risk of coronary heart disease in men. The group that included bran had a lower risk compared to those consuming whole grain. The same authors suggested that bran components would be the key factor in this relationship. Several components concentrated in the bran may work synergistically to bring about the positive health outcomes (Jensen et al. 2004; Liu 2004). These health benefits have partly been attributed to phytochemicals in whole grains.

Among the phytochemicals in grains, phenolic acids are the most abundant while carotenoids are minor components. Phytochemicals in grains complement those in fruits and vegetables and may have synergistic effects in the provision of health benefits (Liu 2004; Slavin 2004). Cereals are also excellent sources of many micronutrients, especially B-vitamins and minerals (Serna-

Saldivar 2010). When use of refined grains increased in the early 20th century, nutritional shortages of B-vitamins resulted in a number of historical global deficiency diseases such as beri-beri and pellagra due to lack of thiamine and niacin, respectively (Bollet 1992). Several literature reported that health benefits of whole grains are related to their rich nutrient content, fiber and phytochemicals (Jensen et al. 2004; Slavin 2004; Slavin et al. 2001). Thus the 2005 Dietary Guidelines for Americans and Health People 2010 recommended consumption of at least three servings of whole grains per day (Liu 2007). The American Association of Cereal Chemists International, (AACC) 1999 described whole grains as follows:

> *''whole grains shall consist of the intact, ground, cracked, or flaked caryopsis, whose principal anatomical components—the starchy endosperm, germ, and bran—are present in the same relative proportions as they exist in the intact caryopsis''.*

As consumers become more aware of the importance of diet and health, the food industry is responding by producing whole grain products that either contain an intact whole grain or reconstituted whole grain. A reconstituted whole grain product contains all the fractions of a whole grain recombined in relative proportions as naturally occurring in the grain kernel (Okarter and Liu 2010). However, individual grain fractions such as whole bran, some parts of the bran (aleurone) and germ have been utilised as food ingredients in the development of grain-based products with increased functional and nutritional potential (Atwell et al. 2007; Hemery et al. 2010; Izydorczyk et al. 2014). The bran and the germ have significant amounts of phytochemicals and nutrients (Jones et al. 2002; Slavin et al. 2001). Therefore, in order to separate grain fractions that have enhanced levels of micronutrients and phytochemicals it is important to have an understanding of the chemical composition of the anatomical / botanical parts of the kernel.

2.5 Chemical composition of cereal grains

Cereal grains are complex biological systems with diverse biochemical constituents that are synthesized and packaged in different grain tissues (Fulcher and Wong 1980). Cereal biochemical constituents include carbohydrates, proteins, lipids, vitamins, minerals and non-nutrient phytochemicals (Hoseney 1986; Liu 2007; Serna-Saldivar 2010; Shahidi and Naczk 2004). The composition and distribution patterns of these components in different types of grains vary widely (Pascoe and Fulcher 2007) and even within the grain kernel (Fardet 2010; Kent and Evers 1969).

2.5.1 Phytochemicals

Phytochemicals are naturally occurring bioactive non-nutrient compounds that are classified into carotenoids, phenolic compounds, alkaloids, nitrogen-containing compounds, and organosulfur compounds (Liu 2004). They are found in fruits, vegetables and grains (Liu 2004). Phenolics and carotenoids are the most commonly studied in cereal grains.

2.5.1.1 Phenolic compounds

Phenolic compounds are the main phytochemicals found in whole grains. They include phenolic acids, flavonoids, anthocyanidins and phytosterols (Liu 2007; Serna-Saldivar 2010; Shahidi and Naczk 2004; Siebenhandl et al. 2007; Zielinski and Kozlowska 2000). Phenolic acids are the most abundant in whole grains (Liu 2007) and are grouped into hydroxycinnamic and hydroxybenzoic acids (Shahidi and Naczk 2004). Hydroxycinnamic acids include ferulic, sinapic, *p*-coumaric and caffeic while hydroxybenzoic acids include *p*-hydroxybenzoic, protocatechuic, vanillic and gallic acids (Liu 2004; Sosulski et al. 1982).

Ferulic acid (trans-4-hydroxy-3-methoxycinnamic acid) (FA) is the most abundant phenolic acid in cereal grains (Abdel-Aal et al. 2001; Maillard and Berset 1995). FA exists in free, soluble and bound forms, in the ratio 0.1:1:100, respectively (Adom and Liu 2002). It is largely bound to arabinoxylans at the O-5 position of the arabinose units (Bunzel et al. 2005). Over 97% of FA in 11 wheat varieties was in bound form (Adom et al. 2003). Of the total bound phenolic acids in barley, 68% was ferulic acid (Andersson et al. 2008). Within the grain, FA is concentrated in the aleurone, pericarp and embryo cell walls. Its characteristic autofluorescence under ultraviolet light (Pussayanawin et al. 1988) was used to localise FA in the aleurone cell wall (Fulcher 1972) as well as its distribution within the grain (Saadi et al. 1998).

Phenolic acid composition of different types of whole grains have been determined (Abdel-Aal et al. 2006; Adom and Liu 2002; Adom et al. 2005; Arranz et al. 2010; Beta et al. 1999; Del Pozo-Insfran et al. 2006; Dykes and Rooney 2007; Sosulski et al. 1982). Different phenolic acids including ferulic, sinapic, *p*-coumaric, caffeic, *p*-hydroxybenzoic, protocatechuic, vanillic and gallic acid are present in these grains in varying concentrations. Phenolic acids are unevenly distributed within the grain (Evers et al. 1999; Fardet 2010), being concentrated in grain outer layers. In wheat, rye and oats, the range of total phenolic acids were 472-1366 µg/g and 651-4527 µg/g in whole grains and bran, respectively (Mattila et al. 2005). Furthermore, dehydrodiferulic acid content was 10 to 20 times more in outer layers (aleurone/bran) than in the starchy endosperm (Lempereur et al. 1998).

2.5.1.1.1 Distribution of phenolic acids in grain fractions

Understanding of the distribution of phytochemicals in grains is critical in guiding the separation of grain fractions rich in these components (Sidhu et al. 2007). Dry fractionation methods,

referred to as mechanical separation such as pearling, debranning, dehulling, abrasive milling, roller milling, sieving and air classification are used to obtain milling fractions with different characteristics (Bellido and Beta 2009; Beta et al. 2005; Hemery et al. 2011; Hemery et al. 2007; Izydorczyk et al. 2014). The challenge with mechanical separation is that the collected fractions are not homogenous. Variations in the characteristics of fractions obtained by different debranning processes are summarised in the review by Hemery et al. (2007). In addition, Hemery et al. (2009) demonstrated that fractions obtained by different mechanical separation techniques comprised blends of different grain outer layers which affect their chemical composition. In contrast, it is assumed that manual dissection most likely yields relatively pure histological parts and are recommended for compositional analysis at laboratory scale (Barron et al. 2007; Brouns et al. 2012). To obtain homogenous fractions, Barron et al. (2007) manually separated wheat grains into seven components and studied their relative amounts, carbohydrate and phenolic acid composition. However, manual separation has disadvantages too. When manual separation is preceded with a soaking step, there is a possibility of loss or redistribution of soluble material (Pomeranz 1988). In addition, manual separation is labour intensive, challenging and time consuming for acquisition of adequate amounts of samples needed for extensive analysis.

As a result evaluation of the distribution and composition of phenolic acids in grains utilising manually separated fractions is limited and mainly focussed on wheat grains (Barron et al. 2007; Parker et al. 2005). Parker et al. (2005) manually separated wheat bran into four layers. Out of the six monomeric phenolic acids that were identified, four that were found in highest concentration included ferulic acid, protocatechuic, *p*-hydroxybenzoic acid and vanillin. Wheat

grain was manually separated into wheat bran, pericarp and aleurone and analysed for phenolic acid composition to identify chemical components for use as biomarkers during fractionation or milling process (Antoine et al. 2004; Hemery et al. 2009). However, not much has been documented on phenolic acid profiles in manually separated fractions from maize, barley and oat grains. Maize (Guo et al. 2013) and maize bran (hulls) (Chiremba et al. 2012) are known for their high ferulic acid and dehydrodimers. Despite the finding that ferulate-phytosterol esters (FPEs) are localised and found in high concentrations in maize aleurone layer (Moreau et al. 2000), little is known about the phenolic acid composition of this fraction.

Research into the distribution and composition of phenolic acids has mainly utilised mechanically separated fractions (Bellido and Beta 2009; Beta et al. 2005; Gallardo et al. 2006; Hernanz et al. 2001; Zhou et al. 2004a; Zhou et al. 2004b; Zielinski and Kozlowska 2000). The fractions analysed in these studies varied widely and included whole bran, hulls, bran shorts, endosperm, middlings (M), milling fractions (F1-F3), aleurone-rich and pericarp-rich. Thus comparing data from different research groups is generally a challenge considering that mechanically separated grain fractions include varying combinations. For example, whole wheat bran contained 48% aleurone, 18% pericarp, 9% starchy endosperm, and 26% of an intermediate layer whereas the aleurone-rich and pericarp- rich fractions were composed of 78% aleurone and 71% pericarp, respectively (Hemery et al. 2009). In addition, barley fine hulls (FNH) obtained after dehulling and sieving comprised germ, pericarp and testa (Liu et al. 2007).

In wheat milling fractions obtained by pearling and roller milling, ferulic acid was found to be higher in whole wheat bran and milling fractions than flour fraction (Beta et al. 2005; Peyron et

al. 2002). The aleurone layer had significantly higher content of ferulic acid, *p*-hydroxybenzoic acid as well as vanilic, syringic and coumaric acid compared to whole grain and bran of Swiss red wheat (Zhou et al. 2004b). Hydroxycinnamic acid profiles and distribution were also determined in bran, germ, middlings and husks of wheat, rye and buckwheat, (Gallardo et al. 2006) and in 3 fractions from 11 barley varieties namely F1, comprising of husk and outer layers; F2, intermediate fraction; and F3, consisting mainly of the endosperm (Hernanz et al. 2001). In the latter study, it was not established which fraction had higher percentage of the aleurone layer. However, a study by Nordkvist et al. (1984) reported that barley composite fractions with high percentage of aleurone cells had the highest concentration of ferulic acid whereas highest concentrations of *p*-coumaric acid were found in fractions with higher percentage of husks. This observation indicates differences in distribution of these components within barley grain.

Secondly, studies on distribution of phenolic compounds have mainly used wheat grains and barley to some extent but rarely oat and maize fractions. Wheat bran has been extensively studied (Kim et al. 2006; Parker et al. 2005; Verma et al. 2009; Zhou et al. 2004a; Zhou et al. 2004b) while maize bran (Chiremba et al. 2012), barley bran (Siebenhandl et al. 2007) and oat bran (Peterson et al. 2001) have received less attention. For maize, phenolic acid profiles have been reported in whole bran and flour fractions (Chiremba et al. 2012) but not in the aleurone layer. In oat, total phenolic and antioxidant activity have been studied in different fractions (Peterson et al. 2001, Gray et al. 2000) while phenolic acid profiles are not well documented.

Overall, phenolic acid composition and distribution have been extensively studied in mechanically separated grain fractions of some grains and in individual grains. The extraction conditions in these studies varied widely, NaOH concentration ranged from 1- 4M and extraction time from 1-16 hours and temperatures ranging from 25-140°C. In addition chemical composition and concentration of phenolic acids are influenced by several factors, including grain type, variety, part of grain sampled, genotype and the environment used for pre-harvest grain production (Adom et al. 2003; 2005; Fernandez-Orozco et al. 2010; Zhou et al. 2004a). Furthermore, distribution and composition of phenolic acids in aleurone layer and other fractions from barley and maize grains are not well documented. Therefore, studies to examine phenolic acid profiles in aleurone layer and other fractions from diverse cereal grains under similar conditions are essential.

2.5.1.2 Carotenoids

Carotenoids are responsible for the yellow, orange and red colours of plants and grains. They are subdivided into hydrocarbons (β-carotene and α-carotene) and xanthophylls, that is, their oxygenated derivatives (lutein, zeaxanthin, β-cryptoxanthin) (Liu 2004). In human health, they are recognised for their provitamin A activity and antioxidant properties (Liu 2004). Carotenoids are a minor constituent in cereals (Irakli et al. 2011). Lutein and zeaxanthin are the most abundant carotenoids while β- and α-carotene are found in minimal amounts (Heinonen et al. 1989; Hentschel et al. 2002; Panfili et al. 2004). Lutein and lutein esters amounted to >90% of the yellow pigment compared to approximately 1% of β-carotene in wheat (Lepage and Sims 1968).

Carotenoid content and composition have extensively been studied in whole grain wheat (Abdel-Aal et al. 2007; Adom et al. 2003; Hentschel et al. 2002; Panfili et al. 2004) and yellow maize or maize (Kean et al. 2008; Kimura et al. 2007; Kurilich and Juvik 1999; Luterotti and Kljak 2010). However, information on carotenoid composition in whole grain oat (Panfili et al. 2004) and barley (Goupy et al. 1999; Heinonen et al. 1989) as well as their fractions is scant.

2.5.1.2.1 Distribution of carotenoids in grain fractions

Similar to phenolic acids, most research on the distribution and composition of carotenoids of wheat grains (Adom et al. 2003; Borrelli et al. 2008; Heinonen et al. 1989; Hidalgo and Brandolini 2008; Panfili et al. 2004; Zhou et al. 2004a) and maize (Kean et al. 2008; Luterotti and Kljak 2010) used mechanically separated fractions. In wheat, the fractions included bran, flour, endosperm, germ and aleurone-rich flour whereas in maize, it was yellow maize bran, maize meal, grit, and flour. These studies reported varying levels of lutein, zeaxanthin and β-carotene based on fresh weight or dry weight basis. The variations were attributed to differences in type of fraction analysed as well as growing condition. In the study by Zhou et al. (2004a) carotenoids composition also differed in wheat bran samples from four different countries indicating effect of environmental conditions on chemical composition. Thus, lutein and β-cryptoxanthin were found in all tested samples, zeaxanthin in six and β-carotene only in four samples. In addition, different extraction solvents such as water saturated butanol, methanol, mixture of potassium hydroxide and ethanol and tetrahydrofuran and extraction methods, hot versus room temperature saponification were used. These studies mostly utilised one cereal and the above variations make comparisons of results a challenge. Therefore comprehensive studies that utilise multiple grains are important to generate a good database for use in selection of raw materials suitable for development of carotenoid-rich fractions.

Although levels of lutein and β-carotene in wheat bran and aleurone rich wheat flour were comparable (Heinonen et al. 1989), separate aleurone fractions were not analysed. It could be inferred that the carotenoids may have been from the aleurone layer fraction. However, in wheat aleurone, carotenoid composition and concentrations are not known. Furthermore, there is a dearth of information on carotenoid composition in manually separated fractions of maize, oat and barley fractions. To understand the distribution and composition of carotenoids in these grains the study utilised manually separated fractions including wheat aleurone layer fraction.

2.5.4 Nutritional components in cereal grains

2.5.4.1 Proteins

In cereals, proteins are the second largest chemical components after carbohydrates. Cereals supply about 50% of the world's protein intake (Serna-Saldivar 2010). Wheat, oat, maize and barley are good sources of proteins but oats have uniquely high amounts. Thus, the range of protein content in flint maize, soft wheat, barley and oats is 9.5 to 12%, 8.0 to12.0%, 7.5 to 15.6 % and 12.4 to 24.4%, respectively (Serna-Saldivar 2010). Proteins in cereals are classified into four groups according to solubility albumins, globulins, prolamins, and glutelins (Delcour and Hoseney 2010**)**. Albumins and globulins are concentrated in the aleurone cells, bran and germ whereas prolamins and glutelins, called storage proteins are restricted to the endosperm (Delcour and Hoseney 2010). However, a recent study on characterisation of wheat (*Triticum aestivum L.*) bran protein reported that some of the glutelins are entrapped in aleurone cells (De Brier et al. 2015).

2.5.4.1.1 Distribution of proteins

Proteins are concentrated in the aleurone layer and germ fraction with low amounts found in the endosperm (Delcour and Hoseney 2010). However, in total, the endosperm contains a high

percentage (70-80%) because it is the largest botanical part of the kernel (Serna-Saldivar 2010). The distribution of protein content in the germ, endosperm and bran of maize kernel is 17-20, 8-9 and 4-6%, respectively (Lawton and Wilson 2003) whereas in wheat kernel, the aleurone layer and germ contain 30% proteins, and the starchy endosperm and bran have approximately 13 and 7%, respectively (Belitz et al. 2009). In addition, air-classified fractions of flour milled from hard red winter (HRW) had protein content that was higher in the outer endosperm (45%) than in the inner endosperm (11%) (Kent and Evers 1969). It may be inferred from these results that the outer endosperm probably encompassed the aleurone layer. Protein content in mechanically separated middlings, bran and endosperm fraction from barley have been reported with the former two fractions containing 2-fold higher levels than the latter (Sullivan et al. 2010). Although there is valuable information on distribution of protein content in wheat, maize and barley, this is obtained from mechanically separated fractions with limited studies utilising manually separated fractions. Thus, Yupsanis et al. (1990) analysed protein content in manually separated barley aleurone layer and in aleurone and endosperm fraction from four maize varieties (Peru 442, yellow dent maize, Coroico and accession of Coroico) (Wolf et al. (1972). The protein content was higher in aleurone than endosperm fractions and the levels were different among the four samples. This variation in protein content was attributed to grain varietal differences.

2.5.4.1.2 Amino acid composition

Protein nutritional quality is the product of several factors, the most important of which are amino acid composition and ratios. There are 8 essential amino acids namely, threonine, valine, leucine, isoleucine, lysine, tryptophan, methionine and phenylalanine. Of the eight essential amino acids (EAA) that are significant in the human diet, most are available to some extent in

different grains (Jensen and Martens 1983; Wolf et al. 1972; Yupsanis et al. 1990). The amino acid composition in wheat aleurone, aleurone cells, wheat bran, and endosperm has been reported (Fulcher et al. 1972a; Jensen and Martens 1983; Tkachuk and Irvine 1969). Jensen and Martens (1983) compared the amino acid composition of manually separated aleurone and endosperm to those in mechanically separated fractions reported by Fulcher et al. (1972a). The amino acid composition in these two studies was similar and EAA were generally higher in aleurone fraction compared to those in the endosperm fraction (**Table 2.2**). Recently, Sullivan et al. (2010) analysed 11 amino acids in mechanically separated barley products which included endosperm, middlings and bran fractions. Although, the amino acid composition in these 3 fractions was similar, higher concentrations were found in middlings, followed by bran and endosperm. It was not established whether the aleurone layer was either part of middlings or the bran. In addition, amino acid composition of maize has also been reported in manually separated germ, endosperm and envelope (comprising tip cap, pericarp and aleurone) (Landry and Moureaux 1980). However, as reported in barley, the aleurone layer was part of composite fraction envelopes. In Yupsanis' study the aleurone layers were mechanically separated from barley (*Hordeum vulgare* L.) and amino acid composition (**Table 2.2**) was performed on isolated globulin proteins (Yupsanis et al. 1990) and not in intact aleurone layer.

In another study, only six essential amino acids were determined in isolated aleurone layer of three maize varieties (Wolf et al. 1972). Furthermore, Sodek and Wilson (1971) determined a full profile on amino acid composition only in the endosperm fractions from different maize varieties (normal, opague-2 and hybrid) and aleurone layer was excluded among the fractions. While the above studies provide valuable information regarding amino acid composition, comparisons are difficult due to differences in sample characteristics. In addition, studies using

barley and maize fall short of utilising intact manually separated aleurone layers and of analysing full amino acid compositional profiles, hence the need for further studies.

Table 2.2 Amino acid composition of botanical components of wheat, maize and barley

	Wheat[a]		Wheat[b]		Maize[c]		Barley[d]
Amino acid	Aleurone	Endosperm	Aleurone	Endosperm	Aleurone	Endosperm	Aleurone
Asparagine	7.9	4.2	9.3	5.7	-[e]	-	8.8
Threonine	2.9	2.2	3.8	3.1	3.6	3.2	3.6
Serine	2.9	2.7	5	6.1	-	-	8.4
Glutamic acid	20.9	35.2	18.3	33.5	-	-	14.9
Proline	6.3	12.9	4.6	11.5	-	-	4.2
Glycine	5.8	3.6	7	4.6	-	-	13.6
Alanine	5.9	3.5	6.5	3.9	-	-	7.3
Cysteine	-	-	-	-	-	-	0.4
Valine	5.3	4.2	5.5	4.1	-	-	6.7
Methionine	1.6	1.6	0.4	0.4	1.6	1.9	0.7
Isoleucine	3.6	4	3.2	3.5	3.2	3.4	3.6
Leucine	6.5	7.3	6.7	7	7.6	14.4	7.5
Tyrosine	3.3	3.7	2.8	3.1	-	-	1.6
Phenylalanine	3.8	5.3	4.2	4.4	-	-	3.9
Histidine	3.4	2	4.3	2.3	-	-	1.7
Lysine	4.8	2.1	5.9	3.1	4.1	1.6	5.2
Arginine	11.1	3.6	12.3	4	6.4	3.6	7.9
Tryptophan	4	2	-	-	-	-	-

[a] Jensen et al. (1983) manually separated fractions
[b] Fulcher et al. (1972) mechanically separated fractions by air-classification and centrifugation
[c] Wolf et al. (1972) manually separated fractions of Coroico maize
[d] Yupsanis et al. (1990) mechanically separated fractions by pearl milling; [e] no value reported

2.5.4.2 Vitamins

Cereal grains are a rich source of the B-vitamins (thiamine, riboflavin, niacin, pyridoxine, pantothenic acid, and folates) and tocopherols (Delcour and Hoseney 2010; Serna-Saldivar 2010). However, cereals lack vitamins A, C and B_{12} (Truswell 2002). B-vitamins are highly concentrated in the aleurone layer and/or scutellum (Delcour and Hoseney 2010). In most cereals, niacin is the most abundant of the B-vitamins, followed by thiamine, pyridoxine,

pantothenic, riboflavin and folate (Batifoulier et al. 2006; Lebiedzin´ska and Szefer 2006; Rohi et al. 2013).

Distribution of B-vitamins within the grain has been studied in scutellum, aleurone and endosperm of maize, wheat and rice. Of the total thiamine content in wheat, 62, 32 and 3% and rice, 47, 34 and 8% were found in the scutellum, aleurone layer and endosperm fractions, respectively (Heathcote et al. 1952). Maize had 60 and 11% of the thiamine present in aleurone and scutellum, respectively (Heathcote et al. 1952). These data not only show differences in distribution of thiamine within the grain but also variation in thiamine content in different types of cereal grains. However, recent data on thiamine content in aleurone layer is available only for wheat species and not in barley and maize aleurone layer which will be analysed in this study. Thus, similar levels of thiamine content were reported in mechanically separated wheat aleurone (14 μg/g) (Buri et al. 2004) and manually separated wheat aleurone (Pomeranz 1988).

Much is known about niacin, one of the major B-vitamins in cereals. Niacin is composed of a pyridine ring which is attached to a carboxylic group. A great percentage of niacin is biologically unavailable to humans since it is in bound form in cereal grains (Ball 2006). To understand the nature of bound niacin, earlier studies isolated niacin from wheat bran and concluded that niacin was either contained in a carbohydrate (Kodicek and Wilson 1960) or was attached to a peptide (Das and Guha 1960). It is assumed that it is the same niacin that was later identified as a protein carbohydrate body (PCB), called a type II inclusion using scanning electron microscope (SEM) and histological staining techniques (Jacobsen et al. 1971). However, Fulcher et al. (1981) described type II inclusions as site for niacin reserve, based on König reaction and using

fluorescence microscope to visualise niacin within the aleurone cells. This same reaction has been used in the colorimetric determination of niacin content in foods. In this reaction, pyridine derivatives react with cyanogen bromide (CNBr) and an aromatic amine such as sulphanilic acid to produce a coloured, polymethine dye (Fulcher et al. 1981). The challenge with this reaction is that CNBr reacts with all '3-pyridine derivatives' (niacin, pyridoxine and pyridoxal) and its specificity is reduced (Rose-Sallin et al. 2001). However, use of HPLC provides more specific results because the compound is identified by comparing its retention time and maximum absorption wavelength to one of a known standard. In addition when HPLC is coupled to mass spectrometry, the identity of the compound is further confirmed through structural data that includes molecular weight (m/z ratio) and fragmentation pattern. Although HPLC has been used to identify and quantify niacin in cereals and cereal food products, LC-MS/MS has been done to confirm its structural identity in fortified Italian pasta (Leporati et al. 2005). Therefore part of this work will elucidate the structure of niacin in isolated aleurone cell contents and aleurone layer using LC-MS/MS.

2.5.4.3 Minerals (macro-and microelements)

Minerals in grains may contribute to the overall mineral content in the diets; however, their contribution is overlooked (O'Dell et al. 1972). Macro-and micromineral elements present in cereals include potassium (K), magnesium (Mg), phosphorus (P), calcium (Ca), zinc (Zn), iron (Fe), copper (Cu), manganese (Mn) and selenium (Se). The most abundant macroelement is phosphorus while calcium is the least (Serna-Saldivar 2010). The concentration of different minerals varies from one type of cereal to another (Lin et al. 2005; Liu et al. 2007; Lott and Spitzer 1980; O'Dell et al. 1972; Salunkhe et al. 1985). Understanding the distribution of

minerals is important because Singh et al. (2014) suggested that mineral location and/or accumulation within the grain tissue may influence its dietary availability.

The distribution of minerals across the grain kernel in wheat, barley and maize is different (Liu et al. 2007; O'Dell et al. 1972; Salunkhe et al. 1985). In wheat and barley, all minerals (higher proportion of phytate) are concentrated in the bran portion whereas in maize, 80% of the minerals are concentrated in the germ fraction (Liu et al. 2007; O'Dell et al. 1972; Salunkhe et al. 1985). Distribution of minerals in grain *in-situ* using x-ray analysis has been reported in barley (Liu and Pomeranz 1975; Ockenden et al. 2004) and to a limited extent in wheat (Lott and Spitzer 1980; Singh et al. 2014) and maize (Lin et al. 2005). Distribution of minerals in barley and wheat has been studied using mechanically separated fractions such as the germ, hulls, abraded fractions (comprising different bran layers [BN-1, BN-2 and BN-3]) and different milling fractions. The major (P, K, Mg, Ca) and minor (Fe, Zn, Cu and Ba) mineral elements recorded were all found in higher concentrations in grain outer layers. Variation in concentration was attributed to differences in genotype (Liu et al. 2007). Lan et al. (2005) also found variation in mineral concentration between wild type (WT) and low phytic acid-1(*lpa*1-1) maize (*Zea mays* L.). Information on mineral element distribution in manually separated fractions is scant. Thus, O'Dell et al (1972) studied the distribution of nutritionally important elements in manually separated germ, endosperm, aleurone and pericarp fractions from maize, rice and wheat. In the study by O'Dell et al. (1972) barley was not included, although there seem to be extensive studies that have analysed mineral distribution in barley using x-ray analysis and mechanically separated fractions. This study examined mineral constituents in manually separated aleurone layer and endosperm fractions from barley, yellow maize and wheat grains.

2.6 Role of microscopy in cereal research

Different microscopic techniques are used to study cereal grain structure and different chemical components (Kalab et al. 1995). Light (bright field) microscope, scanning electron microscope (SEM) transmission electron microscope (TEM) and fluorescence microscope have been an important part of cereal research since the 20th century (Fulcher and Miller 1993). Use of different microscopic techniques is vital in defining structural and chemical interactions within the grain (Fulcher 1986). Microscopy remains a viable tool that helps scientists to visualise the complex nature of grain structure and *in situ* chemical composition at the microlevel, often hidden during conventional chemical analysis. In addition such techniques are less destructive and do not require milling of the grain to reveal the elemental composition of tissues (Singh et al. 2013). The following grain morphological characteristics may be examined: cell wall thickness, size of cells, cell composition, starch granule structure and localisation and distribution of chemical components such as protein fat and phenolic acid (Abdel-Aal et al. 2011; Autio and Salmenkallio-Marttila 2001; Rooney et al. 1983, Saadi et al. 1998). Microscopy is even more advantageous when used together with chemical, physical, and biochemical analysis (Rooney et al. 1983) as it offers an opportunity to show changes occurring during processing and possibly relate what is visible to the chemical concentration especially when using fluorescence microscope.

2.6.1 Fluorescence microscopy

Fluorescence microscopy is based on the fluorescence of organic molecules (Ghiran 2011) which can only absorb light of a specific wavelength (Kapitze 1997). Fluorescence can be measured using either absorbance or reflectance techniques. Guilbault (1989) suggested that the reflectance technique can be used to measure fluorescent compounds directly in grains in solid state. In such

situations unlike in solutions, fluorescence intensity increases linearly and then levels off as predicted by the relationship between fluorescence and concentration of the fluorescent compound shown by the basic equation 1 (Guilbault 1989). Fluorescence intensity (F) may therefore be used to predict differences in the concentration of the fluorophore:

$$F = \Phi I_0 (1\text{-}e^{-\varepsilon bc}) \qquad \textbf{equation 1}$$

where F is the intensity of emitted light, Φ is the quantum efficiency, I_0 is the incident radiant power, ε is the molar absorptivity, b is the path length of the cell and c is the molar concentration of the fluorophore.

Fluorescence microscopy provides resolution, chemical specificity and sensitivity that is unique from other techniques (Autio and Salmenkallio-Marttila 2001; Fulcher and Wong 1980). It has been used widely to evaluate topography, heterogeneity, location and distribution of chemical components that fluoresce in grains *in-situ* (Abdel-Aal et al. 2011; Autio and Salmenkallio-Marttila 2001; Mills et al. 2005). For example, use of fluorescence microscope led to localisation of components such as ferulic acid in wheat aleurone (Fulcher 1972; Morales et al. 2001) and wheat grain milling fractions (Pussayanawin et al. 1988). Symons and Dexter (1996) used fluorescence as an indicator of the presence of pericarp or aleurone to assess the flour refinement process. Recently, Abdel-Aal et al (2010) observed blue autofluorescence in the cell wall of the aleurone layer in canary seeds indicating the presence of ferulic acid.

2.6.1.1 Fluorescence intensity and distribution of ferulic acid

Fluorescence intensity has also been used to examine the distribution of phenolic acids (ferulic acid or FA) in cereal grains and wheat milling fractions (Fulcher and Wong 1982; Pussayanawin et al. 1988; Saadi et al. 1998b) and flavonoids in maize kernels (Sen et al. 1994). Differences in fluorescence intensity brightness on micrographs were interpreted to mean variation in the

concentration of phenolics (FA) (Pussayanawin et al. 1988; Sen et al. 1994). However, these authors concluded that micrograph gave qualitative results. These authors suggested that quantitative assessments were possible if a spectrophotometer was coupled to the fluorescence microscope. Thus, Pussayanawin et al. (1988) later showed that there was a high correlation between relative fluorescence intensity values measured by microspectrofluorometer and ferulic acid content determined by HPLC. This led to the recommendations that fluorescence intensity maps as opposed to fluorescence micrographs would be a useful tool in quantitative measurements of phenolics (Sen et al. 1994). Use of such a technique would enhance our knowledge and understanding of the distribution and concentration of chemical components within grain *in-situ*.

However, fluorescence has mainly been used to localise FA in cereals and fluorescence intensity (FI) to map distribution of phenolic acids (Saadi et al. 1998; Sen et al. 1994). Limited studies have shown the correlation between relative FI values and FA concentration in cereal flours, (Pussayanawin et al. 1988) and pericarp and aleurone layers (Symons and Dexter, 1996). With the aid of digital cameras, fluorescence microscopes have been used to quantify spatial and temporal measurements of fluorescent molecules (Waters 2009). The digital images taken provide fluorescence intensity information, which shows the distribution and local concentration of fluorophores in the specimen (Waters 2009). Differences in concentration of the fluorophores evidenced by heterogeneous brightness(Kask et al. 1999), represents light intensity emitted from a corresponding area in the sample (Baldwin et al. 1997). Such variation in intensity can be shown by fluorescence intensity profile that have numerical values which may be correlated with FA concentration analysed using HPLC. Strong correlations would mean FI values may be an

indicator of FA quantity. Thus such an approach would provide a rapid method for screening grains and requiring minimal sample preparation (Saadi et al. 1998). Therefore there is need for further studies to explore the use of fluorescence intensity profiles to assess and predict ferulic acid concentration.

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CHAPTER 3

Ferulic Acid Fluorescence Intensity Profiles and Concentration Measured by HPLC in Pigmented and Non-pigmented Cereals

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3.1 Abstract

Pigmented and non-pigmented cereals were examined by fluorescence microscopy and local concentrations and distribution of ferulic acid (FA) mapped by fluorescence intensity profiles. Hand dissected fractions (pericarp, aleurone layer and endosperm) were analyzed separately, following microwave-assisted extraction, for FA concentration using high performance liquid chromatography (HPLC). Fluorescence intensity profiles showed that FA distribution varied across the grains and fluorescence intensity values were highest in the outer layers and lowest in the endosperm representing the concentration of the fluorophore. FA concentration in the bran and aleurone layers of pigmented cereals were significantly different ($p < 0.05$) from the non-pigmented cereals, although purple barley and yellow maize had lowest and the highest concentration in both fractions. In all the grains, the endosperm fraction had the lowest amounts of FA. Similar trends were observed in both FA concentration determined by HPLC and fluorescence intensity values across the grain. Overall, a positive and significant correlation ($r=0.421$ $p < 0.001$) was found between FA concentration and fluorescence intensity values. These findings suggest that fluorescence intensity profiles may be a promising approach for assessing FA local distribution and concentrations in the grain.

3.2 Introduction

Visual examination of food is important to consumers, regulators and food processors. However, functional and nutritional components of food are so minute that often they cannot be viewed by the naked eye but only by use of optical microscopes. Fluorescence microscopy has been used to evaluate topography, heterogeneity, location and the distribution of chemical components in grain *in situ* (Abdel-Aal et al. 2011; Autio and Salmenkallio-Marttila 2001; Fulcher et al. 1972; Mills et al. 2005; Sen et al. 1994). Fluorescence microscopy is highly recommended in cereal research due to high resolution, selectivity, sensitivity and potential chemical specificity (Autio and Salmenkallio-Marttila 2001; Wood and Fulcher 1983). Ferulic acid (FA) the most abundant phenolic acid in cereal grains (Adom et al. 2003) fluoresces naturally under UV light (Pussayanawin et al. 1988). This attribute has been used mainly to map the location of FA within cereal grains (Abdel-Aal et al. 2011; Earp et al. 1983; Fulcher and Irving 1986; Pussayanawin et al. 1988)) and evaluate presence of grain outer layers in wheat milling fractions (Pussayanawin et al. 1988; Symons and Dexter 1993). Symons and Dexter (1996) showed that pericarp and aleurone fluorescence were useful indicators in assessing flour refinement. However, a few studies have shown the relationship between FA fluorescence and concentration in grain *in situ* (Sen et al. 1991; Sen et al. 1994). Digital fluorescence images give spatial and intensity information, showing the distributions and local concentrations of fluorophores in the specimen (Waters 2009). Fluorescence intensity is related to concentration of fluorophore and may therefore be used to determine the concentration of the components. However, fluorescence intensity profiles have not been used in examining the distribution and local concentration of FA in grain *in situ*.

Pigmented cereals are a source of natural colourants (Abdel-Aal et al. 2006) and are utilised in new food products. Blue or red maize has been used for production of tortillas (Cortes-Gomez et al. 2005) while purple wheat has been used as a colorant on the surface of bread (Bezar 1982). Anthocyanin content and antioxidant activity have also been studied in pigmented cereals (Bellido and Beta 2009; Choi et al. 2007; Siebenhandl et al. 2007). Studies have shown that pigmented cereals and their cereal products have higher antioxidant activity compared to non-pigmented cereals (Abdel-Aal et al. 2008; Hirawan et al. 2011; Mussi de Mira et al. 2008).

In non-pigmented cereals, phenolic acid content have been investigated by several authors (Adom and Liu 2002; Adom et al. 2003; Beta et al. 2005; Dykes and Rooney 2007; Zielinski and Kozlowska 2000) but only a few studies have included pigmented cereals (Mussi de Mira et al. 2008; Zielinski and Kozlowska 2000). These studies mainly involved the use of whole grain wheat, wheat milling fractions, and some barley (Zupfer et al. 1998). Cereal grains have different compartments that show significant chemical variation (Fulcher 1986). For instance, a wide variation in phenolic acid content was found among seven barley varieties and the differences were attributed to caryopsis structure and composition, such as ferulic acid (FA) substitution frequency in arabinoxylan backbone and increase in aleurone cell-wall volumes (Zupfer et al. 1998). FA has been localized in mature wheat and developing wheat kernels using fluorescence microscopy (Evers and Millar 2002; Fulcher and Irving 1986; Pussayanawin et al. 1988). Recently, FA has been located in canary seeds using fluorescence microscopy (Abdel-Aal et al. 2011). Among pigmented cereals FA was located in pigmented sorghum (Earp et al. 1983). In barley and oats, β-glucans have been located using Calcofluor White M2R New, a fluorescence probe that binds to β-glucans (Miller and Fulcher 1994).

Less attention has focused on comparing the location and distribution of FA in pigmented and non-pigmented kernels using fluorescence microscopy. Grain fractions with enhanced levels of FA are of great interest to both researchers and the food industry for use as functional food ingredients (Malkki 2004) because of their potential health benefits. To optimize use and successfully isolate grain fractions for use as functional food ingredients, there is need to visualise the location and distribution of the functional components of the various grains *in situ*. This study was therefore conducted to compare the location and distribution of ferulic acid in pigmented and non-pigmented cereals and explore the possibility of using fluorescence intensity profiles to predict FA concentration.

3.3 Materials and Methods

3.3.1 Chemicals

HPLC grade hexane, ethyl acetate and methanol, monobasic and dibasic sodium phosphate, acetic acid, 25% glutaraldehyde and Tissue–Tek were purchased from Fisher Scientific (Whitby, ON, Canada). Ferulic acid standard was obtained from Sigma-Aldrich Co. (St Louis, MO, USA).

3.3.2 Grains

Five pigmented and four non-pigmented cereals were used. The pigmented cereals were purple barley, purple wheat and 3 yellow maize varieties (USP1395XR, P1508HR and Dasca maize) while the non-pigmented cereals comprised common barley, Ambassador wheat, Caledonia wheat and MSUD8006 wheat.

3.3.3 Sample preparation

3.3.3.1 Sectioning and fixation for microscopy

Grain ends were removed and grains cut in half or quarter sections of approximately 2-3 mm using a sharp scalpel. Fixation was done as described by Abdel-Aal et al. (2011) with modifications. Briefly the sections were fixed in 5% glutaraldehyde in 0.1M phosphate buffer (pH 7.0) for five days at room temperature and rinsed in 0.1M pH 7.0 phosphate buffer over night at 4°C, wiped on paper towels and stored at -20°C overnight. The frozen and fixed sections were mounted on blocks using Tissue-Tek OCT-compound, an embedding medium for frozen specimen, and held for 10 minutes on a stand in the cryotome chamber at -20°C. The fixed and frozen grain sections were cut into 10 µm slices by a cryotome (Shandon cryotome FSE, Thermo Electron Corporation, Cheshire, UK) with microtome blade knife (Shandon MX35 premier+ 35/80 mm,) (Whitby, ON, Canada). The sections were quickly transferred onto glass slides (Color Frost/Plus microscope slides precleaned) (Whitby, ON, Canada) and were then examined under fluorescence microscope for location and distribution of ferulic acid.

3.3.4 Microscopic imaging and analysis

3.3.4.1 Imaging

The grain cross sections were examined by an upright Carl Zeiss Fluorescence microscope (Zeiss, Axio Imager MI, Jena, Germany) equipped with an X-Cite 120 fluorescence illuminating system, and a pre-aligned intelli-lamp (Hg-mercury) system (EXFO Electro-Optical Engineering Inc., Quebec, Canada). The microscope was set at channel 3 in the multidimensional acquisition mode, contrast manager turned to fluorescence and “reflector” 49 DAPI selected. Natural fluorescence due to FA was observed under UV/49-DAPI fluorescent filter set at 365 nm and > 420 nm excitation and emission wavelengths, respectively using a 10x objective lens (Zeiss Plan-

NeoFluor, 0.3 NA, Jena, Germany). The exposure time were measured and adjusted accordingly. Thus, after switching to fluorescence, the “measure” function was selected to set exposure time. For each specimen the exposure time was adjusted to 180–200 ms ± 20 and thereafter the images were captured by AxioCam HRc Camera using AxioVision 4.8.1 (9-2010) software (Zeiss, Jena, Germany). This procedure was repeated for each specimen under similar conditions.

3.3.4.2 Analysis

The raw digital images were examined for areas showing bluish white fluorescence in the grain cross-section. To show local concentration and distribution of FA, fluorescence intensity profiles were mapped by drawing a line in the raw digital images using curve spline. Curve spline, is a function of image analysis in Axio-Vision Release Software 4.8.1 (9-2010) which shows arbitrary length between two points and differences in fluorescence intensities along the pixels in the image. The intensity value of a pixel is related to the number of the fluorophore present at the corresponding area of specimen in the fluorescence microscope (Waters 2009). It was assumed therefore that fluorescence intensity (grey level values) in the profile indicated differences in ferulic acid concentration across the cereal grain cross-section.

3.3.5. Hand dissection of grain fractions for phenolic acid analysis

The germ, pericarp, aleurone layer and endosperm were separated from whole grains using the procedure of Chrispeels and Varner (1967) as modified by Stewart et al. (1988) with further modifications. Briefly, germ and grain ends were removed with a sharp scalpel and the remaining grain was cut lengthwise. The latter were soaked in 0.1% sodium hypochlorite for 15-20 minutes to sterilize the surfaces and rinsed using sterile, distilled, deionised water. The seeds were placed in 10 cm petri dishes lined with two ashless filter papers moistened with sterile, distilled, deionised water. The petri dishes were wrapped in aluminum foil and kept at 20°C for

two days. The pericarp (outer and inner/seed coat), aleurone layer and endosperm were separated using a sharp scalpel under a magnifying glass. The aleurone layers of all grains were further examined under the microscope to confirm and check cleanliness or purity of the layers. The fractions were stored at -20°C and were freeze dried before chemical analysis. Freeze dried grain fractions were ground into fine flour using a multi-use blade grinder, model PCC770 (Loblaws Inc. MO, Canada) and passed through a 0.5 mm sieve screen and stored at 4°C before analysis.

3.3.6 Microwave assisted extraction of phenolic acids

Phenolic acids were extracted as described by Antoine et al. (2003) with modifications. Briefly 200 mg of ground whole grain or 100 mg of grain fractions (aleurone layer, pericarp, germ and endosperm) were extracted in 4M sodium hydroxide (5 mL and 2.5 mL) using a 45 mL polytetrafluoroethylene (PTFE) acid digestion bomb vessel (Parr Instrument Company, Moline, IL). Nitrogen was added to the mixture, before placing the vessel in a 1400W domestic microwave oven (Diplomat Model D811, Danby, Suweon, Korea). The temperature was set at 170°C, power 8 and samples were digested for 45 seconds or for 30 seconds for maize endosperm. The hydrolysates were cooled and transferred into 250-mL conical flasks and pH adjusted to 1.5 to 2.0 using 6M HCl. The hydrolysate was extracted twice with 20 mL hexane to remove lipids. A separating funnel was used to separate the organic and the aqueous phenolic phase. The aqueous phenolic phase was extracted three times with 45 mL of ethyl acetate. The combined ethyl acetate extracts were dehydrated with 1 g of Na_2SO_4 before evaporation to dryness at 35°C using a Buchi Rotavapor R-205 (Laboratoriums Technik AG, Postfach, Switzerland). The dried phenolic extracts were reconstituted with 2 mL of 50% methanol and stored at -20°C before analysis. The extracts were filtered through a 0.45 µm nylon filter prior to HPLC analysis. All analysis was done in duplicate.

3.3.7 HPLC analysis

Phenolic acid composition in the whole grain and grain fractions was determined using a Waters 2695 HPLC (Waters, Milford, MA) equipped with a photodiode array (PDA), (Waters 996) and an auto sampler (717 Plus, Waters). The analytical column was a reverse phase Shim Pack HRC-ODS, C18 (250 mm x 4.6 mm, 5 µm) (Shimadzu, Kyoto, Japan). The gradient mobile phase consisted of solvent A (0.1% acetic acid in Milli-Q water) and solvent B (0.1% acetic acid in methanol). Phenolic acid separation was accomplished using a 70 min linear solvent gradient at a flow rate of 0.9 mL/min, as follows: 0-10 min 9% B, 11-13 min 14% B, 14-23 min 15% B, 24-27 min 16.5% B, 28-29 min 19% B, 30-35 min 25% B, 36-37 min 26% B, 38-40 min 28% B, 41-45 min 35% B, 46-47 min 40% B, 48-52 min 48% B, 52-58 min 53% B, 59 min 70% B, and 10 min to rinse and equilibrate the column using 9% B. Identification of phenolic acids was accomplished by comparing the retention time of peaks in the samples to those of standards at 320 nm. Quantification of ferulic acid was done using total peak area and substituting in the regression equation (y=491899x-3623.3) of ferulic acid standard curve with concentrations ranging from 0.004 to 1 mg/mL and $R^2 = 0.9997$. Y is the peak area and x is the concentration.

3.3.8 Statistical analysis

All extractions were done in duplicate and reported as mean ± standard deviation (SD). Means were compared by Duncan's multiple range test using SAS 9.2 (SAS Institute Inc., Cary, NC) and significant differences reported at $p < 0.05$. Pearson's correlations were performed to determine the relationship between FA concentration and fluorescence intensity values.

3.4. Results and Discussion

3.4.1 General characteristics of grain outer layers and endosperm

When examining the grain cross sections, subtle and distinct differences were identified in the arrangement of outer layers, size and shape of aleurone cells, thickness of the aleurone cell walls and endosperm cells in pigmented and non-pigmented grains (**Figure 3.1 A-F**). Purple barley and non-pigmented barley both had three-celled aleurone layers with an average size of 48.8 to 96.5 µm. However, the size and shapes of aleurone cell and cell wall thickness were different (**Figure 3.1A & B**). Yellow maize kernels and wheat grains had one-celled aleurone layers. The average size of the aleurone layers ranged from 34.1 to 50.9 µm and 39.3 to 41.54 µm in yellow maize and wheat, respectively. The range of thickness of aleurone cells found in studied grains were comparable to 50 µm reported previously (Delcour and Hoseney 2010). Unlike wheat and barley which had multiple layers in the pericarp, there were only two distinct outer layers in yellow maize (USP1395XR, Dasca and P1508HR). These were the pericarp and aleurone layer and the former had multiples of minute round hole-like structures (**Figure 3.1C**). Cell wall thickness varied widely among the cereals. Yellow maize and non-pigmented wheat (**Figure 3.1C & D**) had thinner cell walls compared to cell walls in purple barley, purple wheat and non-pigmented ambassador wheat (**Figure 3.1E & F**). Even though both pigmented and non-pigmented wheat had several outer layers, in purple wheat the outer layers, particular the outer pericarp had a distinct pattern (**Figure 3.1E**). Cell wall thickness may contribute to differences in ferulic acid content within the aleurone layer, and consequently the total FA content in the different cereal grains.

3.4. 2 Ferulic acid (FA) fluorescence in pigmented and non-pigmented cereal

Bluish white natural fluorescence due to FA was observed and fluorescence brightness differed among the different types of cereal grains and within the grain cross-sections (**Figure 3.1**). While, there was no difference in the location of FA in pigmented (**Figure 3.1 A, C & E**) and non-pigmented (**Figure 3.1 B, D & F**) cereal grains, intense fluorescence was observed in the aleurone layer and specifically in the cell walls compared to endosperm. Other phenolic acids, such as coumaric also auto fluorescence under UV light (Hutzler et al. 1998) may therefore have contributed to the fluorescence intensity. However, the contribution would be minimal, because *p*-coumaric content in the pericarp, aleurone, germ and endosperm of the studied cereals was between 8 to 21-fold, 12 to 21-fold, 3 to 5- and 5-fold lower than the levels of FA, respectively, according to HPLC analysis (Chapter 4). Previous studies have mainly highlighted intense fluorescence in aleurone cell wall of wheat and sorghum (Earp et al. 1983; Fulcher et al. 1972). In the present study apart from the aleurone layer, profound fluorescence was also observed in the sections of pericarp layers in non-pigmented barley (**Figure 3.1B**) and in yellow maize (USP1395XR) (**Figure 3.1C**). In the latter, endosperm cell walls were also distinct probably indicating the differences in of FA content yellow maize endosperm compared to the other cereals.

3.4.3 Fluorescence intensity profiles showing FA local concentrations and distributions

A wide variation in fluorescence brightness was observed across the examined grain cross-section in the digital images (**Figures 3.1 & 3.2**). According to Kask and coworkers such heterogeneous brightness in samples reflects differences in concentration of the molecules shown by distribution of photon count numbers (Kask et al. 1999). Digital images taken under particular spectral conditions are considered as 2D table of numbers, referred to as grey level values,

representing the measured light intensity emitted from a corresponding area of sample (Baldwin et al. 1997). Fluorescence can be measured by transmission or reflectance techniques. Reflectance technique may be used to measure fluorescent compounds directly in grains in solid state (Guilbault 1989). In such situations, unlike in solutions, fluorescence intensity increases linearly and levels off at high concentration (Guilbault 1989). Because grains are in solid state and reflectance technique was used, the present method was based on the assumption that fluorescence intensity value is related to the concentration of the fluorophore in cell walls and within the selected points where the lines were drawn.

3.4.4 Local concentrations and the distribution of FA across the grain

Average ranges of FI values were calculated from four different lines drawn at random at different areas within the pericarp, aleurone and endosperm of each cereal grain to incorporate inherent variation within the grain**.** Although FA distribution trends were similar from outer layer into the endosperm in pigmented and non pigmented cereal, the average ranges of fluorescence intensity (FI) values were different (**Table 3.1**). They were higher (625-7900 and 1200-10500) in the outer pericarp and in aleurone layers (1165-11,500 and 925-9025) compared to FI values in the endosperm (118-1475 and 190-770) in pigmented and non-pigmented cereals, respectively. Amongst the pigmented cereals, yellow maize samples had the highest range of fluorescence intensity values in the pericarp (4150-7900) and the lowest in aleurone layer (1165-4450). In contrast purple wheat had the lowest range of FI values (625-2525) in the pericarp and highest (2200-11,500) in the aleurone layer. Within the non-pigmented cereals, the FI value ranges of were similar except in non-pigmented barley which had higher FI values both in pericarp and aleurone layer. Ranges of fluorescence intensity values were low in the endosperm of all the pigmented and non-pigmented cereal grains.

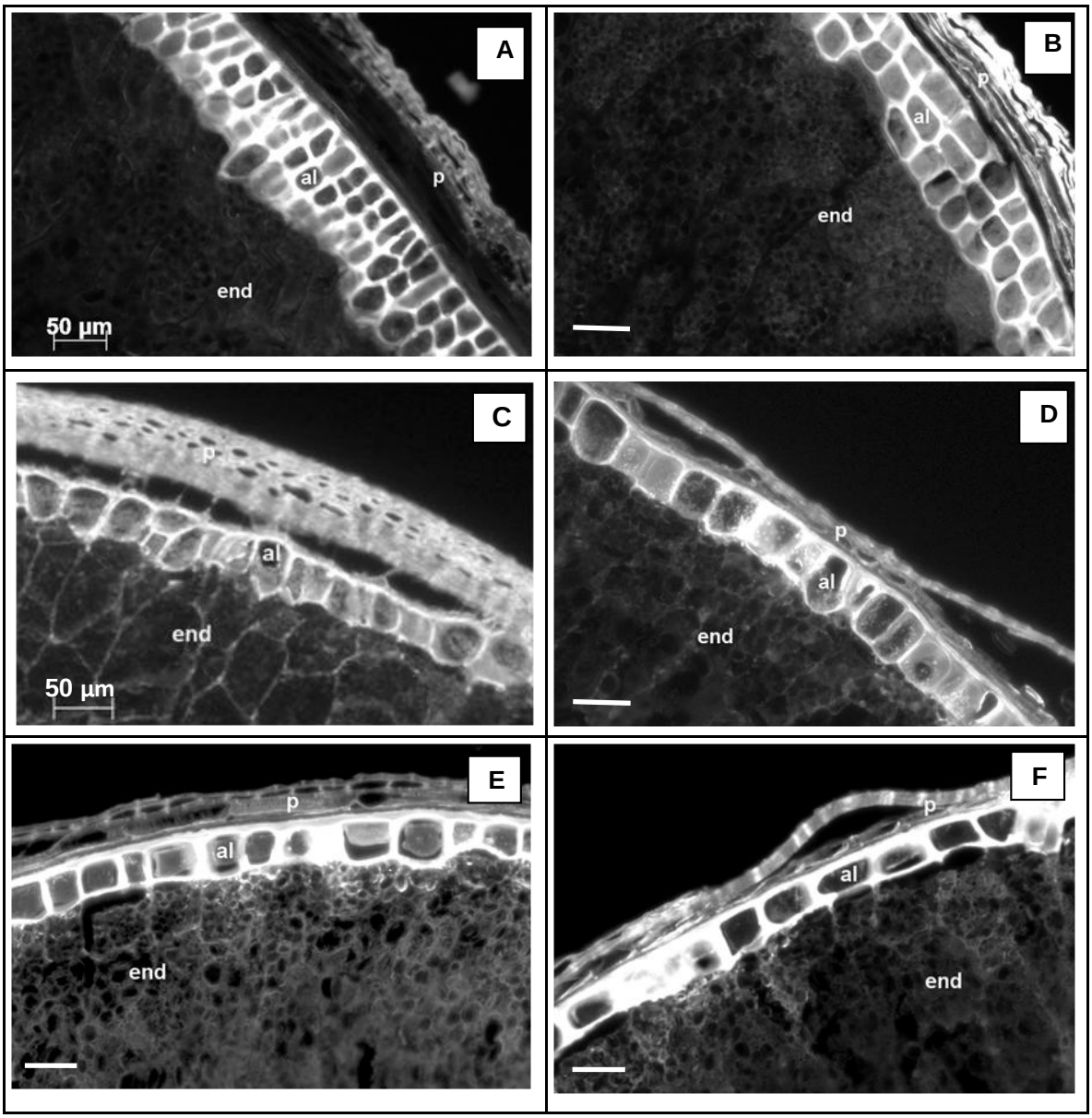

Figure 3.1 Digital images showing location of ferulic acid in the pericarp, aleurone layer and endosperm of pigmented and non-pigmented cereal grain cross-sections of purple barley (A); non-pigmented barley (B); USP1395XR yellow (C); MSUD8006wheat (D); purple wheat **(**E) and Ambassador wheat (F); end= endosperm; al= aleurone; p= pericarp. Bar = 50 µm

Use of fluorescence intensity maps as opposed to fluorescence micrographs were recommended as a useful tool in quantitative measurements (Sen et al. 1994) because micrographs only show qualitative difference, just as digital images do without the fluorescence intensity profiles. The

advantage of fluorescence intensity profiles is the grey level values which show significant differences across the grain. To illustrate this, **line 1** was drawn to map the fluorescence intensity (FI) profiles **A1- F1** from the outer layers into the endosperm of the different grains (**Figure 3.2A-F**). The FI values (grey) increased with increase in brightness of the fluorescence and decreased in less intense sections of the grain suggesting difference in concentration of the fluorescent molecules.

Table 3.1 Range of fluorescence intensity values* (grey) across the grain fractions of pigmented and non pigmented cereals

Cereal type	**Cereal grain sections**		
Pigmented	**Pericarp**	**Aleurone**	**Endosperm**
Purple barley	1050-6925	2725-9375	825-1475
Purple wheat	625-2525	2200-11500	225-1005
Yellow maize			
Dasca	4425-7175	1165-4950	225-575
USP1395XR	4375-7900	2000-4475	188-525
P1508HR	4150-7600	2275-4450	208-438
Overall Pigmented	**625-7900**	**1165-11500**	**118-1475**
Non-pigmented			
Ambassador wheat	2025-6175	2675-7025	233-618
Caledonia wheat	3000-6300	925-5675	220-625
MSUD8006 wheat	1200-5725	1675-6650	190-500
Non-pigmented barley	4250-10500	3125-9025	450-770
Overall non-pigmented	**1200-10500**	**925-9025**	**190-770**

*values are average of 4 different profile lines drawn randomly within the grain fractions , picking lowest and highest intensity levels in the different grain cross-sections.

Within the barley grain, the ranges of fluorescence intensity values (grey) were lower (500 - 5500) in the pericarp in purple barley compared to non-pigmented barley (2000-11500) (**Figure 3.2A1 & B1**). In the aleurone layer, FI value ranges were higher (1000-11,000) in the former than in the latter (1800-5000). Similarly, purple wheat had lower (600-3000) and higher (1000-14,000+) (**Figure 3.2C1**) ranges of FI values than non-pigmented MSUD8006 wheat (**Figure 3.2D1**). It was interesting to note that the opposite was observed in yellow maize where the range

of FI values were higher in the pericarp (2000-9500+) compared to the range in the aleurone (1500-3000) (**Figure 3.2E1**). The lowest FI value in the pericarp of yellow maize varieties was within the FI value range in non-pigmented barley (**Figure 3.2F1**) and was three-fold higher in the pericarp in purple wheat. This indicates that FA concentration in pericarp of maize is much higher compared to levels in the pericarp of non-maize cereals. These results further confirm earlier work in cereals reported in literature (Abdel-Aal et al. 2001; Barron et al. 2007; Zhao et al. 2005).

3.4.5 Fluorescence intensity profiles of lines drawn within the aleurone layer

Additional fluorescence intensity profiles were mapped by drawing a line within the aleurone layer in digital images and labelled **line 2** in **Figure 3.2**. The peaks with highest FI values in the profiles were aleurone cell walls (cw) while the low points represent the areas within the aleurone cells (wal) (**Figure 3.3**). The high intensity values were assumed to reflect high concentration of FA as earlier explained in section 3.4.2. Although they did not indicate intensity values, Bacic and Stone (1981) also reported intense fluorescence in cell walls and lower fluorescence intensity within aleurone cells in wheat and barley grains. About 85-90% of the alkaline–soluble ferulic acid in grain was localised in the insoluble dietary fiber in wheat and rye (Rybka et al. 1993) which is part of the cell walls.

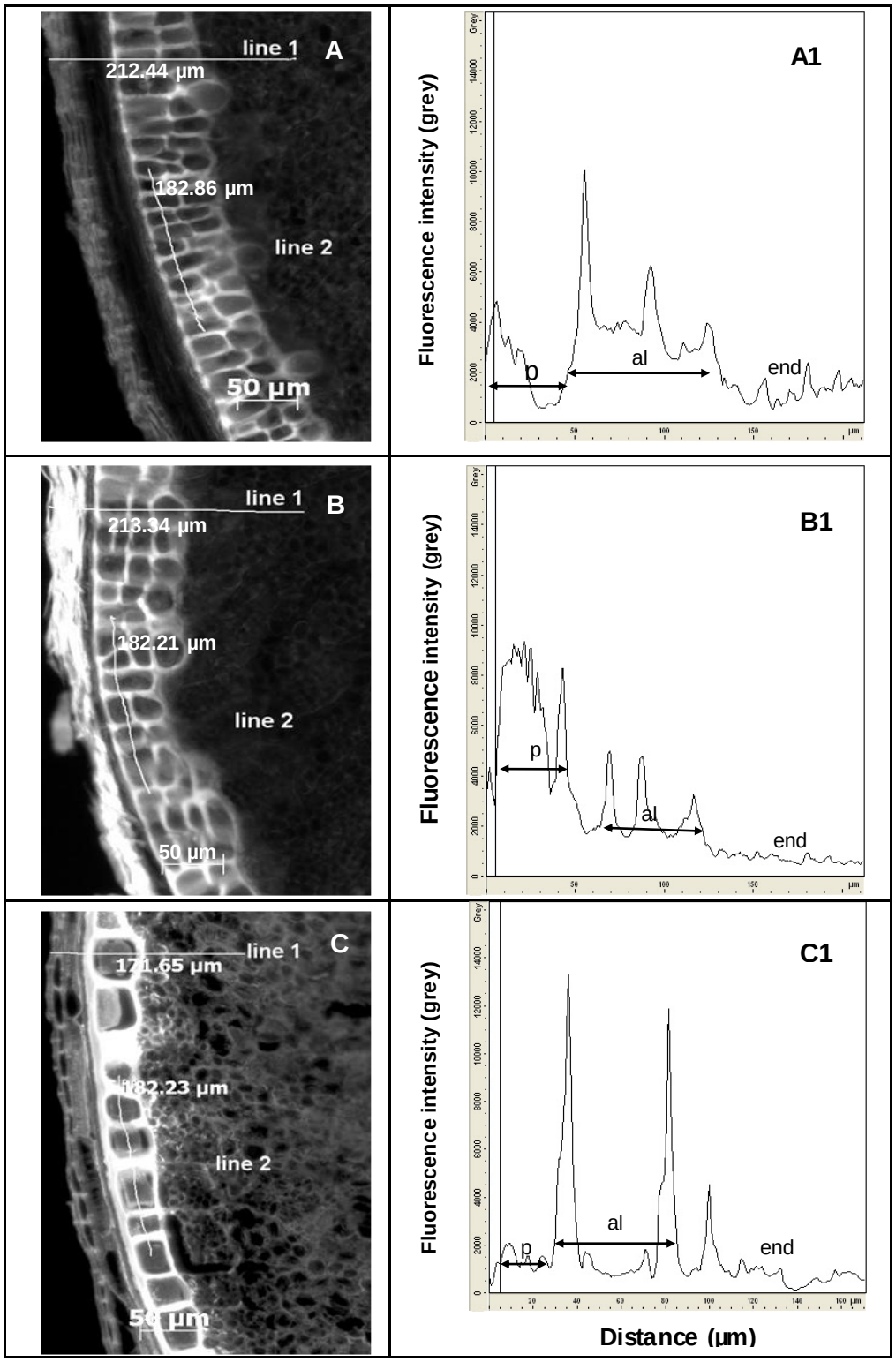

Figure 3.2 Fluorescence intensity profiles corresponding to line 1 drawn in the raw digital images showing the distribution of ferulic acid from outer layers (pericarp and aleurone) into the endosperm; purple barley (A and A1); non-pigmented barley (B and B1); purple wheat (C1 and C2); MSUD8006 wheat (D and D1); USP1395XR yellow maize (E and E1) and Ambassador wheat (F and F1). Bar= 50 µm.

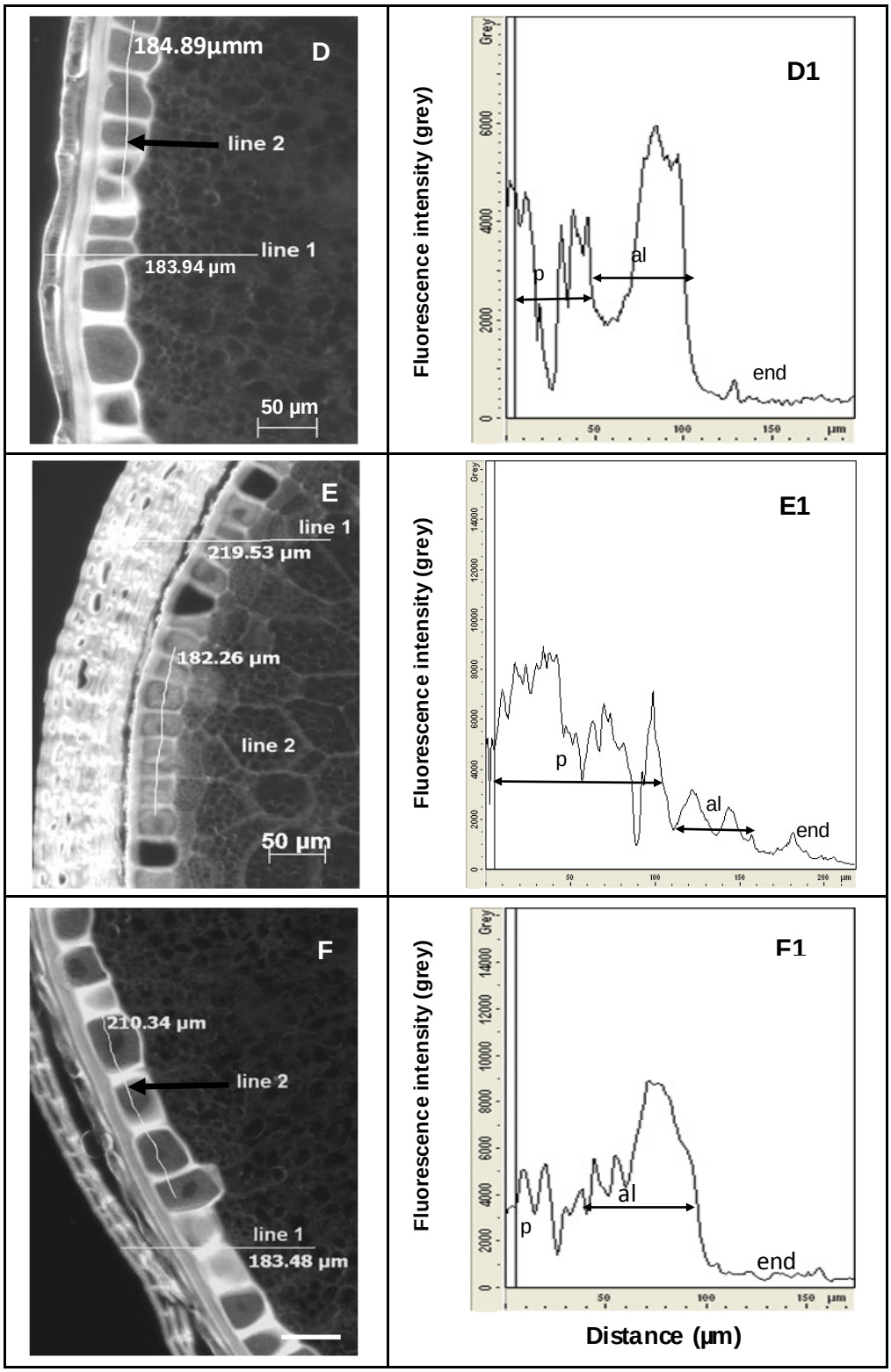

Figure 3.2 (continued). Fluorescence intensity profiles corresponding to line 1 drawn in the raw digital images showing the distribution of ferulic acid from outer layers (pericarp and aleurone) into the endosperm; MSUD8006 wheat (D and D1); USP1395XR yellow maize (E and E1) and Ambassador wheat (F and F1). Bar =50 µm.

Although the minimum FI values in aleurone layer of purple barley (2000-9500) (**Figure 3.3A2**) and non-pigmented barley (2000-7000) (**Figure 3.3B2**) were similar, the overall range was different. In contrast, the FI values in cell walls of purple wheat (**Figure 3.3C2**) were 2 times higher than FI values in non-pigmented wheat (MSUD8006) (**Figure 3.3D2**), signifying higher concentration of FA in aleurone layer of purple wheat compared to non-pigmented wheat varieties. However, compared to FI values in cell walls of yellow maize (USP1395XR) (**Figure 3.3 E2**), non-pigmented wheat had slightly higher values (**Figure 3.3F2**). The difference in the ranges in FI values observed in these grains would be attributed to difference in the section where the lines were drawn, cell wall thickness, type of cereal and probably also showing inherent variation within and among the cereal grains. Fluorescence intensity profiles mapped in this study demonstrated the differences in concentration of FA fluorophore in the cell walls and within the aleurone cell that is often hidden when traditional methods of analysis are used. In addition, the profiles also showed differences in the arrangement and to some extent, size (width) of the aleurone cells.

3.4.6 Quantification of ferulic acid in pericarp, aleurone and endosperm

To test the assumption that fluorescence intensity values may be used to predict FA concentration, chemical analysis was done on hand dissected fractions of the studied grain cereals following microwave-assisted extraction. Generally, FA concentrations were higher in outer layers compared to the endosperm. FA concentration in the pericarp and aleurone layers of pigmented cereals were significantly different ($p < 0.05$) from the non-pigmented cereals, with purple barley having lowest content and yellow maize the highest in both fractions (**Table 3.2**). Compared to non-pigmented barley, FA content in the pericarp and aleurone of purple barley were 1.9- and 0.6 –fold lower, respectively. FA concentrations in the aleurone layers of non-

pigmented barley and purple barley were 3-4 fold lower compared to the other cereals. Hence suggesting that FA concentration in the aleurone layers of barley grains may be generally low. FA concentration in the pericarp of pigmented and non-pigmented wheat was not significantly different ($p < 0.05$); however, in the aleurone layers they were significantly different ($p < 0.05$). Purple wheat aleurone layer had the highest FA content (3617 μg/g) and also had highest fluorescence intensity values. FA content in the aleurone layer of the wheat varieties found in this study was within the ranges reported by Zhou et al. (2004) and Barron et al. (2007). In contrast, FA concentration in the pericarp and aleurone layers of yellow maizes differed significantly ($p < 0.05$) within the maize varieties and when compared to the other cereals. The FA concentration in the maize pericarp was about 6-fold the amounts found in wheat and non-pigmented barley pericarp. Zhao et al. (2005) also found a 6-fold higher FA content in maize bran than in wheat bran, the differences of which were attributed to the complex structure of maize bran. FA concentrations in the yellow maize endosperm were higher and significantly different from the other cereals. Overall FA concentrations in the pericarp of barley, wheat and maize grains were 23.2-, 31.6-and 108.7-fold higher, respectively, than the FA levels in the endosperm.

Similarly higher FA concentrations were found in the aleurone layers of these grains. Similarly, Barron et al. (2007) found higher FA concentration in grain outer layers compared to the starchy endosperm. The endosperm fraction therefore may not be considered as a potential source of FA for use as a functional ingredient.

3.4.7 Relationship between FA concentrations and fluorescence intensity values

Similar trends were observed in ferulic acid concentration determined by HPLC and fluorescence intensity profile values in pigmented and non-pigmented cereals. Higher amounts of FA

concentration and grey level values were found in the pericarp and aleurone layers and low values in the endosperm fraction with distinct variations among the different cereal types (**Table 3.1 & Table 2**). Sen et al. (1991) reported a strong correlation (r = 0.86) between relative fluorescence intensity in ground maize samples detected by microspectrofluorometer and ferulic acids content analysed by HPLC.

In the present study, both pigmented and non-pigmented cereals had lower FI values in the endosperm and FA concentration were also the lowest (**Table 3.2**). However, in the aleurone layers of non-pigmented wheat varieties the FI values were within the same range while purple wheat had the highest FI values range (**Table 3.1**) and a similar trend was found in FA concentrations. The trends of FI values in yellow maize were similar to the FA concentrations in the grain fractions. Thus, high intensity values and FA concentrations were observed in the pericarp and both were low in the aleurone layer.

Table 3.2 Ferulic acid concentration (µg/g*) in the grain fractions (pericarp, aleurone and endosperm)

	Grain Fractions		
Cereal	**Pericarp**	**Aleurone**	**Endosperm**
Pigmented			
Purple barley	1915±17.83^{eA}	1008±3.25^{gB}	116±2.10^{cC}
Purple wheat	3815±29.61^{dA}	3616±15.47^{dB}	118±3.88^{cC}
Yellow maize			
Dasca	14300±447.84^{cA}	4910±166.00^{bB}	183±1.78^{aC}
USP1395XR	18463±590.79^{bA}	3944±83.46^{cB}	172±24.06^{abC}
P1508HR	22437±1018.90^{aA}	5652±10.84^{aB}	152±13.05^{bC}
Non-pigmented			
Ambassador wheat	3194±3.34^{dA}	3367±154.36^{eA}	112±0.56^{cB}
Caledonia wheat	3343±16.22^{dA}	3172±44.91^{eB}	109±0.27^{cC}
MSUD 8006 wheat	3718±117.96^{dA}	3303±59.48^{eB}	107±4.99^{cC}
Non pigmented barley	3698±30.85^{dA}	1651±83.06^{fB}	123±0.00^{cC}

* Values are means of n=2 ±Standard deviation. Values with different lower case and uppercase letters in the same column and same row are significantly different at $p < 0.05$ (Duncan's multiple range tests) among different cereals and within the grain fractions of each cereal

Although the range of fluorescence intensity value in the aleurone layers of pigmented and non pigmented barley were higher than in non-pigmented wheat and yellow maize (**Table 3.1**), lower FA concentration were found in the former (**Table 3.2**).

It would be speculated that fluorescence quenching is occurring in the latter group of cereals especially in yellow maize because carotenoids have been implicated in fluorescence quenching of other compounds (Belefant-Miller et al. 2005). In general, these observations suggest that fluorescence intensity profile values may be a potential technique for demonstrating differences in FA concentration in grain *in situ*.

Despite the similar trends observed in the average ranges of FI values and FA concentrations in the grain fractions positive and significant correlations were only found when all the fractions in all cereals and per cereal type were correlated separately (**Table 3.3**). Thus the overall Pearson correlation for all fractions in the studied cereals was significant ($r = 0.4212$, $p = 0.001$). The weak and significant correlation would be attributed to the wide variations in the FA concentration and fluorescence intensity values across the different cereal types. When correlations for the three fractions were performed in combination for each type of cereal, they were $r = 0.5929$, $p = 0.042$; $r = 0.5849$, $p = 0.002$ and $r = 0.8115$, $p < 0.001$ for barley, wheat and yellow maize, respectively. The correlation was strong and highly significant in yellow maize. The correlation found in yellow maize therefore would be more predictive of the relationship between FA and FI compared to those found in wheat and barley.

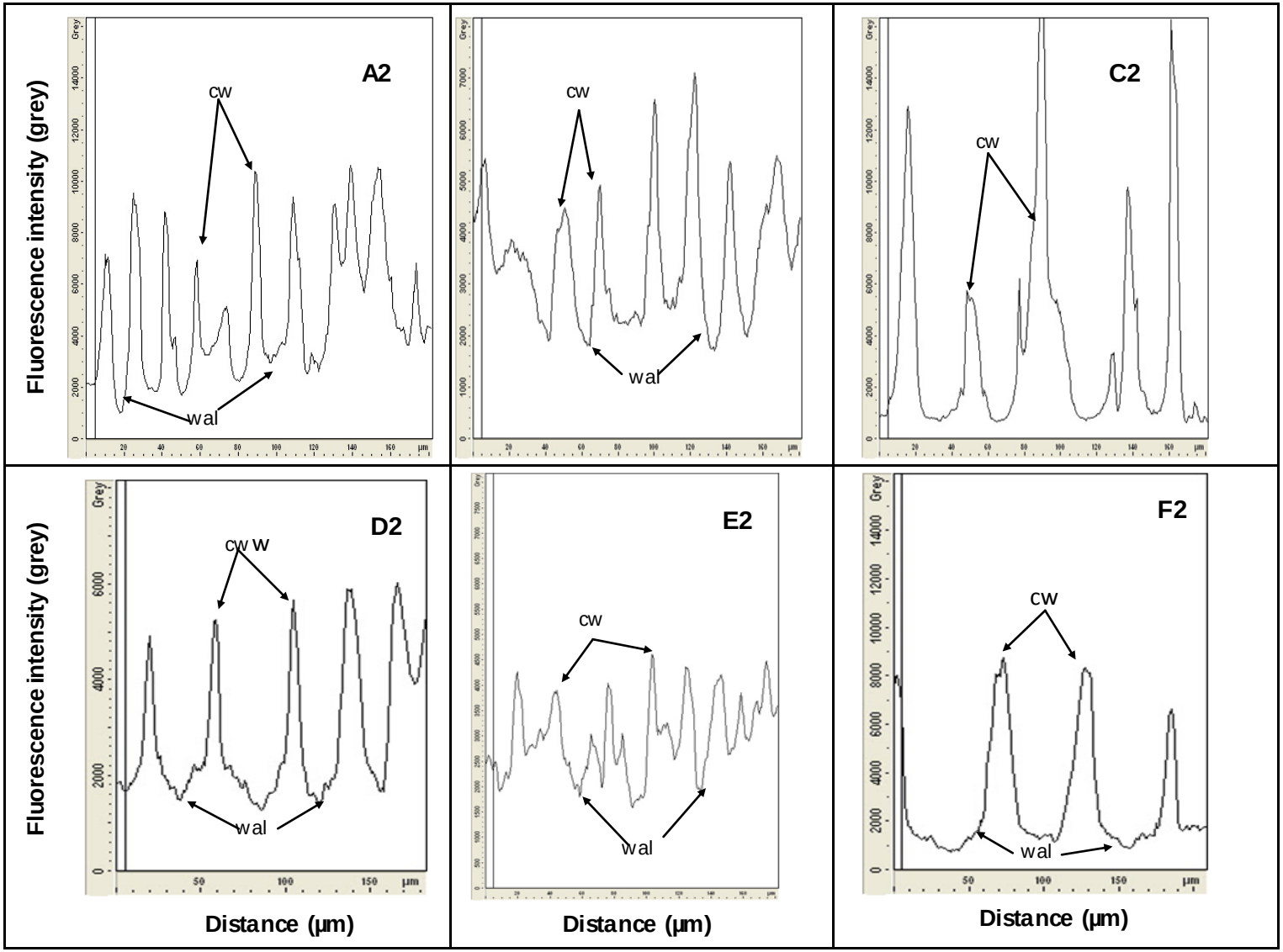

Figure 3.3 Fluorescence intensity profiles showing distribution of ferulic acid within the aleurone layer, line 2 in the raw digital images in Figure 2; purple barley (A2); non-pigmented barley (B2); purple wheat (C2); MSUD8006 wheat (D2); USP1395XR maize (E2) and Ambassador wheat (F2). Peaks represent aleurone cell walls (cw) and low points are areas within the aleurone cell (wal).

Table 3.3 Pearson correlation coefficients for ferulic acid concentration and fluorescence intensity values in different cereal grains and their fractions

Cereal	Barley	Wheat	Yellow maize[2]	All Cereals[1]
Fraction		**r-value (p-value)**		
Pericarp	0.5042(0.49)*	-0.3366(0.41)	0.0152(0.97)	0.3013(0.22)
Aleurone	-0.0854(0.91)	0.5014(0.20)	-0.0645(0.90)	-0.2968(0.23)
Endosperm	-0.7514(0.24)	0.2155(0.60)	-0.0452(0.93)	-0.2700(0.27)
All fractions[2]	0.5929(**0.042**)	0.5849**(0.002)**	0.811(**0.000**)	**0.4211(0.001)**

[1]Correlations were done on data from all cereals (barley, wheat and yellow maize) by fraction and all fractions
[2]Correlations were done on data on individual fractions of each cereal and all fractions together.
*Figures in parentheses are p-values and in bold correlations were significant.

This suggests that there exists a relationship between FA and FI values. On the other hand, for specific grain fractions, both overall and individual cereal-correlations were low or negative but not significant (**Table 3.3**). In contrast, Symons and Dexter (1993) found a strong correlation between aleurone fluorescence to flour refinement in reduction flour and B4 flour. The lack of correlation in the fractions would be attributed to inherent heterogeneity and variability within the whole grains compared to use of ground homogenous samples.

3.5 Conclusion

Use of fluorescence microscopy enabled visualisation of inherent chemical variation within the grain and FA-rich fractions which may be isolated for possible use as functional food ingredients. The present study attempted to show the relationship between fluorescence intensity values and FA concentrations. These findings suggest that there is a relationship between fluorescence intensity values and FA concentration. Hence, fluorescence intensity profiles may be a promising approach to utilise in screening FA concentration and distribution in grain *in situ*. It would be a fast technique compared to the traditional methods. Differences in FA concentration in pigmented and non-pigmented cereals may be attributed to morphological differences other than pigmentation. Among the isolated grain fractions pericarp and aleurone

layer provided concentrated source of FA. Yellow maize aleurone would be an alternative functional food ingredient to the commonly used wheat aleurone layer.

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CHAPTER 4

Comparative Studies on Composition and Distribution of Phenolic Acids in Cereal Grain Botanical Fractions

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4.1 Abstract

The phenolic acid composition and concentration of four manually separated fractions, pericarp, aleurone layer, germ and endosperm fractions as well as whole grains of yellow maize, wheat, barley and oat were analysed by HPLC-MS/MS following microwave assisted alkaline aqueous extraction. Phenolic acid composition in whole grains and their fractions were similar, with minor differences among the grain fractions. Significant differences ($p< 0.05$), however, were observed in phenolic acid concentration among cereal types, within cereal varieties and among grain fractions, with yellow maize exhibiting the highest values. The levels of *p*-coumaric (*p*-CA) and syringic acid (SyA) in the pericarp were 10- to 15- and 6- to 10-fold higher, respectively, in yellow maize than in wheat, barley and oat. In the aleurone layer, sinapic (SA) and vanillic (VA) in yellow maize were about 8-fold and 30-fold more than in wheat. The germ fraction of wheat had 1.8 times more SyA than yellow maize germ. Grain fractions, excluding endosperm had enhanced levels of phenolic acid compared with whole grain. Sinapic acid was more concentrated in pericarp and germ of wheat. *Iso*-ferulic acid was concentrated in the germ of purple barley. Syringic and vanillic acids were concentrated in pericarp and sinapic acid in aleurone layer of yellow maize. These findings are important in understanding the composition and distribution of phenolic acids, and they act as a guide in identification of grain fractions for use as food ingredients. In addition, yellow maize fractions (aleurone and pericarp) may potentially be alternative phenol- rich functional food ingredients in grain-based food products.

4.2 Introduction

Wheat, maize and rice are major grains while oat, barley and rye are minor cereals in the human diet. These cereals are essential sources of energy, proteins, dietary fiber, vitamins, minerals and phytochemicals (Liu 2007). Phytochemicals are naturally occurring bioactive non-nutrient compounds present in plants classified into carotenoids, phenolic compounds, alkaloids, nitrogen-containing compounds and organosulfur compounds (Liu 2004). Phenolic compounds are the major components in cereals (Adom et al. 2003). In the human diet, phenolic compounds from whole grains are a source of antioxidants that have been associated with reduced risks of chronic diseases (Liu 2004) and promoting human health (Dykes and Rooney 2007).

Phenolic compounds include phenolic acids, flavonoids (flavonols, flavanols and anthocyanidins) and tannins (Shahidi and Naczk 2004). Phenolic acids are the most abundant class of phenolic compounds in whole grains. They are further grouped into derivatives of benzoic acid (*p*-hydroxybenzoic, protocatechuic, and vanillic acids) and cinnamic acid (*p*-coumaric, caffeic, ferulic, and sinapic acids). Reports suggest that cereal grains have different composition and distribution of phytochemicals (Evers et al. 1999; Hernanz et al. 2001; Xu et al. 2010; Shao et al. 2014). Most of these are concentrated in grain outer layers (Nordkvist et al. 1984; Kahkonen et al. 1999; Hernanz et al. 2001; Zhou et al. 2004) and in the germ (Sen et al. 1994). For example, in the husk and outer layers of barley grain, 77.7 to 82.3% and 78.0 to 86.3% of the total amounts of phenolics were ferulic and *p*-coumaric acid, respectively (Hernanz et al. 2001). About 83% of the total phenolic content in wheat was found in the germ and bran fractions (Liu 2007) which had higher antioxidant activity compared to the flour fraction (Miller et al. 2000). In contrast, in yellow maize, carotenoids were more concentrated in the endosperm

and aleurone layer while barley, wheat and oat carotenoids were more concentrated in the germ fraction followed by aleurone layer, and the endosperm had the lowest levels (Chapter 5). This suggests that phenolic acids like other phytochemicals are unevenly distributed across the grain (Evers et al. 1999).

Phenolics exist in free and bound forms (Sosulski et al .1982; Adom and Liu 2002; Holtekjolen et al. 2006; Arranz et al. 2010). Holtekjolen et al. (2008) observed that wheat and barley phenolic acids are mainly found in the insoluble fraction (bound). About 85, 75 and 62% of the total phenolics in maize, wheat and oat, and rice were in bound form, respectively (Adom and Liu 2002). Similarly, Arranz et al. (2010) reported higher levels (210mg/100g) of non-extractable polyphenols (NEPP) compared to 57mg/100g of extractable polyphenols (EPP) in cereals. Furthermore, Shao et al. (2014) found that bound phenolic acids in rice bran contributed 90% of total phenolic acids in whole grain whereas free and conjugated phenolic acids in white, red and black rice bran accounted for 41, 65 and 85% of total phenolic acids, respectively.

Several studies have compared phenolic acid profiles in whole grains of wheat (Adom et al. 2003; Dykes and Rooney 2007; Siebenhandl et al. 2007) barley, (Goupy et al. 1999; Hernanz et al. 2001) and maize (Del Pozo-Insfran et al. 2006; Lopez-Martinez et al. 2009; Xu et al. 2010). Phenolic acid profiles of grain fractions have mainly been studied in wheat grain (Nordkvist et al. 1984; Zielinski and Kozlowska2000; Antoine et al. 2004; Zhou et al. 2004; Beta et al. 2005; Parker et al. 2005; Siebenhandl et al. 2007) and to a limited extent, in barley (Nordkvist et al. 1984; Hernanz et al. 2001; Barron et al. 2007). Most of the studies that analyzed phenolic acid profiles in wheat and barley utilised mechanically separated grain fractions with a few using

manually separated fractions. However, studies that utilised the latter mainly focussed on identifying biochemical markers for use during grain milling process (Antoine et al. 2004; Barron et al. 2007; Hemery et al. 2009) rather than comparing phenolic acid composition and distribution. In addition there is limited information on phenolic acid profiles in botanical fractions of yellow maize. Thus, there is a need for comparison of phenolic acid composition and distribution of manually separated grain fractions of wheat, barley, yellow maize and oat under similar experimental conditions.

Understanding the distribution of phytochemicals in the bran, germ and starchy endosperm has become an area of interest (Liu 2007). Knowledge on phenolic acid distribution is essential in identifying and separating grain tissues that may be utilised as functional food ingredients (Antoine et al. 2004; Sidhu et al. 2007) to improve food quality and enhance health benefits. Previous studies have shown that several factors, namely grain type and variety, environmental and growing conditions can influence the presence and distribution of hydroxycinnamic acids (Zielinski and Kozlowska 2000; Adom et al. 2003; Zhou et al. 2004). Therefore the aim of the current study was to investigate the composition and distribution of phenolic compounds in manually separated botanical fractions of yellow maize, wheat, barley and oat relative to their whole grains.

4.3 Materials and methods

4.3.1 Chemicals

HPLC grade hexane, ethyl acetate and methanol were obtained from Fisher Scientific (Whitby, ON, Canada) and 11 phenolic acid standards (gallic, *p*-coumaric, caffeic, vanilllic, sinapic,

ferulic, syringic, protocatechuic, *iso*-ferulic, and *p*-hydroxybenzoic acids) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

4.3.2 Grain samples and sample preparations

Three yellow maize varieties (Dasca, USP1395XR and P1508HR), 2 barley varieties (purple barley and non-pigmented barley), 4 wheat varieties (purple wheat, Caledonia, Ambassador, and MSUD8006) and 1 oat variety were used (Chapter 5). These samples were of commercial value supplied by the cereal industry. USP1395XR and P1508HR were pioneer hybrids, fresh line seeds grown in the US copper belt and Dasca maize is typical Argentina flint maize. The four wheat samples grown in Michigan, USA, were all soft wheat whereas barley samples were hard grains. The samples were manually separated into four fractions, namely the germ, pericarp (consisting of outer pericarp + inner pericarp + testa), aleurone and endosperm as previously described (Chapter 3) following the original procedure of Chrispeels and Varner (1967) modified by Stewart et al. (1988). Fractions were meticulously and homogenously separated with minimal contamination and only the aleurone layers were further examined under fluorescence microscope (supplementary **Figure A, Appendix I**).

4.3.3 Microwave assisted extraction of phenolic acids and HPLC-MS/MS analysis

Phenolic acids were extracted as described by Chiremba et al. (2012a) with some modifications as previously reported (Chapter 3). LC-MS and MS/MS were performed with a quadrupole time of flight (Q-TOF) mass spectrometer (Micromass Waters, Milford, MA, USA) equipped with electrospray ionisation. The instrument was calibrated through a mass range of 100–1000 amu with 2 μg/mL of sodium iodide and operated in negative mode. The full mass spectra were recorded using the capillary voltage of 1.8kV and cone voltage 48V. The flow rates were 900L/h and 50L/h for desolvation gas (N_2) and cone gas, respectively. The desolvation temperature and

source temperature were set at 350 and 150°C, respectively, and MS/MS spectra acquired using collision energy of 20V. The *m/z* ratio and fragmentation patterns were further used to confirm the identified monomeric phenolics and diferulic acids (DFA) in the studied cereal grains and their fractions.

4.3.4 Statistical analysis

All extractions were done in duplicate and reported as mean ± standard deviation (SD). Means were assessed by Duncan's multiple range test using SAS 9.2 (SAS Institute Inc., Cary, NC) and significant differences reported at ($p < 0.05$).

4.4 Results and Discussion

4.4.1 Identification of phenolic acids in whole grains and their botanical fractions

Typical HPLC chromatograms at 280 nm of pure phenolic acid standard mixture (A), aleurone layers of USP1395XR maize (B) and MSUD8006 wheat (C) and the germ fractions (D) and (E) of the same, respectively are shown in **Figure 4.1**. Eight monomeric phenolic acids were separated and identified as *p*-hydroxybenzoic acid (*p*-OH-BA*)*, vanillic acid (VA), caffeic acid (CA), syringic acid (SyA), *p*–coumaric acid (*p*-CA*)*, ferulic acid (FA), sinapic acid (SA) and *iso*-ferulic acid (*iso*-FA*)*. Gallic, protocatcheuic and *o*-coumaric acid were not detected in this study. The identities of detected compounds were confirmed by comparing retention time and UV spectra of the peaks in crude sample extracts with those of commercial standards.

The monomeric phenolic acids were confirmed using LC-MS and MS/MS concurrently to be VA, CA, SyA, *p*-CA, FA, SA and *iso*-FA based on the molecular ions $[M-H]^-$, their fragmentation patterns and by comparison with information in the existing literature (**Table 4.1**).

The LC-MS spectrum of the unknown peak *y* at t_R = 33.5 min in the wheat germ fraction (**Figure 4.1E**) was compared with that of SyA (λ_{max} 273 nm) because it had similar UV-spectra with maximum absorption at 274 nm. The former had a molecular ion with *m/z at* 135, 100% (RI) (Supplementary **Figure B** (a), **Appendix II**) and the latter had a molecular ion with *m/z* at 197, 100% relative intensity (RI) confirming that it was SyA (Supplementary **Figure B** (b), **Appendix II**). Thus the component with *m/z* 135 was likely a degraded product that did not result from SyA because mild conditions of 45 s were used instead of microwaving for 20 min (Liziad et al 2007). Diferulic acids were identified by performing an LC-MS/MS scan at *m/z* 385. **Figure 4.2** shows LC chromatogram of normal barley aleurone (**Figure 4.2a**) highlighting peaks that were assumed to be diferulic acids (DFA) eluting at t_R = 47.02, 52.29 and 55.45 min. These peaks were designated as 8-5´, 5-5' and 8-O-4´, respectively, based on MS/MS fragmentation patterns (**Figure 4.2b & Table 4.1**) (Chiremba et al. 2012a; Guo and Beta 2013) and elution sequence (Bily et al. 2003). Apart from the aleurone layer, these dehydrodimers were also found in the pericarp and germ fractions whereas previous studies identified the same diferulates in whole grain, maize bran, insoluble and soluble fibers (Bily et al. 2003, Chiremba et al. 2012b; Guo and Beta 2013). FA dehydrodimers 8-O-4' and 8-5' are commonly found in cereals (Andreasen et al. 2000; Qiu et al. 2010). Fragmentation patterns of 8-*O*-4'dehydrodimer (DFA3) in barley aleurone (**Figure 4.2b**) and maize pericarp were different from those observed in 8-5' (DFA 1) and 5-5' (DFA 2) dehydrodimers.

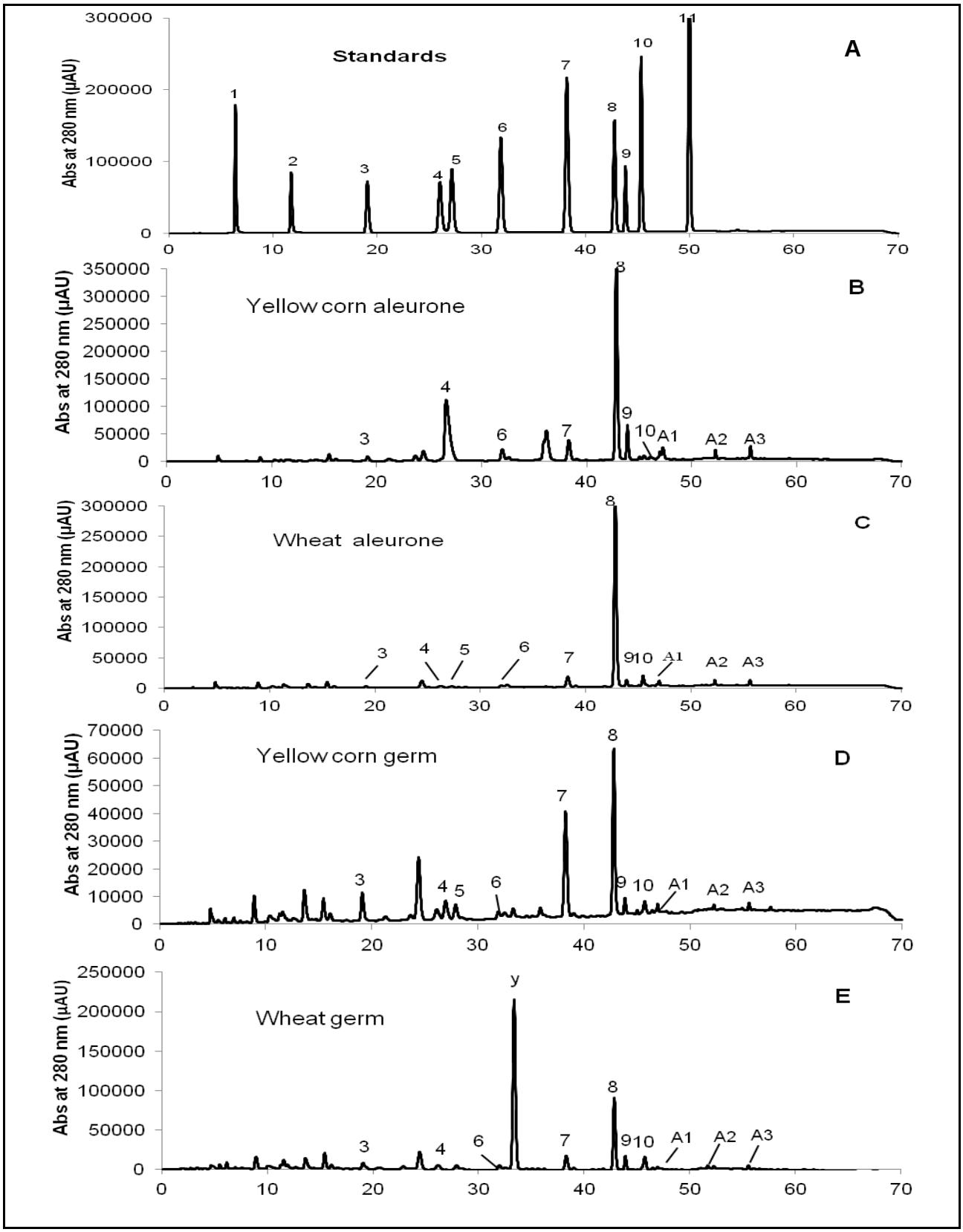
Standards
A
Abs at 280 nm (µAU)
Yellow corn aleurone
B
Wheat aleurone
C
Yellow corn germ
D
Wheat germ
E
A1
A2
A3

Figure 4.1 HPLC chromatograms of phenolic acid standards (A): 1, gallic acid; 2, protocatechuic acid; 3, *p*-hydroxybenzoic acid; 4, vanillic acid; 5, caffeic acid; 6, syringic acid; 7, *p*–coumaric acid; 8, ferulic acid; 9, sinapic acid; 10, *iso*-ferulic acid; and 11, *o*-coumaric acid; aleurone (B, C) and germ (D, E) fractions of Dasca yellow maize and MSUD8006 wheat, respectively.

Apart from the other MS/MS fragments at *m/z* 267, 281, 311, and 341, DFA3 in maize aleurone layer had fragments similar to those observed in monomeric FA, with m/z at 178 149 and 134 due to loss $-CH_3$ (15 Damu), CO_2, (44 Damu) and $-CH_3$ plus CO_2 (59 Damu), respectively. Such a breakdown indicates that cleavage may have occurred at -O- bond between two ferulic acid that make up 8-*O*-4 diferulic acid, hence releasing a monomeric FA that was further fragmented.

4.4.2 Comparing phenolic acid composition and quantities

4.4.2.1 Whole grains

Phenolic acid composition and content of whole grains are presented in **Table 4.2**. Phenolic acid composition in the studied whole grains was generally the same. Six acids (VA, SyA, *p*-CA, FA, SA and *iso*-FA) were found in all whole grains except *p*-OH-BA which was found only in yellow maize. However, levels of phenolic acid content differed significantly ($p< 0.05$) among different cereal types and within cereal varieties (**Table 4.2**). Yellow maize had significantly ($p< 0.05$) higher content of the identified phenolic acids compared to wheat and barley. Oat had the lowest phenolic acid concentration. As previously reported, on the average, ferulic acid was the predominant phenolic acid in all the grains (Adom et al 2003). In this study, *p*-coumaric acid was the second highest phenolic acid in yellow maize, whereas in wheat and barley it was vanillic acid. Highest levels of FA, *p*-CA, *iso*-FA and SyA were found in yellow maize followed by barley, wheat and oat had the lowest (**Table 4.2**). The levels of *p*-CA in yellow maize (0.25 μg/g) reported by Hu and Xu (2011) were lower than values in this study likely due to variety differences and extraction method. In contrast, higher *p*-CA content (1,160 μg/g) in pioneer

hybrid (P3905) was reported by Bily et al. (2003) compared with the levels in USP1395XR and P1508HR. SA and VA were second highest in wheat followed by barley and oat. Among wheat varieties, only FA and VA were significantly different ($p < 0.05$). Purple wheat had highest content of SA and SyA. FA, VA and p-CA were significantly different ($p < 0.05$) in barley varieties. *p*-CA and FA were significantly different ($p<0.05$) among yellow maize varieties. However, *p*-OH-BA, SyA and *iso*-FA were not significantly different between Dasca maize and the two hybrid varieties. Overall yellow maize contained two to five times more total phenolic acids than barley, wheat and oat. Similarly Sosulski et al. (1982) also found that phenolic acids in yellow maize flour were three times more than in flour from wheat, oats, and rice. These results suggest that both grain type and variety influenced phenolic acid content in the studied grains. This observation is in agreement with previous reports that grain type and variety, environmental and growing conditions may influence the presence and distribution of hydroxycinnamic acids (Zielin´ski and Kozłowska, 2000; Adom and Liu 2002; Adom et al. 2003; Zhou et al. 2004). Furthermore, it was observed that phenolic acid concentrations were lower in whole grains compared to grain fractions (**Tables 4.3** and **4.4**) with the exception of the endosperm fraction. This observation suggests that components that are concentrated in fractions that contribute a smaller proportion to whole grain become diluted. In whole grains, the pericarp, aleurone and germ are mixed with the endosperm fraction, which is the largest component of the grain (about 80-86%) but contains the lowest content of phenolic acids. The bran made up of the aleurone and pericarp, constitutes only 10-15 and 8.8 % and the germ only 1.6-3.7 and 12% of the whole kernel, respectively, in wheat, barley and oat and yellow maize (Serna-Saldivar 2010).

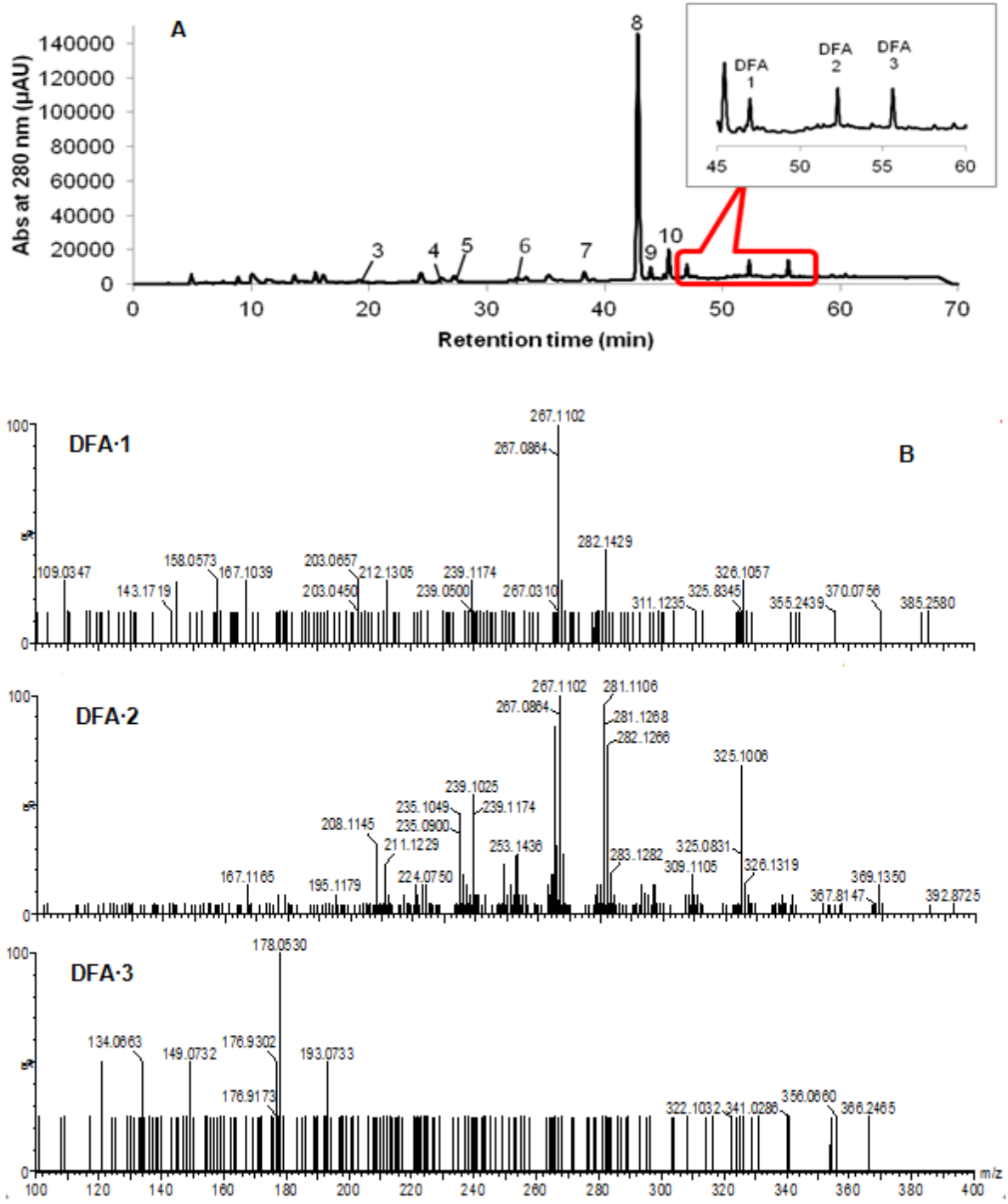

Figure 4.2 Liquid chromatogram of normal barley aleurone layer (a) highlighting 3 diferulic acids and their MS/MS spectra (b) of 8-5' (DFA1), 5-5' (DFA2) and 8-O-4 (DFA3) dehydrodimers

Table 4.1 Peak number (No.), retention time (Rt) (min), MS/MS fragmentation and assignment of identified phenolic acids

Peak No.	Rt (min)	m/z [M-H]⁻	MS/MS Fragment	Identification
3	19.18	136	nd*	*p*-hydroxybenzoic acid
4	26.2	167	167 (123)	Vanillic acid
5	27.4	179	179 (135)	Caffeic acid
6	32.1	197	197(153, 182)	Syringic acid
7	38.2	164	164 (119, 163,)	*p*-Coumaric acid
8	42.7	193	193 (134, 149, 178)	Ferulic acid
9	43.9	223	223 (208, 179, 149)	Sinapic acid
10	45.4	193	193 (134, 149, 178)	*Iso*ferulic acid
A1	47.2	385	267 (341, 326,282, 239, 211)	8-5' DFA
A2	52.3	385	281, 267 (224/211, 239, 282, 325, 341)	5-5' DFA
A3	55.4	385	193 (178, 149, 134, 239, 267, 281, 311, 341)	8-*0*-4 DFA

4.4.2.2 Botanical fractions (pericarp, aleurone, germ and endosperm)

During separation of the fractions, it was observed that although bran layers in cereal grains are the same, their adherences to each other within the grains varied widely. For instance, the intermediate layers (seed coat) in purple wheat were tightly adhered to the aleurone layer, unlike in purple barley and the other wheat varieties. Difficulties in separation of wheat bran layers were also reported by Parker et al. (2005). Despite the challenge, manually separated (hand dissected) fractions had minimal contamination compared to those obtained through mechanical separation.

Phenolic acid composition and concentrations in grain fractions are shown in **Tables 4.3 and 4.4.** The identified phenolic acids included *p*-OH-BA, VA, CA, SyA, *p*-CA, FA, SA and *iso*-FA. Because FA levels in the pericarp, aleurone and endosperm were reported in Chapter 3, only FA levels of the germ fraction will be discussed along with

Table 4.2 Phenolic acid content (μg/g) in whole grains of barley, wheat, oat and yellow maize

	Hydroxycinnamic acids				Hydroxybenzoic acids			
Cereal	***p*-CA**	**FA**	**SA**	***Iso*-FA**	***p-OH-BA***	**VA**	**SyA**	**Total**
Purple barley	96±6.4D	657±4.8F	40±13.77C	40±3.13B	nf[2]	75±16.2DE	61±6.10CD	969
Normal barley	54±3.2E	805±30.6D	61±11.81C	38±1.83B	nf	108±4.7C	66±4.01C	1132
Mean	**75**	**731**	**51**	**37**	nf	**92**	**63**	
Purple wheat	63±0.9E	731±3.9E	114±2.23B	27±0.42D	nf	121±4.5C	55±1.17CD	1111
Ambassador wheat	44±1.7E	600±0.7G	59±0.31C	34±0.71BC	nf	nd[3]	20±0.08E	757
Caledonia wheat	57±1.9E	674±23.3F	47±4.00C	21±1.63D	nf	90±0.9D	21±0.23E	910
MSUD8006 wheat	51±5.5E	750±2.3E	46±0.46C	33±0.08BC	nf	71±1.5E	30±1.98E	981
Average in wheat	**54**	**689**	**66**	**29**	nf	**94**	**31**	
Oat	50±1.5E	367±7.5H	57±1.93C	nd	nf	nd	46±0.89	520
Dasca maize	243±4.0B	1808±12.3B	151±0.15A	33±0.40BC	13±0.95B	229±8.8A	102±13.9B	2579
USP1395XR maize	204±24.9C	1578±3.5C	117±3.85B	31±4.56BC	29±0.11A	191±3.8B	104±14.7B	2254
P1508HR maize	278±6.5A	1977±12.4A	132±0.23AB	67±10.40A	17±0.95B	181±3.6B	140±22.1A	2792
Average in maize	**242**	**1788**	**133**	**44**	**20**	**201**	**116**	

Values are means of n=2 ±standard deviation. Values with different letters in the same column are significantly different at $p<0.05$ (Duncan's multiple range tests) among different cereals. *p*-CA,*p*-coumaric acid; FA, ferulic acid; SA, sinapic acid; *Iso*-FA, isoferulic acid;VA,vanillic acid; SyA, syringic acid and *p*-OH, *p*-hydroxybenzoic acid. nf[2], not found and nd[3], detected but not quantified.

Table 4.3 Phenolic acid content (µg/g) in pericarp and aleurone layer of barley, wheat, oat and yellow maize

		Hydroxycinnamic acids					Hydroxybenzoic acid			
Fraction	**Cereal**	**CA**	**p-CA**	**FA[1]**	**SA**	***Iso*-FA**	***p-OH-BA***	**VA**	**SyA**	**Total**
Pericarp	Purple barley	nf[2]	330±5.92D	1915±17.8F	103±4.73H	69±8.33EF	107±5.27A	394±7.37AB	92±1.1C	2910
	Normal barley	nf	131±0.64E	3698±30.8DE	146±1.06FG	153±20.71BC	28±2.52C	192±12.55DE	166±13.3C	4514
	Mean		**230**	**2807**	**125**	**111**	**72**	**293**	**129**	
	Purple wheat	nf	192±1.44E	3815±29.6D	253±4.11D	102±2.27DE	56±3.91B	486±6.12A	234±8.8C	5138
	Ambassador wheat	nf	169±14.72E	3194±3.3DE	176±2.67EF	158±26.89BC	nd[3]	164±21.08E	218±15.9C	4079
	Caledonia wheat	nf	126±7.66E	3343±16.2DE	199±24.30E	160±12.92BC	nd	185±35.36DE	211±51.83C	4224
	MSUD8006 wheat	nf	179±14.96E	3718±117.9DE	173±11.04EF	138±0.35BCD	nd	206±33.90DE	272±14.9C	4686
	Average in wheat	nf	**166**	**3517**	**200**	**139**		**260**	**234**	
	Oat	nf	165±0.90E	2757.8±2.3EF	124±2.01GH	44±5.46F	29±0.52C	226±16.79DE	176±4.21C	3521
	Dasca maize	nf	2023±24.78C	14300±447.8C	399±8.67C	134±22.13CD	nd	342±78.36BC	889±19.63B	18087
	USP1395XR maize	nf	2464±20.82B	18463±590.8B	520±21.69A	181±38.65B	nd	395±89.39AB	1507±156.5A	23530
	P1508HR maize	nf	3179±175.19A	22437±1018.9A	478±20.70B	395±11.03A	nd	276±50.04CD	1716±321.4A	28481
	Average in maize		**2555**	**18400**	**465**	**237**		**338**	**1371**	
Aleurone	Purple barley	64±1.32C	64±2.64G	1008±3.3G	73±0.39H	39±0.30E	19±1.64H	117±1.70C	39±0.02E	1423
	Normal barley	118±10.53A	73±5.53G	1651±83.1F	153±7.75G	133±1.02BC	36±0.39FG	122±7.86C	47±1.75E	847
	Mean	**91**	**68**	**1329**	**113**	**87**	**28**	**119**	**43**	
	Purple wheat	64±23.51C	120±10.63F	3616±15.5D	108±10.23GH	110±35.71CD	27±6.9GH	178±29.66C	112±0.88CD	4335
	Ambassador wheat	85±2.78BC	185±9.27D	3367±154.4E	337±12.19D	186±13.66A	49±6.43E	123±22.58C	117±0.02C	4449
	Caledonia wheat	78±5.97BC	159±2.00E	3172±44.9E	311±3.36D	166±9.93AB	65±4.18D	135±3.23C	115±0.16CD	4201
	MSUD8006 wheat	82±11.10BC	169±4.57DE	3303±59.5E	225±3.02E	187±14.99A	45±2.83EF	133±8.87C	106±2.45CD	4250
	Average in wheat	**77**	**158**	**3365**	**245**	**162**	**47**	**142**	**113**	
	Oat	103±26.14AB	62±3.52G	748±32.2H	178±13.49EF	35±6.19E	50±5.4E	160±5.34C	91±2.53D	1427
	Dasca maize	nf	492±3.58A	4910±166.0B	2282±62.58A	88±9.60D	152±5.60C	3700±5.28B	337±6.74B	11961
	USP1395XR maize	nf	325±17.70C	3944±83.5C	1391±9.73C	94±12.88D	192±3.15B	3929±317.8B	340±9.93B	10215
	P1508HR maize	nf	420±1.70B	5652±10.8A	2196±8.32B	136±7.50BC	261±10.9A	5101±266.5A	416±1.70A	14182
	Average in maize		**412**	**4836**	**1956**	**106**	**226**	**4243**	**274**	

[*] Values are means of n=2 ±standard deviation. Values with different letters in the same column are significantly different at $p<0.05$ (Duncan's multiple range tests) among different cereals fractions. *p*-CA, *p*-coumaric acid; FA, ferulic acid; SA,sinapic acid; *Iso*-FA, isoferulic acid; VA,vanillic acid; SyA, syringic acid and *p*-OH, *p*-hydroxybenzoic acid. [1]Ferulic acid values from Chapter 3, nf[2], not found and nd[3], detected but not quantified because peak area was below the peak area of lowest standard concentration.

Table 4.4 Phenolic acid content (µg/g)* in germ and endosperm fractions of barley, wheat, oat and yellow maize

		Hydroxycinnamic acids				Hydroxybenzoic acid			
Fraction	**Cereal**	**p-CA**	**FA**	**SA**	***Iso*-FA**	***p-OH-BA***	**VA**	**SyA**	Total
Germ	Purple barley	146±0.94E	571±34.2E	nd[3]	850± 2.38A	40±10.47D	209±0.51C	57±0.88DE	1873
	Normal barley	91±0.64F	558±0.43E	nd	175±3.66B	43±1.73D	141±1.56E	44±2.16E	1052
	Average in Barley	**118**	**565**		**513**	**41**	**175**	**50**	
	Purple wheat	66±0.28G	490±0.43F	334±20.96 C	124±11.09C	126±12.67C	251±20.92A	128±7.15A	1519
	Ambassador wheat	213±5.13C	879±21.3C	656±52.93A	nf	165±5.98BC	173±25.46D	121±39.99AB	2207
	Caledonia wheat	96±3.73F	868±25.2C	222±30.17D	nf	129±11.32C	186±20.57CD	83± 1.10CD	1584
	MSUD8006 wheat	179±1.37D	1054±6.64A	401±3.66B	nf	190±24.88AB	242±0.57BA	133±0.88A	2199
	Average in wheat	**139**	**823**	**403**		**152**	**213**	**108**	
	Oat	65±0.41G	571±22.3E	nd	nf	113±0.06C	173±2.35C	96±1.77BC	1018
	Dasca maize	293±3.03B	846±11.4CD	173±7.61E	nf	145±9.07BC	186±9.15C	58±4.64DE	1701
	USP1395XR maize	357±1.03A	825±0.95D	139±4.99E	nf	239±3.02A	216±1.10B	66±2.79CDE	1842
	P1508HR maize	289±6.25B	919±0.49B	128±0.36E	nf	166±5.14BC	169±4.86C	63±0.4DE	1734
	Average in maize	**249**	**863**	**146**		**183**	**190**	**63**	
Endosperm	Purple barley	29±3.36B	116±2.1CD	nf[2]	nf	nf	nf	nf	145
	Normal barley	25±2.93BC	123±0.00C	nf	nf	nf	nf	nf	148
	Average in barley	**27**	**119**						
	Purple wheat	nd	118±3.9CD	nf	nf	nf	nf	nf	118
	Ambassador wheat	21±2.51CD	112±0.6CD	nf	nf	nf	nf	nf	133
	Caledonia wheat	19±0.17D	109±0.3CD	nf	nf	nf	nf	nf	128
	MSUD8006 wheat	nd	107±5.0CD	nf	nf	nf	nf	nf	107
	Average in wheat	**20**	**111**	nf	nf	nf	nf	nf	
	Oat	20±0.82D	98±1.2D	nf	nf	nf	nf	nf	118
	Dasca maize	40±0.32A	183±1.8A	nf	nf	nf	nf	nf	223
	USP1395XR maize	nd	172±24.1AB	nf	nf	nf	nf	nf	172
	P1508HR maize	27±1.96B	152±13.1B	nf	nf	nf	nf	nf	179
	Average in maize	**33**	**169**						

* Values are means of n=2 ±standard deviation. Values with different letters in the same column are significantly different at $p<0.05$ (Duncan's multiple range tests) among different cereals fractions. *p*-CA, *p*-coumaric acid; FA, ferulic acid; SA,sinapic acid; *Iso*-FA, isoferulic acid; VA,vanillic acid; SyA, syringic acid and *p*-OH, *p*-hydroxybenzoic acid.[1]Ferulic acid values from Chapter 3, nf[2], not found and nd[3], detected but not quantified because peak area was below the peak area of lowest standard concentration.

p-OH-BA, VA, CA, SyA, *p*-CA, SA and *iso*-FA, in all the fractions and FA will be included in the germ fraction.

Phenolic acid concentration differed significantly ($p< 0.05$) among the fractions from the different grains and varieties. In some instances even the distribution of phenolic acids within grain fractions varied widely. For example, on average, SA in wheat varieties was highly concentrated in the germ (403 μg/g), followed by aleurone layer (245 μg/g) and pericarp (200 μg/g). SA levels were higher in wheat than in similar fraction of barley and oat. In contrast, SA was concentrated in the aleurone layer (1956 μg/g) and pericarp (465 μg/g), while the germ had the lowest levels (146 μg/g) in yellow maize. This observation supported that even within the outer layers, phenolic acid are also unevenly distributed. Overall all the phenolic acids were highly concentrated in the outer layers, with significant amount in the germ fractions, whereas the endosperm had negligible levels. Unlike in whole grain, phenolic acid composition in the botanical fractions of yellow maize, wheat, barley and oat differed.

***Pericarp (outer and inner pericarp, testa and nucellar epidermis*):** Seven phenolic acids (*p*-OH-BA, VA, SyA, *p*-CA, FA, SA, and *iso*-FA) were present in the pericarp of all the grains. However, *p*-OH-BA was only quantified in the pericarp of purple barley, normal barley, purple wheat, and oats, whose levels were above 8 μg/g based on the lowest standard concentration of 1.25 μg/mL. For yellow maize pericarp, the quantities are not reported because of the large variation (CV>25%) between the duplicate samples likely due to very low concentrations. Significant differences ($p< 0.05$) were observed in individual phenolic acid content among grain types. Levels of VA, SyA and SA in wheat, VA in barley and VA and SyA in oat were higher

than those of *p*-CA except in yellow maize (**Table 4.3**). Recently, Butsat and Siriamornpun (2010) also reported VA as a dominant phenolic acids in bran and husks of Thai rice. On average, yellow maize pericarp had significantly higher ($p < 0.05$) concentration of *p*-CA (2,556 μg/g) and SyA (1,371 μg/g) than in wheat (166 and 234 μg/g), barley (230 and 129 μg/g) and oat (165 and 176 μg/g) pericarp, respectively. Lower levels of *p*-CA (302 and 133 μg/g) were found in bran of hard and soft maize cultivars (Chiremba et al. 2012b). Both *p*-CA and SyA were 10 times higher in yellow maize than in barley and wheat (**Table 4.3**). Significant differences ($p < 0.05$) were also observed in *p*-CA concentration in barley varieties. Purple barley had 2.5-fold higher *p*-CA than normal barley (**Table 4.3**). However, *p*-CA content within wheat varieties was not significantly different. The range of *p*-CA in wheat varieties in the present study was higher (126 - 192 μg/g) compared to 50-110 μg/g in the manually-separated pericarp of Caphorn and Crousty wheat (Barron et al. 2007). The observed difference in *p*-CA concentration in these studies may not only be attributed to varietal differences, but also to sample preparation and extraction methods. In the present study, phenolics were extracted using 4M NaOH at 170°C for 45 secs with MAE whereas the previous study used 2M NaOH at 35°C for 2 hour. Hence the present study may have enhanced the release of bound *p*-CA from the cell walls. On average, SA content was approximately 2 and 3 times higher in yellow maize varieties (465 μg/g) than in wheat (200 μg/g) and barley (124 μg/g) varieties, respectively. Oat had the lowest levels of SA. The content of phenolic acids in yellow maize implies that its pericarp, although rarely utilised in cereal based food products, is a potential functional food ingredient. However, phenolic acids from maize bran (pericarp) were found to be slightly bioavailable in rats (Zhao et al. 2005), which may affect its utilisation.

Purple wheat had the highest content of VA (485.6±6.12 µg/g) followed by purple barley (394.4±7.37 µg/g) and then oat (226.3±16.79 µg/g). Ambassador wheat had the lowest levels (164.1±21.08 µg/g) of VA. In contrast normal barley had higher concentration of *iso*-FA (152.8±20.71 µg/g) and SyA (166.3±13.3 µg/g) than purple barley. The difference in concentrations of VA, *iso*-FA and SyA differentiated pigmented from non-pigmented cereals in wheat and barley varieties. However, no attempts were made to examine if the variation would be attributed to pigmentation, although the role of phenolic compounds such as anthocyanins in pigmentation of plant foods is well known (Shahidi and Naczk 2004).

Phenolic acids were more concentrated in pericarp than in whole grain. Thus FA and SyA were >3- and 2-fold, 5- and >5-fold and 11 and >9-fold higher in the pericarp than whole grain of barley, wheat and yellow-maize, respectively. SA content was 2 to 3 times higher in yellow maize compared to wheat, barley and oat; however, its concentrations in relation to whole grain in these grains were within the same range (2.2 to 4.3-fold more). In contrast, *iso*-FA and VA, respectively, were more concentrated in the pericarp of wheat varieties up to 7 and 4 times more than in yellow maize. These two phenolic acids were only 4 and 2 times higher than in whole grain of wheat. The high levels of phenolic acids in the pericarp and aleurone are produced in response to pest and pathogen attack (Sen et al. 1994; Wu et al. 2009).

Aleurone layer: Unlike in the pericarp, eight phenolic acids (*p*-BA, VA, CA, SyA, *p*-CA, FA, SA and *iso*-FA) were present in the aleurone layers except for maize where CA was not detected. Difficulties in detecting CA have been reported in other maize samples, such as maize stover (Dobberstein and Bunzel 2010).

The aleurone layer of yellow maize had the highest and significantly different concentration of each of the detected phenolic acids except for *iso*-FA (**Table 4.3**). On average, *iso*-FA was the highest in wheat (162 µg/g), followed by yellow maize (106 µg/g) and barley (87 µg/g). Although *iso*-FA is rarely reported in cereals, it was also found and quantified in the pericarp fractions and only in the germ of barley varieties and purple wheat. Recently, in our lab, Guo and Beta (2013) identified *iso*-FA in whole grains, insoluble fiber and soluble fiber of barley, wheat and maize. (Guo and Beta 2013). I*so*-FA was described as a positional isomer of FA and differentiated from FA based on relative intensities of the MS/MS fragments in the spectra. Similarly, Kuhnert et al. (2010) distinguished FA from *iso*-FA based on difference in relative abundance of the fragments when using different collision energies in MS.

Unlike in the pericarp fraction, yellow maize aleurone had the highest concentration of VA, followed by SA, *p*-CA, SyA and *p*-OH-BA. *p*-OH-BA was quantified in the aleurone layer of all grains. The average content of VA in yellow maize was 4243 µg/g, which was 26-35 times higher than in wheat, barley and oat. Furthermore, yellow maize also had highest content of SA (1956 µg/g) followed by wheat (245 µg/g), oat (178 µg/g) and lastly purple barley (113 µg/g). *p*-CA content was also highest in yellow maize, followed by wheat, barley and oat varieties.

Within the yellow maize varieties, levels of phenolic acids differed significantly ($p<0.05$). Maize variety P1508HR had higher levels of FA, *iso*-FA, VA, SyA and *p*-OH-BA whereas SA and *p*-CA dominated in Dasca. USP1395XR maize had intermediate levels of phenolic acids except for *iso*-FA, VA and SyA which were within same range as Dasca variety. This implies that phenolic acid content in aleurone layer of P150HR differed significantly from USP1395XR as well as

Dasca maize likely due to variety differences as already established by (Adom et al. 2003; Hu and Xu 2011) and genotype-environment interactions (Moore et al. 2005).

Most phenolic acids in the aleurone layer of wheat varieties were within the same range and were not significantly different ($p< 0.05$). Compared to wheat varieties, phenolic acid levels in barley varieties were significantly lower ($p< 0.05$) (**Table 4.3**). In this study, wheat and barley aleurone ranked second and third from yellow maize in terms of phenolic acid concentrations. However, there were distinct and significant ($p< 0.05$) differences in the concentration of SA, *iso*-FA, *p*-OH-BA and CA (**Table 4.3**) between purple barley and normal barley. The observation that phenolic acid levels were significantly different in barley varieties and not in wheat suggests the effects of varietal differences in wheat varieties under investigation were negligible.

The aleurone layer represents the major proportion of grain with beneficial substance for human nutrition (Atwell 2010). Wheat aleurone layer is the most utilised fraction as a functional food ingredient (Brouns et al. 2012) due to its high FA concentration. In this study, apart from the predominant FA, maize aleurone layer also had high concentrations of SA and VA, making it a richer source of phenolic acids compared to wheat aleurone layer. Thus maize aleurone layer can be used as source of substantial phenolic acids if appropriate fractionation technologies are developed to separate the maize aleurone layer, as is currently the case with wheat aleurone layer.

Compared with whole grains, grain fractions were concentrated sources for most of the analyzed phenolic acids. On average, *p*-CA, SyA and VA were 3-, 4- and 1.2-fold more in wheat aleurone

compared with whole grain, respectively. These values are lower than those reported by Zhou et al. (2004) who found that *p*-CA, SyA and VA were 5.5-, 6.5- and 4-fold more in wheat aleurone layer compared to whole grain, respectively. In the present study, FA, *p*-CA and *iso*-FA ranged from 4.4 to 5.6-, 1.9 to 4.2- and 4.0 to 7.9-fold higher, respectively, in wheat aleurone layer compared with whole grain. This variation in phenolic acid content may be attributed to several factors such as varietal difference, fractionation and extraction methods.

Germ fraction: The phenolic acid composition in the germ fractions was different from that observed in the pericarp and aleurone layers (**Tables 4.3** and **4.4**). *p*-OH-BA was present in all the grains. *Iso*-ferulic acid was only detected and quantified in purple barley, normal barley and purple wheat. Although SA was present in barley and oat varieties, it was not quantified because the peak areas were below that of the lowest standard concentration (2 µg/mL) in the calibration curve used in calculating concentrations in the actual sample.

As observed with the other fractions, phenolic acid concentration differed significantly ($p<0.05$) among cereal grains and within cereal varieties (**Table 4.4**). On average, levels of *p*-OH-BA (183 µg/g) and *p*-CA (249 µg/g) were higher in yellow maize than in wheat (152 µg/g and 139 µg/g), respectively. The levels of FA were within the same range in yellow maize (825-919 µg/g) and wheat varieties (879-1054 µg/g). However, FA contributed about 45 and 50% to the total amount of phenolics in wheat and yellow maize, respectively. With respect to FA concentration in wheat germ our data are in contrast to those presented by Barron et al. (2007) who reported higher FA content when amounts found in scutellum and embryonic fraction of wheat were combined. The differences were a result of different grain variety and sample preparation

methods. On the other hand, the levels of FA content in the maize germ are within same range as those reported in literature (Bakan et al. 2003). Thus, the sum of (*Z)*-FA plus *(E)*-FA in the different forms (free, soluble and cell wall bound phenolic acids) found in the maize germ was 861 µg/g, which is comparable to the range of 825 to 919 µg/g in yellow maize germ in the present study. This result suggests that microwave-assisted extraction released the three forms of phenolic acids, hence overcoming the problem of underestimation and long processing times (Adom et al. 2003; Bakan et al. 2003; Qiu et al. 2010). The advantage of using microwave-assisted extraction is that it is a quick method and uses less solvent, hence being cost effective. FA content was also within a similar range in barley and oat germ.

Wheat had highest content of SA (403 µg/g), VA (213 µg/g) and SyA (108 µg/g), followed by yellow maize and barley. Specifically, SA, VA and SyA in wheat varieties were 3-fold, 1.2-fold and 1.7-fold more than in yellow maize varieties. *Iso*-ferulic acid was 4 times more in purple barley than in purple wheat (**Table 4.4**). Compared with barley, oat had significantly higher levels of *p*-OH-BA and SyA ($p < 0.05$) but they had similar content of VA. However VA was significantly lower in barley and oat compared with levels found in wheat. These findings suggest that apart from FA, wheat germ has enhanced levels of the other phenolic acids compared to yellow maize, barley and oat germ. Total phenolic acid content in germ fractions of yellow maize, oat, barley and wheat was 9 to 15-fold higher than their respective endosperm. A similar range of 15 to 18-fold high total phenolic acids in wheat bran/germ fractions has been reported (Adom et al. 2005). Considering the high lipophilic antioxidant activity of wheat bran and germ fractions reported by Adom et al. (2005), it could be speculated that the high levels of phenolic acids contribute towards protection of the germ against lipid oxidation because the

germ is a reservoir of lipids in the grain kernel whereas the endosperm is mainly composed of starch.

Endosperm fraction: Only FA and *p*-CA were detected and quantified in the endosperm of almost all grains but *p*-CA could not be quantified in MSUD8006 wheat and USP1395XR maize. Compared with the other three fractions, the endosperm had the lowest levels of both *p*-CA and FA (**Table 4.4**). FA was 5-fold higher than *p*-CA in yellow maize, wheat, barley and oat and accounted for about 80.0 to 85.5% of the total phenolic acids in the endosperm. *p*-CA content in yellow maize (Dasca and P1508HR) were significantly different ($p<0.05$) and higher than in wheat, barley and oat (**Table 4.4**). The *p*-CA content in wheat endosperm in this study was comparable to those reported by Antoine et al. (2004). In contrast, Barron et al. (2007) found zero *p*-CA in hand dissected endosperm fractions of Caphorn and Crousty wheat. Unlike in the present study, Siebenhandl et al. (2007) detected VA in the flour fractions from wheat and barley but the amounts were low. These differences in *p*-CA content and absence of VA may also be due to difference in wheat varieties and growing conditions which have been reported to influence the chemical composition of the grain (Adom et al. 2003).

4.5 Conclusion

Out of the eight phenolic acids that were identified in this study, five (VA, *p*-CA, FA, SA and SyA) were present in all whole grains and their botanical fractions. Three phenolic acids (*p*-OH-BA, CafA and *iso*-FA) were either absent or present but could not be quantified in some whole grains, pericarp, aleurone and germ of some cereal grains. However, phenolic acid composition of the studied grains was generally the same. The presence of particular phenolic acids in

specific fractions may suggest where they are localised and concentrated. Thus, *p*-OH-BA may be more concentrated in the pericarp, aleurone layer and germ of wheat, barley and oat, and only in the aleurone and germ of yellow maize, whereas CafA was present in aleurone layers of wheat, barley and oat. Phenolic acid concentration varied significantly in whole grains and their botanical fractions, depending on grain type and cereal varieties. Botanical fractions had enhanced levels of phenolic acids. Thus the pericarp, aleurone and germ fractions of all grains had higher concentration of phenolic acids compared with whole grains. Furthermore, there was a wide variation in individual phenolic acids that were more concentrated in specific fractions among the grains. For example, SA was more concentrated in pericarp and germ of wheat, *iso*-FA in barley germ, SyA and VA and SA in pericarp and aleurone layer of yellow maize respectively, indicating uneven distribution within the outer layers. In addition to the commonly known FA and *p*-CA, the high content of VA and SA present in the maize aleurone layer positions it as a potential alternative to commercially available, wheat aleurone layer. Overall, grain fractions, particularly, the aleurone layer, pericarp and the germ have diverse composition and recognisable amounts of the phenolic acids, which may enhance the functional properties of grain-based food products.

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CHAPTER 5

Distribution of Carotenoids in Endosperm, Germ and Aleurone Fractions of Cereal Grain Kernels

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5.1 Abstract

To compare the distribution of carotenoids across the grain, non-maize and maize cereals were hand dissected into endosperm, germ and aleurone fractions. Total carotenoid content (TCC) and carotenoid composition were analysed using spectrophotometry and HPLC. Cereal carotenoid composition was similar; however, concentrations varied significantly ($p < 0.05$). Endosperm fractions had TCC ranging from 0.88 to 2.27 and 14.17 to 31.35 mg/kg in non-maize cereals and maize, respectively. TCC, lutein and zeaxanthin in germ fractions were higher in non-maize cereals than in maize. Lutein and zeaxanthin contents were lower in non-maize cereal endosperms. The aleurone layer had zeaxanthin levels 2- to 5-fold higher than lutein among the cereals. Positive significant correlations ($p < 0.05$) were found between TCC, carotenoids analysed by HPLC and DPPH results. This study is the first to report on carotenoid composition of the aleurone layer. Our findings suggest that the aleurone of wheat, oat, maize and the germ of barley have significantly enhanced carotenoid levels.

5.1 Introduction

Increased interest in functional foods requires more information on the phytochemicals including carotenoids in grain cereals that have health enhancing properties. Carotenoids are among the abundant families of pigments in nature that are responsible for the yellow, orange and red colours of fruits, vegetables and grains. They form part of the antioxidant system in seeds (Howitt and Pogson 2006). Xanthophylls carotenoids, which include lutein and zeaxanthin, are recognised with antioxidant properties (Gentili and Caretti 2011; Leenhardt et al. 2006; Miller et al. 1996). Carotenoids act as radical scavengers and singlet oxygen quenchers (Leenhardt et al. 2006). Epidemiological studies have shown that carotenoid-rich foods reduce the risk of degenerative diseases, such as cancer, cardiovascular diseases, and age-related macular degeneration and also maintain skin health (Burkhardt and Boehm 2007; Rice-Evans 1996; Roberts et al. 2009).

Although a minor component in cereals (Irakli et al. 2011), some grains contain higher and others lower content of carotenoids compared to fruits and vegetables (Abdel-Aal et al. 2002; Humphries and Khachik 2003). However, carotenoid content is an important characteristic in the utilisation of cereals such as durum wheat for pasta production (Hentschel et al. 2002). Several authors have studied carotenoid content and composition in whole grain wheat (Abdel-Aal et al. 2007; Adom et al. 2003; Hentschel et al. 2002; Panfili et al. 2004), yellow maize or maize (Kimura et al. 2007; Luterotti and Kljak 2010) and barley (Goupy et al. 1999). The main carotenoids in cereal grains are lutein and zeaxanthin (Hentschel et al. 2002; Panfili et al. 2004). Lutein was the most abundant carotenoid in eleven wheat varieties (Adom et al. 2003). Okarter et al. (2010) found higher levels of lutein and zeaxanthin in eight diverse, whole wheat varieties

than reported by Adom and others (2003). Zeaxanthin was the dominant carotenoid in maize whereas lutein was the main component in oat, barley, spelt and durum wheat (Panfili et al. 2004). Studies on carotenoid content and composition mainly used durum wheat, bread wheat, specialty wheat (Einkorn, Khorasan) (Abdel-Aal et al. 2007; Abdel-Aal et al. 2002; Hidalgo et al. 2006), yellow maize (Hulshof et al. 2007; Kurilich and Juvik 1999b) while barley and oat are rarely used (Goupy et al. 1999; Panfili et al. 2004). Only a few have studied the distribution of carotenoids in grain kernels and their fractions (Borrelli et al. 2008; Hentschel et al. 2002; Panfili et al. 2004). There is limited or no information on carotenoid composition of the aleurone layer. These fractions can be obtained mechanically at large scale during milling and dry fractionation processes (Antoine et al. 2004) although they are generally lacking in purity. The main objective of this study was therefore to investigate the distribution of total and individual carotenoids in endosperm, germ and aleurone fractions obtained by hand dissection of diverse cereals grains. A secondary objective was to determine the antioxidant activity of carotenoid extracts.

5.2 Materials and methods

5.2.1 Chemicals

Acetonitrile, methyl-t-butyl ether (MtBE) 1-butanol and methanol were purchased from Fisher Scientific (Whitby, ON, Canada). Carotenoid standards, lutein, zeaxanthin and β-cryptoxanthin were purchased from ChromaDex Inc. (Santa Ana, CA) and trolox (S)-(-)-6-hydroxyl-2, 5,7,8-tramethylchroman-2-carboxylic acid and 2,2-diphenyl-1-picrylhydrazyl (95%) from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

5.2.2 Samples

A study was conducted on 7 non-maize; 2 barley varieties (purple and regular), 4 soft wheat varieties (Ambassador, Caledonia, Purple wheat and MSUD8006), 1 oat and 3 yellow maize (USP1395XR, P1508HR and Dasca-flint maize) cereals. Samples were analysed by HPLC and spectrophotometry. These included 10 whole grains, 10 endosperm fractions, 10 germ fractions and 10 aleurone layer (only for HPLC).

5.2.3 Sample preparation

5.2.3.1 Hand dissection/grain fractionating

The grains were hand dissected to separate the outer pericarp (bran), inner layer (aleurone layer), germ and endosperm based on the procedure described by Stewart et al. (1988) with further modifications. Briefly, grain brush ends and germs were removed by a sharp scalpel under a magnifying glass and the seeds cut lengthwise. The grains were soaked in 0.1% sodium hypochloride for 15-20 minutes to sterilize the surfaces, and rinsed using sterile, distilled, deionised water. The seeds were placed in 10 cm petri dishes lined with 2 ashless filters, moistened with 10ml of sterile distilled deionised water. The petri dishes were wrapped in aluminum foil and kept at room temperature (20°C) for two days. The pericarp, aleurone layer and endosperm were separated using a scalpel under a magnifying glass and stored at -20°C. Samples were freeze dried and ground into fine flour using a multi-use blade grinder, model PCC770 (Loblaws Inc. Toronto, ON, Canada) to pass through a 0.5 mm sieve screen. The ground samples were stored at -20°C before extraction and analysis.

5.2.3.2 Estimation of seed fraction proportions

To determine the ratio of the germ, endosperm and bran to whole grain seed, 25 seeds of each grain sample were randomly selected, individually weighed and manually hand dissected under a magnifying glass. The weight of germ was calculated by subtracting weight of the endosperm from weight of endosperm without germ. Isolated germs were randomly weighed to confirm weight found by subtraction. The average weight of whole grain and percentage proportions of each fraction are shown in **Table 5.1**. The percentage proportions were later used to calculate the distribution of the TCC in the grain fractions.

Table 5.1 Whole grain weight and percentage proportion of each fraction

Cereal type	Weight (mg)	% proportion of whole grain		
	Whole	Bran	Germ	Endosperm
Purple barley	45.5	15.7	2.6	81.7
Non-pigmented barley	47.5	14.4	2	83.6
Mean-Barley	**46.5**	**15.1**	**2.3**	**82.7**
Purple wheat	45.7	11.7	1.6	86.8
Ambassador wheat	47.6	12	2	86
Caledonia wheat	50.8	12.2	2.1	85.8
MSU D8006 wheat	48.8	12	1.9	86.1
Mean-Wheat	**48.2**	**12.0**	**1.9**	**86.2**
Oat	38.4	8.7	1.8	89.5
DASCA maize	378.7	5.7	12.1	82
USP1395XR maize	360.6	5.8	11.2	83.0
P1508HR maize	394.6	5.2	10.7	84.1
Mean-Yellow maize	**377.9**	**5.6**	**11.3**	**83.0**

Average weight of different grain cereals (n=25) and % proportion of the grain fractions (bran, germ and endosperm)

5.2.4 Extraction of carotenoids

Carotenoids were extracted according to the method described by Abdel-Aal et al. (2007) with some modifications. Briefly, 200 mg of ground samples (whole grain, endosperm and germ) were mixed with 2 mL of water–saturated butanol in tubes covered with black cap and aluminum

foil in the fumehood. The mixtures were vortexed for 30 secs and carotenoids extracted by shaking for 15 min at 40 rpm using a horizontal rotary shaker (RKVSD, ATR Inc., Laurel, MD, USA). After shaking, the samples were left to stand for 60 min at room temperature in the dark and then vortexed before shaking for another 15 min. Lastly, the samples were allowed to stand for 60 min. About 1.8 mL of extract were transferred into 2 mL brown micro-centrifuge tubes and centrifuged at 4,000xg and 20°C using IEC Micromax Microcentrifuge (Thermo Electron Corporation, Milford, MA, USA). All the procedures were carried out in the dark.

5.2.5 Spectrophotometeric determination of TCC

Supernatants were transferred from micro-centrifuge tubes into semi-micro quartz cuvettes and absorbance measured at 450 nm (average absorbance for carotenoids in wheat and maize) using an Ultraspec 1100 pro, UV/Visible spectrophotometer (Biomicron Ltd. Cambridge, England). All analysis were done in triplicate. Total carotenoid content (TCC) was calculated using the following equation and expressed as µg lutein equivalents per g sample

$$C=2(A/S)(W)\ \mu g/g$$

where C= lutein content, µg/g; A = absorbance reading, S = regression coefficient (the number that express the relationship which is created based on concentration of lutein working standard solutions in µg/mL and the absorbance); 2 = dilution factor (the dilution factor 2 is based on the total extracted volume of 2 mL) and W = sample weight, g (Abdel-Aal et al. 2007; Abdel-Aal and Young 2009).

5.2.6 Determination of carotenoid composition by HPLC

Fresh extractions were done to determine carotenoid composition using the same procedure described above up to the centrifugation step. After centrifuging, the supernatant was filtered through a 0.45 µm nylon disc filter into brown HPLC vials and stored at -20°C overnight before

analysis. HPLC analysis of carotenoid composition included the aleurone layer fraction. The determination of carotenoid composition was done in duplicates according to the method described by Abdel-Aal et al. (2007) with some modifications. Briefly, the chromatographic separation and quantification of carotenoids was carried out on an HPLC (Waters 2695) equipped with a photodiode array detector (PAD) (Waters 996) and auto sampler (Waters 717 plus) (Waters, Milford, MA, USA) using YMC™ carotenoid S-3, 3 µm packing, 4.6x100 mm column (Waters, Milford, MA). The column was operated at 35°C; 20 µL of sample was injected by the auto sampler and eluted with a gradient system consisting of (A) methanol/methyl tert-butyl ether/Milli-Q water (81:15:4,v/v/v) and (B) methyl tert-butyl ether/methanol (90:10, v/v). The flow rate was set at 1 mL/min. The gradient was programmed as follows: 0-9 min, 100-75% A; 10-12 min 0% A; 12-13 min, 0-100% A; and 13-15 min, 100% A. The separated carotenoids were detected and measured at 450 nm. The eluted carotenoids were identified based on similarity of retention time, elution sequence and UV/vis spectra with those of standards as well as literature reports. Lutein and zeaxanthin standards were used for identification and quantification whereas β-cryptoxanthin was only used for identification as specified by manufacturer. Five concentrations in the range of 0.05-0.5 µg/mL and 0.25-2.5 µg/mL per injection of 20 µL were prepared for lutein and zeaxanthin, respectively, to generate regression equations for quantification. The regression equations ($y=5000.7x+1.2333$ and $y=3182.6x+80.362$) showed a linear relationship with R^2 of 0.9999 and 0.9983 for lutein and zeaxanthin, respectively. Y is peak area and x is the concentration.

5.2.7 Determination of antioxidant activity

The DPPH radical scavenging activity of carotenoid extracts was determined according to the method described by Brand-Williams et al. (1995) and modified by Li et al. (2005) with further

modifications. Aliquots of 3.9 mL of 60 µM DPPH in methanol were mixed with 0.1 mL of the carotenoid extracts from whole grain, endosperm, and germ and aleurone layer. The mixtures were held for 30 min under subdued light. The absorbance of DPPH radicals was read at 515 nm against methanol as a blank using an Ultraspec 1100 pro, UV/Visible spectrophotometer (Biomicron Ltd. Cambridge, England). The analysis was done in duplicate. For the standard curve, trolox concentrations ranged from 100 to 700 µmol. The percentage scavenging of DPPH radical was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [(1-A_{sample,t})/(A_{control})] \times 100.$$

where $A_{control}$ is the absorbance of DPPH radical in methanol at 0 min, A_{sample} is the absorbance of DPPH radical + sample extract or standard at 30 min.

5.2.8 Statistical Analysis

The analytical data were reported as mean ± standard deviation (SD) of triplicate determinations for TCC and duplicate determinations for HPLC analysis of independent extractions. One- way analysis of variance (ANOVA) of results was performed using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA). Significant differences among samples for TCC and carotenoid composition were assessed by Duncan multiple range test at $p < 0.05$. Correlations between parameters were assessed by Pearson's correlation test.

5.3 Results and Discussion

5.3.1 Variation in total carotenoid content in whole grain, endosperm and germ

5.3.1.1 Whole grain

Table 5.2 shows total carotenoid content (TCC) expressed as lutein equivalent (mg/kg) in whole grain. Levels of TCC were significantly different ($p < 0.05$) among different cereal types and

within cereal varieties. Non-maize cereals had lower TCC compared to maize. The average TCC was 3.40, 2.57 and 18.19 mg/kg for barley, wheat and yellow maize, respectively. Oat had the lowest TCC (1.8 mg/kg). Among the non-maize cereals, purple barley had highest levels (4.54 mg/kg). TCC of wheat varieties (range 2.11–2.84 mg/kg) was similar to those reported by other authors (Hentschel et al. 2002; Konopka et al. 2006; Panfili et al. 2004). However, Luterotti and Kljak (2010) reported a lower range of TCC (1.1–1.3 mg/kg) in wheat flour. Compared to values in low, yellow-pigmented durum wheat reported by Ramachandran et al. (2010), TCC of wheat varieties in the present study were 2.2 to 2.5-fold lower. Purple barley had 2-fold higher TCC compared to regular barley **Table 5.2**. However, use of colorimetric methods to determine TCC may overestimate the amounts. For instance, TCC was over estimated by 20% in wheat (Abdel-Aal et al. 2007) and only 30–50% of the yellow pigments were carotenoids in durum wheat (Hentschel et al. 2002). Hentschel and others suggested the presence of other unknown pigments contributing to the yellow colour in grains. This may have been the case with purple barley, as not all pigments were carotenoids according to HPLC results to be discussed in Section 5.3.5. TCC of yellow maize in the present study were higher than values reported recently in yellow maize (11–23 mg/kg) (Luterotti and Kljak 2010) although they were within the same range of 19.3– 26.4 mg/kg reported for ten maize varieties (Rios et al. 2009).

5.3.1.2 Endosperm

Non-maize cereal endosperms had significantly lower TCC ($p<0.05$) which ranged from 0.88 to 2.27 mg/kg compared to 14.17 -31.35 (mg/kg) in yellow maize (**Table 5.2**). MSUD8006 wheat and regular barley had the highest and least TCC, respectively. Within the yellow maize, Dasca had the highest TCC. On average, TCC of barley, wheat and oat endosperm were 29.7, 74.7 and

65.5% of total carotenoid content in their whole grain, respectively, implying that TCC was concentrated in the germ of barley.

5.3.1.3 Germ

In contrast, TCC were high in germ fraction of non-maize cereals (6.08–14.77 mg/kg) and low in yellow maize (3.19 – 4.81 mg/kg) **Table 5.2**. The concentrations were significantly different ($p < 0.05$) among all the cereal types. The ratios of TCC in the germ of non-maize cereals to germ of maize cereals were 3.6:1, 2.4:1 and 1.6:1 for barley, wheat and oat respectively. The highest TCC were observed in regular barley germ followed by purple barley, Ambassador wheat, MSUD8006 wheat, purple wheat and oat among the non-maize cereals. The higher content of TCC in germ would be attributed to presence of yellow carotenoid pigments which were found in trace amounts in endosperm of non-maize cereals. One study reported 1.8-fold higher TCC in the germ of durum wheat than in whole grain content (Panfili et al. 2004). In this study, germ carotenoids were 4.0-, 3.5- and 3.3-fold higher in barley, wheat and oat, respectively, compared to TCC of their whole grains. These results show that barley germ is a more concentrated source of carotenoids than wheat germ. Oat germ had about 66.7% of the TCC of wheat germ. These results confirm that in wheat, carotenoids are concentrated in the germ, and suggest that the same is applicable to barley and oat. Barley, wheat and oat germ fractions may therefore be targeted for use as food ingredients with enhanced carotenoid content.

5.3.2 Contribution of each grain fractions to TCC in whole grain

In non-maize cereals, TCC contribution to whole grain varied widely while in yellow maize it was within the same range (**Table 5.2**). TCC contribution ranged from 20.5–70.6% for the endosperm; 3.3– 9.5% for germ and 19.9–72% for bran among non-maize cereals. In yellow maize, TCC contribution was >91.6–106% for endosperm only and between 1.4 and 4.3% for the

germ and bran. Similarly, Weber (1987) reported 90–107% and 1.3–3.6% TCC contributions from horny and floury endosperm and, germ and tip cap of maize inbreds, respectively.

Table 5.2 Total carotenoid content (mg/kg) in whole grain, endosperm, germ and bran fractions

Cereal	Whole grain	Endosperm	Germ	Bran[b]
Purple barley	4.54 ± 0.12d	1.14±0.02 g (20.5)[c]	12.68 ±0.16b (7.2)	3.28 (72.3)
Non-pigmented barley	2.25 ±0.08g	0.88± 0.02h (32.8)	14.77 ±0.23a (3.3)	1.22 (54.2)
Mean-Barley	**3.40**	**1.01**	**13.73**	**2.25**
Purple wheat	2.62 ±0.03f	1.71±0.06 f (56.5)	8.45 ±0.39e (5.3)	1.00 (38.2)
Ambassador wheat	2.11 ±0.04g	1.73± 0.04f (70.6)	9.87 ±0.24c (9.5)	0.42 (19.9)
Caledonia wheat	2.73 ±0.05ef	1.98± 0.02e (62.3)	9.42 ±0.1d (7.3)	0.83 (30.4)
MSU D8006 wheat	2.84 ± 0.06e	2.27± 0.05d (68.7)	8.71 ±0.12e (5.9)	0.72 (25.4)
Mean-Wheat	**2.57**	**1.92**	**9.11**	**0.74**
Oat	1.8 ± 0.05h	1.18± 0.03g (58.9)	6.08 ±0.25f (6.1)	0.63 (35.0)
Dasca maize	26.46 ± 0.18a	31.35± 0.22a (97.2)	3.19 ±0.02h (1.4)	0.37 (1.4)
USP1395XR maize	12.86 ±0.03c	14.17± 0.16c (91.6)	4.81 ±0.14g (4.2)	0.55 (4.3)
P1508HR maize	15.24 ±0.02b	18.79± 0.02d (106)	3.33 ±0.03h (2.4)	none
Mean-Yellow maize	**18.19**	**21.44**	**3.78**	**0.46**

[a]Values are mean ± standard deviation (n=3). Values with a different letter in each column are statistically different at the 5% level (Duncan's multiple range test).
[b] Calculated by subtraction: TCC in whole grain- (TCC in endosperm - TCC in germ) based on seed fraction weight proportions to whole grain.
[c]Figures in parentheses are percentage contribution of fraction to TCC in whole grain.

5.3.2.1 Endosperm

Among the non-maize cereals, wheat and oat endosperm contributed above 50% of TCC to whole grain (**Table 5.2**). Ambassador wheat contributed the highest TCC (70.6%) despite having the lowest TCC in whole grain among the wheat varieties. Worth noting was the low contribution (20.5%) of purple barley endosperm to whole grain. In maize, the endosperm contributed the highest, which is attributed to the yellow pigment responsible for its colour (Coultate 2009). TCC contribution to whole grain was highest for the endosperm since it is the largest fraction.

5.3.2.2 Germ

Although TCC of non-maize cereals was the highest in the germ fraction, its contribution to TCC in whole grain was the lowest compared to contributions from endosperm and bran fractions (by difference). Therefore it may be inferred that, although TCC concentrations in the germ fractions of wheat (7.26–13.33%), barley (5.34–9.48%), and oat (6.11%) were the highest, their contribution to TCC in whole grain are minimal because the germ only constitutes about 1.9–2.0% of whole grain. Yellow maize germ contributed the lowest TCC (1.47–4.20%) to the whole grain. Slightly lower ranges of TCC contribution (1.3–3.6%) of maize germ to whole grain were reported by Weber (1987).

5.3.2.3 Bran

TCC in bran fraction was calculated by subtraction based on bran percentage composition and total carotenoid content in germ, endosperm and whole grain. The percentage contributions were between 19.9–72.3% (63.3% average) in non-maize cereals and 1.4–4.3% (2.9% average) in yellow maize (**Table 5.2**). Although this study found negligible amounts of TCC, Kean et al. (2008) reported 1.77–6.50 mg/kg of carotenoids in yellow maize bran. The latter used mechanically separated bran fraction while our study used hand dissected fractions which were pure with zero to little adulteration of endosperm fraction. TCC from purple barley (72.3%) and purple wheat (38.2%) bran fraction was higher than contributions from the bran of non-pigmented barley and wheat varieties. The high TCC contribution may be attributed to pigmentation in the seed coat of these grains. According to Fratianni et al. (2005), the seed coat contains interfering pigments which would lead to over estimation of the total carotenoid content.

5.3.3 Separation and identification of carotenoids in whole grains and their grain fraction

The short method was chosen assuming that water-saturated butanol extracted mainly polar carotenoids unlike non-polar ones, which are found in minimal amounts in cereal grains. Lutein and lutein esters amounted to about >90% of the yellow pigment compared to approximately 1% of beta-carotene in wheat (Lepage and Sims 1968). Identification of carotenoids was accomplished by comparing the retention times (t_R) in the samples with those of the external standards and the UV–Visible absorption spectra in published literature. In non-maize cereals and maize, two major peaks were identified. A third major peak was found in maize. Minor peaks were also observed in some cereals and their fractions. Compared to peaks in the standard mixture (**Figure 5.1A**), the two major peaks were identified as lutein and zeaxanthin, the primary carotenoids found in cereal grains and grain products (Fratianni et al. 2005; Panfili et al. 2004).

Lutein and zeaxanthin were identified in all whole grain samples. Barley grains had an additional minor peak at t_R 5.9 min (y) (**Figure 5.1B**). In yellow maize, the peak at t_R 8.1 min was identified as beta-cryptoxanthin. Other unknown minor peaks were at t_R 3.3 min (x), 5.9 min (y) and 6.6 min (z) (**Figure 5.1C**). In contrast, only lutein was identified in the endosperm fraction of non-maize cereals (**Figure 5.1D**) while maize endosperm had similar peaks to the ones identified in the whole grain and one additional unknown peak at t_R 7.1 min (w). Lutein and zeaxanthin were detected in the aleurone layers of wheat and oat while only zeaxanthin was detected in barley. However, zeaxanthin was not detected in the endosperm of other non-maize cereals. This finding suggests that the aleurone layer in non-maize cereal may be closely adhering to the bran than the endosperm. In addition to lutein and zeaxanthin peaks, Caledonia

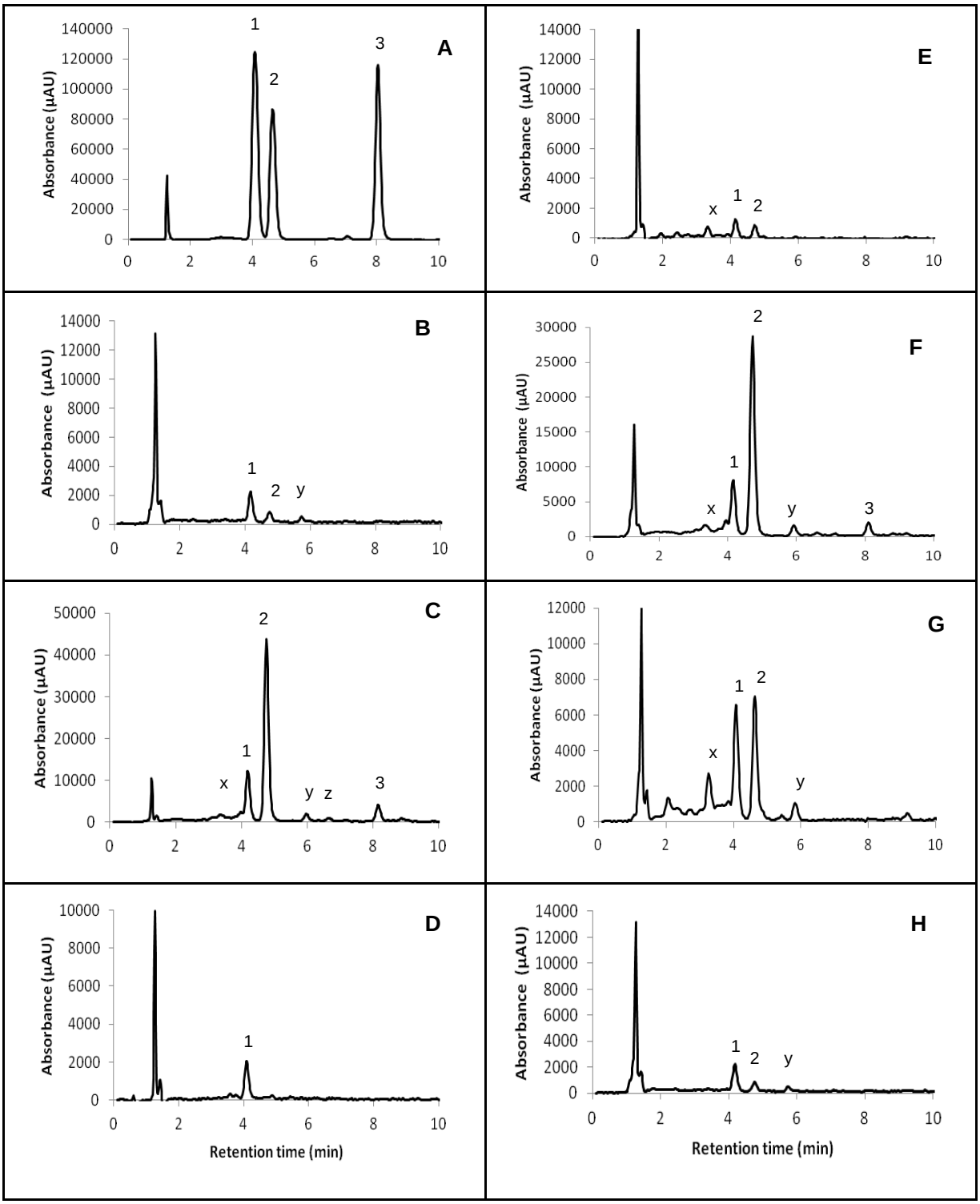

Figure 5.1 LC–UV/Vis chromatograms of carotenoids separated from standard mixture (A); whole grain: purple barley (B) and DASCA maize (C); endosperm: Caledonia wheat (D); aleurone layer: Caledonia wheat (E) and Dasca maize (F) and germ: Caledonia wheat (G) and purple barley (H); 1, lutein; 2, zeaxanthin; 3, β-cryptoxanthin; x, 13-*cis*-lutein; y, 9'-*cis*-lutein and z, 9-*cis* lutein

wheat had an unknown peak at t_R 3.3 min (x) (**Figure 5.1E**). Apart from lutein, zeaxanthin and b-cryptoxanthin, the aleurone layer of yellow maize also had an unknown peak at t_R 5.9 min (y) (**Figure 5.1E**). Lutein and zeaxanthin were identified in all the germ fractions. In addition to these two, non-pigmented barley and Caledonia wheat germ also had minor peaks at t_R 3.3 min (x) and 5.9 min (y) (**Figure 5.1F**), while purple barley germ only had the latter peak (**Figure 5.1H**).These unknown peaks (x, y, z and w) were proposed to be *cis*-isomers of lutein on the basis of UV/Vis spectra and absorption maxima (λ_{max}) reported in literature. The elution sequence of the unknown peaks in the present study was similar to the ones reported by Abdel-Aal et al. (2007) in wheat and maize. Based on this literature, the unknown peaks were suggested to be 13-*cis* lutein, 9-*cis* lutein and 9'-*cis* lutein, respectively. In addition, the observed spectra absorption maxima of 440, 442, 430 and 442 (**Figure 5.2A & B**) of the unknown peaks were comparable to ones reported by Gentili and Caretti (2011). Gentili and Caretti (2011) identified in maize flour, 13-*cis* lutein and 9-*cis* lutein at 420/443/471 and 444/471 nm, respectively. Similarly, *cis*-luteins have been reported in wheat, fruits and vegetables (Humphries and Khachik 2003). In the present study, they were also found in yellow maize and barley.

5.3.4 Quantification of lutein and zeaxanthin in whole grains

Quantitative data were calculated from the linear calibration curves. The concentration of lutein and zeaxanthin in non-maize cereals and yellow maize varied widely and were significantly different ($p < 0.05$).

Lutein ranged from 101–1034 µg/kg in non-maize cereals and 3414–3891 µg/kg in yellow maize (**Table 5.3**). Lutein content was significantly different ($p < 0.05$) among the different cereal types and within the cereal varieties. Lutein was the primary and major component in non-maize

cereals. Among the non-maize cereals, wheat varieties had the highest lutein levels on average. Oat had the lowest levels of lutein. Purple barley had two times more lutein compared to non-pigmented barley. The ranges of lutein content in wheat varieties are comparable to 841–1340 µg/kg in einkorn wheat (Hidalgo et al. 2006).

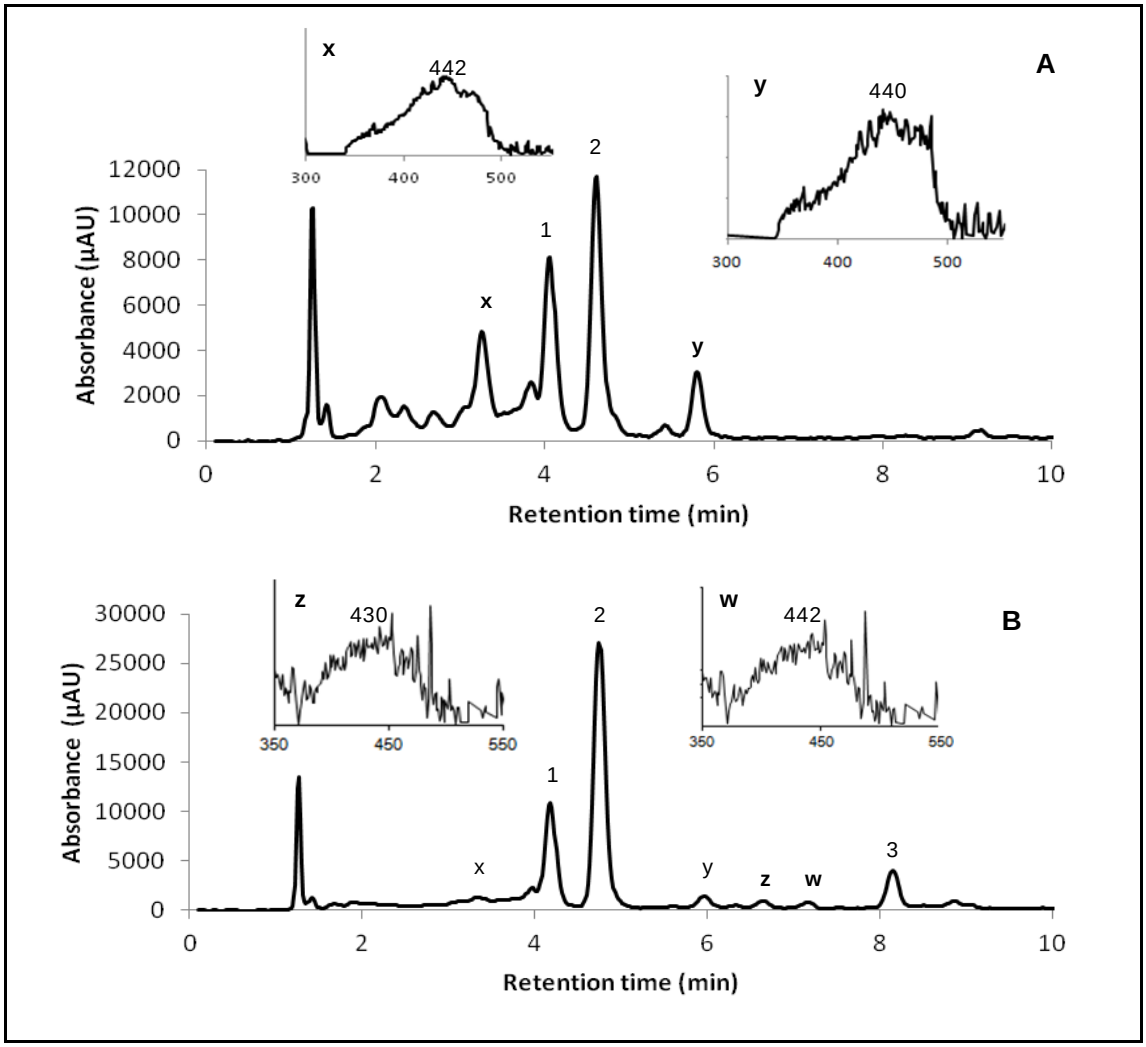

Figure 5.2 LC chromatograms and absorption spectra for non-pigmented barley germ (A) and DASCA maize endosperm (B), showing maximum wavelength absorption spectra at 3.3 min (X); 5.8 min (Y); 6.6 min (Z) and 7.1 min (W)

In wheat, barley and oat grains, lutein was reported as the major and zeaxanthin as the minor xanthophyll (Luterotti and Kljak 2010; Okarter et al. 2010; Panfili et al. 2004). Panfili and others

reported higher amounts of lutein in soft and durum wheat (1310 and 2650 μg/kg), barley (860 μg/kg) and oat (230 μg/kg). Lutein was on average 7.4 times and 4.5 times higher in maize than in barley and wheat, respectively. In non-maize cereals, lutein accounted for 22.2–70.2% of the total carotenoid content. Among wheat varieties, lutein contributed about 52–70%, which was lower than 78–85% reported in Mindum and Thatcher wheat and 80–90% in Einkorn wheat (Abdel- Aal et al., 2002).

Zeaxanthin levels ranged from 356 to 650 and 4593–18,369 μg/kg in non-maize cereals and yellow maize, respectively. Zeaxanthin levels in yellow maize are similar to those reported in maize (Kurilich and Juvik 1999a). In the non-maize cereals, zeaxanthin levels were significantly different ($p < 0.05$) among cereal types but similar within cereal varieties. On average, barley varieties had the highest levels of 637 μg/kg, followed by wheat varieties (438 μg/kg) and oat (356 μg/kg). Zeaxanthin levels were significantly different ($p < 0.05$) among maize varieties, with Dasca having 3.9 times and 2.7 times the amounts in USP1395XR and P1508HR maize. Zeaxanthin levels of 6430 and 120 μg/kg in maize and wheat respectively, were previously reported (Panfili et al., 2004). On average, zeaxanthin levels in barley and wheat were only 4.4% and 6.4% of the amounts found in yellow maize. Furthermore, zeaxanthin accounted for 29.8–77.8% and 55.0–82.5% in non-maize cereals and maize varieties, respectively. Lutein contributed 10–40% (Kimura et al. 2007) and zeaxanthin 30–60% of the total carotenoid in maize (Hulshof et al., 2007).

Differences in lutein and zeaxanthin content in wheat grains and others cereals observed in this study would be attributed to varietal and genetic differences and growing location which affect chemical composition of the cereal grains (Kurilich and Juvik 1999a; Panfili et al. 2004).

5.3.5 Quantification and distribution of lutein and zeaxanthin in grain fractions

Table 5.3 reports carotenoid composition in grain fractions of diverse cereal grains and of particular interest is the aleurone layer which has very limited literature.

5.3.5.1 Aleurone layer

The range of lutein content was lower in non-maize cereal (151–534 μg/kg) compared to 3171–7631 μg/kg in yellow maize aleurone layers. On average, wheat varieties had 2.3–2.4 times higher lutein content than oat and barley. The amount of lutein in barley varieties was not significantly different, in agreement with Fratianni et al. (2005) that pigmentation in seed coat leads to overestimation of TCC. Among maize (average 5360 μg/kg), highest levels of lutein were observed in USP1395XR (7631 μg/kg) and the least in DASCA (3171 μg/kg). However, lutein content was not significantly different among the non-maize cereals ($p < 0.05$). The levels of lutein in oat and barley aleurone layers were 4- and 3 times higher than in endosperm fractions. Similarly in maize, lutein content was higher in aleurone than in the endosperm.

Zeaxanthin levels varied significantly ($p < 0.05$) both in non-maize cereals and maize varieties. Zeaxanthin ranged from 580–1217 μg/kg among the non-maize cereals with higher levels (7232–13,730 μg/kg) in yellow maize. Purple wheat had the highest zeaxanthin among non-maize cereals, followed by oat, Caledonia wheat, Ambassador wheat and MSUD8006 wheat. DASCA maize had the highest levels of zeaxanthin. Zeaxanthin has been reported to be concentrated in

Table 5.3 Carotenoid composition in whole grain and its distribution in grain fractions (μg/kg)[a]

Fraction	Whole grain	Endosperm	Aleurone	Germ	Whole grain	Endosperm	Aleurone	Germ
Cereal type			**Lutein**				**Zeaxanthin**	
Purple barley	699 ± 0.02e	45 ± 0.00f	190 ± 0.00d	2517 ± 0.21b	624 ± 0.02d	nd	nd	3015 ± 0.11d
Regular barley	295 ± 0.02f	66 ± 0.01f	151± 0.06d	2630 ± 0.0ab	651 ± 0.03d	nd	nd	5855 ± 0.00a
Mean –Barley	**497**	**56**	**171**	**2573**	**637**	**-**	**-**	**4435**
Purple wheat	596 ± 0.00e	554 ± 0.01de	534 ± 0.08d	1714 ± 0.01c	543 ±0.02de	nd	1217 ± 0.04d	3233 ± 0.01c
Ambassador wheat	741± 0.01ed	493 ± 0.03e	370 ± 0.03d	1566 ± 0.08c	359 ± 0.01e	nd	629 ± 0.05f	2491 ± 0.06e
Caledonia wheat	905 ± 0.01cd	559 ± 0.03de	350 ± 0.02d	2531 ± 0.15b	411 ± 0.01de	nd	680 ± 0.05ef	3726 ± 0.01b
MSUD8006 wheat	1034 ± 0.02c	624 ± 0.04d	446 ± 0.05d	2816 ± 0.17a	439 ±0.03de	nd	580 ± 0.04f	2927 ± 0.00d
Mean –Wheat	**819**	**557**	**425**	**2157**	**438**	**-**	**776**	**3094**
Oat	101 ± 0.00f	43 ± 0.00f	181 ± 0.02d	1340 ± 0.00d	356 ±0.0e	nd	943 ± 0.11de	1960± 0.13f
DASCA maize	3891 ± 0.21a	3365 ± 0.00c	3171 ± 0.01c	236 ± 0.00e	18369 ±0.19a	13210 ±0.32a	13730 ±0.13a	873 ± 0.04gh
US P139SXR maize	3762 ± 0.16a	3672 ± 0.14b	7631 ± 1.14a	307 ± 0.01e	4593 ±0.11c	7294 ± 0.18b	7993 ± 0.31b	969 ± 0.01g
P1508HR maize	3414 ± 0.07b	3881 ± 0.03a	5277 ± 0.04b	261 ± 0.01e	6674 ±0.23b	7710 ± 0.05b	7232 ± 0.19c	818 ± 0.00h
Mean - Yellow maize	**3689**	**3639**	**5360**	**268**	**9879**	**9404**	**9652**	**886**

[a]Values are mean ± standard deviation (n=2). Values with a different letter in each column are statistically different at the 5% level (Duncan's multiple range test). nd- not detected

the germ fraction of wheat (Panfili et al., 2004) and maize endosperm (Kean et al. 2008). In the present study, zeaxanthin was also the dominant carotenoid in the aleurone layers. In wheat and yellow maize varieties, zeaxanthin content was 1.8-fold higher than lutein content while in oat, it was 5.2-fold higher than lutein. These results indicate that the aleurone layers of wheat, oat and maize cereals have significant levels of lutein and zeaxanthin, which have antioxidant properties in addition to ferulic acid. Aleurone grains in the aleurone layer are surrounded by lipid droplets and carotenoids in this layer may be protecting the membranes from lipid peroxidation. Konopka et al. (2006) suggested that the lipid fraction of grains contains carotenoids which may enhance the antioxidant activity of the aleurone layer.

5.3.5.2 Endosperm

Only lutein was detected in the endosperm fraction of non-maize cereals, ranging from 43 µg/kg (oat) to 624 µg/kg (MSUD8006) and 3171–7631 µg/kg in yellow maize. The average lutein content was 9.9 times higher in wheat than in barley. Although wheat had the highest lutein content among the non-maize cereals, its levels were 6.5 times lower compared to levels in maize. In maize, lutein levels varied significantly among the varieties. Similarly, zeaxanthin levels were significantly ($p < 0.05$) high in DASCA maize (13210 µg/kg).

5.3.5.3 Germ

Lutein and zeaxanthin content in the germ fraction had an opposite trend to what was observed in whole grain, endosperm and aleurone layer. Lutein levels ranged from 1340 to 2816 µg/kg in non-maize cereals and 236-307 µg/kg in yellow maize (**Table 5.3**). On average, barley varieties had the highest levels of lutein (2573 µg/kg) and oat had the least (1340 µg/kg) among the non-maize cereals. Lutein content was much lower in maize germ (268 µg/kg). The proportion of

lutein to total carotenoid in the germ was 30.9-49.0% and 21.3-24.2% in non-maize cereals and maize, respectively.

Zeaxanthin content ranged from 1960 to 5855 μg/kg in non-maize cereals and 818–969 μg/kg in yellow maize. In barley and wheat germ, the average zeaxanthin content was 5.6 and 3.5 times higher than in yellow maize. The levels of zeaxanthin were lower in yellow maize; however, the proportion of zeaxanthin to lutein was high in maize (75.8-78.7%) and low in non-maize cereals (51.0-68.1%). The high concentration of lutein and zeaxanthin in the germ fraction would be attributed to their role as antioxidants and in promoting seed germination (Rogozhin et al. 2001).

Within the germ fraction, the ratio of zeaxanthin to lutein varied among the types of cereal grains. In purple barley and MSUD8006 wheat, the ratio was 1:1 whereas as in oat, Caledonia wheat, Ambassador wheat, purple wheat and non-pigmented barley, the ratio ranged from 1.5:1 to 2.2:1. The ratio was much higher in yellow maize ranging from 3.1-3.7:1 despite the low amounts. Comparing the amounts of lutein and zeaxanthin in the germ fraction to those in the whole grain, results clearly showed that the germ is a more concentrated source of these carotenoids especially zeaxanthin. The ratio of lutein in germ to whole grain ranged between 2 and 3 times in wheat and purple barley. In oat and non-pigmented barley germs, the ratio was very high, 13 and 9 times, respectively. The levels of zeaxanthin content in germ were higher than in whole grains across the cereals and varied widely. It was 9-fold in Caledonia wheat and non-pigmented barley, 7-fold in Ambassador and MSUD8006 wheat, 5.5- to 5.9-fold in oat and purple wheat and 4.8-fold in purple barley. A higher ratio (21 times) of zeaxanthin in germ to zeaxanthin in whole grain was found in soft wheat (Panfili et al., 2004). However, for both lutein

and zeaxanthin, respectively, the ratios in maize were low, 0.06 and 0.04 in Dasca maize, 0.08 and 0.13 in USP1395XR and 0.07 and 0.10 in P1508HR maize. These results suggest that zeaxanthin is mainly localised and highly concentrated in the germ fraction and aleurone layer in non-maize cereals, and in the endosperm and aleurone layer of maize varieties.

Concurring with Panfili et al. (2004), lutein was found in all the grain fractions; however, in the present study it was unevenly distributed. Zeaxanthin was not detected in endosperm of non-maize cereals and barley aleurone layer, but it was more concentrated in germ as previously reported, with some considerable amounts in the aleurone layer. In addition, this study is the first, to the authors' knowledge, to report on lutein and zeaxanthin content in aleurone layer fractions of different cereals. Comparing the amounts of zeaxanthin in grain fractions, the levels in the aleurone layer ranked second from the germ.

5.3.6 Antioxidant activity of carotenoid extracts from whole grain and its fractions

Lutein and zeaxanthin have been reported as important antioxidants (Gentili and Caretti 2011; Leenhardt et al. 2006) and hence the extracts used for HPLC analysis were also examined for their antioxidant activity using DPPH method. DPPH assay measures the reducing ability of antioxidants towards DPPH radical through discolouration, showing the % of DPPH that has been quenched. The results presented are for the scavenging activity at 30 min. There was a wide and significant variation in %DPPH scavenging activity among the different cereal types and their grain fractions ($p < 0.05$) (**Figure 5.3**).

The %DPPH scavenging activity in whole grains was generally low (≤16.4%) in non-maize cereal and slightly higher (≥20 ≤ 23.9%) in yellow maize. The %DPPH scavenging activity in

MSUD8006 and Caledonia wheats were below the lowest scavenging activity (11.9%) of minimum trolox concentration (100 µM). However, a positive correlation was found between scavenging activity and total carotenoid (lutein and zeaxanthin) content ($r = 0.6945$, $p < 0.05$). Similarly, %DPPH scavenging activity in the endosperm was low in non-maize cereals (<5%) cereal and slightly higher in maize (<10%). In the non-maize cereals, the scavenging activity decreased in the following order: oat > Ambassador wheat > -non-pigmented barley, purple barley > purple wheat > MSUD8006 wheat > Caledonia wheat (**Figure 5.3**). Despite this observation, a significant and strong correlation was found between DPPH scavenging activity and carotenoid content analysed by HPLC in the endosperm fraction ($r = 0.9647$, $p < 0.05$).

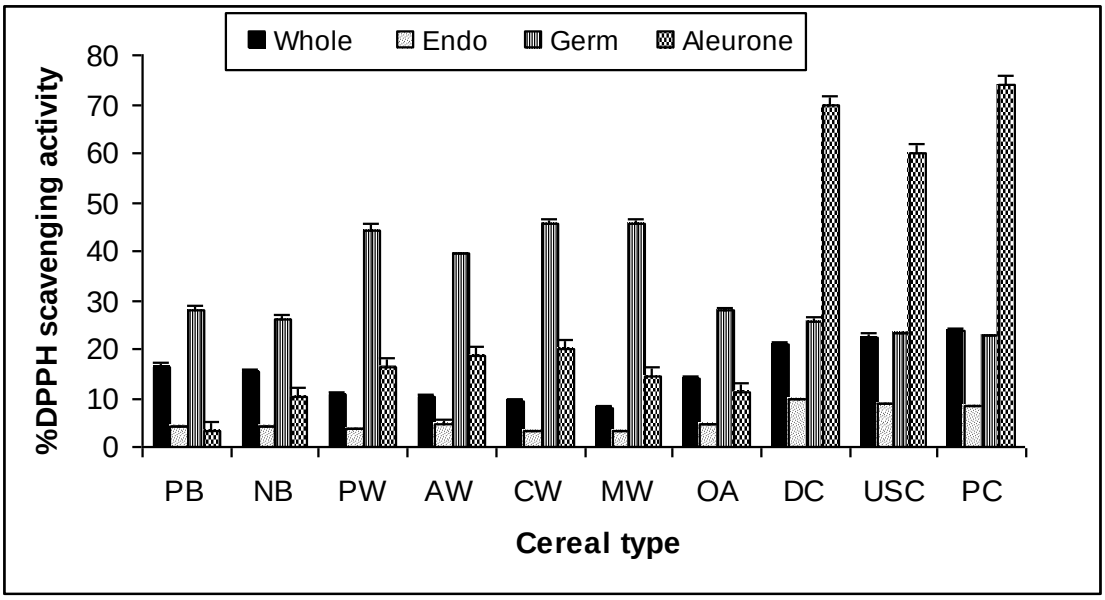

Figure 5.3 %DPPH scavenging activity of WSB extracts from whole grain, endosperm and germ of different cereals. PB, purple barley; NB- regular barley; PW, purple wheat; AW, Ambassador wheat; CW, Caledonia wheat, MW, MSUD8006 wheat; OA, Oat; DC, Dasca maize; USC,USP1395XR maize; PC, P150HR maize. The vertical bars represents the standard deviations (n=2)

The low percentage of DPPH scavenging activity in the endosperm of the non-maize cereals may be attributed to the low total carotenoid content and absence of zeaxanthin. Mortensen and Skibsted (1997) studied the role of carotenoid structure in radical scavenging reactions and reported that zeaxanthin is more reactive than lutein although their order of reactivity only shows slight differences. However, we could not explain why maize endosperm despite its high lutein and zeaxanthin content, had low DPPH scavenging activity compared to the other fractions.

In contrast, %DPPH scavenging activity in the germ fractions showed different trends as was observed in TCC and HPLC analysis results. Compared to the other fractions, scavenging activity was the highest in the germ ranging from 26.2 to 45.5% in non-maize cereal and 22.8–25.8% in yellow maize germ. Within the germ fractions, scavenging activity was the highest in wheat varieties (MSUD8006 and Caledonia) and lowest in maize. The germ of barley varieties, despite having higher amounts of total carotenoid (**Table 5.3**) compared to wheat varieties, had lower DPPH scavenging activity. This discrepancy may have contributed to the weak correlations ($r = 0.4802$) which was significant ($p = 0.03$) observed between %DPPH scavenging activity and lutein and zeaxanthin content.

% DPPH scavenging activity in the aleurone layer of wheat varieties was about a third (31.4%) to half (46.9%) the activity observed in their germ fractions. Barley aleurone layer had the lowest scavenging activity. The high scavenging activity of wheat aleurone may be attributed to approximately 1.2–2 times more zeaxanthin compared to lutein while the low scavenging activity in barley would be due to lack of zeaxanthin and low levels of lutein in the aleurone layer. A positive significant correlation was observed between DPPH scavenging activity and

lutein + zeaxanthin content in aleurone layers ($r = 0.9604$, $p < 0.05$). Zhou et al. (2004) reported highest concentration of 2980 µg/kg (lutein + zeaxanthin) in Australian wheat bran, which also includes the aleurone layer.

These results suggest that carotenoids may be localised in aleurone layer rather than the other bran layers (pericarp, seed coat). There is need therefore for further research to analyse carotenoid content in the other layers of wheat and oat bran. The highest scavenging activity was observed in yellow maize aleurone ranging from 60.0 to 73.8%. This may be attributed to the high levels of lutein in aleurone layer compared to endosperm since average zeaxanthin levels were similar in the two fractions.

5.3.7 Relationship between carotenoid content and %DPPH scavenging activity

Positive and strong correlations were found between carotenoid content and %DPPH scavenging activity as discussed above. In contrast, no correlation was found between antioxidant activity and carotenoid content in methanolic extracts of cereal grains (Choi et al. 2007). Similarly, Thaipong et al. (2006) reported zero and negative correlations between carotenoid content in different fruit juice extracts and their antioxidant activity using DPPH. The positive correlation observed in this study would be attributed to lutein and zeaxanthin levels extracted using the water saturated-butanol. According to Baublis et al. (2000), aqueous extracts from cereal grain products showed significant antioxidant activity. In addition, lutein and zeaxanthin have hydroxyl groups on each ring (Miller et al. 1996) and these polar functional groups render them more accessible to aqueous environments (Rice-Evans 1996). Furthermore, strong correlations were also observed between TCC and carotenoid content determined by

HPLC (**Figure 5.4**). A similar trend in correlation coefficients was also observed in germ, whole grain and endosperm. The correlation was slightly lower in germ fractions (R^2 = 0.8591) compared to that of endosperm (R^2 = 0.9833) and whole grain (R^2 = 0.9656).

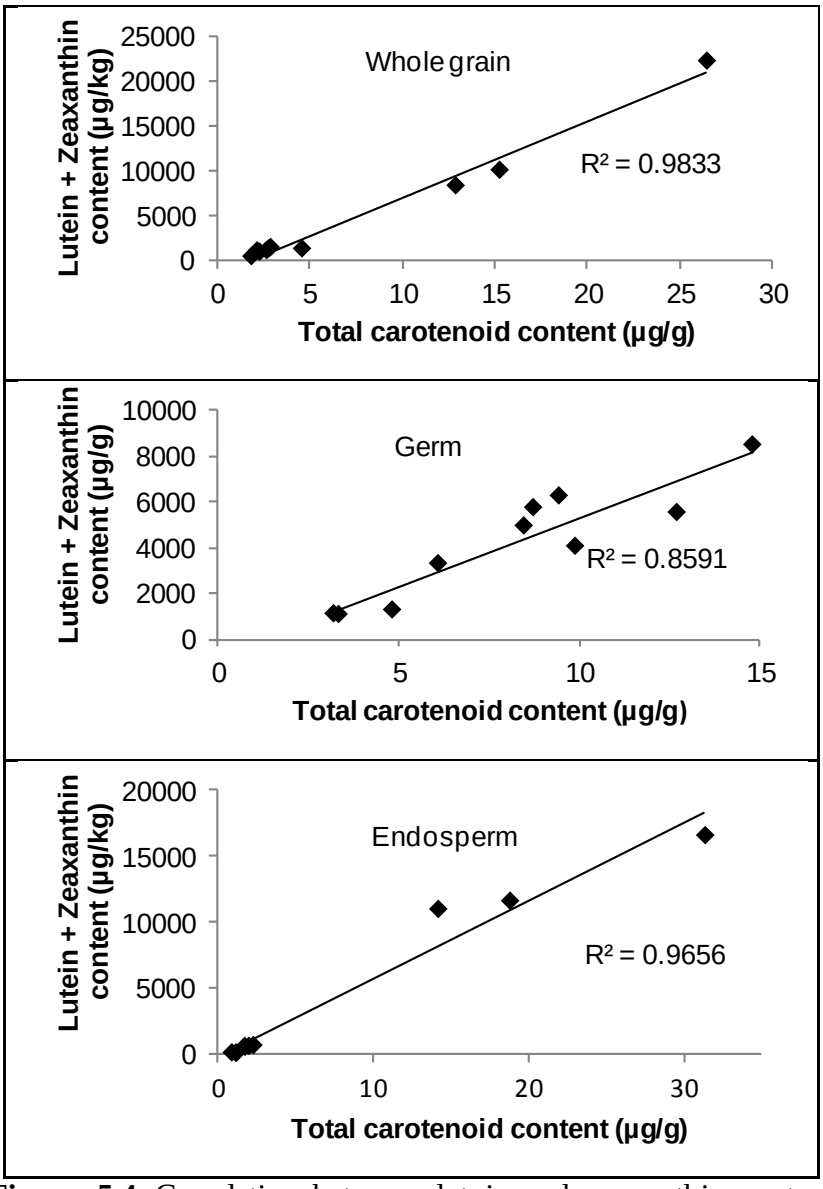

Figure 5.4 Correlation between lutein and zeaxanthin content determined by liquid chromatography and total carotenoid content determined by spectrophotometry

5.4 Conclusion

Carotenoid composition was similar except in yellow maize which also had β-cryptoxanthin. Generally, lutein and zeaxanthin and TCC were all unevenly distributed across the grain kernel. In non-maize cereals, lutein and zeaxanthin were concentrated in the germ unlike in yellow maize where they were concentrated in the endosperm and aleurone layer. This study is the first to report on carotenoid composition of the aleurone layer. Oat, wheat and yellow maize aleurone layer exhibited higher levels of zeaxanthin compared to lutein content. Antioxidant activity of carotenoid in aleurone layer of non-maize cereals was about 50% of the germ, the most concentrated source of carotenoid in cereals. A high correlation between %DPPH scavenging and total lutein and zeaxanthin content was found. Apart from yellow maize endosperm and aleurone layers, the germ and aleurone layers of oat and wheat, and the germ of barley may be utilised as food ingredients with enhanced carotenoid content in development of functional foods for health conscious consumers.

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CHAPTER 6

Variation in Protein, Amino acids, B-Vitamins and Mineral Contents in Aleurone of Wheat, Yellow Maize and Purple Barley

6.1 Abstract

Nutritional composition of wheat aleurone tissue, a commercialised food ingredient has been widely investigated. The purpose of the study was to characterize the nutritional profiles of purple barley (PB) and yellow maize (YC) aleurone tissues as potential food ingredients that can be used as alternatives to wheat aleurone. Manually isolated aleurone and endosperm fractions were analysed for protein, amino acids, minerals and vitamins, B1 (thiamine) and B2 (riboflavin). Protein content in the aleurone layer decreased in the order: PB (18%) > YC (14.5 %) > wheat (13.1%). Compared to wheat, PB and YC aleurone were rich in essential amino acids (EAA), phenylalanine, leucine and methionine. Minerals were generally higher in wheat than in PB and YC, except for Ca which was highest in PB aleurone. Wheat and PB aleurone had the highest and intermediate levels, respectively, of vitamin B1. These results provide useful information on the potential use of PB and YC aleurone as alternative food ingredients to wheat aleurone.

6.2 Introduction

Botanically the aleurone is the outermost component of the starchy endosperm. However, as an industrial product, it is also described as the innermost layer of the bran (Evers et al. 1999; Hoseney 1986; Serna-Saldivar 2010). It is removed together with other parts of the bran layers during the milling process. More importantly, it is where most of the minerals, B-vitamins (Buri et al. 2004; Fardet 2010; Hoseney 1986; Pomeranz 1988), phenolic acids (Antoine et al. 2004; Hernanz et al. 2001) and nutritionally-superior proteins are concentrated within the grain kernel. Cereals provide significant levels of nutrients in the human diet and wheat flour is the major ingredient in bakery products (Serna-Saldivar 2010).

Nutritional composition of wheat aleurone and its use as a food ingredient summarised in reviews by Atwell et al. (2007) and Brouns et al. (2012), indicate that the aleurone layer is a rich source of various nutrients. For example, hand dissected wheat aleurone layers contained 15% of total wheat proteins, 30% of total lysine and 40-60% of total wheat minerals (Pomeranz 1988). Similarly, mechanically separated commercial wheat aleurone layers had high protein content, well balanced essential amino acid, over 80% of total wheat minerals and significant amounts of B-vitamins (Buri et al. 2004). Currently, wheat aleurone is commercialised and largely utilised in enhancing the health benefits of bakery products. Incorporation of 20% aleurone material into white flour produced aleurone bread with similar nutritional benefits as whole wheat flour (Atwell et al. 2007).

In cereals, proteins are the second highest nutritional components after carbohydrates. However, protein concentration varies considerably in commercial wheat supplies, depending on grain type

and variety, and production conditions, among other factors (Serna-Saldivar 2010). Protein nutritional quality is also the product of several factors, the most notable of which are amino acid contents and ratios. Of the essential amino acids (EAA) that are important in the human diet, most are available to some extent in wheat. However, while amino acid composition has been determined for wheat bran, the aleurone layer, aleurone cell contents and endosperm (Fulcher et al. 1972; Jensen and Martens 1983), there is limited information on amino acid composition in grain fractions of barley and maize (Landry and Moureaux 1980; Sodek and Wilson 1971; Wolf et al. 1972; Yupsanis et al. 1990).

Cereals are also an excellent source of many micronutrients, especially B-vitamins and minerals (Hoseney 1986; Serna-Saldivar 2010). These are concentrated in grain outer layers (Hoseney 1986) that are usually removed during conventional milling, thereby reducing the nutritional value of refined flours. Reconstituted whole wheat meal flour, comprising 20% coarse bran, 5 % thin bran and 75 % white flour, had relatively high B-vitamin content (thiamine, riboflavin and pyridoxine) compared to its corresponding white flour (Batifoulier et al. 2006). Aleurone layers are notable for their substantial levels of B-vitamins (niacin, thiamine, riboflavin and pyridoxine). In most cereals, niacin is the most abundant of the B-vitamins, followed by thiamine, pyridoxine, pantothenic, riboflavin and folate (Batifoulier et al. 2006; Lebiedzin´ska and Szefer 2006; Rohi et al. 2013). Thiamine, riboflavin and pyridoxine act as co-enzymes of many metabolic processes, being critical factors in the metabolism of carbohydrates, protein and fats (Ball 2006; Batifoulier et al. 2006). Nutritional shortages of B-vitamins have resulted in a number of historical and global deficiency diseases such as beri-beri and pellagra that are due to lack of thiamine and niacin, respectively. Although deficiency diseases are not a major problem

in developed nations, Rohi et al (2013) suggested that marginal deficiencies may appear with increased consumption of grain products made from refined flours.

Cereals also contain an abundance of minerals, including macro-mineral elements, potassium, magnesium, phosphorus, sodium and calcium and, micronutrient elements, zinc, copper, manganese, selenium and iron among others. In most cereals, phosphorus is the most abundant macro-element and calcium is the least (Serna-Saldivar 2010). Mineral composition in whole maize and normal barley grains and their fractions (endosperm, germ, pericarp and abraded fractions) has been reported (Lin et al. 2005; Liu and Pomeranz 1975; O'Dell et al. 1972; Ockenden et al. 2004; Stewart et al. 1988). In barley, most of these researchers quantified the minerals in aleurone in grain *in-situ* using x-ray analysis with a few having analysed mineral content in isolated aleurone layers of barley and maize (O'Dell et al. 1972; Ockenden et al. 2004).

Overall, little is known on how comparable the nutrient composition of aleurone layer from other cereals is to that of wheat. Moreover, there has been increased interest in use of barley as food either partially or in whole (Baik and Ullrich 2008). Therefore, the primary purpose of this study was to compare the nutritional composition of manually separated fractions of wheat to those of purple barley and maize aleurone layers. A secondary objective was to compare the nutrient composition of these aleurone layers with the endosperm fractions as representatives of refined flours.

6.3 Materials and methods

Chemicals: Sulphuric acid, hydrochloric acid, methanol and formic acid were purchased from Fisher Scientific (Whitby, ON, Canada). Thiamine hydrochloride standard, riboflavin standard, alpha-amylase (CAS no. 9001-19-8), papain (CAS no.9001-73-4) and diastase (CAS no. 9000-92-4) were purchased from Sigma Aldrich (St. Louis, MO, USA).

6.3.1 Samples and sample preparation.

Five grains were used for protein and only three with the highest protein content for each grain type were selected for amino acid, mineral and B-vitamin analyses. The grains included two yellow maize varieties (Dasca, USP1395XR), two barley varieties (purple barley and normal barley) and wheat (MSUD8006). Their characteristics in terms of where they were grown, physical properties (soft/hard) and use were described earlier in Chapter 4. Aleurone layers and endosperm fractions were prepared manually as described previously in Chapter 3.

6.3.2 Determination of protein and amino acids.

Crude protein content was determined using the Kjeldahl Method, AACC (2000) method 46-12.01. Amino acid analysis was accomplished following AOAC (2005) Official Method 994.12, "Amino Acids in Feed" 1995 and Amino Acid analysis of feed constituent described by Andrews and Baldar (1985). Briefly, for all the amino acids, except cysteine, methionine and tryptophan, 6M HCl was added to 100 mg of each sample and hydrolysed at 110°C for 24 h. After cooling, the mixture was neutralised with 25% (w/v) NaOH made up to 50 mL in a volumetric flask, and filtered through a #40 Whatman filter paper. For cysteine and methionine, samples were oxidised with a mixture of formic acid (88%) and H_2O_2 (35%) in proportion of 9:1 (v/v) prior to acid hydrolysis with concentrated HCl at 110°C for 16 h. For tryptophan determination, samples were hydrolysed with an alkaline solution following the method of Hugli and Moore (1972). Then 50

µL of hydrolysates from each sample was analysed using an Amino Acid Analyzer (Model S4300, Sykym, Germany).

6.3.3 Extraction and determination of minerals

Freeze dried samples (1.0 g each) were dry-ashed (at 600°C for 12 hrs), and acid extracts of the ash were prepared in duplicates except for aleurone fractions. The aleurone samples were limited and therefore, they were not assayed in duplicate. In brief, 10 mL of 1% HNO_3 and 5M HCl were added to the ash and digested at 70°C in a water bath for 1 hour. After cooling, the solution was diluted with 50 mL of deionised water in a volumetric flask. About 20 mL of aliquot was filtered through a Q5 filter paper. Macronutrient and micronutrient elements were determined by inductively coupled plasma atomic emission spectrophotometry (ICP-AES) (Varian Inc., USA) as described in Method 985.01(AOAC 2005).

6.3.4 Thiamine and riboflavin analysis

Different solvents and extraction conditions are available for releasing vitamins B1 and B2 from complex food systems. Extraction of vitamins B1 (thiamine) and B2 (riboflavin) from aleurone, endosperm and whole grain was accomplished by the method described by Rodriquez et al. (2012) with some modification. Briefly 5 mL of 0.1N H_2SO_4 were added to 250 mg (500 mg for whole grain) of sample in an amber bottle, vortexed and left to stand for 15-20 minutes at room temperature. The pH of this mixture was adjusted to 4.5 using 2.5M sodium acetate. Then 50 mg of papain and 30 mg each of diastase and α-amylase was added. The solution was incubated in an oven at 37°C for 18 hours, and then diluted to 10 mL with distilled water in a volumetric flask. This mixture was transferred into centrifuge tubes, shaken for 20 minutes using a wrist action shaker (Model 75, Burrell Scientific Pittsburgh, PA, USA) and centrifuged for 10 minutes

at 10,000xg. The supernatant was freeze dried and reconstituted with 2 mL of distilled water. The aliquots were filtered through a 0.45 µm nylon filter before injecting into HPLC.

6.4 Results

6.4.1 Protein content and amino acid composition

Protein contents in aleurone and endosperm fractions are shown in **Figure 6.1.** Compared to wheat, purple barley had significantly ($p < 0.05$) higher protein levels followed by Dasca maize aleurone. Protein content differed significantly ($p < 0.05$) between the aleurone and endosperm fractions in the grains examined. These findings are in agreement with what has been reported previously (Serna-Saldivar 2010; Wolf et al. 1972). Aleurone proteins are highly concentrated in distinct spherical protein storage vacuoles (PSV) or aleurone grains (Bethke et al. 1998). In contrast, the endosperm contains much lower levels of protein, being a tissue that is predominantly starch (Macewicz et al. 2006).

The amino acid composition in the aleurone layer and endosperm of Dasca maize, wheat and purple barley are presented in **Table 6.1**. The two fractions displayed similarities in amino acid composition although levels of most amino acids were higher in the former compared to the latter. Of the essential amino acids, phenylalanine and leucine were relatively high in purple barley and maize than in wheat aleurone. According to FAO recommendations, phenylalanine and leucine are among the 3 EAA that are needed in higher amounts for children aged 2-5 years (**Table 6.1**). In contrast, tryptophan and lysine were higher in wheat than in purple barley and yellow maize aleurone layers. Overall, the levels of four (methionine, valine, threonine and

isoleucine) out of the eight EAA in purple barley and maize were similar to those in wheat aleurone.

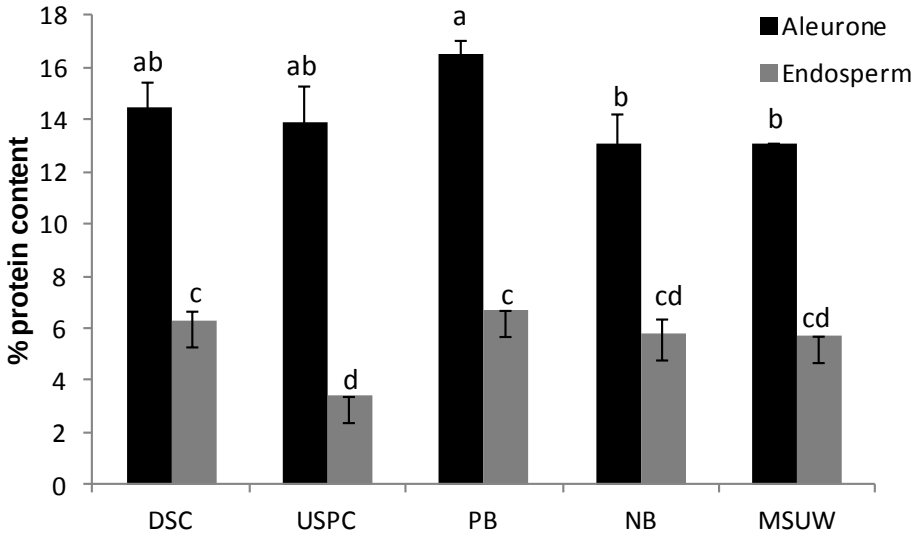

Figure 6.1 Percentage protein content in aleurone and endosperm of maize, barley and wheat. DSC, Dasca maize; USPC, USP1395XR maize; PB, Purple barley; NB, Normal barley and MSUW, MSUD8006 wheat

Based on FAO daily requirements (FAO/WHO 2009), purple barley and maize aleurone layers may provide over 50% of most of the EAA required by children and 100% required by adults. Methionine and leucine in maize and phenylalanine and lysine in purple barley were higher than in wheat endosperm.

Amino acid composition of wheat aleurone in the present study is similar to previous reports (Fulcher 1972; Jensen and Martens 1983). The trends in proportion of most of the amino acids were also similar to those in hand-dissected aleurone reported by Jensen and Martens (1983) although levels of some were lower when compared to mechanically separated wheat aleurone

(Fulcher et al. 1972). The amino acid composition of proteins isolated from barley aleurone cells in a study by Yupsani et al. (1990) was similar to the present study. However, the levels of several individual amino acids were relatively higher (**Table 6.1**) in the study by Yupsani et al. (1990). These variations were likely due to differences in grain varieties, sample characteristics and components used for amino acid analysis. Fulcher et al. (1972) and Yupsani et al. (1990) analysed isolated aleurone cells and isolated protein (globulin) from aleurone cells, respectively, but not whole aleurone layers. In maize aleurone, leucine content was comparatively higher than all the other amino acids. Similarly, moderately higher leucine in relation to the other 5 amino acids in aleurone layer of Coroico maize has been reported (Wolf et al. 1972).

The maize endosperm had the typical characteristic amino acid composition of zein storage proteins (prolamins) (Lawton and Wilson 2003). Previous studies reported similar trends in amino acid composition of zein protein from the maize endosperm fraction (Landry and Moureaux 1980; Sodek and Wilson 1971). Similarly, the wheat endosperm displayed the characteristic amino acid composition typical of glutelin endosperm storage proteins (Wingley and Bietz 1988).

6.4.2 Macro- and micromineral elements.

Table 6.2 shows the content of macro-and micromineral elements in the aleurone layer, endosperm and whole grain. Both macro-and micromineral elements were higher in aleurone than in the endosperm fractions. Major mineral elements (K, Mg and P) were <10 -fold, >30 to 100-fold and >10 to 30-fold higher in maize, wheat and purple barley aleurone layers compared to endosperm fractions, respectively. It is important however to note that the results for mineral composition in aleurone layer fraction be regarded cautiously because only a single analysis was

conducted due to limited sample. Wheat had highest levels of K, Mg, P, which were 2- and 3- to >10–fold higher than in purple barley and maize aleurone, respectively. In contrast, Ca levels were consistently lower in all aleurone layers compared to the other 3 macro elements. However, Ca was 1.3 and 40 times lower in wheat and maize endosperm compared to purple barley, suggesting that purple barley aleurone had relatively high calcium content.

For micronutrient elements, wheat had the highest levels of both Zn (3.47 ppm) and Fe (3.39 ppm) followed by Fe in purple barley (1.88 ppm) and Zn in maize (1.98 ppm) aleurone. Although zinc content in maize was higher than in purple barley, it was approximately 1.7-fold lower than in wheat aleurone. Among endosperm fractions, Fe content in purple barley was significantly different ($p< 0.05$) from wheat and maize. Levels of these elements in grains differ due to growing environments while variation in the aleurone and endosperm fractions would be attributed to differences in accumulation and distribution in various grain compartments. Thus, in wheat and barley, all minerals (higher proportion of phytate) are concentrated in the bran whereas in maize 80% of the minerals are in the germ (Liu et al. 2007; O'Dell et al. 1972; Salunkhe et al. 1985).The latter explains the low levels of both macro-and microelements in maize aleurone and endosperm in comparison to wheat and purple barley fractions. In addition, K, Mg, P and Ca are part of storage material that accumulates as complex salts (Bethke et al. 1998) within protein bodies in the aleurone layer. Ockenden et al. (2004), using EDX and X-ray analysis on barley and maize grains, demonstrated that globoid–crystals in the aleurone layer contained high K, Mg and P and low Ca, Fe, and Zn levels (Lin et al. 2005; Stewart et al. 1988). Low levels of these mineral elements in endosperm would be attributed to lack of protein bodies with electron dense globoid-crystals (Lott and Spitzer 1980). Lin et al. (2005) found also that the

starchy endosperm contained almost no mineral nutrient elements. Singh et al. (2014) suggested that mineral location and accumulation within the grain tissue may influence its dietary availability. According to Singh et al. (2014) it is assumed that the high mineral contents within the aleurone may not be easily bioaccessible because they are enclosed in thick, resistant cell walls.

6.4.3 Thiamine and riboflavin content

Thiamine levels in the aleurone layer, endosperm and whole grain are shown in **Table 6.2**. Riboflavin, however, was not detected in all samples. As reported in previous studies, riboflavin content was much lower compared to thiamine in whole grains, grain products including milling fractions (Batifoulier et al. 2006; Lebiedzin´ska and Szefer 2006). Niacin and thiamine were the most abundant in the aleurone layer as previously reported (Buri et al 2004). Niacin content is presented in Chapter 7; therefore only thiamine content will be discussed.

Thiamine levels were significantly different in the aleurone, endosperm and whole grain ($p< 0.05$). Wheat had the highest levels of thiamine (38 µg/g), which was approximately 2- and >3-fold higher than in purple barley and maize aleurone layers, respectively. Thiamine content in our wheat aleurone was much higher than in mechanically (14 µg/g) (Buri et al. 2004) and manually separated wheat aleurone (16 µg/g) (Pomeranz 1988). Variations in thiamine levels may be due to differences in grain varieties, methods of aleurone separation and extraction and analysis. The use of a cocktail of enzymes (amylase, papain and diastases) may have contributed to release of more thiamine, including the phosphorylated form, since diastases have phosphatase activity (Ndaw et al. 2000). Heat was excluded to avoid partial destruction of thiamine (Rodriguez et al. 2012).

Table 6.1 Amino acid composition in aleurone and endosperm fractions of maize, barley and wheat (%)

	Dasca maize		Purple barley		MSUD8006 wheat		Barley[a]	FAO recommendation[b]	
	Aleurone	Endosperm	Aleurone	Endosperm	Aleurone	Endosperm	Aleurone cell contents	Children (2-5yrs)	Adults
Asparagine	7.7	6.2	5.5	5	7.6	4.1	8.8		
Threonine	3.2	2.7	2.6	2.6	3	2.1	3.6	3.4	0.9
Serine	6.2	5.5	4.9	4.9	5.5	5.7	8.4		
Glutamic acid	16.7	21	27.3	29.8	20.4	35.8	14.9		
Proline	9.6	13.2	15.2	17.3	8.1	15.7	4.2		
Glycine	4.5	2.4	3.2	2.8	5.4	3.2	13.6		
Alanine	8.9	7.9	4.5	3.6	6.5	3.1	7.3		
Cysteine	3.1	1.8	2	1.7	2	1.8	0.4		
Valine	4.3	4.2	4.1	4.2	4.6	3.8	6.7	3.5	1.3
Methionine	1.7	1.4	1.5	0.9	1.4	0.6	0.7	2.5	1.7[d]
Isoleucine	2.5	2.3	2.7	2.7	2.5	2.1	3.6	2.8	1.3
Leucine	10	14	6.6	6.6	6.2	6	7.5	6.6	1.9
Tyrosine	3.3	3.4	2.7	2.3	2.7	2.1	1.6		
Phenylalanine	4.4	4.7	5.4	5.2	4	4.4	3.9	6.3[c]	1.9
Histidine	3.5	3.6	2.6	2.5	3.9	2.7	1.7	1.9	1.6
Lysine	3.7	1.6	3.1	2.6	4.6	2.1	5.2	5.8	1.6
Arginine	5.9	3.5	5.1	3.9	8.6	3.5	7.9		
Tryptophan	0.9	0.6	1.1	1.3	3.2	1.2	nd	1.1	0.5
Sum	100	100	100	100	100	100			
F[e]	17.76	7.56	22.23	8.85	16.12	7.32			

[a] Yupsanis et al (1990)

[b]FAO/WHO (2009), [c]Phenylalanine +tyrosine, [d]Methionine+cysteine

[e]Grams of amino acid per 100mg sample, dry basis

Table 6.2 Macro-and microminerals, thiamine and riboflavin content in aleurone and endosperm fractions

		Macromineral elements[1]					Micromineral elements[1]				B-vitamins[2]	
		Ca	**K**	Mg	Na	P	Cu	Fe	Zn	Mn	Thiamine	Riboflavin
Aleurone[3]	PB	20.77	187.8	91.81	10.25	255.8	0.16	1.88	1.58	0.35	18.48±0.82b	nd
	MSUW	15.64	492.7	196.3	7.85	499.5	0.65	3.47	3.39	0.74	38.27±0.24a	nd
	Dasca	4.96	45.97	19.47	13.69	36.2	0.15	1.07	1.98	0.26	10.27±0.34cd	nd
Endosperm	PB	3.71±0.21c	8.09±0.91e	2.70±0.21d	6.54±1.63	19.19±0.41d	0.16±0.04a	0.38±0.07c	0.49±0.28abc	0.085±0.01c	4.96±0.02fg	nd
	MSUW	3.60±0.09c	7.97±0.89e	1.36±0.01e	6.9±0.10	13.94±0.25e	0.17±0.01a	0.17±0.02d	0.27±0.11bc	0.12±0.09d	5.77±0.25ef	nd
	Dasca	0.635±0.15d	12.95±0.42d	3.27±0.01d	6.9±0.34	11.90±0.08e	0.09±0.01b	0.15±0.01d	0.18±0.01c	0.03±0.01e	4.19±0.23g	nd
Whole	PB	12.99±0.04a	74.33±1.81b	30.86±0.14a	3.32±0.93	89.53±0.58a	0.14±0.01ab	0.95±0.02a	0.92±0.03a	0.50±0.01a	11.50±0.27c	nd
	MSDU	7.135±0.11b	89.74±0.98a	25.95±0.25b	2.96±0.08	78.75±0.79b	0.12±0.00ab	0.69±0.00b	0.73±0.02ab	0.46±0.01b	9.87±0.23d	nd
	Dasca	1.07±0.10d	64.84±1.24c	21.37±0.54c	5.24±2.72	55.0±1.22c	0.11±0.00ab	0.44±0.01c	0.55±0.02abc	0.12±0.00c	7.06±0.07cd	nd

Sample names PB- purple barley, MSUW - MSUD8006 wheat and Dasca maize. [1]Macro and micro mineral elements (ppm) and [2]vitamins (µg/g)
[3]Values for aleurone layer were single analysis for minerals (due to limited amount of sample needed for this analysis) but duplicate for vitamins. Values for endosperm and whole grain are means [(n=2) ± Stdev]. Values with different letters in the columns are significantly different $p<0.05$ (Tukey T-test).

The low levels of thiamine in aleurone layers of purple barley and maize may be due to the difference in how thiamine is distributed within these grains. Earlier studies by Heathcote et al. (1952) found that scutellum of barley and maize contained about 50 to 80% of the total thiamine.

Thiamine levels were significantly ($p< 0.05$) lower in the endosperm fractions compared to aleurone layers. Wheat had relatively higher levels compared to purple barley and maize endosperm. Recently, Rohi et al. (2013) found that wheat endosperm contained only a small portion of total grain thiamine. In further support of our findings, earlier studies on the distribution of thiamine in wheat and rice, reported 62, 32, 3% and 47, 34 and 8% of thiamine content in scutellum, aleurone layer and endosperm fractions, respectively. This variation in levels within the grain fractions may be due to the fact that accumulation of thiamine and pyridoxine in the different layers is likely under genetic control (Batifoulier et al. 2006). Lebiedzin´ska and Szefer (2006) determined thiamine, riboflavin and pyridoxine levels in a variety of cereals and their products. Maize grain and its products had the lowest content of thiamine. However, maize aleurone had higher carotenoid levels compared to wheat aleurone (Chapter 5) and the other grain fractions.

6.4.4 Correlation among thiamine, proteins and phosphorus

Thiamine in foods exists either in free form or bound to macromolecules such as proteins and carbohydrates and phosphorylated forms (Ball 2006). Positive correlations were found between protein and thiamine ($r = 0.5877$) and thiamine and phosphorus ($r =0.9871$) across the analysed samples. Recently, Rohi et al. (2013) also found a significant correlation between protein and thiamine in whole wheat flour. Thiamine binding proteins (TBP), isolated from wheat grains and sesame seeds, are proposed to be synthesised in the aleurone layer where TPB accumulates as

thiamine (Adachi et al. 2000; Watanabe et al. 2004), hence the positive correlation. However, Ndaw et al. (2000) reported that in wheat flour, only 5% of thiamine existed as phosphorylated forms, while 50% was in free form and 45% was bound to proteins. The high correction between thiamine and phosphorus in the present study would be attributed to the fact that these two components are highly concentrated in the aleurone compared to the endosperm, which represent wheat flour reported in the study by Ndaw et al. (2000).

6.5 Conclusion

Based on the above findings, it can be concluded that purple barley and maize aleurone layers had similar amino acid composition but different levels of some EAA amino acids. Purple barley and maize were rich in three EAA namely phenylalanine, leucine and methionine, which are among those that are needed at high levels by children aged between 2-5 years. Thus they may be considered as potential food ingredients in cereal foods meant for children. Wheat aleurone had higher levels of thiamine and minerals, except for Ca that was highest in purple barley aleurone.

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CHAPTER 7

Application of LC-MS-MS to Identify Niacin in Aleurone layers of Yellow Maize, Barley and Wheat Kernels

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7.1 Abstract

Previous studies identified type II inclusions *in-situ* of aleurone layers as niacin reserves and quantified niacin using colorimetric methods. Such identification and quantification was based on the König reaction which lacks specificity because cyanogen bromide reacts with all pyridine derivatives. The aim of this investigation was to define the structure of niacin in aleurone layers and aleurone cell contents of yellow maize, wheat and barley using LC-MS/MS. Aleurone layers were manually separated and cell contents released by ultrasonic processing and the residue and pellets were examined microscopically. Niacin was extracted from the samples by autoclaving in alkali. The extracts were analyzed using HPLC and the structural identity of niacin was confirmed using quadrupole time of flight mass spectrometry in positive mode. Niacin concentration was highest in wheat and lowest in maize aleurone. The MS/MS spectra of pure niacin and the purported niacin peak in sample extracts showed similar fragmentation patterns. The major ion product occurred at *m/z* 80 representing loss of CO_2 and parent ion at *m/z* 124 $(M+H)^+$. These findings define some of the structural characteristics of niacin in aleurone layer of cereal grains, and demonstrate the possibility of using an ultrasonic processor to release cell contents.

7.1 Introduction

Niacin (nicotinic acid), one of the B-complex vitamins, is composed of a pyridine ring which is attached to a carboxylic group. It occurs naturally in various foods both in free and bound forms (Ball 2006). Acid hydrolysis is used to extract and determine free niacin content while determination of total niacin is achieved through use of alkaline hydrolysis (Ball 2006; Windahl et al. 1998). For nutritive analysis, acid hydrolysis may be used, because it releases niacin that is biologically available to humans (Ward and Trenerry 1997). However, in mature cereal grains, niacin is found mainly bound to macromolecules such as polysaccharides, peptides and glycopeptides (Das and Guha 1960; Mason et al. 1973) making it biologically unavailable (Rose-Sallin et al. 2001). Nonetheless studies have shown that alkaline processing of maize during tortilla production releases the bound niacin making it biologically available (Bressani et al. 1961; Christianson et al. 1968). For example, the presence of moderate amounts of niacin in alkaline hydrolysed maize (Christianson et al., 1968) indicated that bound niacin was released during processing. Niacin is concentrated in the aleurone and germ layer of the grain kernel (Ball 2006). These layers are almost all removed during grain processing with some exceptions such as in tortilla production. Paredes-lopez and Saharopulos (1982) demonstrated that the aleurone is still present in the nixtamal (alkali treated kernel) which is the key ingredient of tortillas suggesting that released niacin to be part of the nutrients taken up by consumers. Furthermore, absence of niacin deficiency (pellagra) among Mexican populations whose diet contained a high proportion of maize, consumed as tortillas (Ball, 2006) among other products, further confirms niacin bioavailability.

The aleurone layer, constituting 6-9% of whole grain kernel (Surget and Barron 2005), is composed of 1-3 cell layers depending on type of grain. It is morphologically and functionally distinct and its cells remaining alive in mature dry grains (Fincher 1989). Several studies have been done to understand morphological characteristics and chemical composition of the aleurone layers (Fulcher 1972; Fulcher et al. 1981; Jacobsen et al. 1971; Morrison et al. 1975; Stevens 1973). Recently, Atwell (2010) described the aleurone layer as the outer most layer of the endosperm, whose morphologically distinct characteristics have been revealed through microscopic evaluation. Atwell (2010) highlighted that the layer contains a high concentration of niacin bodies within fibrous cell walls, a phenomenon demonstrated by Fulcher et al. (1981) in wheat, oat and barley grains. Fulcher et al. (1981), referred to niacin bodies as type II inclusions in aleurone grains which were previously identified as protein-carbohydrate bodies (PCB) using either transmission or scanning electron microscopes (Jacobsen et al. 1971; Morrison et al. 1975). These contradictory observations may partly be explained by earlier studies that aimed at understanding the nature and structure of bound niacin in cereal grains. These studies suggested that niacin in wheat bran was either contained in a polysaccharide or attached to a peptide (Das and Guha 1960; Mason et al. 1973). The presence of niacin was acknowledged in these earlier studies. However, to date there is scarcity of studies that seek to elucidate the structure of niacin in cereal grains using advanced techniques such as LC-MS/MS.

Earlier studies used cereal bran to determine the nature of niacin complexes. (Fulcher et al. 1981) studied the chemical composition of cereal bran using different microscopic techniques. Their results gave more insight regarding the localisation of niacin within aleurone layer. On the other hand, manually separated grain fractions have been utilised to study distribution of niacin across

the grain kernel (Heathcote et al. 1952; Teas 1952). These studies reported higher niacin content was found in aleurone layer fraction compared to the other grain fractions. The proportion of nicotinic acid was 60 to 70% and 82.7 % in aleurone layers of maize and wheat, respectively (Heathcote et al. 1952; Teas 1952). In these studies, niacin was determined by the AOAC (2000) microbiological assay. The AOAC microbiological assay is based on the principle that specific microorganisms only reproduce and grow in the presence of specific vitamins (Solve et al. 1994). Although the microbiological assay is both useful and specific, the conventional AOAC procedure is time consuming (Solve et al. 1994). On the other hand, *in-situ* and colorimetric methods of determining niacin are based on the König reaction. In this reaction, pyridine derivatives react with cyanogen bromide (CNBr) and an aromatic amine, sulphanilic acid to produce polymethine dye (Fulcher et al. 1981). The challenge with this method is that apart from CNBr being highly toxic, it reacts with all 3-pyridine derivatives and specificity is reduced (Rose-Sallin et al. 2001).

HPLC methods have been used to determine niacin in fortified foods (Rose-Sallin et al. 2001) such as cereals, meat and selected foods (Ward and Trenerry 1997; Windahl et al. 1998). They have an advantage of increased specificity, reproducibility and reliability because compounds are identified by comparing their retention times and UV spectra to those of known standards. In addition, when an HPLC is coupled with a mass spectrometer (LC-MS/MS), the identity of compounds is further confirmed using structural information, such as the molecular weight (mass to charge ratio (m/z)) and fragmentation patterns. In previous studies, LC-MS/MS has been utilised in the identification of water soluble vitamins in Italian pasta (Leporati et al. 2005) and human plasma (Wang et al. 2010). The niacin reported in coffee grains is endogenous while the

refined pasta contained added niacin. To our knowledge LC-MS/MS has not been used to confirm the structure of niacin in aleurone layer, the major location of this vitamin in cereal grains. In order to confirm the structure of niacin in cereal grains, this paper describes the application of LC-MS/MS to identify niacin in aleurone layers and aleurone cell contents from maize, barley and wheat and the use of an ultrasonic processor to release aleurone cell contents.

7.2 Materials and Methods

7.2.1 Grain samples

Two yellow maize varieties (Dasca, USP1395XR), two barley varieties (purple barley and normal barley) and two wheat varieties (Caledonia and MSUD8006) were used. These cereal grains are of commercial value and their characteristics were described earlier in Chapter 4.

7.2.2 Preparation of aleurone layers and release of aleurone cell contents

Aleurone layers and endosperm were prepared manually as previously described in Chapter 3. Release of cell contents was achieved after soaking ground aleurone layers in distilled water for 30-45 minutes in a sonication tube at 20°C. The tube was immersed in ice-cold water and sonicated with 4 mm probe at 20-23 joules per second for 40 minutes using a Vibra-Cell (^{TM}) high intensity ultrasonic processor (Sonics & Materials Inc., Danbury, CT, USA). After sonication, the mixture was first filtered through a Miracloth filter (Calbiochem, EMD Chemicals, Inc. San Diego, CA, USA). The residue was kept and the filtrate centrifuged for 10 minutes at 600 x g. The first (1^{st}) pellet was kept and the supernatant was centrifuged again at higher centrifugal force of 14000 x g for 30 minutes. The second (2^{nd}) pellet was assumed to have concentrated aleurone cell contents. Part of the residue, 1^{st} pellet and 2^{nd} pellet were examined under bright field using an upright Carl Zeiss microscope (Zeiss, Axio Imager MI,

Jena, Germany). The remaining samples were air-dried overnight and kept refrigerated for niacin extraction. Iodine staining was performed on the 1st and 2nd pellets to check for the presence of starch. This was based on the preposition that isolated aleurone layers are expected to have low starch content (Atwell, 2010) and to ensure that the second pellet was concentrated with cell contents. The aleurone cell contents were identified by high frequency of intact or clumped aleurone grains, the spherical protein-rich structures that are highly characteristic of aleurone cells.

7.2.4 Niacin analysis

7.2.4.1 Chemicals

Niacin standard (purity 99.92%) was obtained from Sigma Aldrich (St. Louis, MO, USA). HPLC grade methanol, formic acid and sodium hydroxide were purchased from Fisher Scientific (Whitby, ON, Canada).

7.2.4.2 Niacin extraction

Alkaline hydrolysis was used to extract niacin from aleurone layers and aleurone cell contents. The method was adapted from Rose-Sallin et al. (2001) and Leporati et al. (2005) with some modifications. Briefly, 100 mg of aleurone layer samples were weighed separately into 25 mL amber bottle. Five millilitres of 0.5M NaOH were added and the suspension was autoclaved at 121°C for 30 minutes. After cooling at room temperature, the pH of the hydrolysate was adjusted to 4.5 with HCl (1M then 0.25M). The hydrolysate was transferred into a 10 mL volumetric flask, and volume made up with distilled water. This mixture was transferred into 25-mL centrifuge tubes, shaken on a vortex and centrifuged at 10,000 x g for 10 minutes. Solid phase extraction (SPE) as described by Ekinci and Kadakal (2005) was used to remove interference and

concentrate the extract using Supelclean^{TM}Envi^{TM}-18 SPE, 500 mg cartridges on a Supelco Separator (Supelco VISIPREP TM ^{24}). The stationery phase was activated by adding 10 mL methanol followed by 10 mL acidified deionised water (pH 4.5). About 5 mL of extract (supernatant) was loaded into the SPE cartridge and components eluted with 5 mL acidified water followed by 10 mL methanol. The eluents were collected and evaporated under nitrogen using a rotary evaporator (IKA RV10, IKA® Works Inc., Wilmington, NC, USA). The dried extracts were reconstituted with 2 mL of the mobile phase, filtered through 0.45µm nylon filters, stored at -20°C prior to HPLC analysis.

7.2.5 HPLC and LC-MS/MS analysis

HPLC analysis was performed using a Waters Alliance 2695 HPLC (Waters, Milford, MA, USA) equipped with a photodiode array (PDA) detector (Waters 996) and an autosampler (717 Plus, Waters). The conditions for eluting niacin were as described by Wang et al. (2010) with modifications. The analytical column was a reversed phase, Phenomenex (150 mm x 4.6 mm, 5 µm) (Gemini 5u C18). The mobile phase consisted of solvent A (0.1% formic acid in Milli-Q water) and solvent B (100% methanol). Niacin separation was achieved using an isocratic gradient 90/10 (V/V) for solvent A and solvent B at flow rate of 0.6 mL/min for 8 minutes and column operated at 20°C. The autosampler was set to 20°C and the injection volume was 20 µL. Niacin was detected at 260 nm. To elucidate the structure of niacin, LC was coupled to quadrupole time of flight mass spectrometer (Micro mass Q-TOF micro^{TM}, Model YB369, Waters, Milford, MA, USA) equipped with an interface of electrospray ionisation (ESI). During the run, the eluents were split into half after the column, 50% was introduced into the inlet of the ESI probe, the other diverted to waste and time of niacin elution blocked for MS/MS analysis. The instrument was calibrated in positive mode with 2 µg/mL of sodium iodide through a mass

range of 100-1000 amu. The flow rates were 900 and 50 L/h for desolvation gas (N2) and cone gas, respectively. After infusing niacin standard solution, interface parameters: ion spray voltage, cone voltage, desolvation and source temperature and collision were optimised and set at 3200V, 40V, 350°C and 120°C, respectively. The fragmentation conditions were optimised by varying the collision energy between 10 and 40eV and were set at 33eV (an average of 35 and 30eV). Mass Lynx V4.1 software was used for data acquisition and processing. The fragmentation pattern was compared to those of pure standards and those reported in literature. The spectrum of positively charged niacin showed the formation of characteristic product ions at m/z 53, 78, 80 and 96.0 (Leporati et al. 2005; Tellstorm et al. 2013). The fragment ion at m/z 80 is considered as the major product ion due to its high relative abundance.

7.3 Results and Discussion

7.3.1 Release of aleurone cell contents

During the process of releasing aleurone cell contents, parts of the residue, 1st pellet and 2nd pellet were sampled and examined under bright field microscopy. Microscopic evaluation of the residue showed that cell contents were released from the aleurone layers using an ultrasonic processor as evidenced by empty cells and broken cell walls shown in **Figure 7.1**. It is expected that isolated aleurone layers have low starch content and no starch is found in the aleurone layers of mature grains (Fincher 1989). Therefore, the presence and absence of starch was examined by staining with 0.1% potassium iodide. A closer evaluation of the residue revealed that it contained starch granules that stained blue-black with an approximate size of 20 µm, empty and aleurone filled aleurone cells (**Figure 7.1a**). The starch granules were probably from underlying endosperm that may not have been thoroughly scraped off during separation. Teas (1952) also reported the presence of starch on manually separated aleurone layers. The residue also had

intact aleurone layer with filled and empty cells (**Figure 7.1b**) as well as framework of aleurone layer most of which were largely emptied cell walls with few containing their contents (**Figure 7.1c**). Similarly yellow maize aleurone residue also had some aleurone layer pieces with both filled and empty cells (**Figure 7.1d**) and empty cells with only cell walls remaining (**Figure 7.1e**).

Although some cells in aleurone layers were still filled, these observations demonstrated that using the ultrasonic processor broke the aleurone layers and in some instances even the cell walls releasing cell contents. **Figure 7.2** shows the 1st pellet from wheat (**Figure 7.2a**) and yellow maize (**Figure 7.2b**) aleurone layers with starch granules stained blue-black. The granules were larger in size compared to the other components. A fragment of a tube cell was also identified in wheat pellet. The second pellet was largely an unstained mass, assumed to be aleurone cell contents. These remained yellowish brown with very few stained starch granules (larger in size) that could be spotted under the microscope (**Figure 7.2c & 7.2d**). The approximate yield of the cell contents was 5-10 mg from 100-150 mg of ground aleurone layers. Compared to long procedure and use of benzene and carbon tetrachloride in the method described by Stevens (1973), use of the Vibra-cell high intensity ultrasonic processors would be rapid and safer method of releasing cell contents.

7.3.2 Optimisation of niacin extraction

Different concentration of NaOH (0.5- 5M) and autoclaving periods (30 minutes to 2 hours) have been used to extract niacin from different fortified food products and natural foods (Leporati etal. 2005; Rose-Sallin et al. 2001; Ward and Trenerry 1997). In order to establish the extraction

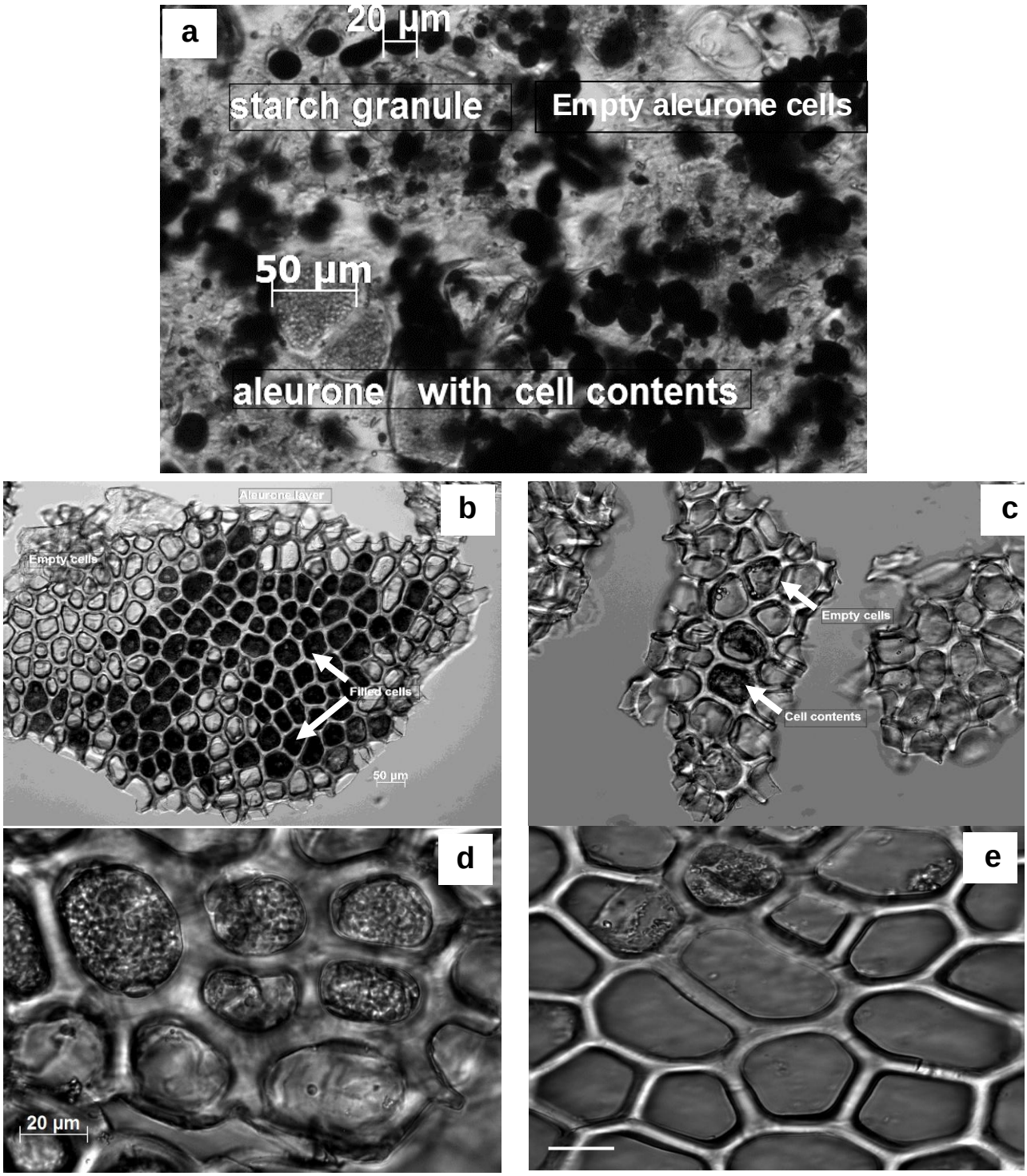

Figure 7.1 Digital image of stained wheat aleurone residue showing stained starch granules (blue-black) and fragments of empty and filled aleurone cells (a); wheat aleurone layer with empty and filled aleurone cells (b); broken aleurone cell walls and empty aleurone cells (c); maize aleurone with filled and empty cells (d) and empty aleurone cells (e).

conditions for this study, different concentrations of sodium hydroxide (0.1, 0.25, 0.5 and 1M) were used in combination with extraction periods of 30 minutes and 1 hour in the autoclave. For preliminary studies, ground commercial aleurone layer was used. There was a wide variation in the peak areas at different concentrations and a distinct decrease was observed when 1M NaOH was used. Use of 0.5M NaOH gave consistent results with minimal difference when autoclaved for 1 hour compared to 30 minutes. The niacin standard was treated as sample using

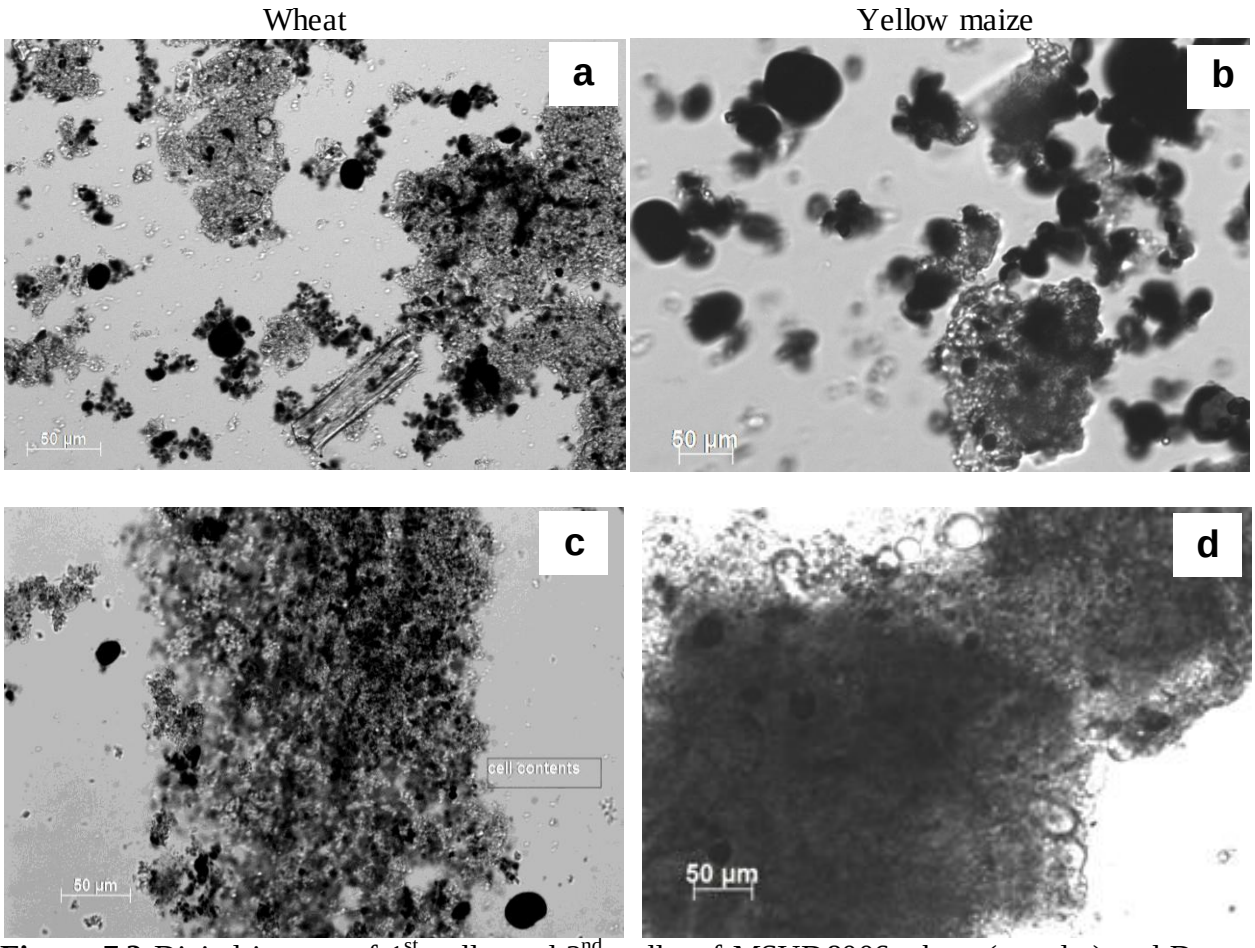

Figure 7.2 Digital images of 1st pellet and 2nd pellet of MSUD8006 wheat (a and c) and Dasca maize (b and d) aleurone layer with 1st pellet showing stained starch granules and cell contents and 2nd pellet assumed to be mass of aleurone cell contents only.

0.5 and 1M NaOH and autoclaved for 30 minutes. The results showed a decrease in peak area of niacin that was extracted using 1M NaOH compared to that in which 0.5M NaOH was used. It was therefore speculated that 1M NaOH may be affecting the extraction or detection of niacin. However, this could not be explained except that when 1M NaOH was added to the sample, the mixture was more viscous than the other concentrations. Based on these preliminary results, 0.5M NaOH and 30 minutes period of autoclaving were chosen and used for niacin extraction.

7.3.3 Identification of Niacin using HPLC-MS/MS

Niacin was identified by comparing retention time, UV-spectra and mass spectra with that of pure niacin standard. **Figure 7.3** shows LC-chromatograms at 260nm of niacin standard (**A**) and aleurone layer extracts from yellow maize (**B**), wheat (**C**), normal barley cell contents (**D**) and aleurone (**E**). These results confirm the presence of niacin in the extracts as they all had a peak eluting at comparable time to that of the niacin standard. Similar peaks were observed in extracts from whole grains and residues. The absorption units on the LC-chromatogram of aleurone cell contents (**Figure 7.3D**) were higher compared to those on LC-chromatogram for normal barley aleurone layer (**Figure 7.3E**) and residue (data not shown). This observation further confirms that niacin is highly concentrated and localised within the aleurone cells in the aleurone layer, in agreement with previous reports (Fulcher et al. 1981; Heathcote et al. 1952). In the endosperm fraction, niacin peak was hardly detectable in wheat varieties whereas minor peaks were present in barley and yellow maize.

To elucidate the structure of niacin, HPLC was coupled to Q-TOF mass spectrometer. LC-MS and MS/MS were run concurrently with the conditions as described in section 5.2.3. The major species produced were protonated molecular ions ($[M + H]^+$). LC-MS spectra and MS/MS

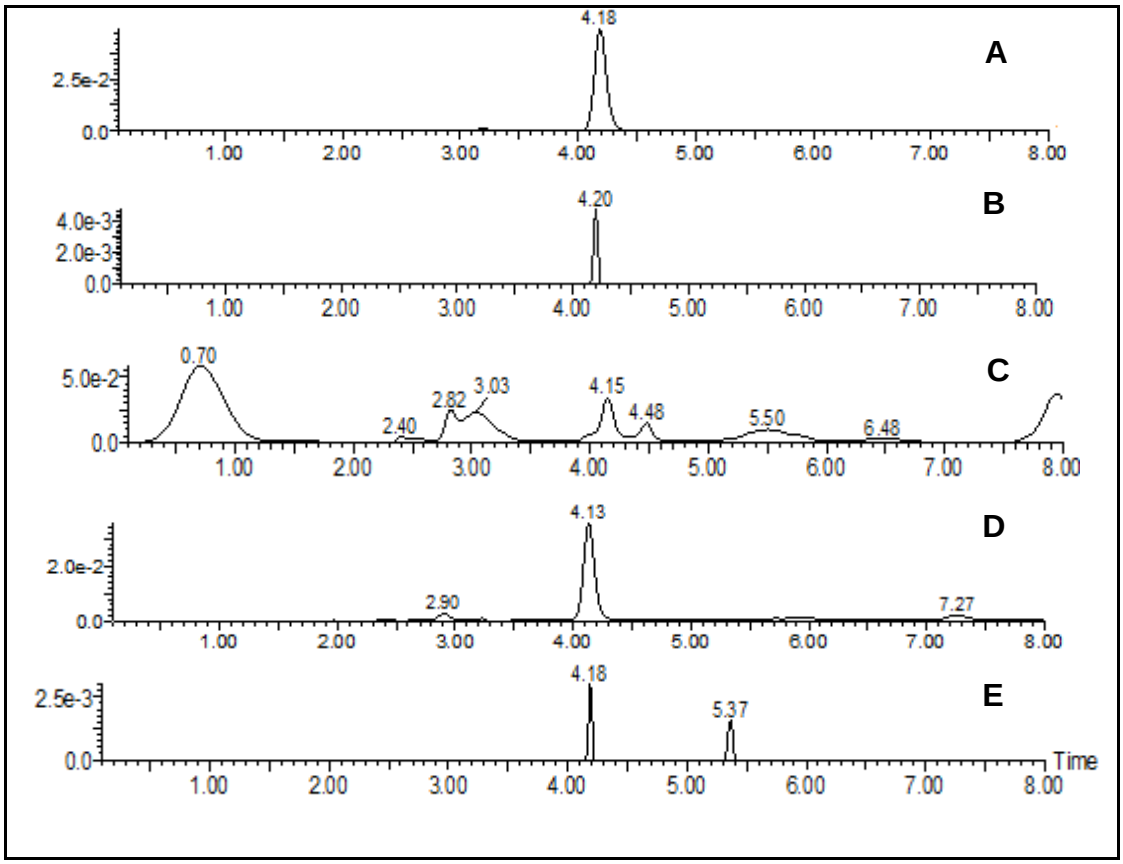

Figure 7.3 LC-chromatogram of niacin standard (A), aleurone layer extract from Dasca maize (B), MSUD8006 wheat (C), normal barley aleurone cell contents (D), and aleurone layer (E).

spectra of niacin standard and aleurone cell analytes are shown in **Figure 7.4a**. The LC-MS spectra for niacin standard (**Figure 7.4a** [**i**]) had one peak with 100% relative abundance and molecule ion *m/z* was 124 $(M+H=123 + 1= 124)^{+}$. The molecular weight of niacin is 123, which yielded 124 due to protonation. A similar pattern was observed in LC-MS spectra for aleurone cell contents analyte (**Figure 7.4a** [**ii**]), confirming that the identified component was niacin. Further fragmentation of this ion (isotope) through MS/MS resulted in a product ion at m/z 80, due to loss of CO_2 (124-44) (**Figure 7.4a** [**iii & iv**]). This had the highest abundance (100%) hence it was considered a major product ion. Other detectable ionic species observed in the standard and aleurone cell contents included fragment ions at *m/z* 53, 68, 78, 96 and 111. Similar

fragmentation patterns were observed in analytes from aleurone layer analytes of barley, wheat and yellow maize (**Figure 7.4b**).

Although relative abundance the major product ion at *m/z* 80 was the same, the abundance of the other fragments ions varied widely. The abundance of the fragment ions at *m/z* 53, 78 and 96 were lower in barley (38%, 48% and 35%) (**Figure 7.4b** [**I**]) and wheat (48, 52 and 60%) (**Figure 7.4b** [**II**]) compared to those in yellow maize (**Figure 7.4b** [**III**]) (70, 70and 80%), respectively. The major product ion of *m/z* 80 plus the other fragment ions with *m/z* 53, 78, 96 have been previously reported during identification of niacin using LC- MS in Italian pasta (Leporati et al. 2005) and human plasma (Wang et al. 2010).

However, studies by Leporati et al. (2005) and Wang et al. (2010) only explained the loss leading to the yield of the major product ion at *m/z* of 80. In the present study, the description of fragment ions at m/z 53, 78 and 96 were based on a report by Tellstorm et al. (2013), who also used a Q-TOF system (Bruker Compact^{TM} QTOF system) to identify nicotinic acid. Tellstorm et al. (2013) used SmartFormula3D software to determine the molecular formula of the precursor and formula of fragment ions and MetFrag to confirm identity of compound within the open source web application. Therefore, according to Tellstorm et al. (2013), fragments at *m/z* 53, 78 and 96 were due to loss of C_2HNO_2 (124-71), CH_2O_2 (124-57) and CO (124-28), respectively. Because similar fragments were also achieved in the present study, it was concluded analytes of aleurone layers had niacin. These results are significant because the molecular information obtained by using LC-MS/MS shows the structure of niacin in isolated aleurone layers and aleurone cell contents.

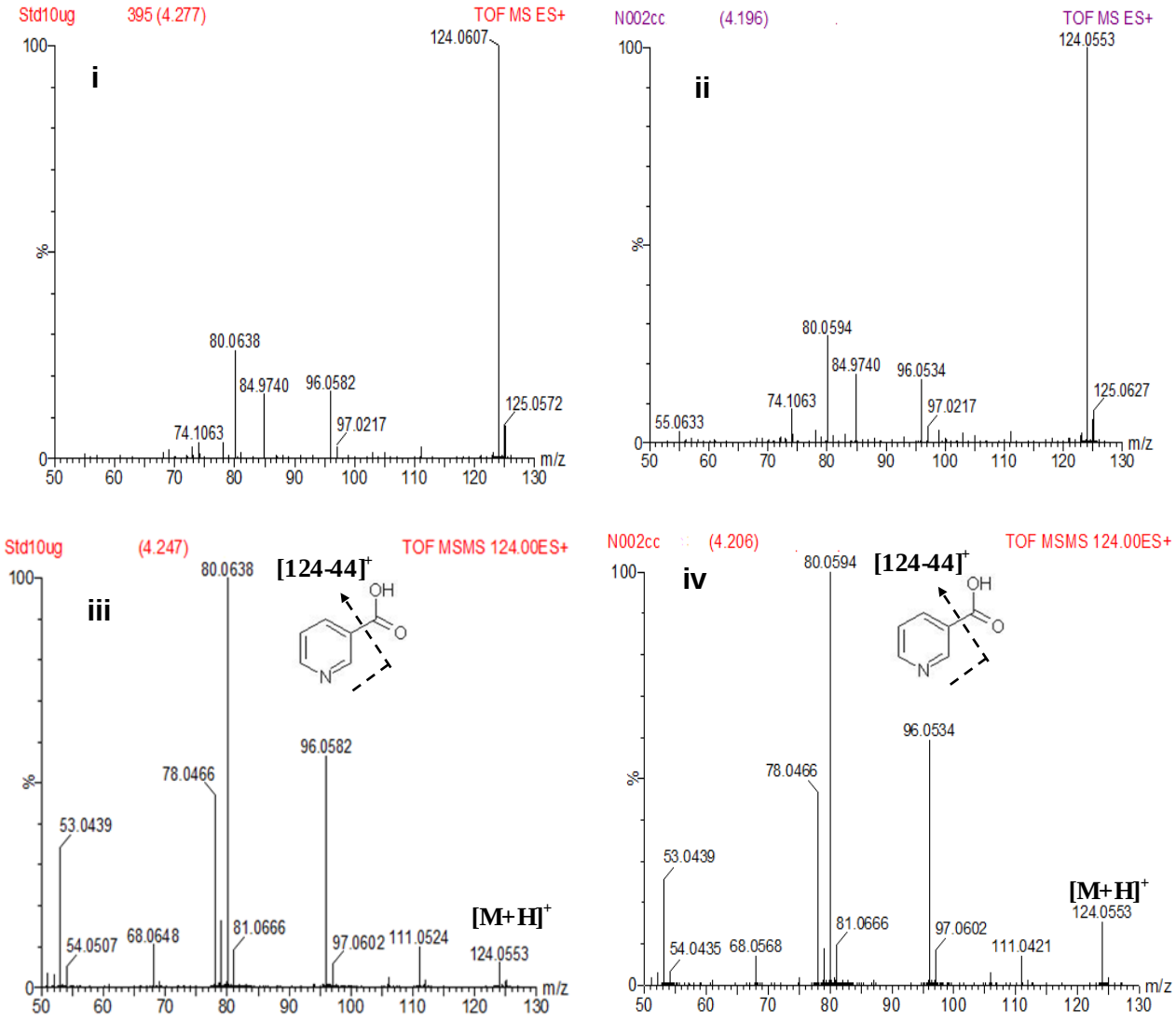

Figure 7.4a LC-MS and MS/MS spectra of niacin standard (i & iii) and aleurone cell contents (ii & iv) showing niacin fragmentation pattern with major fragment ion at m/z 80 in standard and sample extract.

7.3.4 Quantification of niacin in aleurone layers

Total niacin levels in the aleurone layers are shown in **Figure 7.5**. On average, wheat had the highest concentration of niacin (171 µg/g), followed by barley (113 µg/g) and yellow maize aleurone had the lowest levels (87 µg/g). Similarly, among whole grains, maize had lowest niacin content concurring with Carpenter (1983) who reported that mature maize has lower niacin

content than wheat and rice. In the aleurone layers of barley, wheat and maize, respectively, niacin levels were 4-, ≥8- and ≥6- fold higher than those in whole grains.

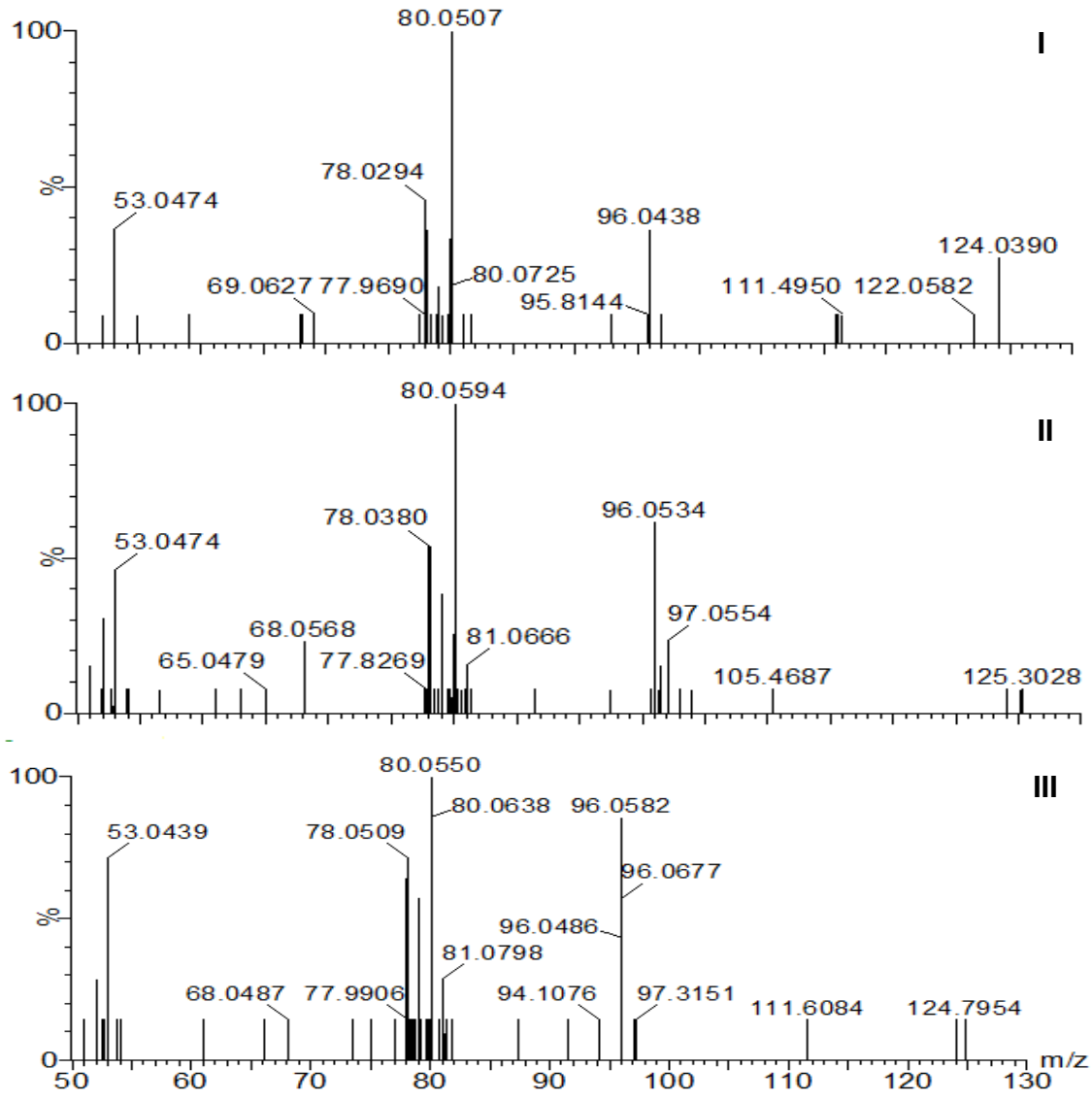

Figure 7.4b MS/MS spectra of niacin in intact aleurone layer of Dasca maize (I), normal barley (II) and MSUD8006 wheat (III) with major fragments at m/z 80 and other distinct fragments at 53, 78, and 96.

Furthermore, compared to isolated aleurone layers , niacin concentrations in released aleurone cell contents were 20- fold higher in normal barley (2630 µg/g) and maize (2003 µg/g) and 5-fold higher in wheat (1077 µg/g) aleurone layers. These values are within the range of 1500 µg/g of niacin in isolated aleurone grains from wheat bran reported by Fulcher (1972).

However, niacin concentrations in aleurone layers reported in the literature seem to be highly variable (Buri et al. 2004; Heathcote et al. 1952; Pomeranz 1988; Teas 1952). The levels of niacin in wheat aleurone layer were 2-and 4-fold lower than 329 µg/g reported by Buri et al. (2004) and 613 and 741 µg/g reported by Heathcote et al.(1952) and Pomeranz (1988). Similarly levels of niacin in yellow maize aleurone layers in the present study were about 4 times lower compared to 371 µg/g reported in literature (Heathcote et al., 1952; Teas, 1952). These variations are likely due to differences in grain type and varieties (Adom and Liu 2002), fraction separation methods, purity of samples, extraction methods and methods of niacin determination. For instance, most of the previous studies used microbiological assay to determine niacin content while the present study used HPLC. Rose-Sallin et al. (2001) determined niacin content in fortified food using both microbiological and HPLC-fluorescence methods. These authors concluded that HPLC values were lower than those reported following microbiology assay, with significant differences being observed in cereal based products. Hence, our approach allows some understanding of such variations. Nonetheless, the ratio of total niacin content in yellow maize aleurone was approximately 2-fold lower than in wheat aleurone. This finding is in agreement with what was reported by Heathcote et al. (1952). These results therefore, suggest that despite varietal differences and use of different measuring techniques the trends in terms of niacin concentration seem to be similar, with maize having the lowest levels.

Niacin is important for the metabolism of carbohydrates and fats and other substances in the body (Ball, 2006). Apart from studies that have shown that bound niacin in maize is released during processing and is bioavailable, there is limited literature on effect of processing on niacin content in wheat and barley. The larger percentage of niacin in bound form and concentrated in aleurone layer may suggest that either whole grains or aleurone layer, added as ingredients, should be processed under alkaline conditions during food product development. There is need therefore for further studies to examine effects of using alkaline conditions on niacin content in food products, to which wheat or barley aleurone fractions may be used as an ingredient.

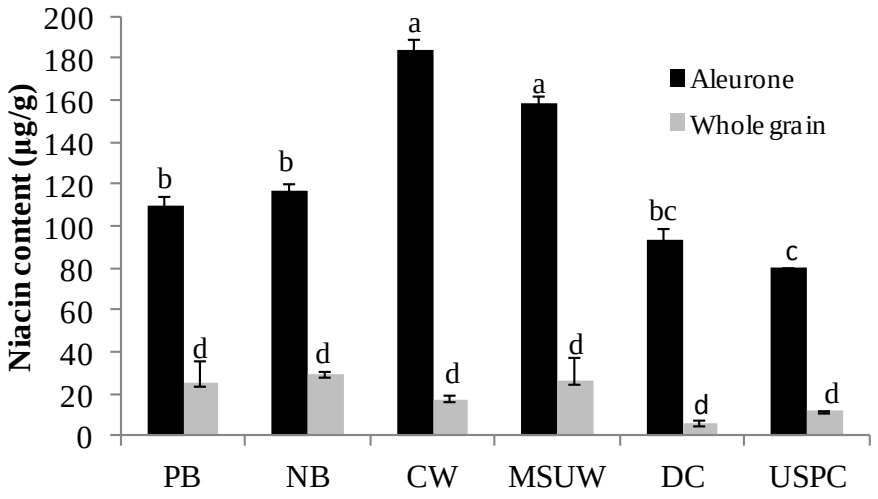

Figure 7.5 Niacin content in whole grains and aleurone layer; the vertical bars represent the standard deviations (n = 2) and bars with different letters are significantly different at $p < 0.05$ (Duncan's multiple range test). PB, Purple barley; NB, normal barley; DC, Dasca Maize; USPC, USP1395XR maize; CW, Caledonia wheat and MSUW, MSUD8006 wheat.

7.4 Conclusion

Use of LC-MS/MS confirmed the structure of niacin in isolated aleurone layers of some selected cereal grains. This was achieved by comparing the fragmentation patterns of pure niacin standard

and those from the extracts and information in literature. The fragments were found to be similar leading to the conclusion that niacin in cereal grain is composed of pyridine ring attached to a carboxyl group as shown by the fragmentation pattern. Thus, the major product fragment ion at m/z 80, indicated cleavage between the benzene ring and the carboxyl group. Furthermore, use of an ultrasonic processor may be a quick method of releasing cell contents from aleurone layers.

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CHAPTER 8

General Discussion and Conclusion

8.1 General discussion

Health benefits of consuming whole grain food products have been associated with micronutrients and phytochemicals. Among the phytochemicals, phenolic acids and carotenoids analysed in this research are known for their role in promoting health by reducing the risks of developing chronic diseases due to their antioxidant properties and other mechanisms. On the other hand, micronutrients such as B-vitamins and minerals, play vital roles in metabolic processes as co-enzymes or catalyst hence contribute to promotion of well being and health of humans (Ball 2006). In addition, about 48% of protein in the diet of an average person comes from cereal grain products (Serna-Saldivar 2010). Thus, the above components are important in human health and work together synergistically within the whole grain (Jacobs 2015; Jacobs and Steffen 2003; Liu 2004).

However, modern populations are to a larger extent consuming wheat-based food products produced from refined endosperm (Hemery et al. 2007; Marquart et al. 2007). Recently there has been a shift and consumers are now concerned about eating healthier foods. The food industry has responded by incorporating either whole grains or grain fraction in grain-based food products to meet consumer needs. However, grain fractions that are obtained through mechanical separation are comprised of blends of different botanical fractions hence affecting their chemical composition (Beta et al. 2005; Hemery et al. 2009; Liu et al. 2007). In order to select grain fractions with enhanced levels of phytochemicals and micronutrients, knowledge of their distribution and composition within the grain is important. Therefore, this research focused on

examining the distribution and composition of phytochemicals (phenolic acids, carotenoids), micronutrients (B-vitamins and minerals), protein and amino acid contents in different varieties of yellow maize, barley, wheat and oat. The overall aim was identification of cereal fractions that are the major contributors of these components that would be perceived as having potential application to the formulation of healthier cereal products.

To achieve the overall aim, the research used manual separation to obtain fractions including pericarp (comprising inner +outer pericarp, testa, and nucellus), aleurone layer, germ and endosperm (Chapters 3 and 4). To ensure purity, the fractions were screened under bright field microscope. Thus, these fractions were assumed to be relatively pure compared to mechanically separated ones and therefore deemed suitable for compositional analysis at laboratory scale. In this research, component identification and quantification in the botanical fractions and whole grains were accomplished by several techniques including fluorescence microscopy, spectrophotometry, HPLC, ICP-AES and LC-MS/MS for confirmation of molecular structures of the identified components.

The findings of this research have demonstrated that we can get a better understanding on the phytochemical and nutritional composition and distribution profiles of diverse types of cereals; hence providing a good database for comparison of these components in these important cereals. In the process the research explored ways of estimating FA concentration using fluorescence intensity profile values for the first time. Furthermore, ultrasonic processors was utilised to release aleurone cell contents and LC-MS/MS applied to confirm the structure of niacin in cereal

aleurone layers for first time. These two approaches showed promising results and may be applied by other researcher working with cereal grains.

In the first study, the distribution of FA, the most abundant phenolic acid was evaluated using two approaches; fluorescence microscopy to examine in grain *in-situ* (Chapter 3) and chemical analysis by HPLC in separated grain fractions (Chapters 3 and 4). These two techniques were instrumental in testing the hypothesis that FI values can be used to estimate FA concentration. The high correlations found between FI values and FA concentrations (Table 3.3, Chapter 3) in pericarp, aleurone and endosperm ($r = 0.5929$, $p = 0.042$; $r = 0.5849$, $p=0.002$ and $r=0.8115$, $p<0.001$) for barley, wheat and yellow maize, respectively, successfully demonstrated that FI values have potential as indicators of FA concentrations in grain *in-situ*. This finding has therefore shown that fluorescence intensity profiles may be useful not only for mapping FA distribution, as was the case previously (Saadi et al. 1998; Sen et al. 1994) but also in estimating FA concentrations in grain i*n-situ*. The uniqueness of this part of study is the attempt to quantitate fluorescence using microscopy. However, several factors affect measurement of fluorescence intensity such as specimen thickness, temperature, signal to noise ratio and exposure time (Baldwin et al. 1997; Waters 2009). In the present study the specimen thickness was the same (10µm) for all the fractions. Exposure time is known to affect the fluorescence intensity as overexposure may cause photobleaching or quenching, affecting the final fluorescence. Even though exposure time was set (Chapter 3), a standardized protocol for looking at fluorescence intensity between sections and varieties, would make such comparisons stronger.

In addition, the digital images acquired through use of fluorescence microscope with high resolution enhanced our understanding of the structure-chemical composition and functional relationship summarised by Serna-Saldivar (2010). One of which is the protective role of pericarp and aleurone layer which contain various chemical components including phenolic acids. These images showed that FA is localised and highly concentrated in the pericarp and cell walls of aleurone layer. The presence of FA and other phenolic acids in these fractions play a protective role against insect and pathogens by providing both physical and chemical barriers (Bily et al. 2003; García-Lara et al. 2004; McKeehen et al. 1999; Santiago and Malvar 2010). García-Lara et al. (2004) investigated the role of pericarp cell wall components in maize weevil resistance in 9 genotypes of tropical maize. Their results showed that FA was among the most important phenolic compound contributing to maize weevil resistance. In wheat, phenolic acids, ferulic and *p*-coumaric have been associated with tolerance to *Fusarium* species during grain development through to maturity (McKeehen et al. 1999).

The second and third experimental chapters (the research) provided information on variation of phytochemical composition in whole grains and fractions of wheat, yellow maize and barley as evidenced by the significant differences in concentrations and the distribution of phenolic acids (Chapter 4) and carotenoids (Chapter 5). This research adds to literature about the compositional information of meticulously hand separated grain fractions (pericarp, aleurone layer, germ and endosperm) of wheat, yellow maize and barley varieties and oat grain fractions. The trend in concentrations of phenolic acids in the grain fractions was to a larger extent similar. The highest concentrations were in the pericarp or aleurone fractions, followed by germ and the endosperm had lowest, in decreasing order yellow maize>wheat>barley.>oat. However, the trend was

different for carotenoid content with wheat, barley and oat having highest concentration in germ, followed by aleurone and endosperm. In contrast, in maize endosperm and aleurone layer, the concentrations were within the same range and germ had the lowest content.

The results also provided more insights in the variation in composition of whole grains versus that of their grain fractions. Thus some components undetectable in some whole grains were detectable in one or two of their grain fractions. This phenomenon was observed both in phenolic acids and carotenoids. For example, CA was only found in aleurone layer of wheat and barley grains (Table 4.3) and not detectable in their whole grains. Alternatively, some components present in fragments were undetectable in the whole grain. For instance, in phenolic acids, for example, *p*-OH-BA, was undetectable in whole grain of wheat, barley varieties and oat (Table, 4.2) but present in their pericarp, aleurone layer and germ fractions (Table 4.3 and Table 4.4). For carotenoids, zeaxanthin was present in whole grains but was not detectable in endosperm of barley, wheat and oat, and barley aleurone layer. The fact that zeaxanthin was not detectable contributes to our understanding of why lutein has been reported as the abundant and dominant carotenoids in wheat, oat and barley in previous studies (Hentschel et al. 2002; Okarter et al. 2010, Adom et al 2010 Panfili et al 2004).

In addition to further confirming the uneven distribution of these components within the grain kernel, it also showed the existence of variation in the distribution and composition of these phytochemicals within the different grain fractions (Chapters 4 and 5). In chapter 5, the study evaluated carotenoid content and composition using HPLC and spectrophotometry. The high correlation in total carotenoid content found by these two methods (Figure 5.4) indicated a good

quantitative agreement in composition. Furthermore, this is the first study to report on carotenoid composition in aleurone layers of different grain cereals, hence adding new information to the body of scientific knowledge.

Although the current focus on whole grains is on understanding phytochemical composition, it is important to recognise that cereals have been a source of energy, proteins and micronutrients (B-vitamins and minerals) in the human diet for millennia. Therefore in chapter 6 the study evaluated selected nutrients (proteins and amino acid composition, vitamins B1, B2, and B3 and mineral constituents) in the aleurone layer, endosperm and whole grains. The aleurone layer and endosperm fractions were chosen to represent the grain fraction with highest and lowest phenolic acids and carotenoids contents with exception of yellow maize endosperm. The objective was to characterise and compare the nutritional profiles of purple barley (PB) and yellow maize (YC) aleurone tissues to that of wheat aleurone layer. Wheat aleurone layer, a commercialised food ingredient is known for its functional and nutritional properties (Atwell, 2010; Brouns, et al. 2012). By analysing the nutritional alongside the phytochemical profile we gain a better understanding of chemical composition of the grain fractions, hence have adequate information on which to base the selection of the fractions. As evidenced in these results, yellow maize aleurone which had highest concentration of most of the phenolic acids (Chapter 4) and carotenoids (Chapter 5) had in general a poor nutritional composition. Thus yellow maize aleurone had lowest amounts of protein, minerals, thiamine and niacin compared to wheat and barley aleurone layers. Despite this shortfall, yellow maize aleurone, may still be considered as an ingredient to improve the functional properties of grain based food products. The study has limitation in that lipids and vitamin E were not analysed.

Niacin is the most abundant B-vitamin in cereals and is highly concentrated in aleurone cells within the aleurone layer. In chapter 7, the study characterised the structure of niacin using LC-MS/MS after alkaline extractions. The results obtained in the present study confirmed the structure of niacin based on molecular weight and fragmentation pattern, information obtained through use of mass spectrometry that is more specific. Earlier studies that attempted to understand the structure of bound niacin in cereal bran described niacin as being contained in a polysaccharide or attached to a peptide (Das and Guha 1960; Mason et al. 1973) and niacin was identified through König reaction, that has reduced specificity (Rose-Sallin et al. 2001). In addition, an ultrasonic processor was utilised for the first time to release aleurone cell contents as opposed to use of solvents with different densities (Stevens 1973). The study reports on total niacin content, which includes the bound form that is biologically unavailable to humans. For nutritive analysis, acid hydrolysis is recommended. Therefore further studies, to determine biologically available niacin in aleurone layer or to evaluate how much niacin would be released using an *in-vitro* model would be essential.

Overall, these study findings provide information that will be useful to the miller, cereal chemists and food technologists as they attempt to process elements of grain or its fractions for use toward the production of functional and nutritious food products. Firstly, such information on phytochemical and nutritional profiles may be used in selection of cereal grain fractions with enhanced levels of phenolic acids, carotenoids, micronutrients and amino acids of interest for use in improving the functional properties of grain-based foods to meet customer needs. Among the studied grain fractions, results obtained on phenolic acids (Chapter 4), carotenoids (Chapter 5) and micronutrients (Chapter 6) most of the components were not only present but also found in

high concentration in the aleurone layer compared to the other fractions. These findings further confirm the fact that the aleurone layer is the major portion of the grain where most of the physiologically beneficial substances are concentrated (Atwell 2010). Yellow maize aleurone layer was unique in that it had enhanced levels of both phenolic acids and carotenoids but had lower levels of vitamin B1 and B3 compared to wheat aleurone. Therefore like commercial wheat aleurone, yellow maize aleurone has potential for use in improving the functional properties of non-whole grain foods. According to a review by Brouns et al. (2012) results of various studies on consumption of whole grains were associated with amount of aleurone present in whole grain; therefore aleurone enrichment would provide beneficial effects as those in whole grain. Furthermore, bread enriched with 20% wheat aleurone flour not only increased the nutritional value to be comparable to that of whole wheat bread but also tasted like white bread (Atwell et al. 2007; Brouns et al. 2012).

This study has generated information on the composition and distribution of phenolic acids, carotenoids and micronutrient in whole grains and grain fractions that would be instrumental in development of mechanical milling processes and or fractionation techniques. Such knowledge of chemical composition is important in obtaining grain fractions with superior levels of valuable phytonutrients and nutritional properties (Hemery et al. 2010; Izydorczyk et al. 2014). Hemery et al. (2011), reported that biochemical composition influenced particle charge, and was successfully employed to separate wheat aleurone from pericarp. Similarly, Izydorczyk et al. (2014) utilised information on beta-glucan rich barley fractions to obtain fiber rich fractions (FRF) using roller milling. Buhler also has developed patent for mechanical separation of commercial aleurone fractions from wheat (Bohm et al. 2011). However, for maize aleurone

layers, such techniques are not yet in commercial use, hence the need for more research to explore and develop mechanical or fractionation methods that would be employed at industrial level.

Knowledge of the contents of lutein, zeaxanthin, FA and other phenolic acids found in the grain fractions is essential. However, failure to understand the effects of processing on and bioaccessibility of these components weakens our assumption of their potential health benefits on human health. Processing is known to have both positive and negative effects (Fulcher and Rooney Duke 2002). Zielinski et al. (2001) studied the effect of hydrothermal processing on bioactive compounds in cereals and found that phenolic acid content increased by 200-300% whereas amounts of tocopherols, tocotrienols and glutathione decreased. Phenolic acids from maize bran (pericarp) were found to be slightly bioavailable (Zhao et al. 2005), however, the bioavailability of phenolic acids in maize aleurone layer is not known.

Recently, Calani et al. (2014) demonstrated that FA from inner part of wheat aleurone was more bioavailable compared with FA from outer part of aleurone. Zaupa et al. (2014) also demonstrated that phenolic acids, thiamine and niacin in inner part of durum wheat were highly bioaccessible compared to outer aleurone fractions. Similarly, higher levels of phenolic acids were bioaccessible in whole-grain bread and bran-rich breads than in white bread with SA being highly bioaccessible compared with FA (Hemery et al. 2010). Effects of processing on carotenoids bioaccessibility have also been reported (Hornero-Méndez and Mínguez-Mosquera 2007). Structure-chemical interactions have different effects during processing (Fulcher and Rooney Duke 2002) and food matrix or chemical components have been implicated as factors

affecting bioaccessibility (Reboul et al. 2006). Further studies are therefore needed to evaluate effect of processing and bioavailability of phenolic acids as well as carotenoids found in maize and barley aleurone layer.

8.2 Conclusion

Fluorescence intensity values may be used to predict differences in ferulic acid content across the grain which can be further confirmed by ferulic acid concentrations determined by HPLC. Thus, fluorescence intensity profiles are one of the possible approaches to be explored further for use in screening of FA content in cereal grains.

The results of this work contribute to our understanding of the distribution of phenolic acids, carotenoids and micronutrients in cereal grains and to further identify suitable grain fractions as food ingredients. Phenolic acids, carotenoids and micronutrients are not only unevenly distributed within the grain, their distribution in yellow maize, wheat, barley and oat varies widely. The study has demonstrated that apart from wheat aleurone layer, a commercialized food ingredient, the maize aleurone fraction has potential as functional food ingredients due to its high phenolic acid and carotenoid concentration. However, cereal aleurone fractions differ in phytochemical composition and concentration. In addition, barley pericarp and germ and yellow maize pericarp may also be considered. As previously reported, wheat aleurone is a rich source of both phenolic acids and micronutrients. However, this study has demonstrated that maize aleurone layer is also rich in phenolic acids and carotenoids and poor in the micronutrients (minerals and the B-vitamins) compared to wheat. Barley aleurone was comparatively higher in nutritional components compared to maize aleurone layer. Such information therefore may act as

a guide in the selection of grain fraction for use as food ingredients to enhance the functional and nutritional properties of grain-based food products.

Furthermore, the study demonstrated that an ultrasonic processing may be used to break cell walls and release aleurone cell contents and the structural identity of niacin, the most abundant B-vitamin in the aleurone layer, was confirmed. Structural information is important in confirming the identity of components of interest and in understanding their functionality.

8.3 Recommendations for further studies

Based on the study results, yellow maize aleurone layer is a potential candidate as an alternative functional food ingredient to the current commercialized wheat aleurone layer; however, further research is needed to:

1) Examine the effect of incorporating corn aleurone layer in grain-based food products on sensory characteristics and processing on phenolic acids and carotenoids content in such food products.
2) Assess bioaccessibility and bioavailability of these phenolic acids, carotenoids and micronutrients in processed products such as muffins, cookies and baked cereal snack products to which corn aleurone layer has been incorporated.
3) Explore fractionation methods that would be employed at industrial level to separate corn aleurone layer.

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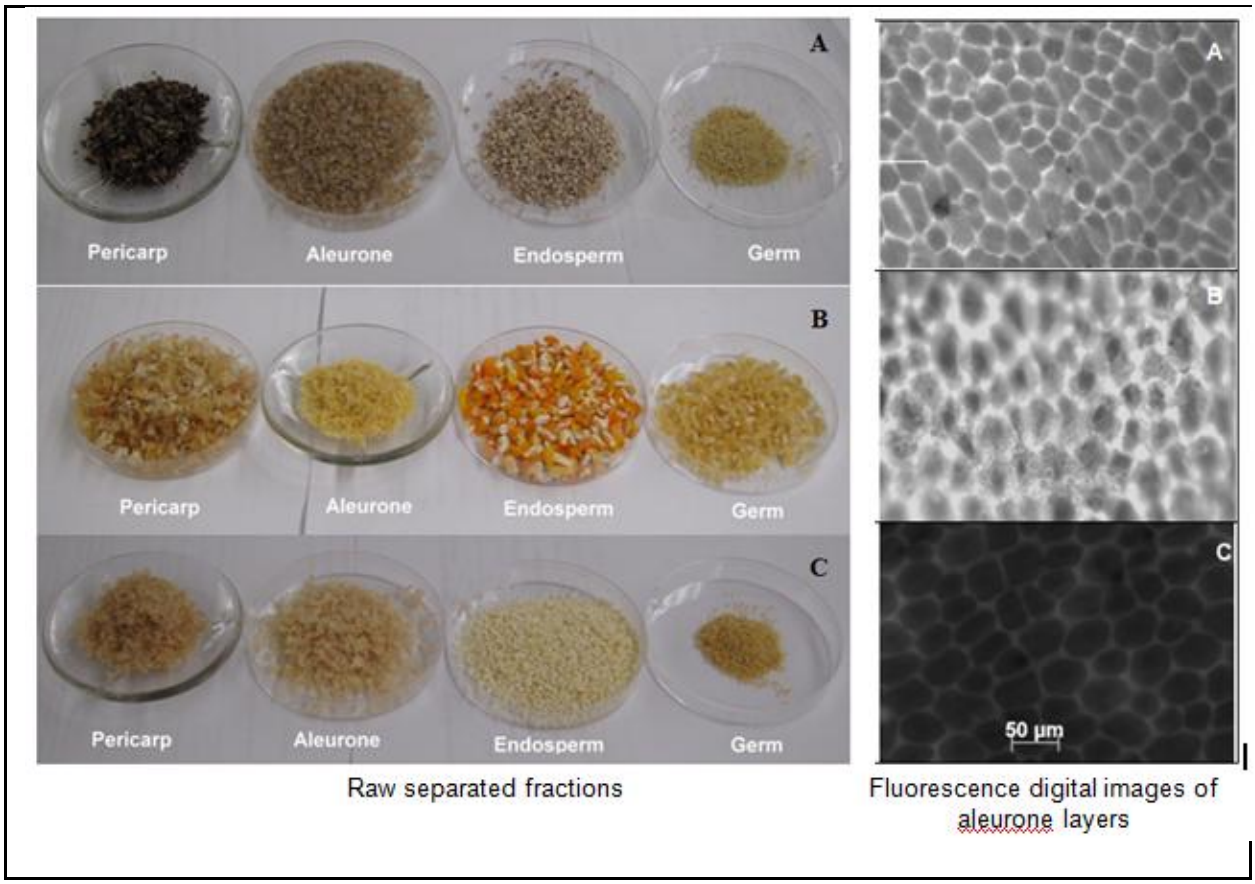
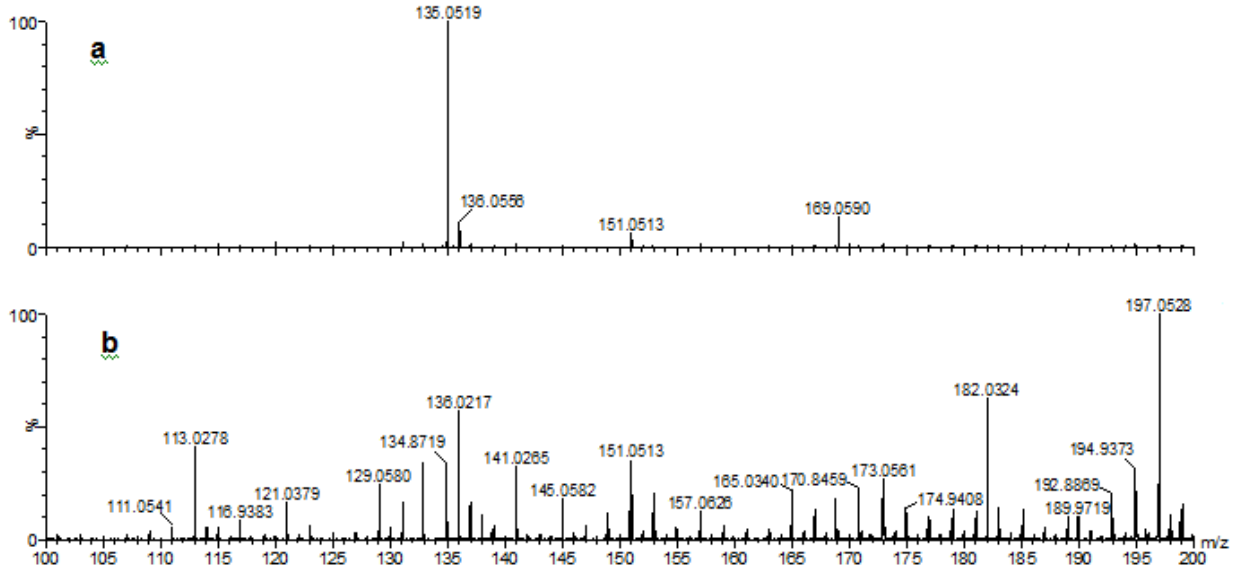
Appendix I



Supplementary Figure A. Raw hand dissected fraction (pericarp, aleurone, endosperm and germ) and fluorescence digital images of purple barley (A), Dasca maize (B) and Ambassador wheat (C)

Appendix II



Supplementary Figure B LC-MS spectra of wheat germ (a) for unknown peak (y) and *(b)* MS/MS spectra for syringic acid (peak 6)