

**Antioxidant Properties of Spaghetti and Infant Cereals and Characterization of Major
Phenolic Compounds by LC/MS Analysis**

By

Rhanissa Hirawan

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ABSTRACT

Consumption of whole grain is linked to reduced risk of chronic diseases including cancer, cardiovascular diseases and diabetes. This is due to the concentrated amount of vitamins, minerals, natural antioxidants and dietary fibre in the outer layers of the grains. There are options regarding consuming grain products and thus consumers need to be informed on the antioxidant properties of one of the common products, pasta. The present study investigated the antioxidant properties of ten samples of commercially available regular- and whole- wheat spaghetti. The antioxidant properties comprise the total phenolic content (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, oxygen radical absorbance capacity (ORAC). The contents of purported natural antioxidants ferulic acid, C-glycosyl flavones and secoisolariciresinol (SDG) were measured by using LC/MS techniques. The TPC of raw and cooked spaghetti were compared. Whole wheat spaghetti showed significantly higher TPC and ORAC. The contents of ferulic acid, C-glycosyl flavones and SDG were significantly higher in whole wheat spaghetti. The TPC was found to decrease 48 – 78% after cooking. The results showed that health-promoting phytochemicals are concentrated in the outer layers of the grain and thus consumption of whole grain is strongly recommended.

Another common grain product is infant cereals. Oxidation damage at the cellular level can also happen in infants. Thus, a balance between oxidants and natural or dietary antioxidants needs to be maintained. Data on the antioxidant properties of commercially available infant cereals are needed. There is also the possibility of improving the antioxidant properties by using pigmented grains in the production of infant cereals. The present study compared the antioxidant properties of commercial infant cereals. Purple wheat and red rice were then utilized to produce

home-made and lab-made infant cereals. Lab-made infant cereals represent the industrial processing method. Pigmented infant cereals showed higher TPC, total anthocyanin content (TAC) and ORAC than the control brown rice and commercial infant cereals. Anthocyanins were found only in purple wheat infant cereals while a C-glycosyl flavone was found at higher levels in purple wheat infant cereals than in red rice infant cereals. Home-made processed infant cereal did not show significant differences in TPC, TAC and ORAC with lab-made ones; however, significant differences were found in the contents of anthocyanins and C-glycosyl flavone. Also, home-made purple wheat infant cereals showed higher cellular antioxidant activity (CAA) than lab-made purple wheat, red rice and commercial infant cereals. Whole purple wheat holds potential to improve the antioxidant properties of infant cereals.

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TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	vi
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF ABBREVIATIONS	xii

CHAPTER 1: Literature review

1.1. Introduction	1
1.2. Spaghetti	2
1.2.1. Wheat in general	2
1.2.2. Durum wheat history and milling	4
1.2.3. Pasta processing	5
1.3. Infant cereal	6
1.3.1. Cereal ingredients	6
1.3.1.1. Purple wheat and red rice	7
1.3.2. Non-cereal ingredients	8
1.3.3. Infant cereal processing	11
1.3.4. Introduction stage	12
1.3.5. Antioxidant protection in infants	13
1.4. Antioxidants in cereal grains as the main ingredients	14
1.4.1. Antioxidants in general	14
1.4.2. Antioxidant polyphenols	15
1.4.2.1. Phenolic acids: ferulic acid and other cinnamic acids	16
1.4.2.2. Flavonoids	17
1.4.2.2.1. C-glycosyl flavones	18
1.4.2.2.2. Anthocyanins	18
1.4.2.3. Lignans: Secoisolariciresinol diglucoside (SDG)	19
1.4.2.4. Other antioxidant polyphenols in rice	20
1.4.3. Epidemiological studies of cereal grain health benefits	21
1.4.4. Antioxidant polyphenols in cereal grains	21
1.4.4.1. Antioxidant polyphenols in durum wheat	22
1.4.5. Antioxidant polyphenols in pigmented cereal grains	22
1.5. <i>In vitro</i> , <i>in vivo</i> studies and LC/MS analysis of red rice and purple wheat	23
1.5.1. Red rice	23
1.5.1.1. Pigment components of red rice	23
1.5.1.2. <i>In vitro</i> studies of red rice	23
1.5.1.3. <i>In vivo</i> studies of red rice	24

1.5.2. Purple wheat	24
1.5.2.1. Pigment components of purple wheat	24
1.5.2.2. In vitro studies of purple wheat	25
1.6. Cell culture assay	25
CHAPTER 2: Antioxidant properties of commercial regular and whole-wheat spaghetti and LC/MS analysis of their C-glycosyl flavones and secoisolariciresinol diglucoside	
ABSTRACT	28
2.1. Introduction	29
2.2. Materials and Methods	32
2.2.1. Samples	32
2.2.2. Chemicals	35
2.2.3. Sample preparation	35
2.2.4. Moisture content determination	35
2.2.5. Extraction	36
2.2.5.1. Acidified ethanol and methanol extraction	36
2.2.5.2. Alkaline hydrolysis	36
2.2.5.3. Aqueous acetone extraction	37
2.2.5.4. Acid hydrolysis	38
2.2.6. Chemical model assays	38
2.2.6.1. Total Phenolic Content (TPC) determination	38
2.2.6.2. DPPH radical scavenging activity determination	39
2.2.6.3. Oxygen Radical Absorbance Capacity (ORAC) determination	39
2.2.7. HPLC and HPLC/MS/MS analyses	40
2.2.7.1. HPLC analysis for ferulic acid	41
2.2.7.2. HPLC/MS/MS analysis for C-glycosyl flavones	41
2.2.7.3. HPLC/MS/MS analysis for SDG	42
2.2.8. Statistical analysis	42
2.3. Results and discussion	43
2.3.1. TPC and antioxidant activity of uncooked, regular versus whole-wheat spaghetti	43
2.3.2. Effects of cooking on TPC of regular and whole-wheat spaghetti	47
2.3.3. Ferulic acid content of uncooked, regular versus whole-wheat spaghetti	50
2.3.4. C-glycosyl flavones contents of uncooked, regular versus whole-wheat spaghetti	54
2.3.5. SDG contents of uncooked, regular versus whole-wheat spaghetti	61
2.4. Conclusion	65
CHAPTER 3: Antioxidant potential of infant cereals produced from pigmented grains in comparison to commercial infant cereals and LC/MS analysis of their anthocyanins	
ABSTRACT	66

3.1. Introduction	67
3.2. Materials and methods	69
3.2.1. Samples	69
3.2.2. Chemicals	73
3.2.3. Sample preparation	73
3.2.4. Moisture content determination	76
3.2.5. Extraction	76
3.2.6. Chemical model assay	78
3.2.6.1. Total Phenolic Content (TPC) determination	78
3.2.6.2. Total Anthocyanin Content (TAC) determination	78
3.2.6.3. Oxygen Radical Absorbance Capacity (ORAC) determination	79
3.2.7. HPLC/MS/MS analysis	79
3.2.8. Cell culture assay	80
3.2.8.1. Cell line	80
3.2.8.2. Cytotoxicity assay	81
3.2.8.3. Dichlorofluorescein diacetate (DCFH-DA) assay	82
3.2.9. Statistical analysis	83
3.3. Results and discussion	83
3.3.1. Home-made and lab-made pigmented grain infant cereal production	83
3.3.2. TPC, TAC and antioxidant activity of uncooked grains, home-made, lab-made and commercial infant cereals	84
3.3.3. HPLC/MS/MS analysis of major peak components in acidified methanol extracts of uncooked grains, home-made and lab-made infant cereals	89
3.3.3.1. Identification	89
3.3.3.2. Quantification	98
3.3.4. Cell culture assay of home-made, lab-made whole purple wheat, unpolished red rice and a commercial infant cereal	103
3.3.4.1. Cytotoxicity assay	103
3.3.4.2. Dichlorofluorescein diacetate (DCFH-DA) assay	105
3.4. Conclusion	109
GENERAL CONCLUSIONS	110
REFERENCES	111

LIST OF TABLES

Table 1.1. Introduction stage and ingredient list of commercial infant cereal samples	10
Table 2.1. TPC, DPPH radical scavenging activity and oxygen radical absorbance capacity of uncooked regular pasta from different brands	46
Table 2.2. TPC, DPPH radical scavenging activity and oxygen radical absorbance capacity of uncooked whole-wheat pasta from different brands	46
Table 2.3. Total phenolic content of uncooked vs. cooked regular pasta from different brands	49
Table 2.4. Ferulic acid content of uncooked regular and whole-wheat pasta	52
Table 2.5. Flavonoid glycosides and SDG contents of regular spaghetti from different brands	60
Table 2.6. Flavonoid glycosides and SDG contents of whole-wheat spaghetti from different brands	60
Table 3.1. Total Phenolic Content (TPC), Total Anthocyanin Content (TAC) and Oxygen Radical Absorbance Capacity (ORAC) of uncooked grains, home-made, lab-made pigmented grain infant cereals and commercial infant cereals	86
Table 3.2. MS/MS identification of five major peaks in acidified methanol extracts of whole purple wheat and unpolished red rice uncooked grains	91
Table 3.3. HPLC quantification of major peaks in acidified methanol extracts of whole purple wheat grain and unpolished red rice uncooked grains, home-made and lab-made infant cereals	100

LIST OF FIGURES

Figure 2.1.	Regular spaghetti (left) compared to whole wheat spaghetti (right).....	34
Figure 2.2.	(a) HPLC chromatograph for ferulic acid standard measured at 320 nm. (b) HPLC chromatograph showing the dominant ferulic acid for No Name regular spaghetti measure at 320 nm. (c) HPLC chromatograph showing the dominant ferulic acid for Primo whole-wheat spaghetti measured at 320 nm.....	53
Figure 2.3.	(a) Full LC chromatogram (0-50 min) of 6-C-glucosyl-8-C-arabinosyl and sinapic acid ester of apigenin-C-diglycosides obtained at 333 nm in whole-wheat spaghetti, PC brand; Extracted ion chromatogram (EIC) of sinapic acid ester of apigenin-C-diglycosides ions at $m/z = 769$ (b) and 6-C-glucosyl-8-C-arabinosyl ions at $m/z = 563$ (c) for whole-wheat spaghetti, PC brand.....	55
Figure 2.4.	MS/MS spectrum of hexosyl-pentosyl apigenin (6-C-glucosyl-8-C-arabinosyl apigenin) in whole-wheat spaghetti, PC brand ($t_R = 26.15$ min).....	56
Figure 2.5.	MS/MS spectrum of sinapic acid ester of apigenin-C-diglycosides in whole-wheat spaghetti, PC brand ($t_R = 31.32$ min).....	57
Figure 2.6.	(a) Full LC chromatogram (0-35 min) of SDG obtained at 280 nm in control spaghetti, Catelli Smart; Extracted ion chromatogram (EIC) of SDG, ions at $m/z = 686$ (b) for control spaghetti, Catelli Smart.....	62
Figure 2.7.	MS/MS spectrum of SDG in Catelli Smart.....	63
Figure 3.1.	Uncooked grains (from left to right: brown grains, partially polished red rice, unpolished red rice and whole purple wheat).....	72
Figure 3.2.	Home-made (left) and lab-made (right) unpolished red rice infant cereals.....	72
Figure 3.3.	Rehydrated infant cereals samples after final freeze-drying process. LBR: lab-made unpolished brown rice. LRR: lab-made partially polished red rice. LRC: lab-made unpolished red rice. B1: Beech Nut, Stage 1. N2: Nestle, Stage 2. O1: Organics for Baby, Stage 1.....	72
Figure 3.4.	Home-made infant cereal production procedure: a. ground cereal; b. dispersion in water; c. heating and stirring; d. storage for analysis.....	75
Figure 3.5.	Lab-made infant cereal production procedure: a. ground cereal; b. toasting; c. dispersion in water; d. enzymatic hydrolysis; e. hot-plate boiling; f. storage (Gil et al., 1991,4).....	76
Figure 3.6.	LC chromatograms (0 – 35 min) of acidified methanol extracts obtained at (a) 280 nm, (b) 320 nm and (c) 520 nm in uncooked whole purple wheat grain with its five major peaks (W1 – W5); at (d) 280 nm and (e) 320 nm in uncooked unpolished red rice grain with its five major peaks (R1 – R5).....	90
Figure 3.7.	EIC of peak W1 with $m/z = 447$ (a) and its MS/MS spectra with its retention time (b) in uncooked whole purple wheat grain.....	93
Figure 3.8.	LC chromatogram of cyanidin 3-O-glucoside standard peak at 16.47 min at 520 nm (a) and MS/MS spectrum of the standard peak with $m/z = 465$ and its retention time (b).....	94
Figure 3.9.	EIC of peak R4 (a) and W3 (b) with $m/z = 563$ and their retention times in uncooked unpolished red rice and whole purple wheat grains, respectively.....	96
Figure 3.10.	MS/MS spectra of peak R4 (a) and W3 (b) with $m/z = 563$ at their retention times in uncooked unpolished red rice and whole purple wheat grains, respectively (top	

figure). Bottom figures are highlights of areas in spectra in the top figure (A, B, C and D) showing the fragment ions of peak R4 and W3..... 97

- Figure 3.11.** The cytotoxicity of home-made whole purple wheat (HPW), lab-made whole purple wheat (LPW), home-made unpolished red rice (HRC), lab-made unpolished red rice (LRC) infant cereals and commercial sample of Heinz Stage 3 (Wheat and Oat Cereal with Banana Raspberry) at concentrations 2 mg/mL and 0.5 mg/mL in confluent FHs 74 Int (normal fetal small intestine cell line). Control was untreated cells at 100% cell viability. Data represent mean $\pm$ SD, n = 8.....104
- Figure 3.12.** AAPH-induced free radical oxidation of DCFH to DCF is represented in fluorescence units by positive (+) control over time. Negative (-) control shows cell conditions without addition of AAPH over time. The treatment by home-made whole purple wheat (HPW), lab-made whole purple wheat (LPW), home-made unpolished red rice (HRC), lab-made unpolished red rice (LRC), commercial Heinz Stage 3 (H3) infant cereals at two difference concentrations; 2 mg/ml (2) and 0.5 mg/mL (0.5) shows fluorescence intensity values in-between the control values.....106
- Figure 3.13.** CAA values of home-made whole purple wheat (HPW), lab-made whole purple wheat (LPW), home-made unpolished red rice (HRC), lab-made unpolished red rice (LRC) and commercial Heinz Stage 3 (H3) infant cereals at different concentrations; 2 mg/mL (2) and 0.5 mg/mL (0.5) (mean $\pm$ SD, n = 8). Different letters indicate significant difference ($p < 0.05$)..... 108

LIST OF ABBREVIATIONS

AAPH	2,2'-azobis (2-amino-propane) dihydrochloride
AUC	Area under the curve
B1	Commercial BeechNut Stage 1 infant cereal
CAA	Cellular antioxidant activity
DAD	Diode array detection
DCF	Dichlorofluorescein
DCFH-DA	Dichlorofluorescein diacetate
DPPH	2,2'-diphenyl-1-picrylhydrazyl
EIC	Extracted ion chromatogram
FL	Fluorescein
H3	Commercial Heinz Stage 3 infant cereal
HPLC	High performance liquid chromatography
HPW	Home-made whole purple wheat infant cereal
HRC	Home-made unpolished red rice infant cereal
LC-MS	Liquid chromatography – mass spectrometry
LBR	Lab-made unpolished brown rice infant cereal
LPW	Lab-made whole purple wheat infant cereal
LRC	Lab-made unpolished red rice infant cereal
LRR	Lab-made partially polished red rice infant cereal
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
N2	Commercial Nestle Stage 2 infant cereal
O1	Commercial Organics for Baby Stage 1 infant cereal
ORAC	Oxygen radical absorbance capacity
Q-TOF	Quadrupole time-of-flight
TAC	Total anthocyanin content
TIC	Total ion chromatogram
TPC	Total phenolic content

CHAPTER 1: Literature review

1.1. Introduction

Cereal grains are staple food in almost all parts of the world with wheat and rice as the most important food grains (USDA, 2010). Consumption of whole grains has been long associated with positive health effects against chronic diseases including cancer, cardiovascular disease, diabetes and obesity (Anderson, 2003; Chatenoud et al., 1998; Jacobs et al., 1998a; Jacobs et al., 1998b; Jacobs et al., 2001; Kasum et al., 2002; Munter et al., 2007; Pauline and Rimm, 2003; Slavin, 2000; Slavin, 2003; Slavin et al., 2001; Soler et al., 2001). However, consumption of whole grain still falls short of the recommended guidelines of three daily servings as shown in surveys in Minnesota, Minneapolis, U.S. (Larson et al., 2010) and in the U.S. (Mancino and Buzby, 2005).

Whole-grain, whole-wheat and novel grain products including spaghetti pasta products have been introduced to answer to these health-demands. However, there is a need for more evidence on health benefits of whole grain including their antioxidant properties in order to increase the daily intake. Higher consumption of dietary antioxidants is associated with decreased risk of chronic diseases (Halliwell and Whiteman, 2004). Natural antioxidants are present in wheat (Adom and Liu, 2002; Naczki and Shahidi, 2006) including durum wheat used in pasta making (Adom et al., 2003; Esposito et al., 2005; Onyencho and Hettiarachchy, 1992). Grain processing and final cooking stages influence the nutritional and antioxidant levels (Abdel-Rahman, 1982; Li et al., 2007b; Lintas, 1998a). Thus, the first objective of the present study was to conduct a comparative study of the antioxidant properties of commercially available regular and whole-wheat spaghetti and also to investigate the effect of cooking on antioxidant levels.

Whole grains are also used to manufacture infant cereals. Infancy is an important stage where one grows most rapidly and oxidation at cellular level occurs due to internal and external sources (Almaas et al., 1997; Friel et al., 2004; Rosenfeld and Davis, 1998; Saugstad, 2001). Antioxidant properties of infant cereals can be improved by the incorporation of pigmented grains that contain anthocyanins in addition to non-pigmented phytochemicals (Abdel-Aal et al., 2006; Cooke et al., 2005; Goffman and Bergman, 2004; Li et al., 2005; Nam et al., 2006; Toyokuni et al., 2002). However, the literature lacks the evidence on the antioxidant properties of infant cereals made from pigmented grains especially using an assay which is more biologically-relevant. Thus, the second objective of this study was to compare the antioxidant properties of pigmented infant cereal prototypes and commercially available infant cereals and also to investigate the different processing effects on antioxidant properties.

1.2. Spaghetti

1.2.1. Wheat in general

Wheat, rice and corn are the most important grains produced worldwide. However, wheat and rice are the most important food grains as corn is mainly used as feed except in Latin America, part of Asia and Africa (USDA, 2010). Evolution of wild grasses found growing in the Eastern Mediterranean areas where climate conditions of winter rains, dry summer and humid fall and spring exist resulted in wheat (Bozzini, 1988). Wheat production spread to the Americas, Europe, Australia, New Zealand, Israel and South Africa and then to the rest of the world. The genus *Triticum* covers all wheats, the types of which include common wheat (used for bread making), durum wheat (pasta) and club wheat (cookies) (Bozzini, 1988).

The wheat kernel structure consists of bran including pericarp and seed coats (14 to 16% of total kernel weight), endosperm including aleurone (81 to 84%) and germ (2 to 3%) (Jones and Ziegler, 1964). The bran in milling includes aleurone while aleurone is part of the endosperm botanically (Hoseney, 1994). Wheat kernels are normally ground to produce refined wheat flour. Refined wheat flour contains most of the starchy endosperm with minor amount of pericarp, seed coats and aleurone layers. The endosperm provides mainly starch (65% to 71%) (Bechtel et al., 1964) and protein (5% to 16%) (Pence et al., 1964). The outer layers are concentrated in minerals, dietary fibres and antioxidant phytochemicals while the germ is concentrated in vitamins B and E (Hoseney, 1994). Consuming the endosperm combined with the outer layers has been found to provide longer satiety than eating the endosperm alone (Slavin, 2003). In Canada, the outer layers remain in “whole-wheat” products, however, the germ part is removed to ensure good shelf-life period as opposed to “whole-grain” products (Food and Drugs Regulation, 2010; Health Canada, 2007).

The chemical composition of wheat including different protein contents results in a variety of wheat end-products. Wheat gluten protein influences the resulting taste and texture of the final products (Kadan et al., 2001). Other chemical characteristics such as (yellow) pigment components and starch content also dictate the end-product type (Bushuk, 1998). Bread and cake products require whiteness while pasta products require yellowness (Zhang and Dubcovsky, 2008). Components leading to the yellowness are xanthophyll (α -dihydroxy carotene or oxidized carotene, particularly lutein and zeaxanthin as the major ones in durum wheat), carotenes, flavones (tricin found in Khapli and Marquis wheats), isomerisation products of carotenoids and decomposition products of chlorophyll (both products have yellow-brownish colour) (Lepage and Sims, 1968; Mares and Campbell, 2001; Parker and Harris, 1964). Most of these pigment

components are found in the germ, followed by endosperm and bran while durum has higher pigment contents than common wheats (Adom et al., 2005; Parker and Harris, 1964; Siebenhandl, 2007). Pigmented varieties have additional components, particularly anthocyanins contributing to the intense colour of the grains (Abdel-Aal and Hucl, 1999 Abdel-Aal and Hucl, 2003; Abdel-Aal et al., 2006; Hossenian et al., 2008; Hu et al., 2007 and Liu et al., 2010).

1.2.2. Durum wheat history and milling

Wheat genomic history is divided into three ploid (basic number of chromosomes making up a genome)-levels which are diploid, tetraploid and hexaploid levels (Bozzini, 1988). The earliest type was diploid wheats (AA and BB) and hybridization resulted in tetraploid wheat (AABB) which includes *Triticum durum* or durum wheat. Hybridization of tetraploid and another diploid wheat (DD) resulted in hexaploid wheats (AABBDD) which include *Triticum aestivum* ssp. *vulgare* or common wheat and *Triticum aestivum* ssp. *compactum* or club wheat. Historical recordings further show that pasta might have had evolved from unleavened bread made from durum wheat that served as food for the ships of Genoa, Italia (Bizzarri and Morelli, 1988). Wheat first entered Canadian agriculture through French colonization in eastern Canada in the early 17th century and durum wheat was first introduced to western Canada, particularly Manitoba due to the severe rust epidemic affecting hard red spring wheat while durum varieties were resistant to the rust epidemic (Matsuo, 1988).

The durum wheat milling process includes separation of wheat kernels from other foreign materials including stones, dampening and tempering of wheat kernels prior to grinding, threshing of hulls that are free-threshing (the hulls separates cleanly), reduction to proper granule size, and cleaning processes to remove remaining soil and chaff that ultimately result in refined

semolina (Bizzari and Morelli, 1988). Semolina requires less water than flour which makes the drying operation more cost and time-efficient (Irvine, 1964). Semolina has texture stability during boiling and soaking and also higher degree of fluidity and lower degree of elasticity compared with bread wheat flour (Bushuk, 1998).

1.2.3. Pasta processing

Some sources suggested the invention of pasta was done by the Chinese and pasta was brought into Italy by Marco Polo; however, other sources denote the Ancient Roman times as the origin of pasta (Baroni, 1988). Four basic shapes of pasta include long pasta (e.g., spaghetti), short or cut pasta (e.g., macaroni), bologna-type pasta (e.g., ribbon pasta), and lastly, “nests” and “skeins” (Baroni, 1988). Pasta drying is an important step where stability and preservability (elimination of bacterial pathogens) are created by removing moisture in the shortest amount of time possible with the greatest care to preserve cooking qualities and flavour (Guler et al., 2002). Steps in the production of whole-wheat or whole-grain pasta are similar to refined pasta although raw materials (whole durum wheat semolina versus refined semolina) differ. Irvine (1964) and Baroni (1988) explained automated processing of pasta consisting of the following steps:

1. Selection of raw materials (durum wheat semolina and water) and mixing them together to form a dough.
2. Passing the dough through a vacuum chamber where all air bubbles are removed and then into kneading worm.
3. Passing the dough through an extrusion head with the desired die.
4. Forcing the product out of the extruder, where it will be surface-dried using hot air blast to harden the surface and thus maintain the shape.
5. Cutting into desired length and hanging using drying rods.
6. Exposure to preliminary and final drying processes which used to take about 15 to 36 hr a decade ago (40 – 60°C) but nowadays takes about 8 to 14 hours (above 60°C).

7. Cooling, collection of final product, packaging and storing at room temperature.

Semolina processing, pasta storage and cooking all influence sensory and nutritional qualities. Most of the B vitamins likely survive processing steps including drying and storage while a significant loss of vitamin E occurs during milling, extrusion and drying (Lintas, 1988a). Vitamins generally tend to be lost due to leaching and degradation during boiling while losses of minerals in cooking water tend to be less (Abdel-Rahman, 1982).

1.3. Infant cereal

1.3.1. Cereal ingredients

Infant cereals comprise either single cereal such as wheat, oat, rice or sorghum, or mixtures of cereals. The composition of infant cereals depends on the introduction stage. Organic whole grain infant cereals are available commercially, for example, organic brown rice infant cereal by Organics for Baby (Lucerne Foods, Calgary, AB). Studies showed higher antioxidant activities of pigmented cereal grains due to the presence of additional components contributing to the pigments (Abdel-Aal et al., 2006; Cooke et al., 2005; Goffman and Bergman, 2004; Li et al., 2005; Nam et al., 2006; Toyokuni et al., 2002). One example of pigmented infant cereals is red rice infant cereal which is available commercially in Indonesia. The pigmented infant cereals are usually home-made with partially polished red rice and white rice as the ingredients. The latter is used to improve taste and flavour profiles.

Phenolic compounds are concentrated in the pericarp layer of grains. They are associated not only with antioxidant activity (Li et al., 2010) but also with astringency, bitter and sour tastes influencing the overall sensory characteristics (Brannan et al., 2001). For example, My Organic Barley Baby Cereal by My Organic Baby, Inc., Canada which had the highest antioxidant activity also scored lowest in aroma quality in comparison to breast milk samples (Li et al.,

2010). Pigment components in red rice might reduce the bioavailability of nutrients such as iron, protein and carbohydrates in human body; however, these issues are usually only associated with individuals with low nutritional levels (Sweeney et al., 2006). The increase in acceptance of a novel solid food occurs more often with breast-feeding cases compared with formula-feeding (Sullivan and Birch, 1994).

High anthocyanin blue and purple wheat are not available commercially. However, they can be made available locally based on the breeding techniques as found by Kniewel et al. in Saskatoon, Canada (2009). Red rice is available commercially. Red rice often exists as difficult weeds to the commercial white rice grown in southern United States because red rice and white rice have the similar genetic structures (Gealy et al., 2002). In other countries, red rice is known as a disease-resistant variety. It is often preferred for its distinctive taste, texture, aroma and appearance. However, rice actually yields higher in temperate areas including Japan, Europe, the United States and Australia compared to tropical areas such as Indonesia, Thailand, India and Philippines (Peng et al., 2004).

1.3.1.1. Purple wheat and red rice

Blue colour wheat originated in *Triticum boeoticum* (also called Einkorn wheat) while purple colour wheat originated in tetraploid durum (*Triticum dicoccum*). Both colour traits infiltrated the common spring wheat gene pool with a long-term process of introgression (Zeven, 1991). A team of scientists from the Crop Development Centre (Saskatoon, Saskatchewan, Canada), Agriculture and Agri-Food Canada (Guelph, Ontario, Canada) and Tohoku National Agricultural Experiment Station (Morioka, Iwate, Japan) studied the possibility of breeding the blue and/or purple pigments into the common spring wheat such that these pigments would be

concentrated in the aleurone (blue pigments) and pericarp (purple pigments, mainly cyanidin 3-glucoside and peonidin 3-glucoside) (Knievel et al., 2009).

Rice varieties are divided into long, medium and short grain types associated with the different physical and chemical characteristics. Other divisions are based on market demands according to either different processes or whether they are aromatic or containing coloured bran (Moldenhauer et al., 2004). Red rice is becoming a unique health product on the market and at the same time, a constant weed problem in the rice fields. Red rice can belong to either *Oryza rufipogon* or *O. sativa ssp. indica* (rice plant producing the common white rice) (Vaughan et al., 2001). The rice grain consists of hull, bran (pericarp, testa, nucellus and aleurone) (10 – 15% of total kernel weight), endosperm (sub-aleurone and starchy endosperm) (82 – 89%) and germ (1 – 3%) (Champagne et al., 2004). The pigment components are located in the testa or in the pericarp of coloured rice (Sweeney et al., 2006). Similar to wheat, polishing produces commercial white rice by removing bran parts. The sub-aleurone or “polish”, germ and a little amount the starchy endosperm are also removed. Unpolished brown rice or pigmented rice is produced when only the hull is removed. The pigmented rice is usually partially polished. Brown rice or unpolished pigmented red rice often has short shelf life due to lipid enzymatic hydrolysis and oxidation during storage (Kim, 2004).

1.3.2. Non-cereal ingredients

Infant cereals often contain fruits found in dehydrated form, flakes or powder. Other common additional ingredients in infant cereals are prebiotics (inulin, oligofructose), iron, ascorbic acid, B vitamins, emulsifier (soybean or sunflower lecithin), whey protein concentrate, honey, plant oils (canola oil, coconut oil, soy oil, palm olein, olive oil, sunflower oil and

safflower oil), dry skim milk or whole milk powder or yogurt powder and beetroot powder for flavour and colour. Iron fortification of infant cereals requires further investigation as iron might act as a pro-oxidant factor if it is not fully absorbed by the baby's gut (Almaas et al., 1997; Fuchs et al., 1993; Michaelsen et al., 1995). Iron availability in infant cereals has been found to be low (Fuchs et al., 1993) and the presence of phytate in the cereals might partially contribute to this (Michaelsen et al., 1995). Supplemental electrolytic iron powder only has 5% absorption compared to ferrous sulphate (Formon, 1987). However, ferrous sulphate is not used in infant cereals due to discolouration, distribution problems of iron within the product and shortened shelf life (Rios et al., 1975). Ferrous sulphate is then used in infant formulas to meet infant needs (Rios et al., 1975). Four mg/l of iron in the form of ferrous sulphate in infant formula is sufficient for babies of 6 months of age and higher levels might cause negative effects (Lonnerdal and Hernell, 1994). One study found iron supplementation to be safe for breast-fed infants (Friel et al., 2003). Another form of iron, haem-iron (from meat, fish and chicken) is easier to be absorbed while these food materials also contain substances that enhance the absorption of non-haem iron (Rios et al., 1975). Table 1.1 shows commercially available infant cereals along with their ingredients.

Table 1.1. Introduction stage and ingredient list of commercial infant cereals

No.	Brand	Stage	Ingredients
1	BeechNut	1	Rice flour Tricalcium phosphate, inulin, fructooligosaccharides, sunflower oil, niacinamide, electrolytic iron, thiamine mononitrate, riboflavin
2	Heinz	1	Rice flour Dicalcium phosphate, safflower oil, soy lecithin, inulin, oligofructose, natural flavour, electrolytic iron, vitamins (niacinamide, riboflavin, thiamine mononitrate)
3	Heinz	2	Oat flour, wheat flour, rice flour Dicalcium phosphate, safflower oil, inulin, oligofructose, reduced iron, vitamins (niacinamide, riboflavin, thiamine mononitrate)
4	Heinz	3	Wheat flour, oat flour Dry skim milk, dried banana flakes, dicalcium phosphate, raspberry puree, safflower oil, black raspberry puree, soy lecithin, beetroot powder, natural flavour, inulin, oligofructose, electrolytic iron, vitamins (niacinamide, riboflavin, thiamine mononitrate)
5	Heinz	3*	Oat flour, rice flour, rye flour, wheat flour, tapioca flour Mango puree, dry skim milk, pineapple puree, pear paste, dicalcium phosphate, safflower oil, soy lecithin, inulin, mango flavour, oligofructose, electrolytic iron, vitamins (niacinamide, riboflavin, thiamine mononitrate), ascorbic acid
6	Milupa	3*	Cereal flours (wheat, rice, oat, barley, rye, corn, millet) Maltodextrin, skimmed milk powder, dehydrated fruits (apple, pear, banana, orange, apricot), vegetable oil (palm, canola, coconut, sunflower), demineralized whey powder, vitamins (niacin, vitamin B1, vitamin B2), iron powder, ascorbic acid
7	Nestle	2	Whole wheat flour, oat flour, barley flour, rye flour, corn, rice flour Dry skim milk, palm olein, prebiotics (oligofructose, inulin), canola oil, coconut oil, sunflower oil, mineral and vitamins (ferric pyrophosphate, nicotinamide, thiamine mononitrate, riboflavin), bifidobacterium lactis cultures
8	Organics for Baby	1	Organic brown rice Sunflower lecithin, iron, riboflavin (vitamin B2), thiamine mononitrate (vitamin B1)
9	PC Organics	3	Organic rice flour Organic apple flakes, organic pear flakes (organic pear, organic rice flour, sunflower lecithin), organic inulin, tricalcium phosphate, electrolytic iron, niacinamide, thiamine mononitrate, riboflavin

*These commercial stage 3 infant cereals were considered as stage 4 in the study based on their ingredients.

1.3.3. Infant cereal processing

Infant cereals can be home-made or industrial-made. Industrial infant cereals are commercially available cereals that have gone through certain processing steps. On the other hand, home-made ones are ground grains made into porridge. Luminal digestion of starch by salivary amylase in the mouth and pancreatic amylase in the stomach does not appear to be fully developed in infants (Mobassaleh et al., 1985). Conventionally, infant cereals are subjected to heat treatments including toasting, boiling, drying and also hydrolysis process to improve flavour and texture qualities, digestibility mainly of starch, safety and shelf life (Fernandez-Artigas et al., 2001). Infant cereal processing steps were described by Gil et al. (1991, 1994) as follows:

1. Selection of a cereal or a mixture of cereals and toasting at 120 – 150°C for a period of 15 – 90 minutes in toasters heated with oil in closed circuit.
2. Cooling to 20 – 40°C through a spiral conveyor with a double jacket for water circulation.
3. Dispersion up to 20 – 40% of solids in water, in a tank with stirring and a double jacket.
4. Heating to 35 – 60°C and a dispersion of an alpha-amylase enzyme (prepared in a small volume of water) is added. The hydrolysis reaction is maintained for 10 – 90 minutes. The concentration of the enzyme used is between 0.2 – 0.4 g/kg of cereals and preferably between 0.2 – 2.0 g/kg.
5. Another thermal treatment at 105 – 130°C for a period of 5 seconds to 2 minutes. The enzyme is inactivated and the gel formation of the non-hydrolyzed starches also takes place. This treatment is carried out in a tubular heat-exchanger with scraped walls from which a rotating drier is fed. A sheet of convenient thickness and dampness is obtained.
6. Sifting of the agglomerated portions of dry cereal dough, forwarding to the area of mixing and pacing where they are mixed only with sugar or with sugar and adapted milk. Vitamin and mineral complex and an aroma, for example vanilla, are added. Dehydrated fruit juice and whole fruit concentrates are added in cereals with fruits.

The heat treatments of toasting, boiling and drying improve flavour quality via Maillard reactions and reduce water content to a safe level (Nout, 1993). Toasting and boiling prepare

protein and starch polymers for hydrolysis in the later stage (Fernandez-Artigas et al., 2001). Enzymatic hydrolysis usually by α -amylase improves starch dispersibility in liquids and starch digestibility (Gil et al., 1991, 1994). It also increases sweetness by increasing glucose content and decreases syneresis effect (Gil et al., 1991, 1994).

1.3.4. Introduction stage

Breast-feeding especially if prolonged provides good nutritional and immunological benefits for infant health including protection against microorganism-caused diarrhoea (Megraud et al., 1990; Victoria et al., 1987). On the other hand, introduction to complementary foods including infant cereals might be crucial before infants reach 6 months of age due to the needs for complementary calories and protein (Harris et al., 1990; Waterlow, 1981). In the late 1900's, solid foods including cereals, strained fruits, vegetables, soups and meats are introduced at as early as 3 months of age (Cone, 1981). Recommended age of introduction for solid foods is 6 months as babies are then physiologically and developmentally ready (Cheung-cheung, 2010). Physiological improvement includes better functioning gut and kidney while developmental improvement includes ability of babies to sit upright and control their head movements (Cheung-cheung, 2010). A similar recommendation is stated by the American Academy of Pediatrics.

Infant cereal products are introduced at different stages. Four stages of introduction are commonly used. Beginner or stage 1 infant cereal is for starter cereal and introduced at 6 months of age. Stage 2, 3, and 4 are infant cereals introduced at age 6, 8 and 12 months or toddlers, respectively. Beginner or stage 1 infant cereals are usually simple, single finely ground grains, while stage 2 infant cereals are a mixture of a few different grains combined with fruits. Stage 3 infant cereals are a mixture of different grains that are less finely ground and might also come in

combination with fruits. Whole grains are mostly found in stage 3 infant cereals (8-month-old babies and older). However, whole grains are also used in some stage 1 infant cereals.

1.3.5. Antioxidant protection in infants

The infancy period (birth to one year-old) is the most important period of life as this is when a child grows most rapidly than any other life period. Thus it is crucial to provide a sufficient amount of required nutrients including proteins, carbohydrate, fats and additional nutrients including phytochemicals that can provide antioxidant protection in infants. There is an increasing evidence that exposure to such phytochemicals in infant diets at an early age might have long-term effects in later life (Lucas, 1998). Newborn infants are subjected to drastically greater exposure to oxygen at their birth compared with the intrauterine conditions (Saugstad, 2001). This exposure might cause free radical injuries that can lead to various pathogenesis conditions (Rosenfeld and Davis, 1998). It is suggested that the mother or the infant might benefit from additions of antioxidants in their diet (Friel et al., 2004). The oxidative stress-related conditions found (especially in premature infants) include necrotizing enterocolitis, bronchopulmonary dysplasia, carcinogenesis and retinopathy of prematurity (Almaas et al., 1997). Other exogenous sources of oxidants are lipid peroxidation in fresh human milk and infant formulas and environmental pollution (Almaas et al., 1997). Endogenous sources include mitochondrial electron transport chain, granulocytes, xanthine oxidase, arachidonic acid pathway and transition metals (Almaas et al., 1997). Newborns are generally lacking natural antioxidant defences (Rosenfeld and Davis, 1998). The balance between oxygen, other free radicals and antioxidant compounds derived naturally and through diet has to be maintained (Saugstad, 1998).

There is no ORAC value provided for infant cereals under the USDA compiled ORAC database (USDA, 2007). Antioxidant and also aroma data on nine commercially available infant cereals and six infant cereals with added breast milk were investigated by Li et al. (2010). Breast milk was used as a control. My Organic Barley Baby Cereal showed the highest antioxidant capacity and the lowest aroma quality compared with the control, breast milk (Li et al., 2010). Five commercial rice infant cereals were found to contain p-coumaric, ferulic and sinapic acid while the four commercial barley infant cereals contained caffeic, p-coumaric, ferulic and sinapic acid (Li et al., 2010).

1.4. Antioxidants in cereal grains as the main ingredient in infant cereals

1.4.1. Antioxidants in general

Free radicals and other reactive species including ROS (oxygen), RNS (nitrogen) and RCS (chlorine) have been associated with chronic diseases including cancer and cardiovascular diseases, age-related diseases and possibly even the aging process by causing oxidative stress and damage (Halliwell and Whiteman, 2004). Seifried et al. (2003) found that the interaction between free radicals and other reactive species and dietary antioxidants is likely dependent on the individual's health status and genetic susceptibilities.

Oxidation in human tissues may occur via oxidative enzymes (for example, cyclooxygenase) and non-enzymatic lipid autoxidation caused by radical chain reaction. Free radical ROS plays a role in human body development including signal transduction (Friel et al., 2004). Biological antioxidants can be enzymatic or non-enzymatic. Enzymatic antioxidants include superoxide dismutase while non-enzymatic antioxidants include antioxidant enzyme cofactor (e.g., selenium), oxidative enzyme inhibitors (e.g. aspirin), transition metal chelators

(e.g. phytic acid), and free radical scavengers (e.g. vitamin C and E) (Sies, 1997). Even though there is a wide scope of antioxidant mechanisms, free radical scavengers or radical chain reaction inhibitors are the most common (Huang, Ou & Prior, 2005).

1.4.2. Antioxidant polyphenols

Polyphenols, as natural antioxidants in plants, play an important role in the plant's growth, reproduction and defence against pathogens, parasites and predators including insect damage (Tan et al., 1991). Some polyphenols such as condensed tannins reduce the bioavailability of protein (Sweeney et al., 2006); however, their antioxidant activity including free-radical scavenging and metal-chelating activities emerge as one of the most intensively researched topics. Polyphenols exist naturally in a wide range from simple phenolic acids to highly polymerized forms. They may be found in conjugated form with one or more sugar residues esterified to their hydroxyl groups (Manach et al., 2004). The sugars (e.g. glucose and arabinose) can be monosaccharides, disaccharides or oligosaccharides (Manach et al., 2004). Other compounds bound to the polyphenols include carboxylic and organic acids, amines and lipids (Rice-Evans, 2001). They might also exist linked together such as ferulic acid dimer. Polyphenols are divided into phenolic acids, flavonoids, stilbenes and lignans (Manach et al., 2004). Phenolic acids include hydroxybenzoic acids such as gallic acid and protocatechuic acid and hydroxycinnamic acids such as *p*-coumaric acid, caffeic acid and ferulic acid (Natella et al., 1999). The cinnamic acid group covers *trans*-phenyl-3-propenoic acids with different group substitution on their ring (Clifford, 2000). Flavonoids are grouped based on the oxidation level of their central C ring and they include flavonols or flavones (which are 3-hydroxyflavone), flavanones, anthocyanidins and proanthocyanidin oligomers of the monomeric flavan-3-ols

(Rice-Evans, 2001; Schijlena et al., 2004). Resveratrol is a stilbene while an example of lignans is secoisolariciresinol which is commonly found in the form of secoisolariciresinol diglucoside in plants including cereals such as wheat and legumes (Qu et al., 2005).

1.4.2.1. Phenolic acids: ferulic acid and other cinnamic acids

Ferulic acid is a ubiquitous plant component which can be found as esters with monosaccharides, disaccharides, plant cell wall fibres, glycoproteins, lignin, and betacyanins (Smith and Hartley, 1983). Two physiological functions of ferulic acid in plants are first, as positive growth factor in plant cell elongation as ferulic acid crosslinks pentosan, arabinoxylans and hemicelluloses (Humberstone and Briggs, 2000). Second, ferulic acid is a germination inhibitor because its crosslinks reduces the cell solubility and thus hydrolytic degradation by endosperm enzymes is hindered (Tan et al., 1991). As an antioxidant, ferulic acid scavenges damaging radicals because its phenolic nucleus and extended side chain conjugation readily forms a resonance stabilized phenoxyl radical while it also suppresses radiation-induced oxidative reactions by absorbing UV (Graf, 1992). The antioxidant functions of ferulic acid in the human body have been related to its affinity to lipid substance (Castelluccio et al., 1996; Kikuzaki et al., 2002; Srinivasan et al., 2007). Esterification of ferulic acid in plant cell walls was investigated (Harris and Hartley, 1976). Bioavailability and dietary intake of ferulic acid were reported by several workers (Bourne and Rice-Evans, 1998; Kanskia et al., 2002; Zhao and Moghadasian, 2008). Levels of ferulic acid in durum wheat (Adom and Liu, 2002; Adom et al., 2003; Lempereur et al., 1997; Onyeneko and Hietiarachchy, 1992) and rice (Adom and Liu, 2002; Norton, 2005; Zhou et al., 2004) have also been reported.

1.4.2.2. Flavonoids

Flavonoids are pigment components (yellow or red or blue) found in nearly all plants. Their chemical structure is based on the backbone of 2-phenylchromen-4-one (2-phenyl-1-benzopyran-4-one). Their *in vitro* antioxidant activities are based on mechanisms including scavenging reactive oxygen species (ROS) (Ishige et al., 2000; Lin et al., 2002; Rice-Evans et al., 1996) and reactive nitrogen species (RNS) (Crespo et al., 2008; Kerry and Rice-Evans, 1999), chelating transition metal ions in a structure-dependant manner (Brown et al., 1998; Sugihara et al., 1999) and inhibiting lipid peroxidation and oxidation of low density lipoprotein (LDL) (de Whalley et al., 1990; Fuhrman and Aviram, 2001; Viana et al., 1996). Inhibition of LDL oxidation is associated with reduced risk of coronary heart disease (Boullier et al., 2001; Kita et al., 2001; Steinbrecher et al., 1990). Flavonoids act as antioxidants by donating an electron to an oxidant and this action depends on the reduction potentials of radicals and the accessibility of the radicals (Rice-Evans, 2001). *In vivo* studies of the antioxidant activity of flavonoids require an understanding of their metabolism within the human body. Earlier studies suggested that dietary flavonoid glycosides would first be hydrolysed by the digestive microflora before being absorbed in the large intestine (Griffiths and Barrow, 1972). Thus the small intestine did not seem to absorb dietary flavonoids (Griffiths and Barrow, 1972). However, recent findings reported the activity of β -glucosidase activity in the human small intestine and in the liver against flavonoid and isoflavonoid glycosides (Day et al., 1998). Another study found that quercetin glycosides were more readily absorbed than the aglycone form in ileostomy patients (Hollman et al., 1995). Also, there were different rates of absorption for different types of flavonoids (quercetin versus rutin) (Manach et al., 1997).

1.4.2.2.1. C-glycosyl flavones

Flavones can be found in the outer tissues of nearly all plants because their formation depends on the action of light (Wellmann, 1971). Even though flavones are yellow in colour, they are not major colouring agents in plants except when they are found in very high concentration complexed with metal (Herrmann, 1976). Examples of flavones are apigenin (4',5,7-trihydroxyflavone), luteolin (3',4',5,7-tetrahydroxyflavone), tangeritin (4',5,6,7,8-pentamethoxyflavone) and chrysin (5,6-dihydroxyflavone). C-glycosyl compounds of apigenin and luteolin occur predominantly in cereal crops and they are found in high concentration in their leaves (Herrmann, 1976). Apigenin has been reported as a chemopreventive agent in mice skin carcinogenesis (van Dross et al., 2003). Isovitexin (6-C-glycosyl apigenin) from rice hull demonstrates antioxidant activity (Ramarthnam et al., 1989).

1.4.2.2.2. Anthocyanins

Anthocyanins are glycosides of polyhydroxy 2-phenylbenzopyrylium or flavylium salts found in cereals, fruits and vegetables (Asen et al., 1972; Abdel-Aal and Hucl, 2003; Hrazdina et al., 1982; Stintzing and Carle, 2004). Anthocyanins in plants act as pigment components especially in colours of flower petals to attract pollinators, seed dispersal agents in seeds and fruits, deterrents against insect or other feeding, protection against UV light irradiation damage (Holton and Cornish, 1995). Anthocyanins showed *in vitro* antioxidant activity (Awika and Rooney, 2004; Stintzing and Carle, 2004; Velioglu et al., 1998; Wang et al., 1997). Anthocyanins extracted from flower petals have been reported to suppress the growth of cultured tumour cells (Kamei et al., 1995) while anthocyanins from red glutinous rice showed anti-tumour effect (Koide et al., 2009). Anthocyanins extracted from berries were found to inhibit oxidation

of human low-density lipoprotein and lecithin-liposome systems *in vitro* (Abuja et al., 1998; Satue-Gracia et al., 1997).

1.4.2.3. Lignans: Secoisolariciresinol diglucoside (SDG)

Lignans are estrogen-like chemicals found in plants. They are derived from phenylalanine via dimerization of substituted cinnamic alcohols (known as monolignols) to a dibenzylbutane skeleton (Lewis and Davin, 1994). These phytoestrogens can act as antioxidants (Dinelli et al., 2007; Kuhnle et al., 2009; Rios et al., 2002; Verma et al., 2009) and antitumour agents in cell culture (Qu et al., 2005). They are ubiquitous in plants including oilseeds, nuts, grains, legumes, berries and fruits. However, lignans are found in the highest concentration in the knots of Norway spruce and linseeds (flax seeds) (Smeds et al., 2007; Zhang and Xu, 2007) and sesame seeds (Fukuda et al., 1986; Smeds et al., 2007). Lignans have also been reported in cereals (Dinelli et al., 2007; Kuhnle et al., 2009; Meagher and Beecher, 2000; Penalvo et al., 2005; Qu et al., 2005; Smeds et al., 2007; Smeds et al., 2009; Verma et al., 2009). The most commonly found lignans are secoisolariciresinol in the glycoside form (secoisolariciresinol diglucoside or SDG), matairesinol, lariciresinol and pinoresinol (Liggins et al., 2000; Milder et al., 2005). SDG is metabolized to secoisolariciresinol, enterodiol and enterolactone in the human body (Heinonen et al., 2001; Prasad, 2000). Using a chemiluminescence assay, the highest antioxidant activity was found in secoisolariciresinol and enterodiol forms compared with SDG and vitamin E control (Prasad, 2000).

1.4.2.4. Other antioxidant polyphenols in rice

Rice contains many antioxidant polyphenolic components (Adom and Liu, 2002) including vitamin E (tocotrienols and tocopherol), oryzanols (Lloyd et al., 2000; Xu et al., 2001) all-*trans*- β -carotene in black/purple and red/brownish rice varieties from various Asian countries (Frei and Becker, 2005), carotenoids in the endosperm of golden (yellow) rice (Schaub et al., 2005) and condensed tannins in the pericarp of brown/red rice (Oki et al., 2002; Reddy et al., 1995; Sweeney et al., 2006). Vitamin E includes fat-soluble α -, β -, γ - and δ -tocopherols and α -, β -, γ - and δ -tocotrienols with α -tocopherol as the major one with the highest biological activity (Herrera and Barbas, 2001; Traber and Atkinson, 2007). The *in vitro* and *in vivo* antioxidant activities of vitamin E have been well documented (Burton and Traber, 1990; Rimm et al., 1993; Traber and Atkinson, 2007; van Acker et al., 1993; Wayner et al., 1987; Xu et al., 2001). Oryzanol was originally thought to be a single component but it is now known to be a mixture of steryl ferulates (Xu and Godber, 1999). Oryzanol may lower blood cholesterol (Rukmini and Raghuram, 1991; Wilkinson and Champagne, 2004) and possess antioxidant activity (Juliano et al., 2005; Wang et al., 2002; Xu and Godber, 2001; Xu et al., 2001). Carotenoids are tetraprenoid organic pigments found in plants and other photosynthetic organisms such as algae, fungus and bacteria. They absorb light energy for photosynthesis and protect chlorophyll from photodamage (Armstrong and Hearst, 1996). Carotenoids are divided into two groups: (1) xanthophylls which contain oxygen; (2) carotenes which contain only hydrocarbons and no oxygen (Simpson, 1983). The antioxidant activities of carotenoids have been reported (Miller et al., 1996; Stahl and Sies, 2003; Terao, 1989). Condensed tannins are water-soluble oligomeric chains of the monomeric flavan-3-ols (flavanols) such as catechin, galocatechin and their epimers (Rice-Evans, 2001;

Stevens et al., 2002). Proanthocyanidins have been reported to have antioxidant activities (Koga et al., 1999; Stevens et al., 2002).

1.4.3. Epidemiological studies of cereal grain health benefits and consumer awareness

An inverse relationship between the consumption of whole grain and grain-based products and risks of chronic diseases (cancer, cardiovascular disease, diabetes and obesity) has been observed in epidemiological studies and well documented in scientific reviews (Anderson, 2003; Chatenoud et al., 1998; Jacobs et al., 1998a; Jacobs et al., 1998b; Jacobs et al., 2001; Kasum et al., 2002; Munter et al., 2007; Pauline and Rimm, 2003; Slavin, 2000; Slavin, 2003; Slavin et al., 2001; Soler et al., 2001). Consumers are becoming aware of the association between antioxidants and cancer according to a survey in the continental United States (Toner, 2004). Consumer awareness of this link was at 54% in 2002 compared to 79% that believes in the association of calcium and osteoporosis. The latter link has been communicated to the public many times and for a longer period (Toner, 2004). Thus, antioxidant content information can enhance the purchase of certain products.

1.4.4. Antioxidant polyphenols in cereal grains

Phenolic acids in cereal grains exist as free, soluble conjugated and insoluble bound forms. The bound forms comprise 85% of total phenolic acids in corn, 75% in oats and wheat, 62% in rice (Adom and Liu, 2002) although the content of free forms have been the most frequently reported in the literature (Adom and Liu, 2002; Kahkonen et al., 1999; Li et al., 2007a; Liu, 2007; Sosulski et al., 1982). Adom and Liu (2002) found the bound forms of phytochemicals to be responsible for the total antioxidant activity in grains (90% in wheat, 87%

in corn, 71% in rice and 58% in oats) (Kim et al., 2006; Liyana-Pathirana and Shahidi, 2006; Naczk and Shahidi, 2006). The bound phytochemicals are capable of reaching the colon. They are part of cell wall materials that are difficult to digest and thus survive stomach and small intestinal digestion mainly to be digested by gut microflora.

1.4.4.1. Antioxidant polyphenols in durum wheat

Durum wheat bran extracts showed greater antioxidant activity compared with soy oil. Phenolic acids that partially contribute to the antioxidant activity were protocatechuic, *p*-hydroxybenzoic, gentisic, caffeic, vanillic, chlorogenic, syringic, *p*-coumaric and ferulic acids (Onyencho and Hettiarachchy, 1992). Esposito et al. (2005) found comparable antioxidant activity of durum wheat bran fractions to those of fruit and vegetable due to the presence of fibre-bound phenolic compounds. Adom et al. (2003) tested the antioxidant activity of 11 diverse wheat varieties including red and white spring durum and found total phenolic content (709.8 – 860.0 μmol gallic acid equivalent/100 g), total flavonoid content (105.8 – 141.8 μmol catechin equivalent/100 g) and also carotenoids (26.41 – 143.46 μg lutein/100 g, 8.70 – 27.08 μg zeaxanthin/100 g, 1.12 – 13.28 μg β -cryptoxanthin/100 g) which likely contribute to the antioxidant activity.

1.4.5. Antioxidant polyphenols in pigmented grains

Pigmented grains likely offer the benefits of higher phytochemical contents than the regular non-pigmented grains. Pigmented cereal crops are often chosen in crop rotation because they are more resistant against pathogens, parasites and predators including insects. Commercially available pigmented grains include red rice (in Indonesia) and purple corn or blue

corn (Tostitos Natural Blue Corn Tortilla Chips, Frito Lay Canada, Ontario, Canada). The antioxidant polyphenols, *in vitro* and *in vivo* studies of the two pigmented grains, red rice and purple wheat are discussed below.

1.5. *In vitro*, *in vivo* studies and LC/MS analysis of red rice and purple wheat

1.5.1. Red rice

1.5.1.1. Pigment components of red rice

Anthocyanin profiles in red rice are relatively simple and they were found mainly in the form of cyanidin 3 – glucoside (67%) followed by peonidin 3 – glucoside and cyanidin glucoside (Abdel-Aal et al., 2006; Hiemori et al., 2009; Hu et al., 2007; Hu et al., 2003; Ryu et al., 1998; Xia et al., 2006). Condensed tannins were also found in red rice (Min et al., 2009).

1.5.1.2. *In vitro* studies of red rice

Red rice was found to have significantly higher phenolic concentrations and antiradical efficiency compared with light-brown and brown rice (Finocchiaro et al., 2007; Goffman and Bergman, 2004). Phenolic concentrations correlate with the free radical scavenging activity (Choi et al., 2007; Goffman and Bergman, 2002). On the other hand, unmilled or partially – processed red and black rice from various South and South-East Asian countries were found to be able to contribute to increased β – carotene intake and provide sufficient amount of essential lipids for absorption and conversion to vitamin A (Frei and Becker, 2005). Pigmented red rice bran was shown to have greater inhibition action against lipid and membrane peroxidation and greater free radical scavenging than non-pigmented rice bran (Nam et al., 2006). Different types of thermal processing were found to effect anthocyanins of pigmented rice with pressure cooking

contributing the most adverse effects on the anthocyanin levels (Hiemori et al., 2009). The study found that protocatechuic acid was likely formed during thermal processing (Hiemori et al., 2009).

1.5.1.3. *In vivo* studies of red rice

Red rice was found to reduce atherosclerotic plaque formation in the aorta caused by hypercholesterolemia, improve serum and liver total antioxidant capacity, improve erythrocyte superoxide dismutase activity, and decrease oxidative stress by lowering liver reactive oxygen species and aortic malondialdehyde *in vivo* (in rabbits) (Ling et al., 2001; Ling et al., 2002). Red rice was found to provide greater protection against Fenton reaction-based tissue injury in rats compared with a white rice diet (Toyokuni et al., 2002). A red-anthocyanin pigmented rice diet increased life span of tumourous animals compared with normal rice or control diet (Cooke et al., 2005).

1.5.2. Purple wheat

1.5.2.1. Pigment components of purple wheat

Cyanidin 3-glucoside is the major anthocyanin in purple wheat followed by cyanidin 3-galactoside, peonidin 3-glucoside, peonidin malonylglucoside and pelargonidin 3-glucoside in purple wheat (Abdel-Aal and Hucl, 1999; Abdel-Aal and Hucl, 2003; Abdel-Aal et al., 2006; Hossenian et al., 2008; Hu et al., 2007). Cyanidin 3-glucoside, peonidin 3-glucoside and some trace amounts of cyanidin rutinoside and peonidin rutinoside were found in the pericarp of purple-seeded wheat (Dedio et al., 1972). Purple wheat pigments are located in the pericarp (Zeven, 1991). Purple wheat also contains anthocyanins, lignans and melatonin which likely

provide health benefits (Hossenian et al., 2008). Lutein and zeaxanthin were found in relatively high levels in wheat endosperm and anthocyanins were found in higher levels in the shorts of blue and purple wheat (Siebenhandl et al., 2007).

1.5.2.2. *In vitro* studies of purple wheat

Li et al. (2005) found that pigmented wheat varieties showed significantly higher DPPH radical scavenging activities compared to the non-pigmented cultivar. HPLC and pH differential methods showed significantly higher anthocyanin levels in heat stressed purple wheat than normal purple wheat (Hossenian et al., 2008). Purple wheat was also found to contain secoisolariciresinol diglucoside (SDG) (770 µg/kg) and melatonin that might contribute health benefits (Hossenian et al., 2008). Original total phenolic content (5.98 mg ferulic acid equivalent/g), ORAC (52.49 mg Trolox equivalent/g) and total anthocyanin content (1.155 mg cyanidin 3-glucoside equivalent/g) in purple wheat bran significantly decreased in heat-treated purple wheat bran and heat-treated purple wheat bran-enriched muffins while the DPPH radical scavenging activity remained the same (Li et al., 2007a). Anthocyanins in purple wheat were found to be completely degraded during brewing to produce antho-beer and thus more studies need to be done of the antioxidant activity of the residual structures after anthocyanin degradation (Li et al., 2007b).

1.6. Cell culture assay

The effect of food consumption on oxidative stress in cells has been investigated (Song et al., 2010; Wolfe and Liu, 2007; Wolfe and Liu, 2008; Wolfe et al., 2008). Chemical model assays describe *in vitro* antioxidant mechanisms including free radical scavenging mechanisms,

however, they do not consider human physiological conditions and the bioavailability and metabolism of the antioxidant components in human body (Liu and Finley, 2005). On the other hand, human and animal studies are costly especially for initial screening of antioxidant levels of the components in foods or dietary supplements (Liu and Finley, 2005).

Cell cultures were then introduced as part of antioxidant assays. Cell cultures can involve normal or cancerous cell lines. The food extract (sample) is added to the cell line as part of the treatment. A cytotoxicity assay is performed to check if the sample treatment (containing alleged antioxidant components) actually causes cell death (Ellis et al., 2010). The assay measures cell viability and proliferation. If cells are viable and proliferate that means cells metabolize actively. This process reduces the yellow MTT reagent (3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) resulting in intracellular purple formazan which is soluble and can be measured spectrophotometrically (MTT Cell Proliferation Assay, ATCC[®], Manassas, VA, USA). The sample absorbance is deducted from the absorbance of the blank containing medium only to eliminate the background absorbance. The sample absorbance is then compared with the absorbance of the control cells which are untreated cells. A lower absorbance than the control cells' indicates a reduced rate of cell proliferation.

The dichlorofluorescein diacetate (DCFH-DA) or cellular antioxidant activity (CAA) assay is the most commonly used method in antioxidant research to detect generalized cellular oxidative stress (Halliwell and Whiteman, 2004; Song et al., 2010; Wolfe and Liu, 2007; Wolfe and Liu, 2008; Wolfe et al., 2008). The following is the proposed principle of the assay (Wolfe and Liu, 2007):

1. Cells are pre-treated with antioxidant extract samples and the dye which is 2',7'-dichlorofluorescein diacetate (DCFH-DA)
 - a. Antioxidants bind to cell membrane and/or pass through into the cells

- b. DCFH-DA passes through the membrane into the cells, cellular esterases separate the DA moiety resulting in the more polar DCFH and this remains in the cells
2. Cells are then treated with AAPH
- a. AAPH passes through membrane into the cells
 - b. AAPH immediately produces peroxy radicals that attacks cell membrane lipids producing more radicals
 - c. AAPH oxidizes the intracellular DCFH to the fluorescent DCF, the absorbance of which can be measured at 527 nm and excitation wavelength of 485 nm

The presence of antioxidants is supposed to prevent the above events 2.b. and 2.c. which are the oxidation of DCFH and cell membrane lipids resulting in the less fluorescent DCF being formed (Wolfe and Liu, 2007).

Culturing of cells may also cause oxidative stress by facilitating the generation of reactive species and by blocking the adaptive cellular process of increasing the internal antioxidant levels (Halliwell and Whiteman, 2004). There is also the possibility of free radical reactions taking place in the culture media (Halliwell and Whiteman, 2004). Thus, the dye DCFH-DA is used at low concentrations and minimal light condition in order to prevent cytotoxicity and photochemical oxidation in the media (producing fluorescent DCF and this could be mistaken for reactive species generation by the cells) (Halliwell and Whiteman, 2004).

CHAPTER 2: Antioxidant properties of commercial regular and whole-wheat spaghetti and LC/MS analysis of their C-glycosyl flavones and secoisolariciresinol diglucoside*

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ABSTRACT

Whole grains contain more vitamins, minerals, natural antioxidants and dietary fibre than regular, refined grain products. Consumption of whole grain products is associated with beneficial health effects. Antioxidant properties of whole grain have been extensively studied as one of the mechanisms in providing these effects. However, the consumers are lacking data on the antioxidant properties of pasta in the market. The present investigation evaluated the antioxidant properties of ten samples of commercially available regular- and whole- wheat spaghetti. The methods employed were total phenolic content (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, oxygen radical absorbance capacity (ORAC). Ferulic acid, flavonoid glycosides and the lignan diglucoside, secoisolariciresinol diglucoside (SDG) contents as contributors to the health promoting properties of whole-wheat spaghetti, were investigated using LC/MS techniques. The effects of cooking on the antioxidant properties of spaghetti were also studied.

Whole wheat spaghetti exhibited significantly higher levels of total phenolic content (1389 µg/g) than regular wheat spaghetti (865 µg/g); however, TPC in both regular and whole wheat spaghetti was 48-78% of the original content after cooking. Whole wheat spaghetti (234

µg/g) had significantly higher content of ferulic acid than regular spaghetti ($p < 0.05$). Flavonoid glycosides present in the regular and whole-wheat spaghetti samples were identified as 6-C-glucosyl-8-C-arabinosyl apigenin and the sinapic acid adduct of apigenin-C-diglycoside. The content of these compounds were found to be significantly higher in whole-wheat spaghetti (16.95 and 15.15 µg apigenin equivalent/g) compared to the regular brands (9.47 and 5.83 µg apigenin equivalent/g). SDG content was also found to be significantly higher in whole-wheat spaghetti (41.8 µg/g) compared to the regular brands (12.9 µg/g). There was a significant difference in ORAC values (10.8 and 18.4 µmol Trolox equivalents/g); however, there was no significant difference in DPPH scavenging activity (1.0 and 2.3 µmol Trolox equivalents/g) among whole wheat and regular spaghetti. These findings lend further support to the notion that phenolic compounds bound with dietary fibre are concentrated in the bran layers of the wheat kernel and hence consumption of whole grain products is strongly recommended to obtain significant levels of health promoting phytochemicals.

2.1. Introduction

Whole-wheat products contain more vitamins, minerals, antioxidants and dietary fibre than regular, refined ones (Baic, 2005). Cardiovascular disease factors such as serum lipids and lipoprotein have been shown to decrease in the Neapolitan area where there is high consumption of pasta, compared to the northern regions (Mariani-Constantini, 1988). Whole-wheat pasta not only prevents chronic diseases such as cardiovascular disease, cancer and diabetes, but also helps maintain body weight by further lowering the glycemic responses (Baic, 2005).

Antioxidant capacity of whole-wheat products is relatively high and even similar to that of fruits or vegetables on a per serving basis (Slavin, 2003). Compounds in wheat known to

exhibit antioxidant properties can be divided into polar phytochemicals primarily, phenolics (Beta, Nam, Dexter & Sapirstein, 2005; Mpofu, Sapirstein & Beta, 2006; Zielinski & Kozłowska, 2000) and non-polar phytochemicals, primarily carotenoids (Adom et al. 2003; Hentsche et al., 2002). The insoluble fibre in durum wheat bran contains 0.5 – 1.0% phenolic acids, as one class of the phenolics (Slavin, 2000). Ferulic acid, esterified to the primary alcoholic group of arabinose side chains, is an example of the phenolic acids found in durum wheat bran (Slavin, 2000; Lintas, 1988b). The fibre is broken down in the colon by microbial enzymes releasing the bound phenolic acids thereby providing antioxidant protection (Slavin, 2003).

Flavonoids, as another class of the phenolic compounds present in wheat grain contribute to the colour, enzyme inhibition, protection against ultraviolet radiation, insect damages and also serve as metal chelating agents (Cuyckens and Claeys, 2004). Flavonoids normally occur in plants as glycosides in which a sugar group (glycone) is covalently bonded to another group (non-sugar group called aglycone) via an *O*-glycosidic bond or *C*-glycosidic bond. Flavonoids including flavones are concentrated in the bran and germ layers of the wheat grain (Adom et al., 2005). Apigenin-6-*C*-arabinose-8-*C*-glucoside, apigenin-6-*C*-glucoside-8-*C*-arabinoside, apigenin-6-*C*-arabinoside-8-*C*-galactoside and apigenin-6-*C*-galactoside-8-*C*-arabinoside are likely to be found either in the free form or esterified to sinapic acid or ferulic acid in wheat germ (Asenstorfer et al., 2006; King, 1962). Apigenin-6-*C*-glucoside-8-*C*-arabinoside was found as the minor flavone-glycoside and apigenin-6-*C*-glucoside-8-*C*-arabinoside as the major flavone-glycoside in hard red spring wheat bran (Feng et al., 1988). Some *C*-glycosyl flavonoid derivatives that were putatively identified in wheat fractions were vicenin, schaftoside and isoschaftoside, also with derivatives of feruloyl or sinapoyl group linked to them (Gallardo et al.,

2006). Asenstorfer et al. (2006) found concentrations of apigenin 6-C-glucoside-8-C-arabinoside and its isomer apigenin 6-C-arabinoside-8-C-glucoside of 8.68 and 10.1 mg naringin/g of germ fractions of an Australian common bread wheat cultivar whole meal.

Lignans occur in plants as natural polyphenols that are natural defence substances. Wheat and rye bran have been reported to have the highest concentration of lignans compared to the other cereals (Smeds et al., 2007). Lignans are also concentrated in the bran layers of the wheat grain (Qu et al., 2005). Plant lignans usually occur as glycosides and the major compound found is secoisolariciresinol diglycoside (SDG) (Meagher and Beecher, 2000). Results from epidemiological studies concluded that high intakes of phytoestrogens including plant lignans are likely associated with lower risk of tumour, cancer and coronary heart disease (Aldercreutz et al., 1982; Heinonen et al., 2001; Qu et al., 2005; Vanharata et al., 1999). The type of diet (Asian versus Western diet) might influence hormone production and also biochemical mechanisms that occur at the cellular level contributing to these diseases (Meagher and Beecher, 2000). Secoisolariciresinol (SECO) was studied in white, whole-wheat, raw and cooked spaghetti by Kuhnle et al. (2009).

Some antioxidants formed during processing contribute to the total antioxidant activity of the grain products (Slavin, 2003). Non-enzymatic browning reactions such as the Maillard reaction can generate some products that display antioxidant properties (Amarowicz, 2009; Manzocco et al., 2001). Food antioxidants are lost in significant amounts as a result of food processing, storage, home handling and cooking (Nicoli et al., 1997). The antioxidant capacity, role in disease prevention and levels generally consumed of several whole grain products have been reported (Marquart et al., 2003; Seal, 2006; Slavin, 2000; Slavin, 2003).

Durum wheat semolina is preferred for production of spaghetti and other pasta products (Marconi & Marcia, 2001). Regular semolina is obtained by repeated grinding and sieving of the durum resulting in maximum yield of the granular endosperm product and little amounts of bran powder. In whole-wheat semolina, the bran is included. Manufacturers employ high-temperature drying technology to obtain better quality pasta in a short period thereby increasing productivity (Anese et al., 1999). Pasta had antioxidant or pro-oxidant properties depending on temperature, time and moisture conditions during the drying process (Anese et al., 1999).

Whole grain pasta products have become available on the market; however, there is little or no literature on the antioxidant profile and specific phytochemical contents of spaghetti derived from whole durum wheat versus semolina. The aim of the present investigation was to conduct a comparative study on the antioxidant properties among brands of regular and whole-wheat spaghetti. The effect of cooking on the phenolic content was also investigated. The C-glycosyl flavone and lignan diglucoside contents of regular- and whole- wheat spaghetti were also determined using LC-MS/MS techniques.

2.2. Materials and methods

2.2.1. Samples

Ten samples of commercial spaghetti were purchased from major supermarkets in Winnipeg (Manitoba, Canada) (Figure 2.1). The spaghetti samples were grouped into regular and whole-wheat spaghetti samples. The regular spaghetti samples contained semolina as the main ingredient.

(1) Five regular spaghetti samples were:

- a. Catelli (Ronzoni Foods Canada Corporation, Montreal, QC)
- b. Splendor (Ronzoni Foods Canada Corporation, Montreal, QC)

- c. Primo (Primo Foods Inc., Toronto, ON)
- d. No Name (Loblaws Inc., Calgary, AB)
- e. Safeway (Canada Safeway Limited, Calgary, AB)

No Name and Safeway showed darker colour than the other regular spaghetti brands and higher fat content based on their Nutrition Facts. The darker colour and higher fat content were likely due to higher bran contamination during semolina production unlike other brands that were lighter in colour.

Catelli Smart (Ronzoni Foods Canada Corporation, Montreal, QC), containing inulin as added fibre to impart the same fibre benefits of whole wheat, was included as a control among the whole-wheat spaghetti even though it is made from refined semolina. It has the same appearance, texture and flavour as that of regular spaghetti.

(2) Four whole-wheat spaghetti samples were:

- a. Catelli (Ronzoni Foods Canada Corporation, Montreal, QC)
- b. Primo (Primo Foods Inc., Toronto, ON)
- c. President's Choice (PC) (Loblaws, Inc., Calgary, AB)
- d. Eating Right (Lucerne Foods, Calgary, AB)

The spaghetti brands had almost the same ingredients (durum semolina and other ingredients for example vitamins, iron and folic acid) with the exception of Catelli Smart which had inulin added, and PC which had durum whole-wheat semolina listed as the only ingredient.

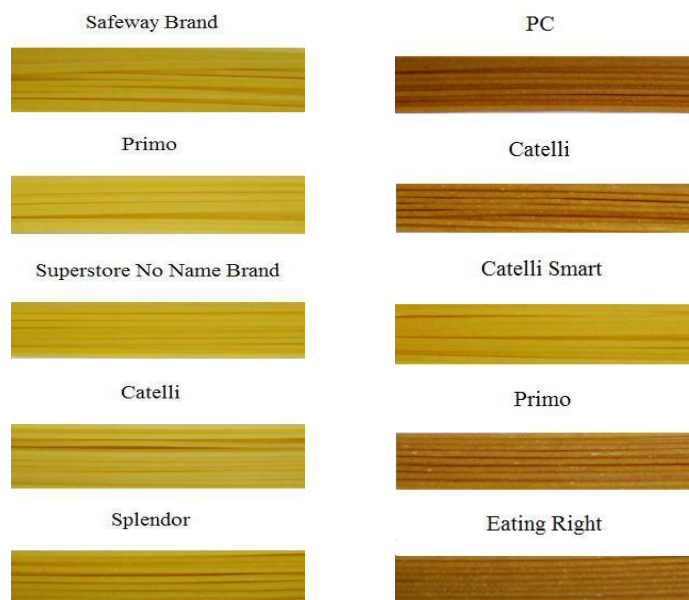


Figure 2.1. Regular spaghetti (left) compared to whole wheat spaghetti (right). Catelli Smart was used as control sample among the whole wheat spaghetti since fiber, in the form of inulin, was added to its semolina to simulate the effects of bran found in whole wheat.

2.2.2. Chemicals

Folin-Ciocalteu's phenol reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH) and ferulic acid standard were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Fluorescein (FL) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Fisher Acros Organics (Morris Plains, NJ, USA).

HPLC grade methanol was used in the extraction. MS grade acetonitrile and acetic acid were used in the LC/MS analysis. HPLC and MS grade solvents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2.3. Sample preparation

Catelli regular, Primo regular, Splendor, Catelli Smart, Catelli whole-wheat and Primo whole-wheat spaghetti samples were cooked as follows: 750 mL of water was brought to boil; 17 g of regular or whole-wheat spaghetti and half teaspoon of salt were then added. The spaghetti was cooked for 12 minutes and 20 seconds (until they achieved *al dente*). Cooked spaghetti samples were frozen prior to freeze-drying (Thermo Electron Corporation, Waltham, MA). A sample mill (Black & Decker, Hunt Valley, MD) was used to grind the raw and freeze-dried cooked spaghetti so as to pass through a screen of 0.42 mm.

2.2.4. Moisture content determination

Ground raw and freeze-dried cooked spaghetti samples were weighed and dried overnight at 100°C in an air oven following the AOAC method 930.15 (1990). All samples were analyzed in duplicate.

2.2.5. Extraction

2.2.5.1. Acidified ethanol and methanol extraction

The extraction of antioxidant components in raw and cooked spaghetti was adapted from methods by Li et al. (2007b). Finely ground spaghetti (1 g) was extracted with ethanol (95%) and 1 M HCl/95% ethanol (v/v, 15/85) (10 mL). The mixture was shaken for 1 h at ambient temperature in a dark room using a wrist action shaker (Burrell, Pittsburgh, PA) and centrifuged at $7,800 \times g$ (10,000 rpm, SS-34 Rotors, RC5C Sorvall Instruments) at 5°C for 15 min. The supernatant was collected using a pipette and transferred into a sample vial. The extractions were done in triplicate. The supernatants were stored in the dark at -20°C for DPPH radical scavenging activity, total phenolic content (TPC). The extraction of antioxidant components in spaghetti samples for oxygen radical absorbance capacity (ORAC) was also adapted from methods by Li et al. (2007b). Finely ground infant cereal (1 g) was extracted with 1 M HCl/methanol (v/v, 15/85) (10 mL). The mixture was sonicated (Bransonic 5510OR-DTH, Branson Ultrasonic Corporation, Danbury, Connecticut, USA) for 45 min, shaken every 5 min and centrifuged at $26,920 \times g$ (15,000 rpm, SSA-34 Rotors, RC5C Sorvall Instruments, Thermo Scientific, Asheville, NC, USA) at ambient temperature for 15 min. The supernatant was collected and centrifuge pellet was washed. Supernatants were stored at -20°C prior to the analysis of TPC, DPPH, and ORAC.

2.2.5.2. Alkaline hydrolysis

For HPLC analysis of ferulic acid, a method adapted from Li et al. (2007b) was used. Sixty millilitres of 4 M NaOH were added to finely ground spaghetti and the mixture incubated at ambient temperature for 4 hours. Nitrogen was infused into the mixture for 5 min every hour

during incubation. The mixture was acidified to pH 1.5 – 2.5 using ice-cold HCl (6 M) prior to centrifugation at 5°C for 20 min at 7,800 x *g* (10,000 rpm, SS-34 Rotors, RC5C Sorvall Instruments). The supernatant was extracted three times using ethyl acetate (70 mL). The organic phase was then dehydrated with sodium sulphate and further dried by using a rotary vacuum evaporator (RE-51 Rotary evaporator, Yamato Scientific America, Inc., Santa Clara, CA). Five mL of 50% methanol was added. The hydrolysate was filtered through a 0.45 µm PTFE filter prior to HPLC analysis.

2.2.5.3. Aqueous acetone extraction

The extraction for HPLC/MS/MS analysis of C-glycosyl flavones was adapted from methods by Li et al. (2007b). Finely ground spaghetti (1g) was extracted with 80% acetone (10 mL). The mixture was sonicated for 45 min (Bransonic 5510OR-DTH, Branson Ultrasonic Corporation, Danbury, Connecticut, USA) and centrifuged at 26,920 x *g* (15,000 rpm, SS-34 Rotors, RC5C Sorvall Instruments, Thermo Scientific, Asheville, NC, USA) at ambient temperature for 15 min. The supernatant was collected and centrifuge pellet was washed. The supernatants were rotary-evaporated at 35°C for about 30 min to complete dryness (IKA® RV10, IKA® Works, Inc., North Carolina, USA). Two mL of 80% methanol was added to reconstitute the samples from the rotary-evaporator followed by sonication. Samples were then micro-centrifuged at 19,319 x *g* (12,000 rpm) for 5 min (IEC Micromax Microcentrifuge, Thermo Fisher Scientific Inc., Waltham, MA), supernatant collected, filtered with 0.45 µm PTFE filter and analyzed using HPLC-DAD and QTOF-MS (Waters Corp., Milford, MA).

2.2.5.4. Acid hydrolysis

The extraction for HPLC/MS/MS analysis of SDG was adapted from methods by Dinelli et al. (2007). To five grams of finely ground spaghetti was added 20 mL of hexane. The mixture was manually stirred until all the dry material was wet and then stirred with stirring bar for an hour. The samples were then vacuum-filtered using Whatman filter paper grade no. 4. The residue was air-dried for about an hour. Sixty mL of 1 M HCl/methanol/distilled water (w/v, 15/75/10) to the residue was then added prior to placing the mixture in shaking water bath at 60°C for 3 hrs. Samples were centrifuged at 7,800 x *g* (10,000 rpm, SS-34 Rotors, RC5C Sorvall Instruments) for 30 min at 20°C. The supernatants were rotary-evaporated at 40°C to a final volume of 1 mL for about 20 min. Samples were then sonicated for 2 min and samples from the rotary-evaporator were reconstituted to 4 mL with distilled water. Samples were micro-centrifuged at 19,319 x *g* (12,000 rpm) (IEC Micromax Microcentrifuge, Thermo Fisher Scientific Inc., Waltham, MA) for 5 min, supernatant collected, filtered with 0.45 µm PTFE filter and analyzed using HPLC/MS/MS.

2.2.6. Chemical model assays

2.2.6.1. Total phenolic content (TPC) determination

The TPC of the extracts from spaghetti samples were measured following modifications of the Folin-Ciocalteu method (Singleton and Rossi, 1965). The Folin-Ciocalteu's phenol reagent was first diluted 10 times and 200 µL of extract added to 1.5 mL of the diluted Folin-Ciocalteu's phenol reagent. Sodium carbonate solution (60 g/L) (1.5 ml) was then added to the mixture. The reaction was allowed to take place at room temperature for 120 min. The absorbance of the solution was measured at 725 nm against a blank of distilled water. Ferulic acid was used as a

standard and the results were reported in μg ferulic acid equivalents/g. All analyses were conducted in triplicate.

2.2.6.2. DPPH radical scavenging activity determination

The DPPH assay (Brand-Williams et al., 1995) was used following the modifications of Beta et al. (2005). Sixty $\mu\text{mol/L}$ of DPPH radical was prepared in 95% ethanol. DPPH solution (3.04 mL) was then added to 160 μL of the spaghetti extracts and the reaction allowed to take place for 30 min. Absorbance (A) of the solution was measured at 515 nm against a blank of 95% ethanol at $t = 30$ min. DPPH radical scavenging activity (%) of both samples and Trolox standards was calculated using the formula: $(1 - [A_{\text{sample}}/A_{\text{control}, t=0}] \times 100)$. A standard curve was created (Trolox concentration vs. %DPPH radical scavenging activity). The Trolox standards were used at 0, 125, 250, 375, and 500 μM . Based on the equation of the standard curve and the %DPPH radical scavenging activity of the samples, the concentration of the samples were calculated. Results were reported in μmol Trolox equivalent/g. All analyses were conducted in triplicate.

2.2.6.3. Oxygen radical absorbance capacity (ORAC) determination

The ORAC values were obtained following modifications of the procedure described in the literature (Huang et al., 2002). The measurement was taken using an FLx800 microplate fluorescence reader (BioTek Instruments, Inc., Winooski, VT) with fluorescence filters at excitation wavelength of 485/20 nm and an emission wavelength of 528/20 nm. The instrument was monitored by the software, KC4 3.0, version 29. A dilution factor of 500 from the supernatant fluid was used for the samples, rutin was used at 10 μM as a control, and Trolox

standards were used at 0 μM , 6.25 μM , 12.5 μM and 50 μM . The remainder of the procedure was according to Li et al. (2007b). The calculation of antioxidant capacity was based on the method used by Huang et al. (2002). A regression equation between the Trolox concentration and the net area under the fluorescence decay curve was constructed. The formula to obtain area under curve (AUC) was as follows:

$$\text{AUC} = 0.5 + \frac{f_1}{f_0} + \dots + \frac{f_i}{f_0} + \dots + \frac{f_{49}}{f_0} + 0.5 \frac{f_{50}}{f_0}$$

where f_0 = initial fluorescence reading at 0 min and f_i = fluorescence reading at time i min. Net AUC = AUC (blank) – AUC (sample). The final ORAC values were expressed as Trolox equivalent and determined according to the standard curve. All analyses were conducted in duplicate.

2.2.7. HPLC and HPLC/MS/MS analyses

High performance liquid chromatography (HPLC) with reverse phase column coupled with UV-Vis photodiode array detector (PAD) is used for separation, identification and quantification of components in food (Robbins, 2003) and non-food research labs while gas chromatography (GC) is used with more volatile compounds. The MS technique used in the present study is time-of-flight analyzer that is also equipped with quadrupole mass filter and collision cell for tandem mass spectrometry analyses. Ion fragmentation patterns were compared with standard fragmentation patterns and literature database for identification.

2.2.7.1. HPLC analysis for ferulic acid

Ferulic acid was quantified in the spaghetti samples by using Waters model 600 pump and controller with Waters 2489 UV/visible detector (Waters Corp., Milford, MA). Analysis was performed with a Gemini 5 μ C18 110A column (150 mm x 4.60 mm) (Phenomenex®, Torrance, CA). A gradient of solvent A (1% acetic acid in water) and solvent B (1% acetic acid in methanol) was used for 22 min at a flow rate of 0.5 mL/min. The gradient was as follows: at 0 min 20% B, 4 min 23% B, 8 – 14 min 25% B, 14 – 19 min 27% B, 19 – 22 min 20% B. Detector was set at 320 nm. Identification of ferulic acid in the spaghetti samples was achieved by comparison of the retention time of the ferulic acid standard and sample spiked with the ferulic acid standard. The HPLC analyses were done in duplicate.

2.2.7.2. HPLC/MS/MS analysis for C-glycosyl flavones

C-glycosyl flavones were identified and quantified in the spaghetti samples by using an HPLC (Separation Module, Waters 2695), a photodiode array detector (Waters 2996) and an autosampler (Waters 717plus), coupled with a quadrupole time-of-flight mass spectrometer (Q-TOF MS) (Micromass, Waters Corp., Milford, MA). Analysis was performed with a Gemini 5 μ C18 110 Å column (150 mm x 4.60 mm) (Phenomenex®, Torrance, CA). A gradient of solvent A (1% acetic acid in water) and solvent C (1% acetic acid in acetonitrile) was used for 50 min at a flow rate of 0.5 mL/min. The gradient was as follows: at 0 min 5% C, 5 min 10% C, 15 min 15% C, 20 min 20% C, 30 min 25% C, 40 min 40% C, 45-50 min 10% C. The photodiode array detector was set at 333 nm. The sample was then introduced into the Q-TOF MS. The Q-TOF MS was calibrated with sodium iodide in the negative mode and full mass spectra were obtained with capillary voltage of 1.8 kV and cone voltage of 45 V. Desolvation gas (N₂) and cone gas

(He) were used at flow rates of 900 and 50 L/hr, respectively. The temperature settings for the desolvation gas and ion source were 350 and 150°C, respectively. Identification of C-glycosyl flavones was achieved by using tandem mass spectrometric technique with collision energy of 30 kV and quantification was done using apigenin standard. The apigenin standard was obtained from Chromadex™ (Irvine, CA, USA) graded Primary Standards (P) for quantitative validation. Its adjusted purity was 97.5% The analyses were done in duplicate.

2.2.7.3. HPLC/MS/MS analysis for SDG

SDG were identified and quantified using the above HPLC/MS/MS instrument and settings (see section 2.2.7.2. above). A gradient of solvent A (1% acetic acid in water) and solvent C (1% acetic acid in acetonitrile) was used for 35 min at a flow rate of 0.5 mL/min. The gradient was as follows: at 0 min 10% C, 5 min 15% C, 20-25 min 40% C, 30-35 min 10% C. Detector was set at 280 nm. The sample was then introduced to the Q-TOF MS. Identification of SDG was achieved by using tandem mass spectrometric technique with a collision energy of 30 kV and quantification was done using SDG standard. The SDG standard was obtained from Chromadex™ (Irvine, CA, USA), extracted from flax and graded Primary Standards (P) for quantitative validation. Its adjusted purity was 97.6% and it was obtained from *Linus usitatissimum*. The analyses were done in duplicate.

2.2.8. Statistical analysis

All data were converted to dry weight basis and reported as means of duplicate or triplicate analyses. One-way analysis of variance of results was performed using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC). Significant differences among sample means

for regular, whole-wheat and regular versus whole-wheat spaghetti samples for each analysis were tested using Fisher's Least Significant Difference (LSD) test at $p < 0.05$.

2.3. Results and Discussion

2.3.1. TPC and antioxidant activity of raw, regular versus whole-wheat spaghetti

TPC was expressed as micrograms of ferulic acid equivalent per gram ($\mu\text{g/g}$) of spaghetti. Ferulic acid, the major phenolic acid in wheat, was used as a standard as previously reported (Beta et al., 2005; Mpofu et al., 2006). TPC of regular wheat spaghetti brands (Table 2.1) was significantly lower than that of whole-wheat spaghetti brands (Table 2.2) ($p < 0.05$). Regular and whole-wheat spaghetti had an average TPC of 865 and 1389 $\mu\text{g/g}$, respectively. Among the brands examined, TPC decreased to about 62% of the content found in whole wheat during production of regular spaghetti. Assuming at least an 8 % loss during durum wheat milling (Borrelli et al., 1999), the outer layers of wheat, particularly the 10 % outer layers (Beta et al., 2005), are well-known to be concentrated in TPC. Regular spaghetti from different brands had TPC ranging from 718 to 927 $\mu\text{g/g}$. Primo and Splendor had the highest and lowest TPC, respectively; however, Catelli, Primo, No Name and Safeway did not show any significant differences at $p < 0.05$. Contrary to regular wheat, whole-wheat spaghetti from different brands ranged from 1263 to 1529 $\mu\text{g/g}$ for Catelli and Primo, respectively (Table 2.2). Catelli Smart, the control sample among the whole-wheat spaghetti, exhibited TPC levels similar to those found in regular spaghetti, an indication that the product differs from whole wheat spaghetti since phenolic phytochemical constituents are mostly bound to fibre in whole wheat.

The DPPH and peroxy radical scavenging activities of spaghetti were expressed as micromole Trolox equivalents per gram ($\mu\text{mol Trolox equiv/g}$) of spaghetti. There were no

significant differences in average DPPH radical scavenging activity for regular (Table 2.1) and whole-wheat (Table 2.2) spaghetti brands. The DPPH radical scavenging activity assay does not work with low pH extracts as they will form precipitation gels which hinder accurate absorbance readings. Thus, samples were extracted with ethanol only while bound phenolics are separated and made available for antioxidant activity readings by acid extraction (Li et al., 2007a). The average DPPH radical scavenging activities for raw regular and whole-wheat spaghetti were 1.6 and 1.5 $\mu\text{mol Trolox equiv/g}$, respectively. Significant differences were observed among spaghetti brands in each of the two categories, with the Safeway and PC brand displaying the highest DPPH radical scavenging activity among the regular and whole-wheat samples, respectively. DPPH radical scavenging activity ranged from 0.94 to 2.32 $\mu\text{mol Trolox equiv/g}$ and decreased in the order: Safeway > Primo > No Name > Splendor > Catelli for regular spaghetti. DPPH radical scavenging activity ranged from 1.08 to 1.87 $\mu\text{mol Trolox equiv/g}$ among the whole-wheat brands. PC and Eating Right had the highest DPPH scavenging capacity that did not differ significantly at $p < 0.05$. In a study by Amarowicz et al. (2002), DPPH radical scavenging of wheat caryopses and embryos was tested. The range fell between 0.6 and 0.8, measured at 20 min at 517 nm and the concentration of the extracts were 2 mg/0.1 mL (Amarowicz et al., 2002). The range of absorbance of the spaghetti samples were 0.3-0.6, measured at 30 min at 515 nm and the concentration of the extracts were 100 mg/0.16 mL. The lower absorbance or higher DPPH radical scavenging activity of the spaghetti samples is likely due to higher concentration of the extracts.

ORAC values ranged from 5.59 for Splendor to 15.9 $\mu\text{mol Trolox equiv/g}$ for Safeway among the regular brands (Table 2.1). Primo had the second highest ORAC value followed by Catelli and No Name. Whole-wheat spaghetti had ORAC values ranging from 11.1 for Catelli

Smart as the control to 21.7 $\mu\text{mol Trolox equiv/g}$ for PC (Table 2.2). Whole-wheat Primo once again had the second highest ORAC value followed by Catelli and Eating Right that did not have significant differences in their ORAC values. The average ORAC values for regular (10.8 $\mu\text{mol Trolox equiv/g}$) and whole-wheat (18.4 $\mu\text{mol Trolox equiv/g}$) (Table 2.1 and Table 2.2) spaghetti were significantly different ($p < 0.05$, LSD value was 2.09 $\mu\text{mol Trolox equiv/g}$). Liyana-Pathirana and Shahidi (2007) reported ORAC values of 48 ± 2 and 100 ± 1 $\mu\text{mol Trolox equiv/g}$ for semolina and whole grain, respectively for one Canadian Western Amber Durum wheat. The respective values were higher than the averages found in this investigation on regular and whole-wheat spaghetti because of processing effects and also genetic and environmental factors (Mpofu et al., 2008; Zhou & Yu, 2004).

Overall, PC and Safeway representing the whole-wheat and regular spaghetti samples, respectively had high total phenolic contents and also highest antioxidant activity as shown by their DPPH radical and peroxy radical scavenging activities. Splendor showed low total phenolic content and the two types of radical scavenging activities. Catelli Smart also showed low total phenolic content and radical scavenging activities and this suggests that addition of inulin fibre in the ingredient did not substitute both fibre and antioxidant benefits of wheat bran.

Table 2.1. TPC, DPPH radical scavenging activity and oxygen radical absorbance capacity of raw regular pasta from different brands ^a

Sample name	TPC (μg equivalent of ferulic acid/g)	DPPH (μmol equivalent of Trolox/g)	ORAC (μmol equivalent of Trolox/g)
Catelli	892 a	0.94 d	10.5 c
Primo	927 a	1.60 b	13.1 b
Splendor	718 b	1.28 c	5.59 e
No Name	897 a	1.77 b	9.15 d
Safeway	892 a	2.32 a	15.9 a
LSD	61.3	0.19	1.07

^a LSD, Least Significant Difference at $p = 0.05$ level of probability. Mean values for regular spaghetti samples having similar letters in the same column are not significantly different.

Table 2.2. TPC, DPPH radical scavenging activity and oxygen radical absorbance capacity of raw whole wheat pasta from different brands ^a

Sample name	TPC (μg equivalent of ferulic acid/g)	DPPH (μmol equivalent of Trolox/g)	ORAC (μmol equivalent of Trolox/g)
Catelli Smart -Control	773 d	1.14 b	11.1 d
Catelli	1263 c	1.36 b	17.2 c
PC	1342 b,c	1.87 a	21.7 a
Primo	1529 a	1.08 b	18.3 b
Eating Right	1423 a,b	1.71 a	16.6 c
LSD	113	0.28	1.01

^a LSD, Least Significant Difference at $p = 0.05$ level of probability. Mean values for whole wheat spaghetti samples having similar letters in the same column are not significantly different.

2.3.2. Effects of cooking on TPC of regular and whole-wheat spaghetti

Since there was no significant difference in antioxidant activity between the regular and whole wheat spaghetti samples, the effect of cooking on antioxidant properties was evaluated by determining the total phenolic content (TPC) for Catelli regular and whole-wheat, Primo regular and whole-wheat, Catelli Smart and Splendor spaghetti samples. Both regular and whole wheat spaghetti showed significant differences in TPC before and after cooking (Table 2.3). There was a 39% overall decrease in TPC of regular and whole wheat spaghetti after cooking. The average TPC differed significantly ($p < 0.05$) for raw (846 $\mu\text{g/g}$) and cooked (505 $\mu\text{g/g}$) regular brands. Catelli Smart, the inulin fiber-enriched spaghetti had a 31% decrease in TPC after cooking. TPC decreased significantly ($p < 0.05$) from 1529 to 844 $\mu\text{g/g}$ after cooking Primo whole wheat spaghetti.

There is very little literature on phenolic content and antioxidant properties of processed durum wheat, particularly the recently introduced whole wheat pasta products. The ferric reduction-antioxidant capacity of four commercial brands of regular spaghetti decreased after cooking (Halvorsen et al., 2006). The antioxidant components (mmol of electrons/hydrogen atoms donated in the redox reaction per 100 g sample), calculated as percentage of the non-processed food ranged from 42 to 63 % for spaghetti cooked by steaming. Although the redox-active compounds in methanol/water (9:1, v/v) extracts were not identified in the study (Halvorsen et al., 2006), it is likely that they were phenolic constituents. Other compounds exhibiting antioxidant activity are known to be present in significant amounts in whole grain products.

Browning reactions in grain-based products may also contribute to antioxidant properties due to the development of melanoidins during the high temperature drying of pasta (Slavin,

2003; Manzocco et al., 2001). These compounds likely survived the final cooking process. The antioxidant properties of pasta depend on the temperature, time and moisture conditions of the drying process (Anese et al., 1999). Cooking with water and also with the presence of oxygen could induce the reaction of lipoxygenase that oxidatively degrades carotenoids pigments in cooked emmer pasta (Borrelli et al., 1999; Fares et al., 2008). However, Fares et al. (2010) found a different trend in cooked durum pasta as there was a stable level of free phenolic acids, an increase in levels of bound phenolic acids and antioxidant activity. The *in vitro* extractability of bound phenolic acids from food matrix might be enhanced during boiling in water (Fares et al., 2010). Fares et al. (2010) enriched their durum pasta samples with debranning fractions. Further investigations on the health implications of the Maillard reaction in pasta drying as well as the characterization of MRPs in cooked spaghetti are still needed.

Table 2.3. Total phenolic content of raw vs. cooked regular pasta from different brands ^a

Regular	µg equivalent of ferulic acid/g	Whole Wheat	µg equivalent of ferulic acid/g
Raw Catelli	892 a	Raw Catelli Smart (C)	773 a
Cooked Catelli	509 b	Cooked Catelli Smart (C)	530 b
LSD	60	LSD	108
Raw Primo	927 a	Raw Catelli	1236 a
Cooked Primo	443 b	Cooked Catelli	831 b
LSD	74	LSD	117
Raw Splendor	718 a	Raw Primo	1529 a
Cooked Splendor	564 b	Cooked Primo	844 b
LSD	47	LSD	119

^a LSD, Least Significant Difference at $p = 0.05$ level of probability. For each brand, mean values of cooked and raw spaghetti having similar letters in the same column are not significantly different.

2.3.3. Ferulic acid content of raw, regular versus whole-wheat spaghetti

HPLC chromatograms of the ferulic acid standard and samples (regular and whole wheat spaghetti) are shown in Figure 2.2. Ferulic acid was detected in two of the regular brands and in all of the whole wheat spaghetti samples (Table 2.4). The ferulic acid content in No Name and Safeway regular spaghetti brands were 44 and 54 $\mu\text{g/g}$ respectively. Ferulic acid was not detected in Catelli, Primo and Splendor. The control (Catelli Smart), with added inulin fiber, had negligible amounts of ferulic acid. Whole wheat spaghetti samples had ferulic acid contents ranging from 214 to 273 $\mu\text{g/g}$. PC had significantly the highest ferulic acid content ($p < 0.05$). Whole wheat spaghetti samples had significantly higher ferulic acid content than regular and inulin-enriched spaghetti samples ($p < 0.05$). The results on ferulic acid content were generally in agreement with the average values of the TPC discussed above.

The free and esterified ferulic acid content for whole grain of Canadian Western Amber Durum was $46.43 \pm 0.13 \mu\text{g/g}$ (Liyana-Pathirana & Shahidi, 2007). Jennah Khetifa (red spring durum) and Cham1 (white spring durum) had total ferulic acid contents of 148 and 303 μmol of ferulic acid/100 g of grain, respectively (Adom et al., 2003). Weidner et al. (1999) found values of 1.99, 13.51, 1.33 and 16.83 $\mu\text{g/g}$ for free, soluble esters, soluble glycosides and total content of ferulic acids in wheat (cv. Alba) caryopses. They also found values of 2.00, 24.91, 3.47 and 30.38 $\mu\text{g/g}$ for free, soluble esters, soluble glycosides and total content of ferulic acids in wheat (cv. Elena) (Weidner et al., 1999). However, whole wheat spaghetti products averaged 234 $\mu\text{g/g}$ in ferulic acid levels (free and bound ferulic acids). Since genetic and environmental variation in ferulic acid content is recognized among wheat varieties (Adom et al. 2003; Mpofu et al., 2006), it is likely that durum wheat pasta products will vary in phenolic composition. Processing durum into semolina led to considerable losses of ferulic acid such that it could not be detected in other

regular brands. Addition of fibre other than the cereal bran gives only the benefits of fibre while whole wheat has the benefits of both fibre (Ferguson and Harris, 1999; Koh-Banerjee and Rimm, 2003) and phenolic phytochemicals (Ferguson and Harris, 1999).

Table 2.4. Ferulic acid content of raw regular and whole wheat pasta^a

Regular	Ferulic acid content (µg/g)		Whole wheat	Ferulic acid content (µg/g)	
Catelli	nd		Catelli Smart (C)	6	d
Primo	nd		Catelli	215	c
Splendor	nd		PC	273	a
No Name	54	a	Primo	214	c
Safeway	44	a	Eating Right	234	b
LSD	9		LSD	10	

^a LSD, Least Significant Difference at $p = 0.05$ level of probability. For each brand, mean values of cooked and raw spaghetti having similar letters in the same column are not significantly different.

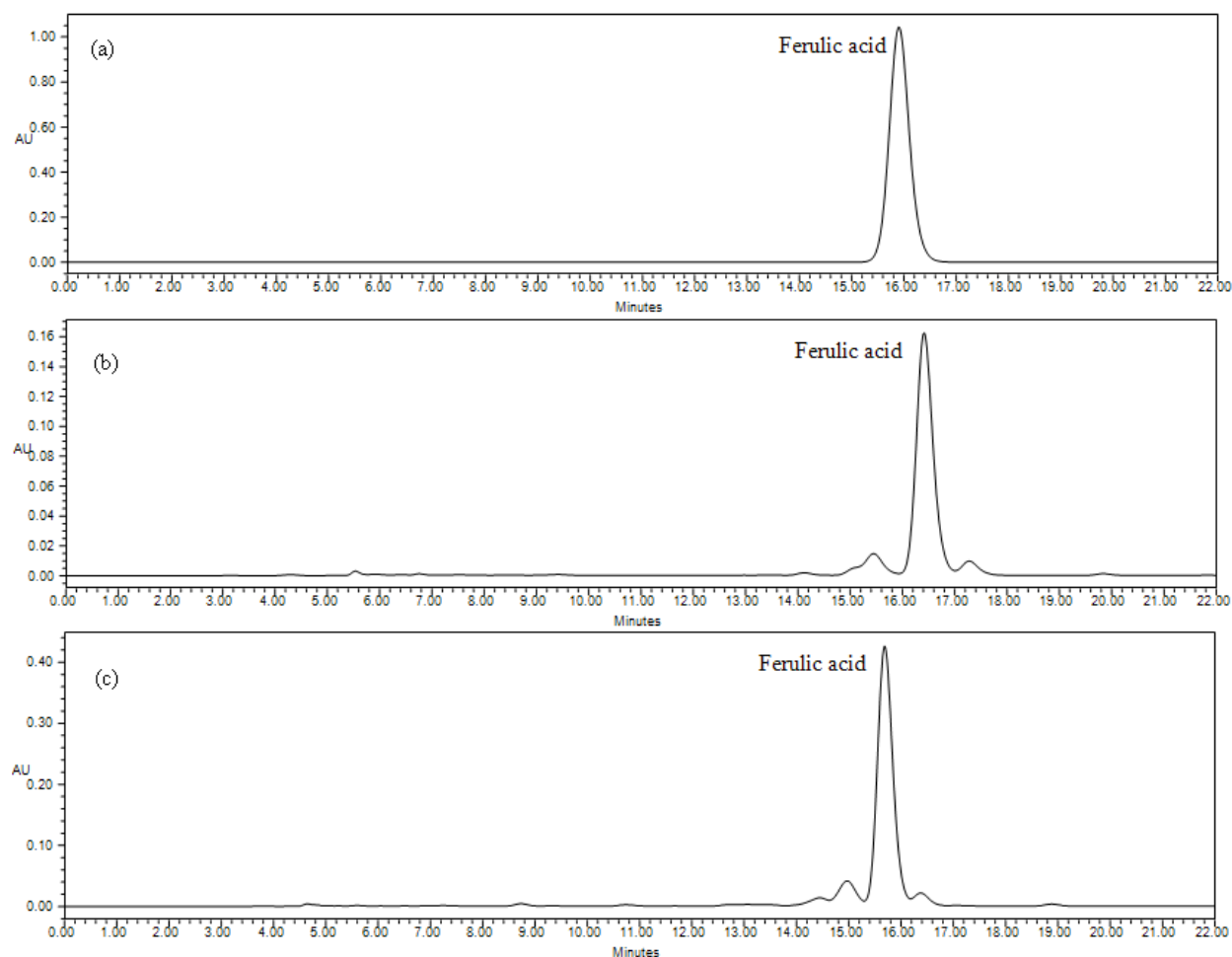


Figure 2.2. (a) HPLC chromatograph for ferulic acid standard measured at 320 nm. (b) HPLC chromatograph showing the dominant ferulic acid peak for No Name regular spaghetti measured at 320 nm. (c) HPLC chromatograph showing the dominant ferulic acid peak for Primo whole wheat spaghetti measured at 320 nm.

2.3.4. C-glycosyl flavone contents of raw, regular versus whole-wheat spaghetti

There were two major peaks of flavonoid glycosides in both the regular and whole-wheat spaghetti samples as shown in Figure 2.3 Peaks with $m/z = 563$ (24.5-26.5 min) (percentage of relative abundance at 24.98 and 26.15 min); MS, $[M - H]^- 563$; MS/MS, $[M - H - 18]^- 545$ (3%), $[M - H - 90]^- 473$ (11%), $[M - H - 120]^- 443$ (13% and 17%), $(A + 113) 383$ (82% and 94%) and $(A + 83) 353$ (100%) with the MS/MS spectrum shown in Figure 2.4 are likely 6-C-glucosyl-8-C-arabinosyl apigenin according to Qiu et al. (2009) (peak with $m/z = 563$ ($t_R = 25.58$ min): MS, $[M - H]^- 563$; MS/MS, $[M - H - 18]^- 545$, $[M - H - 90]^- 473$, $[M - H - 120]^- 443$, $[M - H - 150]^- 413$, $[M - H - 180]^- 383$, $[M - H - 210]^- 353$, $[M - H - 238]^- 325$). According to the relative abundance, both peaks are 6-C-glucosyl-8-C-arabinosyl apigenin (Ferrerres et al., 2003; Qiu et al., 2009). Apigenin-6/8-C-arabinoside-8-C-hexoside isomers were reported in durum wheat varieties by Dinelli et al. (2009). Peaks with $m/z = 769$ (30-32.5 min); MS, $[M - H]^- 769$; MS/MS, $[M - H - 206]^- 563$, $[M - H - 206 - 18]^- 545$, $[M - H - 224 - 90]^- 455$, $[M - H - 224 - 120]^- 425$ with the MS/MS spectrum shown in Figure 2.5 are likely sinapic acid esters of apigenin-C-diglycosides. The loss of 206 m/z from 769 and 563 might represent sinapic acid ester while the loss of 120 m/z might indicate the presence of hexose. Asenstorfer et al. (2006) found two peaks with $m/z = 771.4$ identified using positive ion mode. The two peaks indicated by Asenstorfer et al. (2006) were found to be similar to the sinapic acid ester of the flavone-C-diglycoside identified by King (1962). The spectra of these two peaks have absorption maxima of 272 and 340 nm (Asenstorfer et al., 2006) and this is comparable to the absorption maximum of 333 nm that was found in this study.

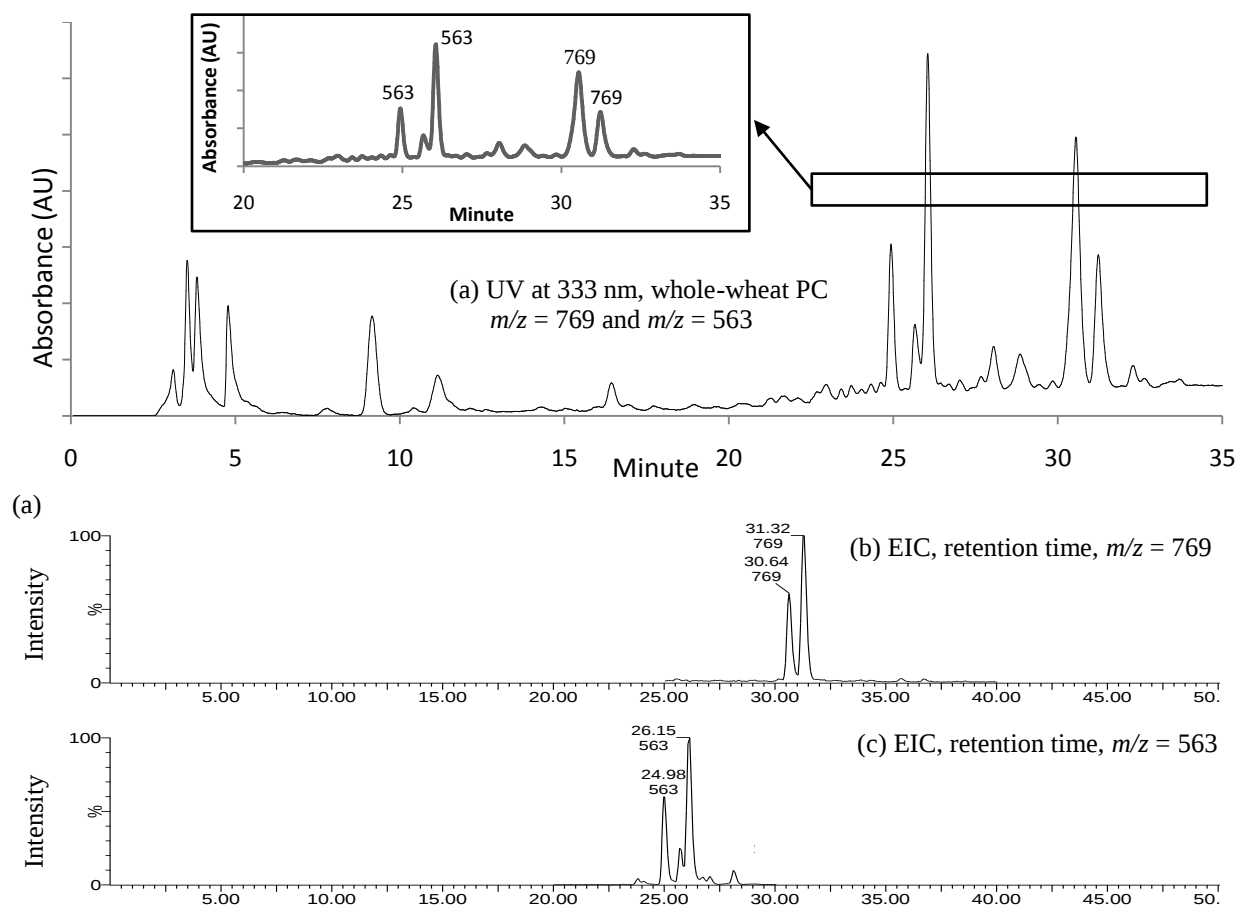


Figure 2.3. (a) Full LC chromatogram (0-50 min) of 6-*C*-glucosyl-8-*C*-arabinosyl and sinapic acid ester of apigenin-*C*-diglycosides obtained at 333 nm in whole-wheat spaghetti, PC brand; Extracted ion chromatogram (EIC) of sinapic acid ester of apigenin-*C*-diglycosides ions at $m/z = 769$ (b) and 6-*C*-glucosyl-8-*C*-arabinosyl ions at $m/z = 563$ (c) for whole-wheat spaghetti, PC brand.

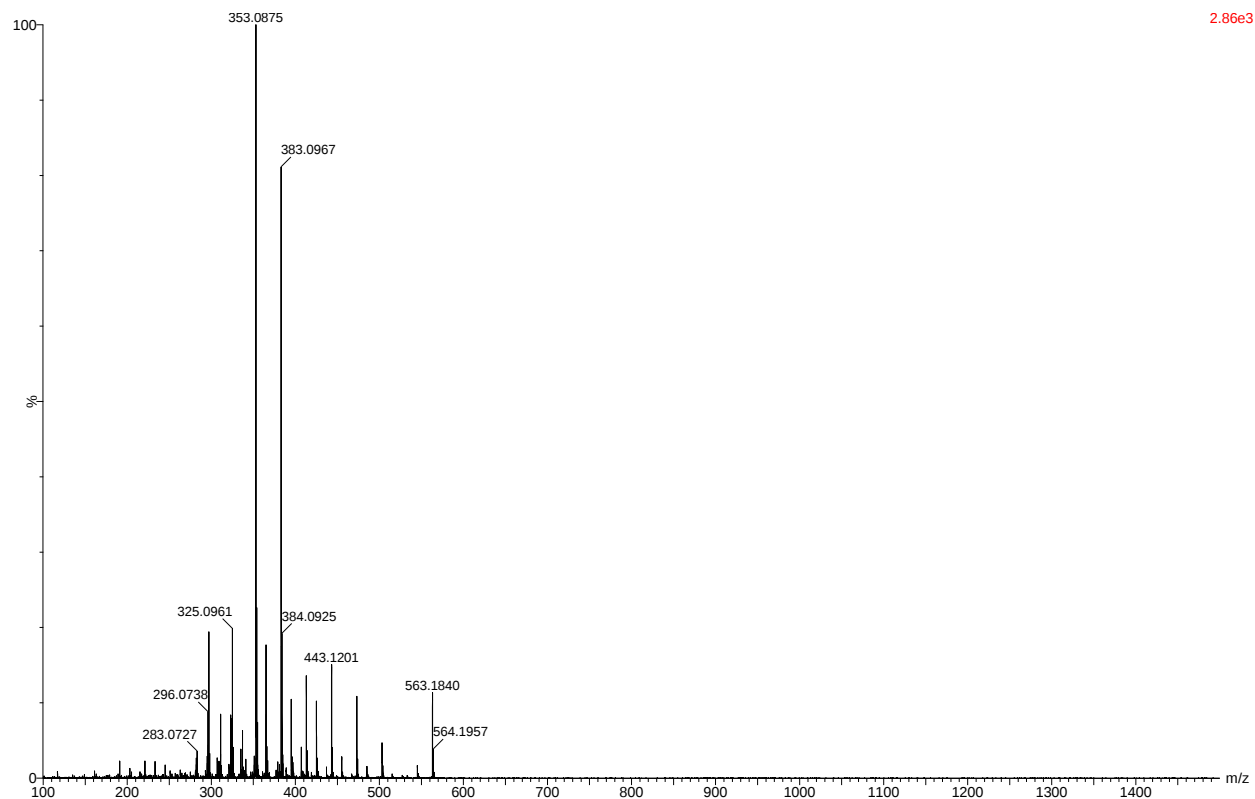


Figure 2.4. MS/MS spectrum of hexosyl-pentosyl apigenin (6-*C*-glucosyl-8-*C*-arabinosyl apigenin) in whole-wheat spaghetti, PC brand ($t_R = 26.15$ min).

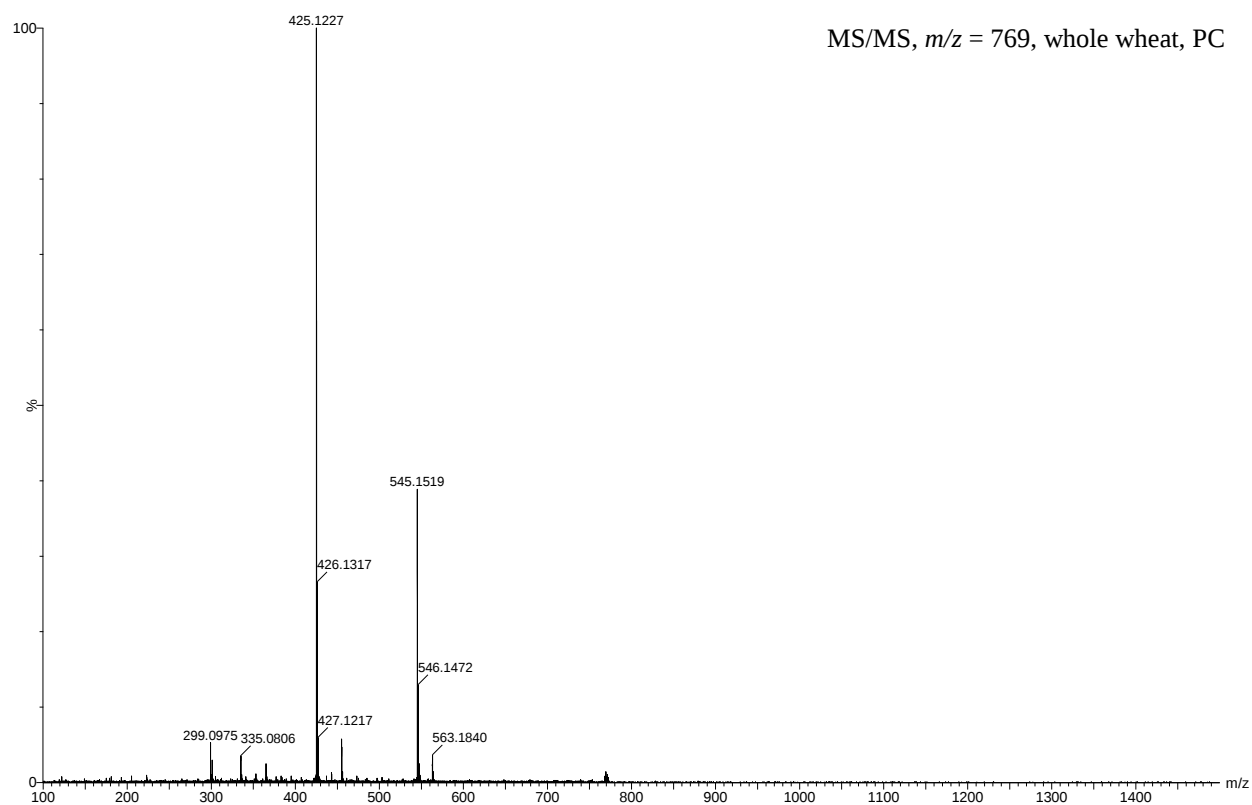


Figure 2.5. MS/MS spectrum of sinapic acid ester of apigenin-C-diglycosides in whole-wheat spaghetti, PC brand ($t_R = 31.32$ min).

Table 2.5 shows flavonoid glycosides contents (μg apigenin equivalent/g) of the regular spaghetti brands. The range of 6-*C*-glucosyl-8-*C*-arabinosyl content in regular spaghetti was 6.92 – 12.11 $\mu\text{g/g}$. The concentration was highest in Safeway, followed by Primo and No Name which did not show significant differences at $p < 0.05$. Splendor had the lowest concentration. The range of sinapic acid ester of apigenin-*C*-diglycosides content in regular spaghetti was 3.88 – 7.55 $\mu\text{g/g}$. The concentration in regular brands ranked as follows: Catelli > Safeway > Primo > No Name > Splendor. A similar trend among the regular spaghetti samples was observed in total phenolic content (TPC) and ferulic acid levels of the same brands. TPC was not significantly different among Catelli, Primo, No Name and Safeway brands, however, it was lower in Splendor. Ferulic acid content was not detected in Catelli, Primo and Splendor; however, it was detected in No Name and Safeway. Higher concentrations of phenolic compounds were found in Safeway and No Name than other brands. The higher contents were likely due to higher bran contamination in the wheat semolina used in making the spaghetti for two brands. The Safeway and No Name spaghetti brands were visually darker in color and both had higher fat content as indicated in the Nutrition Facts than Catelli, Primo and Splendor.

Table 2.6 shows flavonoid glycoside contents of the whole-wheat spaghetti brands (μg apigenin equivalent/g). Catelli Smart was used as a control for the whole-wheat spaghetti samples. The range of 6-*C*-glucosyl-8-*C*-arabinosyl content in whole-wheat spaghetti was 11.76 – 22.39 $\mu\text{g/g}$ and the content was 7.16 μg apigenin equivalent/g in Catelli Smart. PC and Eating Right had higher concentration than Catelli and Primo. The range of sinapic acid ester of apigenin-*C*-diglycosides content in whole-wheat spaghetti was 12.25 – 17.36 $\mu\text{g/g}$ and while Catelli Smart had only 3.88 $\mu\text{g/g}$. PC and Eating Right did not differ significantly and neither did Catelli and Primo as observed with 6-*C*-glucosyl-8-*C*-arabinosyl content.

Catelli Smart showed the lowest concentration for both flavonoid glycosides. Values of TPC and ferulic acid contents of the same brands were recently reported (Hirawan, et al., 2010). Catelli, Primo, PC and Eating Right showed slightly different TPC values while PC and Eating Right showed high ferulic acid content. Catelli and Primo showed lower ferulic acid content. Catelli Smart showed the lowest values for both TPC and ferulic acid analyses. PC has durum wheat semolina listed as the only ingredient and thus higher proportion of bran is likely to be present compared to the other whole-wheat spaghetti brands which may be diluted by other ingredients. Catelli Smart, that is supplemented with inulin fibre, showed the lowest concentration of phenolic compounds compared to whole-wheat spaghetti.

The average content of 6-*C*-glucosyl-8-*C*-arabinosyl was significantly higher in whole-wheat spaghetti (16.95 µg /g) compared to the regular brands (9.47 µg /g) at $p < 0.05$. Levels of the sinapic acid ester of apigenin-*C*-diglycosides were also significantly higher in whole-wheat spaghetti (15.15 µg /g) compared to the regular brands (5.83 µgg) at $p < 0.05$. Whole-wheat spaghetti brands were superior to the regular ones based on the content of flavonoid glycosides.

Table 2.5. Flavonoid glycosides and SDG contents of regular spaghetti from different brands^a

Sample Name	$m/z = 563^*$ (μg equivalent of apigenin/g)	$m/z = 769^+$ (μg equivalent of apigenin/g)	SDG [§] (μg SDG/g)
Catelli	7.86 d	7.20 a	13.8 d
Primo	9.81 c	5.37 b	17.7 bc
Splendor	6.92 e	3.88 c	15.4 cd
No Name	12.1 a	5.17 b	20.2 b
Safeway	10.6 b	7.55 a	24.3 a
LSD	0.29	0.38	2.47

^aLSD, Least Significant Difference at $p = 0.05$ level of probability. Mean values for regular spaghetti samples having similar letters in the same column are not significantly different.

*Hexosyl-pentosyl apigenin or 6-*C*-glucosyl-8-*C*-arabinosyl apigenin

⁺Sinapic acid adduct of apigenin-*C*-diglycosides

[§]secoisolariciresinol diglycoside

Table 2.6. Flavonoid glycosides and SDG contents of whole-wheat spaghetti from different brands^a

Sample Name	$m/z = 563^*$ (μg equivalent of apigenin/g)	$m/z = 769^+$ (μg equivalent of apigenin/g)	SDG (μg SDG/g)
Catelli Smart-Control	7.16 d	3.88 d	13.1 c
Catelli	15.9 b	17.2 a	37.1 b
PC	22.4 a	17.4 a	37.2 b
Primo	11.8 c	12.3 c	34.2 b
Eating Right	17.7 b	13.8 b	58.8 a
LSD	2.43	0.44	10.9

^aLSD, Least Significant Difference at $p = 0.05$ level of probability. Mean values for whole wheat spaghetti samples having similar letters in the same column are not significantly different.

*Hexosyl-pentosyl apigenin or 6-*C*-glucosyl-8-*C*-arabinosyl apigenin

⁺Sinapic acid adduct of apigenin-*C*-diglycosides

2.3.5. SDG contents of raw, regular versus whole-wheat spaghetti

SDG in Catelli Smart, a control sample for the spaghetti brands is shown in Figure 2.6 along with the extracted ion chromatograms showing the SDG peak. The MS/MS spectrum in Figure 2.7 showed $m/z = 686$ (14.5 – 15 min); MS, $[M - H]^-$ 686; MS/MS, $[M - H - \text{Glu} - \text{Glu}]^-$ 362 as also reported by Zhang and Xu (2007).

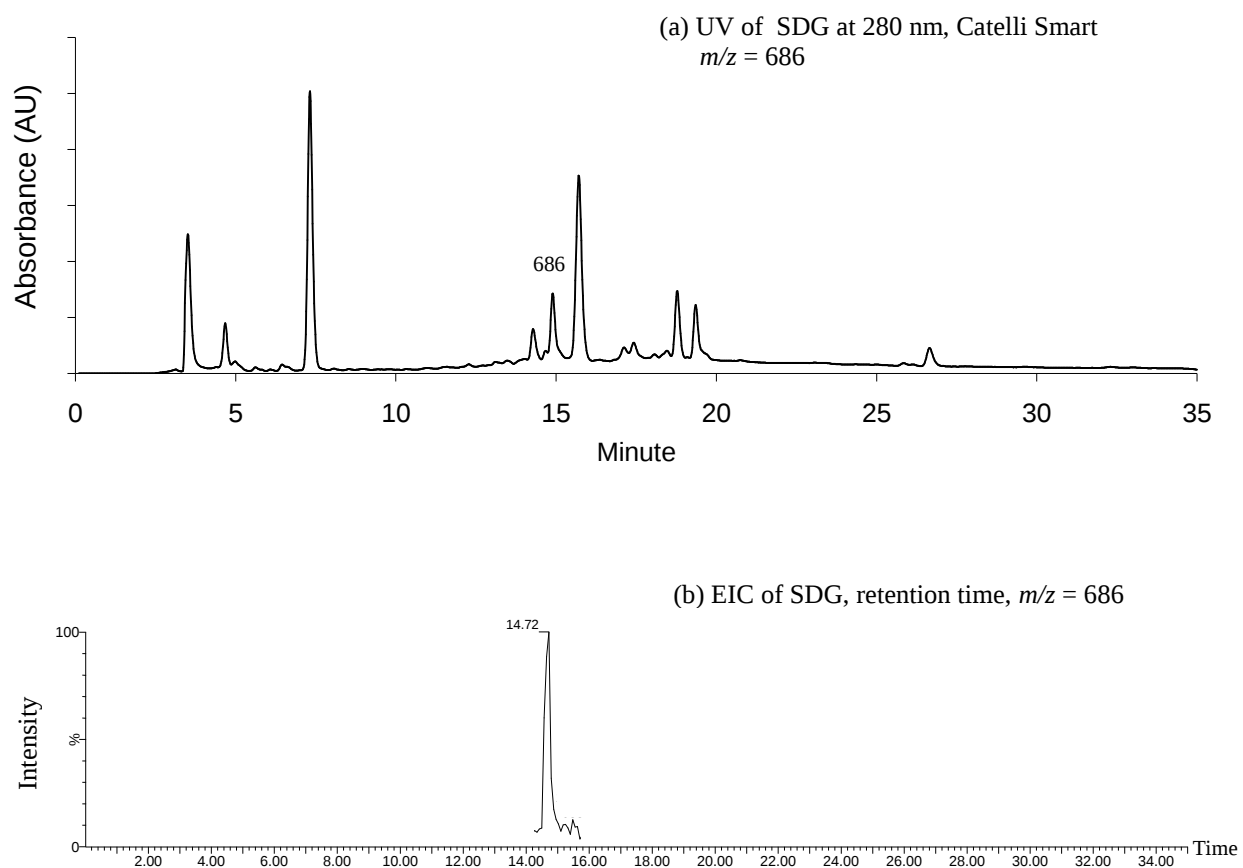


Figure 2.6. (a) Full LC chromatogram (0-35 min) of SDG obtained at 280 nm in control spaghetti, Catelli Smart and (b) extracted ion chromatogram (EIC) of SDG, ions at $m/z = 686$ for control spaghetti, Catelli Smart.

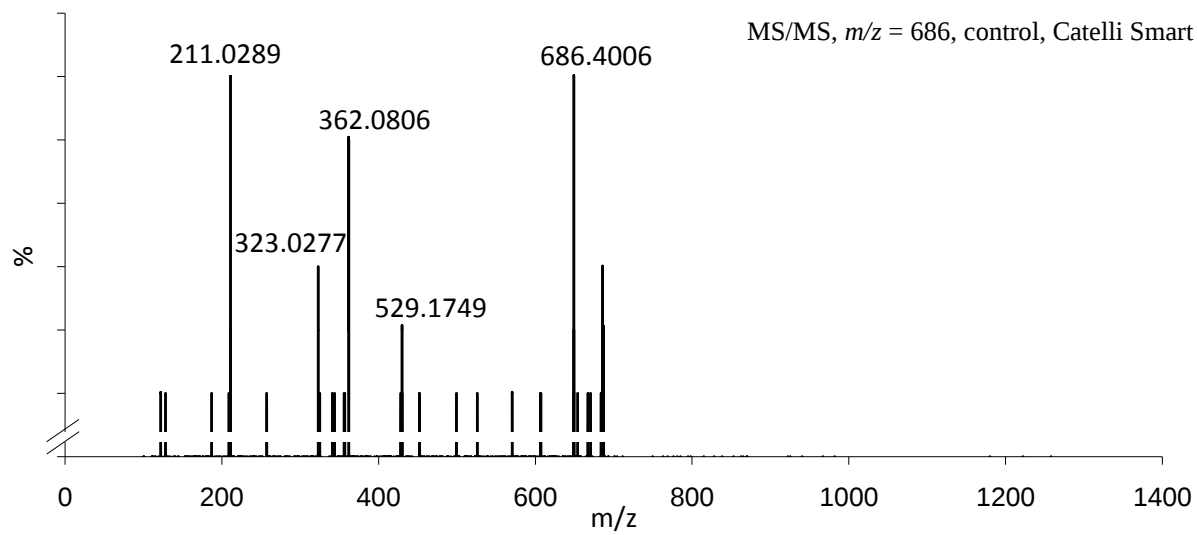


Figure 2.7. MS/MS spectrum of SDG in Catelli Smart.

Table 2.5 shows SDG content of regular spaghetti brands ($\mu\text{g SDG/g}$). SDG content in regular spaghetti ranged from 13.83 – 24.27 $\mu\text{g/g}$. Safeway had the highest concentration among the regular brands. Table 2.6 shows SDG content of whole-wheat spaghetti brands ($\mu\text{g SDG/g}$). The range of SDG content in whole-wheat spaghetti was 34.20 – 58.81 $\mu\text{g/g}$. Eating Right displayed the highest concentration. Catelli, PC and Primo did not show any significant differences. The control, Catelli Smart had the lowest SDG content of 13.08 $\mu\text{g/g}$. Catelli Smart is claimed to be white pasta with the fibre benefit of whole wheat. Antioxidant phenolic compounds are normally bound to fibre in whole wheat and thus by consuming whole wheat products both fibre and antioxidant benefits can be obtained. However, inulin fibre lacks the antioxidants of whole wheat fibre. The average content of SDG was significantly higher in whole-wheat spaghetti brands (41.8 $\mu\text{g/g}$) compared to the regular ones (18.3 $\mu\text{g/g}$), a confirmation that SDG is concentrated in the bran layers of wheat grain. Qu et al. (2005) found SDG concentration of 82.9 $\mu\text{g/g}$ in a wheat bran sample. In the present study, aqueous acid hydrolysis with higher temperature might have increased the efficiency of releasing SDG from the processed wheat product matrix while cultivar and growing conditions of the raw materials might also contribute to the variation in values. The concentration of the secoisolariciresinol aglycone has also been reported. Kuhnle et al. (2009) found secoisolariciresinol content of 3 $\mu\text{g/100 g}$ wet weight in raw whole wheat spaghetti samples as an average value of three spaghetti products while Smeds et al. (2009) found secoisolariciresinol content range of 20.0 – 42.5 $\mu\text{g/100 g}$ in wheat samples.

2.4. Conclusions

Whole-wheat spaghetti had significantly higher TPC, ferulic acid, C-glycosyl flavones, SDG contents and ORAC than the regular spaghetti. Spaghetti brands carried by supermarkets such as No Name and Safeway showed TPC, ferulic acid, C-glycosyl flavones, SDG contents and ORAC than the other regular spaghetti brands due to bran contamination. There were no significant differences in mean DPPH radical scavenging activity assay among the spaghetti samples. Catelli Smart generally showed phenolic phytochemical contents and antioxidant activities similar to regular spaghetti. While whole-wheat spaghetti imparts both fibre and antioxidant phytochemical benefits, Catelli Smart with inulin fibre added as an ingredient impart the benefit of fibre only. An approximately 40% reduction in total phenolic content of both regular and whole wheat spaghetti brands was observed after cooking. In regular brands, the three phytochemicals were present in amounts less than 50% of those found in whole wheat spaghetti. Whole-wheat products generally contain more health-beneficial components than refined grain products.

CHAPTER 3: Antioxidant potential of infant cereals produced from pigmented grains in comparison to commercial infant cereals and LC/MS analysis of their anthocyanins

ABSTRACT

Oxidation damage at the cellular level caused by endogenous and exogenous sources is a threat even at an early stage of life in infants. Thus, proper antioxidant protection is required through balance between oxidants and natural or dietary antioxidants. A comprehensive study is needed to investigate the possibility of improving the commercially available infant cereals based on the antioxidant properties. Pigmented grains of whole purple wheat, unpolished red rice and partially polished red rice and non-pigmented grain of brown rice were processed to produce home-made and lab-made infant cereals. The antioxidant properties were investigated through the measurement of total phenolic content (TPC), total anthocyanin content (TAC), oxygen radical absorbance capacity (ORAC), individual anthocyanins, C-glycosyl flavone and cellular antioxidant activity (CAA).

Home-made and lab-made pigmented infant cereals differed in that lab-made involved longer exposure to higher temperature and enzymatic hydrolysis. Home-made and lab-made unpolished red rice showed higher total phenolic contents and peroxy radical scavenging activity than whole purple wheat infant cereals. However, the latter had higher total anthocyanin contents. Infant cereal processing did not significantly influence the TPC, TAC and ORAC ($p < 0.05$). Pigmented infant cereals generally had higher TPC, TAC and ORAC than the commercial infant cereals showing their potential advantage over the latter. Anthocyanins were identified in whole purple wheat, however, they were not detected in unpolished red rice. Apigenin C-

glycosides and some di-esters of cinnamic acids were found in both whole purple wheat and unpolished red rice. Whole purple wheat showed significantly higher levels of cyanidin 3-glucoside and apigenin C-glycosides than unpolished red rice. Processing significantly decreased the cyanidin 3-glucoside and apigenin C-glycoside contents ($p < 0.05$). Dichlorofluorescein diacetate (DCFH-DA) assay in the cell culture assay showed that whole purple wheat infant cereals had higher CAA than unpolished red rice ones and that home-made infant cereals had higher CAA than lab-made ones. Whole purple wheat infant cereals showed higher antioxidant activity than the commercial infant cereal suggesting a possibility of improving infant antioxidant status by incorporating this grain in their diet.

3.1. Introduction

Infancy is the most important stage in life where one grows most rapidly. Oxidation damage at the cellular level caused by endogenous and exogenous sources is a threat at this early stage of life and thus proper antioxidant protection is required (Almaas et al., 1997; de Lima et al., 2005; Rosenfeld and Davis, 1998; Saugstad, 1998). Balance between oxidants and natural antioxidants or antioxidants obtained through diet must be maintained (Saugstad, 1998). Infant cereals are commonly introduced to infants at 4-6 months of age. Infant cereals may have the antioxidant potential that help maintain the above balance. An inverse relationship between intake of whole grains and their products and the risk of chronic diseases has been shown in epidemiological studies (Anderson, 2003; Chatenoud et al., 1998; Jacobs et al., 1998a; Jacobs et al., 1998b; Jacobs et al., 2001; Kasum et al., 2002; Koh-Banerjee and Rimm, 2003; Munter et al., 2007; Soler et al., 2001). Various forms of phytochemicals in cereal grains (Adom and Liu,

2002; Kim et al., 2006; Liyana-Pathirana and Shahidi, 2006; Naczk and Shahidi, 2006) affect their bioavailability in the human body and protects against oxidative stress.

Commercially available infant cereals come in a wide variety of compositions according to the developmental and growth stage of the infant. Infant cereals mainly comprise of cereal grains but there are additional ingredients such as fruits, prebiotics, added nutrients (iron and vitamins), emulsifier, whey protein concentrate, honey, plant oils, milk and beetroot powder. Issues regarding the use of these semi-solid foods include stage of introduction and allergic reactions. Processing of infant cereals include toasting, boiling, drying and also hydrolysis to improve flavour and texture qualities, digestibility (mainly of starch), safety and shelf life (Fernandez-Artigas et al., 2001). There are innate preferences for sweet and salty flavours while there is usually rejection for sour and bitter flavours (Crook, 1978; Birch, 1999).

Pigmented grains have been shown to have higher antioxidant potential than the regular non-pigmented grains (Cooke et al., 2005; Goffman and Bergman, 2004; Li et al., 2005; Nam et al., 2006; Toyokuni et al., 2002).

Red rice and purple wheat were selected for the model study of pigmented infant cereals. Anthocyanin profiles in black and red rice are relatively simple. They are found mainly in the form of cyanidin 3 – glucoside (Abdel-Aal et al., 2006; Hiemori et al., 2009; Hu et al., 2007; Hu et al., 2003; Ryu et al., 1998; Xia et al., 2006). Cyanidin 3-glucoside is also the predominant anthocyanin in purple wheat, however, purple wheat has a more complicated anthocyanin profile (Abdel-Aal and Hucl, 1999; Abdel-Aal and Hucl, 2003; Abdel-Aal et al., 2006; Dedio et al., 1972; Hossenian et al., 2008; Hu et al., 2007). Condensed tannins are also found in purple and red rice (Min et al., 2009). Red rice and purple wheat have shown antioxidant activities *in vitro* and *in vivo* models (Choi et al., 2007; Cooke et al., 2005; Frei and Becker, 2005; Goffman and

Bergman, 2002; Goffman and Bergman, 2004; Hiemori et al., 2009; Hu et al., 2007; Li et al., 2005; Li et al., 2007a; Li et al., 2007b; Ling et al., 2001; Ling et al., 2002; Nam et al., 2006; Toyokuni et al., 2002).

Studies have investigated the processing of infant cereals with regards to their sugar profile (Fernandez-Artigas et al., 2001) and iron fortification (Almaas et al., 1997; Rios et al., 1975). Gu et al. (2004) estimated the content of proanthocyanidins in infant foods and infant intake of proanthocyanidins. Dicks-Bushnell and Davis (1967) determined the vitamin E content of infant formulas and cereals. The present study aimed to prepare prototype infant cereals using pigmented grains with and without amylase enzymes. Commercial infant cereals were used for comparison. Total phenolic content, total anthocyanin content and total antioxidant activity of the selected commercial infant cereals and the pigmented infant cereals were determined. Key antioxidant phytochemicals in the pigmented infant cereals were identified and quantified. The antioxidant properties of selected samples were also investigated using a cell culture assay.

3.2. Materials and Methods

3.2.1. Samples

Brown rice was used as a control sample representing non-pigmented grain. The samples included:

(1) Raw grains (Figure 3.1):

- a. Raw partially polished red rice: Red Raw Rice, produced by M&M Twins Limited, Scarborough, ON, Canada, imported from Srilanka
- b. Raw unpolished red rice: Red Cargo Rice, produced by BangSue Chia Meng Rice Mill Co. Ltd., Bangkok, Thailand, imported from Thailand
- c. Raw unpolished brown rice: short grain, organic, purchased at Real Canadian Superstore, Winnipeg, MB, Canada

- d. Raw purple wheat: AnthoGrain™ wheat, developed by Crop Development Centre, University of Saskatchewan, Saskatoon, Canada and provided by Infraready Products (1998) Limited, Saskatoon, SK, Canada

The two red rice samples, brown rice and purple wheat samples were tested as is (raw grains) and also made into home-made and lab-made infant cereals.

(2) Infant cereals made from pigmented grains (Figure 3.2 and 3.3):

- a. Home-made and lab-made partially polished red rice
- b. Home-made and lab-made unpolished red rice
- c. Home-made and lab-made unpolished brown rice
- d. Home-made and lab-made whole purple wheat

Nine commercial samples were purchased from major supermarkets in Winnipeg, Canada and they were selected according to their ingredients and introduction stage (Table 1.1).

(3) Commercial infant cereals:

- a. Stage 1:
 - i. Rice Cereal, Beech Nut (Beech-Nut Nutrition Corporation, Canajoharie, NY, USA)
 - ii. Rice Cereal, Heinz (Heinz Canada, North York, ON, Canada)
 - iii. Brown Rice Cereal, Organics for Baby (Lucerne Foods, Calgary, AB, Canada)
- b. Stage 2:
 - i. Mixed Cereal, Heinz (Heinz Canada, North York, ON, Canada)
 - ii. Mixed Grains Cereal, Nestle (Nestle Canada Inc., North York, ON, Canada)
- c. Stage 3:
 - i. Wheat and Oat Cereal with Banana and Raspberry, Heinz (Heinz Canada, North York, ON, Canada)
 - ii. Rice Cereal with Apple and Pear, President's Choice® Organics™ (PC Organics) (Loblaw Inc., Toronto, ON, Canada)
- d. Stage 4:

- i. Multigrain Cereal with Mango, Pineapple and Pear, Heinz (Heinz Canada, North York, ON, Canada)
- ii. Mixed Cereals with Fruits, Milupa, Danone (Van de Water-Raymond LTD, Laval, QC, Canada)



Figure 3.1. Raw grains (from left to right: brown grains, partially polished red rice, unpolished red rice and whole purple wheat).



Figure 3.2. Home-made (left) and lab-made (right) unpolished red rice infant cereals.



Figure 3.3. Rehydrated infant cereals. LBR: lab-made unpolished brown rice. LRR: lab-made partially polished red rice. LRC: lab-made unpolished red rice. B1: Beech Nut, Stage 1. N2: Nestle, Stage 2. O1: Organics for Baby, Stage 1.

3.2.2. Chemicals

Folin-Ciocalteu's phenol reagent, 2,2'-azobis (2-amidinopropane) dihydrochloride (APPH) and ferulic acid standard were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Fluorescein (FL) and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Fisher Acros Organics (Morris Plains, NJ, USA). Alpha-amylase enzyme extracted from *Bacillus subtilis* was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 2',7'-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Invitrogen Canada, Inc. (Burlington, ON, Canada). The cyanidin 3-glucoside standard was purchased from Sigma-Aldrich® (Oakville, ON, Canada). The purity was 99.9%. The apigenin standard was obtained from Chromadex™ (Irvine, CA, USA) graded Primary Standards (P) for quantitative validation. The adjusted purity was 97.5%.

HPLC grade methanol was used in the extraction. Hydrochloric acid for extraction was purchased from Fisher Acros Organics (Morris Plains, NJ, USA). MS grade acetonitrile and formic acid were used in the LC/MS analysis. HPLC and MS grade solvents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

3.2.3. Sample preparation

The production procedures for home-made and lab-made infant cereals made from brown rice, partially polished red rice (red rice 1), unpolished red rice (red rice 2) and purple wheat are summarized in Figure 3.4 and 3.5. The cooking method for home-made pigmented infant cereals was adapted from a method by Edelman (2010). Water (355,5 mL) was added to 64.5 g of ground grain ($\pm$ 20% solid in water) prior to boiling for 10 min while stirring at all times. The mixture was then cooled for 30 min. Infant cereals were frozen prior to freeze-drying (VirTis,

Genesis-Pilot Lyophilizer, SP Industries, Warminster, PA). The processing method for lab-made pigmented infant cereals was adapted from a patented method by (Gil et al., 1991, 1994). Ground grain (50 g) was placed in an air oven and toasted uncovered at 120°C (DL-110A-2 Stabil-Therm Constant Temperature Cabinet, Blue M Electric Co., Blue Island, IL, USA) for 30 min. The sample was then cooled for 30 min and dispersed in 250 mL RT water (20% solid in water) while whisking at all times. A small amount of powdered enzyme (0.02 g of α -amylase, *Bacillus subtilis*) was dispersed in 5 mL of water and added to the grain mixture to allow for enzymatic hydrolysis of starch. The whole mixture was then placed in a 60°C-water bath for 90 min with stirring every 5 min. The mixture was boiled (90°C – 100°C) for 2 min while stirring at all times. The sample was then cooled for 1 hr. Infant cereals were frozen prior to lyophilization (VirTis, Genesis-Pilot Lyophilizer, SP Industries, Warminster, PA).

A sample mill (Black & Decker, Hunt Valley, MD) was used to grind the raw grains and freeze-dried home-made and lab-made infant cereals so as to pass through a screen of 0.42 mm.

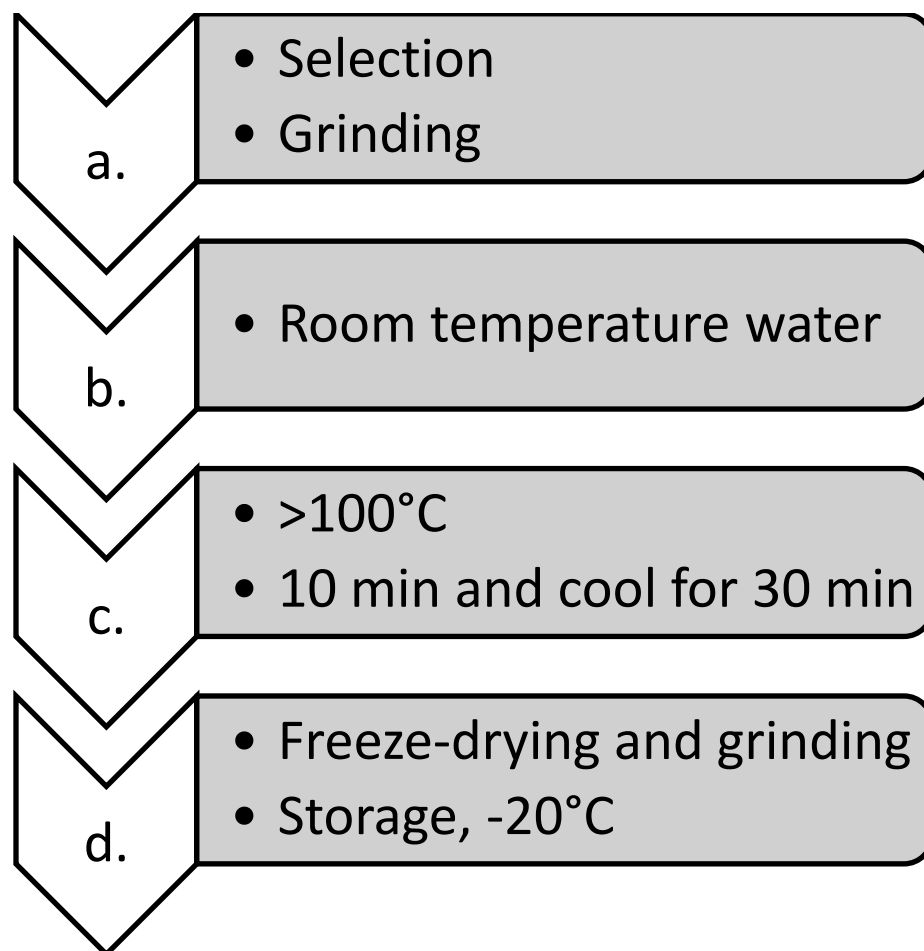


Figure 3.4. Home-made infant cereal production procedure: a. ground cereal; b. dispersion in water; c. heating and stirring; d. storage for analysis.

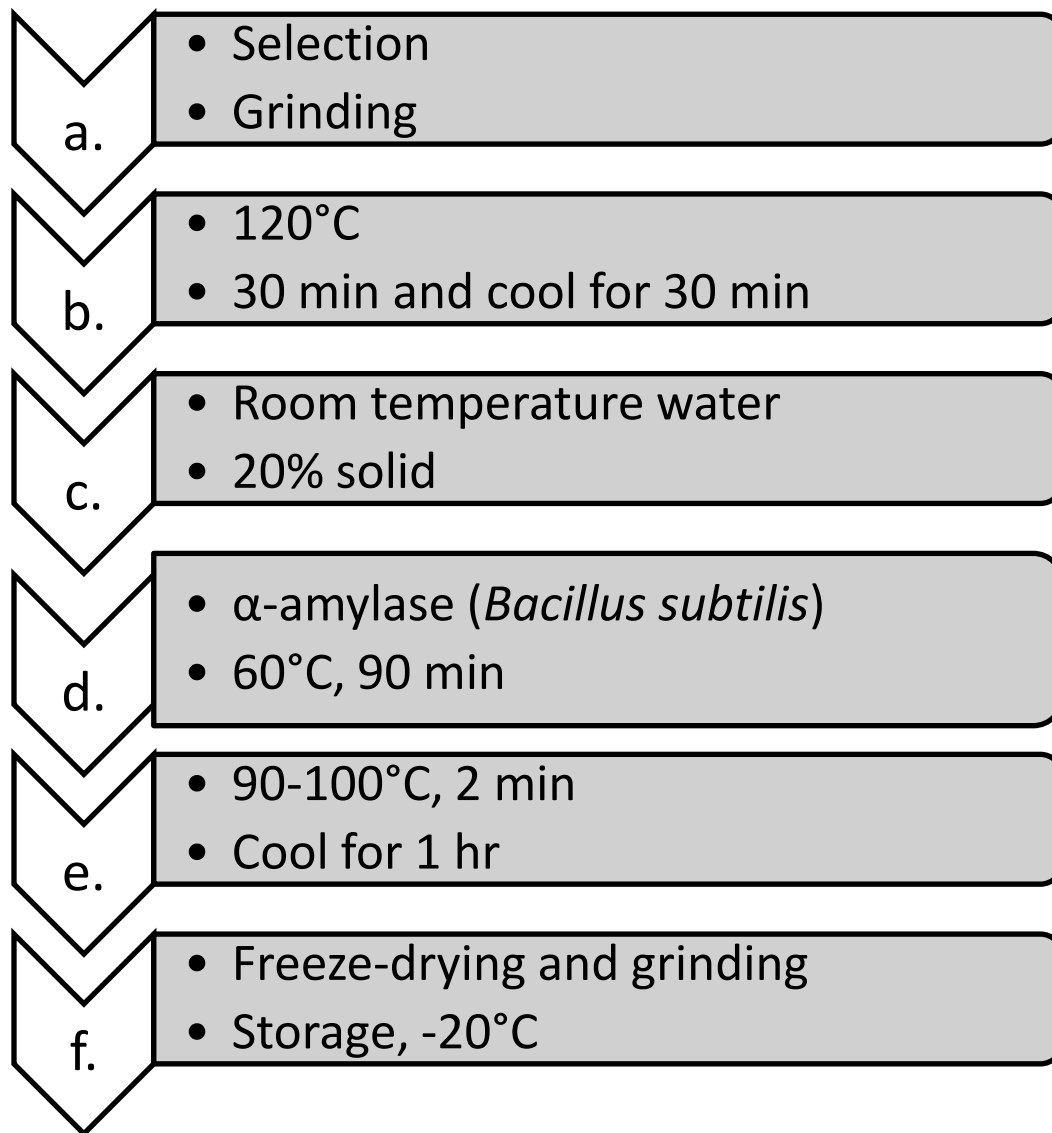


Figure 3.5. Lab-made infant cereal production procedure: a. ground cereal; b. toasting; c. dispersion in water; d. enzymatic hydrolysis; e. hot-plate boiling; f. storage (Gil et al., 1991, 1994).

3.2.4. Moisture content determination

Ground, raw and freeze-dried home-made and lab-made infant cereals were weighed. They were then dried overnight at 100°C in an air oven following the AOAC method 930.15 (1990) for moisture determination. All samples were analyzed in triplicate.

3.2.5. Extraction

The extraction of antioxidant components in raw grains, infant cereals made from pigmented grains and commercial infant cereals was adapted from methods by Li et al. (2007b). Finely ground infant cereals (1 g) were extracted with 1 M HCl/methanol (v/v, 15/85) (10 mL). The mixture was sonicated (Bransonic 5510OR-DTH, Branson Ultrasonic Corporation, Danbury, Connecticut, USA) for 45 min, shaken every 5 min and centrifuged at 26,920 x *g* (15,000 rpm, SSA-34 Rotors, RC5C Sorvall Instruments, Thermo Scientific, Asheville, NC, USA) at ambient temperature for 15 min. The supernatant was collected and the centrifuge pellet was washed. The supernatants were stored at -20°C for determination of total phenolic content (TPC), total anthocyanin content (TAC) and oxygen radical absorbance capacity (ORAC). Extraction for LC/MS analysis and cell culture assay followed the same procedure until this point. The supernatant was recentrifuged to remove precipitation. The supernatant was then rotary-evaporated at 35°C for about an hour (IKA IKA® RV10, IKA® Works, Inc., North Carolina, USA). Two mL of 80% methanol for LC/MS analysis and 2 mL of distilled and deionized water for cell culture assay were added to reconstitute the samples from the rotary-evaporator, sonicated and micro-centrifuged at 12,000 rpm for 5 min (IEC Micromax Microcentrifuge, Thermo Fisher Scientific Inc., Waltham, MA) and filtered with 0.45 µm PTFE

filter. Samples were then analyzed using HPLC-DAD and QTOF-MS (Waters Corp., Milford, MA).

3.2.6. Chemical model assays

3.2.6.1. Total phenolic content (TPC) determination

The TPC of the extracts from infant cereals was measured using a modified method based on a method by Singleton and Rossi (1965). Modifications were as stated in Chapter 2 (Section 2.2.6.1). All analyses were conducted in triplicate.

3.2.6.2. Total anthocyanin content (TAC) determination

The TAC of the infant cereals was determined based on a method by Abdel-Aal and Hucl (1999). Acidified methanol (1 M HCl/methanol, 15/85, v/v) (24 mL) was added to ground samples (commercial samples were used as is) (3 g) and the mixture was shaken manually. The mixture was then adjusted to pH 1 using 6 N HCl. The sample was sonicated (Bransonic 5510OR-DTH, Branson Ultrasonic Corporation, Danbury, Connecticut, USA) for 15 min with shaking every 5 min. The sample was centrifuged at $26,920 \times g$ (15,000 rpm, SSA-34 Rotors, RC% C Sorvall Instruments, Thermo Scientific, Asheville, NC, USA) at ambient temperature for 15 min. The supernatant collected was made to 50 mL with acidified methanol. Acidified methanol was used as the blank and absorbance was read at 535 nm. The absorbance read at 535 nm showed linear relationship with the concentration producing the standard curve of cyanidin 3-glucoside in acidified ethanol. Beer's law was used to calculate the molar absorptivity of cyanidin 3-glucoside. Calculation of total concentration anthocyanins (Abdel-Aal and Hucl, 1999) is as follows:

$$C = (A/\epsilon) \times (\text{vol}/1,000) \times \text{MW} \times (1/\text{sample wt}) \times 10^6$$

where C is concentration of total anthocyanin ($\mu\text{g/g}$), A is absorbance reading, ϵ is molar absorptivity (cyanidin 3-glucoside = $25,965 \text{ cm}^{-1} \text{ M}^{-1}$), vol is total volume of anthocyanin extract (50 mL), and MW is molecular weight of cyanidin 3-glucoside = 449. The calculation is simplified to: $C = A \times 288.21 \mu\text{g/g}$. Analysis was conducted using 4 replicates.

3.2.6.3. Oxygen radical absorbance capacity (ORAC) determination

The ORAC values were obtained using a modified method based on a method by Huang et al. (2002). Modifications were as stated in Chapter 2 (Section 2.2.6.3.). All analyses were conducted in triplicate.

3.2.7. HPLC/MS/MS analysis

Cyanidin 3-glucoside, peonidin 3-glucoside, apigenin 6-C-glucoside-8-C-arabinoside and cinnamic acid esters were separated, identified and quantified in the infant cereals by using an HPLC (Separation Module, Waters 2695) with a photodiode array detector (Waters 2996) and an autosampler (Waters 717plus). The HPLC was coupled to a quadrupole time-of-flight mass spectrometer (Q-TOF MS) (Micromass, Waters Corp., Milford, MA). Analysis was performed with a Gemini $5\mu \text{ C}_{18}$ 110 Å column (150 mm x 4.60 mm) (Phenomenex®, Torrance, CA). A gradient of solvent A (1% formic acid in water) and solvent C (1% formic acid in acetonitrile) was used for 50 min at a flow rate of 0.5 mL/min. The gradient was as follows: at 0 min 5% C, 5 min 10% C, 15 min 15% C, 20 min 20% C, 30 min 25% C, 40 min 40% C, 45-50 min 10% C. The photodiode array detector was set at 280, 320 and 520 nm. The sample was then introduced into the Q-TOF MS. The Q-TOF MS was calibrated with sodium iodide in the negative mode

and full mass spectra were obtained with capillary voltage of 1.8 kV and cone voltage of 45 V. Desolvation gas (N₂) and cone gas (He) were used at flow rates of 900 and 50 L/hr, respectively. The temperature settings for the desolvation gas and ion source were 350 and 150°C, respectively. Identification of C-glycosyl flavones was achieved by using tandem mass spectrometric technique with collision energy of 30 kV and quantification was done using apigenin standard. The analyses were done in duplicate.

3.2.8. Cell culture assay

3.2.8.1. Cell line

The present study used human fetal small intestine cell line (FHs 74 Int, CCL-241™, American Type Culture Collection (ATCC®), Manassas, VA, USA) to further investigate the *in vitro* effects of cereal grain consumption at an human early age in relation to cellular oxidation. This is an epithelial cell line obtained from the small intestine of a female 3 to 4 months-old normal fetal. The growth medium was 5 mL of Dulbecco's Modified Eagle Medium with D-glucose content (an energy source) (DMEM, Invitrogen Canada Inc., Burlington, ON, Canada) supplemented with:

1. 10% fetal bovine serum (a widely used serum-supplement with low level of antibodies and greater growth factors)
2. 10 mg/mL penicillin/streptomycin (bacterial contamination elimination and prevention agents)
3. 200 mM L-glutamine (an essential amino acid for growth factor)
4. 1 mg/mL insulin (a growth factor)
5. 20 mg/mL extra cellular growth factor (EGF)
6. 2.5 nM sodium pyruvate (an additional source of energy and protective agent against hydrogen peroxide)
7. 1 mg/mL human transferrin (an iron-transporting agent as a factor for active cell division)

The growth medium was changed every 5 days. EDTA (0.02%) and trypsin (0.25%) were used during the routine passage of the cell lines. Cells were incubated at 37°C in a 5% CO₂ humidified incubator for optimal growth. EDTA and trypsin solutions were used during routine passage of the cell line to detach and re-suspend cells from the cell culture flask surface. EDTA chelates calcium and prevents calcium-dependent adhesion between cells, prevents clumps of cells in liquid suspension.

3.2.8.2. Cytotoxicity assay

Home-made and lab-made whole purple wheat and unpolished red rice infant cereals and Stage 3 Heinz Wheat and Oat Cereal with Banana and Raspberry infant cereal (Heinz Canada, North York, ON, Canada) were tested for their cytotoxicity using the MTT cell proliferation assay (ATCC[®], Manassas, VA, USA) according to a method by Ellis et al. (2010) with some modifications. Home-made, lab-made and commercial sample water-extracts (500mg/mL) were further diluted with medium to concentrations of 2 mg/mL and 0.5 mg/mL. An infant small intestine cell suspension (100 µL) was seeded in a 96-well plate and maintained until confluent (a concentration of 1×10^5 /mL) for 72 hours. The medium in all wells was then aspirated out, 100 µL of 2 mg/mL and 0.5 mg/mL sample medium-extracts and 100 µL of fresh medium for control wells were added. The blank wells contained medium only. The cells were treated and left in the incubator for 4 hrs. The yellow-coloured MTT reagent (10 µL) was added to all wells including control wells (without removing treatment solutions and medium in order not to disrupt the cells) and left in the incubator for 3 hours. Dots of purple precipitate were visible under the microscope and 100 µL of detergent reagent was added to all wells including controls. The plate was swirled gently. The covered plate was then left in the dark overnight at room

temperature. The plate cover was removed and the plate was measured for absorbance at 590 nm using Opsys MR 96-well plate reader (DYNEX Technologies, Chantilly, VA, USA). Cytotoxicity level (%) was calculated by comparing absorbance to that of controls. Analysis was done in 8 replicates.

3.2.8.3. Dichlorofluoresin diacetate (DCFH-DA) assay

The infant cereals were then tested for their effects in cellular oxidative status using the cellular antioxidant activity (CAA) method by Wolfe and Liu (2007) with some modifications. Home-made, lab-made and commercial sample water-extracts (500mg/mL) were further diluted with medium to concentrations of 2 mg/mL and 0.5 mg/mL. An infant small intestine cell suspension (100 μ L) was seeded in a 96-well plate and maintained until confluent (a concentration of 1×10^5 /mL) for 72 hours. The medium in all wells was then aspirated out, 100 μ L of 2 mg/mL and 0.5 mg/mL sample medium-extracts and 100 μ L of fresh medium for control wells were added. Blank wells contained medium only. The cells were treated and left in the incubator for 1 hr. At 30 min incubation time, 100 μ L of 10 μ M DCFH-DA solution was added to all wells including positive (control with AAPH oxidant added) and negative (control without AAPH oxidant added) controls. The final concentration of the DCFH-DA solution was then 5 μ M. After 1 hr incubation time, all 200 μ L of treatment solutions were removed and 100 μ L of 500 μ M AAPH dissolved in Hank's Buffered Salt Solution (HBSS) was added to all wells except negative control wells (100 μ L of HBSS was added instead). The plate was then immediately placed into a Fluoroskan Ascent FL 96-well plate reader (ThermoLabsystems, Franklin, MA, USA). The temperature was set at 37°C, emission wavelength at 527 nm, excitation wavelength at 485 nm and measurements taken every 15 min for 1 hr. Analysis was

done in 8 replicates. The cellular antioxidant activity (CAA) value was calculated by integrating the area under the curve of fluorescence versus time at each concentration (2 mg/mL and 0.5 mg/mL) of home-made, lab-made and commercial infant cereal sample extracts as follows:

$$\text{CAA value} = \{1 - (\int SA / \int CA)\} \times 100$$

Where *SA* was the area under sample fluorescence versus time curve and *CA* was area under positive control fluorescence versus time curve.

3.2.9. Statistical analysis

All data were converted to dry weight basis and reported as means of duplicate or triplicate analyses. One-way analysis of variance of results was performed using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC). Significant differences among sample means for raw grains, home-made, lab-made and commercial infant cereals for each analysis were tested using Tukey's test at $p < 0.05$. Pearson correlation coefficient was calculated for TPC and ORAC.

3.3. Results and Discussion

3.3.1. Home-made and lab-made pigmented infant cereal production

Home-made infant cereals are often made by mothers keen to ascertain the ingredients fed to their infants. Lab-made formulations were taken to represent industrial made infant cereals. Starch microscopy with bright field showed complete starch gelatinization which means the processing methods sufficiently cooked the pigmented infant cereals. High temperatures (boiling at 90°C – 100°C) partially deactivated α -amylase (Sapirstein, 2009). Freeze-drying likely completely deactivated the enzyme. Wang, Hey and Nail (2004) found significant loss of

α -amylase activity in the α -amylase collapsed material over the freeze-drying process. A collapse is a process that happens during freeze-drying and is defined as “ the loss of microstructure that was established by freezing due to the viscous flow of amorphous material during primary drying (sublimation of ice) or secondary drying (removal of water that did not freeze” (Wang, Hey and Nail, 2004). Rehydrated samples had the same overall texture as commercial samples (Figure 3.3.). Pigmented infant cereals appeared smoother because samples were ground completely while the commercial ones are in the form of flakes. The viscosity of solutions when stirred manually was found to be similar between pigmented infant cereals and commercial ones. The colour of commercial infant cereals is mostly yellowish white while the organic unpolished brown rice has darker yellow colour. The colour of pigmented infant cereals was similar to the original grain colour.

3.3.2. Total phenolic content (TPC), total anthocyanin content (TAC) and antioxidant activity of raw grains, pigmented and commercial infant cereals

The TPC, TAC and antioxidant activity results are reported in Table 3.1 TPC was expressed as micrograms of ferulic acid equivalent (FE) per gram ($\mu\text{g/g}$) of infant cereal. TPC ranged from 532 to 8626 $\mu\text{g/g}$ ($p < 0.05$). Raw unpolished red rice had the highest TPC while Beech-Nut Rice Cereal had the lowest TPC. The average TPC for raw grains and infant cereals were not significantly different ($p < 0.05$). The average TPC for infant cereals was significantly higher than that of the commercial infant cereals ($p < 0.05$). The highest TPC among the sample groups was observed in the unpolished red rice samples in the order: raw grain > home-made infant cereal > lab-made infant cereal ($p < 0.05$). Heinz Stage 3 Wheat & Oat Cereal with Banana & Raspberry had the highest TPC among the commercial sample group.

TAC was expressed as micrograms of cyanidin 3-glucoside equivalent per gram ($\mu\text{g/g}$) of infant cereal. TAC ranged from 2 to 251 $\mu\text{g/g}$ ($p < 0.05$). Raw whole purple wheat had the highest TAC while Heinz stage 4 Multigrain Cereal with Mango, Pineapple & Pear had the lowest TAC. Anthocyanins were not detected in brown rice samples, Organics for Baby Stage 1 Brown Rice Cereal, Beech-Nut Stage 1 Rice Cereal, Heinz Stage 1 Rice Cereal. The average TAC for raw grains and infant cereals were not significantly different ($p < 0.05$). The average TAC for pigmented infant cereals was higher; however, it was not significantly different from that of the commercial infant cereals ($p < 0.05$). The highest TAC among pigmented sample groups was observed in the whole purple wheat samples in the order: raw grain > home-made infant cereal > lab-made infant cereal ($p < 0.05$). Nestle Stage 2 Mixed Grains Cereal and Heinz Stage 3 Wheat & Oat Cereal with Banana & Raspberry had the highest TAC among the commercial sample group.

Oxygen radical absorbance capacity (ORAC) was expressed as micromoles of Trolox equivalent (TE) per gram ($\mu\text{mol/g}$) of infant cereal. ORAC ranged from 5.9 to 87.1 $\mu\text{mol TE/g}$ ($p < 0.05$). Raw unpolished red rice had the highest ORAC while Beech-Nut Rice Cereal had the lowest ORAC value. The average ORAC for raw grains and infant cereals were not significantly different ($p < 0.05$). The average ORAC for infant cereals was significantly higher than that of commercial infant cereals ($p < 0.05$). The highest ORAC value was observed in the unpolished red rice samples in the order: raw grain > home-made infant cereal = lab-made infant cereal ($p < 0.05$). Heinz Stage 3 Wheat & Oat Cereal with Banana & Raspberry had the highest ORAC value among the commercial samples.

Table 3.1. Total phenolic content (TPC), Total anthocyanin content (TAC) and Oxygen radical absorbance capacity (ORAC) of raw grains, home-made, lab-made pigmented infant cereals and commercial infant cereals*

No.	Sample	TPC (μg ferulic acid equivalent/g)*				TAC (μg cyanidin 3-glucoside equivalent/g)*				ORAC (μmol Trolox equivalent/g)*			
1	Whole purple wheat	4469	±	34.6	e	251	±	0.83	a	43.3	±	2.22	f
2	Unpolished brown rice (control)	2079	±	88.2	jk	nd			-	31.3	±	0.44	hi
3	Partially polished red rice	5232	±	136	d	15	±	0.18	h	67.1	±	2.80	c
4	Unpolished red rice	8626	±	207	a	23	±	0.59	e	87.1	±	0.42	a
5	Home-made whole purple wheat	3076	±	55.1	h	185	±	0.49	b	48.2	±	0.21	e
6	Home-made unpolished brown rice	1712	±	64.5	l	nd			-	29.1	±	0.22	i
7	Home-made partially polished red rice	3559	±	62.5	f	9	±	0.27	j	50.4	±	2.26	de
8	Home-made unpolished red rice	6622	±	145	b	20	±	0	f	79.0	±	0.66	b
9	Lab-made whole purple wheat	3390	±	49.2	fg	95	±	0.43	c	51.6	±	0.35	d
10	Lab-made unpolished brown rice	2090	±	52.7	jk	nd			-	37.4	±	0.30	g
11	Lab-made partially polished red rice	3352	±	92.9	fg	11	±	0.47	i	52.4	±	1.13	d
12	Lab-made unpolished red rice	6152	±	98.7	c	22	±	0.55	e	79.0	±	1.07	b
13	Brown rice cereal (Organics for Baby)	1972	±	29.3	l	nd			-	18.7	±	0.53	k
14	Rice cereal (Beech-Nut)	532	±	27.5	n	nd			-	5.9	±	0.04	m
15	Mixed grains cereal (Nestle)	2235	±	57.7	j	39	±	0.68	d	12.5	±	0.17	l
16	Rice cereal with fruits (PC Organics)	3265	±	27.8	gh	10	±	0.23	i	21.1	±	0.19	jk
17	Mixed cereals with fruits (Milupa)	2242	±	15.3	j	17	±	1.22	g	31.9	±	0.44	hi
18	Rice cereal (Heinz)	963	±	34.1	m	nd			-	10.1	±	0.12	l
19	Mixed cereal (Heinz)	2060	±	8.66	jk	4	±	0.14	k	23.9	±	1.19	j
20	Wheat and oat cereal with fruits (Heinz)	4235	±	45.8	e	39	±	0	d	33.8	±	0.48	h
21	Multigrain cereal with fruits (Heinz)	2567	±	47.3	i	2	±	0.15	l	19.8	±	0.49	k

* LSD, Least Significant Difference at $p = 0.05$ level of probability. $n = 3$ (TPC), $n = 4$ (TAC) and $n = 3$ (ORAC). Mean values for samples having similar letters in the same column are not significantly different. nd is not detected.

Among the raw grains, red rice showed the highest TPC and ORAC value while brown rice had the lowest values as expected. Pigmented infant cereals showed the same trend in their TPC and ORAC values as the raw grains in the order: unpolished red rice > partially polished red rice > whole purple wheat > unpolished brown rice. A study found that brown rice had a TPC range of 981 – 1126 µg FE/g and ORAC values ranging from 14 – 18 µmol Trolox equivalent/g (Aguillar-garcia et al., 2007). In the present study, the brown rice had 2079 µg/g and 31.3 µmol TE/g ORAC. Organic brown rice used in the present study might contribute to the higher TPC and ORAC value. The TPC of unpolished red rice found by Shen et al. (2009) was 8353 µg FE/g. The TPC of unpolished red rice (8626 µg FE/g) agreed with the previous finding. Li et al. (2007a) found TPC and ORAC value for acidified methanol extracts of 7973 µg FE/g and 209.72 µmol TE/g of purple wheat bran respectively while the present study found 4469 µg FE/g of whole purple wheat and 43.3 µmol TE/g respectively. The TPC and ORAC value of the present study were lower due to dilution by the endosperm material. The TPC and ORAC values had a good correlation (Pearson correlation coefficient = 0.90). To our best knowledge, this is the first study comparing the total phenolic content and antioxidant activity of purple wheat versus red rice.

Raw, whole purple wheat had the highest TAC while anthocyanins were not detected in raw, unpolished brown rice. Raw, unpolished red rice had significantly higher TAC than the partially polished one, an indication that anthocyanins are concentrated in the bran layer. Pigmented infant cereals showed the same trend in their TAC with the raw grains. TAC of 93.5 (Abdel-Aal et al., 2006) for red rice and 211.9 – 235 µg cyanidin 3-glucoside equivalent/g for blue/purple wheat (Abdel-Aal et al., 2006; Liu et al., 2010) were reported. The present study found TAC of 23 and 251 µg cyanidin 3-glucoside/g for raw, unpolished red rice and whole

purple wheat, respectively. The results from previous findings on purple wheat are in agreement with the results of the present study.

The higher TPC and ORAC values observed in red rice compared to purple wheat in the present study might be due to the components other than anthocyanins. Brown rice contained phenolic acids and other phenolic compounds (Tian et al., 2005; Zhou et al., 2004) while red rice contained condensed tannins (Min et al., 2009) as biological antioxidants (Hagerman et al., 1998) and tocopherols, tocotrienols and γ -oryzanol (Aguillar-Garcia et al., 2007; Xu, Hua and Godber, 2001).

Li et al. (2007a) found that purple wheat bran baked at 177°C for 20 min had high TPC and ORAC values. The present study found decreased TPC and increased ORAC values for lab-made infant cereals subjected to toasting at 120°C for 30 min. However, the overall TPC, TAC and ORAC in raw grains and pigmented infant cereals were not significantly different ($p < 0.05$). Thus for lab-made infant cereals, high heat treatment and enzymatic hydrolysis did not influence the TPC, TAC and ORAC significantly.

The average TPC and ORAC values in raw grains and pigmented infant cereals were higher than that of the commercial ones ($p < 0.05$). However, they were not significantly different in their TAC ($p < 0.05$). Raspberry has high anthocyanin content (Strzalkowska, et al., 1989; Torre and Barritt, 1977; Wu et al., 2006) and thus its presence in Heinz Stage 3 Wheat and Oat Cereal increased the least significant difference value. Pigmented infant cereals had significantly higher TPC, TAC and ORAC than commercial infant cereals and thus, they are potential ingredients in producing infant cereals with higher antioxidant contents.

According to the chemical model assays, unpolished red rice seemed to be a good candidate for producing a new infant cereal type since it had the highest TPC and ORAC and

medium TAC. Lab-made processing did not significantly influence TPC, TAC and ORAC compared with the home-made processing ($p < 0.05$). Heinz Wheat and Oat Cereal with Banana and Raspberry, a stage 3 infant cereal, had the highest TPC, TAC and ORAC among the commercial infant cereals likely due to the raspberry and black raspberry puree contents.

3.3.3. HPLC/MS/MS analysis of major components in acidified methanol extracts of purple wheat and red rice raw grains, home-made and lab-made infant cereals

3.3.3.1. Identification

Figure 3.6 shows the LC chromatograms of the major peaks found in the acidified methanol extracts of raw whole purple wheat and unpolished red rice obtained at different wavelengths of 280 nm, 320 nm and 520 nm. There were five major peaks found, W1 – W5 for purple wheat and R1 – R5 for red rice. The same peaks were not found in the LC chromatogram of red rice sample at 520 nm wavelength. Tentative MS and MS/MS identification of these peaks are listed in Table 3.2. and discussed below.

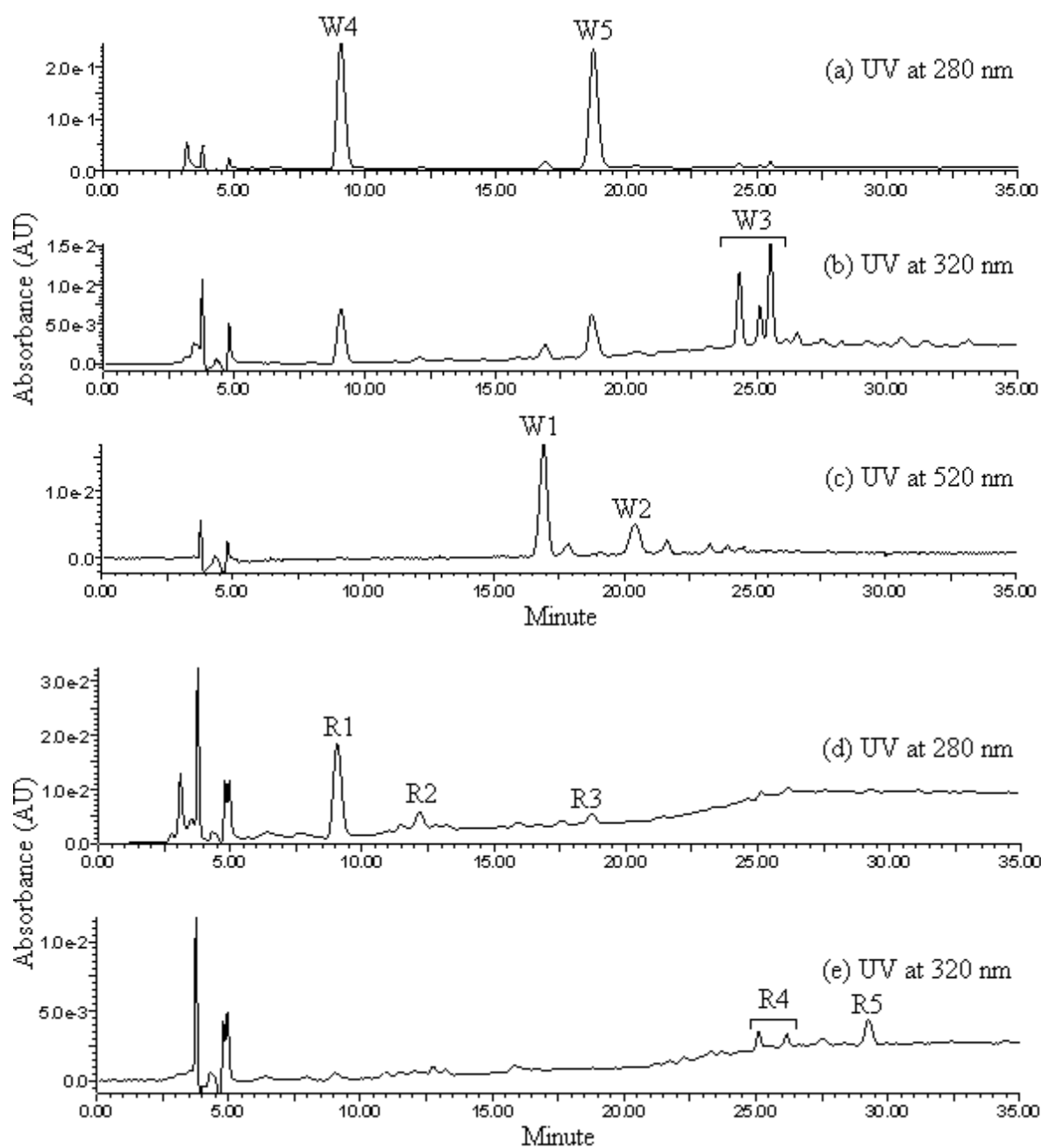


Figure 3.6. LC chromatograms (0 – 35 min) of acidified methanol extracts obtained at (a) 280 nm, (b) 320 nm and (c) 520 nm in raw, whole purple wheat with its five major peaks (W1 – W5); at (d) 280 nm and (e) 320 nm in raw, unpolished red rice with its five major peaks (R1 – R5).

Table 3.2. MS/MS identification of five major peaks in acidified methanol extracts of raw whole purple wheat and unpolished red rice

No.	Retention time (min)	MS, [M-H] [−]	MS/MS fragment ions		Identification
Whole purple wheat					
W1	16.79	447	[M − H − 162] [−]	285	Cyanidin 3-glucoside
W2	20.70	461	[M − H − 162] [−]	299	Peonidin 3-glucoside (Liu et al., 2010)
W3	25.02	563	[M − H − 18] [−]	545	Apigenin 6-C-glucoside-8-C-arabinoside (Dinelli et al., 2009; Liu et al., 2010)
	25.76		[M − H − 60] [−]	503	
	26.14		[M − H − 90] [−]	473	
			[M − H − 120] [−]	443	
			(A + 113)	383	
W4	9.10	515	(A + 83)	353	Dicaffeoylquinic acid (Clifford et al., 2005; Schütz, et al., 2005; Zhang et al., 2007)
			[M − H − 162] [−]	353	
				317	
				191	
				179	
W5	18.28	311		111	Monocaffeoyltartaric acid (Kammerer et al., 2004)
			[M − H − 162] [−]	149	
Unpolished red rice					
R1	12.46	515	[M − H − 162] [−]	353	Dicaffeoylquinic acid (Clifford et al., 2005; Schütz, et al., 2005; Zhang et al., 2007)
				317	
				191	
				179	
R2	13.39	334	[M − H − 162] [−]	171	Dehydrodivanillic acid (Bollag et al., 1982)
R3	19.67	311	[M − H − 162] [−]	149	Monocaffeoyltartaric acid (isomer 1) (Kammerer et al., 2004)
R4	25.73	563	[M − H − 18] [−]	545	Apigenin 6-C-glucoside-8-C-arabinoside (Dinelli et al., 2009; Liu et al., 2010)
	26.73		[M − H − 60] [−]	503	
			[M − H − 90] [−]	473	
			[M − H − 120] [−]	443	
			(A + 113)	383	
R5	30.57	311	(A + 83)	353	Monocaffeoyltartaric acid (isomer 2) (Kammerer et al., 2004).
			[M − H − 162] [−]	149	

Peak W1 of $m/z = 447$ found at 16.79 min in raw whole purple wheat with the extraction ion chromatogram (EIC) and its MS/MS spectra shown in Figure 3.7 is identified as cyanidin 3-*O*-glucoside (molecular mass of 449) ($C_{21}H_{21}O_{11}^+$) as confirmed using the UV and MS/MS spectrum of the kuromanin chloride standard shown in Figure 3.8. The standard, cyanidin 3-*O*-glucoside chloride ($C_{21}H_{21}ClO_{11}$) (485) had $m/z = 465$ and eluted at 16.79 min. In both MS/MS spectra, the product ion $m/z = 285$ shows presence of cyanidin aglycone (287) ($C_{15}H_{11}O_6^+$). Also observed in the MS/MS spectrum of peak W1 was fragmentation of glucoside molecule (162) ($C_6H_{10}O_5^-$). Peak W2 of $m/z = 461$ found at 20.70 min in raw whole purple wheat suggests the presence of peonidin 3-glucoside (463) ($C_{22}H_{23}O_{11}^+$). Fragment ion $m/z = 299$ indicated the presence of peonidin aglycone (301) ($C_{16}H_{13}O_6^+$) and fragmentation of glucoside molecule (162) ($C_6H_{10}O_5^-$) (Liu et al., 2010).

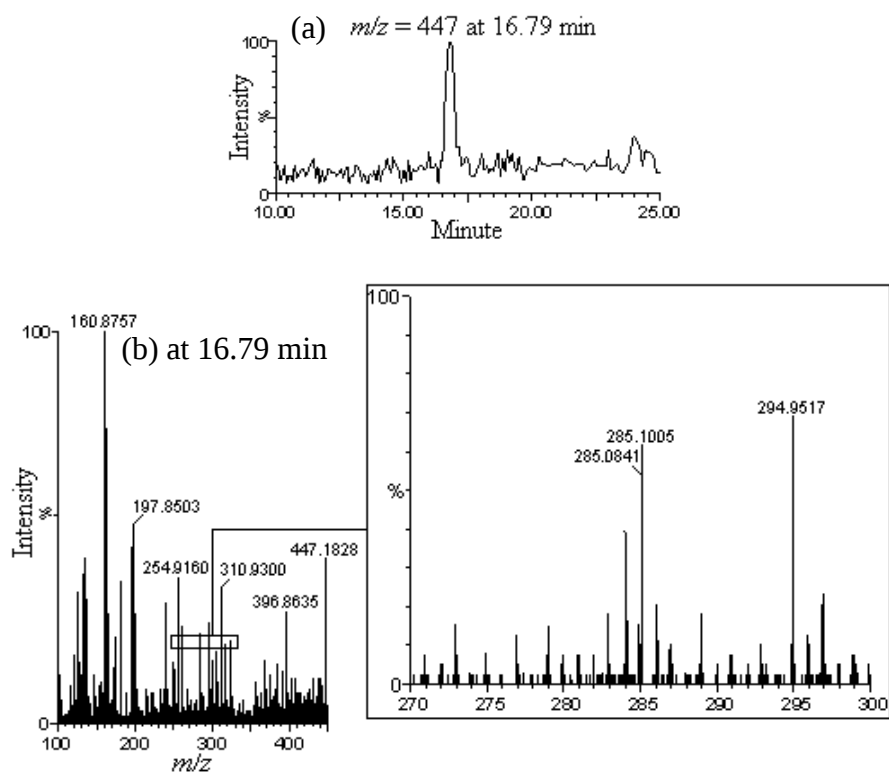


Figure 3.7. Extracted ion chromatogram (EIC) of peak W1 with $m/z = 447$ (a) and its MS/MS spectra with its retention time (b) in raw whole purple wheat.

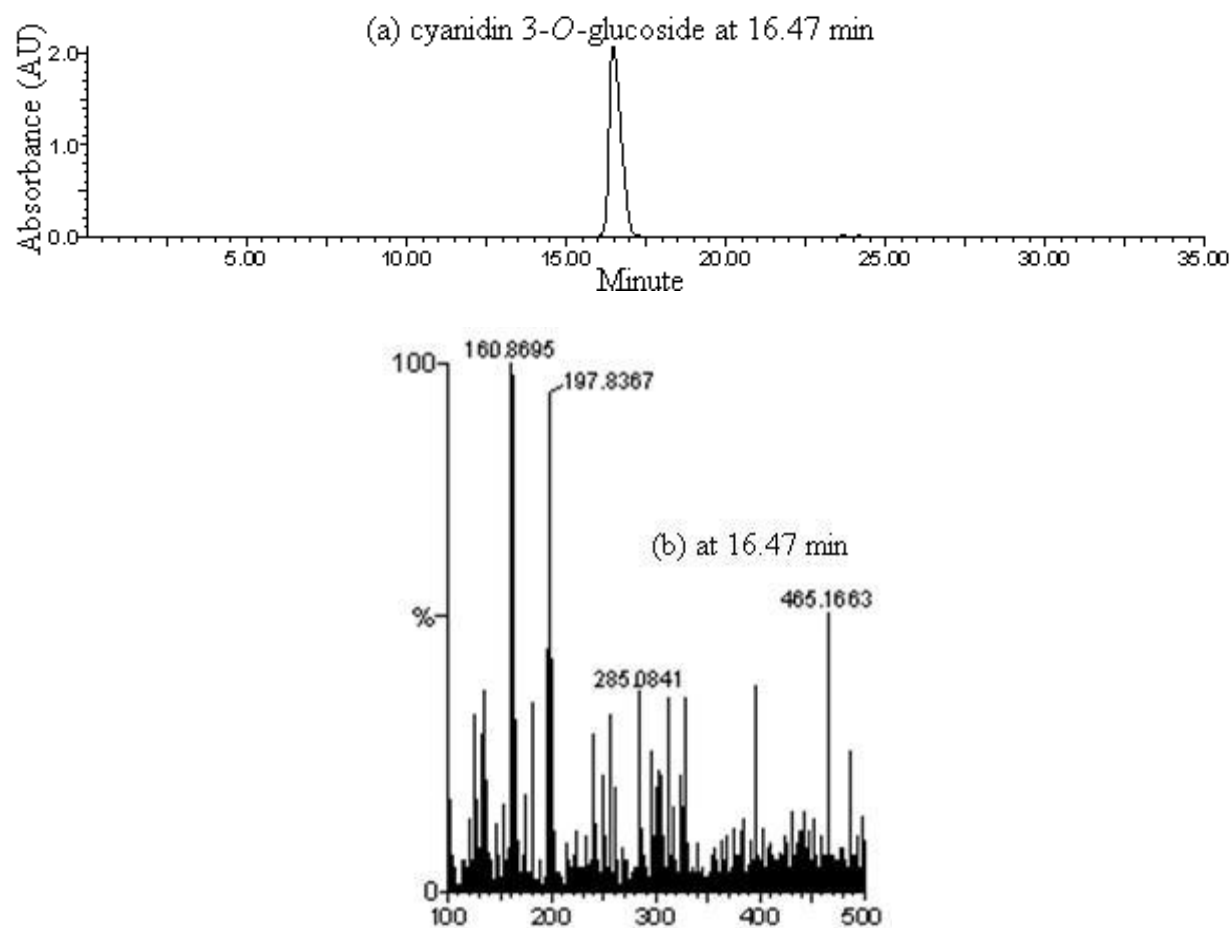


Figure 3.8. LC chromatogram of cyanidin 3-*O*-glucoside standard peak at 16.47 min at 520 nm (a) and MS/MS spectrum of the standard peak with $m/z = 465$ (b).

Peak W3 and R4 of $m/z = 563$ found at 25.73 and 26.73 min in raw unpolished red rice and at 25.02, 25.76 and 26.14 min in raw, whole purple wheat with the EIC (Figure 3.9) and MS/MS spectrum (Figure 3.10) indicated the presence of apigenin 6-*C*-glucoside-8-*C*-arabinoside ($564 = 270 + 162 + 132$). Fragment ions ($A + 83$) and ($A + 113$) at $m/z = 353$ and 383 showed the presence of apigenin aglycone (270) since *C*-glycosyl bonds are rarely completely severed compared with *O*-glycosyl bonds while $[M - H - 18]^-$ at $m/z = 545$ suggests loss of water molecule (Dinelli et al., 2009; Liu et al., 2010). The presence of $[M - H - 60]^-$, $[M - H - 90]^-$ and $[M - H - 120]^-$ at $m/z = 503$, 473 and 443, respectively, suggests fragmentation of glucoside (162) and arabinoside molecules (132) since this fragmentation pattern is likely due to cross-ring cleavages in their residues and also since *C*-glycosyl bonds have been found only at 6- and 8- positions (Dinelli et al., 2009; Liu et al., 2010).

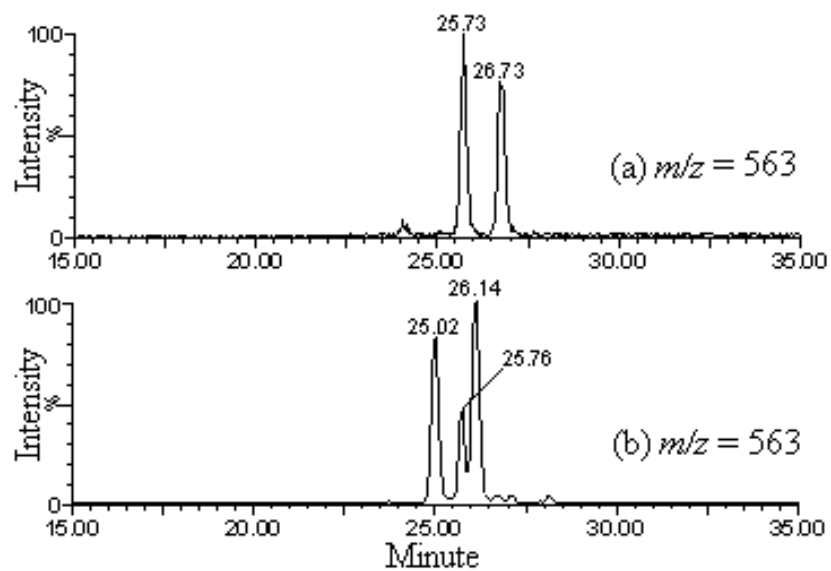


Figure 3.9. EIC of peak R4 (a) and W3 (b) with $m/z = 563$ and their retention times in raw, unpolished red rice and whole purple wheat, respectively.

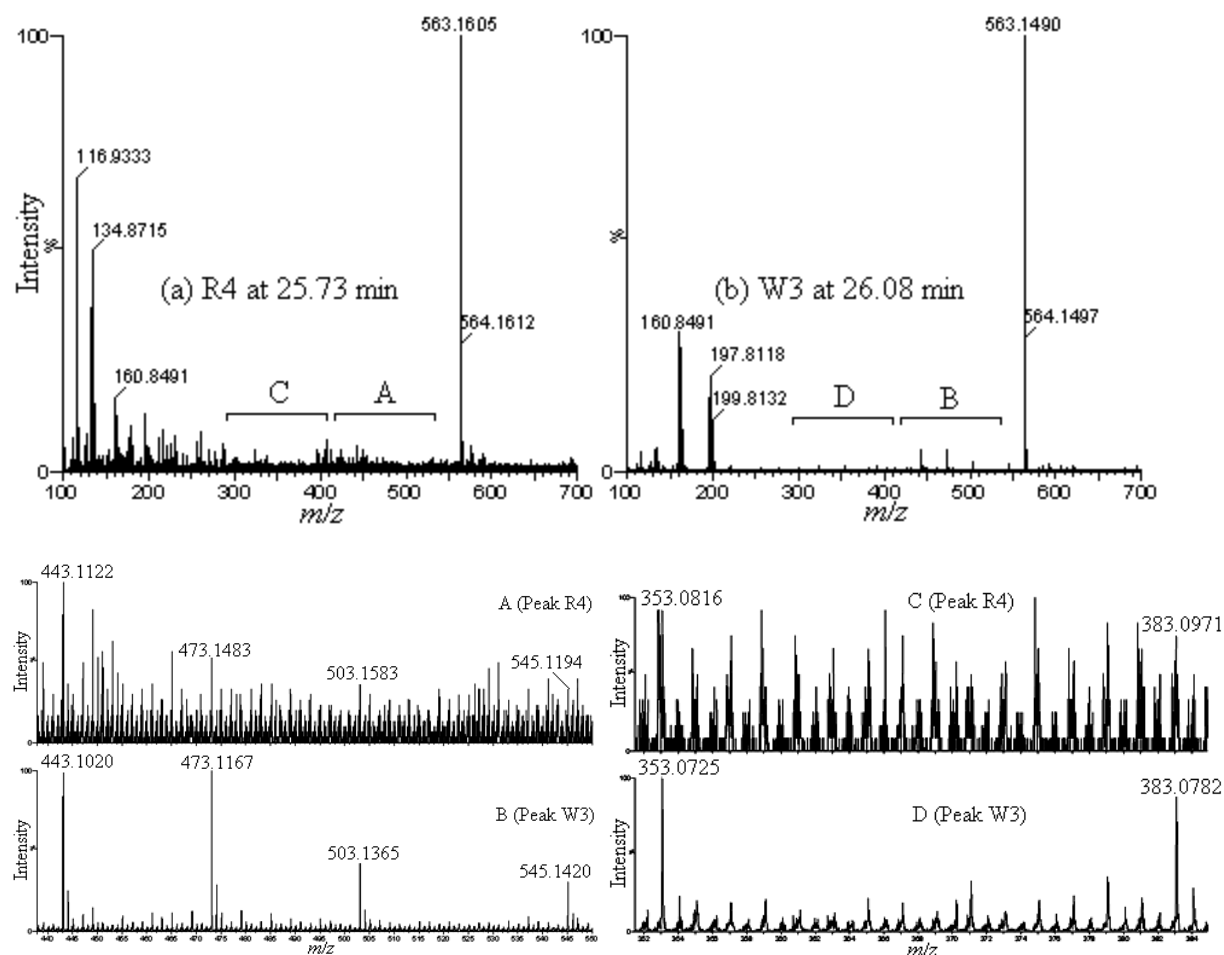


Figure 3.10. MS/MS spectra of peak R4 (a) and W3 (b) with $m/z = 563$ at their retention times in raw, unpolished red rice and whole purple wheat, respectively (top figure). The bottom figures are highlights of areas in the spectra at the top figure (A, B, C and D) showing the fragment ions of peak R4 and W3.

Peak W4 and R1 of $m/z = 515$ found at 9.10 min in raw whole purple wheat and at 12.46 min in raw, unpolished red rice was identified as dicaffeoyl quinic acid ($516 = \text{caffeic acid } 180 + \text{caffeic acid } 180 + \text{quinic acid } 192 - \text{water molecules } 36$) ($\text{C}_{25}\text{H}_{24}\text{O}_{12}$). The fragment ion $[\text{M} - \text{H} - 162]^-$ at $m/z = 353$ indicates the release of one caffeic acid moiety (Clifford et al., 2005; Schütz, et al., 2005; Zhang et al., 2007). The fragment ions found at $m/z = 191$ and 179 suggests the presence of quinic acid (192) ($\text{C}_7\text{H}_{12}\text{O}_6$) and caffeic acid (180) ($\text{C}_9\text{H}_8\text{O}_4$), respectively (Clifford et al., 2005; Demiray et al., 2009; Harbaum et al., 2007; Schütz, et al., 2005). Peak W5 and R3 of $m/z = 311$ found at 18.28 min in raw, whole purple wheat and at 19.67 min in raw, unpolished red rice suggest the presence of monocaffeoyl tartaric acid ($312 = \text{caffeic acid } 180 + \text{tartaric acid } 150 - \text{water } 18$) ($\text{C}_{13}\text{H}_{12}\text{O}_9$) (Kammerer et al., 2004). The fragment ion $[\text{M} - \text{H} - 162]^-$ at $m/z = 149$ shows a caffeic acid (180) ($\text{C}_9\text{H}_8\text{O}_4$) moiety and loss of tartaric acid (150) ($\text{C}_4\text{H}_6\text{O}_6$) (Kammerer et al., 2004; Schütz, et al., 2005). Peak R5 of $m/z = 311$ found at 30.57 min in raw unpolished red rice suggests the presence of another isomer of monocaffeoyl tartaric acid. Peak R2 of $m/z = 333$ found at 13.39 min in raw, unpolished red rice indicates the presence of a vanillic acid dimer which is dehydrodivanillic acid (334) (2,2'-dihydroxy-3,3'-dimethoxy-5,5'-dicarboxybiphenyl) ($\text{C}_{16}\text{H}_{14}\text{O}_8$) (Bollag et al., 1982). The fragment ion $[\text{M} - \text{H} - 162]^-$ at $m/z = 171$ shows a vanillic acid moiety ($168 + 3$) ($\text{C}_8\text{H}_8\text{O}_4$).

3.3.3.2. Quantification

Anthocyanins were quantified at 520 nm while apigenin C-glycosides (as apigenin equivalent) were calculated at 320 nm wavelength. Results are shown in Table 3.3. Dicaffeoylquinic acid, dehydrodivanillic acid and monocaffeoyltartaric acid were not quantified due to unavailability of commercial standards. Cyanidin 3-glucoside in purple wheat samples

ranged from 4.50 to 25.5 µg/g. Apigenin 6-C-glucoside-8-C-arabinoside ranged from 17.4 to 21.9 µg/g and 3.16 – 11.4 µg/g in purple wheat and red rice samples, respectively. Cyanidin 3-glucoside content was significantly ($p < 0.05$) higher in raw purple wheat followed by home-made and lab-made infant cereal. The same trend was found in the levels of apigenin 6-C-glucoside-8-C-arabinoside in raw grains and infant cereals. The average apigenin C-glycoside content was significantly ($p < 0.05$) higher in purple wheat (19.5 µg/g) compared to red rice (7.43 µg/g).

Cyanidin 3-glucoside and peonidin 3-glucoside were found in Charcoal purple wheat while red, yellow and white wheat grains did not contain any anthocyanin (Liu et al., 2010). Cyanidin 3-glucoside and peonidin 3-glucoside were found in purple wheat and red rice in another study (Abdel-Aal et al., 2006). Abdel-Aal (2006) found cyanidin 3-glucoside contents of 14.0, 20.3 and 4.0 µg/g in red rice, blue and purple wheat, respectively. The present study did not find any anthocyanin in the variety of unpolished red rice grain. Values of TAC were comparable between purple wheat in the present study and blue wheat in a study by Abdel-Aal (2006).

Table 3.3. HPLC quantification of major peaks in acidified methanol extracts of whole purple wheat grain and unpolished red rice uncooked grains, home-made and lab-made infant cereals

No.	Sample	Concentration (µg/g)							
		Cyanidin 3-glucoside				Apigenin 6-C-glucoside-8-C-arabinoside ^a			
Whole purple wheat									
1	Uncooked grain	25.5	±	0.74	a	21.9	±	0.07	a
2	Home-made infant cereal	18.6	±	0.29	b	19.2	±	0.28	b
3	Lab-made infant cereal	4.50	±	0.00	c	17.4	±	0.04	c
Unpolished red rice									
4	Uncooked grain	-				11.4	±	0.27	a
5	Home-made infant cereal	-				7.74	±	0.46	b
6	Lab-made infant cereal	-				3.16	±	0.09	c

^aConcentration of apigenin 6-C-glucoside-8-C-arabinoside as µg/g apigenin equivalent

Apigenin 6-*C*-glucoside-8-*C*-arabinoside was found in raw commercial regular and whole-wheat samples as well as in other cereal grains including Charcoal purple wheat (Liu et al. 2010), wild rice (Qiu et al., 2009), durum wheat (Dinelli et al., 2009) and common bread wheat (Asenstorfer et al., 2006). This is the first time that this compound has been quantified in purple wheat and red rice raw grains and infant cereals. Apigenin 6-*C*-glucoside-8-*C*-arabinoside and its isomer apigenin 6-*C*-arabinoside-8-*C*-glucoside were identified and quantified with concentrations of 8.7 and 10.1 mg/g naringin equivalent in an Australian common bread wheat germ as the major yellow colour components (Asenstorfer et al., 2006). Regular spaghetti contained 6.9 – 12.1 µg/g apigenin equivalent and whole wheat spaghetti contained 11.8 – 22.4 µg/g apigenin equivalent of apigenin 6-*C*-glucoside-8-*C*-arabinoside (section 2.3.4.). Whole wheat spaghetti showed generally similar contents with raw, whole purple wheat and its infant cereals. This means that apigenin 6-*C*-glucoside-8-*C*-arabinoside was likely not destroyed during processing including high temperature treatments. Approximately 3-times higher contents of apigenin 6-*C*-glucoside-8-*C*-arabinoside were found in purple wheat compared with red rice. As far as we know, apigenin 6-*C*-glucoside-8-*C*-arabinoside has not been previously reported in red rice grain. Apigenin 6-*C*-glucoside-8-arabinoside (schaftoside), its isomer apigenin 6-*C*-arabinoside-8-*C*-glucoside (isoschaftoside) and other apigenin glycosides were found in rice phloem (part of the plant stem) sap extracts acting as feeding inhibitors and resistance factors to insect damage (Stevenson et al., 1996) and in rice leaves (Kim et al., 2008).

The decrease of cyanidin 3-glucoside contents was 5% and 82% in the processing of raw, whole purple wheat to home-made and lab-made infant cereals, respectively while a decrease of 12% and 21% was found in the apigenin 6-*C*-glucoside-8-*C*-arabinoside content. In red rice, the apigenin 6-*C*-glucoside-8-*C*-arabinoside content decreased by 32% and 72%. Processing did not

affect TPC, TAC and ORAC significantly (section 3.3.2.); however, individual content of cyanidin 3-glucoside and apigenin 6-C-glucoside-8-C-arabinoside was affected significantly. Higher and longer-exposure temperature treatment in the processing of lab-made infant cereal likely contribute to the higher loss of cyanidin 3-glucoside and apigenin 6-C-glucoside-8-C-arabinoside contents compared with the home-made one. Cooking using pressure cooker, rice cooker and gas range was found to thermally degrade cyanidin 3-glucoside; however, as a result of the same thermal treatment, protocatechuic acid was produced (Hiemori et al., 2009). The level of this particular phenolic acid with *in vitro* antioxidant activity was found to be 2.7 to 3.4 times higher after cooking (Hiemori et al., 2009). Ling et al. (2001) found that the consumption of 0.3 g of red rice powder/g of rabbit body weight in one diet exhibited inhibition against atherosclerotic plaque formation and increased antioxidant status while Toyokuni et al. (2002) found that the consumption of 0.5 g of cooked red rice or 0.3 g of uncooked red rice/g of rat body weight in one diet exhibited protection against Fenton reaction-based renal lipid peroxidation. A normal person with 60 kg would eat 1 cup of rice at approximately 185 g of red rice in one Asian-style diet and this equals to 0.003 g of red rice/g of body weight. According to this comparison, the normal consumption is lower than required. Pro-oxidant factors were used in the diets and thus requiring higher level of consumption of red rice than in a normal diet. Incorporation of red rice bran powder in a diet might help increase the consumption level.

3.3.4. Cell culture assay of home-made, lab-made whole purple wheat, unpolished red rice and a commercial infant cereal

3.3.4.1. Cytotoxicity assay

MTT Cell Proliferation Assay (ATCC[®], Manassas, VA, USA) was used to evaluate the cytotoxicity of home-made, lab-made whole purple wheat, unpolished red rice and commercial Heinz stage 3 infant cereals. Figure 3.11 shows the cytotoxicity of the sample treatments with different concentrations (2 mg/mL and 0.5 mg/mL) represented by cell viability compared to the untreated control (control having 100% viability). The cell viability of sample-treated cells ranged from 71 – 91% and the viability of those treated with commercial samples was 70% (2 mg/mL) and 85% (0.5 mg/mL). Since the home-made and lab-made infant cereals behaved similarly or better than the commercial samples, pigmented infant cereals were considered to pose no toxicity against the fetal small intestine cell lines ($p < 0.05$).

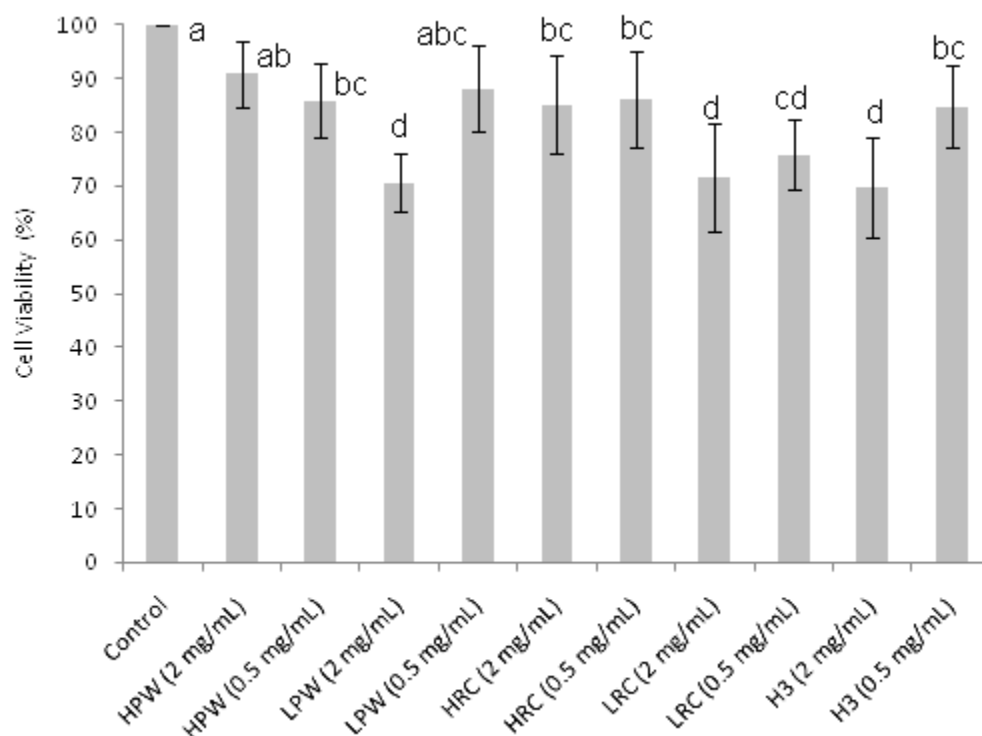


Figure 3.11. The cytotoxicity of home-made whole purple wheat (HPW), lab-made whole purple wheat (LPW), home-made unpolished red rice (HRC), lab-made unpolished red rice (LRC) infant cereals and commercial sample of Heinz Stage 3 (Wheat and Oat Cereal with Banana Raspberry) at concentrations 2 mg/mL and 0.5 mg/mL in confluent FHs 74 Int (normal fetal small intestine cell line). Control was untreated cells at 100% cell viability. Data represent mean $\pm$ SD, $n = 8$.

3.3.4.2. Dichlorofluorescein diacetate (DCFH-DA) assay

Figure 3.12 shows the reaction of the sample-treated normal fetal small intestine cell lines against the AAPH-induced free radical oxidation. AAPH induced the oxidation of the dye DCFH to DCF. The fluorescence intensity of DCF can be measured as representation of oxidation rate. Positive (+) control shows the fluorescence intensity of the AAPH oxidation over time. Negative (-) controls were cells to which AAPH was not added and were used to illustrate the conditions without any oxidation-inducer. The treatments using home-made whole purple wheat (HPW), lab-made whole purple wheat (LPW), home-made unpolished red rice (HRC), lab-made unpolished red rice (LRC), commercial Heinz Stage 3 (H3) infant cereals at two different concentrations, 2 mg/ml (2) and 0.5 mg/mL (0.5), showed inhibition of oxidation as their fluorescence intensities were higher than the negative control; however, they were lower compared with the positive control over time.

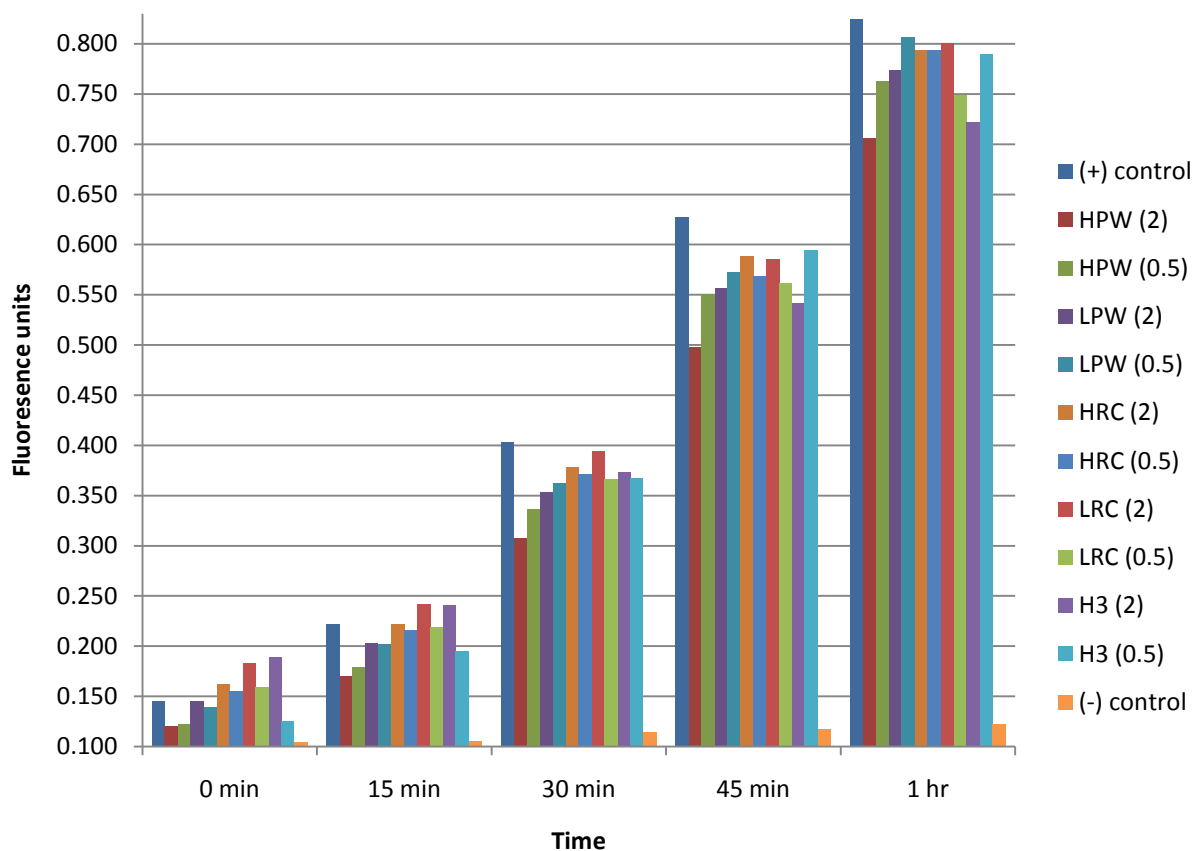


Figure 3.12. AAPH-induced free radical oxidation of DCFH to DCF is represented in fluorescence units by positive (+) control over time. Negative (-) control shows cell conditions without addition of AAPH over time. The treatment by home-made whole purple wheat (HPW), lab-made whole purple wheat (LPW), home-made unpolished red rice (HRC), lab-made unpolished red rice (LRC), commercial Heinz Stage 3 (H3) infant cereals at two different concentrations, 2 mg/ml (2) and 0.5 mg/mL (0.5), shows fluorescence intensity values in-between the control values.

Cellular antioxidant activity (CAA) was then calculated based on the fluorescence intensity of infant cereals and AAPH-treated cells (Figure 3.13). The CAA ranged from 15.3 to 30.4 CAA units ($p < 0.05$). Home-made whole purple wheat infant cereal at 2 mg/mL showed the highest antioxidant activity followed by the same sample at 0.5 mg/mL. Lab-made unpolished red rice gave the lowest CAA at 2 mg/mL. Other pigmented infant cereals at 0.5 mg/mL and commercial Heinz Stage 3 at both concentrations did not differ significantly in their CAA. Thus, home-made whole purple wheat infant cereal showed evidence of possible improvement of infant diet through its CAA. Longer exposure to higher temperature in lab-made and commercial infant cereals might contribute to loss of cellular-effective antioxidants.

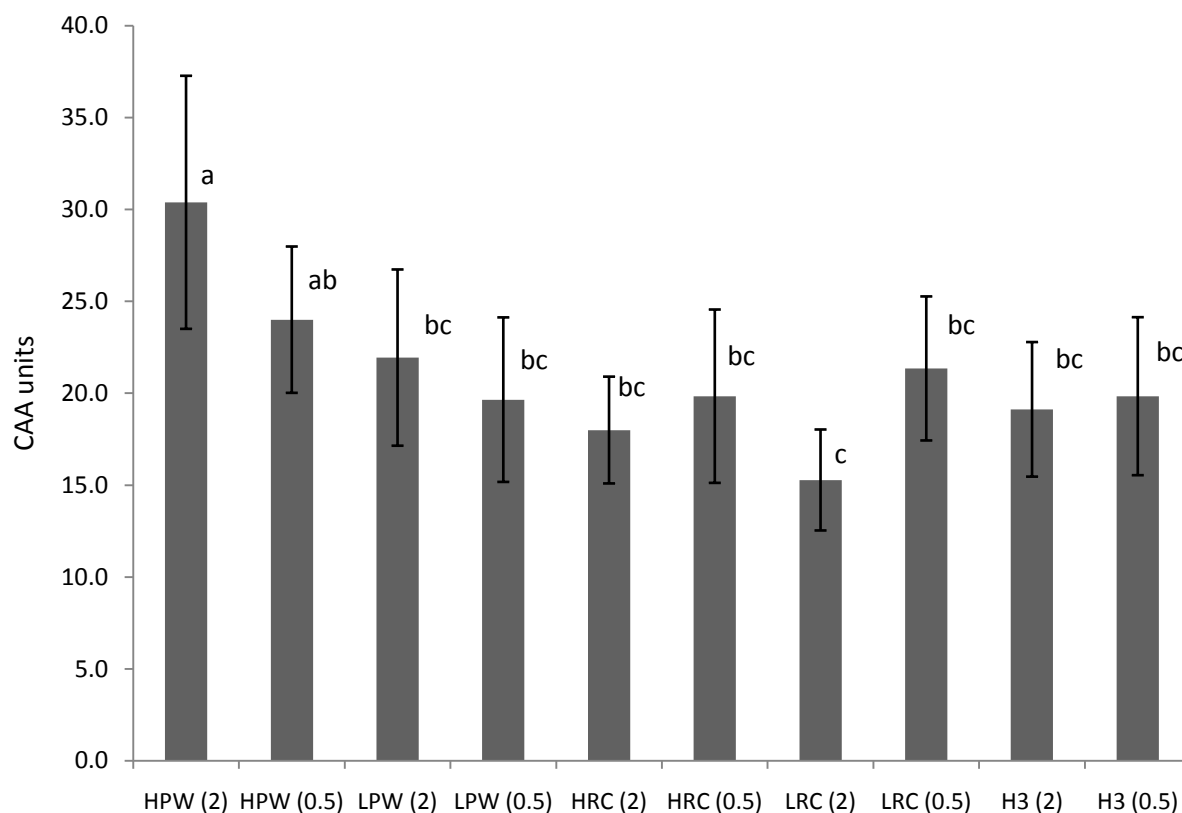


Figure 3.13. CAA values of home-made whole purple wheat (HPW), lab-made whole purple wheat (LPW), home-made unpolished red rice (HRC), lab-made unpolished red rice (LRC) and commercial Heinz Stage 3 (H3) infant cereals at different concentrations, 2 mg/mL (2) and 0.5 mg/mL (0.5) (mean $\pm$ SD, $n = 8$). Different letters indicate significant differences among raw grains and infant cereals ($p < 0.05$).

3.4. Conclusion

Pigmented infant cereals including whole purple wheat, unpolished and partially polished red rice were produced in the present study using two different methods of home-made and lab-made. Lab-made processing involved longer exposure to higher heat-treatment and enzymatic hydrolysis of starch. Processing completely gelatinized starch granules and deactivated α -amylase enzyme. The texture was generally similar to commercial infant cereals. Red rice had significantly higher TPC and ORAC compared to purple wheat while purple wheat had significantly higher TAC than red rice ($p < 0.05$). Home-made and lab-made infant cereals did not show significant differences in TPC, TAC and ORAC ($p < 0.05$). Pigmented infant cereals had significantly higher TPC and ORAC than commercial infant cereals ($p < 0.05$). Home-made infant cereals had significantly higher cyanidin 3-glucoside and apigenin 6-C-glucoside-8-C-arabinoside contents than lab-made ones ($p < 0.05$). Home-made purple wheat had significantly higher CAA than lab-made one, red rice and commercial infant cereals ($p < 0.05$). Based on our findings, more research on home-made whole pigmented infant cereals may give definitive answers as to whether they are superior to the commercial ones.

GENERAL CONCLUSION

The present study investigated the antioxidant properties of spaghetti products including the commercially available regular refined wheat or white pasta, whole-wheat pasta and inulin-added white pasta. No-name products were found to have higher antioxidant activity. Inulin fibre-added spaghetti might not provide all the benefits of whole-wheat. Whole-wheat provides not only fibre but also the antioxidant components bound as non-soluble part of the wheat bran. The present study also provided a prototype of pigmented, whole cereal grain infant cereals (utilizing red rice and purple wheat grains) and compared them with commercial infant cereals on their antioxidant properties. Red rice infant cereals had higher TPC, TAC and ORAC than the brown rice infant cereal control and the commercial infant cereals ($p < 0.05$). There were no significant differences in TPC and ORAC between raw grains and infant cereal products and among the processed products ($p < 0.05$). Cyanidin 3-glucoside was present in purple wheat infant cereal. Purple wheat infant cereal had significantly higher contents of C-glycosyl flavones than red rice infant cereal ($p < 0.05$). Home-made purple wheat infant cereal had significantly higher cyanidin 3-glucoside, C-glycosyl flavones and CAA than its lab-made counterpart ($p < 0.05$). Purple wheat and red rice infant cereals had significantly higher TPC, TAC, ORAC, cyanidin 3-glucoside, C-glycosyl flavones and CAA than the commercial infant cereals ($p < 0.05$) and thus suggesting the possibility of improving infant health through their consumption.

Recommendations for future studies

Further investigation on the antioxidant properties of home-made pigmented infant cereals compared to non-pigmented commercial infant cereals is needed. Pigmented grains can also be used in the production of pasta and a comparative study of their antioxidant properties against the non-pigmented commercial pasta needs to be done. Observations on the aroma and flavour properties of pigmented pasta and infant cereals and means of improvement are required.

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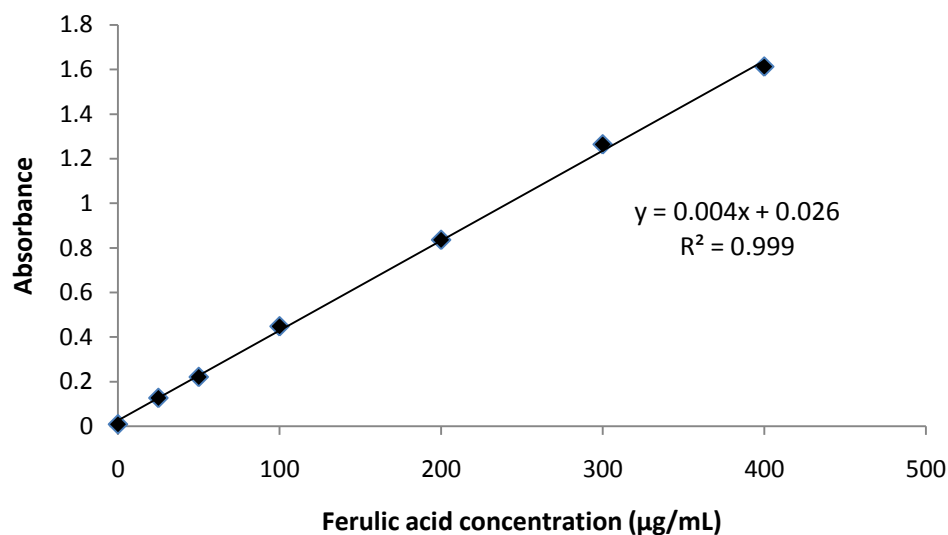
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APPENDIX

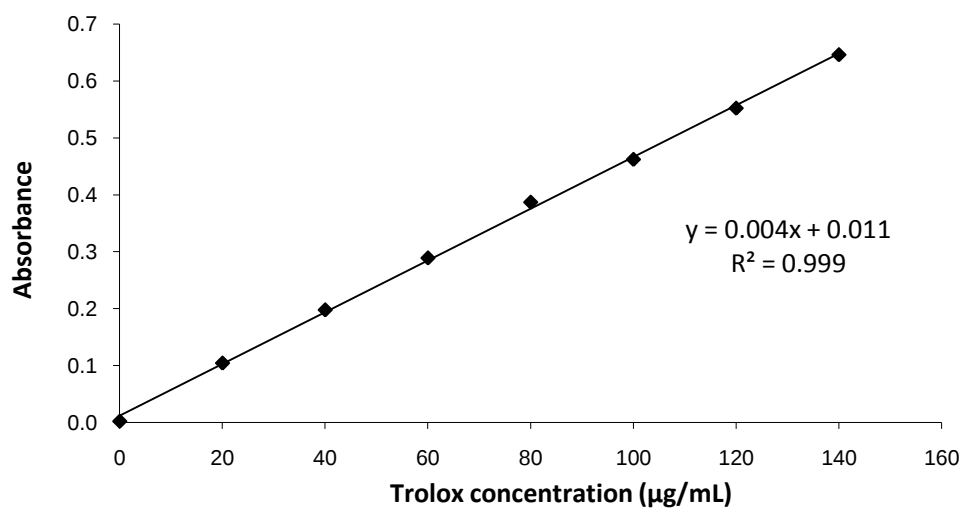
A.1. Standard Curves

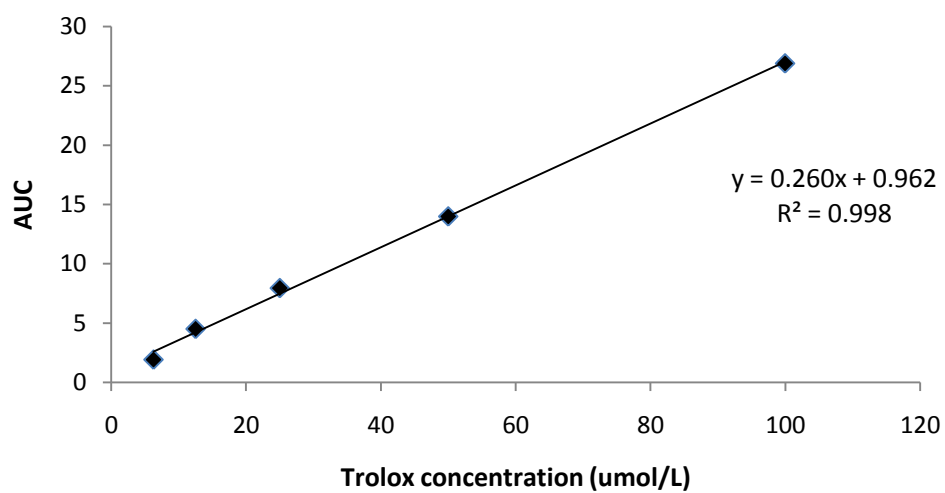
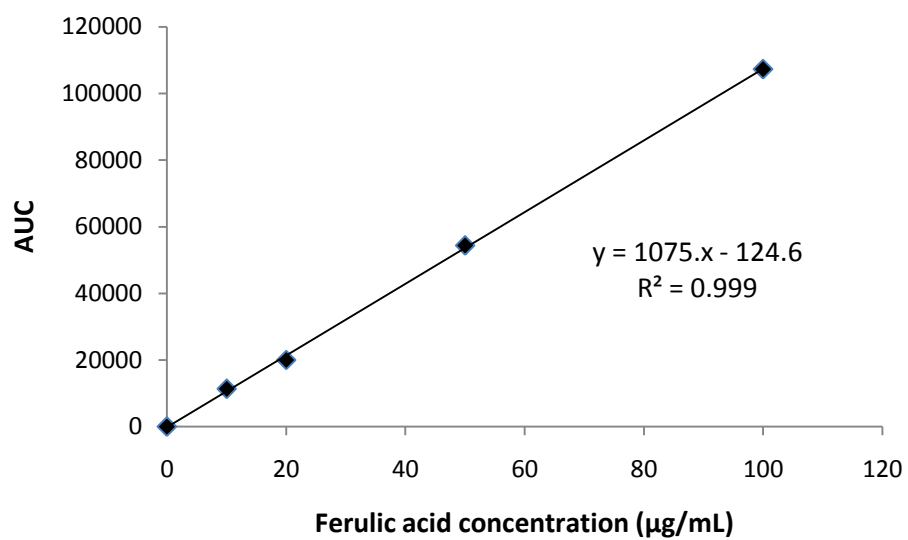
CHAPTER 2: Antioxidant properties of commercial regular and whole-wheat spaghetti and LC/MS analysis of their C-glycosyl flavones and secoisolariciresinol diglucoside

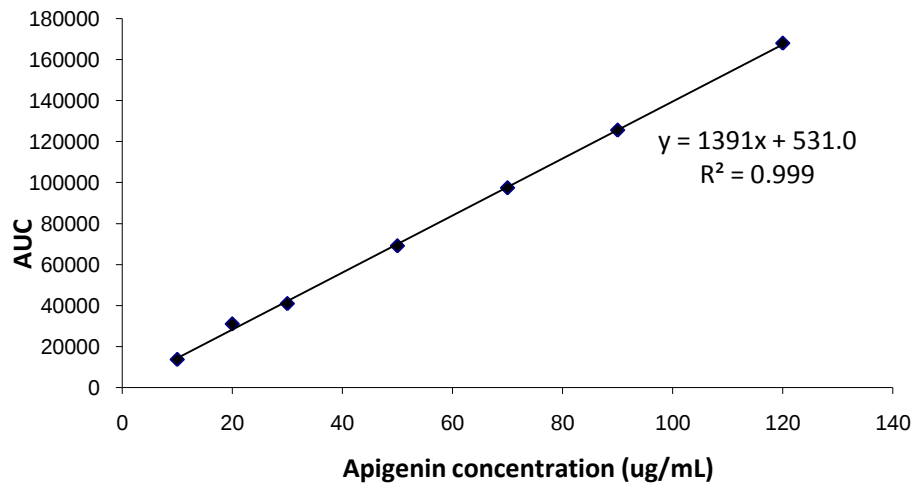
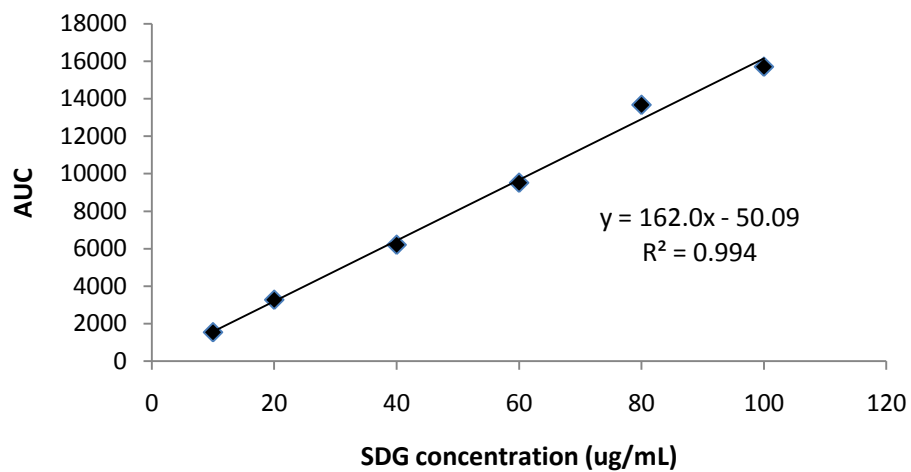
A.1.1. Ferulic acid standard curve for TPC determination



A.1.2. Trolox standard curve for DPPH radical scavenging activity determination

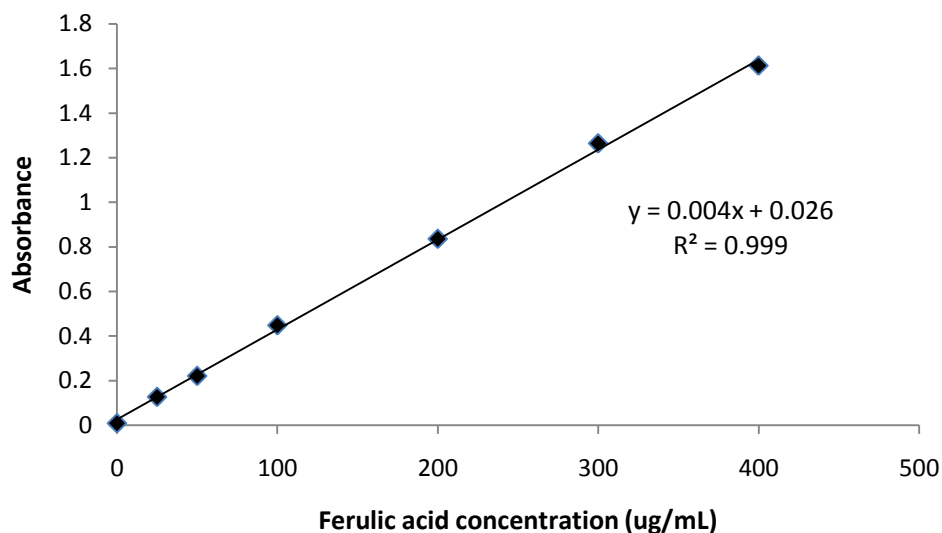


A.1.3. Trolox standard curve for ORAC determination**A.1.4. Ferulic acid standard curve for HPLC quantification analysis**

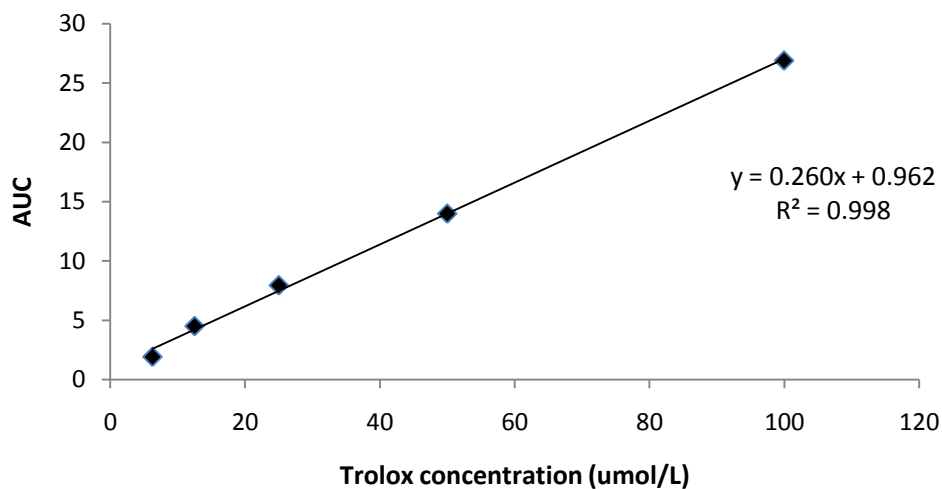
A.1.5. Apigenin standard curve for HPLC quantification analysis**A.1.6. SDG standard curve for HPLC quantification analysis**

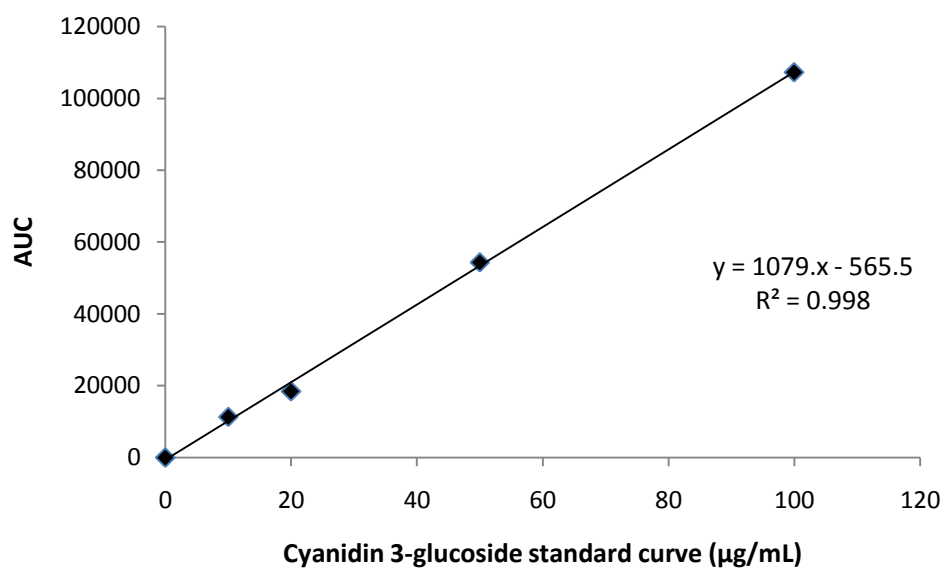
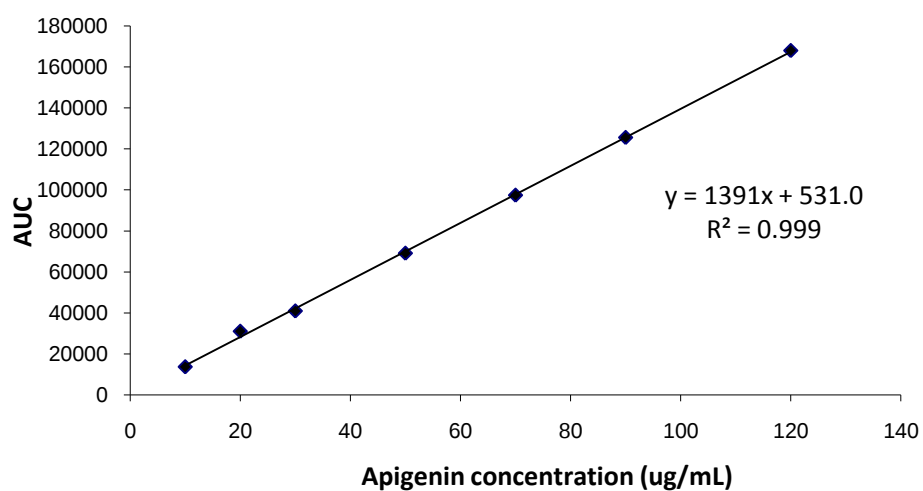
CHAPTER 3: Antioxidant potential of infant cereals produced from pigmented grains in comparison to commercial infant cereals and LC/MS analysis of their anthocyanins

A.1.7. Ferulic acid standard curve for TPC determination



A.1.8. Trolox standard curve for ORAC determination



A.1.9. Cyanidin3-glucoside standard curve for HPLC quantification analysis**A.1.10. Apigenin standard curve for HPLC quantification analysis**

A.2. HPLC Chromatogram

CHAPTER 2: Antioxidant properties of commercial regular and whole-wheat spaghetti and LC/MS analysis of their C-glycosyl flavones and secoisolariciresinol diglucoside

A.2.1. Whole-wheat Primo spaghetti spiked with ferulic acid (1 mg/mL)

