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Phenolic acid composition and antioxidant capacity of acid and alkali hydrolysed wheat bran fractions

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ABSTRACT

Phenolic acid concentrations, profiles and antioxidant capacity of acid and alkali hydrolysates from the bran of six wheat cultivars representing six Canadian market classes were determined. Aqueous ethanol was used to extract the free phenolics (FP) and diethyl ether to extract the insoluble bound phenolics released after acid and alkaline hydrolysis of the bran. Folin–Ciocalteu (FC) reagent was used to estimate the total phenolic content and HPLC-UV to detect and quantitate 14 phenolic acids and one lignan. trans-Ferulic acid was the dominant acid in the bran extracts but mass spectrometric analysis showed tryptophan to be dominant in the FP extracts. The antioxidant capacity of individual phenolic acids and extracts was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidant assays. The FP extracts had the lowest average antioxidant capacity and the hydrolysates the highest. Based on the concentration of each phenolic acid in the extracts, and the antioxidant capability of each phenolic standard, trans-ferulic acid was the dominant contributor (66.4–95.5%) to antioxidant capacity of the wheat bran extract.

1. Introduction

Wheat is a major component of human and animal diets and accounts for approximately 30% of total grain consumption with >600 million metric tonnes consumed globally in 2005/2006 (USDA, 2007). Besides meeting caloric needs, studies have shown that a whole wheat diet can reduce the incidence of human diseases such as type 2 diabetes, cardiovascular disease and colon cancer (Astorg et al., 2002; Willcox, Ash, & Catignani, 2004; Zhou, Su, & Yu, 2004). Attempts to identify compounds responsible for reduced disease incidence (Astorg et al., 2002; Drankhan et al., 2003; Maziyadixon, Klopfenstein, & Leipold, 1994) suggest that the health benefits of whole grain consumption may arise from a synergistic action of several components within the wheat grain (Sang et al., 2006). However, it is known that some of these diseases may be initiated or be exacerbated by cellular and molecular damage caused by reactive oxygen species and free radicals. Collectively, phenolic acids within the wheat grain are strong antioxidants and may alleviate oxidative stress by quenching or neutralising reactive species, thereby reducing cellular damage or death (Andreasen, Kroon, Williamson, & Garcia-Conesa, 2001; Zhou & Yu, 2004). Besides acting as antioxidants, phenolics can be beneficial to health by chelating metal ions (Liyana-Pathirana & Shahidi, 2006), stimulating antioxidative (Moore, Liu, Zhou, & Yu, 2006) and detoxifying enzymes (Yoshioka, Deng, Cavigelli, & Karin, 1995) and inhibiting transcription factors that initiate and promote tumour growth (Natarajan, Singh, Burke, Grunberger, & Aggarwal, 1996; Yoshioka et al., 1995).

Structurally, phenolics in wheat can be subdivided into acids derived from either benzoic acid or cinnamic acid (Kim, Tsao, Yang, & Cui, 2006). Vanillic and salicylic acids, for example, are derivatives of benzoic acid while ferulic acid, the dominant phenolic acid in a wheat grain, and caffeic acid are derivatives of cinnamic acid (Abdel-Aal et al., 2001). Phenolic acids are predominantly found in the outer bran layer of a wheat grain (Beta, Nam, Dexter, & Sapirstein, 2005; Gallardo, Jimenez, & Garcia-Conesa, 2006; Moore et al., 2006). Though termed secondary metabolites, phenolics play an essential role in protecting plants from UV radiation (Stalikas, 2007), inhibiting pathogens (Abdel-Aal et al., 2001) and providing structural integrity to the cell wall (Klepacka & Fornal, 2006). The majority of phenolics in a wheat grain are insoluble and bound by ester and ether linkages with polysaccharides, such as arabinoxylan and lignin, in the cell wall (Liyana-Pathirana & Shahidi,

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2006; Renger & Steinhart, 2000), while a smaller portion is soluble (Stalikas, 2007). The bran layer is highly stratified not only in phenolic composition, but also in the degree of ester and ether bonds and the compounds to which the phenolics are cross-linked (Parker, Ng, & Waldron, 2005; Renger & Steinhart, 2000). These bran traits vary among wheat cultivars (Beaugrand, Croner, Debeire, & Chabbert, 2004), so that the dissolution of bran into its components in the different regions of the gastrointestinal tract, when consumed and digested, will vary (Renger & Steinhart, 2000). Therefore, it is essential to establish a complete profile of phenolic acids within the wheat bran prior to, or concurrent to, studying the effect of wheat bran in reducing the incidence of human diseases.

Phenolics are commonly extracted by alkaline hydrolysis (Krygier, Sosulski, & Hogge, 1982; Stalikas, 2007) and very limited information is available regarding the effects of acidic and enzymatic hydrolysis (Kim et al., 2006; Stalikas, 2007), both of which are more representative of how bran is broken down during digestion within the human body. A possible reason for this is that acidic hydrolysis at elevated temperatures results in the loss of some phenolics (Krygier et al., 1982). Therefore, after removing free phenolics (FP), bran of six different Canadian wheat cultivars was hydrolysed with HCl and NaOH, at room temperature, to obtain a comprehensive profile of total and individual phenolic acids within the bran. A vast majority of the reports for detecting and identifying phenolic acids use HPLC with UV detection. In the present work, HPLC-UV was used along with mass spectrometry to verify the phenolic acids within wheat bran extracts. In addition, we have attempted to study the contributions of selected phenolic acids to the overall antioxidant capacity of wheat bran extracts.

2. Materials and methods

2.1. Materials

All wheat cultivars were grown in 2005 in a uniform field nursery at the University of Saskatchewan, Kernen Research Farm, Saskatoon, Saskatchewan, Canada. The cultivars and classes of wheat used were: Katepwa, Canada Western Red Spring (CWRS); AC Crystal, Canada Prairie Spring Red (CPSR); AC Vista, Canada Prairie Spring White (CPSW); Glenlea, Canada Western Extra Strong (CWES); AC Avonlea, Canada Western Amber Durum (CWAD); and AC Andrew, Canada Western Soft White Spring (CWSWS) (Tables 1–5). The phenolic acid standards of 3,4-dimethoxycinnamic, caffeic, chlorogenic, ellagic, trans-ferulic, *p*-coumaric, and sinapic (all cinnamic acid derivatives); and the benzoic acid derivative standards of 4-hydroxybenzoic, gallic, gentisic, protocatechuic, salicylic, syringic and vanillic acids were all purchased from Sigma–Aldrich Chemical Co. (Oakville, ON, Canada). The lignan secoisolariciresinol diglycoside (SDG) was purchased from Chromadex (Irvine, CA, USA). Potassium persulphate, the Folin–Ciocalteu reagent, tryptophan, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma–Aldrich (Oakville, ON, Canada). All solvents were purchased from VWR International (Mississauga, ON, Canada) and were of American Chemical Society grade.

2.2. Sample preparation

All of the wheat samples (100 g) were tempered to a moisture content of 15% except for the durum wheat, which was tempered to 16%. Milling was performed using a Brabender Quadrumat Junior (Brabender GmbH and Co., KG, Duisburg, Germany) according to AACC Method 26–50 (American Association of Cereal Chemists, 2000).

2.3. Extraction of free phenolics

Bran free phenolics were extracted essentially using procedures reported previously (Adom, Sorrells, & Liu, 2003; Naczk & Shahidi, 1989) with slight modifications. Four replicates (1 g each) of wheat bran, were individually mixed for 10 min with 80% (v\v) cold ethanol then centrifuged at 2500*g*. This process was repeated, the supernatants pooled and rotary evaporated to <5 ml and reconstituted to 5 ml with water and frozen at −20 °C until further analysis within a three-month period.

2.4. Acidic and alkaline hydrolysis

The phenolics were extracted as previously described (Krygier et al., 1982) with slight modifications for acid hydrolysis: 75 ml of 6 M HCl were mixed with 1 g of wheat bran residue, which previously had the FP removed, and the container was purged with nitrogen so as to minimise the oxidation of phenolic compounds. The mixture was shaken on a rotary shaker at 2000 rpm for 4 h, and the pH was adjusted to two with 10 M NaOH. Diethyl ether (100 ml) was added to the mixture, the container inverted 11 times and then centrifuged at 1000*g* for 10 min. The supernatant was removed and the process repeated with 75 ml of ethyl acetate. The supernatants were pooled, evaporated to <5 ml, reconstituted to 5 ml with water and frozen at −20 °C until further analysis within a three-month period.

The phenolics liberated by acid hydrolysis were significantly less than the phenolics liberated by alkaline hydrolysis. Therefore, the acid hydrolysed wheat bran was subjected to an additional alkaline hydrolysis step. The procedure for alkaline hydrolysis was similar to that for acidic hydrolysis, except that 75 ml of 2 M NaOH were used and the pH was adjusted to two with 6 M HCl.

Table 1
Phenolic acid composition of free phenolics from bran of six wheat cultivars. Mean values ± standard deviation ($n = 4$) as determined by HPLC-UV and Folin–Ciocalteu (FC) assay. Results are expressed as μg per gram bran, except for the FC assay where it is expressed as μg ferulic acid equivalent per gram of wheat bran. ND is for non-detectable.

Aqueous alcohol soluble phenolic acids (μg g^{-1} bran)	Katepwa	AC Crystal	AC Vista	Glenlea	AC Avonlea	AC Andrew
3,4-Dimethoxycinnamic	0.4 ± 0.2	0.8 ± 0.5	0.8 ± 0.3	0.6 ± 0.3	2.1 ± 1.1	1.6 ± 0.01
4-Hydroxybenzoic	13.4 ± 1.9	9.0 ± 2.9	10.8 ± 4.0	8.3 ± 2.5	10.0 ± 1.3	4.5 ± 1.0
Caffeic	0.9 ± 0.4	0.4 ± 0.3	1.8 ± 0.2	0.8 ± 0.5	0.9 ± 0.7	1.6 ± 0.9
p-Coumaric	<0.2	<0.2	<0.2	<0.2	ND	ND
trans-Ferulic	8.2 ± 1.8	19.5 ± 7.4	15.4 ± 7.2	11.4 ± 5.0	12.1 ± 0.9	16.8 ± 9.5
cis-Ferulic	ND	0.04 ± 0.02	ND	1.7 ± 1.2	ND	0.7 ± 0.4
Protocatechuic	7.2 ± 1.5	13.3 ± 3.9	10.5 ± 3.8	8.7 ± 5.5	6.8 ± 1.0	8.9 ± 1.4
Sinapic	3.4 ± 0.5	5.7 ± 2.3	4.2 ± 2.0	2.7 ± 0.8	3.7 ± 0.6	5.7 ± 2.3
Salicylic	8.6 ± 2.7	11.2 ± 4.9	8.3 ± 3.9	6.4 ± 1.9	0.8 ± 0.5	6.4 ± 2.1
Syringic	2.5 ± 0.7	2.6 ± 1.1	2.2 ± 1.1	1.8 ± 1.2	2.5 ± 0.5	2.3 ± 0.9
Vanillic	8.1 ± 1.7	4.4 ± 2.2	4.8 ± 2.7	3.6 ± 2.6	8.5 ± 1.5	ND
Total phenolics (HPLC-UV)	52.7 ± 8.5	66.8 ± 16.3	58.7 ± 23.5	45.8 ± 6.3	47.4 ± 4.5	48.4 ± 14.4
Total phenolics (FC)	1666.0 ± 114.8	1670.2 ± 281.9	1660.4 ± 238.2	1453.6 ± 155.1	1136.4 ± 153.7	1467.4 ± 289.8

Table 2
Phenolic acid composition of acid hydrolysed bran of six wheat cultivars. Mean values ± standard deviation ($n = 4$) as determined by HPLC-UV and Folin–Ciocalteu (FC) assay. Results are expressed as μg per gram bran, except for the FC assay where it is expressed as μg ferulic acid equivalent per gram of wheat bran. ND is for non-detectable.

Acid hydrolysed phenolic acids (μg g^{-1} bran)	Katepwa	AC Crystal	AC Vista	Glenlea	AC Avonlea	AC Andrew
3,4-Dimethoxycinnamic	ND	ND	ND	ND	ND	ND
4-Hydroxybenzoic	ND	ND	ND	ND	ND	ND
Caffeic	20.6 ± 4.2	28.6 ± 0.6	22.3 ± 15.7	14.8 ± 8.0	10.1 ± 5.2	5.2 ± 1.4
p-Coumaric	ND	ND	ND	ND	ND	ND
trans-Ferulic	1208.1 ± 105.1	1443.0 ± 18.2	1926.6 ± 664.1	1239.8 ± 240.6	780.2 ± 41.3	649.0 ± 327.2
cis-Ferulic	31.2 ± 14.1	18.5 ± 0.7	31.0 ± 23.4	21.7 ± 1.2	17.2 ± 2.5	17.5 ± 21.0
Protocatechuic	ND	ND	ND	ND	ND	ND
Sinapic	34.5 ± 8.6	79.3 ± 3.5	40.1 ± 14.1	82.4 ± 28.4	30.5 ± 5.4	13.7 ± 3.7
Salicylic	178.9 ± 30.2	189.7 ± 13.1	325.8 ± 215.8	515.9 ± 181.9	76.7 ± 14.3	37.8 ± 4.9
Syringic	20.6 ± 7.5	27.7 ± 5.0	28.3 ± 15.4	40.9 ± 16.3	10.5 ± 2.6	12.8 ± 4.9
Vanillic	31.1 ± 4.2	44.6 ± 10.4	ND	35.1 ± 12.8	ND	8.6 ± 1.0
Total phenolics (HPLC-UV)	1525.2 ± 155.1	1831.5 ± 4.5	2373.9 ± 870.0	1950.5 ± 519.2	925.2 ± 64.5	744.6 ± 625.8
Total phenolics (FC)	1896.5 ± 82.0	2825.5 ± 616.8	2180.8 ± 746.9	1563.2 ± 339.4	1444.3 ± 356.2	1078.0 ± 87.0

Table 3
Phenolic acid composition of alkaline hydrolysed bran of six wheat cultivars. Mean values ± standard deviation ($n = 4$) as determined by HPLC-UV (UV) and Folin–Ciocalteu (FC) assay. Results are expressed as μg per gram bran except for the FC assay where it is expressed as μg ferulic acid equivalent per gram of wheat bran. ND is for non-detectable.

Alkaline hydrolysed phenolic acids (μg g^{-1} bran)	Katepwa	AC Crystal	AC Vista	Glenlea	AC Avonlea	AC Andrew
3,4-Dimethoxycinnamic	ND	ND	ND	ND	ND	ND
4-Hydroxybenzoic	ND	ND	ND	ND	ND	ND
Caffeic	ND	ND	ND	ND	ND	ND
p-Coumaric	282.7 ± 16.6	133.2 ± 22.6	278.1 ± 67.4	476.8 ± 58.0	289.7 ± 77.1	172.3 ± 81.8
trans-Ferulic	1660.6 ± 469.4	838.3 ± 303.1	954.0 ± 475.2	1191.5 ± 366.6	1491.2 ± 283.8	1388.3 ± 387.6
cis-Ferulic	46.2 ± 10.9	28.4 ± 12.2	91.6 ± 88.8	47.0 ± 18.3	39.3 ± 22.3	45.1 ± 44.7
Protocatechuic	ND	ND	ND	ND	ND	ND
Sinapic	134.3 ± 124.8	173.1 ± 115.4	67.3 ± 16.1	207.8 ± 138.3	151.4 ± 80.2	110.0 ± 88.2
Salicylic	173.4 ± 146.4	50.4 ± 19.9	96.9 ± 68.8	157.1 ± 81.3	78.2 ± 30.3	109.8 ± 95.3
Syringic	ND	ND	ND	ND	ND	ND
Vanillic	90.6 ± 43.0	ND	63.4 ± 24.0	51.3 ± 16.8	59.1 ± 9.7	70.2 ± 45.1
Total alkaline phenolics (UV)	2387.7 ± 713.0	1223.4 ± 290.5	1551.3 ± 474.8	2131.5 ± 535.5	2108.9 ± 270.7	1895.8 ± 606.8
Total alkaline phenolics (FC)	4043.6 ± 287.1	3165.2 ± 289.8	3309.5 ± 509.7	4017.8 ± 216.1	2792.8 ± 571.8	2784.1 ± 676.4
Total (free + alkaline) (UV)	2440.8 ± 486.3	1290.2 ± 330.5	1610.0 ± 508.4	2177.2 ± 706.8	2156.3 ± 478.2	1944.2 ± 517.6
Total (free + alkaline) (FC)	5704.6 ± 271.3	4835.3 ± 359.4	4969.9 ± 537.6	5471.4 ± 344.6	3929.1 ± 573.9	4251.6 ± 844.9

Table 4
Phenolic acid composition of re-hydrolysed bran of six wheat cultivars. Mean values ± standard deviation ($n = 4$) as determined by HPLC-UV (UV) and Folin–Ciocalteu (FC) assay. Results are expressed as μg per gram bran, except for the FC assay where it is expressed as μg ferulic acid equivalent per gram of wheat bran. ND is for non-detectable.

Alkaline hydrolysed phenolic acids (following acid hydrolysis) (μg g^{-1} bran)	Katepwa	AC Crystal	AC Vista	Glenlea	AC Avonlea	AC Andrew
3,4-Dimethoxycinnamic	ND	ND	ND	ND	ND	ND
4-Hydroxybenzoic	ND	ND	ND	ND	ND	ND
Caffeic	ND	ND	ND	ND	ND	ND
p-Coumaric	226.7 ± 71.4	72.3 ± 55.4	289.0 ± 135.4	330.0 ± 59.1	175.0 ± 34.0	225.7 ± 21.6
Ferulic	1188.1 ± 89.5	793.3 ± 317.9	1294.8 ± 122.5	1328.7 ± 19.2	890.9 ± 139.0	1194.2 ± 27.8
Ferulic isomer	27.1 ± 5.9	14.9 ± 0.6	44.7 ± 32.2	8.0 ± 8.7	31.5 ± 9.9	33.4 ± 4.7
Protocatechuic	ND	ND	ND	ND	ND	ND
Sinapic	39.8 ± 6.5	77.8 ± 8.5	101.2 ± 73.4	83.1 ± 10.1	ND	27.6 ± 0.9
Salicylic	143.3 ± 25.6	92.2 ± 1.9	29.8 ± 5.4	148.0 ± 28.5	14.3 ± 0.4	24.6 ± 0.0
Syringic	52.9 ± 9.3	47.9 ± 0.8	69.0 ± 27.6	65.4 ± 8.1	15.0 ± 1.8	38.8 ± 2.5
Vanillic	63.4 ± 12.3	52.1 ± 0.7	37.9 ± 3.0	66.8 ± 11.7	24.4 ± 7.2	38.4 ± 10.5
Total alkaline phenolics (UV)	1741.4 ± 196.5	1150.5 ± 348.1	1866.4 ± 340.4	2060.9 ± 78.1	1150.4 ± 170.6	1582.6 ± 73.7
Total alkaline phenolics (FC)	1851.0 ± 344.9	1187.3 ± 202.9	1697.9 ± 445.4	2229.2 ± 644.8	1447.6 ± 173.2	1623.1 ± 152.5
Total (free + acid + alkaline) (UV)	3319.2 ± 137.0	3048.8 ± 327.1	4299.1 ± 1148.1	4057.2 ± 545.2	2123.0 ± 174.9	2375.6 ± 522.0
Total (free + acid + alkaline) (FC)	5413.6 ± 353.7	5682.9 ± 702.5	5539.1 ± 1013.1	5245.9 ± 444.3	4028.3 ± 417.5	4168.5 ± 346.1

2.5. Folin–Ciocalteu (FC) determination of phenolic content

Phenolic concentrations in the extracts were determined as described (Singleton, Orthofer, & Lamuela-Raventós, 1999). Briefly, bran extracts were oxidised with the Folin–Ciocalteu reagent. To neutralise the reaction, a sodium tartrate solution was added and the samples were placed in a 40 °C water bath for 30 min. The absorbance of the solution was measured at 765 nm and phenolic concentrations were determined against external standards of ferulic acid. The phenolic content is expressed as μg of FAE g^{-1} bran.

2.6. Phenolic acids composition by HPLC-UV/MS

Phenolic acid analysis was performed using liquid chromatography with UV detection and confirmed with mass spectrometry. The samples and standards (10 μl) were injected using an Autosampler attached to a HPLC (Agilent 1100, Santa Clara, CA, USA) consisting of a 1100 pump and diode array detector. The phenolic acids were separated on a reverse phase C_{18} Atlantis T3 analytical column 2.1 × 100 mm with a 3 μm particle size (Waters, Milford, MA, USA). The sample tray temperature was 10 °C while the column

Table 5
Antioxidant capacity of alcohol soluble, acid, alkaline and alkaline re-hydrolysed (after acid hydrolysis) bran of six wheat cultivars. Mean values ± standard deviation ($n = 4$). Antioxidant activity is expressed as percent discolouration of 2,2-diphenyl-1-picrylhydrazyl (DPPH) or in μmol of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent. ABTS is 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid).

Radical quenching capacity of extracts (% discolouration)	Katepwa	AC Crystal	AC Vista	Glenlea	AC Avonlea	AC Andrew
DPPH discolouration (%)						
Alcohol soluble (free phenolics)	8.7 ± 2.1	13.6 ± 3.5	16.2 ± 0.1	11.6 ± 4.0	9.2 ± 2.0	5.2 ± 3.9
Acid hydrolysed	18.6 ± 0.6	26.6 ± 6.1	20.1 ± 3.2	12.3 ± 2.6	13.2 ± 2.3	9.7 ± 1.0
Alkaline hydrolysed	17.4 ± 3.0	16.3 ± 2.1	16.2 ± 0.8	17.3 ± 5.0	15.5 ± 0.8	15.0 ± 1.4
Alkaline re-hydrolysis	13.6 ± 2.9	12.9 ± 0.6	15.8 ± 2.4	13.1 ± 1.5	13.1 ± 1.2	16.6 ± 2.6
ABTS (μM Trolox equivalent)						
Alcohol soluble (free phenolics)	3.4 ± 0.4	3.4 ± 0.05	3.8 ± 0.3	4.4 ± 1.2	2.3 ± 0.4	2.9 ± 0.6
Acid hydrolysed	8.3 ± 0.3	8.6 ± 0.9	8.9 ± 0.9	7.1 ± 0.9	7.4 ± 0.9	6.0 ± 0.4
Alkaline hydrolysed	10.3 ± 0.3	9.2 ± 1.2	10.9 ± 0.3	10.5 ± 0.05	8.6 ± 0.2	9.1 ± 0.1
Alkaline re-hydrolysis	7.8 ± 0.6	5.9 ± 1.2	8.1 ± 0.1	8.9 ± 0.7	7.9 ± 0.5	7.7 ± 0.3

temperature was 23 °C. Eluent A was Milli Q purified water while eluent B was acetonitrile, both containing 0.1% formic acid (v/v). The solvent gradient, at a flow rate of 200 μl min^{-1}, was as follows: 0 min 100% A; 30 min 85% A; 50 min 50% A; 55 min 50% A; 60 min 0% A; 65 min 100% A; 80 min 100% A. The mass spectrometer was a QStar XL (AB Sciex Instruments, Foster City, CA, USA) hybrid quadrupole TOF operated in the negative ion mode (ES^-). Mass spectra were acquired continuously over a mass range of 100–1000 at a scan rate of 1 s with no delay. The capillary voltage was −3200 and the temperature of the drying gas, air, was 400 °C. The phenolic acids were detected at either 280 or 320 nm by comparison to the retention times of standards and confirmed by the $[M–H]^-$ ion mass. The phenolics were quantified by comparing the peak area of the samples with the peak area of the external calibration curves for all of the phenolic acids (all $r^2 < 0.99$). Cis-ferulic acid was quantified against standards of trans-ferulic acid.

2.7. Determination of antioxidant activity

2.7.1. DPPH

Antioxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) was measured as previously described (Beta et al., 2005). The extracts (0.1 ml) were reacted with 3.9 ml of a 6×10^{-5} mol l^{-1} of DPPH solution. Absorbance of the extracts was determined on a DU 800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA) at time 0 and 30 min at an absorbance (A) of 515 nm. Antioxidant activity was determined as percent discolouration = (1 − [A of sample at 30 min/A of control at time 0]) × 100. The data for all antioxidant assays is reported as % discolouration ± standard deviation (SD).

2.7.2. ABTS–Trolox

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) equivalent antioxidant capacity (TEAC) assay was performed as previously described (Re et al., 1999). The $ABTS^{\cdot+}$ cation was generated in dark at room temperature by reacting a 7 mM solution of ABTS with 2.45 mM potassium persulphate (final concentration) for 16 h. The resulting $ABTS^{\cdot+}$ was diluted with ethanol to give an absorbance reading of 0.7 ± 0.02 at 734 nm. The sample, 10 μl, was reacted with 1 ml of $ABTS^{\cdot+}$ solution and absorbance (A) measured at 734 nm after 1 min. Trolox equivalency of the samples was calculated by comparing with a standard curve prepared with Trolox ($r^2 = 0.98$).

2.8. Statistical analysis

All analyses were conducted in four repetitions and data reported as means ± SD. Data has been corrected for sample weight and moisture and is on a dry mass basis. Analysis of variance was performed using Sigma Stat 2.03 (Sysstat Software Inc., San Jose, CA, USA) for Windows, and significant differences ($p < 0.05$) among means were determined using the Tukey's multiple range test set at $\alpha = 0.05$.

3. Results and discussion

3.1. Free phenolics

The free phenolics were extracted from bran of all six wheat cultivars with 80% aqueous ethanol. Phenolic contents (μg g^{-1} bran) were measured with the FC assay and the phenolic composition was determined by HPLC-UV. The average free phenolics (FP) content (FC assay) was 1509.0 ± 208.4, with the lowest being 1136.4 for the durum cultivar Avonlea, while the common wheat cultivars Katepwa, AC Crystal and AC Vista gave similar results of approximately 1660 μg g^{-1} FAE (Table 1). The FP content represented 28–35% of the total FC assayed phenolic content. Comparing the present study with previous ones is difficult because researchers use different wheat cultivars, variously pre-treat the flour or bran and use a variety of solvents to extract the "free phenolics" (Adom et al., 2003; Liyana-Pathirana & Shahidi, 2006; Zhou & Yu, 2004). We had previously, using the same protocol as described here, extracted FP from the bran of 51 wheat cultivars and found the average for the six studied here to be 1280 ± 304, with 854 ± 265 for Avonlea and 1650 ± 206 μg g^{-1} GAE for Katepwa (Verma, Hucl, & Chibbar, 2008). Although the FC assay is very useful in determining phenolic concentrations, it does have disadvantages in that it is not specific and may not react with some phenolics within the extract so that phenolic concentrations may be underestimated (Stalikas, 2007). Conversely, the FC reagent can be reduced by some sugars and proteins, cyclic organic compounds and chelating agents leading to overestimation of phenolic concentrations (Peterson, 1979).

To accurately determine concentrations of individual phenolics, these were separated by HPLC and each fraction was quantified by UV absorption. In the FP extract of the six wheat cultivars, HPLC-UV analysis showed that the dominant identifiable phenolic acid was trans-ferulic, and it accounted for 15.6–34.7% of the total identifiable FP content (Table 1). Other phenolic acids comprised a significant but varying portion of the total identifiable pool: protocatechuic (13.7–19.9%), 4-hydroxybenzoic (9.3–25.4%), vanillic (non-detectable (ND) – 17.9%) and salicylic acid (1.7–16.7%). Some phenolics, such as caffeic, *p*-coumaric, 3,4-dimethoxycinnamic, sinapic, and syringic were minor components in the FP extract, while others, a lignan, secoisolariciresinol diglycoside (SDG) and gallic acid, were not detected. An isomer of ferulic acid, cis-ferulic acid with a deprotonated mass m/z of 193.1 and an elution time of 47.7 min as compared to 40.4 min for trans-ferulic acid,

was identified with mass spectrometry. The average total FP content (addition of all of the HPLC-UV detected phenolic acids) was 53.3 ± 8.1 μg g^{-1} bran and ranged from 45.8 ± 6.3 μg for Glenlea to a high of 66.8 ± 16.3 μg for AC Crystal. These values compare favourably with earlier findings despite differences in sample types, preparation and extracting solvents (Hatcher & Kruger, 1997; Liyana-Pathirana & Shahidi, 2007; Sosulski, Krygier, & Hogge, 1982).

The FP content measured by the FC method was nearly 28 times that measured by HPLC-UV analysis. A primary reason for this large discrepancy between the two assays is that many of the compounds that are part of an extract's HPLC-UV chromatogram profile are unknown and therefore not quantified. It was found that two deprotonated species $[M–H]^-$ with m/z of 625.2 and 203.1, which eluted at ~17.5 and 22.5 min, respectively, were major components of the 280 nm absorbance chromatogram. Since the elution time did not match with any of the phenolic standards, neither were quantified. The 203.1 ion, for example, absorbed very strongly at 280 nm and comprised 24.7 (AC Vista), 27.9 (AC Crystal), 35.6 (Glenlea), 39.1 (AC Avonlea), 39.8 (AC Andrew) and 46.3% (Katepwa) of the total area of chromatographic peaks detected at 280 nm. In the chromatograms of the same samples at 320 nm, trans-ferulic acid comprised only 12.2 (AC Vista) and 8.5% (Katepwa) of the total area of chromatographic peaks observed. By comparison to a standard, the 203.1 ion was identified, based on both the elution time and $[M–H]^-$ with m/z of 203.1, as tryptophan (MW 204.1). Tryptophan occurs in both free and protein-bound forms and is concentrated in the wheat bran, with a recent study reporting free tryptophan levels of 1287.9 μg g^{-1} bran, though the wheat cultivar was not specified (Morales, Acar, Serpen, Arribas-Lorenzo, & Gokmen, 2007). Defatting solvents, at least acetone, may not remove the free tryptophan from the wheat bran (Comai et al., 2007). This finding suggests that researchers are including tryptophan as part of the phenolic acid pool, especially if the FC assay is used for quantification, since the FC reagent will react with tryptophan (Lowry, Rosebrough, Farr, & Randall, 1951). Besides the ions mentioned above, the FP extract also contained significant amounts of deprotonated ions with m/z of 172.1, 187.1, 203.1, 237.0, 452.2 and 563.2. The identification of these compounds is outside the scope of the present work, but it does indicate that wheat bran extract is extremely complex and in addition to the identifiable phenolic acids, the extracts may include amino and fatty acids (Gallardo et al., 2006; Krygier et al., 1982), prolamines and unidentified phenolic and acidic compounds.

3.2. Acid and alkali-labile phenolics

Studies have established that the majority of the phenolics present in the wheat grain are insoluble and bound to cell wall components. In this work, acidic and alkaline hydrolysis, at room temperature, were compared to determine the phenolic acid constitution in the bran of six different wheat cultivars. In the bran hydrolysed with HCl, the average FC measured phenolic yield was 1831.4 ± 617.1 and ranged from 1078.0 ± 87.0 to 2825.5 ± 616.8 μg g^{-1} bran (Table 2). The average phenolic yield determined by HPLC-UV was 1558.5 ± 625.8 with a range of 744.6 ± 625.8–2373.9 ± 870.0 μg g^{-1} bran (Table 2) and was similar to that calculated with the FC assay. Alkaline hydrolysis (Table 3) liberated, on average, 3352.2 ± 564.5 μg g^{-1} bran (FC) phenolics that ranged from 2784.1 ± 676.4 for AC Andrew to 4043.6 ± 287.1 μg g^{-1} bran in Katepwa. The HPLC-UV determined concentrations of alkali-labile phenolics ranged from 1895.8 ± 606.8 (AC Andrew) to 2387.7 μg g^{-1} bran (Katepwa) (Table 3). Overall, alkali hydrolysis liberated nearly twice the amount of phenolics as acid hydrolysis, as determined by the FC assay. However, according to HPLC-UV, there was little difference between the acid ($\bar{x}$ = 1558.5 μg g^{-1} bran) and alkaline ($\bar{x}$ = 1883.1 μg g^{-1} bran) hydrolysis in terms of identifiable phenolic acids liberated. Ferulic acid was the dominant phenolic acid in both of the hydrolysates and made up from 55.9% to 87.2% of the total identifiable phenolic pool. The average amount of ferulic acid that was acid-labile was 1207.8 ± 462.2 while 1254.0 ± 318.2 μg g^{-1} bran was labile under alkali conditions. However, comparing the ratio of the yield of ferulic acid to other phenolics within the extracts shows that on average ferulic acid comprised 79.0% of the total acid-labile pool, but only 66.6% of the alkali-labile fraction. In other words, 21% of the total acid-liberated components were phenolics other than ferulic acid but in the alkali-liberated fraction the other phenolics comprised 33.4% of the fraction. Therefore, on average, a larger portion of phenolic compounds within the bran are alkali-labile and a smaller portion acid-labile.

Alkali hydrolysis was more efficient in releasing vanillic, cis-ferulic and sinapic acids from bran as compared to acid hydrolysis. Syringic acid was not detectable in the base-hydrolysed samples but was in the acid-hydrolysed fraction, suggesting that the optimum method to liberate this phenolic is by acidic hydrolysis. Similarly, caffeic acid, which was found in the acid-labile fraction, was not detected in the alkali fraction, supporting Krygier et al. (1982) who reported a 66.7% loss of caffeic acid when hydrolysed in 2 M NaOH at room temperature. However, *p*-coumaric acid, which was not detected in the acid-labile fraction, was measured at very high levels in the alkali-labile fraction indicating that saponification is required to liberate it from its conjugates. This is consistent with a previous study (Krygier et al., 1982) where, using 1 M HCl for 30 min at 100 °C, the authors reported a 72.9%, 78.0%, 87.3% and 91.7% loss of standards of *p*-coumaric, trans-ferulic, caffeic and trans-sinapic acids, respectively. There seemed to be no loss in trans-ferulic acid at room temperature as the average trans-ferulic acid yield from both hydrolysis methods was similar. However, the amount of trans-ferulic acid removed by acid and base for the same cultivars were different (Tables 1–3), suggesting differences in ferulic acid conjugates, hence differences in lability, in bran.

Overall, the concentrations of total phenolics obtained in this study are in the range of previous ones where bran and similar hydrolysis conditions were used (Abdel-Aal et al., 2001; Beta et al., 2005; Verma et al., 2008). In terms of acid hydrolysis, Kim et al. (2006), utilising red wheat bran hydrolysed with HCl at 95 °C for 1 h, reported HPLC-UV determined phenolic fraction of 323.6 μg g^{-1} bran of which only 34.4 μg was ferulic acid. This supports Krygier et al. (1982) and their report of losses in ferulic acid under high temperatures conditions. The concentrations of ferulic acid in bran in the present study are similar to or slightly lower than those found in other studies where saponification was used. Using gas–liquid chromatography for detection and quantitation, Abdel-Aal et al. (2001) measured ferulic acid levels ranging from 2552 to 3215 μg g^{-1}. Gallardo et al. (2006), using bran that was a byproduct of the milling industry and containing no albumen but some husks, reported 4000 μg g^{-1} ferulic acid. Kim et al. (2006) reported ferulic acid content of two wheat cultivars ranging from 1359 to 1934 μg g^{-1} bran. The differences in ferulic acid content in wheat, and therefore overall phenolic content are thought to have a genetic basis (Abdel-Aal et al., 2001; Stalikas, 2007) but with strong environmental influences (Moore et al., 2006).

Due to the yield of phenolics from the acid hydrolysed bran being nearly half that of the saponified bran, the acid-hydrolysed bran was subjected to alkaline hydrolysis. The re-hydrolysis released, on average, an additional 1672.7 ± 355.2 μg g^{-1} bran of phenolics as measured by the FC assay and 1592.0 ± 376.2 μg g^{-1} bran as quantitated by HPLC-UV (Table 4). Results show that phenolic acids such as *p*-coumaric, vannilic, and sinapic require alkaline hydrolysis, salicylic requires acid hydrolysis and syringic requires both acid and base digestion. Re-hydrolysis with NaOH also released a significant

amount of additional ferulic acid, ranging from 793.3 ± 317.9 (AC Crystal) to 1328.7 ± 19.2 μg g^{-1} bran (Glenlea). The average ferulic acid released in total (acidic + base follow up) was 2322.8 ± 554.2 μg g^{-1} bran and ranged from 1671.1 in Avonlea to 3221.4 μg g^{-1} bran in AC Vista. The additional recovery of ferulic acid by re-hydrolysis suggests differential binding of ferulic acid to cell wall components, with about half of the ferulic acid being acid-labile and the remaining portion alkali-labile. The present work suggests that to obtain a comprehensive profile of phenolic acids present within wheat bran, several hydrolysis schemes should be used, to prevent an underestimation of phenolic acids content and constitution.

3.3. Antioxidant capacity

DPPH· and the $ABTS^{\cdot+}$–Trolox equivalent (TE) assays were used to measure the antioxidant capacity of all of the extracts as well as individual phenolic standards. The FP extracts had the lowest DPPH· reducing capacity with an average of 10.8 ± 3.9%, while the acid-labile fraction had the highest average with 16.8 ± 6.2% (Table 5). Individually, the FP extract from AC Andrew was the weakest at reducing DPPH· (5.2 ± 3.9%), while the acid-labile fraction from AC Crystal had the highest measured reducing capacity (26.6 ± 6.1%). In terms of the $ABTS^{\cdot+}$–Trolox assay, the extract with the highest average capacity was the alkali-labile fraction with a TE of 9.8 ± 0.9 μmol g^{-1} bran and again the lowest was the FP extract with 3.4 ± 0.7 μmol TE g^{-1} bran. The lowest TE was 2.3 ± 0.4 μmol g^{-1} and was obtained from the durum AC Avonlea FP extract, while the highest was 10.9 ± 0.3 μmol g^{-1} from the alkali-labile fraction obtained from AC Vista.

It is difficult to compare the results obtained in this study to those of previous studies because of the range of environmental factors and wheat cultivars used, varying testing methods and extraction solvents (Zhou & Yu, 2004). While the use of only one solvent may be favoured, the solubility of individual phenolics varies and the results may not be representative of the true phenolic content of a wheat cultivar. As well, solvents are unlikely to remove the insoluble bound phenolics, so the antioxidant capacity of this dominant portion of phenolics will be significantly underestimated (Liyana-Pathirana & Shahidi, 2006; Serpen, Capuano, Fogliano, & Gokmen, 2007). Environment and genotype are also strong factors (Moore et al., 2006) so even comparisons of the phenolic contents of the same wheat cultivar grown in different locations, or years, become difficult to evaluate. Whether any of these antioxidant assays are applicable as surrogates of physiological or biochemical reactions that occur within the body is still a matter of debate (Liyana-Pathirana & Shahidi, 2006; Serpen et al., 2007). Regardless, Zhou and Yu (2004) used a variety of solvents for extraction from Akron and Trego wheat bran and reported TE against the ABTS radical ranging from 3.1 to 15.3 μmol g^{-1} bran. Similarly, using the bran of seven different wheat cultivars, the authors reported $ABTS^{\cdot+}$ scavenging capacity ranging from 17.5 to 19.7 μmol TE g^{-1} bran (Zhou et al., 2004), which is higher than reported here, but may be due to differences in extraction solvents. Gallardo et al. (2006) reported 16.4 μmol TE g^{-1} bran for extracts removed with 50 °C water, but only 5.5 for methanol extracts. With respect to DPPH· quenching capacity, we previously used methanol to extract antioxidants from the same six cultivars studied here and reported values ranging from 12.3 for AC Avonlea to 18.1 for Glenlea, which are very close to the present values despite the use of different solvents for extraction. Recent studies (Beta et al., 2005; Moore et al., 2006; Verma et al., 2008) have shown a strong correlation between total phenolic content and antioxidant capacity.

The difference in antioxidant capacity of different wheat cultivars could be due to their different phenolic acids composition. We measured the ability of 50 μg ml^{-1} standards of all phenolic acids and a lignan SDG to reduce the DPPH and the ABTS radicals (Table 6). Overall, except SDG, the phenolics reduced DPPH· by 20.1 ± 25.7% and $ABTS^{\cdot+}$ by 0.4 ± 0.3 μmol TE. Separating the phenolics into cinnamic and benzoic acid derivatives showed there to be little difference, on average, between the two groups in reducing DPPH· (18.0 ± 16.9% and 22.2 ± 33.8%, respectively) or $ABTS^{\cdot+}$ (0.3 ± 0.2 and 0.4 ± 0.4 μmol TE, respectively). Statistically, the correlation between DPPH· and $ABTS^{\cdot+}$ was weak ($r = 0.5$, $p = 0.08$) indicating variability in the activity of the phenolics against the DPPH and ABTS radicals. Individually, ferulic acid was ineffective in reducing DPPH· and this may explain the relatively weaker correlation between DPPH· and ferulic acid observed. The benzoic acid derivatives showed both the strongest (gentisic and gallic acid) and weakest (vanillic and salicylic) reducing power against DPPH· suggesting that the differences in reactivity or quenching of the radicals are due to difference in structure and the type and number of substituents on the phenyl ring (Lien, Ren, Bui, & Wang, 1999; Rice-Evans, Miller, & Paganga, 1996). The antioxidant capacity of phenolic acids and how their structures and substituents play a role are obviously complex (Lien et al., 1999; Rice-Evans et al., 1996). However, it should always be considered that when a phenolic acid donates one or several protons, the phenolic acid itself becomes a free radical and unless it is stable, it has the potential to cause oxidative damage to the cell. This aspect of wheat phenolics has not been well studied and should be investigated in future studies.

The contribution of an individual phenolic acid to total wheat bran antioxidant activity is obtained by multiplying the concentration of each known phenolic acid with the appropriate value obtained by the standard against both of the radicals. The concentration of caffeic acid, for example, is multiplied by 26.9 whereas ferulic acid by 8.1 (Table 6) so that the contribution is weighted according to phenolic concentration and effectiveness against the radical. The activity of all of the known phenolics is added and the contribution of each phenolic is calculated as a percentage of the total phenolic activity pool. For DPPH discolouration, ferulic acid contributed from 66.4% (AC Crystal, Table 3) to 92.1% (AC Avonlea, Table 4) of the overall antioxidant capacity of all of the hydrolysed extracts. In AC Crystal, sinapic acid made 14% of the phenolic pool but contributed 29.4% towards the antioxidant activity, because it is more than twice as effective an antioxidant as ferulic acid against the DPPH assay. With respect to reducing $ABTS^{\cdot+}$, the contribution of ferulic acid ranged from

Table 6
Antioxidant activity of 50 μg ml^{-1} phenolic acid standards. Antioxidant activity is expressed as percent discolouration of 2,2-diphenyl-1-picrylhydrazyl (DPPH) or in μmol of 6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid (Trolox) equivalent. ABTS is 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). Negative number indicates increased colouration of the radical solution.

Phenolic acid standard (50 μg ml^{-1})	DPPH (discolouration (%) at 30 min)	ABTS (μM Trolox equivalent at 1 min)
3,4-Dimethoxycinnamic	1.2	−0.02
Caffeic	26.9	0.39
Chlorogenic	22.0	0.17
Ellagic	49.0	0.12
trans-Ferulic	8.1	0.64
p-Coumaric	1.5	0.47
Sinapic	17.4	0.41
3,4-Dihydroxybenzoic	17.2	0.48
4-Hydroxybenzoic	4.1	−0.019
Gallic	60.2	1.17
Gentisic	78.9	0.48
Salicylic	−6.4	0.003
Syringic	7.3	0.45
Vanillic	−5.8	0.35
Secoisolariciresinol diglycoside (SDG)	9.8	0.22

68.1% (Glenlea, Table 3) to 95.5% (AC Crystal, Table 2). The contribution of ferulic acid was lower in Glenlea because of higher concentrations and contributions by *p*-coumaric acid (20.0%) and sinapic acid (7.6%). The contribution of other identified phenolic acids to overall extract antioxidant capacity in the hydrolyzate extracts was as follows ($DPPH^{\cdot}$:$ABTS^{\cdot+}$): vanillic acid (0–0:0.7–3.0); caffeic acid (2.4–4.9:0.5–1.1); *p*-coumaric acid (1.3–5.0:5.5–20.0); cis-ferulic acid (the activity of trans-ferulic acid standards was used) (1.1–7.4:1.3–6.9); sinapic acid (4.0–29.4:1.2–10.3); salicylic acid (0–0:0.006–0.2); and syringic acid (1.0–4.2:0.9–3.5).

There are several assumptions made when calculating the contribution of individual phenolic acids to the overall antioxidant pool. First, it is assumed, that there is little to no contribution to antioxidant capacity by other unknown compounds within the extract. This is in some instances, such as for the FP extracts examined here, unlikely to be true especially if the identified phenolic acids are a very small proportion of the total phenolics. In the FP extracts, where a significant amount of tryptophan was found, the identified phenolics were only 3.5% of the FC measured phenolics. Tryptophan and its metabolites are known to be strong antioxidants, exceeding equimolar concentrations of ascorbate or Trolox (Christen, Peterhans, & Stocker, 1990; Elias, McClements, & Decker, 2005), so any calculations based on the minor identified phenolic components may be highly misleading. Second, it is assumed that a combination of phenolics is unlikely to reduce the radicals to a greater extent than the individual phenolic acid. There is evidence this may be true as Gallardo et al. (2006) found that a mixture of phenolic standards similar to that found in an extract reduced the $ABTS^{\cdot+}$ radical comparably to that of an extract. In another recent study, the authors reported no synergistic effect when two standards of phenolic acids were combined and the activity measured against both $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ (Zhou, Yin, & Yu, 2006). A third assumption is that the quenching capacity remains linear even at the very high concentrations of phenolics present in the wheat bran. Nevertheless, in samples such as the insoluble bound fractions that are relatively clean and in which ferulic acid dominates, assignment of antioxidant contribution may be valuable.

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