JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Phytochemical Profile of Main Antioxidants in Different Fractions of Purple and Blue Wheat, and Black Barley

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Two pigmented wheat genotypes (blue and purple) and two black barley genotypes were fractionated in bran and flour fractions, examined, and compared for their free radical scavenging properties against 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation (Trolox equivalent antioxidant capacity, TEAC), ferric reducing antioxidant power (FRAP), total phenolic content (TPC), phenolic acid composition, carotenoid composition, and total anthocyanin content. The results showed that fractionation has a significant influence on the antioxidant properties, TPC, anthocyanin and carotenoid contents, and phenolic acid composition. Bran fractions had the greatest antioxidant activities (1.9–2.3 mmol TEAC/100 g) in all four grain genotypes and were 3–5-fold higher than the respective flour fractions (0.4–0.7 mmol TEAC/100 g). Ferulic acid was the predominant phenolic acid in wheat genotypes (bran fractions) while *p*-coumaric acid was the predominant phenolic acid in the bran fractions of barley genotypes. High-performance liquid chromatography analysis detected the presence of lutein and zeaxanthin in all fractions with different distribution patterns within the genotypes. The highest contents of anthocyanins were found in the middlings of black barley genotypes or in the shorts of blue and purple wheat. These data suggest the possibility to improve the antioxidant release from cereal-based food through selection of postharvest treatments.

KEYWORDS: Anthocyanins; carotenoids; phenolics; colored grain; *Hordeum*; *Triticum*

INTRODUCTION

Since their domestication, cereal grains have become staple foods providing protein, carbohydrates, and fiber. In recent years, minor bioactive compounds, for example, polyphenols and carotenoids, have attracted more and more interest from both researchers and food manufacturers as health-promoting and disease-preventing effects were found in both in vitro and in vivo studies. It appears that phytochemicals linked to the fiber skeleton, in addition to or instead of the fiber itself, are responsible for the reduced risk of various diseases associated with oxidative stress, such as cancer and cardiovascular and neurodegenerative diseases (*1*). Because vitamins C and E are absorbed in the upper segments of the intestine, polyphenols constitute together with carotenoids the only dietary antioxidants present in the colon in valuable concentrations (*2, 3*). In cereals, the predominant phenolic acid is ferulic acid, representing up to 90% of total polyphenols (*4*). Almost all ferulic acid is found in the pericarp and aleurone layer (*5, 6*), mainly ester-linked to polymers in the plant cell wall (*7, 8*). Free phenolics account only for a small percentage of the total phenolic content, for example, 16–28% in whole wheat flours (*9*). The inverse association between whole grain consumption and incidence of certain chronic diseases could be explained by the release of phenolics in the colon (*10, 11*). Supporting such a hypothesis, an apparent increase of antioxidant release during enzymatic incubation was found in in vitro studies (*12, 13*).

Other phenolic compounds present in valuable amounts in pigmented cereals are anthocyanins (*14*). Anthocyanins in wheat and barley are found either in the pericarp or in the aleurone

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10.1021/jf072021j CCC: $37.00
Published on Web 09/26/2007

layer, causing purple and blue hues of kernel color, respectively. Genes for purple pericarp are derived from tetraploid Ethiopian wheat (*Triticum aethiopicum* Jakubz.), while blue aleurone is caused by alien genes from diploid wild einkorn wheat (*Triticum boeoticum* Boiss.) or tall wheatgrass [*Agropyron elongatum* (Host) P. Beauv.] (*15*). "Black" (deep purple) hues of wheat kernel color can be due to the combination of anthocyanin genes for purple pericarp and blue aleurone (*16*). The black pigmentation of the lemma and pericarp of barley (*Hordeum vulgare* L.), however, is described to be due to a melanin-like pigment (*17*), which may overlap purple or blue hues due to anthocyanins. The black kernel color due to melanin-like pigment is unknown in wheat species.

Potential health benefits, such as the ability to act as antioxidants, immunoenhancers, and inhibitiors of premalignant lesions, have been demonstrated for carotenoids as well as for phenolics. Grains high in carotenoids may protect against oxidative damage, as carotenoids function as free radical scavengers. Lutein and zeaxanthin are responsible for the coloration of the macula lutea ("yellow spot") of the retina, the area of maximal visual acuity. Hence, dietary lutein and zeaxanthin are supposed to protect against age-related macular degeneration and cataract (*18, 19*). There is also the possibility that lutein and zeaxanthin act together with other carotenoids and phytochemicals against cancer, cardiovascular risk, and other diseases (*20, 21*).

A specific extraction methodology to determine phytochemicals in cereals has not yet been established. Most research groups focused on the evaluation of free phenolic acids after extraction of wholemeal samples using aqueous methanol, ethanol, or acetone where a distinct correlation between phenolic content and antioxidant activity was found (*6, 9, 16, 22–25*). Only recently, researchers started to determine the content of bound phenolic compounds by alkaline hydrolysis, acid hydrolysis, or enzymatic digestion (*9, 12, 13, 22–29*). The majority of literature deals with American-grown common wheat. Only a few papers focus on grain varieties of European origin (*23, 24, 30–32*), pigmented grains (*14, 16*), or the distribution of phytochemicals in the kernel (*6, 16, 24*).

Although black, blue, or purple pigmented grains are currently produced in small amounts, they hold promise as valuable raw materials for the production of functional foods or natural food colorants. It was therefore the aim of this work to investigate the distribution of phytochemicals (total phenolics, anthocyanins, carotenoids, and phenolic acids) in different millstreams of four pigmented cereal genotypes and to determine their antioxidant capacities.

MATERIALS AND METHODS

Chemicals and Reagents. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 2,4,6-tripyridyl-*s*-triazine (TPTZ), trifluoroacetic acid, potassium persulfate, sodium hydroxide, Folin–Ciocalteau reagent, phenolic acid standards, and carotenoid standards (lutein and zeaxanthin) were purchased from Sigma-Aldrich (Vienna, Austria). Acetonitrile was obtained from VWR (Vienna, Austria). Ethyl acetate, tetrahydrofuran, methanol, and ethanol were purchased from Carlo Erba (Milan, Italy). All chemicals and solvents used in the study were of high-pressure liquid chromatography (HPLC)-grade.

Grain Samples and Sample Preparation. Purple pericarp Ethiopian wheat (PPW) BVAL-258034 (*T. aethiopicum*), blue aleurone wheat (BAW) BVAL-214025 (*T. aestivum*), two-rowed black barley (BB2) SG-8822.20-2R, and six-rowed black barley (BB6) SG-8822.20-6R (*H. vulgare*) were selected to study differences in their phytochemical profiles and antioxidant capacities. The plant materials were grown in the same conditions in 2004 at the BOKU Experimental Station in Raasdorf (16°35′E, 48°14′N); BB2, BB6, and PPW are of spring growth type and were sown in March 2004, whereas BAW is of winter growth type and was sown in October 2003. All samples were milled using an AQC 109 laboratory mill (Agromatic AG, Laupen, Switzerland), in which the sifting system of the mill was removed, and the samples were subsequently fractionated by a sieving machine (Promylograph LS apparatus, Max Egger Apparatebau, St. Blasen, Austria) equipped with Nytal polyamide flour sieves (Sefar Holding Inc., Thal, Switzerland) of the respective mesh widths into bran (>900 μm), shorts plus coarse middlings (>600 μm for barley, >400 μm for wheat), hereafter simply termed shorts, middlings (>180 μm), and flour (<180 μm). Each fraction was stored at −20 °C until usage.

Extraction of Total Phenolic Compounds. Total phenolic compounds (i.e., free and bound phenolics) were extracted as described (*6*), with minor modifications. Briefly, 0.15 g of flour was digested with 3 mL of 2 M sodium hydroxide at room temperature overnight under nitrogen gas with vortex mixing from time to time. The mixture was brought to pH 3.5 with 3 M citric acid. After centrifugation, supernatants and residues were extracted separately three times with ethyl acetate. The combined ethyl acetate fractions were evaporated to dryness. Phenolic compounds were dissolved in 50% methanol and subsequently used for the determination of antioxidant capacity, of phenolic acid composition, and total phenolic content.

Determination of Phenolic Acids. Phenolic acids were quantified using an RP-HPLC procedure employing a Phenomenex Luna 250 mm × 4.6 mm, 5 μm (HPLC Services, Breitenfurt, Austria) column. Gradient elution was conducted with 0.05% trifluoroacetic acid in water (A) and 0.05% trifluoroacetic acid in acetonitrile (B). The solvent gradient was delivered using a LC-10ADVP pump (Shimadzu, Korneuburg, Austria) and was programmed as follows: at 0 min, 10% B; increasing from 3 to 15 min to 15% B; 25 min, 20% B; 30 min, 40% B; 36–40 min, 80%; decreasing thereafter to 10% B within the next 4 min; and equilibrated before the next injection. The flow rate was 1.0 mL/min. Analyte detection was at 260 nm for vanillic acid and at 280 nm for *p*-coumaric acid and ferulic acid using a SPD-M10AVP photodiodearray detector (Shimadzu, Korneuburg, Austria). Data signals were acquired and processed on a PC running the LC Solution Multi software (Shimadzu, Korneuburg, Austria). Phenolic acids were extrapolated from pure phenolic acid standard curves. Twenty microliter injections were made in each run, and peak areas were used for all calculations.

The recovery of ferulic acid was determined as an addition of defined concentration of ferulic acid (pure compound) to either starch (inert matrix) or sample matrix. Both experiments were conducted in triplicate and routinely added to each experiment. The recovery defines the percentage of ferulic acid in the sample (sample matrix + standard) after subtracting the amount of the added ferulic acid from the total amount of ferulic acid in the matrix. The recoveries for bound ferulic acid analyses were 95 ± 2.3% (n = 3).

Determination of Total Phenolic Content (TPC). The TPC of extracts was determined using the Folin–Ciocalteau reagent. An appropriate dilution of extracts (100 μL) was added to 0.5 mL of freshly diluted 10-fold Folin–Ciocalteau reagent. A 0.8 mL amount of sodium carbonate solution (75 g/L) was added to the mixture after 2 min of reaction time. The absorbance of the resulting blue color was measured at 755 nm against a blank after 5 min of reaction at 50 °C. Ferulic acid was used as the standard, and TPC was expressed as mg ferulic acid (FA) equivalent per 100 g.

Determination of Anthocyanins. The determination of the total amount of anthocyanins (TAC) was done using the reported spectrophotometric method (*33*). Anthocyanins were extracted with acidified methanol (methanol and 1 M HCl, 85:15, v/v), and the absorbance was measured after centrifugation at 535 nm against a reagent blank. Cyanidin-3-glucoside (Extrasynthese, Genay, France) was used as standard pigment, and TAC was expressed as mg cyanidin-3-glucoside per 100 g.

Determination of Carotenoids. The determination of carotenoids was done according to the reported procedure (*31*) with some minor modifications. In brief, magnesium carbonate was added to 2 g of

Table 1. TPC and Phenolic Acid Concentrations (mg/100 g) of Pigmented Wheat and Barley

genotype/millstream	TPC (mg/100 g)[a]	ferulic acid	vanillic acid	*p*-coumaric acid
PPW				
bran + shorts	822.23 a ± 75.26[b,c]	398.61 a ± 7.97	16.36 a ± 1.32	14.30 a ± 0.70
middlings	145.15 b ± 9.45	53.18 bc ± 1.80	2.65 bc ± 0.10	1.46 c ± 0.03
flour	81.16 b ± 14.05	18.01 c ± 2.57	0.99 c ± 0.01	0.46 d ± 0.05
wholemeal	197.35 b ± 15.08	85.17 b ± 1.66	3.51 b ± 0.09	2.43 b ± 0.03
BAW				
bran	761.64 a ± 18.19	350.33 a ± 3.15	9.98 a ± 0.14	45.66 a ± 0.40
shorts	619.98 b ± 17.53	304.42 b ± 5.54	6.48 b ± 0.21	20.46 b ± 0.74
middlings	183.41 c ± 19.51	71.43 c ± 0.83	2.30 c ± 0.21	3.18 c ± 0.23
flour	64.65 d ± 6.53	15.11 d ± 2.22	1.01 d ± 0.16	0.63 d ± 0.11
BB6				
bran	921.64 a ± 11.15	200.24 a ± 3.24	11.73 a ± 0.33	336.70 a ± 4.23
shorts	572.94 b ± 20.39	182.28 a ± 8.63	4.68 b ± 0.31	35.73 b ± 2.77
middlings	169.21 c ± 38.54	37.96 b ± 4.90	2.34 c ± 0.28	6.69 c ± 1.04
flour	123.14 c ± 40.68	17.93 b ± 0.44	1.54 c ± 0.01	2.47 c ± 0.01
BB2				
bran	821.38 a ± 61.25	201.69 a ± 2.55	8.18 a ± 0.31	256.47 a ± 1.02
shorts	651.66 b ± 34.84	187.68 a ± 8.67	4.44 b ± 0.17	47.45 b ± 1.18
middlings	314.16 c ± 8.00	87.26 b ± 1.33	2.57 c ± 0.08	14.00 c ± 0.41
flour	106.16 d ± 29.23	21.85 c ± 6.47	0.93 d ± 0.05	4.58 d ± 0.30

[a] TPC is expressed as ferulic acid equivalents. [b] Mean ± SD. [c] Means followed by different letters are significantly different ($p < 0.05$) within the respective genotype.

sample. Afterwards, the samples were extracted with 10 mL of methanol/tetrahydrofuran (1:1, v/v), vortexed for 2 min, and homogenized for 5 min using an Ultra-Turrax (Jankel & Kunkel, Staufen, Germany). This extraction was repeated until a colorless residue remained. The combined organic phases were rotary evaporated under reduced pressure until dryness. The residue was redissolved in 1.4 mL of methanol/tetrahydrofuran (1/1, v/v), using an ultrasonic bath for 5 min. Lutein and zeaxanthin in sample extracts were quantified using a Develosil 5 μm RP-Aqueous C-30 250 mm × 4.6 mm column (Phenomenex, Torrance, United States). Gradient elution was conducted with initial conditions of 15% solvent B; 30 min, 35% B; 35 min, 78% B; and 40 min, 15% B, where solvent A consisted of MeOH:H_2O (95:5) and solvent B consisted of MBTE at a flow rate of 1 mL/min. This was delivered using a LC-10ADVP pump (Shimadzu, Japan). A Shimadzu SPD-M10AVP photodiodearray detector was used for detection at 440 nm. Data signals were acquired and processed on a PC running the LC Solution Multi software (Shimadzu, Japan). Twenty microliters of sample extracts was injected into the HPLC system, and peak areas were used for all calculations. The carotenoid content of sample extracts was extrapolated from standard curves and expressed as mg per 100 g.

Determination of Total Antioxidant Capacity. The extracts of total phenolic compounds were analyzed for their total antioxidant capacity by Trolox equivalent antioxidant capacity (TEAC) and ferric reducing antioxidant power (FRAP) assays. Moreover, the antioxidant capacity of anthocyanin extracts, obtained with acidified methanol, was measured by the FRAP assay.

TEAC Assay. The method is based on the ability of antioxidant molecules to quench the long-lived $ABTS^+$, a blue-green chromophore with characteristic absorption at 734 nm, as compared with that of Trolox, a water-soluble vitamin E analogue. The addition of antioxidants to the performed radical cation reduces it to ABTS, causing a decolorization. A stable stock solution of $ABTS^+$ was produced by reacting a 7 mmol/L aqueous solution of ABTS with 2.45 mmol/L potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12–16 h before use (*34*). On the day of analysis, an $ABTS^+$ working solution was obtained by diluting the stock solution in ethanol to an absorbance of 0.70 ± 0.02 AU at 734 nm, verified by a HP 8453 diode array spectrophotometer (Hewlett-Packard, Waldbronn, Germany), and used as the mobile phase in a flow-injection system (*35*). Twenty microliters of extracts, adequately diluted depending on their presumed activity, was injected in the system. Results were expressed as TEAC in mmol of Trolox per 100 g.

FRAP Assay. The FRAP assay is based on the reduction of the Fe^{3+}–TPTZ complex to the ferrous form at low pH. This reduction is monitored by measuring the absorption change at 595 nm (*36*). Briefly, 40 μL of sample extract was placed in each well of a 96-well plate and mixed with 260 μL of the FRAP reagent. Absorption was measured at 595 nm in a Tecan plate reader (Tecan, Salzburg, Austria) after 30 min of incubation at 37 °C. The FRAP reagent was prepared daily and consisted of 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ in a ratio of 10:1:1 (v/v/v). FRAP values were obtained by comparing the absorption change in the test mixture with doses obtained from increasing concentrations of Fe^{3+} and expressed as mmol of Fe^{2+} equivalents per 100 g.

Statistical Analysis. Data were reported as means ± standard deviations (SDs) of at least duplicate independent extractions. All statistical analysis was carried out using the SAS software package (release 9.1) (SAS Institute, Cary, NC). Analysis of variance was performed by the general linear model (GLM) procedure. Multiple mean comparisons were carried out at the 5% significance level using the REGWQ option (Ryan–Einot–Gabriel–Welch test). A Pearson correlation test was performed using the CORR procedure.

RESULTS AND DISCUSSION

Flour Extraction. The milling yields (relative amount of each millstream in percent) of each investigated wheat and barley genotypes were as follows: PPW provided 0.9, 11.8, 44.6, and 42.7% of bran, shorts, middlings, and flour, respectively; BAW provided 7.5, 14.6, 22.9, and 55.0%; BB6 provided 7.3, 6.3, 59.1, and 27.3%; and BB2 provided 8.5, 9.5, 57.5, and 24.5%. PPW resulted in a very low yield of the bran fraction. Because of the limited sample size, it was decided to combine the bran and the shorts fraction for further analysis. Instead, a wholemeal fraction of PPW was used to evaluate the effectiveness of fractionation and extraction.

Phenolic Content of Grain Varieties. The TPC of grains has often been underestimated as bound phenolics are not extracted with aqueous solutions of methanol, ethanol, and acetone (*9*). We therefore determined TPC after total digestion with NaOH to release the bound forms. The TPC and phenolic acid concentrations of the investigated material are presented in **Table 1**. Generally, TPC decreased with decreasing fraction size and was highest for the bran fraction of BB6 with 921.64 mg and lowest for the flour fraction of BAW with 64.65 mg FA equivalents per 100 g. Differences in the phenolic acid composition were found between barley and wheat genotypes. Ferulic acid was, as expected, the main phenolic acid in the

Table 2. TAC and Color of Extracts of Pigmented Wheat and Barley

genotype/millstream	TAC (mg/100 g)[a]	TAC contribution (%)[d]	color
PPW			
bran + shorts	16.86 a ± 0.68[b,c]	58.96	dark red
middlings	2.56 b ± 0.06	31.40	red
flour	0.82 c ± 0.01	9.64	orange-pink
wholemeal	3.40 b ± 0.10		deep red
BAW			
bran	22.58 a ± 0.05	22.79	purple
shorts	24.55 a ± 1.14	48.28	purple
middlings	5.29 b ± 0.44	16.32	bluish pink
flour	1.70 c ± 0.12	12.61	light pink
BB6			
bran	15.87 a ± 1.89	34.96	brown
shorts	6.17 b ± 0.51	6.19	brownish orange
middlings	2.43 c ± 0.14	43.32	orange-red
flour	1.21 c ± 0.01	9.95	yellowish
BB2			
bran	8.85 a ± 0.09	23.33	brown
shorts	5.45 b ± 0.12	16.11	brownish
middlings	2.96 c ± 0.08	52.88	orange-red
flour	1.02 d ± 0.02	7.68	yellowish

[a] TAC is expressed as cyanidin-3-glucoside equivalents. [b] Mean ± SD. [c] Means followed by different letters are significantly different ($p < 0.05$) within the respective genotype. [d] [TAC × fraction yield (%)/calculated TAC in wholemeal] × 100.

wheat samples, whereas *p*-coumaric acid was the predominant phenolic acid in the bran fraction of both black barley lines. Because the bran fraction of the black barley samples consisted of both pericarp and hulls (lemma and palea), we suppose that the high content of *p*-coumaric acid is due to the hulls, whereas the high content of ferulic acid is derived from the pericarp. Hence, it is suggested that the composition of phenolic acids in barley caryopses is different to that of their covering hulls. This may be confirmed by the data of the shorts fraction, which consisted mainly of pericarp and aleurone layer particles. The ferulic acid content is not significantly lower than in the bran fraction, whereas the content of *p*-coumaric acid is 5–10 times lower ($p < 0.0001$). High *p*-coumaric acid concentrations have as well been found in brewers' spent grain, the product of malted barley undergoing a hot water extraction, whereas wheat bran contained 28 times less *p*-coumaric acid (*37*).

The content of ferulic acid of wholemeal PPW (85 mg/100 g) is in agreement with other studies (*22*, *25*). As for PPW sample, we analyzed the wholemeal flour and the three milling fractions; on the basis of the percentages of yield of the respective milling fractions, we calculated the "expected" wholemeal total phenolic content by multiplying the TPC content of the respective milling fractions by the milling yield and subsequently adding the values to a predicted/expected wholemeal TPC content. Then, we compared this predicted value against the really measured values for the wholemeal flour sample, obtaining an excellent accordance for TPC (203 vs 197 mg FA equiv/100 g), total ferulic acid content (82.02 vs 85.17 mg/100 g), total vanillic acid content (3.68 vs 3.50 mg/100 g), and total *p*-coumaric acid content (2.66 vs 2.43 mg/100 g).

Total Anthocyanin Content. TAC varied between the genotypes and was significantly influenced by the flour fraction ($p < 0.001$) (**Table 2**). The two lines of black barley differed in their anthocyanin concentration, and both barley anthocyanin profiles differ from purple and blue wheat genotypes, exhibiting different shades of red, purple, or brown colors. Using an extraction solvent at low pH will favor the extraction and stability of the present anthocyanins. However, the color of investigated black barley genotypes is probably a result of copigmentation between anthocyanins and melanin-like pigments. Such pigments, which often mask the purple pigmentation of anthocyanins, are brown/black polymers of high molecular weight, which rarely occur in plants, and withstand almost all solvents (*38–40*).

TAC ranged from 0.82 mg/100 g in PPW flour to 24.55 mg/100 g in BAW shorts. For PPW, BB2, and BB6, the significantly highest TAC values were found in the bran, whereas for BAW, bran and shorts showed similar amounts of anthocyanins. This result is due to the different distribution of the pigments. In PPW and the black barleys, the pigments are located in the pericarp or lemma and pericarp, respectively, both contributing mainly to the bran fraction. In BAW, the anthocyanins are located in the aleurone layer, which contributed to both the bran and the shorts fraction. In general, the flour fractions contained the lowest yield of anthocyanins (0.8–1.7 mg), resulting in a low TAC recovery ranging from 7.7 to 12.6%. The low TAC values of the flour fractions of all genotypes are comparable to our previous obtained results for hard red winter wheat cv. Saturnus (<0.9 mg) (*41*).

Total anthocyanins were calculated according to the milling yield, which indicated the relative quantity of each mill stream produced from a given grain weight. The "expected" total anthocyanins based on wholemeal (3.63 mg/100 g) were similar to the measured wholemeal fraction of PPW (3.40 mg/100 g). The highest TAC on wholemeal basis was calculated for BAW with 7.4 mg/100 g grain, followed by PPW, BB6, and BB2 with 3.4, 3.3, and 3.2 mg/100 g grain, respectively. TAC of PPW is in agreement with our own previous findings (4.2 mg/100 g) and with data recently published for purple wheat Konini (3.8 mg/100 g) (*14*) but differ from earlier published data for Konini, ranging from 6.1 to 15.3 mg/100 g (*42*). The TAC of BAW was significantly lower than values reported for the Canadian blue aleurone wheat Purendo-38, for which TACs of 13.9–16.4 mg (*42*) and 21.2 mg/100 g (*14*) were reported. These data reveal that both genetic and environmental factors influence the TAC of pigmented grains. The purple pericarp trait of Konini was introgressed from tetraploid Ethiopian wheat. Hence, genes for anthocyanin pigmentation in Konini and PPW are most probably the same. Variation in TAC of Konini as reported (*14*, *42*) is most likely due to variation in environmental factors. The genetic background is different with respect to blue aleurone wheat. Purendo-38 carries the gene introgression from *A. elongatum* (*43*), while the blue aleurone trait of BAW was introgressed from *T. boeoticum*. Despite the environmental variation, the significant difference in TAC could be related to the different alien genes for the blue aleurone trait, which would suggest that the alien gene from *A. elongatum* accounts for a more efficient anthocyanin biosynthesis.

Total Carotenoid Content. All four grain samples tested in this study contained significant levels of lutein and zeaxanthin. Under the chosen analytical conditions, separation of lutein and zeaxanthin was only possible if the total amount of both isomers exceeded 3 μg/g. As in none of the analyzed fractions, the isomers exceeded this value, results are expressed as the sum of lutein and zeaxanthin (**Figure 1**). The highest content in wheat was found in the PPW bran + shorts fraction (0.146 mg/100 g), whereas the highest contents in black barley were observed in the BB6 shorts (0.164 mg/100 g) and BB2 middlings (0.150 mg/100 g). Fractionation of the milled grain samples revealed that the carotenoids are distributed differently in the tested genotypes. Significant differences between the milling fractions have been found for BB2 ($p = 0.01$) and BAW ($p < 0.05$); for BB6, the differences were significant at $p = 0.05$.

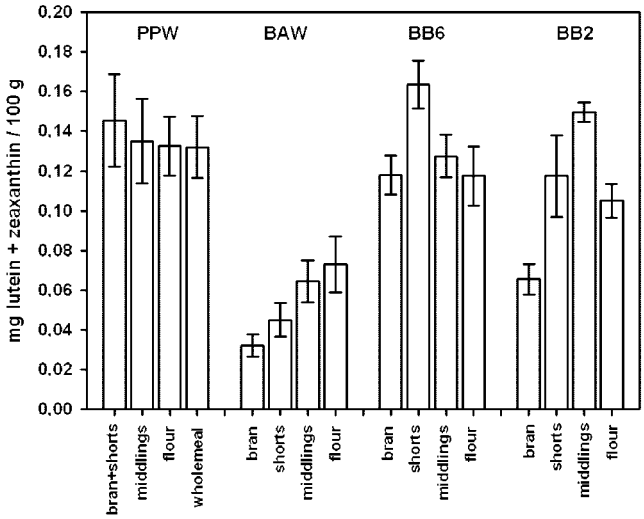

Figure 1. Lutein and zeaxanthin contents of millstreams of pigmented wheat (PPW and BAW) and barley (BB6 and BB2) (mean ± SD; $n = 3$).

Conversely, all fractions of PPW contained similar amounts of carotenoids ($p = 0.88$). Consequently, on the basis of the milling yield of PPW, only 42% of the total grain carotenoids was recovered in the flour fraction. The flour fraction of BAW in contrary contained more than double the amount of the bran, and the higher concentration in the flour fraction points to a higher concentration in the wheat endosperm. In BAW, 63% of the total grain carotenoids derives from the flour fraction. In the black barley genotype BB6, 51% of the total grain carotenoids were recovered in the flour fraction, whereas in BB2 20% of the total grain carotenoids are located in the endosperm and in larger parts in the middlings (67%).

Generally, the analysis of the carotenoid content of cereals is influenced by several factors, such as the milling procedure, the extraction rate, and the solvent used. In addition, origin, growing conditions, and genetic background of cereals affect the carotenoid content. Thus, difficulties arise in the comparison of previous reports. Our results of PPW support earlier observations in European and Canadian durum wheat cultivars that carotenoids are more or less uniformly distributed in the wheat grain (*31*, *44*, *45*). On the contrary, the situation for common wheat is less clear. The higher total carotenoid content in the endosperm was demonstrated in our study for BAW, which is in agreement with findings for Canadian soft white spring wheat AC Reed (*45*). However, these contrast with findings for three U.S. soft winter wheats and Canadian hard red winter wheat Katepwa, where higher carotenoid contents were reported for the bran/germ fractions (*6*, *45*).

Total Antioxidant Capacity. Total phenolic extracts of wheat and barley fractions were examined and compared for their free radical scavenging activities against ABTS radical cation ($ABTS^{\bullet+}$). All fractions showed $ABTS^{\bullet+}$ scavenging capacity (**Table 3**). In general, the TEAC of the bran was 3–5-fold higher than that of the respective flour, and a significant difference in the radical scavenging capacity was detected among genotypes for middlings ($p < 0.01$) and flour ($p < 0.001$) but not for bran ($p = 0.22$) and shorts ($p = 0.13$). In the case of BAW and BB2, all millstreams differed from each other, whereas for BB6 and PPW, bran and shorts and middlings and flour, respectively, were not significantly different. Generally, the $ABTS^{\bullet+}$ scavenging capacity decreased with decreasing particle size of the millstreams.

Table 3. Total Antioxidant Capacity of Pigmented Wheat and Barley after Extraction of Phenolics

genotype/millstream	TEAC (mmol/100 g)[a]	TEAC contribution (%)[d]
PPW		
bran + shorts	1.91 a ± 0.19[b,c]	31.63
middlings	0.72 b ± 0.11	42.09
flour	0.47 b ± 0.02	26.27
wholemeal	0.63 b ± 0.03	
BAW		
bran	2.11 a ± 0.06	18.81
shorts	1.75 b ± 0.05	30.48
middlings	0.92 c ± 0.05	25.12
flour	0.39 d ± 0.00	25.60
BB6		
bran	2.28 a ± 0.18	13.98
shorts	1.93 a ± 0.18	10.19
middlings	1.20 b ± 0.10	59.70
flour	0.70 c ± 0.03	16.09
BB2		
bran	2.14 a ± 0.06	17.07
shorts	1.60 b ± 0.05	14.26
middlings	1.03 c ± 0.02	55.53
flour	0.57 d ± 0.00	13.09

[a] Total antioxidant capacity is expressed as mmol of Trolox equivalents (TEAC). [b] Mean ± SD. [c] Means followed by different letters are significantly different ($p < 0.05$) within the respective genotype. [d] [TEAC × fraction yield (%)/calculated TEAC in wholemeal] × 100.

When the milling yield of PPW was considered, applying the same approach described for total phenolic content (see above), good accordance was found for the "expected"/computed TEAC of PPW on wholemeal basis as compared with the analyzed value of the wholemeal flour (0.76 vs 0.63 mmol/100 g). Therefore, the total TEAC of the other tested genotypes was calculated in the same way. The highest total $ABTS^{\bullet+}$ scavenging capacity was calculated for BB6, followed by BB2 and BAW, with 1.2, 1.1, and 0.8 mmol/100 g, respectively. In black barley, the middlings contributed 55–60% of total TEAC, whereas shorts, middlings, and flour contributed similarly (25–30%) to TEAC of BAW. For PPW, the highest contribution was observed for the middlings (**Table 3**). Highly significant ($p < 0.0001$) correlations were obtained for TEAC with ferulic acid ($r = 0.84$), for TEAC with TPC ($r = 0.96$), and for ferulic acid with TPC ($r = 0.88$). Comparable correlation coefficients for phenolic compounds and antioxidant activity were obtained for New York-grown durum and common wheat (*9*), whereas an earlier study with three Colorado-grown common wheats reports no correlation between the TPC and the radical scavenging capacity (*46*).

FRAP was determined for both acidified methanol extracts and extracts after alkali treatment, and results are expressed as Fe^{2+} equivalents (**Table 4**). The highest FRAP value was observed for BB6 bran among extracts after alkali treatment, followed by BB2, BAW, and PPW. This order is in accordance with the TEAC values of the same extracts. The $ABTS^{\bullet+}$ scavenging capacity was significantly correlated to FRAP ($r = 0.90$, $p < 0.0001$). Grain fractions differed in their FRAP values regardless of extraction–solvent, and the FRAP value of extracts after alkali treatment was as expected greater (2–5-fold) than that of the corresponding methanol extract. The methanol extracts detect free phenolic acids and anthocyanins in their active form, whereas in extracts after alkali treatment also bound phenolic acids are released. A highly significant correlation was found for FRAP values from extracts after alkali treatment and acidified extracts ($r = 0.73$, $p < 0.0001$).

Table 4. Ferric Reducing Antioxidative Power (FRAP) of Pigmented Wheat and Barley

genotype/millstream	FRAP (mmol Fe^{2+} equiv/100 g)	
	extract after saponification	acidified methanol
PPW		
bran + shorts	9.40 a ± 1.05[a,b]	1.95 a ± 0.04
middlings	2.74 b ± 0.63	0.66 b ± 0.05
flour	2.75 b ± 0.31	0.41 c ± 0.01
wholemeal	1.82 b ± 0.15	0.74 b ± 0.01
BAW		
bran	9.50 a ± 0.61	2.05 a ± 0.02
shorts	5.55 b ± 0.42	1.95 b ± 0.04
middlings	2.77 c ± 0.14	0.83 c ± 0.02
flour	1.56 c ± 0.00	0.38 d ± 0.02
BB6		
bran	14.13 a ± 0.61	12.81 a ± 0.61
shorts	5.62 b ± 0.15	6.74 b ± 0.39
middlings	3.15 c ± 0.29	2.29 c ± 0.09
flour	2.60 c ± 0.02	1.07 c ± 0.01
BB2		
bran	11.46 a ± 0.93	3.86 b ± 0.15
shorts	5.86 b ± 0.32	4.44 a ± 0.18
middlings	2.90 c ± 0.17	2.51 c ± 0.07
flour	2.10 c ± 0.05	0.92 d ± 0.03

[a] Mean ± SD. [b] Means followed by different letters are significantly different ($p < 0.05$) within the respective genotype.

In conclusion, significant differences in the content of anthocyanins and carotenoids and in reducing power were observed between genotypes and between the different millstreams of the respective plant material. From the results of this study and from others previously carried out, it can be concluded that genetic factors have a major impact on the phytochemical profile and the antioxidant activity. Postharvest treatments such as fractionation of the raw material have the potential to create flour blends rich in phytochemicals with high antioxidant capacity. Using selected genetic resources, it should be possible for cereal breeders to supply new "multipurpose/multifunctional" cultivars to the food industry in the future. Because of the possibility of multifunctional usage, for example, white or yellow flour for the bakery or noodle industry, respectively, and anthocyanin or melanin pigmented bran for functional foods or natural colorants, such new cultivars should be of added value for the producer and manufacturer. To make breeding programs more efficient in this context, close collaborations between breeders, food chemists, and food technologists are necessary.

ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; BAW, blue aleurone wheat; $ABTS^{\bullet+}$, ABTS radical cation; BB2, two-rowed black barley; BB6, six-rowed black barley; cv., cultivar; HPLC, high-pressure liquid chromatography; FA, ferulic acid; FRAP, ferric reducing/antioxidant power; PPW, purple pericarp Ethiopian wheat; SD, standard deviation; TAC, total anthocyanin content, TEAC; Trolox equivalent antioxidant capacity; TPC, total phenolic content; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; TPTZ, 2,4,6-tripyridyl-s-triazine.

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Received for review July 5, 2007. Accepted August 27, 2007.

JF072021J