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Antioxidant Activity-Guided Fractionation of Blue Wheat (UC66049 *Triticum aestivum* L.)

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ABSTRACT: Antioxidant activity-guided fractionation based on three in vitro antioxidant assays (Folin−Ciocalteu, TEAC, and leucomethylene blue assays) was used to identify major antioxidants in blue wheat (UC66049 *Triticum aestivum* L.). After consecutive extractions with solvents of various polarities and multiple chromatographic fractionations, several potent antioxidants were identified by NMR spectroscopy and mass spectrometry. Anthocyanins (delphinidin-3-glucoside, delphinidin-3-rutinoside, cyanidin-3-glucoside, and cyanidin-3-rutinoside), tryptophan, and a novel phenolic trisaccharide (β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-(4-hydroxy-3-methoxyphenyl)-β-D-glucopyranoside) were the most active water-extractable constituents. However, anthocyanins were found to be major contributors to the overall blue wheat antioxidant activity only when the extraction steps were performed under acidic conditions. Alkylresorcinols were among the most active antioxidants extractable with 80% ethanol in the TEAC assay. However, this may be due to a color change instead of a bleaching of the ABTS radical. Ferulic acid was found to be the major antioxidant in alkaline cell-wall hydrolysates. The contents of the most active antioxidants were determined.

KEYWORDS: *blue wheat, anthocyanins, ferulic acid, alkylresorcinols, tryptophan*

INTRODUCTION

Blue wheat is a wheat variety containing high levels of anthocyanins, leading to a unique color of the aleurone. In contrast, other colored grains such as purple wheat, blue corn, and black rice have colored pericarps.[1] Although few products containing blue wheat are currently available, there is considerable interest in establishing blue wheat as a food ingredient or for the production of natural colorants. Wheat in general is a good source of dietary antioxidants (AOs),[2,3] which are assumed to exhibit health benefits because several chronic diseases are associated with oxidative stress.[4,5] The accumulation of anthocyanins in blue wheat indicates that different biochemical pathways are stimulated or suppressed as compared to other wheat varieties. Only a few studies so far have compared the AO activities of colored grains to those of noncolored varieties and investigated the phytochemical profile in more detail. Li et al. reported that whole grain flour of black wheat exhibited higher AO activity in the DPPH assay than purple and white wheat; however, blue wheat whole meal showed least activity.[6] The AO activities of the bran extracts followed a similar order; purple wheat bran extracts were significantly more active than white wheat bran extracts but still less active than black wheat bran extracts. In another study, purple wheat extracts were also shown to exhibit higher DPPH and ORAC values than red and white wheat extracts.[7] The ORAC values for purple barley were lower than those for black and yellow barley in both bran and whole meal extracts.[8] However, even though previous studies gave valuable insights into the array of potentially active compounds in the investigated extracts, it is still not known which individual compounds are mostly responsible for the observed effects. Hu et al.[9] as well as Abdel Aal and co-workers[10] fractionated blue wheat and reported that the anthocyanin-rich fraction was mainly responsible for the in vitro AO activity. Hu et al. also compared it with another fraction containing nonanthocyanin phenolic compounds and found that the activity of the anthocyanin-rich fraction exceeded the activity of the phenolic fraction.[9] Reports on the contribution of individual compounds to the AO activity of blue wheat have, however, not been reported yet. The aim of this study was to determine the most active AOs in blue wheat by performing activity-guided fractionation with three different in vitro AO assays.

MATERIALS AND METHODS

Materials. Blue wheat (UC66049 *Triticum aestivum* L.) was grown in St. Paul, MN, in 2007. All organic solvents were of HPLC grade; the water was double-distilled. Formic acid, potassium persulfate, and Folin−Ciocalteu (FC) reagent were purchased from Fisher Scientific (Fair Lawn, NJ). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Acros (Pittsburgh, PA), and benzoylleucomethylene blue was bought from TCI (Portland, OR). Delphinidin-3-glucoside and cyanidin-3-glucoside were purchased from Polyphenols Laboratories (Hanaveien, Norway).

Extraction. Blue wheat (2.5 kg) was ground to a particle size of <0.5 mm in a Retsch ZMI mill (Newtown, PA) and consecutively extracted with 80% ethanol, acetone, and ethyl acetate. The whole meal was extracted three times in a shaker at 37 °C using a flour to solvent ratio of 1:4 (w/v) for 2 h per extraction. The flour was dried under a nitrogen stream between extractions with different solvents until the weight remained constant. Extracted triglycerides were removed from acetone and ethyl acetate extracts by precipitation at −78 °C. After extraction, the residual flour was destarched (Termamyl amylase in 0.08 M phosphate buffer at pH 6, 100 °C for 30 min). Cell-wall bound phytochemicals were liberated from the destarched material by saponification with degassed 2 M NaOH at a w/v ratio of 1:4.

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The reaction was carried out for 18 h while protected from light. The alkaline hydrolysate (AH) was then acidified to pH <2 and extracted with ethyl acetate (three times). The solvents of each extraction step were evaporated under reduced pressure at <45 °C. The 80% ethanol extract (118 g) was fractionated into a water-soluble (WSE) and a water-insoluble portion (WISE) by extraction with water (200 mL, three times) and then extracting the water-insoluble residue with acetone (100 mL, three times). The WSE was freeze-dried to yield 46.8 g. Yields for the other extracts were 5.1 g (WISE), 8.9 g (acetone extract, AE), and 0.7 g (ethyl acetate extract, EE), respectively. The AH yielded 4.6 g.

Aliquots (3 g) of the WSE were redissolved in water and applied onto a column (25 cm, 3 cm i.d.) filled with 100 g of Amberlite XAD-2 and eluted with water (300 mL, two times; collected eluates will be referred to as WSE-1 and WSE-2), 50% MeOH (300 mL, two times; WSE-3 and WSE-4), and 100% MeOH (300 mL, two times; WSE-5 and WSE-6). MeOH was removed by rotary evaporation and water by freeze-drying. All flasks were wrapped in aluminum foil to protect them from light.

AO Assays. Three AO assays were employed: the FC assay, the Trolox equivalent antioxidant capacity (TEAC) assay, and the leucomethylene blue (LMB) assay. All results are expressed as the average of triplicate measurements and are given per milligram of sample (crude extract or per fraction). The FC assay was performed as described by Dewanto et al.[11] All values are expressed as the mean of microgram gallic acid equivalents (GAEs) per milligram of sample. The TEAC assay was performed as reported by Re et al.[12] with the $ABTS^{\bullet+}$ solution being produced with the help of potassium persulfate. Results are expressed in micromolar Trolox equivalents (TEs) per milligram of sample. The LMB assay was performed according to the method of Lindenmeier et al.[13] Results are expressed as millimolar TEs per milligram of sample.

HPLC Experiments. All analytical HPLC experiments were performed on a Shimadzu LC-6AD system equipped with an SIL-10AF autosampler and an SPD-M20A photodiode array detector. Semipreparative HPLC was performed on a Shimadzu LC-20AT low-pressure gradient pump and an SPD-M20A photodiode array detector. A Phenomenex (Torrace, CA) Luna phenyl-hexyl column (250 × 10 mm i.d., 5 μm) was used in all HPLC fractionation experiments.

Fractionation. An aliquot (170 mg) of the most active (see AO Assays) Amberlite eluate (WSE-4) was further fractionated by semipreparative HPLC at 30 °C using a flow rate of 3 mL/min. A linear gradient with 1 mM trifluoroacetic acid (TFA) and MeOH (from 15 to 70% MeOH in 45 min) was employed. The eluted compounds were monitored at 280 as well as 325 nm and collected in 4 min time intervals over 40 min. Yields for these HPLC fractions were as follows: 16.6 mg (WSE-4-I), 51.7 mg (WSE-4-II), 1.0 mg (WSE-4-III), 43.4 mg (WSE-4-IV), 5.2 mg (WSE-4-V), 5.1 mg (WSE-4-VI), 18.1 mg (WSE-4-VII), 12.8 mg (WSE-4-VIII), 4.4 mg (WSE-4-IX), and 6.8 mg (WSE-4-X). WSE-4-II and WSE-4-IV were subsequently fractionated on the same HPLC system with 1 mM TFA and MeOH (from 5 to 50% MeOH in 32 min for WSE-4-II; for WSE-4-IV, from 5 to 15% MeOH over 5 min and from 15 to 50% MeOH in 30 min). Both gradients used a flow rate of 3 mL/min and a temperature of 30 °C; the effluent was monitored at 280 nm. WSE-4-II subfractions were collected in 4 min intervals with the exception of the main peak eluting between 11.0 and 12.8 min, which was collected individually. The amount of 51.7 mg of WSE-4-II yielded 6.2 mg (WSE-4-IIa), 2.5 mg (WSE-4-IIb), 1.5 mg (WSE-4-IIc), 24.1 mg (WSE-4-IId), 7.0 mg (WSE-4-IIe), 2.5 mg (WSE-4-IIf), 2.0 mg (WSE-4-IIg), and 1.0 mg (WSE-4-IIh). WSE-4-IV contained one main peak, which eluted between 13.8 and 15.3 min, and was collected individually as the fourth subfraction. The residual effluent was collected in 4 min intervals for subsequent AO assays. Yields of 70 mg were 2.8 mg (WSE-4-IVa), 1.0 mg (WSE-4-IVb), 1.0 mg (WSE-4-IVc), 50.6 mg (WSE-4-IVd), 3.7 mg (WSE-4-IVe), 3.0 mg (WSE-4-IVf), 1.5 mg (WSE-4-IVg), and 1.3 mg (WSE-4-IVh). Molecular masses and structures of the most active AOs found in WSE-4-II and WSE-4-IV were determined by LC-MS and 1D- and 2D-NMR.

An alternative extraction procedure involving 80% ethanol containing 0.3% TFA was performed, too. Fractions of this extraction are denoted with an "*a*" to distinguish them from fractions obtained under neutral pH conditions. Except for the different pH of the solvent, all other conditions were the same as for the extraction with 80% ethanol. Blue wheat (500 g) was extracted three times for 2 h at 37 °C. The pooled extracts were dried under vacuum and re-extracted three times with 0.3% aqueous TFA in water for 1 h each. The thus obtained WSE*a* was lyophilized, reconstituted in 0.3% TFA, and applied onto Amberlite XAD-2. Fractionation conditions were the same as for the WSE except that all eluents contained 0.3% TFA. Amberlite eluate WSE*a*-4 was then concentrated under reduced pressure and fractionated on the same semipreparative HPLC system with the same gradient program as specified above for WSE-4, except that 0.3% TFA was used instead of 1 mM TFA. Fractions WSE*a*-4-VI and WSE*a*-4-VII were subsequently fractionated with gradients based on those of Cuevas Montilla et al.[14] using 87% H_2O/3% ACN/10% formic acid (v/v/v) as phase A and 40% H_2O/50% ACN/10% formic acid (v/v/v) as phase B and a 1.5 mL/min flow rate. The two main peaks in fraction WSE*a*-4-VI were obtained at 40 °C using the following gradient: from 6 to 20% B in 20 min, hold for 5 min, from 20 to 37% B in 10 min, hold for 5 min, followed by a rinsing step. Subfractions of WSE*a*-4-VII were collected in 10 min intervals at 35 °C with a gradient from 6 to 20% B in 20 min, hold for 5 min, from 20 to 37% B in 10 min, followed by a rinsing step. Their yields were 2.9 mg (WSE*a*-4-VIIa), 2.0 mg (WSE*a*-4-VIIb), 3.2 mg (WSE*a*-4-VIIc), 4.8 mg (WSE*a*-4-VIId), and 9.0 mg (WSE*a*-4-VIIe). Four UV−vis active anthocyanins present in the most active subfraction were collected from WSE*a*-4-VIIc with the same HPLC method.

All other fractionations described from here on were performed on the extracts obtained under neutral conditions. The WISE was dissolved in 95% aqueous MeOH and isocratically fractionated at 40 °C by semipreparative HPLC using 95% MeOH/5% water as eluent and a flow rate of 2.5 mL/min. The effluent was monitored at 280, 295, and 435 nm. Peaks eluting at 10.5 min (WISE-1), 11.9 min (WISE-2), and 13.1 min (WISE-3) were collected individually. The two most active AO peaks, WISE-1 and WISE-2, were dried and redissolved in 500 μL of acetone-d_6 for NMR spectroscopy. Their masses were determined after silylation with 10 μL of pyridine and 40 μL of BSTFA by GC-MS (Hewlett-Packard HP 6890 GC system with HP 5973 MS detector) on a Restek (Bellefonte, PA) MXT-5 column (30 m × 0.25 mm, 5 μm film thickness). The temperature program was adapted from that of Ross et al.:[15] 70 °C (initial), hold for 1 min, 40 °C/min to 200 °C, hold for 2 min, 20 °C/min to 300 °C, hold for 14 min, 50 °C/min to 380 °C, hold for 5 min. The helium flow rate was 1 mL/min, the inlet temperature was 275 °C, and the transfer line was held at 300 °C. Electron-impact mass spectra were recorded at 70 eV. The GC elution times were 14.8 min (WISE-1) and 16.1 min (WISE-2).

An aliquot (250 mg) of the AH was dissolved in tetrahydrofuran and subjected to fractionation by isocratic size exclusion chromatography using tetrahydrofuran as eluent (1 mL/min) on a Tosoh (Grove City, OH) TSK gel column (7.8 mm × 30 cm, 5 μm) with detection at 280 nm at room temperature (Hitachi L6200A Intelligent Pump, Spectra 200 detector). Three main peaks were collected: peak AH-1 from 5.4 to 6.1 min with a yield of 105 mg; peak AH-2 from 6.1 to 7.1 min (63 mg); and peak AH-3 from 7.1 to 9.9 min (90 mg). One minor fraction (4.7 mg) collected the remaining eluent after the third main peak. AH-3 was further fractionated by semipreparative phenylhexyl HPLC, using a linear gradient of 1 mM TFA and MeOH with the MeOH concentration ramping from 27 to 90% in 40 min. The temperature was kept at 40 °C, and the flow rate was 3 mL/min. Its most active subfraction (AH-3-IV) was further separated by HPLC as described earlier.[16] Constituents were identified by comparison to standard compounds via analytical HPLC, and the structures were confirmed with NMR spectroscopy.

LC-MS Experiments. For all LC-MS experiments, a Luna phenylhexyl HPLC column (250 × 4.6 mm i.d., 5 μm) was used. Data for WSE-4-IId and WSE-4-IVd were recorded on an Agilent HP series 1100 HPLC coupled to a Waters Micromass Q-TOF (Quattro Micro, Waters, Milford, MA). HPLC gradients consisted of eluents MeOH and 0.1% aqueous formic acid (v/v). The gradient was the same as specified for the purification of those compounds, except that 0.1% formic acid was used instead of 1 mM TFA. MS ionization conditions were as follows: desolvation temperature, 250 °C; source temperature,

100 °C; capillary voltage, 3 kV; cone voltage, 30 V. Mass spectra were recorded in negative ionization mode. Analysis of anthocyanin peaks 1−4 in fraction WSE*a*-4-VII was performed on a Waters Micromass ZQ quadrupole detector coupled to a Shimadzu LC-10AD VP chromatograph. Separation was achieved employing a modified method as reported by Laokuldilok[17] using a mixture of 75% H_2O, 20% MeOH, and 5% formic acid for isocratic elution. The MS parameters were adapted from those of Hosseinian et al.[18] The capillary voltage was 3 kV; the cone voltage, 30 V; the desolvation temperature, 400 °C; and the source temperature, 150 °C; the spectra were recorded in positive electrospray ionization mode.

NMR Experiments. Structure characterization by NMR spectroscopy was performed on a Bruker Avance 700 MHz NMR spectrometer equipped with a triple-resonance $^1H\{^{13}C/^{15}N\}$ pulsed field 5 mm gradient cryoprobe. Spectra of *β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl-(1→6)-(4-hydroxy-3-methoxyphenyl)-*β*-D-glucopyranoside (WSE-4-IId) were recorded in D_2O containing 5% acetone-d_6, and the acetone signals were set to 2.22 ppm (^1H) as well as to 30.89 and 215.94 ppm (^{13}C).[19] Tryptophan (WSE-4-IVd) was measured in DMSO-d_6, anthocyanin spectra (WSE*a*-4-VIIc peaks 1 to 4) were recorded in MeOH-d_4 containing 0.3% TFA-*d*, and alkylresorcinols (WISE-1 and WISE-2) as well as the main and minor peaks in AH-3-IV (ferulic and *p*-coumaric acid, respectively) were measured in acetone-d_6.

Quantification of the Most Active AOs. All contents are reported per gram of dry whole meal. The whole meal water content was determined by extracting 0.5 g of whole meal (in triplicate) with 10 mL of MeOH for 18 h. An aliquot of 0.5 mL of the extract was weighed and injected into Aquatest cma, a Karl Fischer coulometric titrator (Photovolt, Indianapolis, IN).

All active constituents were quantified by analytical RP-HPLC. If isolated compounds were used for external standard curves, their purity was first verified by NMR spectroscopy or GC-MS. A Phenomenex Luna phenylhexyl HPLC column (250 × 4.6 mm i.d., 5 μm) was used for the quantification of WSE-4-IId and tryptophan as well as free and esterified *p*-coumaric and ferulic acid. Quantification of alkylresorcinols and anthocyanins was performed on a Phenomenex C18(2) HPLC column of the same dimensions. For quantification of free *p*-coumaric and ferulic acid, WSE-4-IId, and tryptophan, ground blue wheat (2 g) was extracted three times with 15 mL of chilled 80% ethanol. The solution was made up to 50 mL, and aliquots were dried under nitrogen and redissolved in H_2O for the quantification of WSE-4-IId and tryptophan or 50% MeOH/H_2O for *p*-coumaric and ferulic acid quantification. Tryptophan and WSE-4-IId were quantified against an external standard curve of commercially obtained tryptophan or isolated WSE-4-IId using the following HPLC-gradient (45 °C; 0.8 mL/min; eluents 1 mM TFA and MeOH): 0−7 min, hold 5% MeOH; 7−15 min, from 5 to 10% MeOH; 15−20 min, from 10 to 15% MeOH; 20−30 min, from 15 to 95% MeOH; hold for 6 min, from 95 to 5% MeOH in 3 min. Ferulic acid and *p*-coumaric acid were quantified according to the method of Dobberstein et al.[16] Esterified ferulic and *p*-coumaric acids were liberated from the cell wall by addition of degassed 2 M NaOH (10 mL) to 200 mg of the dried flour after 80% ethanol extraction. The HPLC gradient was (45 °C, 1 mL/min; eluents 1 mM TFA, MeOH and ACN) as follows: 0−10 min, hold 0% MeOH and 13% ACN; 10−20 min, from 0 to 2.5% MeOH and from 13 to 18% ACN; 20−30 min, from 18 to 25% ACN, from 2.5 to 5% MeOH; 30−32 min, from 5 to 25% MeOH and from 25 to 50% ACN; 32−35 min, from 25 to 0% MeOH and from 50 to 13% ACN; hold for 5 min. Alkylresorcinols were quantified according to the method of Knödler et al.[20] with some modifications. Whole meal (3 g) was extracted twice with ethyl acetate for 2 h with 40 mL. The extract was dried under nitrogen and redissolved in 500 μL of MeOH/H_2O/acetone 90:5:5 (v/v/v). An aliquot (50 μL) of this mixture was quantified at 45 °C against external standard curves of isolated compounds purified with the HPLC gradient as used for quantification (see below). The gradient used a flow rate of 0.6 mL/min and eluents MeOH, ACN, and H_2O: 0−5 min, hold 87% MeOH and 13% H_2O; 5−20 min, from 87 to 91% MeOH and from 13 to 9% H_2O; hold for 2 min; 22−28 min, hold 91% MeOH and from 0 to 2.5% ACN and from 9 to 6.5% H_2O; 28−35 min, from 91 to 95% MeOH, from 2.5 to 4% ACN, and from 6.5 to 1% H_2O; 35−40 min, from 95 to 98% MeOH, from 4 to 0% ACN, and from 1 to 2% H_2O; 40−48 min, from 98 to 87% MeOH and from 2 to 13% H_2O; hold for 2 min.

Anthocyanins were quantified following a modified extraction protocol as published by Kim et al.[21] Whole meal (200 mg) was extracted three times with 80% MeOH containing 0.3% TFA (first extraction, 2 mL, 4 h; second extraction, 1 mL, 2 h). After centrifugation at 4000 rpm for 5 min, the supernatant was dried and reconstituted in 250 μL of a H_2O/ACN/formic acid 78.5:12.5:10 (v/v/v) solution. Following another centrifugation step at 3000 rpm for 5 min, the solution was analyzed by RP-HPLC on a Luna C18(2) column (250 × 4.6 mm i.d., 5 μm). The gradient was the same as for the fractionation of WSE*a*-4-VI and WSE*a*-4-VII described above, but the flow rate was 0.5 mL/min. Detection and quantification were performed at 520 nm against a standard curve of commercially obtained anthocyanins in the case of glucosides or anthocyanins that were isolated by semipreparative HPLC in the case of the rutinosides.

RESULTS AND DISCUSSION

Extraction Procedure. The extraction started with 80% ethanol to extract polar compounds while excluding soluble polysaccharides, which were not a focus of our study and were found to interfere with the dried extract's resolubility in previous experiments. Although AO activity was described for certain polysaccharides, especially from mushrooms,[22] arabinoxylans and other soluble polysaccharides from cereal grains are not assumed to be AOs. However, AO active compounds can be attached to polysaccharides, as described later. Because the 80% ethanol extract was a mixture of mostly hydrophilic, but also some lipophilic, compounds, it was divided into a water-soluble (WSE) and water-insoluble (WISE) extract before the initial AO activity testing. The WSE was fractionated over Amberlite XAD-2 to separate aliphatic compounds from aromatic compounds. Whereas the first can be eluted with water, most compounds containing aromatic structural units require some organic modifier, for example, 50 or 100% MeOH, to be eluted from Amberlite XAD-2. After extraction with 80% ethanol, the whole meal was extracted with solvents of decreasing polarity, namely, acetone and ethyl acetate. Triglycerides were precipitated from the WISE, acetone extract (AE), and ethyl acetate extract (EE) at −78 °C because they do not contribute to an extract's AO activity, but interfere with its solubility. After extraction with organic solvents, an alkaline hydrolysis of cell-wall bound compounds was performed. Potent AOs such as hydroxycinnamic acids are ester-linked to cell-wall polysaccharides and thus cannot be obtained by simple extraction with organic solvents.[23,24] After acidification, the alkaline hydrolysate (AH) was extracted with ethyl acetate and the extract was dried.

AO Testing. Because all AO assays have limitations, using at least two assays is strongly recommended.[25] We performed three in vitro assays to analyze the AO activity of the obtained extracts: the Folin−Ciocalteu (FC), Trolox equivalent antioxidant capacity (TEAC), and leucomethylene blue (LMB) assays. The first two assays measure reducing properties, whereas the LMB assay detects reducing as well as radical-scavenging AOs.[13] This test uses the Fenton reaction to generate radicals and oxidize linoleic acid present in a buffered emulsion. AOs with reducing and radical scavenging properties can inhibit this reaction at several points. All samples were tested at the same concentrations, even though more abundant constituents may contribute more to the AO activity of the parent extract or fraction. However, we wanted to find the most active AOs, not the most predominant. In some cases, the most potent fractions had to be appropriately diluted to fall within the standard curve.

If a fraction or an extract was not active at a tested concentration, no efforts were made to determine whether this sample would be active at higher concentrations. Such attempts would not be in accordance with the activity-guided principle, which focuses only on the most active compounds and fractions. For the same reason, the focus of this study was not to determine absolute Trolox equivalents (TE) or gallic acid equivalents (GAE), but to achieve a ranking of the tested samples. Some studies report TEAC values of samples containing phenolic compounds over a longer time period than the usual 6 min, for example, 24 h.[26] It was observed that certain compounds react with $ABTS^{\bullet+}$ for a longer period than 6 min,[25,27] in contrast to Trolox, with which the reaction is quickly terminated. Because we used the TEAC to provide a ranking of samples and not for quantitative evaluation, measurements were terminated after 6 min, as usual in the TEAC. However, the activity ranking of the samples did not change when data after 6 min or after 2 h were compared (data not shown). The results for the crude extracts are summarized in Table 1. The AH exhibited the highest AO activity in all assays, with values 2−3 times higher than the second most active sample. From a physiological point of view, compounds present in the AH might be cleaved from their attached polysaccharides in the human large intestine by microbial esterases.[28] It is widely accepted that these compounds can then be at least partially reabsorbed from the large intestine and, thus, contribute to plasma AO capacity. However, the liberated compounds can also be metabolized by gut microorganisms,[29,30] and these metabolites may have different AO and other biological activities.[29] Thus, we were mostly interested in compounds that can potentially be absorbed in the small intestine. Therefore, the second most active fraction, one of the Amberlite XAD-2 eluates (WSE-4, eluted with 50% MeOH), was also further analyzed. In addition, the WISE was further studied even though it showed high activity only in the TEAC assay. In this assay ethanol was used as solvent, and it is likely that the WISE's poor solubility in water reduced its activity in the other assays. Moreover, the results in the TEAC were not due to a mere bleaching of the reagent, but to a change from a green-colored to a yellow-colored solution. Figure 1 shows the visible spectra of $ABTS^{\bullet+}$ and the WISE/$ABTS^{\bullet+}$ reaction product.

Table 1. Antioxidant Activity of Crude Blue Wheat Extracts[a]

	GAE in FC	TE in TEAC	TE in LMB
WSE-1	no activity	no activity	no activity
WSE-2	no activity	no activity	no activity
WSE-3	78.7 ± 3.3	5.8 ± 0.3	0.3 ± 0.008
WSE-4	183.0 ± 3.8	11.2 ± 1.0	0.4 ± 0.02
WSE-5	75.4 ± 1.1	5.0 ± 0.1	0.3 ± 0.02
WSE-6	80.9 ± 2.1	8.5 ± 0.1	0.3 ± 0.02
WISE	no activity	11.8 ± 0.5	no activity
AE	no activity	no activity	no activity
EE	no activity	no activity	no activity
AH	277.0 ± 17.0	37.9 ± 1.4	1.2 ± 0.1

[a]The results of the Folin−Ciocalteu (FC) assay, the Trolox equivalent antioxidant capacity (TEAC), and the leucomethylene blue (LMB) assay are reported as μg gallic acid equivalents (GAE), μM Trolox equivalents (TE), and mM Trolox equivalents (TE), respectively. All values are reported per mg of sample. Data are expressed as the mean ± SD (n = 3).

Fractionation Procedure. *Activity-Guided Fractionation of the AH.* An aliquot of the AH was fractionated by size exclusion chromatography, and the effluent was monitored at 280 nm, yielding three main fractions. A fourth fraction collected all late-eluting compounds (Figure 2). The third fraction (AH-3) was found to be most active in all three AO assays, as shown in Figure 2, which displays relative AO activities; that is, the most active fraction was set to 100%. AH-3 was assumed to contain low molecular weight compounds present in the AH such as monomeric hydroxycinnamic and other monomeric phenolic acids. Subsequent fractionation was performed by using semipreparative phenylhexyl HPLC with a gradient developed for the analysis of cell-wall bound UV-active constituents.[16] However, subfractions were collected in time intervals to ensure that potential AOs without UV activity would not be missed. The chromatogram showed one main UV-active constituent, and the subfraction AH-3-IV, which contained this peak, was the most AO active fraction in all three assays. Individual collection of all UV-active constituents of this particular fraction, comparison with UV spectra of standard compounds, and ^{1}H NMR spectra revealed that the main peak was *trans*-ferulic acid and that *trans-p*-coumaric acid was present as a minor component. Ferulic acid accounted for 58% of the subfraction AH-3-IV's weight and, for example, 79% of subfraction AH-3-IV's TEAC activity, demonstrating again its dominance as an AO in the AH of grain cell walls. We therefore quantified the contents of free acid and ester-linked ferulic and *p*-coumaric acid present in the whole flour. The content of *trans*-ferulic acid was 2.96 ± 0.13 μg/g flour in the free form and 713.18 ± 29.07 μg/g in the ester-linked form, whereas *trans-p*-coumaric acid was present at 40.40 ± 1.98 and 67.73 ± 4.22 μg/g in the free and ester-linked forms, respectively (Table 2). The importance of ferulic acid as an AO in whole grains has long been recognized,[31,32] and studies on blue wheat bran detected it as one of the most abundant phenolic compounds when both free[9] and total, that is, free and esterified, phenolics[33] were analyzed. As for the AO activity of cell-wall bound constituents, it was shown that dehydrodimers of ferulic acid[34,35] give higher TEAC values on a molar level than the ferulic acid monomer. For instance, 5,5-dehydrodiferulic acid had a value of 2.19 TEAC equiv versus 1.96 for monomeric *trans*-ferulic acid.[36] However, because mixtures of unknown compounds are obtained by activity-guided fractionation, it is possible to compare fractions only on a weight basis. When the different molecular weights of ferulic acid dehydrodimers and the ferulic acid monomer are considered, our results are in agreement with reported data. One of the most active dimers, 8,8-noncyclic dehydrodiferulic acid, was reported to have more than twice the AO activity of ferulic acid.[37] However, this particular dehydrodimer was not detected in the most active fraction of blue wheat AH. Besides potential liberation and reabsorption from the colon, cell-wall bound

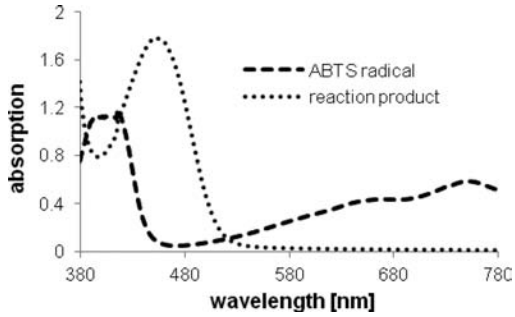


Figure 1. Visible spectra demonstrating the color change of $ABTS^{\bullet+}$ as induced by the water-insoluble extract (WISE) from blue wheat.

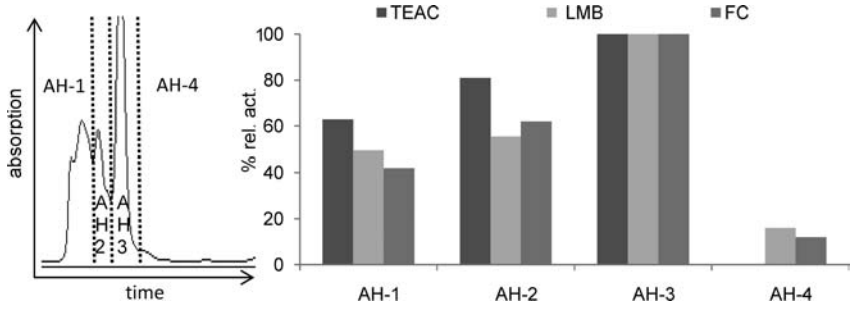
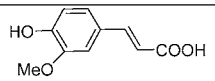
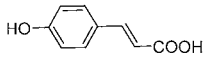
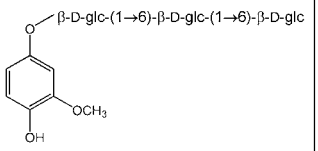
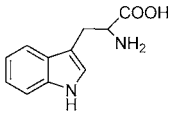
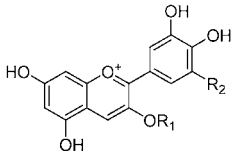
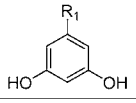


Figure 2. Fractionation of the alkaline hydrolysate (AH) by size exclusion chromatography and relative antioxidant activities of its fractions in the Trolox equivalent antioxidant capacity (TEAC), leucomethylene blue (LMB), and Folin−Ciocalteu (FC) assay. The chromatogram was recorded at 280 nm.

Table 2. Structures and Contents of Most Active Blue Wheat Antioxidants[a]

Compound	Structure	Amount in whole meal[b] [μg/g]
AH-3-IV, ferulic acid		Free: 2.96±0.13 Esterified: 713.18±29.07
AH-3-IV, *p*-coumaric acid (minor constituent of the fraction)		Free: 40.40±1.98 Esterified: 67.73±4.22
WSE-4-IId, β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-(4-hydroxy-3-methoxy-phenyl)-β-D-glucopyranoside		273.72±10.53
WSE-4-IVd, tryptophan		230.86±16.94
R_1 = glucose, R_2 = OH: WSE*a*-4-VIIc peak 1, delphinidin-3-glucoside (D3G) R_1 = rutinose, R_2 = OH: WSE*a*-4-VIIc peak 2, delphinidin-3-rutinoside (D3R) R_1 = glucose, R_2 = H: WSE*a*-4-VIIc peak 3, cyanidin-3-glucoside (C3G) R_1 = rutinose, R_2 = H: WSE*a*-4-VIIc peak 4, cyanidin-3-rutinoside (C3R)		D3G: 9.28±0.34 D3R: 33.10±0.92 C3G: 4.05±0.09 C3R: 18.53±0.29
$R_1 = C_{19}H_{39}$: WISE-1, 5-nonadecyl-benzene-1,3-diol $R_2 - C_{21}H_{43}$: WISE-2, 5-heneicosyl-benzene-1,3-diol		WISE-1: 154.46±6.85 WISE-1: 208.93±4.36

[a]Data are expressed as the mean ± SD (n = 3). [b]Data are given on a dry weight basis.

phenolic compounds are discussed to exert their AO effects in the colon and thus play a role in the observed risk reduction of chronic diseases by whole grains.[38]

Activity-Guided Fractionation of the 80% Ethanol, Water-Soluble Extract (WSE-4). The most active Amberlite XAD-2 eluate, WSE-4, was partially fractionated by semipreparative phenylhexyl HPLC. Figure 3 shows the relative AO activity of the subfractions. WSE-4-II was most active in two of the assays, whereas WSE-4-IV was most active in the FC assay but showed only minor activity in the other two assays. Thus, both fractions were further fractionated, and the subfractions were tested again; however, only those assays were performed in which the parent fractions showed dominant activity. Both WSE-4-II and WSE-4-IV contained one major peak in their chromatograms, and these compounds were found to be mainly responsible for the observed activities. Their structures were elucidated by LC-MS and NMR. The major WSE-4-II peak (WSE-4-IId) contained a compound with a molecular mass of 626 and a sum formula of $C_{25}H_{38}O_{18}$. The ^{1}H NMR spectrum indicated a phenolic trisaccharide, with one sugar moiety linked to an aromatic moiety that had two more substituents exhibiting a +M or +I effect (all phenolic hydrogens had shift values below 7 ppm). One of the substituents was identified as a methoxyl group by HMBC cross coupling of the methyl protons into the aromatic ring. The coupling constants of the anomeric protons indicated that all monosaccharide units were pyranoses in β-conformation. Only one unit had the characteristic ^{1}H and ^{13}C chemical shifts of a free C6 group, indicating 1→6 linkages between the monosaccharides. Several of the carbohydrate protons were overlapping, impairing determination of coupling constants. However, the assigned chemical shifts were in good agreement with published data for gentiotrioses.[39] ^{1}H and ^{13}C chemical shifts are given in Table 3. The nature of the carbohydrate moieties was confirmed by acidic cleavage of the glycosidic linkages and

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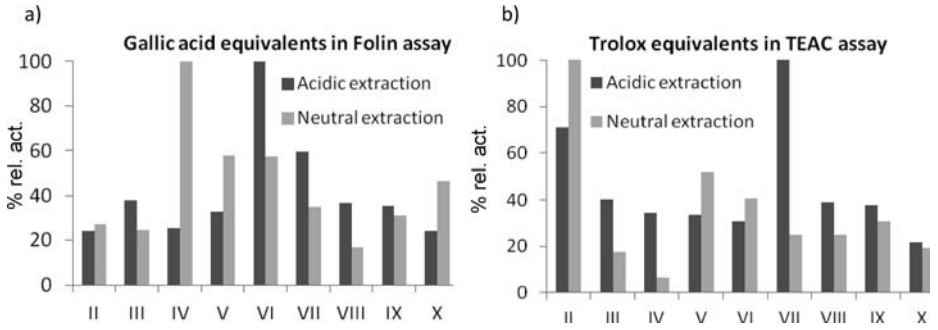

Figure 3. Relative antioxidant activities of subfractions from WSE-4, obtained under neutral conditions, and WSE*a*-4, obtained under acidic conditions, in the Folin–Ciocalteu (FC) assay (a) and the Trolox equivalent antioxidant capacity (TEAC) assay (b). Subfractions I of both extracts did not show antioxidant activity and were therefore not included.

Table 3. Shift Values (Parts per Million) and Coupling Constants (in Parentheses) (Hertz) for Compound WSE-4-IId[a]

	aromatic ring		glucose 1		glucose 2		glucose 3	
position	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C	δ ^{1}H	δ ^{13}C
1		151.7	5.05, d (7.8)	102.3	4.48, d (8.0)	103.9[b]	4.41, d (8.3)	103.9[b]
2	6.85, d (2.4)	103.8	3.54, dd (7.8/9)	74.1	3.27, dd (8.0/8.4)	74.3	3.27, dd (8.3/8.3)	74.3
3		148.9	3.59, dd (9.3/9.3)	76.7[b]	3.42, dd (8.4/8.4)	76.7[b]	3.44, dd (8.3/9.4)	76.7[b]
4		141.6	3.50, dd (9.3/9.5)	70.7	3.38, m	77.0[b]	3.32, dd (9.4/9.4)	70.8
5	6.88, d (8.6)	116.1	3.82, m	76.5	3.47, m	70.6	3.37, m	77.0[b]
6	6.71, dd (8.6/2.4)	109.4	3.90, dd (5.7/11.6)	69.9	3.78, dd (4.8/11.4)	69.6	3.66, dd (6/11.9)	61.9
			4.17, dd (4.3/11.6)		4.17, dd (4.3/11.4)		3.88, m	
OMe	3.87	56.6						

[a]Not all coupling constants could be determined because of overlapping signals. [b]Overlapping signals.

alditolacetate generation according to Blakeney[40] and analysis of the derivatized monosaccharides by GC-FID, where only glucose was detected (data not shown). Thus, WSE-4-IId was identified as β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-(4-hydroxy-3-methoxyphenyl)-β-D-glucopyranoside, but will be referred to as WSE-4-IId throughout the text (see Table 2). To the best of our knowledge, this compound has not been described as a major in vitro AO in whole wheat before. WSE-4-IId was shown to be present at a concentration of 273.72 ± 10.53 μg/g whole meal. Because the quantification procedure involved only extraction with chilled 80% ethanol, drying, and reconstitution in water, followed by RP-HPLC analysis, it is unlikely that the compound was artificially created.

WSE-4-IVd was shown to have a molecular mass of 204 and a sum formula of $C_{11}H_{12}N_2O_2$. Interpretation of the NMR spectra identified the compound in WSE-4-IVd as tryptophan. This amino acid was previously shown to react in the FC assay, which is not specific for phenolic compounds.[41] The reagent also reacts with nonphenolic, reducing food constituents such as ascorbic acid or Maillard reaction products.[42] This assay was even initially developed to detect amino acids.[43] The TEAC and FC assays were often shown to correlate well;[41] however, this correlation may be lost if samples contain compounds that are active in only one of the assays, such as tryptophan. Tryptophan is known to have a low oxidation potential and, thus, has been reported to show activity in in vitro AO assays. However, it should not be considered as an AO in the traditional sense. According to the generally accepted definition of Halliwell,[44] an AO is present at low concentration compared to an oxidizable substrate and prevents or delays the oxidation of this substrate. This substrate could, for instance, be a protein, and hence tryptophan is part of the biomolecules that an AO is meant to protect. In fact, tryptophan residues are among the sites on a protein most susceptible to oxidation. The loss of tryptophan in the course of protecting another biomolecule would not be beneficial, as an essential amino acid is lost in the process. The amount of free tryptophan in the blue wheat sample was determined to be 230.86 ± 16.94 μg/g whole meal, which is higher than in some studies[45,46] but within the range reported by others.[47] Differences between wheat varieties may be responsible for the wide range of reported values.

Because the color of the crude WSE-4 obtained from Amberlite XAD-2 indicated that it contained anthocyanins, it was surprising that they were not in the most active fractions. Several studies have found that extracts from colored grains are more active in AO assays than extracts from noncolored grains and attributed this difference to the higher anthocyanin content of colored grains. However, one study[48] found that the most AO active compounds in colored rice were not anthocyanins when neutral extraction conditions were applied. It should be noted that fractions with dark red color, indicating anthocyanins, were active in the AO assays; however, WSE-4-IId and tryptophan showed even higher activity in these assays. Our procedure, which was developed in ongoing efforts to compare different wheat varieties, involves extractions with aqueous solvents that were not initially acidified. Anthocyanins are more stable at a low pH. It is thus possible that they were partially destroyed during the workup, even though the deep-red color of several semipreparative HPLC fractions indicated that at least some anthocyanins survived the procedure.

To investigate whether the pH had influenced the anthocyanin contribution to the overall AO activity, the WSE isolation was repeated under acidic conditions (pH <2 in all extraction and fractionation steps). This pH was chosen as a compromise between anthocyanin stability and the stability of the used separation media. Decreasing the pH resulted in

different chromatograms as well as in different AO rankings of the fractions (Figure 3). One fraction (WSE*a*-4-VII) was most active in the TEAC and LMB assays, whereas fraction WSE*a*-4-VI was the most active fraction in the FC assay. WSE*a*-4-VI contained one main peak, which was identified as tryptophan by ^{1}H NMR. WSE*a*-4-VI also contained a minor peak with absorption at 520 nm, which was identified as delphinidin-3-glucoside (D3G) by comparing the ^{1}H NMR spectrum with a D3G standard. Its concentration was ca. 20 times less than that of tryptophan, and its FC activity was 204 GAE/mg (tryptophan, 242 GAE/mg). Because tryptophan shows little activity in the TEAC and LMB assays (1.1 and 0.06 Trolox equiv/mg, respectively), it is assumed that D3G was mainly responsible for WSE*a*-4-VI's activities in these two assays. As for WSE*a*-4-VII, it contained several peaks with absorption either at 280 nm only or at both 280 and 520 nm. Thus, we performed one additional fractionation with eluent collection in time intervals. It was found that subfraction WSE*a*-4-VIIc was the most active in both the TEAC and LMB assays (Figure 4). It contained four peaks, all of

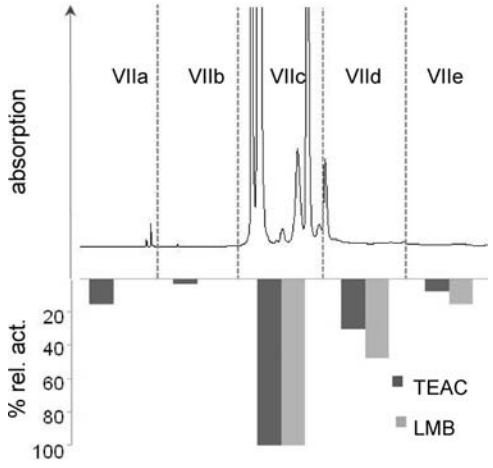

Figure 4. Chromatogram of the separation of WSE*a*-4-VII recorded at 520 nm and relative antioxidant activities of the subfractions in the leucomethylene blue (LMB) and Trolox equivalent antioxidant capacity (TEAC) assays.

which absorbed at 520 nm. They were identified by LC-MS and NMR. The first peak was found to be D3G (vis λ_{max} 524 nm, m/z 465, ^{1}H NMR spectrum matched the D3G standard). The three other peaks were identified as delphinidin-3-rutinoside (D3R), cyanidin-3-glucoside (C3G), and cyanidin-3-rutinoside (C3R). Their m/z values were 611, 449, and 595, respectively. These four anthocyanins were previously identified as the main anthocyanins of a different blue wheat variety by using standard compounds and LC-MS data.[10] With 0.75 mg, D3R was the most abundant compound obtained from WSE*a*-4-VIIc, followed by 0.62 mg of C3R (of 3.22 mg of WSE*a*-4-VIIc). This relationship reflected the amounts of these four anthocyanins in whole meal. Rutinosides were the dominant glycosides, with D3R being the main anthocyanin (33.10 ± 0.92 μg/g), followed by C3R (18.53 ± 0.29 μg/g). The amounts of D3G and C3G were lower, 9.28 ± 0.34 and 4.05 ± 0.09 μg/g, respectively, which contrasts with previous work,[10,49] in which glucosides were found to be the most abundant anthocyanins.

Activity-Guided Fractionation of the 80% Ethanol Extractable, Water-Insoluble Extract (WISE). The WISE was fractionated by using phenylhexyl HPLC. Monitoring the wavelengths 284, 292, and 435 nm seemed to be of special interest, because the color of the extract indicated that it contained tocopherols and carotenoids. However, test runs on an analytical system produced only a few and comparably small peaks at these wavelengths, which mostly coeluted at the beginning of the separation. However, two peaks with a UV maximum at 280 nm and a third peak showing absorption at 435 nm, indicative of a carotenoid, could be separated from these coeluting peaks. Whereas the rest of the compounds were collected in time intervals, these peaks were therefore collected individually and termed WISE-1, WISE-2, and WISE-3. WISE-1 and WISE-2 were shown to be responsible for the observed color change of the ABTS radical (see above and Figure 1) in the TEAC assay. They gave higher TEs than the putative carotenoid WISE-3, which was, in accordance with the activity-guided principle, not investigated further. The NMR spectra of the isolated compounds were very similar and indicated an aromatic moiety and an aliphatic chain. Two of the aromatic protons resonated at 6.25 and 6.18 ppm, indicating a substituent with either a +M or +I effect such as a hydroxyl group. Because protons and carbons in saturated aliphatic parts of a molecule give overlapping and less distinct signals, complete structure assignment was performed by interpreting MS data in addition to NMR spectra. GC-MS after silylation revealed molecular masses of 528 and 542, respectively, indicating homologues differing by only an ethylene group. Comparison with published MS data[50,51] and NMR data of a homologue[52] identified the two compounds as alkylrecorcinols with chain lengths of C19 and C21 (5-nonadecylbenzene-1,3-diol and 5-heneicosylbenzene-1,3-diol, respectively). Alkylresorcinols have been shown to possess weak AO activity in vitro.[53,54] Their amphiphilic structure allows them to interact with biological membranes. Their AO activity in membranes is thought to be the result of competitive inhibition; that is, they can compete for hydrogen abstraction with polyunsaturated fatty acids.[52] It should be noted that their hydrogen-donating and peroxyl radical-scavenging abilities are comparably low,[52] which is in agreement with our data. Their AO activity in the LMB assay was low. The contents of 5-nonadecylbenzene-1,3-diol and 5-heneicosylbenzene-1,3-diol were determined to be 154.46 ± 6.85 and 208.93 ± 4.36 μg/g, respectively, which is in good agreement with literature data for other wheat varieties.[55,56]

In summary, we have determined the main contributors to the blue wheat AO activity as assessed in three in vitro assays. Some AOs were active in only one assay, whereas others exhibited activity in all three tests, highlighting again the need to perform more than one in vitro test. The fact that we found a novel in vitro AO is indicative that not all major AOs in whole grains have been identified yet. Other potent AOs may not have been discovered by our approach. Because we tested all extracts and fractions at the same concentrations, certain constituents that were present in only trace amounts yet have high activity may have been overlooked here.

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ABBREVIATIONS USED

ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; ACN, acetonitrile; AH, alkaline hydrolysate; AO, antioxidant; BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; C3G, cyanidin-3-glucoside; C3R, cyanidin-3-rutinoside; D3G, delphinidin-3-glucoside; D3R, delphinidin-3-rutinoside; DPPH, 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl; FC, Folin−Ciocalteu; GAE, gallic acid equivalents; LMB, leucomethylene blue; ORAC, oxygen radical absorbance capacity; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity; TFA, trifluoroacetic acid; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; WISE, water-insoluble extract; WSE, water-soluble extract.

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