Bioactive compound and antioxidant activity distribution in rollermilled and pearled fractions of conventional and pigmented wheat varieties

TITLE

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RUNNING TITLE

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ABSTRACT

In this study, the chemical composition of pigmented wheats (yellow, purple and blue types), and the distribution of the bioactive compounds in their roller-milled and pearled fractions, were compared with conventional wheats (red and white types).

Roller-milling promoted the recovery of total dietary fiber, β-glucans, phenolic acids and anthocyanins in the bran fraction which resulted also in a higher total antioxidant activity than the refined flour. Conversely, lutein resulted mainly concentrated in the refined flour. In the same way, the distribution pattern in the pearled fractions differ depending on the bioactive considered. The study highlights that a careful selection of the most appropriate fractionation process should be performed to produce flours rich in bioactive compounds. Roller-milling resulted useful for the production of refined flours rich in xanthophylls, with particular emphasis to the yellow-grained wheats. Contrarily, pearling could be more useful in the valorization of the health potential of anthocyanin-pigmented varieties.

KEYWORDS

Pigmented wheats, Grain-fractionation technologies, Total dietary fiber, β-glucans, Antioxidant capacity, Phenolic acids, Carotenoids, Anthocyanins

1. INTRODUCTION

Whole grain cereals are an important source of bioactive compounds, and increasing evidence from clinical and epidemiological studies suggests that the regular consumption of wheat, as whole grain, might reduce the risk of developing chronic diseases (Borowicki, Stein, Scharlau & Glei, 2010; Fardet, 2010; Belobrajdic & Bird, 2013).

Phenolic compounds occur abundantly in cereals, and are related to the antioxidant activity of the grain (Adom & Liu, 2002). Phenolic acids are the main phenolic compounds in cereals, and they constitute one of the major and most complex groups of phytochemicals (Li, Shewry & Ward, 2008). In addition to phenolic acids, other bioactive compounds with antioxidant activity, such as anthocyanins and carotenoids, may also be present in cereal kernels. Anthocyanins and carotenoids both are classified as pigments, and are responsible for the characteristic blue-purple and yellow-orange hue of kernels, respectively. The content of these phytochemicals is generally limited in conventional white- or red-grained wheat varieties (Carson & Edwards, 2009), while they occur more consistently in the so-called pigmented varieties. These types of common wheat varieties, which are characterized by purple, blue or yellow grains, are actually produced in small amounts, but growing interest has recently been shown in the genetic development of novel pigmented varieties (Jaafar, Baron, Siebenhandl-Ehn, Rosenau, Böhmdorfer & Grausgruber, 2013; Martinek, Škorpík, Chrpová, Fučík & Schweiger, 2013). In fact, these unconventional varieties might be important sources of biologically active phytochemicals, and as a result, they could be valuable raw materials for the production of functional foods (Ficco et al., 2016; Li, Pickard & Beta, 2007; Pasqualone et al., 2015).

Nevertheless, the use of wheat varieties that are naturally rich in bioactive compounds should be combined with a suitable grain processing technology, in order to preserve the bioactives and to produce functional ingredients. Previous studies, performed on conventional common wheat varieties, have shown that the bioactive compounds are

79 mainly concentrated in the outer layers of the grain, and that their distribution within the
80 kernel differs according to the class of nutrients (Sovrani et al., 2012). Consequently, the
81 conventional roller-milling process, which promote the removal of the outer layers of the
82 kernel in the bran fraction, causes a great decrease in the nutritional value of the refined
83 flour (Siebenhandl et al., 2007). Another fractionation technology that was proposed as an
84 alternative strategy to valorize the outer layers of the kernel was the pearling process
85 (Hemery, Rouau, Lullien-Pellerin, Barron & Abecassis, 2007). Wheat millers are
86 increasingly using the pearling process before roller milling, since it improves the efficiency
87 of the milling process by removing the outer layers of the kernel through an abrasive
88 scouring (Campbell, Webb, Owens & Scanlon, 2012). Moreover, the degree of pearling
89 could be carefully modulated in order to separate the outermost fractions, which could be
90 characterized by a higher content in contaminants and coarse fiber, from the intermediate
91 fractions, which offer potentially high health benefits (Sovrani et al., 2012; Blandino et al.,
92 2015).
93 The aim of this work was to characterize the chemical composition of roller-milled and
94 pearled fractions obtained from common wheat varieties, characterized by different kernel
95 colors (red, white, yellow, purple and blue), in order to evaluate the best fractionation-
96 technology able to valorize the health potential of pigmented varieties in the production of
97 functional ingredients rich in bioactive compounds.

2. MATERIALS AND METHODS

2.1 Grain samples

The present study has analyzed the milled and pearled fractions of five wheat varieties (*Triticum aestivum* L.). These wheat varieties included:

- PR22R58: red-grained wheat, provided by Pioneer Hi-Bred Italia S.r.l (Italy);
- Whitebear: white-grained wheat, provided by C&M Seeds (Ontario);
- Bona Vita: yellow-grained wheat, provided by Osivo a. s. (Slovakia);
- Rosso: purple-grained wheat, provided by Saatbau (Austria);
- Skorpion: blue-grained wheat, provided by the Agricultural Research Institute Kromeriz, Ltd. (the Czech Republic).

2.2 Analysis of the kernel traits

Thousand kernel weight (TKW) was determined on three 100-kernel sets of each sample, using an electronic balance. Test weight (TW) was determined by means of a Dickey-John GAC2000 grain analysis meter (Dickey-John Corp., Auburn, IL), using the supplied program, after validation with reference materials.

2.3 Wheat grain roller-milling and pearling

Grain samples were processed in order to obtain both roller-milled and pearled fractions.

The roller-milled fractions were obtained using a laboratory-scale mill (Labormill 4RB, Bona, Monza, Italy), after tempering, according to the grain variety. After milling, two fractions were analyzed: the bran and the refined flour. On average, the milling yield (relative amount of refined flour) was 48±1%.

Six pearled fractions of the kernels were obtained through the incremental pearling of the wheat varieties, according to the approach described by Sovrani et al. (2012). The pearling

consisted of consecutive passages of kernels or pearled kernels in an abrasive-type grain testing mill (Model TM-05C, Satake, Tokyo, Japan). Starting from unprocessed grain samples (5 kg), the kernels were initially pearled to remove 5% of the original grain weight, and this resulted in a first fraction (0-5% w/w). The remaining kernels were then pearled to remove a second fraction of 5% (5-10% w/w). The pearling process was repeated to remove a third, fourth and fifth fraction (designed fractions of 10-15%, 15-20%, 20-25% w/w). The pearling process was performed at a constant speed (55 Hz), thus the estimation of the time necessary in order to remove 5% of kernel weight at each pearling passage was experimentally quantified for each variety. The pearling process was then monitored by means of a time control, and after each pearling session, the laboratory pearler was cleaned thoroughly to minimize equipment contamination. The residual 75% of the kernel (25-100% w/w) was also collected.

The residual pearled kernels were milled by means of a laboratory centrifugal mill (Model ZM-100, Retsch, Haan, Germany) equipped with a 1-mm sieve. The same process was performed also for the unprocessed grain samples in order to obtain a wholegrain flour. Prior to the β-glucan analyses, all the samples were ground in an oscillatory mill (particle size < 500 μm) (Mixer mill MM440, Retsch GmbH, Hann, Germany), and were also sieved (particle size < 250 μm) to determine the total antioxidant activity. All the samples were stored at -25°C, before the chemical analyses were performed.

2.4 Chemical analyses

2.4.1 Chemicals

Acetonitrile (CHROMASOLV®Plus, ≥99.9%), dichloromethane (CHROMASOLV®, ≥99.9%), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,6-di-*tert*-butyl-4-methylphenol (BHT, ≥99.0%), ethanol (CHROMASOLV®, ≥99.8%), ethyl acetate (CHROMASOLV®, ≥99.8%), formic acid (≥95.0%), hexane (CHROMASOLV®, ≥97.0%), (±)-6-hydroxy-2,5,7,8-

150 tetramethylchromane-2-carboxylic acid (Trolox, 97%), hydrochloric acid (HCl, 37.0%),
151 methanol (CHROMASOLV®, ≥99.9%), potassium hydroxide (KOH, 90.0%), sodium
152 hydroxide (NaOH, ≥98.0%), *tert*-butyl methyl ether (MTBE, CHROMASOLV®, ≥99.9%)
153 and phenolic acid standards (caffeic acid ≥98%, chlorogenic acid ≥95%, ellagic acid ≥95%,
154 *p*-hydroxybenzoic acid ≥99%, *p*-coumaric acid ≥98%, protocatechuic acid ≥97%, sinapic
155 acid ≥98%, syringic acid ≥95%, *trans*-ferulic acid ≥99% and vanillic acid ≥97%) were
156 purchased from Sigma-Aldrich (St. Louis, Missouri, US). Anthocyanin standards (cyanidin-
157 3-*O*-glucoside, delphinidin-3-*O*-glucoside and peonidin-3-*O*-glucoside) and carotenoid
158 standards (lutein, zeaxanthin, β-cryptoxanthin and β-carotene) were purchased from
159 Extrasynthese (Lyon, France).
160
161 **2.4.2 Proximate composition analysis**
162 The moisture content, determined in order to express the results on a dry weight (dw)
163 basis, was obtained using a Sartorius MA30 thermo-balance (Sartorius AG, Goettingen,
164 Germany). The total nitrogen content and total protein content (conversion factor: 5.70)
165 were obtained according to the Kjeldahl method by means of a Kjeltec system I (Foss
166 Tecator AB, Höganäs, Sweden). The ash content was determined in a muffle furnace
167 according to the AOAC (1990) procedure. The total dietary fiber (TDF) and β-glucan
168 contents were determined by means of the Megazyme total dietary fiber analysis kit and
169 the Megazyme mixed-linkage β-glucan assay kit, respectively.
170
171 **2.4.3 Determination of the Total Antioxidant Activity (TAA)**
172 TAA was determined employing DPPH radical scavenging method (direct measurement
173 on solid samples), as previously described in (Sovrani et al., 2012). Samples were
174 opportunely weighed (0.5-20 mg, to obtain a final inhibition percentage in the 35-65%

range); then, 700 μl of water and 700 μl of a DPPH methanolic solution (100 μM) were added. The reaction was carried out in the dark under stirring at 20°C and 1000 rpm (Thermomixer comfort, Eppendorf, Germany) for 25 min. The samples were promptly centrifuged for 1 minute at 17530 g, and the absorbance was measured at 515 nm after exactly 30 minutes. A control solution, without the ground sample, was tested under the same conditions, in order to calculate the DPPH inhibition percentage of the samples. The final results were expressed as mmol of Trolox equivalents (TE)/kg of sample (dw) through a calibration curve (linearity range: 2.8 - 33.5 nmol; r^2: 0.982).

2.4.4 Extraction of the free and bound phenolic acids and quantification by means of RP-HPLC/DAD

Fifty milligrams of each sample were suspended in 1 mL of a methanol:water 80:20 (v/v) mixture, vortexed for 10 sec, and then extracted in an ultrasonic bath (Bransonic 1510, output 42 kHz, Branson Ultrasonics, Danbury, CT) for 2 min. The extracts were centrifuged at 17530 g for 2 min, and pellets were extracted another two times, according to the method described above. The supernatants were collected and used for the chromatographic analyses of the free phenolic acids.

The pellet remaining after the extraction of the free phenolic acids was hydrolyzed for 3.5 hours under a nitrogen stream, and in continuous agitation, by adding 10 mL NaOH 1 M. After acidification to pH 2.3 with HCl, the bound phenolic acids were extracted with 20 mL of ethyl acetate. The extraction was repeated three times. The combined supernatants were evaporated to dryness, and then reconstituted in 2 mL of methanol.

The extracts were centrifuged at 17530 g for 2 min, and analyzed using an HPLC system (Shimadzu LC-20A Prominence, Shimadzu Italia, Milan, Italy), coupled to a SPD-M20A diode array detector. Separations were carried out using a 150 x 2 mm, 5 μm, Luna C18(2) column (Phenomenex, Torrance, CA), protected by a guard column containing the same

phase; the column temperature was set at 30°C. The mobile phase consisted of 0.1% v/v formic acid in water (solvent A) and 0.1% v/v formic acid in methanol (solvent B), and the following operating gradient was used: 0-30 min, 5-17.5% B; 30-40 min, 17.5-30% B; 40-45 min, 30-100% B. Finally, the mobile phase was brought to 5% B in 10 minutes, and this was followed by 15 minutes of equilibration. The mobile phase flow rate was 0.4 mL/min, and the injection volume was 20 μL for the free phenolic acid extracts and 1 μL for the bound phenolic acid extracts. The hydroxycinnamic acids (ferulic acid, caffeic acid, *p*-coumaric acid, sinapic acid, chlorogenic acid) and hydroxybenzoic acids (vanillic acid, *p*-hydroxybenzoic acid, protocatechuic acid, ellagic acid, syringic acid) were identified using the retention times and UV/Vis spectra of their respective standards. Phenolic acid standards were prepared and diluted to different concentrations, in order to obtain calibration curves for quantification purposes. The hydroxycinnamic acids and hydroxybenzoic acids were quantified at 330 nm and 280 nm, using ferulic acid and protocathecuic acid as reference compounds, respectively (injection volume: 1 μL; linearity range: 0.05-5 μg/mL for hydroxycinnamic acids [5-300 μg/mL in the case of bound ferulic acid]; 0.5-10 μg/mL for hydroxybenzoic acids).

2.4.5 Extraction of the anthocyanins and quantification by means of RP-HPLC/DAD

Each sample (1 g) was extracted using 8 mL of ethanol acidified with HCl 1 N (85:15, v/v) for 30 min. The absorbance was measured after centrifugation at 17530 g for 2 min at 540 nm, as reported by Siebenhandl et al. (2007). The total Anthocyanin Content (TAC) was expressed as mg cyanidin-3-*O*-glucoside (Cy-3-glc) equivalents/kg of sample (dw).

The identification and quantification of the individual anthocyanins was performed using the previously described chromatographic system. Separations were carried out using a 250 x 4.6 mm, 4 μm, RP SynergiTM 4 μm Max-RP 80 Å LC Column (Phenomenex, Torrance, CA), protected by a guard column containing the same phase; the column

temperature was set at 30 °C. The mobile phase consisted of water:formic acid:acetonitrile [87:10:3, v/v; (solvent A)] and water:formic acid:acetonitrile [40:10:50, v/v; (solvent B)], and the following operating gradient was used: 0-20 min, 6-20% B; 20-35 min, 20-40% B; 35-40 min, 40-60% B; 40-45 min, 60-90% B; 45-50 min isocratic 90% B. Finally, the mobile phase was brought to 6% B in 0.5 minutes, and this was followed by 22.5 minutes of equilibration. The mobile phase flow rate was 0.5 mL/min, and the injection volume was 15 μL. Cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and peonidin-3-*O*-glucoside were identified using the retention times and UV/Vis spectra of the individual authentic standard molecules; delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-rutinoside and peonidin-3-*O*-rutinoside were identified on the basis of literature data (Abdel-Aal, Young, & Rabalski, 2006; Abdel-Aal, Abou-Arab, Gamel, Hucl, Young, & Rabalski, 2008; Knievel, Abdel-Aal, Rabalski, Nakamura & Hucl, 2009; Liu, Qiu & Beta, 2010). Quantification of the individual compounds was performed at 520 nm, using glucoside forms of anthocyanins (rutinosides were quantified as equivalents of the corresponding glucosides); calibration curves were obtained at six different concentration levels (injection volume: 5 µL; linearity range: 0.005-10 µg/mL).

2.4.6 Extraction of the carotenoids and quantification by means of RP-HPLC/DAD

Each sample (0.3 g) was extracted for 6 min at 85°C with 95% ethanol, containing 1 g/L BHT. The extracts, including solids, were hydrolyzed with 125 μL of KOH (1g/mL) at 85°C for 10 min, chilled on ice and 3 mL of cold deionized water was then added. This was followed by the addition of 3 mL of hexane, containing 1 g/L BHT. The test tubes were then vortexed and centrifuged at 1200 g for 10 minutes. The extraction was repeated four times, and the combined supernatants were evaporated to dryness under a nitrogen stream, and then reconstituted in 75 μL of dichloromethane.

252 The extracts were analyzed by means of a high performance liquid chromatograph Agilent
253 1200 Series (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 1200
254 Series diode array detector. Separations were carried out using a 250 x 4.6 mm, 3 μm,
255 C30 carotenoid YMC column (YMC Co., Kyoto, Japan); the column temperature was set at
256 25°C. The mobile phase consisted of methanol:MTBE:water [81:15:4, v/v; (solvent A)] and
257 MTBE:methanol [91:9, v/v; (solvent B)]. The following operating linear gradient was used:
258 0-45 min, 0-50% B; 45-60 min, 50-100% B. Finally, the mobile phase was brought to 0% B
259 in 10 minutes, and this was followed by 15 minutes of equilibration. The flow rate of the
260 mobile phase was 1.0 mL/min, and the injection volume was 5 μL. Carotenoids were
261 identified using the retention times and the UV/Vis spectra of their respective standards
262 (lutein, zeaxanthin, β-cryptoxanthin and β-carotene). Carotenoid standards were also
263 prepared and diluted to different concentrations to obtain calibration curves for
264 quantification purposes (linearity range: 0.1-100 μg/mL for lutein and zeaxanthin; 0.3-40
265 μg/mL for β-cryptoxanthin and β-carotene). The quantifications were performed at 450 nm.
266
267 **2.5 Color analyses**
268 The chromatic analyses were performed using a Minolta Chroma Meter reflectance
269 spectrophotometer (Model CR-400, Minolta Co., Osaka, Japan). A 45 mm diameter Petri
270 dish was loosely filled with a ground subsample, and the dish was then tapped gently until
271 the sample was levelled and no gaps were apparent through the base of the dish. The *L**,
272 *a** and *b** color values were determined directly by the instrument.
273
274 **2.6 Statistical analyses**
275 All the analyses were performed at least in triplicate checking that the variation coefficient
276 was lower than 10%. One-way analysis of variance (ANOVA) was applied in order to

277 compare the contents of the bioactive compounds in wholegrain flours on the basis of the
278 wheat genotype, and in the different roller-milled and pearled fractions of each wheat type.
279 The REGW-Q test was performed for multiple comparisons. A 0.05 threshold was used to
280 reject the null hypothesis.
281 Statistical analyses were carried out by means of SPSS for Windows statistical package,
282 Version 22.0 (SPSS Inc., Chicago, Illinois).
283

3. RESULTS AND DISCUSSION

3.1 Kernel traits and the bioactive compound contents of the wholegrain flours

Although the most commonly cultivated wheat varieties are the red- and white-grained ones, the application of pigmented wheat varieties for the production of functional foods has drawn the attention of both researchers and the food industry in the last few years (Ficco et al., 2016; Li et al., 2007; Pasqualone et al., 2015; Yu & Beta, 2015; Zanoletti et al., 2017). In this study the chemical composition of pigmented wheat varieties (yellow, purple and blue types) was analyzed and compared with conventional wheat varieties (red and white types). The compared cultivars showed differences both in their physical characteristics, in the chemical content and in the color of their wholegrain flours (Table 1 and 2).

The wholegrain flour of pigmented wheat varieties showed levels of total dietary fiber (TDF), β-glucans, total antioxidant activity (TAA), free (FPAs) and cell wall-bound (CWBPAs) phenolic acids similar to the ones of conventional wheat varieties, as well as significant concentrations of anthocyanins and xanthophylls, which were absent or limited in the red- and white-grained varieties. As far as the anthocyanin-pigmented varieties are concerned, the purple type showed a higher overall total anthocyanin content (TAC) than the blue one. The main carotenoid detected in all the samples was lutein, and this was followed by zeaxathin, while β-cryptoxantin and β-carotene were not detected. The concentration of lutein was on average 6.6 times higher than that of zeaxanthin, and the yellow variety was the one characterized by the highest content of this xanthophyll.

The chromatic characteristics of the wholegrain flours of both the conventional and pigmented varieties were determined in order to demonstrate that the analyzed grains differed in their color. As expected, the different bioactive compound profiles resulted in significant differences in the lightness (L^*), and in the red (a^*) and blue (b^*) indexes of the wholegrain flour (Table 2). The wholegrain flour of the white variety was characterized by

310 the highest lightness value and the lowest redness one. Differently, the purple and the blue
311 varieties showed the lowest lightness value, and were characterized by the highest
312 redness and blueness values, respectively. The wholegrain flour of the yellow variety was
313 characterized by the highest yellowness, in accordance with its high lutein content.
314
315 **3.2 Bioactive compound distribution in the roller-milled fractions**
316 The analysis of the roller-milled fractions clearly shows that the bioactive compounds are
317 unevenly distributed in the kernels of both conventional and pigmented wheat varieties
318 (Table 3). In particular, roller-milling promoted the recovery of proteins, TDF, β-glucans,
319 FPAs and CWBPAs in the bran fraction. Consequently, regardless the variety considered
320 the bran fraction resulted in a TAA 10 and 3 times higher than the one of the refined flour
321 and the wholegrain flour, respectively. This result was in accordance with previous studies
322 that mainly ascribe TAA of wheat grains to phenolic compounds (Beta, Nam, Dexter &
323 Sapirstein, 2005). As far as the individual phenolic acids are concerned, only free and
324 bound ferulic acid and bound ellagic acid were identified in the refined flour, while a more
325 complex profile was observed in the bran fraction (the main phenolic acids quantified are
326 reported in Table S1). Considering the two anthocyanin-pigmented varieties, their bran
327 fraction was characterized on average by a total anthocyanin content 23 and 2 times
328 higher than the one of the refined flour and the wholegrain flour, respectively. Not many
329 data are currently available on the distribution of carotenoids in wheat kernels. Previously,
330 it has been observed that lutein was concentrated more in the endosperm than in the bran
331 of einkorn and durum wheat, while the opposite trend has been observed in common
332 wheat (Abdel-Aal, Young, Rabalski, Hucl & Fregeau-Reid, 2007). Conversely, Siebenhandl
333 et al. (2007) observed a higher concentration of total carotenoids in the refined flour than
334 in the bran fraction of a blue-grained wheat variety. In the present study, lutein was always
335 mainly concentrated in the refined flour than in the bran fraction, with the only exception

being the blue variety, which did not show any significant difference between its roller-milled fractions. As far as the yellow-grained variety is concerned, the concentration of lutein observed in the refined flour is slightly higher (+12%) than the one observed in the wholegrain flour. Zeaxanthin, whose concentration was always lower than that of lutein, showed an opposite distribution pattern.

In accordance with the composition and distribution of bioactive compounds, regardless the variety considered the bran fraction showed lower lightness and higher redness values than the refined flour (Table S2).

The present study clearly confirms that the roller-milling process results in the concentration of the most nutritional compounds in the bran fraction of both conventional and pigmented wheat varieties. This result is particularly relevant for the anthocyanin-pigmented wheat varieties because of the removal of most of the anthocyanins in the bran fraction. Several studies proposed the use of the wheat bran of both conventional (Sobota, Rzedzicki, Zarzycki & Kuzawińska, 2015) and pigmented wheat varieties (Li et al., 2007) for the production of cereal-derived products enriched in bioactive compounds. At the same time, roller-milling removes the parts of the grain known or suspected to be detrimental to foods, in relation to safety, processing and acceptability by the consumers (Zhang & Moore, 1999; Cheli et al., 2010). Thus, especially for anthocyanin-pigmented varieties, instead of reject or maintain totally the bran fraction, greater attention needs to be paid to optimize the processing of kernels in order to ensure bioactive preservation and at the same time the removal of the fractions suspected to be detrimental for food production.

Contrarily to all the other bioactive compounds analyzed in the present study, lutein resulted mainly concentrated in the refined flour than in the bran fraction. Thus, the application of the conventional roller-milling process could be considered useful for the

361 production of refined flours naturally rich in xanthophylls, with particular emphasis to the
362 yellow-grained varieties.
363
364 **3.3 Bioactive compound distribution in the pearled fractions**
365 In agreement with data observed for the roller-milled fractions, the analyses performed on
366 pearled fractions showed an unevenly distribution of bioactive compounds in the grains of
367 both conventional and pigmented wheat varieties. Moreover, the distribution patterns in the
368 pearled fractions differ depending on the class of bioactive considered (Table 4).
369 In accordance with previous studies (Jerkovic, Kriegel, Brander, Atwell, Roberts & Willows,
370 2010; Sovrani et al., 2012), the protein and β-glucan contents were found to be higher in
371 the intermediate pearled fractions of all the varieties. Contrarily, the TDF, TAA, FPAs and
372 CWBPAs were generally detected at the highest concentration in the outermost layers of
373 the kernel, and decreased moving toward the inner layers. Individual phenolic compounds,
374 in both free and cell wall-bound form, generally presented the highest content in the first
375 two pearling fractions (corresponding to the 0-10% of the kernel weight). The main
376 compounds identified and their relative content was quite similar in the different wheat
377 varieties; the bound form of ferulic acid resulted the most representative (Table S3).
378 Similar distribution patterns were previously observed in the pearled fraction of
379 conventional wheat varieties (Sovrani et al., 2012). The TAA in the first two pearled
380 fractions, which correspond to the outermost layers of the kernels, resulted similar to the
381 one observed in the bran fraction and even 3 times higher than the one observed in the
382 wholegrain flour.
383 The distribution patterns of total anthocyanins in the purple and blue varieties are shown in
384 Figure 1. Anthocyanins were found to be located in specific layers of the kernel. In
385 particular, the purple-grained variety was characterized by the highest TAC in the 0-5%
386 fraction (579 mg Cy-3-glc equivalents/kg dw). The total anthocyanin content of this fraction

387 resulted about 6 times higher than the one observed in the wholegrain flour, and even 2
388 times higher than the one observed in the bran fraction. The second 5-10% pearled
389 fraction still showed a high TAC (425 mg Cy-3-glc equivalents/kg dw), about 4 and 1.5
390 times higher than the one observed in the wholegrain flour and in the bran fraction. The
391 blue-grained variety showed the highest total anthocyanin content in the 10-15% fraction
392 (211 mg Cy-3-glc equivalents/kg dw), about 3 and 1.5 times higher than the one observed
393 in the wholegrain flour and in the bran fraction. In accordance to previous studies (Bottega,
394 Caramanico, Lucisano, Mariotti, Franzetti & Pagani, 2009; Jerkovic et al., 2010; Shetlar,
395 Rankin, Lyman & France, 1947; Singh & Singh, 2010; Sovrani et al., 2012), pearling up to
396 the 5% level on average remove most of the outer pericarp, while at 5-10% and 10-15%
397 levels the aleurone layer is removed. Thus, the results were in agreement with previous
398 studies that showed that anthocyanins are mainly concentrated in the pericarp and in the
399 aleurone layer on purple- and blue-grained varieties, respectively (Abdel-Aal & Hucl, 1999;
400 Zeven, 1991).
401 The analysis of individual anthocyanins (Table 5) showed that the two varieties differ not
402 only for the total anthocyanin content, but also for the anthocyanin profile. The results were
403 in accordance with previous studies performed on other pigmented varieties (Bartl et al.,
404 2015; Knievel et al., 2009). The main anthocyanins in the purple wheat were peonidin and
405 cyanidin glycosides, while delphinidin glycosides were not detected. Contrarily, the main
406 anthocyanins detected in the blue-grained variety were delphinidin-3-*O*-rutinoside and
407 cyanidin-3-*O*-rutinoside, although cyanidin-3-*O*-glucoside, delphynidin-3-*O*-glucoside and
408 peonidin-3-*O*-rutinoside were also present. These results make possible assume that the
409 difference observed in the color of these two varieties could be probably ascribed not only
410 to different anthocyanin concentrations, but also to the different compositional profiles. In
411 fact, delphinidin glycosides, which are responsible for a characteristic blue color, were only

412 detected in the blue-grained variety, while cyanidin and peonidin glycosides were detected
413 above all in the purple-grained variety.
414 In accordance with the distribution observed in the roller-milled fractions, lutein was found
415 to be mainly concentrated in the internal layers of the kernel. Regardless the variety
416 considered, the concentration of lutein in the residual pearled kernel resulted similar to the
417 one observed in the refined flour. Contrarily, the distribution of zeaxanthin changed
418 according to which variety was considered.
419 Pearled fractions showed significant difference in their chromatic characteristics as
420 observed for the roller-milled fractions (Table S4).
421 Previous studies proposed the pearling process as an alternative technology in order to
422 select and valorize the intermediated fractions of conventional red-grained varieties, which
423 are rich in bioactive compounds, allowing at the same time the removal of the parts of the
424 grain that could be detrimental for technological quality and safety (Liyana-Pathirana, C.,
425 Dexter, J., & Shahidi F., 2006; Delcour, Rouau, Courtin, Poutanen & Ranieri, 2012;
426 Sovrani et al., 2012). The present study shows that this technology could be applied
427 indifferently both to conventional and pigmented wheat varieties in order to concentrate the
428 bioactive compounds, such as TDF, β-glucans and phenolic acids, that are generally
429 present in the outer layers of all the wheat kernels. Moreover, this study shows that the
430 use of the pearling process could be the best way in order to enhance the exploitation of
431 the health-promoting potential of purple- and blue-grained cereals, by means of a selection
432 of anthocyanin-rich fractions. The resultant fractions could be mixed with a refined flour to
433 incorporate as much as bioactive compounds in a final ingredient which could be used for
434 the production of both baked products (Blandino et al., 2013; Blandino et al., 2015) and
435 pasta (Zanoletti et al., 2017).
436
437

4. CONCLUSIONS

This study highlights that even if pigmented wheat varieties are an excellent source of bioactive compounds, a careful selection of the most appropriate fractionation process should be performed in order to produce flours naturally rich in bioactive compounds. In fact, the application of the same process to different types of wheat varieties could lead to conflicting results. The conventional roller-milling process could be useful to produce a refined flour rich in lutein starting from yellow-grained varieties. On the contrary, the same process applied to anthocyanin-pigmented varieties results in the loss of the main health benefits associated to the use of these varieties, because of the removal of most of the anthocyanins from the refined flour. The pearling process could be a much more useful tool in order to valorize the latter varieties.

Further studies will be necessary for the development of supply chains based on pigmented wheat varieties, in order to understand whether these varieties could be able to compete with conventional ones in terms of yields and quality performance. Finally, pilot and industrial scale tests should be performed in order to evaluate the potential application of the pearling process to anthocyanin-pigmented varieties in order to produce new ingredients with added value for consumer health.

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593

FIGURE CAPTION

Figure 1. The TAC in the pearled fractions of the purple (cv. Rosso) and the blue (cv. Skorpion) wheat varieties. The results are expressed on a dw basis. The reported data are the means of three values; values with different letters differ significantly, according to the REGW-Q test (the ANOVA level of significance is <0.001).

TABLES

Table 1. Kernel traits and chemical composition of the wholegrain flours of conventional and pigmented wheat varieties.

Wheat type	Variety name	TKW g	TW kg/hL	Proteins %	Ash %	TDF %	β-glucans %	TAA mmol TE/kg	FPAs[1] mg/kg	CWBPAs[2] mg/kg	TAC mg Cy-3-glc/kg	Lutein mg/kg	Zeaxanthin mg/kg
Red	PR22R58	43.6 c	75.6 d	9.1 e	1.27 c	14.1 a	1.01 a	6.65 a	1.10 d	889 c	n.d.	2.18 b	0.15 d
White	Whitebear	46.7 b	82.8 a	11.3 d	1.56 b	15.7 a	0.99 a	6.94 a	4.71 a	776 e	n.d.	1.07 e	0.37 a
Yellow	Bona Vita	37.0 d	79.0 c	15.1 a	1.48 b	12.8 a	0.96 a	6.61 a	4.33 ab	800 d	n.d.	3.62 a	0.34 b
Purple	Rosso	46.4 b	80.4 b	12.4 c	1.58 b	15.1 a	0.66 b	7.57 a	3.71 b	1071 b	102 a	1.67 c	0.34 b
Blue	Skorpion	50.1 a	74.6 e	13.0 b	1.91 a	12.7 a	0.53 c	6.47 a	2.35 c	1093 a	72 b	1.24 d	0.29 c
SEM		0.4	0.3	0.1	0.08	1.6	0.06	0.99	0.58	8	5	0.11	0.02
P (F)		<0.001	<0.001	<0.001	<0.001	0.109	<0.001	0.172	<0.001	<0.001	0.001	<0.001	<0.001

TKW, thousand kernel weight; TW, test weight; TDF, total dietary fiber; TAA, total antioxidant activity; FPAs, free phenolic acids; CWBPAs, cell wall-bound phenolic acids; TAC, total anthocyanins.

Data are expressed on a dw basis. Means followed by different letters are significantly different, according to the REGW-Q test (the ANOVA level of significance is shown in the table).

n.d., not detected.

SEM, standard error of the mean.

[1] sum of the FPAs determined by means of RP-HPLC/DAD

[2] sum of the CWBPAs determined by means of RP-HPLC/DAD

610 **Table 2.** Chromatic characteristics of the wholegrain flours.

Wheat type		Color					
		*L**		*a**		*b**	
Red		83.6	c	1.5	b	10.4	b
White		86.4	a	0.8	d	9.9	c
Yellow		84.3	b	1.4	b	12.2	a
Purple		78.8	e	3.1	a	7.0	d
Blue		80.7	d	1.0	c	6.7	e
SEM		0.4		0.1		0.1	
P (F)		<0.001		<0.001		<0.001	

611 Means followed by different letters are significantly different, according to the REGW-Q test (the ANOVA
612 level of significance is shown in the table).

613 SEM, standard error of the mean.

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615

Table 3. Chemical composition of the roller-milled fractions of conventional and pigmented wheat varieties.

Wheat type	Roller-milled fraction	Proteins %		Ash %		TDF %		β-glucans %		TAA mmol TE/kg		FPAs[1] mg/kg		CWBPAs[2] mg/kg		TAC mg Cy-3-glc eq/kg		Lutein mg/kg		Zeaxanthin mg/kg	
Red	Bran	12.9	a	3.94	a	31.6	a	1.44	a	21.71	a	4.35	a	2640	a	n.d.		1.85	b	0.28	a
	Refined flour	7.79	b	0.37	b	2.1	b	0.60	b	1.98	b	0.88	b	92	b	n.d.		2.41	a	0.09	b
	P (F)	0.02		0.02		0.6		0.05		1.13		0.25		6				0.11		0.01	
	SEM	<0.001		<0.001		0.001		<0.001		<0.001		<0.001		<0.001				0.002		<0.001	
White	Bran	14.5	a	3.79	a	26.6	a	1.29	a	20.57	a	12.22	a	2633	a	n.d.		0.99	b	0.49	a
	Refined flour	10.1	b	0.42	b	3.3	b	0.46	b	2.15	b	0.99	b	137	b	n.d.		1.12	a	0.11	b
	P (F)	0.04		0.03		0.5		0.02		0.83		0.18		1				0.04		0.01	
	SEM	<0.001		<0.001		0.001		<0.001		<0.001		<0.001		<0.001				0.007		<0.001	
Yellow	Bran	16.5	a	4.08	a	30.7	a	1.69	a	18.85	a	15.97		2818	a	n.d.		2.69	b	0.73	a
	Refined flour	14.7	b	0.25	b	3.5	b	0.43	b	1.97	b	n.d.		86	b	n.d.		4.11	a	0.13	b
	P (F)	0.1		0.07		0.5		0.02		0.58		-		7				0.23		0.01	
	SEM	0.004		0.001		0.001		<0.001		<0.001		-		<0.001				0.001		<0.001	
Purple	Bran	17.0	a	4.19	a	30.5	a	0.81	a	21.78	a	14.00	a	3314	a	271	a	1.60	b	0.76	a
	Refined flour	10.7	b	0.3	b	4.9	b	0.39	b	2.09	b	1.40	b	100	b	7	b	1.95	a	0.07	b
	P (F)	0.1		0.01		1.2		0.04		1.55		0.88		4		16		0.14		0.02	
	SEM	<0.001		<0.001		0.003		<0.001		<0.001		<0.001		<0.001		<0.001		0.021		<0.001	
Blue	Bran	15.3	a	4.47	a	33.6	a	0.66	a	21.69	a	17.42	a	3991	a	140	a	1.60	a	0.91	a
	Refined flour	12.6	b	0.39	b	3.9	b	0.35	b	2.01	b	0.68	b	111	b	11	b	1.28	a	0.05	b
	P (F)	0.04		0.02		1.4		0.05		1.95		0.65		7		3		0.24		0.17	
	SEM	<0.001		<0.001		<0.001		<0.001		<0.001		<0.001		<0.001		<0.001		0.134		0.002	

TDF, total dietary fiber; TAA, total antioxidant activity; FPAs, free phenolic acids; CWBPAs, cell wall-bound phenolic acids; TAC, total anthocyanins.

The results are expressed on a dw basis. Means followed by different letters are significantly different, according to the REGW-Q test (the ANOVA level of significance is shown in the table).

n.d., not detected.

SEM, standard error of the mean.

[1] sum of the FPAs determined by means of RP-HPLC/DAD

[2] sum of the CWBPAs determined by means of RP-HPLC/DAD

Table 4. Chemical composition of the pearled fractions of conventional and pigmented wheat varieties.

Wheat type	Pearled fraction	Proteins	Ash	TDF	β-glucans	TAA	FPAs[1]	CWBPAs[2]	Lutein	Zeaxanthin
		%	%	%	%	mmol TE/kg	mg/kg	mg/kg	mg/kg	mg/kg
Red	0-5%	8.7 c	3.05 c	51.7 a	0.99 c	18.26 a	4.32 bc	2327 a	1.31 d	0.20 b
	5-10%	9.7 b	3.07 c	38.3 b	1.32 b	20.09 a	5.96 a	2086 b	1.35 d	0.21 b
	10-15%	10.9 a	3.17 b	32.9 c	1.52 a	19.40 a	5.43 ab	1679 c	1.64 c	0.24 ab
	15-20%	11.1 a	3.29 a	25.5 d	1.64 a	12.86 b	4.48 bc	1668 d	1.76 bc	0.22 ab
	20-25%	10.7 a	2.64 d	21.3 e	1.48 ab	10.55 b	5.30 ab	1419 e	2.03 b	0.25 a
	25-100%	9.0 c	1.17 e	9.5 f	0.60 d	3.65 c	3.71 c	449 f	2.55 a	0.15 c
	SEM	0.4	0.04	1.8	0.12	2.14	0.92	6	0.24	0.03
	P (F)	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	<0.001	<0.001	<0.001
White	0-5%	11.0 d	5.04 b	65.1 a	1.19 c	22.62 a	12.90 a	3040 a	0.73 c	0.88 a
	5-10%	15.4 b	5.21 a	31.1 b	1.77 a	20.95 a	9.58 b	2892 b	0.94 b	0.90 a
	10-15%	15.9 a	4.14 c	26.9 bc	1.45 b	11.19 b	9.53 b	2026 c	1.05 ab	0.77 b
	15-20%	15.1 c	3.17 d	25.3 c	1.16 c	9.54 b	6.53 c	1697 d	1.10 a	0.70 b
	20-25%	15.4 b	2.58 e	18.5 d	0.99 d	7.21 c	6.50 c	1312 e	1.14 a	0.60 c
	25-100%	10.5 e	0.96 f	8.8 e	0.24 e	2.21 d	3.01 d	361 f	1.01 ab	0.20 d
	SEM	0.2	0.07	2.2	0.15	3.15	0.87	7	0.12	0.08
	P (F)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Yellow	0-5%	13.4 d	4.25 a	59.0 a	1.65 b	24.41 a	14.02 a	3088 a	1.37 c	0.78 b
	5-10%	20.6 b	3.82 b	35.1 b	2.26 a	20.34 b	9.67 b	3052 b	3.10 b	0.98 a
	10-15%	21.8 a	2.99 c	23.4 c	1.90 b	16.08 c	7.59 c	2199 c	3.65 ab	0.83 b
	15-20%	20.7 b	2.35 d	16.9 d	1.13 c	9.26 d	5.23 d	1533 d	3.74 a	0.61 c
	20-25%	20.8 b	1.95 e	13.8 de	1.01 c	6.41 e	3.88 d	1079 e	3.70 a	0.49 d
	25-100%	13.8 c	0.88 f	9.3 e	0.24 d	2.43 f	1.75 e	526 f	4.12 a	0.23 e
	SEM	0.2	0.07	2.5	0.23	3.11	1.24	20	0.49	0.06
	P (F)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Purple	0-5%	11.4 d	3.11 c	49.8 a	0.56 b	26.40 a	16.69 a	2632 b	1.12 b	0.75 a
	5-10%	12.5 c	3.60 a	42.0 b	0.83 a	23.63 b	16.28 a	2800 a	1.20 b	0.66 bc
	10-15%	14.6 b	3.62 a	34.3 c	0.84 a	17.48 c	11.38 b	2623 b	1.62 a	0.73 ab
	15-20%	15.2 a	3.49 b	26.0 d	0.82 a	12.03 d	8.98 c	1863 c	1.66 a	0.60 c
	20-25%	15.1 a	3.14 c	21.9 d	0.82 a	9.34 e	7.31 d	1797 d	1.62 a	0.49 d
	25-100%	12.5 c	1.47 d	12.1 e	0.22 c	3.15 f	2.63 e	733 e	1.59 a	0.19 e
	SEM	0.3	0.08	1.8	0.17	1.61	1.2	12	0.15	0.08
	P (F)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Blue	0-5%	9.9 f	3.57 c	59.9 a	0.52 c	23.11 a	11.16 a	5388 a	0.93 c	0.52 b
	5-10%	14.9 d	4.31 a	37.6 b	1.09 a	16.64 b	6.16 b	3452 b	1.15 b	0.66 a
	10-15%	17.7 b	4.59 a	27.8 c	1.03 a	15.25 b	5.10 bc	2913 c	1.36 ab	0.67 a
	15-20%	18.1 a	3.98 b	21.2 d	0.85 b	10.92 c	4.13 c	2243 d	1.51 a	0.63 a
	20-25%	17.5 c	3.32 c	15.8 e	0.82 b	8.04 d	5.13 bc	1890 e	1.46 a	0.51 b
	25-100%	12.5 e	0.98 d	8.0 f	0.24 d	2.48 e	2.51 d	622 f	1.32 ab	0.20 c
	SEM	0.2	0.27	1	0.17	2.92	0.95	11	0.18	0.09
	P (F)	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

TDF, total dietary fiber; TAA, total antioxidant activity; FPAs, free phenolic acids; CWBPAs, cell wall-bound phenolic acids; TAC, total anthocyanins.

The results are expressed on a dw basis. Means followed by different letters are significantly different, according to the REGW-Q test (the ANOVA level of significance is shown in the table).

SEM, standard error of the mean.

[1] sum of the FPAs determined by means of RP-HPLC/DAD

[2] sum of the CWBPAs determined by means of RP-HPLC/DAD

Table 5. Anthocyanin profile in the pearled fractions of the purple- and blue-grained wheat varieties.

Wheat type	Pearled fraction	Cy-3-glc		Cy-3-rut		Dn-3-glc		Dn-3-rut		Pn-3-glc		Pn-3-rut	
		mg/kg											
Purple	0-5%	4.15	a	3.62	a	n.d.		n.d.		1.03	a	9.36	a
	5-10%	2.72	b	2.89	b	n.d.		n.d.		0.72	b	6.72	b
	10-15%	1.47	c	1.76	c	n.d.		n.d.		0.47	c	3.71	c
	15-20%	0.95	d	1.15	d	n.d.		n.d.		0.29	d	2.81	d
	20-25%	0.71	d	0.58	e	n.d.		n.d.		0.20	d	1.44	e
	25-100%	0.13	e	0.11	f	n.d.		n.d.		n.d.		0.13	f
	SEM	0.13		0.32						0.06		0.53	
	P (F)	<0.001		<0.001		-		-		<0.001		<0.001	
Blue	0-5%	1.32	c	5.62	c	1.47	c	4.62	c	n.d.		0.40	c
	5-10%	3.75	a	12.62	a	3.06	ab	10.26	a	n.d.		0.98	ab
	10-15%	3.79	a	12.85	a	3.34	a	10.85	a	n.d.		1.13	a
	15-20%	2.55	b	10.56	b	2.77	b	8.94	b	n.d.		0.92	b
	20-25%	2.69	b	10.33	b	2.85	b	9.17	b	n.d.		0.98	ab
	25-100%	0.18	d	1.15	d	0.37	d	1.15	d	n.d.		0.30	c
	SEM	0.33		1.08		0.32		0.81				0.12	
	P (F)	<0.001		<0.001		<0.001		<0.001		-		<0.001	

Cy-3-glc, cyanidin-3-O-glucoside; Cy-3-rut, cyanidin-3-O-rutinoside; Dn-3-glc, delphinidin-3-O-glucoside; Dn-3-rut, delphinidin-3-O-rutinoside; Pn-3-glc, peonidin-3-O-glucoside; Pn-3-rut, peonidin-3-O-rutinoside.

The results are expressed on a dw basis. Means followed by different letters are significantly different, according to the REGW-Q test (the ANOVA level of significance is shown in the table).

n.d., not detected

SEM, standard error of the mean.

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