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Bioactive compound content, antioxidant activity, deoxynivalenol and heavy metal contamination of pearled wheat fractions.

Running title: Functional compounds and contaminants in pearled wheat fractions

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deoxynivalenol, heavy metals.

Abbreviations: Cd, cadmium; DF, dietary fibre; DON, deoxynivalenol; dw, dry weight; FPA, free phenolic acids; Pb, lead; TAA, total antioxidant activity; TE, trolox equivalents.

1 **Abstract**

2 Wheat kernels are naturally rich in antioxidant compounds, that are mainly present in
3 the outer bran layers and which are removed during milling. Unfortunately, several
4 contaminants are concentrated in the external layers. The pearling process, which
5 progressively and carefully debrans the outer layers of wheat, could provide new
6 functional food ingredients. The aim of the current study was to determine the
7 content of functional compounds and the mycotoxin and heavy metals contamination
8 of fractions derived from the sequential pearling of wheat kernels.

9 The pearling consisted of consecutive passages of 3 wheat varieties to remove 5% of
10 the original grain weight. Totally, five consecutive fractions were obtained starting
11 from the outer layer until the inner kernel that designated as 0-5, 5-10, 10-15, 15-20,
12 20-25%, respectively. The remaining 75% of the inner kernel was also collected.
13 Dietary fibre, free phenolic acid and total antioxidant activity decreased progressively
14 from the external to the internal layers. However, the 5-10% fraction was richer in β -
15 glucan content than the external one (0-5%). Heavy metals were only found in the
16 most external fraction. Deoxynivalenol contamination decreased from the external to
17 the internal layers: 64% of total contamination of kernel was found in the 0-5 and 5-
18 10% fractions.

19 The 10-15% kernel fraction offered the best compromise between high nutritional
20 value and low contamination risk.

21

22 **1. Introduction**

23 Increasing demands about healthier foods has intensified the interest of consumers
24 in phytonutrients (Liyana-Pathirana, Dexter & Shahidi, 2006). The addition of
25 antioxidants to food systems may increase the nutritional profile and the shelf life of
26 products, and thus reduce waste and nutritional loss by inhibiting and delaying
27 oxidation. Recently, much attention has been paid to replacing synthetic antioxidants
28 with natural alternatives. Cereals are an important source of bioactive compounds
29 and some of them, such as phenolic compounds (phenolic acids, lignans and
30 flavonoids) show a marked antioxidant activity (Liyana-Pathirana & Shahidi, 2006).
31 The increased consumption of plant-derived phenolics has been associated with a
32 reduced risk of degenerative and chronic diseases (Dykes & Rooney, 2007).
33 Moreover, a diet containing cereals improve the content of other bioactive
34 compounds, such as dietary fibre (DF) and micronutrients (Hemery, Rouau, Lullien-
35 Pellrin, Barron & Abecassis, 2007).

36 The protective effects of cereal fibres depend on their solubility: soluble fibre,
37 particular β -glucans, can reduce blood cholesterol, while insoluble fibres shorten the
38 transit time through the intestinal tract, decreasing the contact between carcinogens
39 and the epithelial cells in the colon (Fardet, Rock & Rémésy, 2008).

40 Whole wheat flour is richer in protein, phenolic acids and DF than commercial white
41 flour; thus, whole grain flour results in higher antioxidant activity than the refined flour
42 (Liyana-Pathirana & Shahidi, 2007), since bran fractions are removed in traditional
43 milling operations. Unfortunately, the outer layers of the wheat kernel are also the
44 most subjected portions into contamination by natural, such as mycotoxins,
45 principally deoxynivalenol (DON), or synthetic contaminants, such as heavy metals
46 cadmium (Cd) and lead (Pb), and pesticides (Cheli et al., 2010). Thus, whole flour

47 chances more than white flour to cross the limits established by law for both natural
48 and synthetic contaminants. Moreover, whole grain foods are not so attractive to
49 consumers, because the higher bran content in whole grain flour reduces the sensory
50 value of the end-use products: the high fibre content is the main cause of the
51 negative technological properties of whole grain bread, with a reduction in loaf
52 volume, an increase in crumb firmness and a dark color (Zhang & Moore, 1999).
53 Therefore, a grain fractionation technology is needed in order to separate efficiently
54 the negative and positive aspects. This will let to produce new flour mixes and
55 ingredients with technologically optimized functional and nutritional attributes.

56 The pearling (debranning) of wheat, before roller milling, is becoming increasingly
57 accepted by wheat millers as a means of improving milling performance, since it
58 sequentially removes the outer kernel bran layers through an abrasive scouring and
59 increases the efficiency of the milling process (Dexter & Wood, 1996). The average
60 concentrations of DON and heavy metals are more efficiently reduced by pearling
61 than by milling (Cheli et al., 2010). Nevertheless, this process, which involves the
62 external layer of kernels, is responsible for the loss of high nutritional content. A
63 previous study demonstrated that the phenolic content, which is closely highly
64 correlated to the total antioxidant activity (TAA), progressively decreases as the
65 pearling progresses through the aleurone layer into the inner parts of the kernel
66 (Liyana-Pathirana et al., 2006). In fact, the typical grain fraction removed by pearling
67 (before milling) contains more than 40% of the total phenolic content of the whole
68 kernel (Beta, Nam, Dexter & Sapirstein, 2005).

69 However, the degree of pearling could be efficiently modulated in order to separate
70 the external bran fractions, which are characterized by a high sanitary risk and
71 coarse fibre, from the cereal fractions with potential high health benefits. An

72 alternative strategy to the use of whole flour, in order to maximize health benefits of
73 wheat-based products, could be to enrich conventional flour with wheat bran
74 fractions, obtained from sequential pearling, as they are characterized by a higher
75 antioxidant activity and phytonutrient content, but lower risk from the contaminant
76 content (Hemery et al., 2007). For this purpose, it is necessary to evaluate not only
77 the distribution of the phytonutrients and TAA in wheat grain in more detail, but also
78 the content of the contaminants in different and deeper pearled fractions.

79 The aim of this study was to determine the wheat kernel pearled fractions, obtained
80 from progressive pearling, with the highest nutritional value, considering the free
81 phenolic acids (FPA), DF and β -glucan contents and TAA, and the lowest natural and
82 synthetic contaminant contents, in order to use them as functional food ingredients.

83

84 **2. Materials and methods**

85 **2.1 Wheat grain pearling**

86 Three commercial winter wheat varieties (*Triticum aestivum* L.), Bolero, Bologna and
87 Taylor, were collected from homogeneous lots of each cultivar, cultivated in the
88 2010-2011 growing season in Alessandria (44° 57' N, 8° 29' E; altitude of 121 m; in
89 a deep and acid loamy soil - Aquic Frugiudalf) and stored in vertical silos. All the
90 compared cvs were seeded after an autumn ploughing (30 cm) and disk harrowing to
91 prepare a proper seedbed. Planting was held in 12 cm wide rows at the end of
92 October at a seeding rate of 450 seeds m⁻². For Bologna and Taylor cvs, a total of
93 180 kg N ha⁻¹ was applied to wheat fields as granular ammonium nitrate fertilizer. On
94 the other hand, field cultivated with cv Bolero received a total of 140 kg N ha⁻¹. The
95 amount of ammonium nitrate was split equally between tillering and stem elongation
96 stages for each cv. None fungicide was applied at wheat heading to control Fusarium
97 Head Blight (FHB). Wheat fields were harvested in early-mid July with a combine-
98 harvester and kernels of each cv were stored separately. The wheat varieties were
99 characterized by hardness, color and technological qualities (Tab. 1), on the basis of
100 the ISQ method for quality classification of common wheat, proposed by Foca et al.
101 (2007). As far as FHB infection and DON contamination is concerned, Bologna cv is
102 classified as moderately resistant, while cv Bolero and Taylor are classified as
103 moderately susceptible (Mayerle, Pancaldi, Haidukowski, Pascale & Ravaglia, 2007).
104 Moreover, the environmental conditions from anthesis to harvest observed in the
105 growing area were slightly favorable to FHB and to DON contamination.

106 Six fractions of kernels from each variety were obtained through incremental
107 pearling, following the approach proposed by Beta et al. (2005). The pearling

108 consisted of consecutive passages of wheat and pearled wheat in an abrasive-type
109 grain testing mill (TM-05C model, Satake, Tokyo, Japan) at a constant speed of 55
110 Hz. The pearling process was monitored by time control. After each assay, the
111 laboratory pearler was thoroughly cleaned by means of dust aspiration and
112 compressed air, to minimize equipment contamination. Initially, a 500 g portion of
113 each unprocessed wheat was sub-sampled from a 5 kg sample, and the remaining
114 4.5 kg was pearled. Starting from unprocessed grain, kernels were initially pearled to
115 remove 5% of the original grain weight, and this resulted in a first fraction (0-5%).
116 The remaining kernels were then pearled to remove a second fraction of 5% (5-10%).
117 The pearling process was continued until a third, fourth and fifth fraction (designed
118 10-15%, 15-20%, 20-25%, respectively) plus a residual 75% of the kernel (25-
119 100%), were collected.

120 A total of seven samples were obtained from each variety: the whole unprocessed
121 wheat and the 0-5%, 5-10%, 10-15%, 15-20%, 20-25%, 25-100% fractions, obtained
122 through the pearling process. The whole wheat samples and the residual 75% of the
123 unprocessed kernels were milled using a laboratory centrifugal mill (ZM-100; Retsch,
124 Haan, Germany) with a 1 mm opening. Then, both the milled and pearled samples
125 (500 g) were ground to pass through a 0.5 mm screen and stored at -25°C before the
126 chemical analyses.

127

128 2.2. Chemicals

129 Total Dietary Fibre and Mixed-Linkage β -Glucan kits for enzymatic determinations
130 were supplied by Megazyme (Megazyme International Ireland Ltd, Wicklow, Ireland).
131 Methanol (HPLC grade) and formic acid (50%, LC-MS grade) were purchased from
132 Sigma-Aldrich (Milan, Italy). Water was obtained from Milli-Q instrument (Millipore

133 Corp., Bedford, MA, USA). Antibody-based immunoaffinity columns were supplied by
134 VICAM (Waters Corporation, Watertown, MA, USA). All the other chemicals and
135 solvents were of a reagent-grade level and were also purchased from Sigma–Aldrich
136 (Milan, Italy).

137

138 2.3 Chemical analyses

139 2.3.1. Proximate composition analysis

140 The moisture, protein, ash, total DF and β -glucan contents were determined on
141 ground whole kernels and their pearled fractions. The moisture content, determined
142 in order to express the results on a dry weight (dw) basis, was obtained using a
143 Sartorius MA30 thermo-balance (Sartorius AG, Goettingen, Germany). The total
144 nitrogen content and total protein content (conversion factor: 5.70) were obtained
145 according to the Kjeldahl method, using Kjeltex system I (Tecator, Sweden). The ash
146 content was determined in a muffle furnace according to the AOAC (1990)
147 procedure. The total dietary fibre was measured using the Megazyme total dietary
148 fibre analysis kit, according to the enzymatic-gravimetric method proposed by
149 Prosky, Asp, Schweizer, DeVries, and Furda (1988); the determination was
150 performed employing the Fibertec 1023 system (FOSS Italia S.p.A., Padova, Italy). β -
151 glucan determination was performed using the Megazyme mixed-linkage β -glucan
152 assay kit, according to the instructions provided by the producer.

153

154 2.3.2. Extraction of free phenolic acids (FPA)

155 Prior to the extraction of the FPA, samples were ground in a oscillatory mill (Mixer
156 Mill MM440, Retsch GmbH, Hann, Germany) and sieved to obtain fine flours

157 (particle size < 250 µm). Fifty milligrams of each sample were suspended in 1 mL of
158 a MeOH/H₂O 80:20 (v/v) mixture, vortexed for 10 seconds and then extracted in an
159 ultrasonic bath (Bransonic 1510, output 42 kHz, Branson Ultrasonics, USA) for 2
160 minutes. The extracts were centrifuged at 14000 rpm for 1 min (Microcentrifuge 5417
161 R, Eppendorf Italia, Milan, Italy) and pellets were extracted another two times,
162 according to the method described above. Supernatants were collected and used for
163 the chromatographic analyses. All the samples were extracted in triplicate.

164

165 **2.3.3. Determination of free phenolic acids by means of RP-HPLC/DAD**

166 Phenolic acid separation was performed using the Shimadzu LC-20 A Prominence
167 HPLC system (Shimadzu Italia, Milan, Italy) equipped with an LC-20AB pump
168 system, a SIL-20-A auto-injector, a CTO-20A column oven, and a SPD-M20A diode
169 array detector. The used column was an Ascentis RP-amide (150 x 2,1 mm i.d., with
170 a particle size of 3 µm, Supelco, Bellefonte, PA, USA) which was maintained at 27
171 °C. The mobile phase consisted of water/formic acid 0.1% (v/v) (eluent A) and
172 methanol/formic acid 0.1% (v/v) (eluent B), and the following elution programme was
173 used: isocratic 2.5% B (10 min), from 2.5% to 12% B (25 min), from 12% to 100% B
174 (31 min), from 100% to 2.5% B (2 min), isocratic 2.5% B (5 min), The total running
175 time was 73 min and was conducted at a constant flow-rate of 400 µL/min.
176 Chromatograms were recorded at two different wavelengths (280 and 330 nm).

177 The phenolic acids were tentatively identified through a comparison with the retention
178 times and UV-vis spectra of individual standard molecules (gallic acid, protocatechuic
179 acid, syringic acid, *p*-hydroxybenzoic acid, caffeic acid, chlorogenic acid, ellagic acid,
180 ferulic acid); the quantification was performed on the basis of calibration curves (6
181 different concentration levels; linearity range: 0.5 – 5.5 µg mL⁻¹) obtained using the

182 corresponding standards. The previously described phenolic extracts were directly
183 injected into the chromatographic system (injection volume: 15 μ L).

184

185 **2.3.4. Determination of the total antioxidant activity (TAA)**

186 The TAA was determined adapting the classical DPPH radical scavenging method
187 (Locatelli, Gindro, Travaglia, Coisson, Rinaldi & Arlorio, 2009) to the QUENCHER
188 approach (direct measurement of antioxidant activity on solid samples suggested by
189 Gökmen, Serpen and Fogliano, 2009). Exactly 10 milligrams of ground whole kernels
190 and pearled fractions (particle size < 250 μ m) were weighted, then 700 μ L of
191 methanol and 700 μ L of a DPPH^{*} methanolic solution 100 μ M were added. The
192 samples were vortex-mixed and the reaction was then carried out in the dark under
193 stirring at 20 °C and 1000 rpm (Thermomixer comfort, Eppendorf, Milan, Italy) for 25
194 min. The samples were promptly centrifuged for 1 min at 14000 rpm (Microcentrifuge
195 5417 R, Eppendorf Italia, Milan, Italy) and the absorbance at 515 nm was then
196 measured after exactly 30 min of reaction (on attainment of the steady state), using a
197 Kontron UVIKON 930 Spectrophotometer (Kontron Instruments, Milan, Italy). A
198 control solution (700 μ L of methanol and 700 μ L of DPPH^{*} methanolic solution 100
199 μ M) was tested under the same conditions, in order to calculate the DPPH^{*} inhibition
200 percentage of the samples. The final results were expressed as mmol of trolox
201 equivalents (TE) per kg of sample (dw) through a calibration curve (linearity range:
202 4–60 nM; $r^2 = 0.982$).

203

204 **2.3.5. DON contamination**

205 The DON content was analysed using a high performance liquid chromatography
206 (HPLC-MS-MS) method (range 20-1000 μ g kg^{-1}). Samples of 25 g each were

207 extracted with 100 mL of water in a blender at a high speed for 30 minutes; the entire
208 extract was then filtered and collected. Antibody-based immunoaffinity columns
209 (DON testTM WB Columns VICAM) were utilised for cleanup of the sample extracts.
210 Before the sample was loaded, the column was conditioned with 1 mL of deionized
211 water. 1 mL of the sample was loaded on the previously conditioned immunoaffinity
212 column at a rate of approximately 1-2 drops s⁻¹. The column was washed with 5 mL
213 of distilled water. DON was eluted from the column with 2 mL of methanol. DON was
214 quantified by the injection of 10 µL of diluted eluate into the HPLC-MS-MS system,
215 which consisted of a Varian 212-LC Chromatography Pump and a 310-MS TQ Mass
216 Spectrometer. The analytical column was a reverse Varian Polaris C18-A (100 x 2.00
217 mm, 3 µm) while the mobile phase was a mixture of methanol and water fed at a flow
218 rate of 0.2 mL min⁻¹.

219

220 **2.3.6. Heavy metal content**

221 The Cd and Pb analyses were performed according to the method of the Italian
222 Organization for Standardization (UNI EN 14083, 2003). Aliquots of the samples (500
223 mg on a dry-matter basis) were dissolved in 5 mL of concentrated nitric acid and 2
224 mL of 30% v/v hydrogen peroxide, then heated under reflux in a stoppered quartz
225 vessel placed in a microwave oven. The solution was diluted to 25 mL in a volumetric
226 flask with ultra-pure water. Samples were analysed by graphite furnace atomic-
227 absorption spectrometry (GFAAS; Analyst 700, Perkin Elmer Corporation, USA). All
228 the metal concentrations were determined by autosampler injection of the aqueous
229 solution into a graphite furnace.

230

231 2.4. Statistical analysis

232 All the analyses were performed in triplicate, with the exception of the heavy metal
233 content, performed in one replicate. The results are reported as the mean of the
234 three replicates; the coefficients of variation were < 10%. The analysis of variance
235 (One-way ANOVA) was applied for each variety to compare the protein, total DF, β -
236 glucan, FPA, ash and DON contents and the TAA in the whole grain and in the
237 different pearled fractions. The residual normal distribution was verified using the
238 Kolmogorov-Smirnov test, while variance homogeneity was verified using the Levene
239 test. Multiple comparison tests were performed according to the Student-Newman-
240 Keuls test on treatment means. The SPSS for Windows statistical package, Version
241 17.0 (SPSS Inc., Chicago) was used for the statistical analysis.

242

243 **3. Results**

244 **3.1. Bioactive compounds in the whole kernel**

245 The protein, ash, total fibres, β -glucan and FPA contents, and the TAA determined
246 for the grain whole kernels are reported in Table 1. Except for the TAA, ANOVA
247 showed significant differences ($P<0.05$) between the wheat varieties used in this
248 study. According to the ISQ quality classification, the grain protein content of Bolero
249 cultivar was significantly lower than that of the Bologna and Taylor cultivars,
250 respectively. The total DF was significantly higher in the Bolero and Taylor cvs than
251 in the Bologna one. Compared to the other two varieties, Bolero showed significantly
252 lower and higher contents of β -glucans and FPA, respectively.

253

254 **3.2. Bioactive compounds of the pearled fractions**

255 The bioactive compounds content in the fractions obtained from the sequential wheat
256 pearling is reported in Tables 2 and 3. ANOVA showed highly significant differences
257 ($P<0.001$) for the proteins, total fibre and β -glucan content, and for the TAA
258 determined in the different pearled fractions (Tab. 2).

259 The 10-15% fraction showed the highest protein content in all three varieties, while
260 the concentration significantly decreased towards both the internal and the external
261 layers. Only for the Bolero cultivar, there were no significant differences between the
262 5-10% and 10-15% fractions. The Bolero cv showed a significantly higher protein
263 concentration in the more external layers (0-5%) than endosperm (25-100%), while
264 no significant differences were observed between the 0-5% and 25-100% fractions of
265 the Bologna and Taylor cultivars.

266 The total DF was predominant in the outermost layers (0-5%) for all three varieties.
267 Each successive pearling passage significantly decreased the fibre content towards
268 the inner layer. The 5-10% fractions of Bolero, Bologna and Taylor cultivars showed
269 a 37%, 35%, and 33% reduction in DF content compared to the corresponding 0-5%
270 fractions, respectively. The fibre content of Bolero cultivar in the 10-15% and 15-20%
271 fractions was 3.2 and 2.7 times higher than that of endosperm (25-100% fraction),
272 respectively. On the other hand, on average, the 10-15% and 15-20% fractions of the
273 Bologna and Taylor cvs had a 4.3 and 3.4 times higher content of total fibre than the
274 25-100% fraction, respectively. The 5-10% fraction contained the highest β -glucan
275 concentration for all of the three varieties tested. In the next inner pearling passage
276 (fraction 10-15%), a significant reduction in the β -glucan contents by 12%, 22% and
277 12% was perceived for the Bolero, Bologna and Taylor cvs, respectively. The β -
278 glucan content significantly decreased from this fraction to the inner layers at each
279 subsequent pearling. Moreover, the outer fraction (0-5%) in all three varieties showed
280 a significantly lower concentration of β -glucans than the 10-15% fractions. Cv. Bolero
281 showed a significantly higher β -glucan content in the 15-20% fractions than in the 0-
282 5% one, while the outermost layer of the Bologna cv showed a significantly lower
283 concentration of these compounds. No significant differences were observed for the
284 Taylor cv between the 0-5 and 15-20% fractions.

285 The highest TAA for the Bologna and Taylor cvs, was found in the outermost 0-5%
286 fraction, then TAA decreased significantly after each progressive pearling towards
287 the inner layers. On average, for these varieties, TAA was 14%, 23%, 41%, 56% and
288 87% lower than the 0-5% fraction, for the 5-10%, 10-15%, 15-20%, 20-25% and 25-
289 100% fractions, respectively. The pearling passage between the 10-15% and 15-20%
290 fractions resulted in the first important loss of TAA. On the other hand, there were no

291 significant differences in TAA values between the 0-5% and 5-10% fractions of the
292 Bolero cv. Then, from the 5-10% fraction, TAA decreased significantly after each
293 progressive pearling towards the inner layers.

294 The content of some phenolic acids (ferulic, chlorogenic, *p*-hydroxybenzoic, syringic,
295 protocatechuic and caffeic acid) present in the pearled wheat fractions in their free
296 form is reported in table 3. Among the standard molecules employed for the
297 chromatographic analysis, ellagic acid was not clearly recognized in the samples,
298 while gallic acid was only identified in the 0-5% fraction (30, 26 and 48 $\mu\text{g kg}^{-1}$ for the
299 Bolero, Bologna and Taylor cvs, respectively). Ferulic acid was the predominant
300 phenolic acid, followed by chlorogenic and caffeic acids. *p*-Hydroxybenzoic, syringic
301 and protocatechuic acids were found in lower concentrations and were not detected
302 in all the pearled fractions.

303 ANOVA showed highly significant differences ($P < 0.001$) for all the free phenolic acids
304 detected in the different wheat fractions. The total FPA content for the three varieties
305 decreased from the outer fractions to the endosperm. The total FPA content for
306 Bologna cv significantly decreased at each successive pearling from the outer
307 fractions towards the inner layers. Each pearling fraction for Taylor cv showed a
308 significantly different total FPA concentration, with the exception of the 15-20% and
309 20-25% fractions. On the other hand, no significant differences were observed
310 between the 0-5% and 5-10%, or between the 15-20% and 20-25% fractions
311 obtained from Bolero cv. On average, the free ferulic acid and the total FPA were
312 27% and 30% lower in the 10-15% fraction than in the 0-5% one, respectively.

313

314 3.3. Ash, heavy metals and DON contamination of pearled fractions

315 The ash, heavy metal and DON contents of in the wheat fractions are reported in
316 Table 4. ANOVA showed highly significant differences ($P<0.001$) for the ash content
317 and the DON contamination.

318 The highest ash content for the Bolero and Taylor cvs was found in the 5-10%
319 fraction, followed by the 0-5% one, then ash decreased significantly after each
320 progressive pearling towards the inner layers. The ash concentration in the 0-5% and
321 5-10% fractions was not significantly different for the Bologna variety. The ash
322 content of the three varieties was on average 5.4, 5.6, 4.5, 3.5 and 2.9 times higher
323 in the 0-5%, 5-10%, 10-15%, 15-20% and 20-25% fractions compared to the
324 endosperm residue (25-100%), respectively.

325 Among the heavy metals, Pb was not found in any of the pearled wheat fractions,
326 while Cd concentration was only detected in the outermost layer (0-5%) of the
327 Bologna and Taylor cvs.

328 Kernels from the Bolero and Taylor varieties resulted to be contaminated by DON,
329 while the data for the Bologna cv were always below the detection limit. The highest
330 DON contamination in both varieties was found in the outermost fraction (0-5%), and
331 the DON content then decreased significantly after each progressive pearling
332 towards the inner layers. On average, the DON content decreased by 49, 19, 9, 5
333 and 4 times in the 0-5%, 5-10%, 10-15%, 15-20% and 20-25% fractions compared to
334 the endosperm residue (25-100%), respectively.

335

336 **4. Discussion**

337 The presented data have shown clearly how the concentration of bioactive
338 compounds is greater in the outer layers of wheat grain, but their distribution in each
339 pearled fraction is different considering the classes of nutrients.

340 Shetlar, Rankin, Luman and France (1947) reported that outer pericarp, the inner
341 pericarp, the testa and the aleurone layer, respectively represents 3.9, 0.9, 0.7, and
342 9.0% of the kernel weight. Therefore, according also to data reported by Bottega,
343 Caramanico, Lucisano, Mariotti, Franzetti and Pagani (2009); Jerkovic, Kriegel,
344 Brander, Atwell, Roberts and Willows (2010) and Singh and Singh (2010), pearling
345 up 5% level on average removed most of the outer pericarp, while at 5-10% and 10-
346 15% level the aleurone layers were removed.

347 The protein content is higher in the 10-15% fraction for all three varieties, confirming
348 data reported by Jerkovic et al. (2010), who found a much greater concentration and
349 diversity of protein functions in the microdissected intermediate layers (testa and
350 nucellar tissue), corresponding to this pearled fraction, than in the other bran layers.

351 Sequential pearling has shown that, in common wheat, the total DF decreased
352 progressively from the external to internal layers. This reduction was higher in the
353 hard varieties (cvs Bologna and Taylor) than soft one (cv Bolero). In common wheat
354 bran, the values for total DF was almost 4 times higher that of the whole grain (Sidhu,
355 Al-Hooti & Al-Saqer, 1999). Dexter and Wood (1996) reported that the pearling of
356 common wheat reduces the insoluble and soluble fibre content compared to
357 unprocessed grain by 57% and 30%, respectively. The outermost tissues are rich in
358 insoluble DF, while the aleurone layer in particular results in a high soluble DF
359 content (Parker, Ng & Waldron, 2005). Moreover, the fibre present in the most
360 external bran layers is relatively coarse, whereas the fibre near the aleurone layer is

361 finer (Noort, van Haaster, Hemery, Schols & Hamer, 2010).

362 The β -glucans, components of the soluble fibre, were higher in the middle fractions
363 (5-20%), and peaked in the 5-10% pearled fraction, while lower in the more external
364 layers (0-5%) had a lower β -glucan content. Several authors have reported that the
365 aleurone layer in common wheat contains higher levels of β -glucans than whole grain
366 (Hemery et al., 2007). A reduction of 12% in the β -glucan content of unprocessed
367 wheat grain has been observed after pearling processes (Dexter & Wood, 1996).
368 Barley is rich in β -glucans and the highest concentration of β -glucans was found in
369 the middle fractions, followed by bran, while the lowest content were found in the
370 flour (Sullivan, O'Flaherty, Brunton, Gee, Arendt & Gallagher, 2010).

371 The sequential removal of the external layers through pearling resulted in a decrease
372 in FPA with concurrent lower TAA values. The highest observed concentration of
373 FPA in the outer kernel fractions (0-15%) confirms the data reported by Beta et al.
374 (2005) on the concentration of the total phenol compounds in the wheat kernel.
375 Liyana-Pathirana et al. (2006) reported that total bran-rich fractions possess higher
376 total phenolic compounds and TAA than starch-rich fractions. Some authors have
377 shown that the aleurone layer is richer in antioxidant compounds than the other bran
378 tissues, mainly due to its high content of phenolic acids (Buri, von Reding & Gavin,
379 2004). This diversity in the phenolic composition between the bran layers probably
380 reflects differences in the biosynthetic and turnover mechanism, related to
381 arabinoxylan synthesis, as suggested by Parker et al. (2005). Liyana-Pathirana and
382 Shahidi (2006) reported that soft wheat has a higher total phenolic content and TAA
383 than hard wheat. In agreement with these results, in the present study it has been
384 found that Bolero, a soft variety, has a higher total FPA content than the Bologna
385 (medium hard) and Taylor (hard) cvs.

386 Fardet et al. (2008) reported that ferulic acid represents approximately 46-67% of the
387 total phenolic acids in wheat, and that it is found associated with polysaccharides,
388 mainly arabinoxylans, in aleurone cell walls. Our results show that ferulic acid
389 occurred in about 65% of the total FPA analyzed. Liyana-Pathirana and Shahidi
390 (2007) reported that although ferulic acid is dominant in cereal grains, caffeic acid
391 shows a higher antioxidant activity. This information explains the similar TAA levels
392 of the three varieties examined, although Bolero has a higher total FPA and ferulic
393 acid content, while Taylor, with a lower total FPA content, showed a 4-times higher
394 caffeic acid concentration. Moreover, many insoluble components of foods may exert
395 antioxidant properties and esterified phenolic acids in particular can contribute to the
396 total antioxidant activity of cereals (Serpen, Gökmen, Pellegrini & Fogliano, 2008).
397 The direct procedure used in this work to determine TAA allowed the contribution of
398 both the soluble (e.g. free phenolic acids) and insoluble (e.g. bound phenolic acids)
399 antioxidants to be measured. Moreover, the use of an aqueous methanolic solution
400 (methanol/water 50:50, v/v) to perform the DPPH[•] method (so adding to the solid
401 samples 700 μ L of water and 700 μ L of DPPH[•] methanolic solution; see Materials
402 and methods section) allowed to obtain a TAA about 20% greater than that obtained
403 by the method using pure methanol as solvent. These additional results (data not
404 shown) confirm that the use of water as one of the solvents in the mixture helps
405 radicals to diffuse better into the particles of the sample, thus increasing the
406 interaction between the radicals and the antioxidants (Gökmen et al., 2009). Even if
407 an increase of TAA was observed employing aqueous methanolic solutions, the
408 relative antioxidant activity of the samples confirmed results obtained using pure
409 methanol, thus indicating a general TAA decrease during the progressive pearling
410 process.

411 The outermost kernel layers had the highest DON contamination which decreased
412 from the external to the internal layers. According to Lancova et al. (2008), the levels
413 of DON in bran can be two or more times higher than in whole wheat kernels,
414 indicating the concentration of this mycotoxin in the outer part of the kernel. In both
415 laboratory studies and industrial milling systems, the application of pearling before
416 milling has led to flours with lower DON contents (Cheli et al., 2010). Our data
417 confirm that DON decreases moving from the external to the internal layers following
418 a biphasic behavior: a high reduction was observed in the first pearling steps and this
419 was followed by a slower decrease. The residual grain after pearling of the first two
420 outer fractions (0-5 and 5-10%) contained around 64% of the total DON quantity of
421 the whole grains tested in this study. In previous experiment the DON contamination
422 was reduced after a 10% grain mass loss in durum wheat of 15% (Cheli et al., 2010)
423 and 45% (Rios, Pinson-Gadais, Abecassis, Zakhia-Rozis & Lullien-Pellerin, 2009).
424 For barley, a grain mass loss of 15%, reduced the DON contamination of 34%
425 (House, Nyachoti & Abramson, 2003) and in common wheat, with a 18% of mass
426 removal, DON contamination was reduced of 42% (Trenholm, Charmley, Prelusky &
427 Warner, 1991).

428 Moreover, heavy metals were only found in the most external fraction. Milling
429 reduced heavy metal contents in flour or semolina and increased their contents in the
430 by-products, derived principally from the pericarp layers (Oliver, Gore, Moss & Tiller,
431 1993). In the study of Cubadda, Raggi, Zanasi and Carcea (2003) the milling of
432 durum wheat determined an average reduction of 31% and 12% for Cd and Pb,
433 respectively. Cheli et al. (2010) reported that although no significant differences were
434 found between unprocessed wheat and pearled wheat, either in conventional milling
435 or in pearling before milling, Cd and Pb were concentrated in shorts and flour shorts.

436 The external pearled wheat fractions also resulted in the highest ash content. House
437 et al. (2003) and Rehman, Ahmand, Bhatti, Shafique, Ud Din and Murtaza (2006)
438 reported a 29% and 19% lower ash content after removal of 15% of the mass grain of
439 barley and wheat, respectively. Dexter and Wood (1996) reported that the pearling
440 process on durum wheat significantly reduced the ash content by 36% and 16% in
441 pearled kernels and semolina, respectively.

442 In addition, Laca, Mousia, Diaz, Webb, and Pandiella (2006) and Bottega et al.
443 (2009) established that the number of bacteria and moulds present in wheat grains,
444 and located in the outer pericarp, can be conspicuously reduced by pearling.

445 The TAA, the β -glucan, the total FPA and the DON contents (mean values of the
446 three wheat cultivars) are summarised in Fig. 1, with the objective of showing the
447 kernel fractions that offer the best compromise between high nutritional value and
448 low contamination risk. The 10-15% pearled fraction has shown to greatly reduce the
449 DON content, compared to the outer fractions, and has not been contaminated by
450 heavy metals. At the same time, this pearled fraction preserved an acceptable high
451 nutritional content, since it maintained high protein and β -glucan concentrations, and
452 the loss of total FPA content and TAA was not so high compared to the richer
453 external layers. Moreover, the total DF in this pearled fraction remained high, while
454 only the coarse fibre was removed with the outermost bran layer (Esposito, Arlotti,
455 Bonifati, Napolitano, Vitale & Fogliano, 2005). This could constitute an important
456 technological aspect, since bread containing coarse particle size bran is considered
457 less acceptable in sensory quality than breads containing medium-fine particle size
458 bran (Zhang & Moore, 1999). As far as the major chemical components of wheat
459 kernel is concerned, Bottega et al. (2009) and Singh and Singh (2010) reported that
460 pearling level lower than 10% guaranteed low starch and protein losses in the waste

461 and, at the same time, noticeably reduced the detrimental components of kernel
462 (ash, microbial contamination).

463 On the basis of the data collected, it is possible to state that the sequential pearling
464 of wheat kernels confirms to be an interesting dry-fraction technology, which can
465 produce bran fractions with high concentration in aleurone and intermediate material
466 rich in phytochemicals. This material can be used to transform a flour by-product into
467 a high nutritional value food ingredient (Hemery et al., 2007). The present study can
468 be considered as a first contribution towards individuating the most useful pearled
469 grain fractions for this purpose, although more research, also using the measurement
470 of biochemical markers found in wheat grain tissue (Hemery et al., 2009) is still
471 necessary.

472

473 **5. Conclusion**

474 Our study has confirmed the results of other previous reports, that investigated the
475 content of various contaminants and bioactive compounds in pearled wheat fractions
476 separately. Among the wheat varieties and the pearled fractions compared in this
477 experiment, the kernel fraction that offers the best compromise between high
478 nutritional value and low contamination risk is the 10-15% fraction. Furthermore, in
479 grain lots with a low contaminant presence in the outer part of the grains, the 5-10%
480 fraction could also be separated and recovered. On the basis of these results, the
481 pearling process could be an important way of valorizing the wheat bran layers of
482 kernel, as a natural source of bioactive compounds, separated from detrimental
483 components, in order to develop nutritionally enhanced ingredients and products. In
484 fact, the wheat bran layers, instead of being totally rejected or maintained as
485 happens in the traditional milling process for refined or whole flour, respectively,
486 could be progressively and carefully separated, through the pearling process. The
487 most external fractions, with higher risks because of the presence of natural and
488 synthetic contaminants, could then be discarded, while the fractions with a low
489 sanitary impact, but high nutritional value, could be reinserted into the flour or used
490 as a functional ingredient.

491 The replacement of wheat flour with the selected pearling fraction could be an
492 important way of enriching wheat-based products in bioactive compounds and of
493 reducing the sanitary risks associated to the use of bran layers.

494

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502

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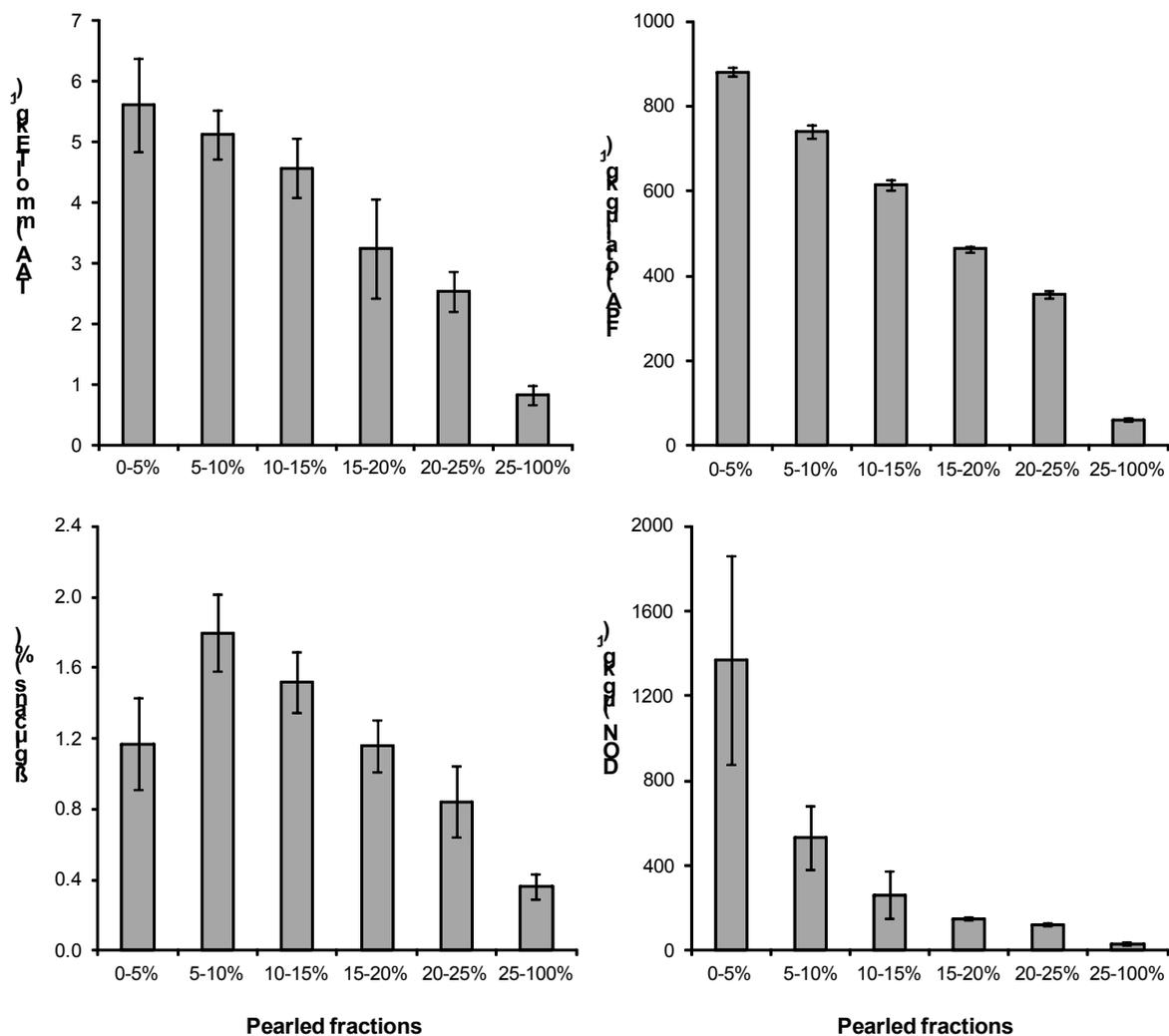
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616

617 **Figures**

618 **Fig. 1.**

619 TAA and FPA, β -glucan and DON contents in pearled wheat fractions. The reported
620 data are mean values of the three wheat varieties.



621
622

623 The error bars indicate the standard deviation between the wheat varieties.

624

625

627 **Tables**

628 **Tab. 1.**

629 Technological characteristics and protein, ash, DF, β -glucan and FPA^a contents and TAA levels of wheat varieties.

Variety	hardness	ISQ ^b	Colour	Proteins (%)	Ashes (%)	DF (%)	β -glucans (%)	FPA (mg kg ⁻¹)	TAA (mmol TE kg ⁻¹)
Bolero	soft	wheat for biscuits	white	12.6 c	1.8 ab	11.9 a	0.5 b	17.4 a	1.3 a
Bologna	medium-hard	superior breadmaking wheat	red	13.7 b	1.6 b	10.2 b	0.7 a	14.8 b	1.3 a
Taylor	hard	improver wheat	red	14.7 a	1.9 a	12.0 a	0.6 a	13.9 b	1.5 a
<i>P</i> (F)				< 0.001	0.013	0.015	0.038	0.003	0.126
sem ^c				0.09	0.05	0.12	0.03	0.43	0.05

630

631 Results are expressed on a dw basis for whole kernel. Means followed by different letters are significantly different (the level of significance is shown in the
632 table).

633 ^a total: sum of FPA determined by means of RP-HPLC/DAD

634 ^b Foca et al., 2007

635 ^c sem: standard error of mean

636

637

638

639

640

641 **Tab. 2.**

642 Protein, DF and β -glucan contents and TAA in pearled wheat fractions.

Variety	Pearling fractions	Proteins (%)	DF (%)	β -glucans (%)	TAA (mmol TE kg ⁻¹)
Bolero	0-5%	13.5 d	58.0 a	0.8 d	4.8 a
	5-10%	20.0 a	36.4 b	1.5 a	5.1 a
	10-15%	20.5 a	25.8 c	1.4 b	4.4 b
	15-20%	19.4 b	21.7 d	1.1 c	2.6 c
	20-25%	17.3 c	15.3 e	0.6 e	2.3 c
	Residue 25-100%	10.6 e	8.1 f	0.4 f	0.9 d
	<i>P</i> (F) sem ^a	< 0.001 0.23	< 0.001 0.45	< 0.001 0.08	< 0.001 0.19
Bologna	0-5%	11.7 d	58.3 a	1.4 c	5.5 a
	5-10%	17.4 c	37.8 b	1.9 a	4.7 b
	10-15%	19.6 a	24.4 c	1.5 b	4.2 c
	15-20%	18.2 b	19.7 d	1.1 d	2.8 d
	20-25%	17.5 c	12.9 e	0.9 e	2.4 e
	Residue 25-100%	12.0 d	5.6 f	0.3 f	0.7 f
	<i>P</i> (F) sem ^a	< 0.001 0.21	< 0.001 0.85	< 0.001 0.06	< 0.001 0.12
Taylor	0-5%	13.7 e	61.5 a	1.3 c	6.6 a
	5-10%	21.1 c	40.9 b	1.9 a	5.6 b
	10-15%	22.7 a	30.4 c	1.7 b	5.1 c
	15-20%	22.2 b	23.3 d	1.3 c	4.3 d
	20-25%	21.8 d	20.2 e	1.0 d	2.9 e
	Residue 25-100%	13.5 e	7.1 f	0.4 e	0.9 f
	<i>P</i> (F) sem ^a	< 0.001 0.14	< 0.001 0.53	< 0.001 0.05	< 0.001 0.14

643

644 Results are expressed on a dw basis. Means followed by different letters are significantly different (the level of significance is shown in the table).

645 ^a sem: standard error of mean

646

Tab. 3. Free phenolic acid^a content in pearled wheat fractions.

Variety	Pearling fractions	total ($\mu\text{g kg}^{-1}$)	ferulic ($\mu\text{g kg}^{-1}$)	chlorogenic ($\mu\text{g kg}^{-1}$)	caffeic ($\mu\text{g kg}^{-1}$)	syringic ($\mu\text{g kg}^{-1}$)	<i>p</i> -hydroxybenzoic ($\mu\text{g kg}^{-1}$)	protocatechuic ($\mu\text{g kg}^{-1}$)
Bolero	0-5%	959 a	620 a	95 c	60 b	77 a	65 a	12 a
	5-10%	876 a	517 b	188 a	78 a	57 b	29 b	6 b
	10-15%	718 b	435 b	160 b	67 b	38 c	14 c	4 c
	15-20%	483 c	313 c	105 c	36 c	29 d	nd d	nd d
	20-25%	396 c	260 c	82 d	32 c	23 e	nd d	nd d
	Residue 25-100%	94 d	53 d	29 e	4 d	8 f	nd d	nd d
	<i>P</i> (F)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	sem ^b	156.0	39.4	5.5	3.8	3.1	1.8	0.6
Bologna	0-5%	788 a	538 a	71 b	67 a	43 a	25 a	18 a
	5-10%	572 b	403 b	80 a	36 b	27 b	11 b	14 b
	10-15%	500 c	394 b	46 c	22 c	24 b	nd c	13 c
	15-20%	395 d	326 b	32 d	15 d	18 c	nd c	5 d
	20-25%	261 e	215 c	22 e	8 e	13 d	nd c	3 e
	Residue 25-100%	23 f	23 d	nd	nd f	nd e	nd c	nd f
	<i>P</i> (F)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	sem ^b	29.1	29.9	3.1	1.5	1.8	0.5	0.5
Taylor	0-5%	999 a	464 a	57 b	211 a	146 a	67 a	6 a
	5-10%	773 b	411 ab	75 a	171 b	85 b	26 b	4 b
	10-15%	626 c	345 bc	73 a	124 c	57 c	24 bc	3 cd
	15-20%	511 d	289 cd	60 b	88 d	49 c	21 c	4 bc
	20-25%	416 d	234 d	52 b	66 e	41 c	20 c	3 d
	Residue 25-100%	68 e	43 e	13 c	5 f	7 d	nd d	nd e
	<i>P</i> (F)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
	sem ^b	46.2	32.1	3.8	10.2	6.4	1.7	0.4

647

648

Results are expressed on a dw basis. Means followed by different letters are significantly different (the level of significance is shown in the table).

649 ^a total: sum of FPA determined by RP-HPLC/DAD. nd: not detected

650 ^b sem: standard error of mean

651

652 **Tab. 4.**

653 Ash, DON and Cd contents in pearled wheat fractions.

Variety	Pearling fractions	Ashes (%)	Cd (mg kg ⁻¹)	DON (µg kg ⁻¹)
Bolero	0-5%	4.5 b	nd	1789 a
	5-10%	4.7 a	nd	636 b
	10-15%	4.0 c	nd	341 c
	15-20%	3.0 d	nd	157 d
	20-25%	2.5 e	nd	128 d
	Residue 25-100%	1.1 f	nd	28 e
	<i>P</i> (F)	< 0.001		< 0.001
	sem ^a	0.06		32.6
Bologna	0-5%	5.7 a	0.05	nd
	5-10%	5.6 a	nd	nd
	10-15%	4.4 b	nd	nd
	15-20%	3.3 c	nd	nd
	20-25%	2.5 d	nd	nd
	Residue 25-100%	0.8 e	nd	nd
	<i>P</i> (F)	< 0.001		
	sem ^a	0.06		
Taylor	0-5%	5.5 b	0.07	949 a
	5-10%	6.0 a	nd	426 b
	10-15%	5.0 c	nd	185 c
	15-20%	4.1 d	nd	147 cd
	20-25%	3.7 e	nd	118 d
	Residue 25-100%	1.2 f	nd	36 e
	<i>P</i> (F)	< 0.001		< 0.001
	sem ^a	0.05		20.8

654

655 Results are expressed on a dw basis. Means followed by different letters are significantly
 656 different (the level of significance is shown in the table).

657 nd: not detected. The quantification limit was 20 µg kg⁻¹ for DON and 0.05 mg kg⁻¹ for Cd.

658 ^a sem: standard error of mean

659

660