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Privat P. Lasme, Francois-Xavier F.-X. Oury, Christophe C. Michelet, Joel Abecassis, Frédéric Mabile, et al.. A study of puroindoline b gene involvement in the milling behavior of hard-type common wheats. Cereal Chemistry, 2012, 89 (1), pp.44-51. 10.1094/CCHEM-08-11-0096 . hal-00964214

HAL Id: hal-00964214

<https://hal.science/hal-00964214>

Submitted on 30 Jul 2024

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1 **A study of puroindoline b involvement in the milling behavior of hard type**
2 **common wheats**

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21
22
23 List of Abbreviations

24 cv., cultivar

25 DArT, Diversity Array Technology

26 EBA, Endosperm Breakage Ability

27 HI, Hardness Index

28 FAR, Farina

29 NIL, Near-Isogenic Line

30 NIRS, Near Infrared Spectroscopy

31	PSI, Particle Size Index
32	SKCS, Single Kernel Characterization System
33	TFY, Total Flour Yield
34	TW, Test Weight
35	TKW, Thousand Kernel Weight
36	
37	Keywords
38	Hardness, near-isogenic lines, vitreousness, endosperm reduction, environmental conditions
39	

40 **ABSTRACT**

41

42 Differences in the milling behavior among hard type common wheat (*T. aestivum*) cultivars
43 are well known by the millers. Among them, the French Soissons cultivar which contains the
44 *Pinb-D1d* allelic form of puroindoline b gene is particularly distinguished for its high milling
45 value. Near-isogenic lines differing by the allelic forms of puroindoline b gene, *Pinb-D1d* or
46 *Pinb-D1b* (one of the most frequent alleles found in the European wheat population), were
47 constructed. Grain characteristics obtained after wheat cultivation in distinct environmental
48 conditions were compared between near-isogenic lines and Soissons, as well as their
49 fractionation behavior. Results showed that near-isogenic lines containing the *Pinb-D1d* allele
50 displayed lower values of grain hardness and vitreousness than the corresponding lines,
51 containing the *Pinb-D1b* allelic form, in the same cultivation conditions. Both the genetic
52 background and the environmental conditions appeared to affect the grain texture. Measured
53 SKCS hardness index values of the samples under study were found to be correlated with the
54 vitreousness values. Studies of the milling behavior helped to point out that grain vitreousness
55 is an important factor acting on endosperm breakage ability whatever the genetic background
56 of the wheat. Our results also demonstrated that, at similar levels of vitreousness, the
57 endosperm of Soissons could be more easily reduced than other wheat lines.

58

59

60 **Introduction**

61 Hardness and vitreousness are both characteristics of the endosperm from common wheat
62 grains that are recognized to affect their mechanical properties (Haddad et al 1999, 2001) and
63 thus play a role in the grain milling behavior (Greffeuille et al 2006, 2007). Indeed,
64 mechanical properties of the endosperm are known to affect the flour particle size
65 distribution, the degree of starch damage and the water absorption capacity of flours.

66 A number of methods have been developed in order to evaluate the mechanical resistance of
67 the endosperm (Turnbull and Rahman 2002). These methods are either based on the size of
68 the particles after grinding (*i.e.* particle size index (PSI)) or more related to the required
69 crushing force to break the grains as determined with Single Kernel Characterization System
70 (SKCS) developed by Martin et al (1993). PSI is a direct measurement of the percentage of
71 particles under a specific size (75 μm) which can be generated after grain grinding and is used
72 to classify wheats in the two classes soft or hard depending on the obtained PSI values
73 (Williams and Sobering 1986). Near-infrared reflectance spectroscopy (NIRS) is often used
74 instead of PSI (Saurer 1978) to classify wheats based on the distinct scattering at specific
75 wavelengths of ground grains which differ by hardness and is calibrated with grains
76 distinguished by PSI. However, SKCS allows to classify wheats according to the required
77 force for grain breaking and defines four ranges based on the kernel texture characteristics
78 (Gaines et al 1996). A hardness index (HI) is calculated to express the average crushing
79 resistance of the grain sample.

80 The endosperm hardness is found to be mainly genetically controlled by the Hardness (Ha)
81 locus on the short arm of Chromosome 5D (Chantret et al 2005) and particularly linked to the
82 expression of wild-type or variant allelic forms of two genes encoding specific proteins called
83 puroindolines A and B (PinA and PinB) which are found to be located at the starch granule
84 and protein network interface (Feiz et al 2009). These proteins are mainly found in the starchy

85 endosperm (Wiley et al 2007), but some authors have also found one of them (PinB) in the
86 aleurone layer (Caparelli et al 2005; Dubreil et al 1998). However, Lesage et al (2011)
87 studying their expression during grain development confirmed their endosperm location.
88 Indeed expression of the wild alleles of these genes (*Pina-D1a*, *Pinb-D1a*) leads to a soft
89 mechanical behavior of the grain endosperm whereas absence or mutations in one of the
90 genes results in a hard phenotype (Giroux and Morris 1998; Lillemo and Morris 2000;
91 Beecher et al 2002; Wanjugi et al 2007). Several possible mutations in the puroindoline genes
92 have been identified and recently reviewed by Morris and Bhave (2008). But the most
93 frequent in Europe corresponds to a point mutation within the gene encoding PinB (*Pinb-*
94 *D1b*) resulting to a Gly to Ser substitution in position 46 of the corresponding protein
95 sequence (Huang and Röder 2005; Lillemo and Morris 2000). Nevertheless other QTL for
96 hardness have also been pointed out as reviewed in Turnbull and Rahman (2002) and Bhave
97 and Morris (2008). It is also recognized that some variation of the hardness level and thus of
98 the milling performance could be due to environmental factors. Indeed temperature and light
99 intensity during grain filling and the rate of grain drying (Parish and Halse 1968) were
100 pointed out to affect other characteristics of the endosperm texture known as vitreousness.
101 Differences in vitreousness could be determined visually by the level of translucence or using
102 new developed methods, i.e. a machine vision (Symons et al 2003) or dual energy X-ray
103 imaging (Neethirajan et al 2007), as demonstrated with durum wheats.
104 Relationships between the Ha locus and vitreousness have been suggested previously (Nelson
105 et al 1995) but were not confirmed by other authors (Weightman et al 2008).
106 A number of mutations in puroindoline genes were found to affect the milling behavior as
107 well as the flour properties (Chen et al 2007; Greffeuille et al 2006; Hogg et al 2005; Ma et al
108 2009; Martin et al 2001), none of them examined the respective contribution of the genetic
109 versus the environmental factors on wheat grain fractionation behavior.

110 In this paper, near-isogenic lines (NILs) containing either the most frequent allele of
111 puroindoline b gene in cultivated French wheats, *Pinb-D1b*, (found for example in Caphorn
112 cultivar (cv.)), or containing the type d allele of puroindoline b gene, *Pinb-D1d*, (found in
113 Soissons cv. which is well known for its high flour producing level), were grown in different
114 environments in order to study the respective effect of puroindoline genotype, site and
115 nitrogen fertilization on the endosperm ability for size reduction in relation to the endosperm
116 texture.

117

118

119 **Materials and Methods**

120

121 **Wheat material and field conditions**

122 Three pairs of near-isogenic lines (NIL1 to NIL3) produced by UFS (Fr.) and differing by the
123 allelic forms (b or d) of *Pinb-D1* gene were used in this study. The near-isogenic lines (NILs)
124 were obtained from two different crosses and after selection of the two allelic forms at the
125 F6:F7 step (F7 siblings drawn from the same F6 parent plant). Genetic similarity within NILs
126 was confirmed using diversity array technology (DArT) markers (Akbari et al 2006)
127 generated by Triticarte Pty.Ltd (www.triticarte.com.au).

128 The wheat cultivar Soissons was used as a reference sample. All the genotypes were grown in
129 France in four different locations: Cappelle (50°29' N/3°10' E), Clermont-Ferrand (45°47'
130 N/3°8' E), Maule (48°54' N/1°51' E) and Orgeval (48°55' N/1°58' E) and harvested in
131 summer 2008. In each location, two types of nitrogen fertilization were used, the first one (N
132 condition) corresponded to intensive farming with nitrogen fertilization around 300 kg/ha
133 adjusted to high yield objectives (around 9t/ha depending on the location), and taking into
134 account the residual nitrogen remaining in soil at early spring. In the second one (N+
135 condition), an additional nitrogen dose (50 kg/ha) was added at flowering.

136 Grain from these different samples (48 samples from near-isogenic lines and 8 samples from
137 Soissons) were collected and cleaned to remove broken kernels or impurities and stored at
138 4°C before milling.

139

140 **Measurement of physical and biochemical characteristics of the grain**

141 Test weight (TW) and thousand-kernel weight (TKW) were measured according to the AACC
142 Method 55-10 (AACC 2000) and the method NF EN ISO 520 (Janv. 2011,

143 www.boutique.afnor.org), and expressed in kg/hl or in g respectively. NIRS Hardness was
144 evaluated using a Percon NIRS apparatus (AACC method 39-70A, AACC 2000)
145 Grain vitreousness was assessed by analysis of kernel cross sections obtained using a Pohl
146 grain cutter (Versuchs and Lehranstalt, Brauerei, Berlin, Germany). Depending on the
147 percentage of vitreous surface in the analyzed grains, they were classed into 5 groups (*i.e.*
148 grains which displayed less than 25 % of vitreousness were classed in the first group, those
149 with around 25 % of vitreousness constituted the second group, those with around 50%, 75 %
150 or 100 % of vitreousness were classed in the third, four and fifth group respectively). The
151 number of grains in each class (N1, number of grains in class 1 for example) was multiplied
152 by the corresponding factor of vitreousness in the considered class and the percentage of
153 vitreousness in the grain sample was calculated using the following equation.

154 Percentage of vitreousness = $(N1*0.00 + N2*0.25 + N3*0.50 + N4*0.75 +$
155 $N5*1)/(N1+N2+N3+N4+N5)$, where the sum of the analyzed grains in each class, N1 to N5,
156 was around 500.

157 A Perten single-kernel characterization system (SKCS) 4100 (Perten Instruments North
158 America INC, Springfield, IL) was used to collect data on 100 kernels and to calculate a mean
159 hardness index (HI) using AACC method 55-31 (AACC 2000). This method also allows to
160 determine grain weight which was found to be highly correlated with TKW measured using
161 the ISO method ($r= 0.84$).

162 Grain moisture and grain protein content were determined according to AACC methods 44-16
163 and 39-35, respectively and expressed in percentage (AACC 2000).

164

165 **Milling behavior characterization**

166 Five hundred grams of cleaned grain was conditioned to 16 % moisture and milled after 24 h
167 at room temperature using a patented prototype (French Patent Chopin, INRA, ARVALIS, N°

168 0905572, 02/20/2009) developed in order to evaluate the wheat milling value. This milling
169 device is equipped with two break rolls, one sizing and one reduction roll. This milling
170 process thus allows the production of two break flours, one sizing and one reduction (in two
171 steps) flours as well as a coarse bran, a fine bran and a short fraction. Sieves of 200 μm mesh
172 were used to isolate the break and sizing flours whereas a 160 μm mesh was used to isolate
173 the reduction flour. Each collected fraction was weighed at each step and expressed either as a
174 percentage of the total grain weight or percentage of the charged product weight at each step.
175 Incomplete milling was also undertaken to analyze more specifically behavior of the inner
176 endosperm part. In this case, coarse endosperm particles, also named farina as defined in
177 International Association of Cereal Science and Technology ICC dictionary
178 (<http://old.icc.or.at/cgi-bin/dic/anetdic.cgi?lang=fr>) obtained after the first break rolls (FAR1)
179 were recovered and directly reduced at the sizing step, skipping the second break roll. A mass
180 ratio between flour produced at the sizing step from coarse endosperm particles obtained at
181 the first break roll (FAR1 in incomplete milling) and FAR1 was determined (= EBA1) to
182 more specifically study the endosperm behavior in size reduction. Similarly the ratio between
183 sizing flour produced during milling from coarse endosperm particles obtained after the two
184 breaking steps (FAR2) and FAR2 was determined to study the endosperm ability to break
185 (=EBA2).

186

187 **Particle size distribution analysis**

188 Particle size distribution of farina or resulting flour was analyzed with a laser beam particle
189 size analyzer (Coulter Co, Miami, FL) using ethanol as a solvent and the sonication mode.
190 Distribution was expressed as the percentage in volume according to the particle size.

191

192 **Statistical analyses**

193 Analysis of variance, comparisons of means using Tukey's test (confidence interval 90%) and
194 simple Pearson's correlations were performed using XLStat software (2008 version 7.03,
195 Addinsoft, Paris, Fr.).

196

197 **Results and discussion**

198

199 **NILs construction and grain characterization**

200 In order to test the impact of two of the most frequent alleles (*b* and *d*) of *Pinb-D1* found in
201 French cultivated wheats and located on chromosome 5DS, near-isogenic lines were produced
202 from two different crosses. Three pairs of lines (NIL1 to NIL3) were retained and analyzed
203 for genetic similarity using diversity array technology (DArT) markers. As expected,
204 similarity appeared higher between NIL2 and NIL3 which were derived from the same cross
205 (14.1% differences revealed using the DArT markers), whereas NIL1, coming from a
206 different cross appeared more distant (26.1 and 27.3% differences with NIL2 and NIL3,
207 respectively according to DArT markers). Within NIL1, NIL2 and NIL3, differences between
208 the 2 genotypes, which either present the *Pinb-D1b* allelic form where Gly46 is replaced by
209 Ser (Lillemo and Morris 2000) or the *Pinb-D1d* allelic form where Trp44 is replaced by Arg
210 (Day et al 2006), were equal to 1.4, 0.7 and 0.9% , respectively, according to DArT markers,
211 and thus lower than the theoretical level (=3.12 %) of residual heterozygosity at this selection
212 step.

213

214 **Table 1**

215

216 All of the samples were grown in four distinct locations and with two distinct levels of
217 nitrogen fertilization in order to increase the variability of conditions under which grains were
218 produced, thus increasing the probability of obtaining distinct grain texture. Mean values and
219 standard errors of main grain characteristics are summarized in Table 1 for each NIL and were
220 compared with cv. Soissons grown in similar conditions. TKW and TW mean values were
221 found to be around 45 g and 79.5 kg/hl for NIL1 and NIL2, respectively whereas NIL3

222 exhibited higher values for TKW (49.5 g) and slightly higher values (80 kg/hl) for TW. No
223 differences were observed taking into account both of the allelic forms. But very low
224 variability (less than 10%) was observed between samples for these two characteristics
225 (especially for TW), as also found for Soissons, which displays lower TKW but TW values
226 similar to those of NIL1 and NIL2.

227 However, NIRS and SKCS hardness, as well as vitreousness, were found to display high
228 variability. Therefore, the experimental plan appeared to be relevant to study the effect of
229 grain texture on wheat grain milling behavior. As expected due to the puroindoline genotype
230 of the considered samples, NIRS hardness values correspond to hard wheat type. Hardness
231 and vitreousness of NIL samples were generally found to display higher values compared to
232 Soissons samples grown in similar conditions. Furthermore, NIL1 appeared to display the
233 highest SKCS hardness as well as the highest level of vitreousness in comparison with the
234 other near-isogenic lines. Mean values for hardness and vitreousness appeared to be
235 significantly higher in genotypes displaying *Pinb-D1b* compared with those with *Pinb-D1d*
236 (except for NIL3), despite similar protein content in grains.

237 Thus, the effect on grain characteristics of the background genotype, or *Pinb-D1* allele (*b* or
238 *d*), as well as of the growth location and nitrogen level, were further analyzed (Table 2).

239

240 **Table 2**

241

242 TKW was found to be mainly affected by the genetic background whereas TW was mostly
243 affected by the growth location even if both genetics and the environment were found to act
244 on these grain characteristics. Interestingly NIRS hardness was found to depend mainly on
245 *Pinb-D1* allele and was also affected by the combination of location and nitrogen amount.
246 Indeed NIRS hardness was already known to depend on the *Pinb-D1* allele when lines

247 displaying a wild type form (*Pinb-D1a*), corresponding to soft phenotype, were compared to
248 lines containing a mutated form corresponding to hard phenotype (Morris and Massa 2003).
249 But, the effect of the *Pinb-D1* allele among the hard wheat types is unexpected as well as the
250 variability of its effect according to the growth conditions. In comparison with NIRS
251 hardness, the measured SKCS hardness index was found to be mainly influenced by the
252 growth location and genetic background. Similar influence of growth location and genetic
253 background was also observed for vitreousness. Therefore for these set of genotypes, the
254 SKCS hardness index seems to take into account the vitreousness differences between
255 samples better than NIRS hardness measurements. This could be due to the differences
256 between NIRS and SKCS measurements. Indeed SKCS HI value determines the required
257 force for grain breaking and helps to define four ranges based on the kernel texture
258 characteristics (Gaines et al 1996), which depend both on the genetic background and the
259 growth conditions, whereas NIRS hardness is more related to the size of the products obtained
260 after grinding. Therefore relationships between these grain characteristics appeared important
261 to analyze. Furthermore, protein content of the samples under study did not appear to be
262 correlated with the puroindoline allele but to depend on the NIL type. As expected, protein
263 content was also found to depend on both the growth location and the nitrogen level. Indeed,
264 Daniel and Triboi (2000) have already demonstrated the effect on nitrogen addition and
265 temperature conditions on grain protein content.

266 The relationships between grain characteristics were therefore studied using Pearson's test.
267 The highest correlation between the analyzed grain characteristics was found between SKCS
268 hardness index and vitreousness ($r = 0.92$), as well as with the protein content ($r = 0.84$),
269 whereas NIRS hardness was significantly correlated with these grain parameters but at a
270 lower level (r around 0.4). Morris and Massa (2003) also compared different texture
271 measurements (i.e. NIR hardness, SKCS and PSI) and already pointed out the lower

272 relationship between NIR hardness determination and SKCS HI within a hardness class in
273 comparison with the relationship established when cultivars from both hard and soft classes
274 were taken into account. As SKCS measurement is more related to compression tests and
275 produces a four-class frequency distribution of hardness data for each sample, this hardness
276 index value more clearly reflect the intrinsic sample variability as already pointed out by
277 Morris et al (2008) similarly to the vitreousness measurement. The narrower genetic
278 background of the studied population could also explain these correlations. However
279 relationships between SKCS HI and vitreousness were already reported in common wheat
280 ($r=0.7$; Orucevic et al 2008) or durum wheat ($R^2 = 0.99$; Sissons et al 1999). In barley,
281 relationships between SKCS and light transfectance measurement as well as visual
282 assessment of mealiness were also pointed out (Gamlath et al 2005 ; Psota et al 2008). TW
283 was also found to be significantly and positively correlated with vitreousness and hardness
284 either measured with NIRS or SKCS, as well as with the protein content. By contrast, TKW
285 was negatively correlated with vitreousness and SKCS HI and protein content. Indeed an
286 increase in the protein level appears to be correlated with an increase of vitreousness which
287 affects the grain density, as already reported in durum wheat (Samson et al 2005), but
288 probably occurs inversely to the grain size. Similar negative correlation between TKW and
289 SKCS hardness index was also pointed out by Gazza et al (2008) studying wheat grains
290 grown in distinct locations.

291

292 **Endosperm breakage ability (EBA) and grain milling behavior**

293 All of the grain samples were milled in order to study their behavior depending on the grain
294 nature and characteristics. The effect of the endosperm structure on size reduction by grinding
295 was particularly examined. Therefore, the main values measured were: percentage of coarse
296 endosperm particles, known as farina, produced at the first break roll (FAR1) or after two

297 breaking steps (FAR2), the ratio between flour produced from farina and corresponding farina
298 obtained either at the first break roll (EBA1) or after two break rolls (EBA2), and the total
299 flour yield (TFY). A variance analysis (Table 3) was performed in order to study the effect of
300 the genetic background, *Pinb-D1* allele, and environmental conditions on the retained
301 parameters.

302

303 **Table 3**

304

305 Production of farina as well as its size reduction was found to be mainly related to the wheat
306 growth location and genetic background. The effect of the *Pinb-D1* allele was also found to
307 be significant but at a lower level than the other parameters. Indeed endosperm from wheat
308 grains carrying the *Pin-b-D1d* allele showed a slightly higher ability to break compared with
309 grains carrying the *Pinb-D1b* allele. The effect of nitrogen addition in soil was also found to
310 be significantly related to the farina production and to the endosperm ability to produce flour
311 at the first break (EBA1). TFY was also related to the wheat growth location and the genetic
312 background. But in this case the genetic background appears as the predominant factor.

313 In order to better understand the grain milling behavior, relationships between the studied
314 parameters and the grain characteristics were analyzed (Table 4).

315

316 **Table 4**

317

318 Clear positive relationships between the ability of coarse endosperm particles to produce flour
319 at the sizing step and TFY were found. Indeed EBA1 could help to explain 50 % of the TFY
320 which is in accordance with previous studies (Greffeuille et al 2007). As expected however,
321 production of farina which is inversely proportional to the flour production at breaking stage

322 appears to be negatively correlated to the TFY. Analysis of relationships between milling
323 behavior and grain characteristics clearly pinpointed the influence of vitreousness on the
324 production and size reduction of farina. Indeed an increase of grain vitreousness appears to
325 increase the farina yield and negatively affects its breakage ability, leading therefore to flour
326 yield reduction. Therefore as location and genetic background were found as the main factors
327 contributing to vitreousness (Table 2), they were also found to be significantly related to the
328 size reduction ability of farina (Table 3). As SKCS hardness index and the protein content
329 were found to be highly related to vitreousness, it was not surprising that similar correlations
330 could be observed between the milling analyzed parameters and these grain characteristics.
331 Results also showed more significant correlations at the first breaking step where farina
332 originated mainly from the inner part of the endosperm (EBA1). If *Pinb-D1* allele was found
333 significantly related to the milling grain behavior as measured by the farina and flour yields
334 and the farina breakage ability, its influence appears less than the site of cultivation and the
335 genetic background. As this influence of puroindoline allele could simply be due to
336 differences in vitreousness (Tables 1 and 2) between grains carrying either *Pinb-D1b* or *Pinb-*
337 *D1d*, deeper analysis between EBA1 and vitreousness was carried out on the overall set of
338 samples and compared with the cv. Soissons (carrying *Pinb-D1d*) grown in the same
339 conditions (Fig. 1).

340

341 **Figure 1**

342

343 Results confirmed the existence of a negative linear relationship between the endosperm
344 ability for size reduction and grain vitreousness whatever the sample analyzed. It also pointed
345 out the major effect of the genetic background differences in comparison with the carried
346 puroindoline allele. A Fisher test enabling the pair-wise comparison of the linear regressions

347 (Tomassone et al. 1983) showed that they were significantly different ($p < 0.001$), except the
348 regressions for NIL2 and NIL3, which indeed derived from the same parental cross.
349 Furthermore, coarse endosperm particles (farina) from the first breaking step (FAR1) from cv.
350 Soissons grains were found to display a higher ability for size reduction (expressed by EBA1)
351 than the near-isogenic lines even if grains of similar vitreousness were compared. Similar
352 conclusions were also obtained if vitreousness values were replaced by the grain protein
353 content or SKCS hardness index (data not shown).

354

355 **Study of the particle size before and after farina reduction**

356 In order to further study the differences in farina ability for size reduction among the
357 examined wheat grain samples, size distribution profiles of farina from the first breaking step
358 (FAR1) were analyzed before and after sizing reduction. For this study, only samples grown
359 under a standard nitrogen amount on the sites leading to the most contrasted grain
360 vitreousness were retained. These locations were Cappelle, where samples displayed
361 vitreousness between 20 and 45%, and Maule where grain vitreousness was between 50 and
362 70%. Typical curves of particle size distribution from farina before and after reduction are
363 shown in Fig. 2.

364

365 **Figure 2**

366

367 Before reduction, size of the farina particles were between 300 and 2000 μm and centered
368 around 800 μm as illustrated in Fig. 2A with NIL1-*Pinb-D1b*. However comparison of the
369 mean size of farina particles clearly pointed out differences between the two locations (Table
370 5). Indeed, the location which was shown to lead to the most vitreous samples (Maule) was
371 found to correspond to the largest farina particles. Significant differences between mean

372 particle size of farina from the distinct near-isogenic lines was also observed, NIL3 displaying
373 the lowest mean particle size. No distinction between lines with the different alleles of *Pinb-*
374 *DI* was pointed out even if potential interactions between genetic background and *Pinb-DI*
375 was revealed using variance analysis ($p < 0.05$).

376

377 **Table 5**

378

379 After farina breakage, distribution of the particle size was enlarged and showed a complex
380 profile between 5 and 2000 μm (Fig. 2A). Particles from the most vitreous grains (grown in
381 Maule location) appeared less reduced compared to the other grain samples whatever the
382 genotype, however, main differences between curves were difficult to pinpoint. Therefore, a
383 mathematical model was further used in order to fit the data of particle size distribution and
384 determined their main characteristics for each sample. The experimental curve between
385 volume according to the size of the particles displayed four discrete peaks, the model was thus

386 considered as the sum of four Gaussian-like shaped functions, $F(x) = \sum_{i=1}^{i=4} F_i(x)$ where F_1, F_2

387 F_3, F_4 correspond to large, medium-size, small and finest particles, respectively. Each
388 function was expressed with the following equation:

389
$$F_i(x) = D_i \cdot \exp\left(-\frac{1}{2} \left(\frac{\log(x) - \log(\bar{m}_i)}{\log(\sigma_i)}\right)^2\right),$$

390 where D_i corresponds to the peak height, x to the particle size, \bar{m}_i to the mean particle size of
391 the sub-population *i.e.* the particle size at the peak of each Gaussian-like curve, σ_i the
392 deviation around the mean particle size. Values for D_i, \bar{m}_i , were first estimated from the
393 experimental curve and σ_i first fixed to 0.5. Then, these parameters were adjusted by
394 minimization of the differences between the area under the experimental data curve $\Phi(x)$ and
395 the mathematical model using the solver function of Excel (Microsoft Office 2007). The

396 following function, $\min \left[\sum_{x \in R} |F(x) - \Phi(x)| \right]$, where R was the set of observed particle size values,
 397 was used to minimize the differences between data and the model curve for large and
 398 medium-size particle peaks whereas $\min \left[\sum_{x \in R} |F(e_x) - \Phi(e_x)| \right]$ was used to minimize differences
 399 due to the small and the finest particles as it takes into account the number of the particles
 400 instead of the volume and was thus more adapted to these type of particles. This model was
 401 found to fit correctly the data curves as illustrated in Fig. 2B, and to help the determination of
 402 \bar{m} , σ and D for each type of particle. Indeed, root mean squared errors were found between
 403 0.09 and 0.14 for curves corresponding to particles of farina before breakage and between
 404 0.02 and 0.08 for curves corresponding to particles after farina breakage by the sizing step.
 405 Table 5 summarizes the results obtained for mean particle size of the farina particles in each
 406 peak obtained after farina breakage. It also reports the percentage of the total particle volume
 407 in each peak. Analyses of the volume of particles distributed in the four peaks deduced from
 408 the curve of each sample after farina size reduction revealed that around 20 to 28% of the
 409 material was not reduced with the equipment used. As no differences were observed between
 410 samples, this could correspond to the grinding limit of the reduction cylinder. Sixty to seventy
 411 percent of the volume of particles were recovered in the peak of medium-size particles after
 412 the sizing step. The other peaks, corresponding to small and finest particles, represented 6-8
 413 % and 1.5-3 %, respectively. Comparison of the mean size of particles in the major peak
 414 (medium-size particles) from distinct samples revealed a larger size of particles obtained from
 415 grains grown in the location (Maule) where grains displayed a higher vitreousness. Moreover,
 416 significant differences between NIL were also pointed out. Medium-size particles of NIL 2
 417 were found to display the lower mean value (182.5 μm) compared to NIL 3 (190.8 μm) and
 418 NIL1 (222.3 μm) which was in accordance with the greatest ability of NIL 2 to be reduced, as
 419 expressed by the endosperm breakage ability (EBA1) values (%) shown in Fig. 1. Indeed in

420 the prototype used for milling, separation of flour particles from farina occurred on a sieve of
421 200 μm mesh, therefore differences of the EBA1 values could be due to differences of the
422 mean medium-particle size obtained after size reduction of farina from the distinct NIL or
423 from samples grown at distinct locations. Thus, if differences in location leading to distinct
424 level of vitreousness mainly impact on endosperm breakage ability as measured by EBA1, the
425 effect of genetic background also appeared as an important factor to consider. Indeed
426 comparison of the mean medium-particle size values obtained from NILs with those obtained
427 after farina reduction from Soissons cv. clearly pointed out the lower particle size value from
428 this cultivar (mean size = 167.3) which is in accordance with the highest ability of the
429 endosperm from these grains to be reduced as measured by the ratio of flour to the farina
430 production (EBA1 in Fig. 1).

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433 **Conclusions**

434 The growing location was found to affect the grain vitreousness level and differences in
435 vitreousness were found depending on the *Pinb-D1* allele of the near-isogenic line (grains
436 from lines containing the *Pinb-D1d* allele were found to be less vitreous).

437 The increase in vitreousness of samples with a similar genetic background was found to
438 deeply affect the endosperm ability for size reduction and thus the efficiency of flour
439 production from farina. Therefore in order to select the sample displaying the best ability for
440 flour production among a set of grain samples from the same hard wheat cultivar, millers have
441 to choose the one with the lowest vitreous grains. The potential of SKCS hardness index
442 measurement to evaluate the wheat grain vitreousness within hard wheats has also been
443 highlighted. Furthermore, differences in endosperm breakage ability appeared to be mainly
444 due to the genetic background rather than to the *Pinb-D1* allele and the higher endosperm
445 ability for size reduction from Soissons grains was pointed out. The size of the medium-size
446 particles obtained from farina size reduction appears as a critical parameter to relate with this
447 better endosperm size reduction ability and higher flour yield.

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451 **Acknowledgments**

452

453 This work has received funding from the FSOV (Fund to support plant selection, Paris, Fr.)
454 and from a French research project between INRA (National Institute of Agronomic
455 Research, Paris) and UFS (French union of breeders, Paris, Fr.), ANMF (National Association
456 of French Flour-milling, Paris, Fr.), ARVALIS - Institut du végétal (Paris), IRTAC (Paris),
457 Kraft Foods (Saclay, Fr.), Chopin Technologies (Villeneuve la Garenne, Fr.), Bühler
458 (Villepinte, Roissy, Fr) and ENILIA-ENSMIC (Surgères, Fr.). P. Lasme has also been partly
459 financed by the French Research Minister.

460 We are also grateful to S. Alquié and N. Beaubras (INRA non permanent staff) for their
461 measurement of grain vitreousness, to G. Maraval and M. Chaurand (UMR IATE,
462 Montpellier) for their help in the management of the Chopin prototype and T. M. Lasserre
463 (UMR IATE, Montpellier) for her help in the particle size measurements. Additionally, we
464 would like to thank S. Destercke (UMR IATE, Montpellier) and T. Andon (one-person
465 business SIRET: 514 905 983 00011) for helpful reading and corrections.

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NILs/Cultivar		TKW (g)	TW (kg/hl)	NIRS hardness	SKCS- HI	Vitreousness (%)	Protein content (%)
NIL1							
<i>Pina-D1a/Pinb-D1b</i>	mean	45.3 a	79.9 ab	82.4 b	61.7 d	62.0 d	13.4 c
	SD	1.8	0.8	3.8	10.1	15.5	1.1
<i>Pina-D1a/Pinb-D1d</i>	mean	44.9 a	79.4 a	73.3 a	55.6 c	54.3 c	13.0 c
	SD	2.7	1.1	7.2	10.4	15.8	0.9
NIL2							
<i>Pina-D1a/Pinb-D1b</i>	mean	45.8 a	79.6 ab	80.3 ab	47.7 ab	44.4 b	11.6 a
	SD	2.3	1.1	7.7	7.4	16.1	0.7
<i>Pina-D1a/Pinb-D1d</i>	mean	45.4 a	79.4 a	73.1 a	45.4 a	40.6 a	11.4 a
	SD	3.0	0.7	10.9	10.0	16.8	1.0
NIL3							
<i>Pina-D1a/Pinb-D1b</i>	mean	49.9 b	80.4 b	83.8 b	49.9 b	53.3 c	12.1 b
	SD	2.3	1.1	9.8	12.6	20.5	1.3
<i>Pina-D1a/Pinb-D1d</i>	mean	49.3 b	80.1 ab	77.1 ab	50.0 b	51.5 c	12.1 b
	SD	3.9	1.1	11.8	12.2	20.5	1.5
Soissons							
<i>Pina-D1a/Pinb-D1d</i>	mean	42.9	79.3	64.9	41.4	42.1	12.4
	SD	2.9	1.3	6.0	9.1	14.4	1.2

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Table 1: Mean values and standard deviations (SD) of main grain characteristics from three pairs of near isogenic lines (NIL1 to NIL3) differing by *Pinb-D1* allele and the French cultivar *Soissons*. Each distinct sample was grown in four locations in 2008, with two levels of nitrogen fertilization. Each line corresponds therefore to eight distinct grain samples. Identical letters indicate homogeneous groups provided by Tukey's test. TKW = thousand-kernel weight; TW = test weight; NIRS = near-infrared reflectance spectroscopy; and SKCS HI = single-kernel characterization system hardness index.

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Studied factor	df	TKW	TW	NIRS hardness	SKCS-HI	Vitreousness	Protein content
Genetic background (NIL)	2	96.4***	2.7**	ns	623.1***	999.3***	12.0***
Pinb-D1 allele	1	ns	ns	697.7***	92.7**	233***	ns
Location	3	65.3***	9.1***	295.0***	1320.8***	3962.1***	10.4***
Nitrogen	1	ns	ns	275.6**	73.8**	413.7***	8.3***
NIL* Pinb	2	ns	ns	ns	38.0*	35.4**	ns
NIL* Location	6	8.1**	ns	ns	54.0***	77.0***	0.9***
Location* Nitrogen	3	ns	ns	334.4***	27.9*	60.5***	0.7**
Model fitting R ²		0.92	0.83	0.84	0.97	0.99	0.96

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Table 2: Effect of genotype, *Pinb-D1* allele (*b* or *d*), growth location and nitrogen level on grain characteristics of the three pairs of near-isogenic lines. *, **, * indicate significance at the 0.05, 0.01, 0.001 probability levels, respectively, ns nonsignificant effect. Values are mean squares. Only significant interactions are pointed out.**

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Studied factor	df	FAR1	FAR2	EBA1	EBA2	TFY
Genetic background (NIL)	2	8.4***	8.7***	211.6***	98.1***	7.2***
Pinb-D1 allele	1	2.4*	4.2*	35.9***	20.9*	0.9*
Location	3	42.7***	55.0***	229.8***	137.3***	3.2***
Nitrogen	1	20.5***	43.4***	9.1***	ns	ns
NIL*Pinb				2.8*	ns	0.7**
NIL* Location	2	1.8***	5.1*	4.0***	ns	0.5**
Pinb* Location	6	1.3*	ns	1.7*	ns	ns
NIL * Nitrogen	2	ns	3.4*	ns	ns	ns
Location* Nitrogen	3	2.8***	ns	ns	ns	ns
Model fitting R ²		0.96	0.92	0.99	0.90	0.92

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Table 3: Effect of genotype, *Pinb-D1* allele (*b* or *d*), growth location and nitrogen level on grain milling characteristics of the three near-isogenic lines. FAR1 = percentage of coarse endosperm particles (farina) obtained after the first break roll; FAR2 = percentage of farina obtained after two breaking steps; EBA1 = endosperm breakage ability (the ratio between flour produced from farina and the corresponding source farina) of FAR1; EBA2 = endosperm breakage ability of FAR2; TFY = total flour yield; NIL = near-isogenic line; df = degree of freedom; *, **, and *** indicate significance at the 0.05, 0.01, and 0.001 probability levels, respectively; and ns = nonsignificant effect. Values are mean squares. Only significant interactions are pointed out.

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Variables	FAR1	FAR2	EBA1	EBA2	TFY
TKW (g)	-0.33	-0.19	0.34	0.20	0.60
TW (kg/hl)	0.60	0.64	-0.55	-0.57	-0.03
NIRS hardness	0.50	0.47	-0.43	-0.41	-0.14
SKCS-HI	0.80	0.79	-0.92	-0.82	-0.73
Vitreousness (%)	0.90	0.86	-0.91	-0.79	-0.59
Protein content (%)	0.88	0.78	-0.85	-0.68	-0.63
FAR1 (%)					
FAR2 (%)	0.85				
EBA1	-0.79	-0.76			
EBA2	-0.61	-0.69	0.86		
TFY (%)	-0.49	-0.42	0.71	0.59	

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644 **Table 4: Relationships between grain characteristics and milling behaviour assessed**
645 **with Pearson's correlations.** Significant correlations with $P < 0.01$ are marked in bold.
646 Correlations are only presented once in the table. Reported values are r . FAR1 = percentage
647 of coarse endosperm particles (farina) obtained after the first break roll; FAR2 = percentage
648 of farina obtained after two breaking steps; EBA1 = endosperm breakage ability (the ratio
649 between flour produced from farina and the corresponding source farina) of FAR1; EBA2 =
650 endosperm breakage ability of FAR2; TFY = total flour yield; TKW = thousand-kernel
651 weight; TW = test weight; NIRS = near-infrared reflectance spectroscopy; and SKCS
652 HI = single-kernel characterization system hardness.

			FAR1 before reduction		Particles from FAR1 reduction after sizing rolls							
			Particle size (µm)	Particle volume (%)	Large particles		Medium- size particles		Small particles		Finest particles	
	Grain vitreousness				Particle size (µm)	Particle volume (%)	Particle size (µm)	Particle volume (%)	Particle size (µm)	Particle volume (%)	Particle size (µm)	Particle volume (%)
Genetic background												
NIL1 (n=4)	Mean	51.9 ^b	836.8 ^b	98.8 ^a	909.9 ^a	20.7 ^a	222.3 ^c	70.1 ^a	30.2 ^a	8.0 ^b	4.7 ^a	1.5 ^a
	SD	20.7	17.5	1.7	43.6	3.9	29.7	3.8	5.9	1.6	0.4	0.2
NIL2 (n=4)	Mean	43.9 ^a	859.9 ^c	97.8 ^a	942.6 ^a	23.1 ^a	182.5 ^a	69.3 ^a	26.2 ^a	6.0 ^a	5.2 ^a	2.3 ^b
	SD	22.4	16.9	1.6	13.4	3.3	37.7	2.8	5.4	1.3	0.3	0.5
NIL3 (n=4)	Mean	49.2 ^b	812.7 ^a	96.6 ^a	870.4 ^a	26.4 ^a	190.8 ^b	64.8 ^a	25.8 ^a	6.7 ^{ab}	4.8 ^a	2.1 ^{ab}
	SD	30.7	32.5	1.0	36.2	5.2	33.6	5.0	5.0	2.0	0.3	0.4
Soissons (n=2)	Mean	41.8	809.6	96.5	906.0	28.1	167.3	62.1	24.0	6.8	5.2	3.0
	SD	24.9	24.1	0.9	80.8	4.9	30.4	4.7	3.0	0.9	0.7	0.7
Location												
Cappelle (n=6) 20 % < v < 45 %	Mean	27.1 ^A	821.7 ^A	96.9 ^A	921.4 ^A	26.1 ^A	166.6 ^A	66.2 ^A	23.2 ^A	5.9 ^A	4.8 ^A	2.2 ^B
	SD	5.9	31.5	1.6	23.2	4.1	22.3	4.0	2.4	1.3	0.3	0.6
Maule (n=6) 50 % < v < 70 %	Mean	69.5 ^B	851.2 ^B	98.6 ^A	893.8 ^A	20.7 ^A	227.4 ^B	69.9 ^A	31.6 ^B	7.8 ^B	5.0 ^A	1.7 ^A
	SD	6.2	19.2	1.2	55.9	3.4	16.2	4.2	3.9	1.6	0.4	0.2

654 **Table 5: Mean values and standard deviations (SD) of particle size (µm) and percent volume of particles from farina (FAR1) before and**
655 **after reduction obtained after milling of the different grain samples (NIL1 to NIL3) differing by *Pinb-D1* allele and the French cultivar**
656 ***Soissons* grown in two distinct locations leading to contrasted vitreousness (v). Identical letters indicate homogeneous group provided by**
657 **Tukey's test applied separately for the two factors, NIL = near isogenic line.**

658 **Figure Captions**

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660 **Figure 1:** Correlation between grain vitreousness and endosperm size reduction ability at first
661 break (EBA1). Data are from Soissons which carries *Pinb-D1d* (*); NIL1 carrying either
662 *Pinb-D1b* (□) or *Pinb-D1d* (■); NIL2 carrying either *Pinb-D1b* (○), or *Pinb-D1d* (●); NIL3
663 carrying either *Pinb-D1b* (△), or *Pinb-D1d* (▲). Equations of regression lines for the
664 different genotypes are, respectively:

665 Soissons: $y = -0.20x + 49.4$; $r^2 = 0.90$

666 NIL1: *Pinb-D1b*: $y = -0.21x + 41.1$; $r^2 = 0.96$; *Pinb-D1d* : $y = -0.24x + 43.6$; $r^2 = 0.95$

667 NIL2: *Pinb-D1b*: $y = -0.19x + 44.6$; $r^2 = 0.83$; *Pinb-D1d* : $y = -0.26x + 47.7$; $r^2 = 0.76$

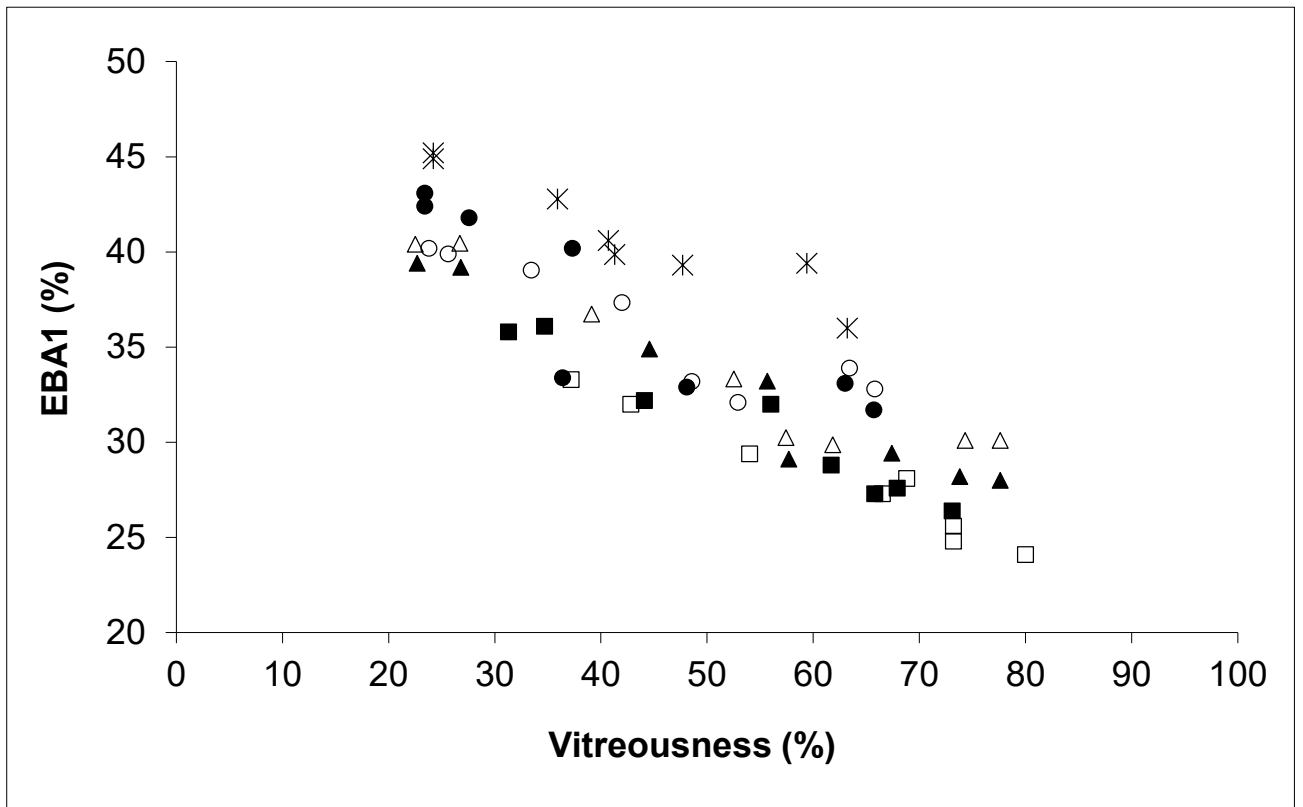
668 NIL3: *Pinb-D1b*: $y = -0.22x + 44.7$; $r^2 = 0.94$; *Pinb-D1d* : $y = -0.21x + 45.0$; $r^2 = 0.90$.

669

670 **Figure 2:** Percentage of volumes of farina particles according to their size before (solid
671 symbols) and after sizing step (open symbols). Experimental data (A) from NIL1-*Pinb-D1b*
672 grown in two distinct locations (without nitrogen addition) which results in vitreous (squares)
673 or non vitreous endosperm (diamond-shaped). Example of modeling curves (B) for the
674 vitreous sample of NIL1-*Pinb-D1b* (squares). Squares are the experimental data, four peaks
675 are generated by mathematical treatment of the data and correspond either to large particles
676 (dashed line), medium-size particles (unbroken line), small particles (dotted line) and to the
677 finest particles (not shown at this representation scale as it only corresponds to less than 1.3%
678 of the particle total volume).

679

680 Figure 1

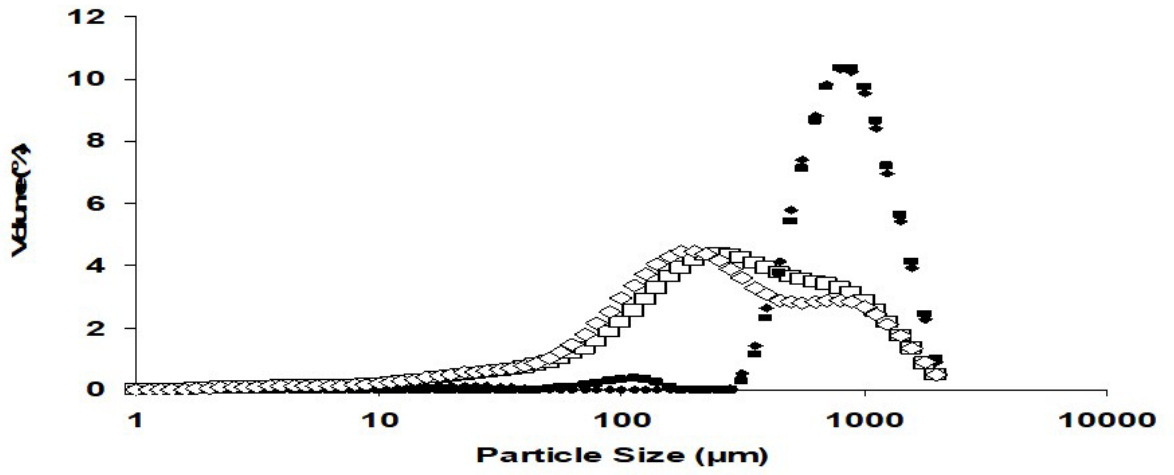


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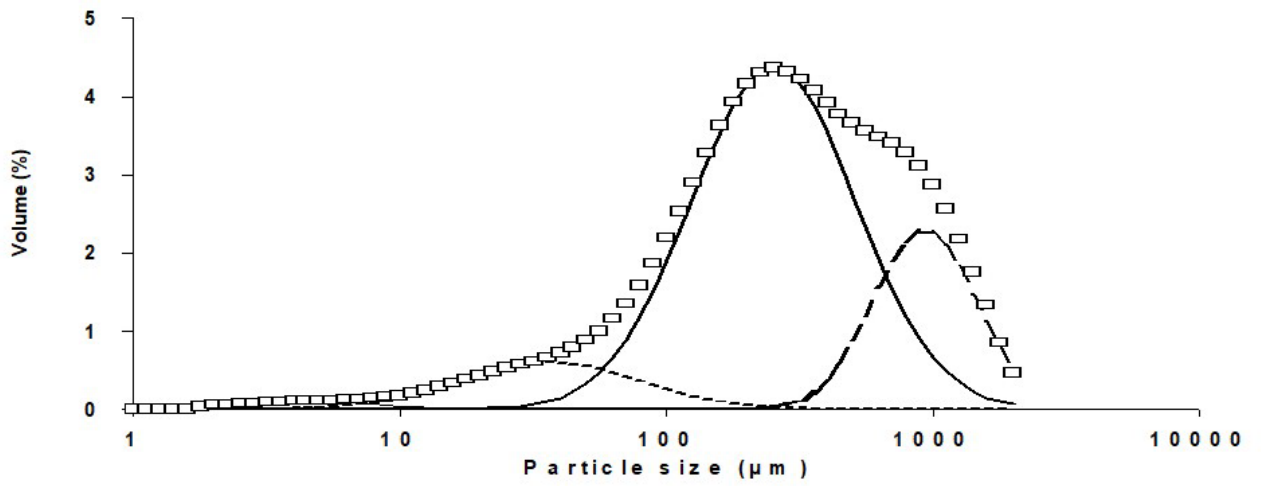
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684 Figure 2

A)



B)



685