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1 **Cadmium distribution in mature durum wheat grains using dissection, laser**
2 **ablation-ICP-MS and synchrotron techniques**

3

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34 **Abstract**

35 Understanding how essential and toxic elements are distributed in cereal grains is a
36 key to improving the nutritional quality of cereal-based products. The main objective
37 of this work was to characterize the distribution of Cd and of nutrients (notably Cu,
38 Fe, Mn, P, S and Zn) in the durum wheat grain. Laser ablation inductively coupled
39 mass spectrometry and synchrotron micro X-ray fluorescence were used for micro-
40 scale mapping of Cd and nutrients. A dissection approach was used to quantitatively
41 assess the distribution of Cd and nutrients among grain tissues. Micro X-ray
42 absorption near-edge spectroscopy was used to identify the Cd chemical environment
43 in the crease. Cadmium distribution was characterized by strong accumulation in the
44 crease and by non-negligible dissemination in the endosperm. Inside the crease, Cd
45 accumulated most in the pigment strand where it was mainly associated with sulfur
46 ligands. High-resolution maps highlighted very specific accumulation areas of some
47 nutrients in the germ, for instance Mo in the root cortex primordia and Cu in the
48 scutellum. Cadmium loading into the grain appears to be highly restricted. In the
49 grain, Cd co-localized with several nutrients, notably Mn and Zn, which challenges
50 the idea of selectively removing Cd-enriched fractions by dedicated milling process.

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53 **Main finding of the work**

54 This work provides the first high-resolution maps of Cd distribution in durum wheat
55 grain and, thereby, adds to our understanding of the processes involved in Cd loading
56 and storage in cereal seeds.

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59 **Keywords**

60 Cadmium; durum wheat grain; LA-ICP-MS; μ XRF; μ XANES

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67 **1. Introduction**

68 Cadmium (Cd) is a heavy metal contaminant whose toxicity to humans has long been
69 recognized. The European Food Safety Authority (EFSA) established a provisional
70 tolerable weekly intake for Cd of 2.5 $\mu\text{g kg}^{-1}$ body weight based on the long-term
71 harmful effects of low doses of Cd on human health (EFSA 2011). Though the
72 average dietary exposure is below this level in the European population, some
73 subgroups including children, young women and vegetarians are more likely to
74 frequently exceed it (Clemens *et al.* 2013). In Europe, almost one third of dietary
75 exposure to Cd is via consumption of cereals (EFSA 2012). Durum wheat has a
76 greater tendency to accumulate Cd in grains than common wheat and other cereals
77 (McLaughlin and Singh 1999; Grant *et al.* 2008). The cadmium concentration is often
78 above 0.1 mg kg^{-1} in durum wheat grains and can sometimes exceed the regulation
79 limit of 0.2 mg kg^{-1} set by the European Union (EC 2008). Therefore, particular
80 attention should be paid to reducing the level of Cd in durum wheat-based products
81 (e.g. semolina, pasta).

82 Substantial efforts have already been made to understand how Cd is taken up,
83 transported, and allocated to the grains in cereal crops (Clemens and Ma 2016;
84 Maccaferri *et al.* 2019). However, little information is available on how Cd is
85 distributed within the grain, even though the differential accumulation of an element
86 among grain tissues may mirror its internal loading, storage and functions within the
87 grain (Lombi *et al.* 2009). For instance, the transfer of zinc (Zn) from maternal to
88 filial tissues was proposed as the main barrier to Zn loading into wheat grains, based
89 on the much lower concentration of Zn measured in the endosperm than in the
90 vascular bundles in the crease (Wang *et al.* 2011). In the same way, the involvement
91 of sulfur (S) complexes in the loading of iron (Fe) into the grain was proposed based
92 on the co-localization of these two elements in the nucellar projection (Singh *et al.*
93 2013). A clear picture of how Cd is distributed among tissues and how it is co-
94 localized with nutrients in the grain is thus a key to developing agronomic strategies
95 to improve the food safety of durum-wheat based products. Among nutrients, those to
96 primarily focus on are Fe, Mn and Zn as they share transporters and loading pathways
97 with Cd, and S since Cd has a high affinity for thiol groups and is often bound to S-
98 containing ligands in plant tissues (Clemens *et al.* 2013).

99 Significant advances in imaging techniques make it possible to obtain high definition

100 elemental maps of wheat grains (Lombi *et al.* 2011). For instance, synchrotron micro
101 X-ray fluorescence (μ XRF) has been used to investigate the distribution of P, S, Mn,
102 Fe, Cu and Zn in bread wheat (Singh *et al.* 2013; Ajiboye *et al.* 2015; De Brier *et al.*
103 2016). Laser ablation inductively coupled mass spectrometry (LA-ICP-MS) can also
104 be used to identify the distribution of metals. This technique has been widely used in
105 recent years to map trace elements such as As, Cu, Fe, Mn, Ni, Pb and Zn in grains of
106 bread wheat (Lombi *et al.* 2009; Wu *et al.* 2013; Van Malderen *et al.* 2017), sunflower
107 (Pessôa *et al.* 2017), maize (Imran *et al.* 2017) as well as in cocoa beans (Thyssen *et*
108 *al.* 2018). The chemical analysis of dissected tissues is another way to investigate the
109 distribution of elements in plant grains (Pieczonka and Rosopulo 1985; Kutman *et al.*
110 2011; Detterbeck *et al.* 2016). Chemical analysis provides quantitative information at
111 the scale of the whole grain whereas imaging can only show the distribution of
112 elements in a section of the grain.

113 Only a few studies have attempted to map the distribution of Cd in grains. This is
114 probably because the level of Cd accumulation in plant grains is usually too low to be
115 accurately quantified by imaging (Clemens and Ma 2016). Van Malderen *et al.* (2017)
116 tried to map Cd in bread wheat grain with a Cd concentration of $0.05 \mu\text{g g}^{-1}$ but the
117 signal intensity of Cd in some parts fell to the background level, preventing the
118 precise delimitation of areas in which Cd accumulated. The only informative maps of
119 Cd distribution were imaged on Cd-contaminated rice grains (Basnet *et al.* 2014; Yan
120 *et al.* 2019) and in sunflower (Pessôa *et al.* 2017). In rice, Basnet *et al.* (2014)
121 observed that Cd was distributed throughout the grain whereas, in sunflower, Pessôa
122 *et al.* (2017) observed that Cd preferentially accumulated in the cotyledons. Today, a
123 high-resolution map of Cd distribution in durum wheat grain is still lacking.

124 In the present study, LA-ICP-MS was set up to produce images of the localization of
125 Cd and of six mineral nutrients (Cu, K, Mn, Mo, P and Zn) in transversal and
126 longitudinal sections of mature durum wheat grains (including the embryo) at the
127 micrometer scale spatial resolution. Synchrotron μ XRF and micro X-ray absorption
128 near-edge spectroscopy (μ XANES) were also used to provide distribution maps of Cd
129 and of nutrients (including Fe and S) at a sub-micrometer lateral resolution, and Cd
130 speciation, respectively, particularly in the crease area. In addition to high-resolution
131 maps, a grain dissection experiment was performed to provide quantitative
132 information on Cd and nutrient concentrations in dissected grain tissues. The

133 objectives of the study were to (i) precisely characterize the spatial distribution of Cd
134 in durum wheat grain, (ii) investigate the differences between the distribution of
135 mineral nutrients based on current knowledge of their loading, storage and functions
136 in the grain, and (iii) examine if there are Cd-enriched tissues that could be removed
137 by adequate milling processes without having too much impact on the nutritional
138 value of the durum wheat flour.

139

140 **2. Materials and methods**

141 *2.1. Grain production*

142 Mature grains of durum wheat (*Triticum durum* cv. *Sculptur*) were harvested from
143 hydroponically grown plants exposed to 100 nM Cd(NO₃)₂. The composition of the
144 nutrient solution and the hydroponic culture procedure are the same as those described
145 in Yan *et al.* (2018) (for details see supplementary comment S1). The average
146 concentration of Cd in the grains was $2.5 \pm 0.1 \mu\text{g g}^{-1}$ dry weight (DW). As a control
147 for mapping, grains with a background level of Cd ($0.0024 \pm 0.0019 \mu\text{g g}^{-1}$ DW) were
148 collected from plants grown under the same conditions but with no Cd added to the
149 nutrient solution. Neither Cd toxicity nor mineral deficiency symptoms was observed
150 in plants over the whole culture period. The spikes were freeze-dried at -60 °C for 72
151 hours before the grains were harvested. All the grains used in this experiment were
152 taken from this pool, whose characteristics are summarized in supplementary Table
153 S1.

154

155 *2.2. Preparation of thin sections by cryomicrotomy*

156 Grains were soaked for 5 to 6 hours at 4 °C. For LA-ICP-MS, grains were then
157 embedded in O.C.T compound (tissue-teck) at low temperature (~ -30 °C) and 40 μm-
158 thick transversal (across root or leaf primordia of the embryo) as well as longitudinal
159 (alongside the crease) sections were prepared by cryomicrotomy (Leica CM 1950).
160 The thin sections were directly deposited on glass slides and air dried. Before LA-
161 ICP-MS analysis, the structural integrity of the grain section was checked under the
162 microscope (Fig. 1-2, supplementary Fig. S1). For μXRF and μXANES, 16 μm-thick
163 sections were cut at ~ -50 °C with a cryomicrotome (Leica LN22) cooled with liquid
164 nitrogen. The frozen sections were sandwiched between two Ultralene[®] films, and
165 mounted directly on the sample holder immediately before measurement. They were

166 kept frozen (-170 °C) during transfer to the analysis chamber and throughout the
167 synchrotron measurements using a passively cooled liquid nitrogen cryostat.

168

169 2.3. LA-ICP-MS imaging

170 Elemental analysis of the grain section was achieved by coupling an NWR 213 laser
171 ablation system equipped with a TV2 ablation cell (ESI, Fremont, CA) with a 7700
172 cs ICP-MS (Agilent, Tokyo, Japan) with Pt cones. A helium flow of 800 mL min⁻¹ was
173 used to transport the ablated material directly into the dry plasma of the ICP-MS. ICP-
174 MS parameters (torch position, carrier gas flow rate and ion lense voltage) were tuned
175 for each set of experiments by ablating the 612 NIST glass reference material while
176 monitoring the ²³⁸U and ²³²Th signals. Detection limits of monitored isotopes are
177 commonly in the range of 0.0001-0.1 µg g⁻¹ (Becker 2002). The grain sections were
178 ablated using a Nd:YAG laser source at 213 nm with a 20 Hz repetition frequency, a
179 10 µm x 10 µm square laser beam, a fluency of 12 J cm⁻² (20% of delivered energy),
180 and a scan speed of 10 µm s⁻¹. Samples were entirely ablated in a line-by-line scan
181 mode with a distance between lines of 20 µm (non-ablated lines). ¹¹⁴Cd and other
182 isotopes (³¹P, ³⁹K, ⁵⁵Mn, ⁶³Cu, ⁶⁴Zn, ⁹⁸Mo) were measured with a sampling time of 1 s
183 while maximizing the integration time for ¹¹⁴Cd (0.59 s). A csv file reporting isotope
184 intensities versus time (then converted to the position at the surface of the sample)
185 was recorded for each ablated line. A specific program was designed and coded to
186 merge the files and build the maps of the different isotopes. For each map, the relative
187 intensity was calculated by dividing the raw signal by the maximum value.

188

189 2.4. Synchrotron µXRF imaging and µXANES analyses

190 Micro-XRF and Cd L_{III}-edge µXANES were performed on the ID21 scanning X-ray
191 microscope at the European Synchrotron Radiation Facility (ESRF, Grenoble, France).
192 The X-ray beam was monochromatized with a Si(111) two-crystal monochromator
193 and focused using Kirkpatrick-Baez mirrors on the sample with a lateral resolution of
194 0.4 µm (V) x 1.4 µm (H). Measurements were made in a vacuum and in cryogenic
195 conditions using a cryostat passively cooled with liquid nitrogen (Cotte *et al.* 2017).
196 To avoid potassium interference (K-edge at 3608 eV) with Cd L lines, chemical
197 mapping was performed in two stages: Cd-S-P-Mg at 3570 eV and Fe-Mn-Ca-K at
198 7200 eV. In both cases, maps were obtained by scanning the samples with a 2 µm step

199 (1 μm for the crease) and a counting time of 100 ms per point. The fluorescence
200 signal was collected with a Silicon Drift Diode XRF detector (SGX Sensortech with
201 an active area of 80 mm^2) and normalized by the incident photon intensity. Elemental
202 mass fractions were calculated from fundamental parameters with the PyMca software
203 package, applying pixel-by-pixel spectral deconvolution to hyperspectral maps
204 normalized by the incoming flux (Sole *et al.* 2007). The incoming flux was monitored
205 using a drilled photodiode previously calibrated to 3570 and 7200 eV. In PyMCA, the
206 reference incoming flux was set at 5×10^{10} photons s^{-1} , and XRF detector parameters
207 were set to an active area of 0.7746 cm^2 and 4.65 cm sample to XRF detector distance.
208 Active area and sample to detector distance were calibrated using an AXO standard
209 (<http://www.axo-dresden.de/products/highprecision/reference.htm>) that contains Fe
210 and Pd as reference to the elements analysed in the wheat grains. The sample matrix
211 was assumed to be cellulose/ice at a density of 1.5 g cm^{-3} . The sample thickness was
212 set at 16 μm using a cryomicrotome. In order to filter out the pixels with poor signal
213 to noise, each pixel was tested to have a signal in the detector element's region of
214 interest $\geq 3.29 \times \sqrt{\text{signal} + \text{background}}$. After filtering the poor signal quality
215 pixels, the calculation of limit of detection (LOD) was performed for each pixel and
216 the highest LOD calculated was used to set the color scale bars in the elemental maps.
217 The LOD maps and the elemental maps are shown in supplementary Fig. S4 and S5.
218 The LOD values obtained were (in mg g^{-1}) 0.16 for Cd, 0.31 for S, 1.95 for P, 32 for
219 Mg, 0.41 for Fe, 0.47 for Mn, 2.35 for Ca and 7.02 for K.

220 Cadmium L_{III} -edge μXANES spectra of the areas of interest of the grain section were
221 recorded in fluorescence mode using a SDD XRF detector (SGX Sensortech with an
222 active area of 80 mm^2) in the range 3520 and 3590 eV with an energy step of 0.5 eV
223 and a counting time of 100 ms. The normalized spectra were compared to Cd-
224 reference compounds we collected previously on the same beamline (Isaure *et al.*
225 2006; Isaure *et al.* 2015; Penen *et al.* 2017) and simulated by linear combination fits
226 (LCFs) of the Cd references (fingerprint approach). The quality of the fit was
227 estimated by the normalized residual sum of squares (*Nss*) and the precision on the
228 proportion of Cd species was estimated to be 10% as described in Isaure *et al.* (2017).

229

230 2.5. Grain dissection and analysis of dissected grain fractions

231 Five fractions of wheat grain were considered: (i) the outer periphery (*OP*), which is

232 loosely attached to the grain and mainly corresponds to the pericarp epidermis,
233 hypodermis, and degenerated parenchyma (Bechtel *et al.* 2009), (ii) the inner
234 periphery (*IP*) comprising the remaining layers that strongly adhere to the endosperm
235 (cross and tube cells, seed coat, nucellar remnants and the aleurone layer), (iii) the
236 germ (*GM*) including the scutellum and the embryo axis, (iv) the starchy endosperm
237 (*SE*), and (v) the crease (*CR*) tissues comprising the periphery layers embedded in the
238 crease of the grain. Because the *IP* parts stick tightly to the starchy endosperm, it was
239 impossible to experimentally separate the whole *IP* from the whole *SE* while being
240 sure that their respective mineral contents were not affected by cross-contamination.
241 Consequently, the concentrations of the element were determined on a subsample of
242 the core of the starchy endosperm free from *IP* contamination. The quantity of a given
243 element in the *IP* was estimated by calculating the difference between the quantity in
244 the whole grain and the sum of the quantities in *OP*, *GM*, *CR* and *SE*. The
245 concentration was then derived by dividing by the biomass of the *IP* (for details see
246 supplementary comment S2).

247 The elemental composition of the fractions was determined as follows. A mixture of
248 0.8 mL of 30% H₂O₂ and 0.2 mL of 67.5% HNO₃ was added to each sample fraction.
249 The mixture was left at room temperature overnight for preliminary digestion.
250 Digestion was accomplished by heating the mixture in an oven using a temperature
251 gradient of 60 °C for 30 min, 80 °C for 45 min, and 100 °C for 90 min. After cooling
252 and filtering through 0.2-µm polycarbonate membrane filters, the solutions were
253 diluted to 10 mL with ultrapure water. The concentrations of Cd, Zn, Cu, Mn, Mo, and
254 Fe in the digests were determined by ICP-MS (7700x, Agilent Technologies), and
255 those of Ca, K, Mg, and P were determined by ICP-OES (ACTIVA, Horiba Jobin
256 Yvon) by the central analytical service of the University of the Basque Country. The
257 results were validated using procedural blanks and the NIST Standard Reference
258 Material 1573a. The recovery rates of reference values were higher than 90% for all
259 elements.

260

261 **3. Results**

262 *3.1. LA-ICP-MS elemental imaging in durum wheat grains*

263 The maps of cadmium (¹¹⁴Cd), zinc (⁶⁴Zn), copper (⁶³Cu), manganese (⁵⁵Mn),
264 molybdenum (⁹⁸Mo), potassium (³⁹K), and phosphorus (³¹P) distributions are shown

265 for the cross sections at the level of the leaf (Fig. 1) or root (Fig. 2) primordia and for
266 the longitudinal section alongside the crease (supplementary Fig. S1). The germ,
267 crease, grain periphery, and starchy endosperm (*SE*) were identified on the different
268 maps. Two layers were visible at the grain periphery: the outer (*OP*) and the inner (*IP*)
269 periphery. In the germ, it was possible to distinguish the scutellum facing the
270 endosperm and the embryo axis, including the coleoptile and coleorhiza surrounding
271 the leaf and root primordia.

272 The starchy endosperm generally had the lowest intensities and the *IP*, the germ and
273 the crease had the highest, regardless of the element. In detail, the *OP* had a detectable
274 signal mainly for Cd, Mn and Zn. Except at the bottom of the grain close to the root
275 primordia (Fig. 2), high abundances of all the elements, particularly K, P, Cd, Mn and
276 Zn, were found in the *IP* layers. In the crease, which is visible only on the radial
277 cross-sections (Fig. 1, 2), the signal intensities of Cd, Zn and Mn was very high but
278 the exact location varied with the element and with their position along the embryo
279 axis. On the cross section made at the level of the shoot primordia (Fig. 1), Cd was
280 abundant in all the crease layers, Zn was more abundant in the innermost layers,
281 presumably the modified aleurone layer, whereas Mn was more abundant in the
282 outermost layers, presumably the nucellar projection, the pigment strand and the
283 vascular bundle area. Hotspots of Cd, Mn and Cu were identified on the cross section
284 at the level of the root primordia (Fig. 2) and attributed to the nucellar projection
285 and/or the pigment strand. Zinc was highly abundant in the crease tissues on each side
286 of the endosperm cavity, but the signal intensity of Zn was lower in the location
287 assumed to be the pigment strand and/or in the vascular bundle area.

288 In the germ, the scutellum was particularly homogeneously enriched in P, Cu, K and
289 to a lesser extent in Cd (Fig. 1-2, supplementary Fig. S1). In the scutellum, Zn was
290 preferentially localized in the part facing the endosperm whereas Mn tended to
291 preferentially accumulate on the opposite side, facing the shoot or root primordia,
292 although to a lesser extent. In the embryo axis, the root cortex (and possibly the
293 epidermis) showed stronger signals of Cd<Zn<Mn<Mo. Markedly high intensities of
294 molybdenum were observed in the periphery of the root primordia (Fig. 2,
295 supplementary Fig. S1). Finally, the starchy endosperm had always the lowest signals.
296 The lower abundances of elements in the *SE* than in the peripheral layers of the grain
297 were clearly visible on the maps and in the signal intensity profile from the inside to

298 the outside of the grain (supplementary Fig. S2). The difference between these and the
299 other parts was (Zn, Mn, P, K) > (Cu, Mo) > Cd.

300 The accumulation of Cd in the *SE* was also visible when we compared the map of Cd
301 in a contaminated grain with that in the uncontaminated control sample
302 (supplementary Fig. S3). Figure 1 and supplementary Fig. S2 suggest a gradient in Cd
303 intensity *IP* > outer endosperm neighboring *IP* > core of the starchy endosperm. It can
304 be seen that the Cu signal in the endosperm was slightly higher around the crease (Fig.
305 1). Tentative grouping of the elements according to the most discriminating hotspots
306 could be as follows: Cd, Mn and Zn accumulated particularly in the crease; P, Cu and
307 K showed a stronger and more homogeneous accumulation in the scutellum; Mo was
308 preferentially located in the periphery of the root primordia.

309

310 3.2. Synchrotron μ XRF elemental imaging

311 Elemental maps of a transversal section with a zoom on the crease are shown in
312 supplementary Fig. S4 and S5 and tricolor maps in Fig. 3. The highest concentration
313 of Cd was clearly located in the crease, where its concentration locally reached 600
314 $\mu\text{g g}^{-1}$. The high resolution lateral map of the crease pinpointed a “curved zone”
315 assumed to be the pigment strand (Drea *et al.* 2005) where Cd was particularly
316 concentrated (red arrows in Fig. 3). Several hotspots where Cd and P co-localized
317 were observed in the vascular bundle area (white arrows in Fig. 3). In contrast, no Cd
318 was detected elsewhere in the grain, probably because the concentration of Cd was far
319 lower in the other grain parts (endosperm, germ and peripheral tissues), as suggested
320 by LA-ICP-MS maps. Sulfur was mainly located in the crease - in the vascular bundle
321 area but also in the pigment strand and in the adaxial nucellar projection - as well as
322 in the root primordia and in the endosperm, particularly in the outward endosperm, in
323 line with results obtained by De Bier *et al.* (2016). High concentrations of P were
324 found in the aleurone layer, where P-enriched vacuoles were visible, and in the
325 scutellum. Iron was concentrated in the vacuoles of the aleurone cells (like P) and in
326 the abaxial nucellar projection. The outward scutellum was also enriched in iron.
327 Manganese showed a different pattern with preferential localization in the vascular
328 bundle area, in the outer periphery and, to a lesser extent, in the root primordia.
329 Calcium was distributed mainly as spots in the endosperm and in the outer periphery,
330 while K was mainly located in the aleurone layer and in the germ (notably in the

331 scutellum).

332

333 3.3. Cadmium speciation by Cd L_{III}-edge μ XANES

334 Cadmium μ XANES was performed on the distinct areas of the crease and compared
335 to Cd-references to probe the Cd ligands. Spectra collected on the various Cd+P
336 hotspots were averaged and showed an edge with a peak characteristic of O ligands
337 (supplementary Fig. S6). The global spectrum was satisfactorily reproduced by 62%
338 Cd-O and 27% Cd-cystein with O ligands provided by carboxyl ligands from organic
339 acids. Although phosphate ligands produced poorer spectral agreement, it was
340 difficult to unambiguously exclude these ligands. The averaged spectrum collected in
341 Cd hotspots in the pigment strand was fitted with 43% Cd-O and 42% Cd-cystein
342 while the averaged spectrum collected in the (Cd, S)-diluted areas of the vascular
343 parenchyma was fitted with 27% Cd-O and 63% Cd-cystein. In contrast, no Cd
344 absorption edge was detected in the nucellar projection, in the aleurone layer or in the
345 endosperm because of their much lower concentration of Cd.

346

347 3.4. Concentrations and amounts of elements in the dissected grain parts

348 Figure 4 shows how the concentration of each element differed among the different
349 grain parts. None of the elements studied were homogeneously distributed in the
350 wheat grain. Three groups were distinguished among grain parts. The concentrations
351 of *SE* were the lowest of all elements, clearly lower than the corresponding
352 concentration of the whole grain (normalized concentration <1). The concentrations
353 of the second group of grain tissues including the *GM* and *IP* were the highest of all
354 the elements, 2.2 to 8.5 times higher than the concentration of the whole grain.
355 Concentrations in the third group, *OP* and *CR*, were between the two, either above or
356 below the concentration of the whole grain, depending on the element. It is worth
357 noting that the crease had by far the highest concentrations of Cd, Mn and Zn, with
358 concentrations respectively 16.6-, 19.2- and 17.7-fold higher than the corresponding
359 concentration of the whole grain. Concentrations obtained by μ XRF and chemical
360 analyses of dissected tissues differed but were not inconsistent since μ XRF gives a
361 local concentration in an area of a few μm^2 while chemical analyses provides
362 information on a bulk tissue that can be highly heterogeneous. Generally, the ranking
363 of grain tissues according to the concentrations of a given element was consistent with

364 LA-ICP-MS and μ XRF maps. In addition, analysis of dissected tissues revealed a
365 tendency for concentrations of Cd, Cu and Mo to be relatively the highest in the *SE*.
366 This characteristic may also be true for S, as suggested by μ XRF maps (Fig. 3), but its
367 concentration was not determined in dissected grain tissues in the present study.
368 Supplementary Fig. S7 shows how the amount of each element was partitioned among
369 the different parts of the grain. *SE+IP* were the two main fractions that together
370 contained between 52% (Mn) and 89% (Mg) of the total quantity of the grain. Hence,
371 69% of Cd in the grain was in *SE+IP*. Although *IP* represents only 10% of the grain
372 dry weight, it accounted for between 22% (P) and 78% (Mg) of the total quantity of
373 the element (30% of Cd). Conversely, *SE*, which is a main grain fraction based on
374 biomass (83% of total, on average) accounted for only 25% (Ca) to 54% (Mo) of the
375 total (39% of Cd). This reflects the high concentration of elements in the *IP* versus
376 their very low concentration in the *SE*. The crease, which only accounted for 0.9% of
377 grain DW, contained more than 15% of the Cd, Mn and Zn in the grain. The germ
378 accounted for 3% of the grain biomass but contained more than a quarter of total P
379 and 15-18% of total Cu, Mn, and Fe. The germ accumulated only 7% of total Cd. This
380 is both the lowest contribution of the germ to the total quantity of an element and the
381 lowest pool of Cd in the grain.

382

383 **4. Discussion**

384 *4.1. Is nutrient accumulation in the crease evidence for their highly regulated loading*
385 *into the seed?*

386 In cereals, inorganic elements are mainly transported to the grain via the phloem
387 (Frick and Pizzolato 1987). They are unloaded from the phloem all along the crease,
388 transported through the apoplast and symplast of the chalaza and nucellar projection
389 cells towards the endosperm cavity, a space filled with water and nutrients that are
390 absorbed by the modified aleurone cells of the endosperm (Wang *et al.* 1995; Patrick
391 and Offler 2001; Borg *et al.* 2009; Yu *et al.* 2015). In the early growth stage of the
392 grain, it is possible that nutrients are also delivered to the endosperm from the whole
393 pericarp through the seed coat by the dorsal and lateral bundles (Fisher 1990).
394 However, this process is limited in time because the dorsal and lateral bundles
395 degenerate and because the seed coat cuticulizes. The crease consequently becomes
396 the main entry route for nutrients to the seed. In the crease region, part of the chalaza,

397 namely the pigment strand, differentiates into an apoplastic barrier through the
398 accumulation of hydrophobic substances and cell wall lignification. One function of
399 the pigment strand may be to limit the backflow of nutrients (but not of water) from
400 the seed to the maternal tissue. The seed is then isolated from the pericarp, seed coat
401 and nucellar remnants (Zee and O'Brien 1970). Nutrients can still be actively
402 delivered to the seed via the symplastic route thanks to the transfer cells (Wang *et al.*
403 1995) that enable control of the delivery.

404 Metal transporters coded by the genes of metal homeostasis are not completely
405 specific and can transport Zn, Fe, Mn, Cd, Pb and Cu with variable affinity,
406 explaining why the distribution of some metals in grains is similar, as is the case of
407 Mn and Zn here. For example, HMA(1,2,3,10) pump Cd/Zn/Co/Pb across the
408 membrane against their electrochemical gradient whereas HMA(4 to 9) operate on
409 Cu/Ag (Tauris *et al.* 2009; Bashir *et al.* 2016). From the transversal sections of LA-
410 ICP-MS maps obtained in our work, and more specifically at the shoot primordia
411 level, it is possible to infer that Zn was more efficiently transferred from the phloem
412 to the aleurone layer than Mn whose signal was higher in maternal tissues than in the
413 aleurone layer. Based on current knowledge of putative metal-chelates in phloem
414 (Harris *et al.* 2012; Khan *et al.* 2014; Flis *et al.* 2016), one possible explanation is that
415 yellow strip-like transporters can transport the Zn-nicotianamine (Zn-NA) chelates
416 from the phloem to the endosperm cavity and/or from the endosperm cavity to
417 aleurone cells (Palmgren *et al.* 2008) but no - or very little - Mn, which has a much
418 lower affinity for NA than Zn (pK= 8.8 for Mn, pK= 17.4 for Zn; Benes *et al.*, 1983).

419

420 *4.2. Relationship between the distribution and the function of inorganic nutrients in* 421 *durum wheat grain*

422 The endosperm is the reserve tissue of the seed but was seen to have the lowest
423 concentrations of elements. This apparent paradox is however sound from a functional
424 point of view. Indeed, at maturity, and therefore during germination, *SE* is a dead
425 tissue and if inorganic nutrients were stored in *SE*, their concentrations would be very
426 high so that transport by diffusion could cover the requirements resulting from the
427 growth of the embryo. Given the size of the endosperm (80% of the seed DW), high
428 concentrations also mean huge amounts of inorganic nutrients, which may not be
429 available. High concentrations of inorganic elements in the endosperm may also affect

430 starch synthesis and proper protein folding in which starch granules are embedded
431 (Mills *et al.*, 2005). Therefore, it is functionally more efficient to store inorganic
432 nutrients in the aleurone layer (Regvar *et al.* 2011) because at germination, the
433 aleurone cells are alive and can actively transfer nutrients to the germ (Bechtel *et al.*
434 2009). Aleurone cells (which are part of the *IP* fraction) have countless globoids of
435 phytic acid combined with metal elements such as Zn, Mn, Cu, Fe, and Mg (O'Dell *et al.*
436 1972; Cakmak 2007; Ficco *et al.* 2009; De Brier *et al.* 2016) allowing the storage
437 of both P and metals. The germ also contained high concentrations of elements, which
438 can be interpreted as a starter pool for germination and for the initial growth of the
439 seedling tissues. Although *SE* had the lowest concentrations of elements, likely
440 reflecting the concentrations required for its metabolism, *SE* was on average the
441 second pool of elements based on quantities, due to the important contribution of this
442 tissue to the grain DW. Compared to the *IP*, *SE* is a significant pool of elements that
443 can be mobilized more slowly, along with the mobilization of C and N reserves of the
444 starch/protein matrix.

445 It is striking that the great majority of Cu accumulation took place in the scutellum
446 and secondarily in the *IP*. This matches the distribution of P and is consistent with the
447 high affinity of Cu for phytates, higher than that of Cd and Mn but equivalent to that
448 of Zn (Crea *et al.* 2008). The ability of copper to change its redox state in biological
449 conditions makes the control of its reactivity particularly important. Furthermore, in
450 plants, excluding Cu sorbed onto the root apoplast, most Cu is contained in the
451 plastocyanin of chloroplasts for photosynthesis, which is not among the plant's top
452 priorities during early germination. From a functional point of view, it makes sense
453 that Cu is stored in the scutellum and not in the embryo axis contrary to Zn and Mn,
454 which are involved in many more enzymes during early germination, in particular
455 those required for growth, protein synthesis, cell elongation, ion absorption and
456 resistance to stress (Broadley *et al.* 2007; Millaleo *et al.* 2010). For example, Zn is
457 required as a cofactor in over 300 enzymes (Palmgren *et al.* 2008). Interestingly, Zn
458 and Mn were highly concentrated in root primordia, which are the first organs to grow.
459 Molybdenum, which is required for both nitrogen assimilation and phytohormone
460 synthesis (Bittner 2014), was also intensely located in the root cortex primordia.
461 Hence, the dissection data and the maps suggest particularly well integrated control of
462 distribution among the grain tissues consistent with the fact that metals are essential

463 for plant metabolism but also highly reactive and possibly toxic.

464

465 4.3. Cadmium distribution, loading and storage in durum wheat grain

466 To our knowledge, this is the first time that Cd has been mapped in wheat grain. In
467 general, the distribution of Cd in the grain closely mirrored that of Zn and was
468 characterized, notably, by very strong accumulation in the crease. The results of
469 dissection showed that the concentration of Cd was on average 16-fold higher in the
470 crease than in the whole grain, reaching $40 \mu\text{g g}^{-1}$. The LA-ICP-MS and μXRF maps
471 of the cross sections showed that Cd was not evenly distributed inside the crease and
472 that distribution varied with the cutting axis. The μXRF distribution map obtained
473 from a cut made at the level of root primordia showed that, on this axis, the vast
474 majority of Cd accumulated in maternal tissues, notably in the pigment strand where it
475 co-localized with S, and where its concentration locally reached $600 \mu\text{g g}^{-1}$. This
476 suggests that Cd loading in nucellar projection cells strongly limits its allocation to
477 the seed, perhaps even more so than that of Mn.

478 Speciation of Cd by μXANES suggested various possible Cd ligands in the crease,
479 probably corresponding to different forms of Cd transport or storage. It seems that Cd
480 is nearly half bound by thiols in the Cd-enriched pigment strand while in (Cd, S)-
481 diluted areas of the vascular parenchyma this proportion increases to 63%. Assuming
482 that the speciation of Cd in the vascular parenchyma mirrors that in the phloem
483 vessels, this would mean that Cd is significantly associated with S ligands in the
484 phloem of durum wheat, in agreement with the literature. Indeed, thiols were
485 identified as Cd ligands in the phloem of the Cd hyperaccumulator *Arabidopsis*
486 *halleri* and of the Cd non-accumulator *Arabidopsis lyrata* (Isaure *et al.* 2015). Thiol
487 ligands were also found in the phloem of *Brassicacea napus* where Cd was assumed
488 to be transported mainly as phytochelatin-Cd and glutathione-Cd complexes
489 (Mendoza-Cozatl *et al.* 2008). Based on this result, one possible explanation for the
490 strong accumulation of Cd in the maternal tissues of the crease is that transporters
491 involved in metal loading in the nucellar projection and/or metal unloading in the
492 endosperm cavity (such YSL and ZIP proteins) do not carry any (or only very few)
493 metals bound to thiol-containing peptides such as Cd.

494 LA-ICP-MS maps showed that Cd was relatively more abundant in the endosperm
495 than most other elements. The dissection results confirmed that the normalized

496 concentration of Cd in the endosperm was higher than that of most nutrients, notably
497 Fe and Zn, and showed that 40% of the total Cd in the grain was stored in the *SE*. This
498 pattern may be related to the protein network of the *SE* that embeds starch granules
499 including glutenins and gliadins (Mills *et al.* 2005). Indeed, Cd has a high affinity for
500 S-containing compounds and, accordingly, the distribution pattern of Cd shown in Fig.
501 1 mirrors that of S in Fig. 3. In rice grains, Cd is associated with S-containing amino
502 acids including cysteine and methionine (Wei *et al.* 2017). The thiol groups of these
503 amino acids are involved in the disulfide bonds that control protein folding and may
504 be preferential sites of Cd binding and retention thereby explaining the relatively high
505 concentration of Cd in the endosperm. Consistent with this hypothesis, the inward
506 decreasing gradient of Cd in the endosperm may reflect the protein gradients in the *SE*,
507 which are more concentrated in the subaleurone layer than in the inner parts (Tosi *et*
508 *al.* 2011).

509

510 *4.4. Development of new milling technologies to reduce the level of Cd in durum* 511 *wheat products*

512 New milling technologies for grain could improve the sanitary quality of durum
513 products by removing the grain tissues that are rich in Cd. However, since there is no
514 tissue in the grain of durum wheat that selectively accumulates Cd, a choice will need
515 to be made between reducing the concentration of Cd and maintaining the nutritional
516 quality of the grain. If the priority is to reduce the grain Cd concentration, for instance
517 because the grain Cd concentration is high or because the grains are intended to be
518 consumed by a population particularly exposed to Cd, the best option would be to
519 remove all fractions except the starchy endosperm, which means producing “white
520 semolina”. According to the calculations made based on our dissection experiment
521 (supplementary Table S2), this would reduce the concentration of Cd by more than 50%
522 but would simultaneously cause huge nutrient losses, particularly of Mg, Fe, Mn and
523 Zn. If the priority is to reduce the grain Cd concentration while minimizing the
524 concomitant loss of inorganic nutrients, the best option would be to selectively
525 remove the outer periphery and the crease, which means saving the *SE+IP* (with or
526 without the germ) for the downstream transformation. This would reduce the Cd
527 concentration by 24% while limiting the loss of P, K, Mg, Cu, Fe and Mo to less than
528 10%. Only the concentrations of Mn (-29%) and Zn (-21%) would be significantly

529 reduced.

530

531 **5. Conclusions**

532 In this study, we combined two high-resolution imaging approaches (LA-ICP-MS and
533 μ XRF) with a dissection approach to describe the distribution of Cd and seven
534 inorganic nutrients in durum wheat grain. The three approaches made it possible to
535 identify high accumulation of Cd in the crease, and inside the crease, mainly in
536 maternal tissues. Loading of Cd into the grain is therefore largely constrained, as is
537 that of Fe and Zn. To control the accumulation of Cd in the grain without affecting
538 that of Zn and Fe (or vice versa), the limiting stages of their loading into the grain and
539 the associated physiological processes will have to be identified. The similarities
540 observed between the μ XRF maps of Cd and S distribution, particularly in the
541 endosperm, as well as the results of μ XANES showing that Cd is mainly linked to
542 sulfur ligands in the vascular bundle area, suggest that sulfur fertilization could be a
543 way to reduce the allocation of Cd to the grain as well as its accumulation in the
544 endosperm in durum wheat. Molecular speciation measurements of Cd such as those
545 conducted by Persson et al. (2016) will deepen the understanding of processes behind
546 endosperm Cd accumulation. The preferential accumulation of Cd in certain grain
547 tissues also opens the way for reducing the Cd content of durum wheat-derived
548 products by adapting milling processes. However, because Cd accumulates in the
549 same parts of the grain as several nutrients (including Zn) these adaptations will also
550 alter the nutritional quality of the resulting flour.

551

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Figure captions

Figure 1. LA-ICP-MS distribution maps of ^{114}Cd , ^{64}Zn , ^{63}Cu , ^{55}Mn , ^{98}Mo , ^{39}K and ^{31}P in mature durum wheat grains sectioned transversally at the position of the leaf primordia. The color scale represents different signal intensities, blue corresponds to the lowest relative intensity and yellow to the highest. The optical image (upper left) shows the different grain tissues visible in this transversal section: *OP*, outer periphery (likely the outer pericarp); *IP*, inner periphery (likely the aleurone layer, the seed coat, the inner pericarp and other maternal peripheral layers); *SE*, starchy endosperm; *col*, coleoptile; *lp*, leaf primordia; *sl*, scutellum; and within the crease (*cr*) region: *mal*, modified aleurone layer; *np*, nucellar projection; *vb*, vascular bundle area.

Figure 2. LA-ICP-MS distribution maps of ^{114}Cd , ^{64}Zn , ^{63}Cu , ^{55}Mn , ^{98}Mo , ^{39}K and ^{31}P in mature durum wheat grains sectioned transversally at the position of the root primordia. The color scale represents different signal intensities, blue corresponds to the lowest relative intensity and yellow to the highest. The optical image (upper left) shows the different grain tissues visible in this transversal section: *OP*, outer periphery (likely the outer pericarp); *SE*, starchy endosperm; *coz*, coleorhiza; *rp*, root primordia; *sl*, scutellum; *cr*, crease.

Figure 3. Synchrotron μXRF tricolor distribution maps of Cd-S-P, Cd-Mg-P, Fe-Mn-Ca and Fe-Mn-K in mature durum wheat grains sectioned transversally at the position of the root primordia. A more detailed map shows the distribution of Cd and P within the crease and the areas where μXANES spectra were collected (white arrows indicate typical positions of P and Cd hotspots, red arrows indicate typical positions of Cd hotspots, black arrows indicate typical Cd diluted areas). The optical image (upper left) shows the different grain tissues visible in this transversal section: *OP*, outer periphery (likely the outer pericarp); *SE*, starchy endosperm; *al*, aleurone layer; *rp*, root primordia; *sl*, scutellum. Within the crease (upper right), *vb* stands for vascular bundle area, *ps* for pigment strand and *np* for nucellar projection (either abaxial or adaxial). The maps of Cd, S, P and Mg were collected at an incident energy of 3570 eV, a step size of 2 μm (1 μm for the crease area) and a dwell time of 100 ms per point. The maps of Fe, Mn, Ca and K maps were collected at an incident energy of

7200 eV, a step size of 2 μm and a dwell time of 100 ms per point. Tricolor maps are displayed as quantitative maps in mg g^{-1} .

Figure 4. Concentrations of P, Ca, K, Mg, Cd, Cu, Fe, Mn, Mo, and Zn measured in the following dissected grain tissues: germ (*GM*), outer periphery (*OP*), inner periphery (*IP*), starchy endosperm (*SE*) and crease (*CR*). The concentration of a given element in a tissue was normalized by dividing the concentration of the tissue by that of the whole grain.