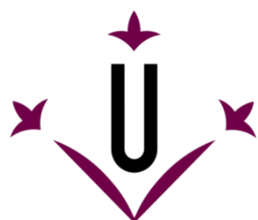

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1 # The fate of several trichothecenes and zearalenone during
2 roasting and enzymatic treatment of cereal flour applied in
3 cereal-based infant food production

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9 ## Abstract

10 Cereal-based baby food production process is expected to have an impact on the initial
11 level of *Fusarium* mycotoxins that can contaminate the raw materials. The aim of the
12 present study was to investigate the changes of some of these toxins during roasting and
13 the treatment with amylolytic enzymes, usually applied during the production process.
14 Three different cereal flours contaminated with *Fusarium graminearum* were
15 considered (barley, wheat and oat). The results did not show significant changes in the
16 concentration of any of the studied mycotoxins (up to 5% change in deoxynivalenol
17 concentration after the enzymes were added). The acetyl-deoxynivalenol also showed
18 slight modifications as a result of the applied processes, however their statistical
19 significance was not proved. Zearalenone and T-2 and HT-2 toxins remained almost
20 unaltered throughout the study.

21

22 ## Keywords

23 Cereal-based baby food, HPLC-MS/MS, modified mycotoxins, glucoamylase, α-
24 amylase.

1. Introduction

Cereal-based products are one of the most important contributors to human diet. Besides their energetic value they are also rich in health promoting compounds such as dietary fiber, antioxidants, B group vitamins and minerals (Gani, SM, FA, & Hameed, 2012). However, also various residues and contaminants may be contained in cereal products. One of the predominant risks associated with their consumption is the exposure to mycotoxins. The major mycotoxins occurring in cereals are *Fusarium* toxins. They are responsible for the contamination with deoxynivalenol (DON), nivalenol (NIV), T-2 and HT-2 toxins, zearalenone (ZEN), fumonisins (FBs) in maize and small cereals, which besides serious consequences on human and animal health, also cause a huge economic impact for the agriculture sector of many countries (Lee & Ryu, 2017; Marroquín-Cardona, Johnson, Phillips, & Hayes, 2014).

One of the "emerging" food safety issues related to mycotoxins in cereals are the modified forms. They can be produced by the *fungi* or be a part of the plant defense mechanism, which is represented by the activity of plant detoxification enzymes (Berthiller et al., 2013). Considering their proven bioavailability and toxicity in humans and animals, their co-occurrence with parental forms can increase the adverse effects associated to each mycotoxin (De Saeger & van Egmond, 2012). Also, there are several studies investigating their formation from the perspective of an industrial food production process (Freire & Sant'Ana, 2018; Schwarz, 2017; Vidal, Morales, Sanchis, Ramos, & Marín, 2014). As opposite to the parental forms, the modified mycotoxins are not regulated by the European Union legislation. Considering the occurrence and toxicity data, FAO/WHO Joint Expert Committee on Food Additives (JECFA) extended the provisional maximum tolerable daily intake (PMTDI) from DON to a group of DON, 3-acetyl-deoxynivalenol (3AcDON) and 15-acetyl-deoxynivalenol (15AcDON), the information regarding deoxynivalenol-3-glucoside (DON-3-Glc) being insufficient to consider its inclusion in the group (JECFA/FAO, 2011). European Food Safety Authority (EFSA) recently assessed the risk to human and animal health related to the presence of DON, 3AcDON, 15AcDON and DON-3-Glc, estimating the occurring relative concentrations of the modified forms to the parental form as 10, 15 and 20%, respectively (Knutsen et al., 2017). Also, the report concludes that although the European population exposure to DON and its modified forms is below the acute reference dose (ARfD) of 8μg/kg bw a regular exceedance of the TDI (1μg/kg bw) can present a potential health concern.

59 Infants are the population group which is the most sensitive to mycotoxin exposure.
60 Moreover, according to EFSA scientific opinion related to the human and animal health
61 risk associated to the presence of mycotoxins, in the case of ZEN, DON and its
62 modified forms, 3AcDON, 15AcDON and DON-3-Glc, infants showed the highest
63 acute and chronic dietary exposure compared to other groups of population (EFSA,
64 2014; Knutsen et al., 2017). Commonly used cereals for baby food production are oats,
65 wheat, barley, maize, rye, triticale, sorghum, millet and pseudo-cereals, such as quinoa,
66 buckwheat and amaranth (Klerks et al., 2019). Besides the already mentioned *Fusarium*
67 mycotoxins, cereals were found to contain their modified forms (Bryła et al., 2016;
68 Gottschalk, Barthel, Engelhardt, Bauer, & Meyer, 2007; Lee & Ryu, 2017; Pleadin et
69 al., 2013). The maximum levels for *Fusarium* mycotoxins in cereals and cereal-based
70 baby food are stipulated by the Commission Regulation No 1881/2006 and its annex:
71 750 and 200 μg/kg for DON in flour and cereal based baby food, respectively; 75 and
72 20 μg/kg for ZEN in cereal flour and cereal-based infant food, respectively (European
73 Commission, 2006). Commission Recommendation 165/2013/EU on the presence of T-
74 2 and HT-2 toxin in cereals and cereal products stipulates indicative levels for the sum
75 of the two toxins at 200, 100, and 50 μg/kg for oats, maize, and other cereals,
76 respectively as well as 15 μg/kg for cereal-based foods for infants and young children,
77 (European Commission, 2013).
78 From the technological perspective, cereal-based baby food production process is
79 relatively simple, however the safety requirements are more demanding compared to
80 other production processes in the food industry (European Commission, 2006a). It is
81 characterized by four main steps: flour roasting, pre-gelatinization and enzymatic
82 hydrolysis, enzymes inactivation and drying. Considering the raised questions related to
83 the safety of the enzymes in food products (*e.g.* allergies), the producers of cereal-based
84 baby foods are intending to eliminate the amylolytic treatment from the production
85 process. Nonetheless, no information is available regarding the nutritional
86 characteristics of a product obtained this way. Thus, the present study will focus on two
87 production stages: (1) flour roasting and (2) pre-gelatinization and enzymatic hydrolysis
88 steps. Flour roasting usually takes place at a temperature range between 105°C and
89 120ºC, with a duration ranging from 20 min to 40 min. This step is important for the
90 modulation of the organoleptic characteristics of the cereal flour and also improves its
91 dispersibility in water during the next production step (Fernández-Artigas, Guerra-
92 Hernández, & García-Villanova, 1999). Pre-gelatinization represents the loss of

93 crystalline structure of the starch and its swelling, process that makes it available to the
94 amylolytic enzymes. From the perspective of the possible mitigation effect on
95 mycotoxin contamination of the abovementioned production steps, there are no studies
96 specifically dedicated to cereal-based baby food products, however some available
97 publications deal with the fate of mycotoxins during roasting and enzymatic production
98 steps in other foodstuffs (*e.g.* malting, brewing, breadmaking) (Hazel & Patel, 2004;
99 Lancova et al., 2008; Pietri, Bertuzzi, Agosti, & Donadini, 2010; Vidal et al., 2014).
100 Yumbe-Guevara, Imoto, & Yoshizawa (2003) studied the thermal degradation of DON,
101 ZEN and NIV during the roasting of barley kernels and flour. They observed the first
102 significant effect on the reduction of mycotoxins at 180 ºC treatment, achieving up to 90
103 and 80% reduction in DON (11 min treatment) and NIV (60 min treatment),
104 respectively. For ZEN, no significant effect on toxin concentration was observed at 140
105 and 160ºC, however 60 min at 220ºC were needed to achieve an 85% reduction in
106 barley flour. With regard to the effect of the enzymatic reactions applied in food
107 industry, α-amylase was proven to lead to up to 10% increase in DON (Vidal,
108 Ambrosio, Sanchis, Ramos, & Marín, 2016), result also found by Zachariasova,
109 Vaclavikova, Lacina, Vaclavik, & Hajslova (2012) during brewing an breadmaking.
110 This increase was partially explained by the cleavage of DON-3-Glc under the
111 enzymatic activity in both studies mentioned above, factor to be possibly considered for
112 the initial contamination of the selected batches of raw materials. For the loss of T2-
113 toxin parallel to increased values for HT2-toxin in grain analysis it is assumed that the
114 conversion is an enzyme mediated process due to deacetylation of T2-toxin (Maul,
115 Pielhau, & Koch, 2014). Also, enzymes are suggested to have a potential for mycotoxin
116 mitigation when part of processing operations, nonetheless it cannot be considered for
117 dealing with the products that do not comply with the existing regulation (Karlovsky et
118 al., 2016). Consequently, various thermal or enzymatic processed may interfere in
119 different ways with the *Fusarium* mycotoxin content of a processed grain sample.
120 The present study aims to identify the impact of roasting and enzymatic hydrolysis
121 processes on DON, acetylated DON (3- and 15AcDON), DON-3-Glc, ZEN and T-2 and
122 HT-2 toxins in *Fusarium* infected cereal flour under typical conditions used in infant
123 food production. The three most frequently used cereal flours will be used for the
124 experiment: oats, wheat and barley. The treatment parameters will be set as close as
125 possible to the ones used during the cereal-based baby food production process. Results

126 will help understand the possible changes in mycotoxin concentration during these
127 production steps considering the compositional differences in the used cereal flours.
128

129 ## 2. Materials and methods

130 ### 2.1 Chemicals and reagents

131 All reagent grade mycotoxin standards (DON, 3-Ac-DON, 15-Ac-DON, ZEN, T-2 and
132 HT-2 toxins) including the unlabeled and the U-[^{13}C]-labelled were purchased from
133 Romer Lab Diagnostic (Tulln, Austria). Methanol and acetonitrile as well as magnesium
134 sulfate, formic acid and ammonium formate were bought from Sigma-Aldrich (Merck
135 KGaA, Darmstadt, Germany). The amylolytic enzymes (α-amylase and
136 amyloglucosidase) were kindly provided by Hero Spain (Alcantarilla, Spain). Malic
137 acid and sodium hydroxide were purchased from VWR Chemicals (Radnor,
138 Pennsilvania, USA); sodium chloride, glucose and sodium potassium tartrate were
139 bought from Fisher Scientific (Hampton, New Hampshire, USA); sodium azide and
140 sodium citrate dihydrate were acquired from Scharlab (Sentmenat, Spain). The 3,5-
141 dinitrosalicylic acid (DNS) was bought from Acros Organics (New Jersey, USA), the
142 citric acid was acquired from PRS Panreac (Barcelona, Spain) and the starch was
143 purchased from Sigma Aldrich (St. Louis, Missouri, USA).

144 ### 2.2 Preparation of mycotoxin contaminated flour

145 Three different cereal flours were purchased for the experiment from a local
146 supermarket in Lleida (Spain), namely barley, oat and wheat flour. A part of each was
147 inoculated with *Fusarium graminearum* strain (F.46) obtained from the strains
148 collection of the Food Technology Department of the University of Lleida.
149 Before inoculation the flours (500 g portions) were put in ISO bottles and sterilized in
150 an autoclave. Afterwards, the flours were aseptically transferred to Petri dishes and 2
151 mL of sterile MilliQ water was added to each Petri dish, which were later stored
152 overnight at 4ºC to achieve a water activity of 0.99 (Aqualab, Serie 3 TE, Decagon
153 Devices, Washington, USA). Then, 1 mL of spore suspensions of *F. graminearum* (10^6
154 spores/mL) was sprayed on each dish. Petri dishes with barley, wheat and oat flour were
155 then incubated at 25 °C for 21 days. Afterwards, the contaminated flours were dried at
156 40 °C, homogenized and DON and ZEN levels were determined.
157 To obtain the required mycotoxin concentration, the contaminated flour was mixed with
158 uncontaminated one in certain proportions. For each of the treatments a separate flour

159 mix was prepared. Table 1 regroups the initial concentrations of the analyzed
160 mycotoxins in the three matrices.

161 **2.3 Flour roasting**

162 The roasting of the contaminated flour was performed using a convection oven
163 (Eurofred, Barcelona, Spain). To confirm that the right temperature was applied
164 throughout the entire treatment time, a temperature logger (Plug&Track, Progres Plus,
165 Willems, France) was used inside the oven. For each designed setup (temperature *vs*
166 time), 50g of contaminated sample were weighted and placed in aluminum trays
167 (32x21x5cm) in order to form a very thin layer (approximately 1 cm) to ensure that the
168 oven temperature is easily reached inside the sample. Two temperature levels were
169 chosen, namely 105 and 120ºC with a treatment length of 30 and 40 min each. Every
170 sample was processed in triplicate and collected at the end of the programmed time. A
171 total of 12 samples of each flour type (barley, wheat and oat) were obtained. The treated
172 samples were cooled down and stored in a dry place at room temperature until their
173 analysis.

174 **2.4 Enzymatic treatment**

175 Before proceeding to the enzymatic treatment of the cereal flour, it was roasted at 105ºC
176 for 30 min. Then, the obtained roasted flour was cooled and 30g samples were prepared
177 by mixing a respective amount of the contaminated flour with the roasted blank flour.
178 The mix was placed in a 100 mL beaker and the enzymes (a-amylase and glucoamylase)
179 were added. The available information of the used doses in cereal-based baby food
180 production process is scarce and non-specific, thus it was decided to add the chosen
181 enzymes simultaneously at the same dose in each sample: each of the enzymes was
182 added in two dosages to the samples: 2.1 and 4 g of enzyme/kg of cereal flour. The
183 53ºC temperature was chosen for the present study as it corresponds to the temperature
184 allowing the activity of both enzymes. Once the flour and enzyme mix was ready, 70
185 mL of distilled water at 53ºC was added and the slur was put into a water bath for 10,
186 50 and 90 min. Samples for each treatment were prepared in parallel and per triplicate
187 for every designed setup. When the time expired, the samples were rapidly cooled in an
188 ice bath, frozen and lyophilized (Telstar LyoBeta 15, Terrassa, Spain). The obtained
189 lyophilized samples were then packed individually and stored in a dry place at room
190 temperature until analysis.

2.5 Assessment of the enzymatic activity

The activity of the added α-amylase and glucoamylase was quantified throughout the experiment to evaluate if there were differences in starch hydrolysis related to each studied matrix.

2.5.1 α-amylase activity

The activity of the α-amylase used during the experiment was determined according to Vidal et al. (2016). Briefly, 1.5 g of treated flour was weighted in 12 mL polypropylene tubes and 10 mL of extraction solvent (0.2M malic acid:0.35M sodium hydroxide:0.2M sodium chloride:0.003M sodium azide, 1:1:1:1, *v/v/v/v*) was added. The obtained mix was incubated in a water bath for 20 min at 40ºC. Then, 0.5 mL of the supernatant was mixed with 1.5 mL substrate (1g of starch in 20 mL of 1M sodium hydroxide, 10 mL of 1.06M sodium and potassium tartrate and 70 mL water). It was left to interact for 3 min at 25ºC, followed by the addition of 1 mL of DNS reagent (1g of DNS, 20 mL of 2M sodium hydroxide, 10 mL of 1.06 M sodium and potassium tartrate and 70 mL water) and boiled in a water bath for 5 min. The whole mix was cooled down in a cold-water bath and 10 mL of distilled water were added. The absorbance of the obtained solution was measured in an UV-visible spectrophotometer (Helios γ, Thermo Electron Corporation, Waltham, Massachusetts, USA) at a wavelength of 540 nm. The results were compared to a glucose standard curve with concentrations ranging from 1 to 10 mg of glucose/mL (r^2=0.99). The corresponding dilution factor was applied to the analysed samples.

2.5.2 Glucoamylase activity

Glucoamylase activity in all the samples was evaluated according to the method described by Puri et al. (2013). The enzymatic activity was expressed in international units (IU), one IU being defined as µmol of glucose per min and milliliter under the standard assay conditions using a glucose standard curve. Briefly, 1g of treated flour was added to 10 mL of 0.1M citrate buffer (pH=5) and stirred at 250 rpm for 30 min. Then, the mix was centrifuged at 5000 rpm for 20 min (Hettlich Universal 320R, Tuttlingen, Germany) and 1 mL of supernatant was mixed with 1mL of 1% maltose in 0.1M citrate buffer (pH=5). The obtained extract was then incubated in a water bath for 30 min at 45ºC. The reaction was stopped by adding 3 mL of DNS reagent (composition described above) and submitted to a boiling water bath for 15 min. One mL of 40% solution of sodium potassium tartrate and 1 mL of distilled water were added prior to

224 cooling the extract to room temperature. Afterwards, absorbance was measured at 575
225 nm using an UV-visible spectrophotometer and the results were compared to a standard
226 curve using glucose at concentrations between 1 to 10 mg of glucose/mL (r^2=0.99),
227 applying the appropriate dilution factor.

228 **2.6 Mycotoxin extraction, detection and quantification**

229 *2.6.1 Sample preparation*

230 The sample preparation method is based on the draft WI 00275287 of the pre-norm
231 FprN 17279 (DIN, 2018), describing a LC-MS/MS screening method developed under
232 CEN/TC 275/WG5. Briefly, 5g of sample were weighted in a 50 mL polypropylene
233 tube, mixed with 20 mL acetonitrile/water (50:50, *v/v*), shaken for 30 min (Multi Reax,
234 Heidolph Instruments GmbH & Co.KG, Schwabach) and centrifuged for 30 min at 4500
235 rpm (Microfuge R, Beckman Coulter, Brea, California, USA). Then, 1 mL of the
236 supernatant was taken and mixed with 100 µL of the internal standard solution and
237 100µL of MiliQ water. Afterwards, 250 mg of anhydrous magnesium sulfate was
238 added, vortexed for another 30 seconds, centrifuged for 5 min at 14000 rpm (Microfuge
239 R, Beckmann, Krefeld, Germany) and 200 µL of supernatant were diluted with 400 µL
240 water and submitted to HPLC-MS/MS analysis.
241 In order to minimize the losses of the more hydrosoluble analytes during phase
242 separation step, the sample preparation before injection for the initial screening of the
243 mycotoxins followed only the first four steps of the protocol described above, namely
244 mixing the 5g of sample with 10 mL water and 10 mL acetonitrile, followed by shaking,
245 centrifuging and taking 1 mL of the supernatant for injection into the LC-MS/MS.

246 *2.6.2 LC-MS/MS analysis*

247 A Shimadzu Nexera X2 HPLC system equipped with a binary pump and a thermostatic
248 autosampler (Kyoto, Japan), coupled with a triple quadrupole mass spectrometer
249 QTRAP 6500+ (SCIEX, Framingham, Massachusetts, USA) were used. Data
250 acquisition and processing was achieved using Analyst® and MultiQuant® software
251 (SCIEX, Framingham, Massachusetts, USA). Separation was achieved on Restek
252 Raptor Fluoro phenyl 100x2.1 mm, 5µm column (Bellefonte, Pennsylvania, USA).
253 The mobile phase consisted of water (A) and methanol (B), both containing 0,1%
254 formic acid and 300 mg/L ammonium formate, which was supplied at a gradient with a
255 flow rate of 0.5 mL/min. The initial gradient was kept at 2% B for 0.8 min, after 4 min
256 B was increased to 53%, after 6 min B was set at 60%, after 11 min B was 95%,

257 followed by 1.5 min washout at 95% B and a 5 min equilibration period at the initial
258 conditions. Total run time was 17.5 min.
259 The detector was operated in both positive and negative electrospray ionization (ESI)
260 modes under multiple reaction monitoring (MRM). Operating ESI conditions were
261 setup as follows: curtain gas: 40 psi, gas 1: 60 psi, gas 2: 35 psi, collision gas flow:
262 medium and source temperature: 300 °C. Ion spray voltage (IS) was set at 4500 V and -
263 4000 V in positive and negative ionization mode respectively. Two characteristic ions
264 were chosen for the assessment of the mycotoxins in the samples for quantification and
265 for qualitative confirmation of the analytes. To compensate for matrix effect, stable
266 isotope dilution assay was performed using U-[^{13}C]-labelled mycotoxins. Internal
267 standard concentrations were 35.6 ng/mL for DON, 34.3 ng/mL for ZEN, 8.6 ng/mL for
268 3-AcDON, 4.3 ng/mL for 15-AcDON, 2.9 ng/mL for T2 and 2.9 ng/mL for HT2 in the
269 final sample submitted to LC-MS analysis. The recovery rate for DON, ZEN, AcDON,
270 T2 and HT2 toxin was determined by tenfold spiking and analysis of blank oat samples.
271 The intraday precision calculated from these spiked samples was ranging from 2.5 %
272 for DON up to 12.7 % for 15-AcDON. Table 2 additionally regroups the data
273 concerning the selected ion transitions, the parameters of the mass detector specific for
274 each fragment ion as well as the individual recoveries and limits of detection (LOD) and
275 quantification (LOQ). LOD was determined by spiking blank samples and the LOQ was
276 derived from it according to Wenzl, Haedrich, Schaechtele, Robouch, & Stroka (2016).
277 Rather high spiking levels of T-2 and HT-2 toxins compared to the levels found in the
278 samples led to obtention of higher than expected LOD and subsequently LOQ for both
279 analytes; however, semi-quantitative results are shown.

280 **2.7 Data Evaluation**

281 MultiQuant™ software (SCIEX, Framingham, Massachusetts, USA) was used for the
282 LC-MS/MS data handling and evaluation of the analyzed samples. ANOVA analysis
283 was applied to the obtained results to determine the significance ($p=0.05$) of the
284 observed changes.

285 **3. Results and discussion**

286 **3.1 Initial screening of mycotoxins**

287 Barley, wheat and oat flours used in the present study were intentionally contaminated
288 with a *Fusarium graminearum* strain, mainly producing DON and ZEN. Reports also
289 suggested the ability of *F. graminearum* strains of producing some of their modified

290 forms, such as 3-Ac-DON, 15-Ac-DON, α-zearalenol (α-ZEL), β-zearalenol (β-ZEL)
291 and others (Hagler, Mirocha, Pathre, & Behrens, 1979; Mirocha, Abbas, Windels, &
292 Xie, 1989). Besides the abovementioned "expected" mycotoxins and their modified
293 forms, the study seeks to evaluate the possible presence of the "emerging" mycotoxins,
294 such as enniatins, beauvericin, sterigmatocystin, which might occur in high frequency
295 or high concentration in cereals and cereal products originating from a natural field
296 contamination (Gruber-Dorninger, Novak, Nagl, & Berthiller, 2017).
297 To achieve this aim and decide on the mycotoxins that, additional to DON and ZEN,
298 should be focused on in this study, an initial screening of mycotoxins was performed on
299 two groups of samples for each matrix (barley, wheat and oat): (i) samples before
300 treatment and (ii) samples treated with a dosage of 4.0 g of enzyme/kg of flour, during
301 90 min. This screening was also aiming to search for the presence of DON-3-Glc,
302 previously reported as being formed as a result of plant enzymatic activity (Ronald
303 Maul et al., 2012; Vidal et al., 2016).
304 The initial screening omitting the phase separation step that would discriminate the
305 most polar compounds led to the identification of the presence of several emerging
306 mycotoxins, mainly produced by *Fusarium* species. The mycotoxins found in all the
307 samples were DON, 3-Ac-DON, 15-Ac-DON, and ZEN. T-2 and HT-2 toxins were
308 only found in oat flour, both before and after the enzymatic treatment. In the case of the
309 "emerging" mycotoxins, enniatin B (ENN B) was present in all the samples, however it
310 showed a relatively low signal intensity. Beauvericin (BEA) was found in barley and
311 oat samples but not in wheat. Sterigmatocystin and enniatin B1 (ENN B1) were only
312 present in oat samples, however also in very low amounts. The findings of the initial
313 screening of mycotoxins were in accordance to the occurrence studies available (Bryła
314 et al., 2016; Lindblad et al., 2013; Santini, Meca, Uhlig, & Ritieni, 2012).
315 At the end of the present screening, it could be seen that the analyzed samples presented
316 a certain heterogeneity, especially in contamination with "emerging" mycotoxins, which
317 brought the work to the need of focusing the study to the quantification of the most
318 abundant following analytes: DON, 3-and 15-AcDON, ZEN and, in the case of oat
319 flour, T-2 and HT-2 toxins were included, but the data need to be considered as only
320 semi-quantitative due to their low content levels.

321 **3.2 Roasting**

322 In food processing, roasting is an important step in flavor modulation and enhancement
323 through caramelization and Maillard reactions taking place on the surface of the
324 product. Besides, cereal roasting is one of the processes thought as potentially being
325 able to reduce the initial mycotoxin charge. Table 1 regroups the initial concentration of
326 mycotoxins in the prepared contaminated flours which were submitted to roasting.
327 Figure 1 shows the changes in mycotoxin concentration in the three flours after
328 applying each combination of time and temperature.

329 For DON, no significant change in concentration was observed in none of the three
330 matrices studied. Although in barley the effect of the treatment was more pronounced at
331 105ºC after 30 and 40 min roasting ($p=0.05$), the observed trend is not aligned with the
332 one in wheat and oat flours (no significant change in the level of mycotoxin). Similarly,
333 no change of DON content during roasting was reported several times (Milani &
334 Maleki, 2014; Yumbe-Guevara et al., 2003), showing that at least a heat treatment of
335 160ºC for 15 min is needed in order to achieve a reduction of 10% in bread and biscuits
336 baking. However, Kaushik (2015) in its review reported studies showing no effect of
337 baking in breadmaking process on DON concentration nor after 30 min at 170ºC, nor
338 after 30 min at 205ºC.

339 There are many recent studies investigating the fate of DON during food processing
340 (Malachova et al., 2010; Pronyk, Cenkowski, & Abramson, 2006; Vidal et al., 2014),
341 however regardless its frequent co-occurrence with 3-AcDON and 15-AcDON, studies
342 on the alteration of these DON conjugates contents are scarce (Wu & Wang, 2016). In
343 the present work, 15-AcDON was present in the three matrices, however 3-AcDON
344 (Fig. 2a) was present only in barley, probably due to small differences in toxin
345 production of the fungus used for flour treatment related to individual matrix
346 composition of barley, wheat and oat (Mirocha et al., 1989). 3-AcDON showed a very
347 similar trend of a slight toxin level decrease like DON in barley (reduction of the level
348 of the toxin after 30 min at 105ºC and its increase after 40 min at the same temperature),
349 which can be explained by its conversion into the parent compound. Nonetheless, the
350 low absolute amounts of 3-AcDON compared to DON and its presence exclusive for
351 treated barley flour cannot doubtlessly confirm this assumption. No significant
352 differences related to the applied treatment are observed for 15-AcDON in any of the
353 flours.

354 ZEN shows a decreasing tendency during the roasting process at both temperatures,
355 however none of the changes in concentration were significant. This matches the
356 previous studies investigating ZEN thermal stability during roasting, which had proven
357 its high resistance to temperature treatment (Kabak, 2009; Yumbe-Guevara et al., 2003).
358 Besides the *F. graminearum* toxins coming from the intended contamination in the
359 laboratory, the analysis of the oat flour identified the presence of T-2 and HT-2 toxins.
360 Due to the low levels identified, the derived content data have to be considered as a
361 semi-quantitative assessment. The toxins were proven as relatively stable with only
362 slight changes compared to the initial level found before the treatment was applied. The
363 result shows more stability compared to the study performed by Kuchenbuch, Becker,
364 Schulz, Cramer, & Humpf (2018), where the decrease of the toxins during roasting
365 represented approximately 5%, however their experiment included two previous
366 operations which led to the main decrease in both toxins. Perhaps, an intended
367 contamination with these toxins could help better explain their fate during roasting.
368 Despite the expected effect of the relatively long treatment time compensating for the
369 low roasting temperature from the perspective of mycotoxins reduction, no significant
370 impact of the roasting process on mycotoxin levels was observed.
371 Additionally, no significant correlation between different mycotoxins was recorded.

372 ### 3.3 Enzymatic treatment

373 There are no studies available on the impact of enzymatic processes on *Fusarium*
374 mycotoxin levels during baby food production process, however enzymes are also
375 commonly used in food production processes either with the aim of achieving the
376 specific characteristics of the final product (*e.g.* brewing) or for the improvement of the
377 technological quality and shelf life (*e.g.* breadmaking, production of fruit juice)
378 (Juodeikiene, Basinskiene, Vidmantiene, & Bartkiene, 2009; Kostelanska et al., 2011;
379 Malachova et al., 2011; Vidal et al., 2016).
380 Two enzymes were used in the present study (α-amylase and glucoamylase) at two
381 doses (2.1 and 4.0 g of enzyme/kg of flour). According to the usual conditions of such
382 enzymatic treatments the first samples have been taken after homogeneous slurry has
383 been obtained, *i.e.* after 10 min of incubation with the enzymes. Table 3 regroups the
384 information on the units of glucose per minute transformed as a result of the presence of
385 the amylolytic enzymes in 1 g of flour. It can be seen that the activity changes
386 depending on the matrix used, which is probably related to the differences in the

387 structure of the starch in the different cereals which has implications on the efficacy of
388 the reaction (Gupta, Gigras, Mohapatra, Goswami, & Chauhan, 2003).
389 Figure 3 illustrates the changes in concentration of DON, 15-AcDON and ZEN during
390 enzymatic hydrolysis caused by two amylolytic enzymes in barley, wheat and oat
391 flours. The obtained results indicate a relatively unchangeable levels of DON in the
392 three matrices (barley, wheat and oat) and even a slight decrease when 2.1 g of
393 enzyme/kg flour was added (between 1.5 and 4%), however it is not proved statistically
394 significant. One similar study of Vidal et al. (2016) reports on the increase in DON (up
395 to 10%) due to α-amylase activity, mainly as a result of their release from the cereal
396 matrix, however the temperature used for bread fermentation was 30 and 45ºC, which
397 could have been the reason of the different result obtained.
398 In the case of 15-AcDON, which was found in all of the three studied flours, a deviating
399 behavior during the process was observed, manifesting a slight increasing trend in
400 barley flour and decreasing in wheat and oat flour. For barley flour, the tendency was
401 more pronounced at a higher enzyme concentration. Wheat flour was characterized by a
402 higher and clear decrease of 15-AcDON at 2.1 g of enzyme/kg flour dose compared to
403 the 4.0 g/kg after 90 min treatment. In the oat flour, a trend of 15-AcDON decrease over
404 time similar to wheat flour was registered, however at a much smaller extent. 3-AcDON
405 which was found only in barley flour, was following a very similar trend to its isomer
406 15-AcDON during the enzymatic treatment (Fig. 2b). There are no available studies
407 focusing on these modified forms of DON which the present work could relate to in
408 order to explain the observed, although small, changes in concentration during the
409 enzymatic process applied.
410 ZEN content remained almost unchanged throughout the whole process at both doses of
411 the enzymes applied in barley, wheat and oat flours. However, the high level of ZEN
412 present in the flour samples might prevent the detection of small absolute alterations
413 throughout the processes. Thus, more studies are needed in order to identify more
414 certainly the behavior of the toxin during enzymatic processes in food industry.
415 T-2 and HT-2 toxin contents in oat did not undergo any significant change at none of
416 the two applied doses of enzymes (Figure 4). Also, their identified low levels were of no
417 concern from the safety perspective investigated by the present study. There are no
418 available studies showing a possible effect of a treatment with amylolytic enzymes on
419 the levels of these toxins and no release from the matrix could be observed in the
420 present study. Other enzymes (such as esterase) are however proven to have the ability

421 of converting T-2 toxin into HT-2 toxin (Lattanzio, Visconti, Haidukowski, & Pascale,
422 2012).
423 A confirmatory small-scale experiment repeating the enzymatic treatment at 4.0 g
424 enzyme/kg flour and 90 min incubation time was performed in the three studied flours
425 (Table 4). All the results obtained before were confirmed and the same tendencies were
426 shown but for DON, which exhibited an increase of approximately 4.5 and 10% in
427 wheat and barley, respectively, nonetheless its concentration in oat flour remained
428 stable after the applied treatment. In general, the obtained data goes in line with an
429 already published report on DON and its 3-glucoside (Simsek, Burgess, Whitney, Gu, &
430 Qian, 2012).

431 ## 4. Conclusion

432 Cereal based baby food products are an important part of the diet for a very sensitive
433 group of population. The safety of these products is a priority for the food industry,
434 especially regarding their contamination with mycotoxins. The present study
435 investigated the change of concentration of *Fusarium* mycotoxins during two steps of
436 the production process: flour roasting and treatment with amylolytic enzymes
437 (glucoamylase and α-amylase).
438 Roasting did not have any significant impact on none of the analyzed mycotoxins, most
439 probably due to the relatively low temperatures applied during the process. It is
440 important to mention also the stability of the acetylated forms of DON, which were still
441 present in the flours after the treatment was applied. Thus, also in treated infant food
442 samples conventional analytical methods do not cover this additional source of toxic
443 potential as acetylated DON remains modified and does not add to DON following
444 deacetylation. The 15-AcDON showed a slight increase in barley flour and a relatively
445 small decrease in oat and wheat flours, however these changes could not be confirmed
446 after the statistical evaluation of the data. ZEN remained stable throughout both
447 treatments applied. The repeated small-scale enzymatic treatment (using the highest
448 enzyme amount and the longest treatment time) only confirmed and strengthened the
449 obtained results.
450 In summary, it can be concluded that the cereal based baby food production process
451 does not lead to a significant decrease of *Fusarium* mycotoxins during the studied steps.
452 Also, no conversion of the acetylated DON into DON was observed. More extreme
453 physical parameters could enhance the impact on the studied mycotoxins, however the

454 applicability of the new conditions from the technological (i.e. cost, yield, organoleptic
455 properties) and safety perspectives (i.e. acrylamide formation) should be considered
456 first. However, also no additional toxin amounts were deliberated from naturally
457 contaminated matrix due to the applied techniques. This results in the need of a
458 particularly careful evaluation of the quality of the raw materials with regards to
459 mycotoxin contamination, especially considering that there are more strict requirements
460 in the legislation (EC 1881/2006) concerning the maximum levels for mycotoxins in
461 cereal-based infant food as compared to cereals intended for human consumption.

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466

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645

646

Figure 1

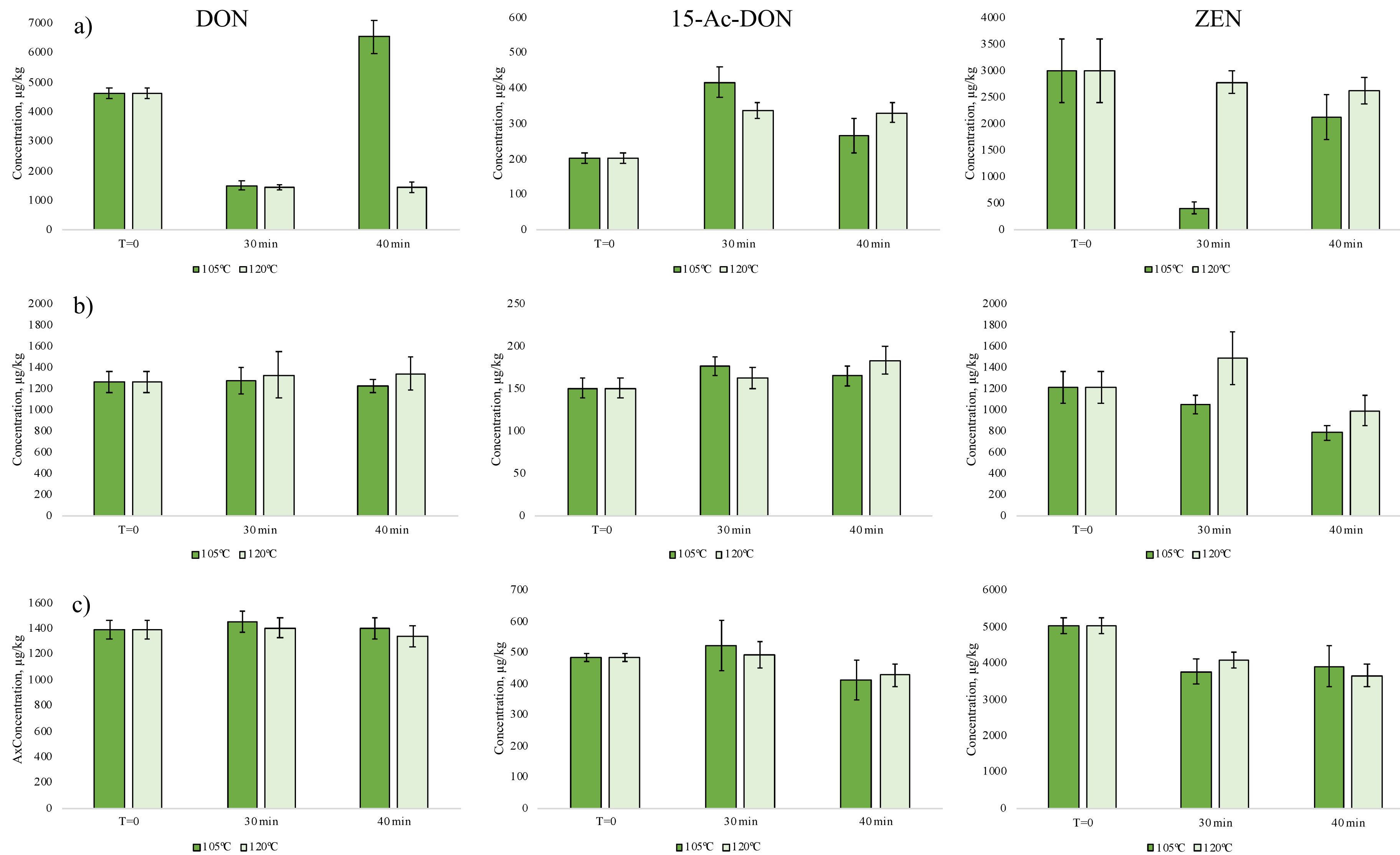

Figure 1: Concentrations of deoxynivalenol (DON), 15-acetyl-deoxynivalenol (15-Ac-DON) and zearalenone (ZEN) (±SD) in barley (a), wheat (b) and oat (c) during roasting at 105 and 120ºC for 30 and 40 min.

Figure 2

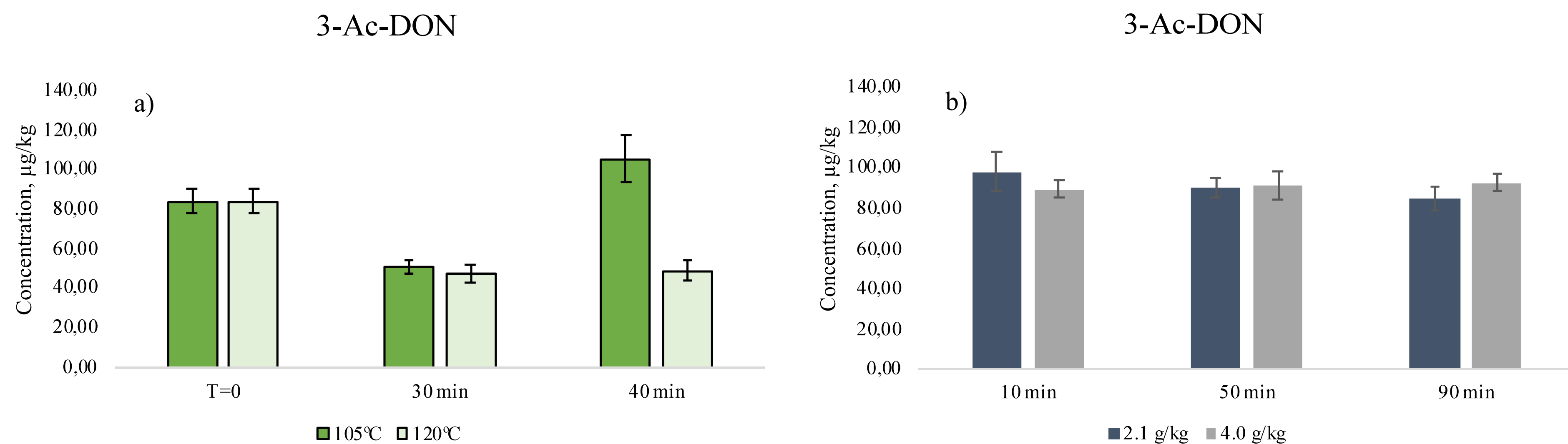

Figure 2: Changes in 3-acetyl-deoxynivalenol (3-Ac-DON) concentration (±SD) during barley roasting at 105 and 120ºC for 30 and 40 min (a) and during enzymatic treatment at a dose of 2.1and 4.0 g/kg during 10, 50 and 90 min (b).

Figure 3

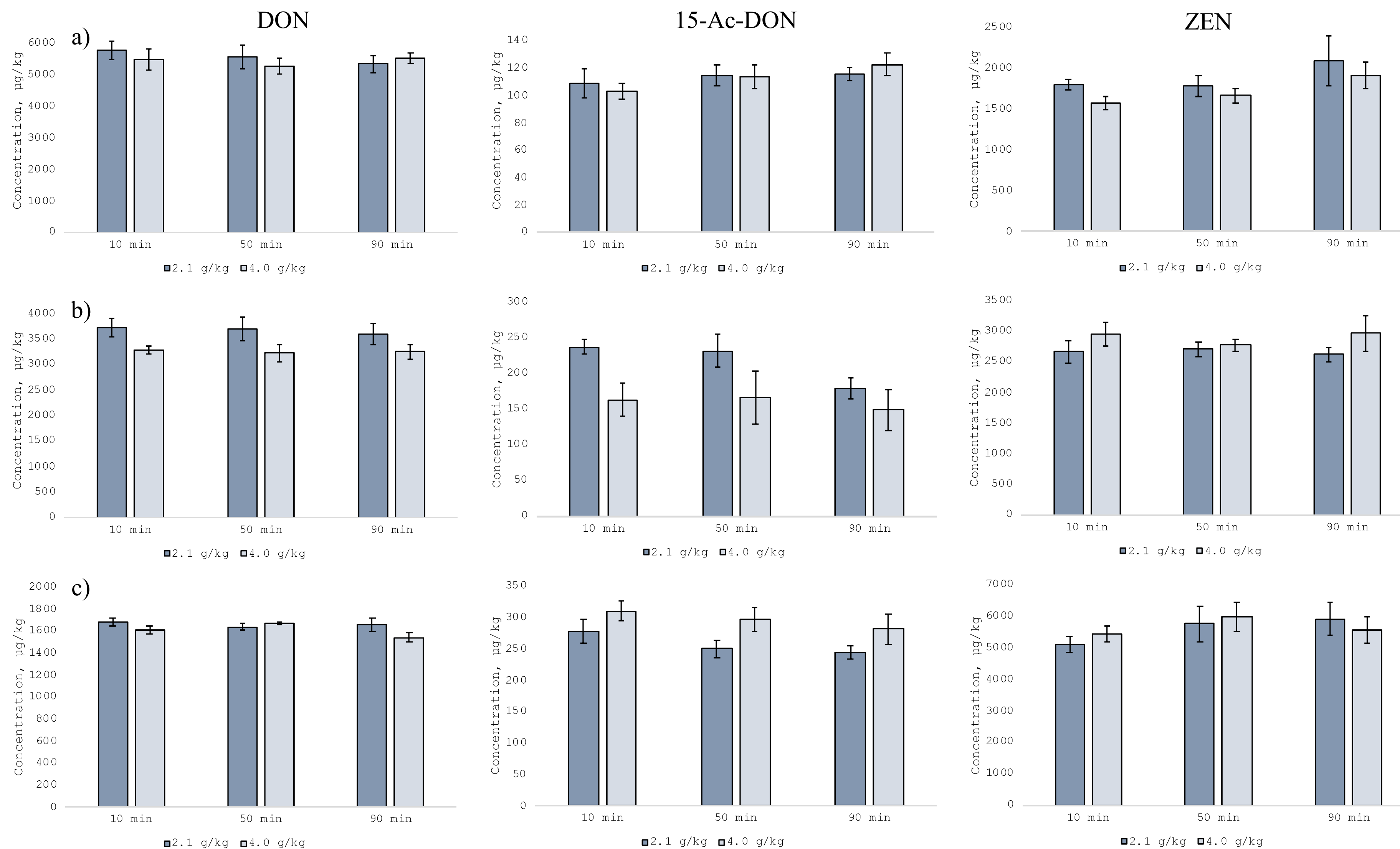

Figure 3: Concentrations (±SD) of deoxynivalenol (DON), 15-acetyl-deoxynivalenol (15-Ac-DON) and zearalenone (ZEN) in barley (a), wheat

(b) and oat (c) during enzymatic treatment (α-amylase and glucoamylase) at a dose of 2.1 and 4.0 g/kg during 10, 50 and 90 min.

Figure 4

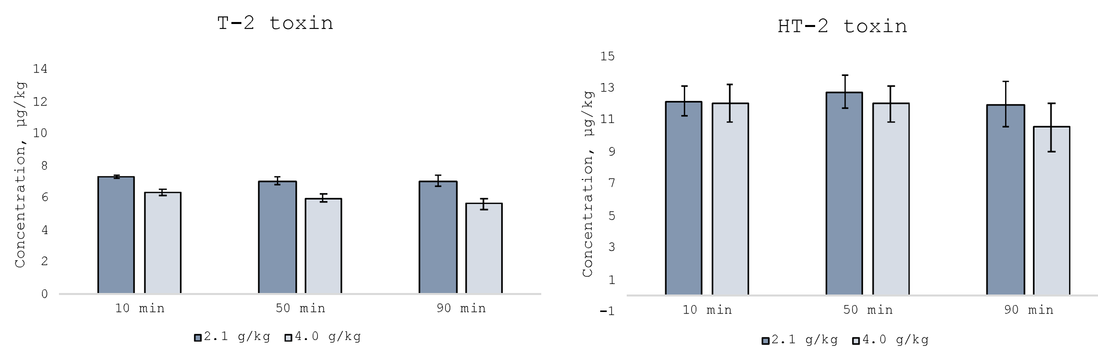

Figure 4: Concentrations of T-2 and HT-2 toxins in oat during enzymatic treatment at a dose of 2.1 and 4.0 g/kg during 10, 50 and 90 min. HT-2 toxin evaluated referring to LOD.

Table 1

Table 1: Initial mycotoxin concentrations obtained in barley, wheat and oat flour

Matrix	Mean concentration, µg/kg±SD						
	DON	DON-3-Glc	3AcDON	15AcDON	ZEN	T-2 toxin	HT-2 toxin
Barley	4610±180	n.d.	83.5±6.2	201±14.7	2991±593	n.d.	n.d.
Wheat	1254±100	n.d.	n.d.	150±12	1209±152	n.d.	n.d.
Oat	1390±72	n.d.	n.d.	482±13.3	5027±219	1.4±0.07	3.85±0.29

n.d. = not detected; DON=deoxynivalenol; DON-3-Glc=deoxynivalenol-3-glucoside; 3AcDON=3-acetyl-deoxynivalenol; 15AcDON=15-acetyl-deoxynivalenol; ZEN=zearalenone;

Table 2

Table 2: Acquisition parameters used for the targeted analytes and recovery rates calculated from the analysis of spiked blank oat flour.

Mycotoxin	Retention time, min	ESI[1] mode	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)	Declustering potential (V)	Spiked concentration [µg/kg]	Recovery, %	LOD [µg/kg]	LOQ [µg/kg]
DON	1.4	ESI+	297.1	**249.0**[4] 203.0	**17** 21	**66** 66	699.8	99.7	25.9	85.4
3-AcDON	3.4	ESI+	339.1	**231.0** 203.1	**15** 19	**31** 31	101.7	105.7	48.4	160
15-AcDON	3.3	ESI+	356.1	**137.0** 321.0	**21** 17	**31** 31	100.2	95.0	3.3	10.8
ZEN	5.9	ESI-	317.1	**131.0** 175.0	**-40** -34	**-75** -75	92.2	91.7	1.1	3.5
T2	5.2	ESI+	484.2	**305.1** 215.0	**17** 23	**51** 51	100.0	97.2	1.0	3.3
HT2	4.7	ESI+	442.1	**263.0** 215.0	**17** 19	**41** 41	100.0	111.6	11	36.3

DON=deoxynivalenol; 3-Ac-DON=3-acetyl-deoxynivalenol; 15-Ac-DON=15-acetyl-deoxynivalenol; ZEN=zearalenone; [1]Electrospray ionization;
LOD= limit of detection; LOQ=limit of quantification

Table 3

Table 3: Activity of α-amylase and glucoamylase in barley, wheat and oat flours after each sampling step during enzymatic treatment.

Sample treatment		**Enzymatic activity, mg glucose/g sample*min**			
		α-amylase		Glucoamylase	
Barley		*2.1 g/kg*[a]	*4.0 g/kg*	*2.1 g/kg*	*4.0 g/kg*
	10 min	ND[b]	ND	0.26±0.01	0.29±0.01
	50 min	ND	0.05±0.007	0.21±0.02	0.18±0.03
	90 min	0.03±0.003	0.14	0.19±0.01	0.19±0.01
Wheat					
	10 min	0.79±0.06	1.11±0.05	0.2±0.01	0.22±0.01
	50 min	1.07±0.04	1.22±0.07	0.23±0.01	0.24±0.01
	90 min	1.28±0.09	1.42±0.1	0.23±0.01	0.21±0.02
Oat					
	10 min	3.55±0.38	1.16	0.1±0.007	0.09
	50 min	4.54±0.25	0.88±0.1	0.11±0.01	0.10±0.01
	90 min	5.06±0.04	0.76±0.07	0.12±0.01	0.1±0.01

[a] dose of enzyme added to the flour for the treatment 2.1 and 4.0 g of enzyme/kg of flour.
[b] ND=not detected

Table 4

Table 4: Mycotoxin concentration after repeating the enzymatic treatment (4.0 g enzyme/kg flour, 90 min)

Analyte	Process step	Concentration, µg/kg±SD		
		Barley	Wheat	Oat
Deoxynivalenol	B.T.[a]	4728	1225	911
	E.T.[b]	5217±101	1280±26.5	920±68
3-Acetyl-deoxynivalenol	B.T.	84.7	<LOD	79.2
	E.T.	98.8±2,4	<LOD	76.6±5.4
15-Acetyl-deoxynivalenol	B.T.	179	178	237
	E.T.	209±34	77±16.1	233±33
Zearalenone	B.T.	1606	1380	1931
	E.T.	2151±141	1329±79	2087±271
HT-2 toxin	B.T.	<LOD	<LOD	<LOD
	E.T.	<LOD	<LOD	<LOD
T-2 toxin*	B.T.	<LOD	<LOD	2.03
	E.T.	<LOD	<LOD	1.8±0.2

[a] B.T. = before treatment; [b] E.T. = enzymatic treatment applied; LOD = Limit of Detection; * Values evaluated referring to the LOD.