Transfer of *Fusarium* mycotoxins from malt to boiled wort

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Abstract

The fate of deoxynivalenol, deoxynivalenol-3-glucoside, 3- and 15-acetyl-deoxynivalenol, zearalenone, α- and β-zearalenol and fumonisins (fumonisin B_1 and fumonisin B_2) through mashing and wort boiling was studied. Three different mycotoxin contamination scenarios were considered. In almost all samples an increase in the level of mycotoxins in wort was observed during mashing followed by a decrease after just 30 min of the process, with levels remaining constant until the end of boiling. Deoxynivalenol and its metabolites were reduced to their initial level contained in the malt before mashing, or even lower, however in none of the samples they were completely eliminated. Zearalenone was not quantitated at the end of boiling, although there was a significant initial level of ZEN. β-zearalenol remained unaltered during the process. Fumonisins were reduced by between 50 and 100 per cent during mashing and boiling.

Keywords: deoxynivalenol, zearalenone, fumonisins, masked mycotoxins, HPLC-MS/MS

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1. Introduction

Cereal grains might be contaminated by moulds both in the field and during storage. Apart from commodity losses, fungal contamination may represent a safety risk because of the synthesis of mycotoxins by certain strains. Barley, which is the main ingredient in beer production, is predominantly exposed to fungal infestation throughout FHB disease (Fusarium Head Blight) (Baenziger, Fredy, Nopsa, & Bockus, 2015). The main *Fusarium* mycotoxinogenic species involved in FHB in barley are *F. graminearum, F. avenaceum, F. culmorum* and, to a lesser extent, *F. proliferatum* (Nielsen, Cook, Edwards, & Ray, 2014). They are known as mainly being deoxynivalenol (DON), zearalenone (ZEN) and fumonisins (FBs) producers (Beccari, Senatore, Tini, Sulyok, & Covarelli, 2018). An important aspect that has been investigated during recent years are the modified forms of mycotoxins, which initially might be formed by the plant or the fungus (Berthiller et al., 2013; Rychlik et al., 2014; Zachariasova, Vaclavikova, Lacina, Vaclavik, & Hajslova, 2012). Concern was raised because of their proven bioavailability and toxicity in humans and animals (Freire & Sant'Ana, 2018). Considering the abovementioned, researchers began to be concerned with both their co-occurrence with parental forms and their origin in the final product (Kostelanska et al., 2011; Lancova et al., 2008; Zachariasova et al., 2008). Modified forms of mycotoxins are not yet included in the current European Union legislation, however, the FAO/WHO Joint Expert Committee on Food Additives (JECFA) has extended the tolerable daily intake for DON to 3- and 15-acetyldeoxynivalenol (3- and 15-ADON) (JEFCA/FAO, 2011). In a recently published EFSA scientific opinion on the risk of sum exposure of the population to DON and its metabolites, the important contribution of acute exposure was attributed to DON on different age groups (from 50 to 90 %) (Knutsen et al., 2017). Nonetheless, in the long run (chronic exposure), the same document concluded the considerable additive health impact of DON metabolites (3 and 15 ADON and DON-3-Glc). ZEN and its metabolites, α- and β-zearalenol, (α- and β-ZEL, respectively) represent a

higher risk for public health compared to DON metabolites, especially if their level is closer to the upper bound scenario defined by EFSA (up to 2.2 fold the tolerable daily intake) (European Food Safety Authority, 2014).

Another aspect to be considered with regard to *Fusarium* mycotoxins is their resistance to physical parameters applied in food industry and possible subsequent transfer to the final product (Wolf & Bullerman, 1998). Besides that, other chemical or physical transformation might occur, depending on the applied production operations (Vidal, Morales, Sanchis, Ramos, & Marín, 2014; Zachariasova et al., 2008, 2012). The brewing process includes several main steps: mashing, wort boiling, fermentation, maturation, stabilization and packaging (with or without previous pasteurisation), the first three representing a significant impact on the levels of mycotoxins possibly present in malt (Pascari, Ramos, Marín, & Sanchís, 2018). The present study will be focusing on two brewing stages (mashing and boiling processes), giving a detailed vision from the inside of each of them. Mashing consists of mixing coarse ground malt with a large amount of water (approximately 170 g per litre) under specific temperatures to activate all the enzymes present in malt, namely: 45-50 ºC is set to ensure protein hydrolysis, 60-65 ºC lead to maltose production, and 75-78 ºC activate α-amylases (Kunze, 2006). Then, malt grist is removed from the wort and after adjusting the relative density of the wort with water (final density should vary between 1005 and 1010 kg/m^3) boiling starts, lasting from 45-60 min up to 3h depending on the beer style to be obtained (Pascari et al., 2018).

Beer production implies the use of five main commodities, namely barley, water, hops, yeast and adjuncts (e.g. maize, sugar syrup, unmalted cereals etc.). Besides barley, mentioned above, beer adjuncts can represent another source of mycotoxins, of particular note being maize (Marin et al., 2013), which is proven to be susceptible mainly for *Aspergillus* section *Flavi* (aflatoxin producers), *F. proliferatum* and *F. verticillioides* (FBs producers) infestation.

Hops added during the boiling stage may also be subject to fungal invasion with subsequent mycotoxin accumulation. However, the study performed by Vaclavikova et al. (2013) did not find hops as a significant source of mycotoxins in brewing wort because of the relatively low quantity added to beer.

According to previously published studies, a transfer of mycotoxins from raw materials to beer is possible due to their relatively high resistance to physical treatments applied during brewing (Inoue, Nagatomi, Uyama, & Mochizuki, 2013; Pascari et al., 2018). Nevertheless, several stages of the production scheme are proved to decrease the initial mycotoxin contamination levels (Lancova et al., 2008). In the EU, maximum allowed mycotoxin levels are regulated by the Regulation EC 1881/2006 with its subsequent updates and amendments. The applicable limits in barley are set as follows: 1250 µg/kg for DON and 100 µg/kg for ZEN. Beer is subjected only to legal limits in FBs content (maximum 400 µg/kg for the sum of fumonisin B_1 and B_2) in the case maize is used as an ingredient, other toxins not being included. This result in the hypothesis that brewing process may lead to a reduction in mycotoxin content.

This study aims to identify the impact of the obtention of brewing wort on mycotoxin contamination, a special focus being given to mashing and boiling parameters. The fate of DON, ZEN and their metabolites was investigated because of their frequent occurrence in malting barley: DON levels range from 69.9 to 602.3 µg/kg, ZEN varies from 181.2 to 204.4 µg/kg (Bolechova et al., 2015), DON-3-Glc and ADONs have an average occurrence of 2 and 1 µg/kg, respectively (Malachova et al., 2010). Taking into account the lack of available studies on the fate of α- and β-zearalenol in brewing and their possible production by some *Fusarium* spp. (Bottalico et al., 1985), these two metabolites were also included in the study. FBs are less occurring in barley, however their incidence in maize is significantly frequent (94,7 %) (Manova & Mladenova, 2009) and in relatively high concentrations in maize grits

used in brewing (from 1146 to 3194 µg/kg) (Pietri, Bertuzzi, Agosti, & Donadini, 2010). The different levels of mycotoxin contamination found in barley and in beer (Piacentini, Savi, Pereira, & Scussel, 2015; Rodríguez-Carrasco, Fattore, Albrizio, Berrada, & Mañes, 2015) leads to the conclusion that an efficient strategy is applied in the field to discard heavily contaminated barley, which, in combination with the mitigation potential of the production process, might reduce the health concern related to this product from the perspective of mycotoxin contamination.

Considering the different mycotoxin producing fungal strains, different sources of contamination (from infected malt or added maize) and the possible impact of the contamination level on mycotoxin transfer, three scenarios will be tested: two scenarios of grain inoculation with mycotoxin producing *Fusarium* strains and one of spiking with mycotoxin standard solutions. Results will help understand the interactions between mycotoxins, malt and beer matrices that might take place and possible mitigation strategies to be considered in the future. One of the strengths of the present study is that it better simulates natural contamination scenarios compared to a study where only spiking with standard solutions of the raw materials is performed.

2. Materials and methods

2.1 Materials

Mycotoxin standard solutions of DON, DON-3-Glc, 3- and 15-ADON, ZEN, α-ZEL), β-ZEL, FB1 and FB2 were purchased from Romer Lab Diagnostic (Tulln, Austria). Water was obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). Methanol and acetonitrile (HPLC grade) were purchased from Scharlab (Sentmenat, Spain), and ammonium formate was purchased from Sigma-Aldrich (St. Louis, MO, USA).

125 **2.2 Preparation of mycotoxin contaminated grains**

126 Three origins of contamination were studied: two sources of contamination with *Fusarium*
127 *graminearum* (F.46) and *F. proliferatum* (F2.333) (infected malt and maize) and
128 contamination by spiking the malt with mycotoxins standard solutions. Mycotoxin producing
129 fungal strains were taken from the strains collection of the Food Technology Department of
130 the University of Lleida.

131 Before inoculation with mycotoxin producing strains, malt and maize grains were disinfected
132 according to Andrews, Pardoel, Harun, & Treloar (1997). Briefly, a batch of grains (500 g)
133 was immerged into 0.4% chlorine solution for 2 minutes and then abundantly rinsed with
134 sterile distilled water. Then, all the grains were placed in an hermetically closed ISO bottle
135 and left overnight with the addition of sterile water for grain hydration (approximately 300
136 and 340 mL/kg for maize and malt, respectively) at 4 °C to achieve a water activity of 0.99
137 (Aqualab Series 3 TE, Decagon Devices Inc., Washington, USA). Three different batches of
138 contaminated grains were prepared: one batch of malt grains and one of maize grains were
139 contaminated with *Fusarium graminearum* (DON and ZEN producer) and another batch of
140 maize grains was contaminated with *Fusarium proliferatum* (FBs producer). For this, the
141 humidified grains were aseptically transferred to Petri dishes and 1 mL of spore suspensions
142 of *F. graminearum* or *F. proliferatum* (10^6 spores/mL) was sprayed on each dish. Petri dishes
143 with malt and maize were then incubated at 25 °C for 30 days. Afterwards, the contaminated
144 grains were dried at 40 °C, homogenized and DON, ZEN and FB1 levels were determined ().

145 To obtain the desired mycotoxin contamination level of the malt, spiking was performed by
146 adding to ground malt DON, ZEN and FB1 standard solutions at two different levels each,
147 ensuring the best possible homogenisation for the entire batch. Spiking levels for each toxin
148 were chosen at approximately a half of the legally allowed limit (A1) and at the maximum
149 allowed limit (A2) (Table 1) (European Commission, 2006). For the inoculated malt and

150 maize, an amount of the above *Fusarium* infected grains with the identified concentration of
151 DON, ZEN and FB1 was added to uncontaminated malt. Worth noting is that in both fungal
152 contamination scenarios, malt was the mashed substrate. In the cases when maize was added,
153 its role was only to increase the amount of fermentable sugars present in wort after the
154 mashing stage. Considering the heterogeneity of mycotoxin accumulation in cereals, various
155 batches were prepared (Table 1). All the samples were prepared in triplicate for each
156 concentration level. In order to ensure that the desired concentration was achieved, the mixes
157 were prepared individually for all replica (250 g of malt mashed each time). Samples coding
158 was performed considering the source of contamination, namely the "B" was attributed when
159 the contamination was coming from inoculated malt, the "M" when it was coming from
160 inoculated maize (the mashed malt was free of mycotoxins), the "A" for the spiked samples
161 and "Blank" stands for mashing the uncontaminated malt.

162 **2.3 Wort production and sampling**

163 A coarse grinding of malt was performed and, in the case where contaminated maize was
164 added, maize kernels were finely ground and mixed with mycotoxin free malt. For each
165 designed setup, 250 g of contaminated malt, or malt and maize adjunct, were mashed with
166 1300 mL of water (50% distilled water, 50% deionized water in order to avoid an increase of
167 the pH of the sweet wort). Once mixed, the mashing process was started by holding the
168 temperature of the mix at 45 °C for 15 min (M15min), then at 65 °C for 1 hour (M75min) and
169 finally at 75 °C for another 15 min (M90min), using an induction plate (PI 4750, Murcia,
170 Spain) and ensuring a frequent periodical homogenization of the mashed volume. Samples of
171 sweet wort and spent grains were taken at each temperature change. In order to make sure
172 that all the starch was transformed into fermentable sugars, the iodine test was performed,
173 consisting of adding a few drops of 0.1M KI solution in a tube containing 5 mL of wort and
174 observing the change in colour (the colour changes to blue if starch is present). Afterwards,

175 the wort was decanted and the density was adjusted to 1005-1010 kg/m^3 with distilled water.
176 Hops were added (10 g of hop pellet/L of wort) and the wort was intensively boiled for 1.5 h,
177 samples being collected every 30 min (B30min, B60min and B90min). All the samples were
178 stored at -18 °C until their analysis. The process was repeated per triplicate for each malt
179 sample prepared (Table 1).

180 **2.4 Mycotoxin extraction, detection and quantification**
181 Considering the complexity of the two obtained matrices (spent grains and wort), two
182 extraction procedures were used, according to previously validated methods.

183 ***2.4.1 Spent grains***
184 All solid samples were dried at 40 °C for 24 h before analysis. Mycotoxin extraction of the
185 spent grains was performed according to Juan et al. (2017), with slight changes. Briefly, 2 g
186 of dried and ground sample was weighted in 50 mL polypropylene tubes, mixed with 10 mL
187 of extraction solvent (acetonitrile:water, 84:16, v/v) and shaken at 200 rpm for 60 min (Infors
188 AG GH-4103, Bottmingen, Switzerland). The mix was then centrifuged at 2336 g for 10 min
189 (Hettich Universal 320R, Tuttlingen, Germany). Five millilitres of supernatant were
190 evaporated under a gentle nitrogen stream (40 °C). The dry extract was resuspended in 1 mL
191 of methanol:water (70:30, v/v) and filtered (PTFE syringe filter, 0.22 µm) before the
192 UHPLC-MS/MS analysis (see 2.4.3).

193 ***2.4.2 Wort***
194 For the liquid samples, the QuEChERS (acronym for Quick, Easy, Cheap, Effective, Rugged
195 and Safe) method developed by Rodríguez-Carrasco et al. (2015), with several modifications,
196 was applied. Ten millilitres of sweet wort were mixed with 10 mL of acetonitrile in a 50 mL
197 polypropylene tube. After mixing it well for 30 seconds, QuEChERS (Phenomenex,
198 California, USA) dehydrating salts were added (4 g of magnesium sulphate, 1 g of sodium
199 chloride, 1 g of sodium citrate tribasic dihydrate, 0.5 g sodium citrate dibasic sesquihydrate)
200 and shaken intensively for 1 min. Afterwards, the tubes were centrifuged at 1413 g for 3 min

and 7 mL of supernatant were taken for a pre-mixed clean-up by dispersive solid phase extraction (Phenomenex, California, USA) in a 15 mL tube containing 1.2 g of magnesium sulphate, 0.4 g of PSA (primary secondary amine), 0.4 g of C18 and 0.4 g of activated carbon. It was vortexed for 1 min and centrifuged for 1 min at 1413 g. Finally, 3 mL of supernatant were evaporated under a gentle nitrogen flow (40 °C). The dry extract was also resuspended in 1 mL of methanol:water (70:30, v/v) and filtered (PTFE syringe filter, 0.22 µm) before the UHPLC-MS/MS analysis (see 2.4.3).

2.4.3 LC-MS/MS analysis

An Agilent Series 1290 RRLC system (Santa Clara, California, USA) equipped with a binary pump (G4220 A) and a thermostatic autosampler (G1330 B), coupled to a triple quadruple mass spectrometer Agilent 6460 A was used for sample analysis. Data acquisition and processing was performed using MassHunter® software (Agilent, Santa Clara, California, USA). Separation was achieved on Agilent Zorbax Plus C18 100x2.1 mm, 1.8 µm column (California, USA).

The mobile phase consisted of methanol (A) and an aqueous solution of ammonium formate at 5mM (B), which was supplied in gradient at a flow rate of 0.2 mL/min. The initial gradient was set at 25 % A, increased to 100 % by the minute 3.75 and maintained during the following 2.25 min. Within the following 0.5 min it was dropped again to 25 % A and maintained at this proportion until the next injection. The injection volume was 5 µL and column temperature was maintained at a constant 25 °C. Total run time was 7.5 min.

Operating ESI (electrospray ionisation) conditions were set up as follows: capillary voltage 3500 V, cone voltage 500 V, nebulizer pressure 45 psi, source temperature 325 °C, cone gas flow 5 L/min, drying gas (nitrogen) temperature 400 °C, drying gas flow 11 L/min. The mass spectrometer was operated in both positive and negative electrospray ionisation modes (ESI) in the multiple reaction monitoring (MRM) mode. The most abundant characteristic product

ion was used for quantification and the rest of the product ions served for qualitative confirmation of the analyte (European Commission, 2002). Linearity was checked using both external calibration and matrix-matched calibration plots. The limit of detection (LOD) was considered as three times the signal of blank noise and the limit of quantification (LOQ) as ten times the signal to noise ratio. Table 2 regroups the data concerning the selected ion transition, the settings of the mass detector and the limits of detection and quantification in the injected solutions. The concentration factors during extraction were 1 and 3 for solid and liquid samples, respectively.

2.5 Statistical analysis

All weight and volume changes during wort obtention were recorded in order to present the results in µg of toxin and to allow comparison among the transfer rates of *Fusarium* mycotoxins throughout the entire mashing and boiling processes. A post hoc multiple comparison of means, Tukey HSD test, was performed to identify significant changes in mycotoxin content between different stages of production, using JMP Pro 13 Software (SAS Institute, New York, USA). A value of 1/2 LOQ was attributed to the non-quantitated samples. For greater precision, the results from malt mashing were analysed separately from the results obtained after wort boiling.

3 Results and discussion

3.1 Blank and spiked malt

In order to obtain complete information regarding the process, the malt used in the present study was checked for all toxins of interest before mashing. It was found to contain only DON-3-Glc, other toxins being below the LOQ (Table 1, fig. 1). Nonetheless, by the end of the mashing process, the levels of DON and DON-3-Glc were significantly increasing in the wort with a simultaneous decrease of DON-3-Glc in the spent grains. This suggested their release from the matrix (particularly obvious for DON, as it was not found in the malt before mashing), due to the increasing temperature and the relative length of the process (90 min of

252 mashing). DON-3-Glc behaviour was very similar in the blank and A2 samples. It was not
253 present in A1 sample initially, nor did it appear during the mashing process, either in spent
254 grains or in the wort. A possible explanation of this is heterogeneity in the initial DON-3-Glc
255 contamination of the barley before malting which may be lower compared to the A2 and
256 blank samples, which together with a lower conversion rate of DON to DON-3-Glc in barley
257 (Freire & Sant'Ana, 2018) did not lead to the formation of the masked form DON-3-Glc
258 (Kostelanska et al., 2009; Medina et al., 2006). A significant increase of DON-3-Glc content
259 in the wort was identified after the last stage of mashing (15 min at 75 ºC). Considering that
260 no decrease of DON was observed after the same production step, no conversion occurred
261 (Lancova et al., 2008). From the above, it can be concluded that the enzymatic activity
262 (macromolecules' hydrolysis) and the high temperature might be the most important catalysts
263 of DON-3-Glc transfer from malt to wort. Boiling led to a 26% reduction in DON level
264 compared to its initial concentration before mashing. DON-3-Glc quantities decreased, with
265 respect to the level after mashing, down to its initial concentration.

266 In the lower spiking concentration (A1 sample), ZEN was found only in the wort during
267 mashing, with no significant changes between stages, and <LOQ after the first 30 min of
268 boiling (B30min). On the contrary, each step of mashing led to a significant increase in ZEN
269 compared to its initial quantity with 700% in the A2 sample. Boiling in the A2 sample was
270 followed by a significant decrease in ZEN after the first 30 min of the process (B30min) until
271 it reached its initial level in malt grist. This result confirms those obtained by Inoue et al.
272 (2013), where an almost 20 % decrease of ZEN was observed in the spent grains.

273 FB1 did not transfer to wort in the A1 sample, and was, as a result, eliminated with the spent
274 grains. Worth noting is that its level in the A2 sample almost tripled in the wort after 15 min
275 at 75 ºC (M90min) compared to the initial quantity present in malt, a similar tendency being
276 observed later in the inoculated samples (M5-M7). Boiling showed a significant reduction of

almost 50 % of FB1 with respect to the initial contamination level, after the first 30 min of the process.

Inoue et al., (2013) performed a study spiking the malt with 14 different mycotoxins and investigating their fate during brewing. Only the results we obtained in the A2 sample correlate with the ones they observed in their study. The different behaviour could be due to the lower spiking concentration that was chosen in the present study for the A1 sample. In Table 1 (Supplementary materials), the data on mycotoxin content is presented in µg of toxin, which makes possible the comparison between the two physical states implied in the process. Altogether, from the obtained results, spiking with mycotoxin standard solution does not take into account the complexity of mycotoxigenic *fungi* propagation into the grain (in the case of fungal contamination) which mainly defines the transfer from malt to wort in *Fusarium* inoculated samples (Freire & Sant'Ana, 2018; Kostelanska et al., 2011).

3.2 Transfer of DON and its metabolites in *Fusarium* contaminated malt and maize

Two possible sources of contamination were considered: mycotoxin contaminated malt (Fig. 2) and maize (Fig. 3). A study of the evolution of DON and its metabolites during mashing and boiling was performed, while also defining initial levels of contamination (Table 1).

As expected, DON passed from malt grist to the wort during mashing process (Lancova et al., 2008). Interestingly, a significant reduction of DON in the spent grains during the first stage of mashing (15 min at 45 ºC) was observed (93 % in B1, 86 % in B2, 94 % in B3, 86 % in B4 and 38 % in B5) , while the other stages did not significantly influence the level of DON contained. The same, but increasing, tendency was observed in the sweet wort in all samples, however in the B5 sample an even greater increase was observed after 60 min at 65 ºC (35 %). In all the samples, except for B1 sample (Supplementary materials, Table 2), the amount of DON found in the wort was twice the initial amount contained in the malt grist and in the

302 case of the samples in which the contamination came from maize (Supplementary materials,
303 table 3) this increase was even higher (2.5 folds the initial content). The increased amount of
304 DON released into the wort is probably due to the fact that the contact with water and the
305 enzymatic activity during mashing causes DON to be unbound from malt's matrix
306 macromolecules (Kostelanska et al., 2011). The different extracted amounts in B and M
307 samples could be explained by the fact that the maize was ground into a smaller particle size
308 than the malt, which increased the contact surface and, as a result, also aided DON transfer
309 into the water. Boiling led to a reduction in DON levels compared to the last stage of mashing
310 (15 min at 75 ºC), the most significant change occurring after 30 min of boiling, except for
311 the samples B2, B4 and M4 where an even more significant reduction took place after 60 min
312 of boiling. In none of the studied scenarios was DON entirely eliminated during boiling,
313 however a level significantly lower than the initial was achieved (up to 60% decrease of
314 DON compared to its level before mashing) in malt contaminated samples (Supplementary
315 materials, tables 2 and 3). This reduction might be related either to its sedimentation with
316 proteins and impossibility of extraction by the method used in this study (Schwarz, 2017) or
317 its chemical modification under temperature action (Rychlik et al., 2014), however
318 supplementary research has to be done to confirm this statement.

319 DON-3-Glc production is linked either to plant metabolism (Lemmens et al., 2005) or to
320 enzymatic activity during food processing (Vidal et al., 2014). DON distribution in the grain
321 is associated with proteins and β-glucans (Nishio, Takata, Ito, & Tanio, 2010), whose
322 hydrolysation during mashing would lead to its release into the wort (Kostelanska et al.,
323 2011). DON-3-Glc was found in the malt grist in only two samples of the present study (B5
324 and M2). At this stage, its origin in the samples certainly derives from the malted barley,
325 probably infested in the field (Medina et al., 2006). Similar to DON, DON-3-Glc in the B5
326 sample gradually augmented in wort during mashing, a three-fold increase of DON-3-Glc

registered in the wort after the last treatment (15 min at 75 ºC), accompanying a significant decrease of the toxin in the spent grains during mashing (almost 100 % decrease compared to the level before mashing). In the M2 sample no transfer was observed possibly because of the low amount of DON-3-Glc found. Converting the data from concentration to µg of toxin (Supplementary materials, Tables 2 and 3) demonstrates the transfer from the malt matrix to the sweet wort.

3- and 15-ADON production is a function of the fungal strain responsible for contamination (Gauthier et al., 2010), which explains the presence of these metabolites in almost all the samples before mashing, although in relatively low levels (Figs. 2 and 3). Both 3-ADON and 15-ADON were transferred to the wort during mashing (Supplementary materials, tables 2 and 3). For the contamination derived from malt (B1-B5), the decrease of 3-ADON was only significant after 15 min at 45 ºC, followed by an increase in the sweet wort and maintaining almost same level until the end of the mashing process, with a subsequent, statistically relevant, reduction during the boiling process in all the samples. 15-ADON was found only in the sweet wort at each mashing stage with no significant change up to the end of the boiling process, except for the B3 sample where it was found <LOQ in the steps following the 60 min at 65 ºC of the mashing stage. Regarding the evolution of 3- and 15-ADON in the samples with contaminated maize adjunct (M1-M4), the two toxins showed a more similar behaviour between them, registering an almost 100 % decrease in the spent grains. In the samples where the amount of 3-ADON was higher (M3 and M4) the quantity found in the wort after 15 min at 45 ºC was almost double the initial level in the malt grist (Supplementary materials, Table 3). 15-ADON showed a similarly significant increase in the wort during mashing, however, contrary to 3-ADON, the boiling process did not lead to a reduction in its level, except for the M2 sample, the only one in which both toxins were <LOQ at the end of the process. All the similarities in the changes in the acetylated forms of DON with their

352 parental form were probably due to their sharing of physical and chemical properties (Nagl &
353 Schatzmayr, 2015).

354 In summary, both DON and its metabolite levels increased in wort, accompanied by a
355 significant reduction in the spent grains. Interestingly, in almost all samples, this change
356 occurred during the first mashing stage (15 min at 45 ºC), all the following mashing stages
357 not having a significant impact on mycotoxins level, except for a few samples (B2, B5 and
358 M1). Studies on DON distribution in the kernels showed a positive correlation with ash and
359 protein content, which is higher in the brans due to pericarp and aleurone tissues (Trigo-
360 Stockli, Deyoe, Satumbaga, & Pedersen, 1996). This fact makes the extraction of DON from
361 a contaminated kernel an important issue for analysis (Zheng, Richard, & Binder, 2006).
362 Nevertheless, the aim of enzymatic activity at 45 ºC during the mashing process is the
363 hydrolysis of β-glucans and proteins; this could lead to a weakening of the bounds between
364 the toxins and the malt matrix and, together with its water solubility, result in an increase in
365 DON and its metabolite content in the wort. From the data presented in supplementary
366 material section, it can be seen that 29 to 59.6 % of DON remained in the wort after boiling
367 in the case when contamination was coming from the infected malt (B1-B5 samples), 59 to
368 106.7 % of DON were found in the wort of the samples when contaminated maize grits were
369 added (M1-M4) and 26.6 and 58.3 % of DON in the case when malt was enriched with DON
370 standard solution. Regarding DON modified forms, besides their low incidence in the
371 analysed samples, a high variability in the results was observed with 31 and 92 % maximum
372 remained in the final wort for DON-3-Glc and 3-ADON, respectively. 15-ADON increased
373 from <LOQ to a maximum of 4,8 µg/L in all the samples coming from malt and maize grits
374 contaminated with *F. graminearum*. Previously published studies have found a correlation
375 between DON and DON-3-Glc accumulation, presumably due to the enzymatic activity
376 (Lancova et al., 2008), however no such correlation was proven in the present research. Also,

previous research identified *S. cerevisiae* spp. potential to adsorb mycotoxins (up to 20 % of DON can be removed with yeast residue) (Pascari et al., 2018).

3.3 Transfer of ZEN and its metabolites in *Fusarium* contaminated malt and maize

In the samples where the contamination came from malt (Fig. 2), the amount of ZEN before mashing was almost always very high (except for the B5 sample), nonetheless no such quantity of ZEN was found in the following stages of mashing and boiling.

In most of the samples, the amount of ZEN in the wort was double that in the spent grains at the three stages of mashing, however no significant increase was registered during the process (Supplementary materials, table 2). Although ZEN proved to have a low water solubility (Bennett, Klich, & Mycotoxins, 2003), the higher amount in the wort could be explained by the high temperatures and prolonged contact with water during the mashing process (Mastanjević et al., 2018). The level of ZEN in the wort reached after 15 min at 45 ºC did not change significantly by the end of the mashing process. The boiling process led to an significant reduction of ZEN in the wort compared to its initial quantity, especially in the higher contamination levels (up to 99% reduction compared to the amount of ZEN before boiling). It is worth noting that in the sample with the lower level of ZEN (B5), the reduction during boiling reached 89 % compared to the ZEN level before the beginning of the process.

In the case of ZEN derived from maize adjuncts (Fig. 3), the amount of the toxin found in the malt grist before mashing was always related to the amount quantified in the spent grains and the sweet wort. A significant reduction in the spent grains was registered after 15 min at 45 ºC in all the samples, though considering the high variability within ZEN contamination, it is difficult to draw any conclusions from this. Moreover, considering that ZEN distribution in the kernel is similar to DON's (Trigo-Stockli et al., 1996), the mashing process might have a

401 partially similar impact beyond the physical parameters of mashing which stimulates the
402 elution of matrix components into the wort (Lancova et al., 2008). There is no proven
403 knowledge on the mechanism of ZEN reduction during boiling, especially considering its
404 thermal stability, and deeper chemistry studies are necessary to complete these findings.

405 α- ZEL was found only in two of the contamination scenarios (M3 and M4) at low levels,
406 only in the wort with no significant changes during malting, but <LOQ after 30 min of
407 boiling up to the end of the process. β-ZEL, on the contrary, was present in almost all
408 samples (except M2) containing contaminated maize before mashing started. The production
409 of these two metabolites might been attributed to their synthesis by the fungal isolate
410 responsible for the contamination (Bottalico et al., 1985), on one hand, and to ZEN
411 metabolization by *Saccharomyces cerevisiae* cells during fermentation, on the other
412 (Sørensen & Sondergaard, 2014). β-ZEL was predominantly found in the wort of the
413 samples where the contamination came from malt and no stage of the process led to a
414 significant change in its quantity. In the two samples that contained α- ZEL, the levels were
415 low and remained nearly constant, while boiling led to a decrease down to <LOQ. β-ZEL in
416 the samples with contaminated maize adjunct showed an increasing trend during mashing
417 followed by a decrease during boiling, but never more than 28% compared to the amount
418 before boiling and not statistically significant. No correlation was found between the initial
419 content of β-ZEL and the reduction rate. There are no studies available on the stability of
420 ZEN metabolites during food processing; however, considering that the main metabolite of
421 yeast activity of ZEN transformation is β-ZEL (lower toxicity compared to ZEN (De Saeger
422 & van Egmond, 2012)), together with the results of this study (no α- ZEL present in the wort
423 after boiling), mashing and boiling processes have been shown to lead to a detoxification of
424 the product from ZEN. Finally, in almost all samples, ZEN reduction reached approximately
425 99 % in the wort at the end of the boiling process.

3.4 Transfer of fumonisins B1 and B2 in *F. proliferatum* contaminated maize

The most probable source of fumonisins in beer are maize adjuncts (Marin et al., 2013). Figure 4 represents the data of changes to the FB1 and FB2 during mashing and boiling. There is a slightly different trend between the samples M1 to M4 and M5 to M7, which may be explained by their co-occurrence with other mycotoxins, as can be seen in Table 1, however there is no evidence to confirm the statement. In the samples from M1 to M4, both FB1 and FB2 were transferred to the spent grains during mashing (no change in concentration in the spent grains and <LOQ in the wort), which suggests their elimination before boiling and decontamination of the product. Nonetheless, the samples M5 to M7 confirm its water soluble properties (Pietri et al., 2010) through the significant increase of FB1 and FB2 in the wort throughout mashing (each of the three stages was described by a significant reduction of FB1 and FB2 in the spent grains with a subsequent significant increase in the wort) (Supplementary materials, table 4). After the first 30 min of boiling, there was a 90 to 100 % reduction in both toxins, which correlates with Pietri et al. (2010) results. There is no evidence on the impact of the enzymatic activity on fumonisins content, nonetheless its increase during this process cannot be solely explained by its solubility in warm to hot water (45 to 75 ºC), suggesting the enzymes may have the indirect effect of facilitating hydrolysation from macromolecules. At the end of boiling process, a 90 % FB1 reduction was observed in the wort containing it before the process started. Also, the different fate of FB1 and FB2 during wort production might be influenced by their co-occurrence with DON and ZEN in the M1-M4 samples, which is not the case for M5-M7 samples, where only FBs are present. Nevertheless, the obtained data are not sufficient to support either of the two abovementioned assumptions and further studies need to be performed to investigate these results.

4 Conclusion

From the three contamination scenarios prepared in the present study, some interactions between the process and the level of mycotoxins were identified. Samples spiked with standard mycotoxin solutions (DON, ZEN and FB1) showed changes in contamination similar to the samples in which the contamination was coming from infected malt. DON and its metabolites had a similar behaviour, showing a significant transfer from malt to wort during mashing. Moreover, an increase of the extracted amount of toxins was observed through the process, the most significant being observed after 15 min at 45 ºC. Nonetheless, however great the rise, boiling always led to a reduction down to the initial level (in samples with the addition of the contaminated maize) or even lower (samples containing contaminated malt). Despite the abovementioned decrease, DON is still of a special concern for brewing because of its 30 to 60 % remaining in the wort after boiling, which could withstand the following production steps.

Almost 100 % reduction in ZEN levels was observed in all the samples (just 30 min of boiling have a significant impact). Very low incidence of α- ZEL was registered along with its complete elimination by the end of the process. β-ZEL was a little more abundant compared to its stereoisomer and showed a low reduction rate at the end of the process.

Both FB1 and FB2 showed transfer into the wort during mashing, however on low levels of contamination it was almost completely removed with the spent grains. Nevertheless, there is a need for more in-depth studies in order to prove this change in FBs levels.

In summary, the crucial stages that induced significant change in mycotoxins levels were the first 15 min at 45 ºC (increase of transfer of mycotoxins into the wort) and the first 30 min of boiling (decrease of mycotoxin level in the wort). Considering the aforementioned, boiling is a crucial step in mycotoxins mitigation but, taking into account that the majority of the mycotoxins are not completely reduced even after 90 min of boiling, more research should be

476 carried out in order to study the changes to mycotoxins and the levels thereof through the
477 next stages of brewing (fermentation, fining and maturation) and identify if there is a
478 reduction in these toxins as expected by European legislation (different maximum allowed
479 limits in raw materials and final products).

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486 6 Conflict of interest

487 Declaration of interest: none.

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Declaration of interest

The authors declare no conflict of interest

Table(s)

Table 1: Initial mycotoxins concentrations obtained in malts for wort production

Contamination scenario	Malt samples ID	Mean concentration, µg/kg± SD							
		DON	***ZEN***	***FB1***	***FB2***	***DON-3-Glc***	***3ADON***	***15ADON***	***β-ZEL***
Mycotoxins coming from *F. graminearum* contaminated malt	B1	78±38	4217±1821	ND	ND	<LOQ	2.8±2	<LOQ	<LOQ
	B2	103±17	4969±1189	ND	ND	<LOQ	4.4±0.4	<LOQ	<LOQ
	B3	107±19	5201±827	ND	ND	<LOQ	5±0.8	<LOQ	<LOQ
	B4	209±5	6542±741	ND	ND	<LOQ	9.2±0.8	<LOQ	<LOQ
	B5	1271±1	989	ND	ND	1032±0.4	<LOQ	<LOQ	<LOQ
Mycotoxins coming from the added *F. proliferatum* contaminated maize	M1	12±4	1482±341	43±2	<LOQ	<LOQ	2.02±0.6	<LOQ	6±9.5
	M2	28±6	2605±373	84±28	64±16	<LOQ	3.5±1	4±6	<LOQ
	M3	74±2	5264±772	230±52	200±92	<LOQ	7.8±0.5	14.2±3.5	22±6
	M4	136±35	7053±496	351±103	328±68	<LOQ	15±3.3	13.4±2.1	38.8±7.8
	M5	ND	ND	189±6	84±12	<LOQ	<LOQ	<LOQ	<LOQ
	M6	ND	ND	310±99	200±84	<LOQ	<LOQ	<LOQ	<LOQ
	M7	ND	ND	758±8	332±16	<LOQ	<LOQ	<LOQ	<LOQ
Artificial contamination with mycotoxins standard solutions	A1	50±3	<LOQ	118±7	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	A2	750±1	75	400±0.5	<LOQ	120±0.4	<LOQ	<LOQ	<LOQ
Blank		<LOQ	<LOQ	<LOQ	<LOQ	124±0.2	<LOQ	<LOQ	<LOQ

*LOQ=limit of quantification; ND= not defined, DON=deoxynivalenol; ZEN=zearalenone; FB1=fumonisin B_1; DON-3-G= deoxynivalenol-3-glucoside; 3-ADON = 3-acetyl-deoxynivalenol; 15-ADON = 15-acetyl-deoxynivalenol; β-ZEL= β-zeralenol. The LOQ was: 3 µg/kg for DON, 6 µg/kg for DON-3-Glc, 1 µg/kg for ZEN, 0.5 µg/kg for 3-ADON, 15-ADON and β-ZEL, 45 µg/kg for FB1 and FB2.

Table(s)

1. Table 2: LC-MS/MS acquisition parameters (MRM mode) used for the analysis of targeted mycotoxins

Mycotoxin	Retention time, min	ESI[1] mode	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)	Fragmentor voltage (V)	LOD[2] (µg/L)	LOQ[3] (µg/L)
DON	2.8	ESI+	297.2	**231.1**[4] 203.1	15 15	65	0.9	3.0
DON-3-Glc	2.7	ESI-	476.2	**297.1** 248.9	5 25	85	1.8	6.0
3-ADON	3.7	ESI+	339.2	**231.1** 203.0	15 25	105	0.15	0.5
15-ADON	3.7	ESI+	339.2	**321.1** 261.1	5 5	125	0.15	0.5
ZEN	5.0	ESI-	317.2	**131.0** 273.1	25 25	185	0.3	1.0
α-ZOL	5.0	ESI-	319.2	**129.9** 174.0	40 40	125	0.15	0.5
β-ZOL	4.8	ESI-	319.2	**174.0** 160.0	40 40	125	0.15	0.5
FB1	4.2	ESI+	722.4	**352.1** 334.1	30 40	175	13.6	45
FB2	4.7	ESI+	706.5	**336.4** 318.4	35 45	125	13.6	45

[1]ESI=electrospray ionisation, [2] LOD=limit of detection; [3] LOQ=limit of quantification; The given LOD and LOQ are corresponding to the injected solutions. [4] Product ions in bold were used for quantification, others for qualitative analysis; DON=deoxynivalenol; DON-3-Glc=deoxynivalenol-3-glucoside;; 3-ADON=3-acetyl-deoxynivalenol; 15-ADON= 15-acetyl-deoxynivalenol; ZEN= zearalenone; α-ZOL= α-zearalenol; β-ZOL= β-zearalenol; FB1= fumonisin B1; FB2=fumonisin B2.

Figure(s)

Click here to download Figure(s): figure 1-Blank, a1, a2.docx

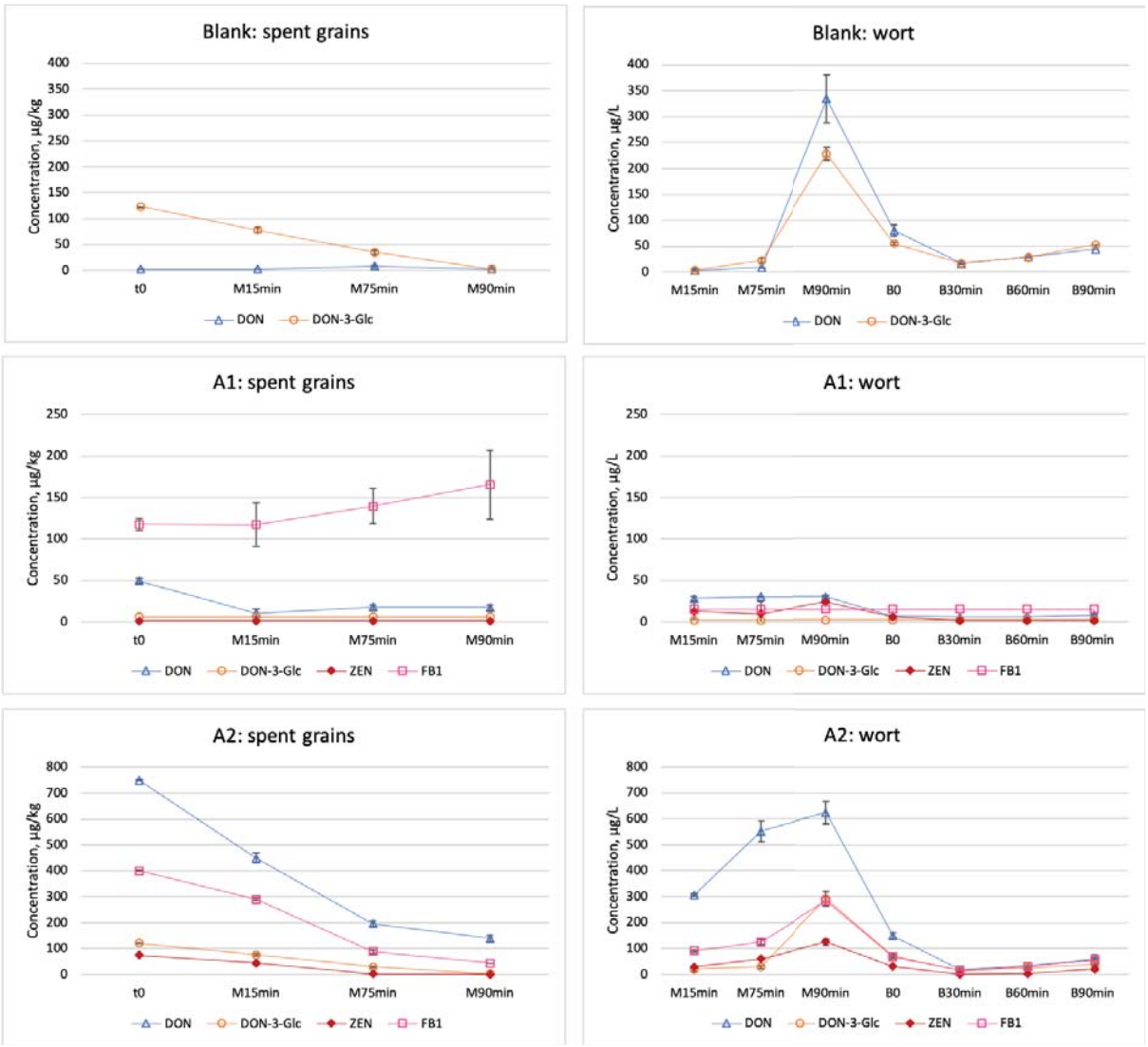

Figure 1: Evolution of mycotoxins concentration originated from malt spiked with standard solutions of DON, ZEN and FB1 throughout mashing (M15min, M75min and M90min) and boiling processes (B0, B30min, B60min and B90min) in spent grains (µg/kg) and wort (µg/L). LOQ was attributed to the values <LOQ. Spent grains: 3 µg/kg for DON, 6 µg/kg for DON-3-Glc, 1 µg/kg for ZEN and 45 µg/kg for FB1. Wort: 1 µg/L for DON, 2 µg/L for DON-3-Glc, 0.3 µg/L for ZEN and 15 µg/L for FB1.

Figure(s)

Click here to download Figure(s): Figure 2. B1-B5.docx

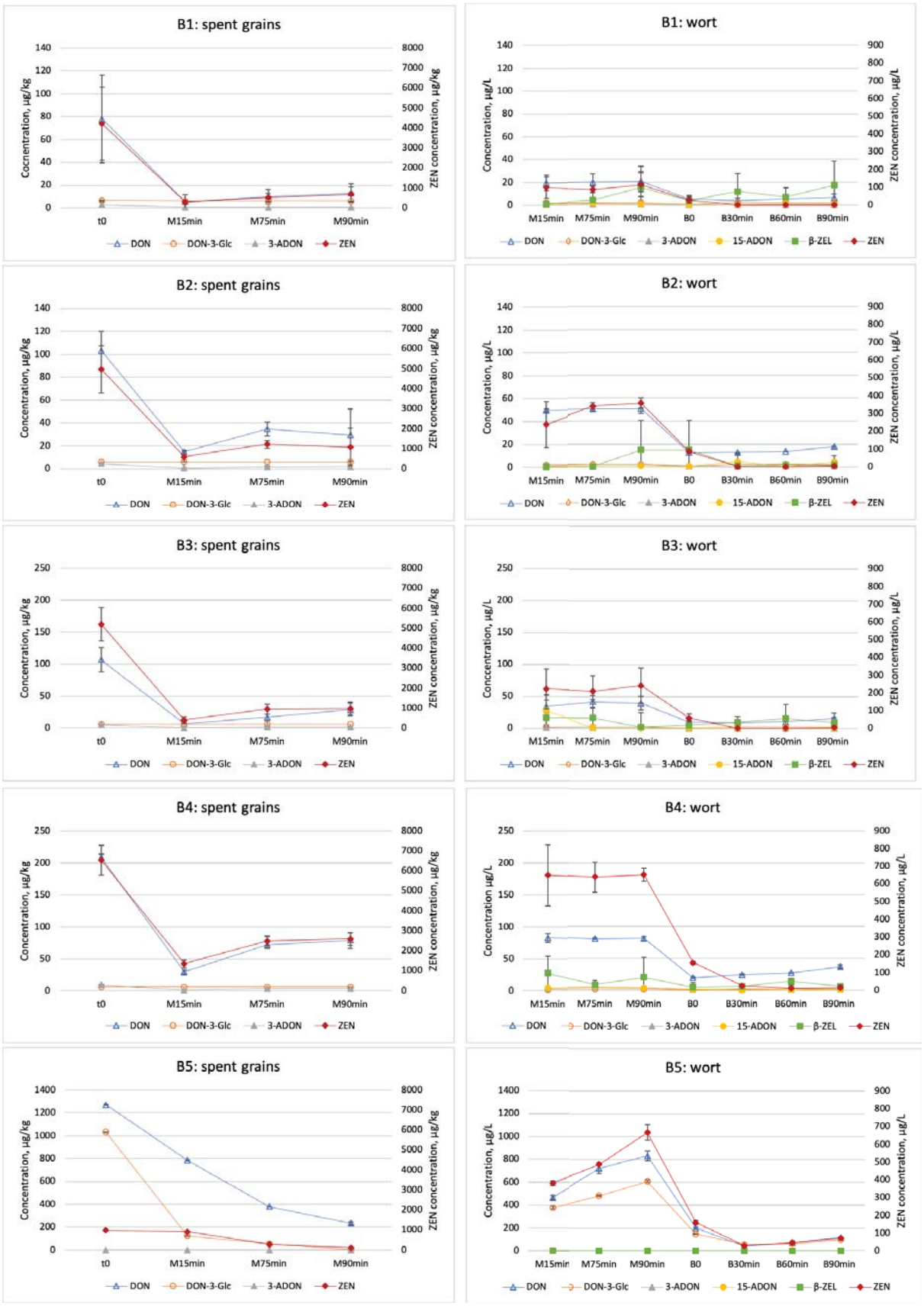

Figure 2: Evolution of deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3-Glc), 3 and 15-acethyl-deoxynivalenol (3ADON and 15ADON, respectively), β-zearalenol (β-ZEL) and zearalenone (ZEN) concentrations originated from the *F. graminearum* contaminated malt throughout mashing (M15min, M75min and M90min) and boiling processes (B0, B30min, B60min and B90min) in spent grains (µg/kg) and wort (µg/L). LOQ was attributed to the values <LOQ. Spent grains: 3 µg/kg for DON, 6 µg/kg for DON-3-Glc, 1 µg/kg for ZEN, 0.5 µg/kg for 3ADON, 15ADON and β-ZEL, 45 µg/kg for FB1. Wort: 1 µg/L for DON, 2 µg/L for DON-3-Glc, 0.3 µg/L for ZEN, 0.17 µg/kg for 3ADON, 15ADON and β-ZEL, 15 µg/L for FB1.

Figure(s)

Click here to download Figure(s): figure 3-m1-m4.docx

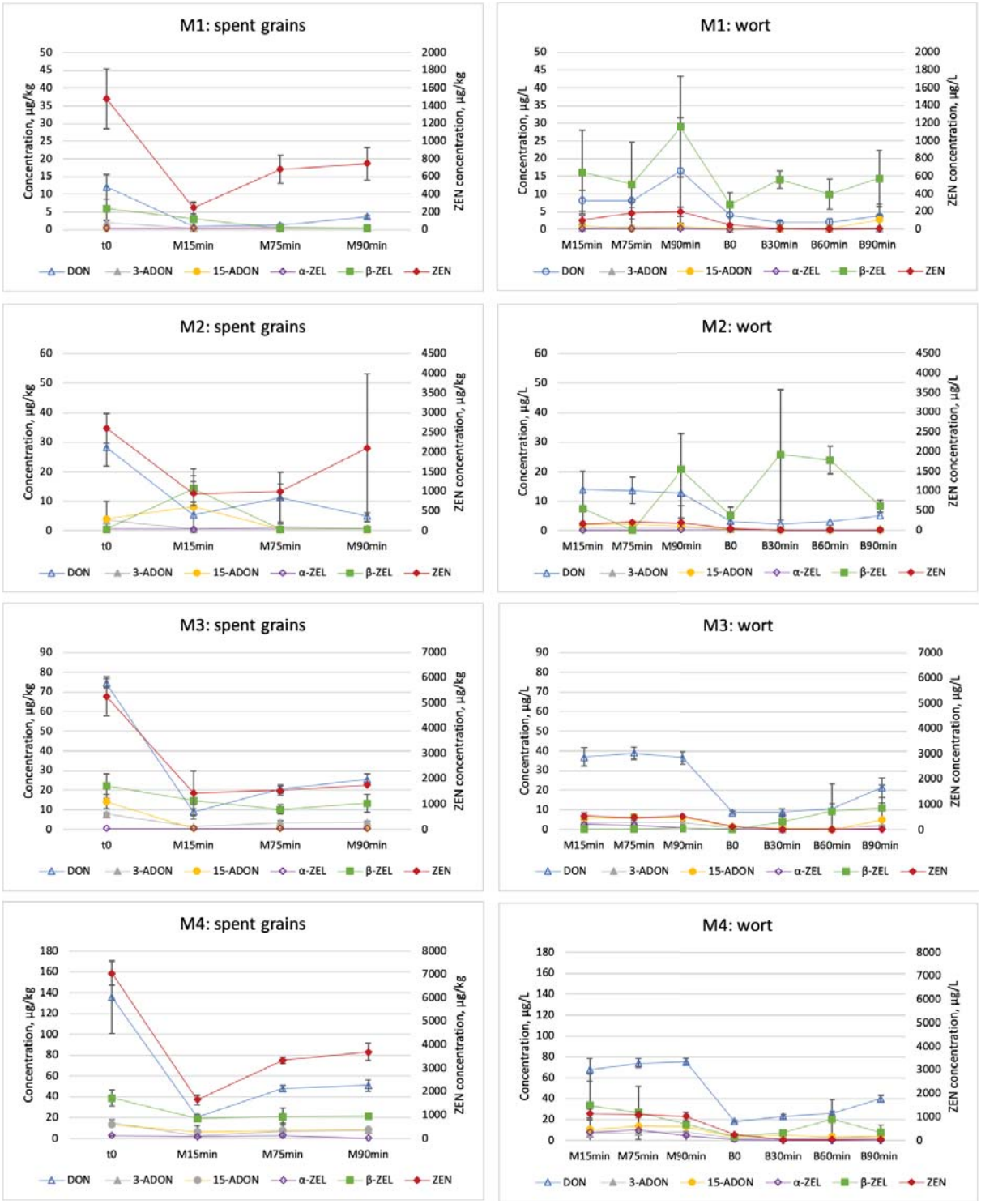

Figure 3: Evolution of deoxynivalenol (DON), 3 and 15-acethyl-deoxynivalenol (3-ADON and 15-ADON, respectively), α- and β-zearalenol (α-ZEL and β-ZEL, respectively) and zearalenone (ZEN) concentrations originated from the *F. graminearum* contaminated maize added to the malt throughout mashing (M15min, M75min and M90min) and boiling processes (B0, B30min, B60min and B90min) in spent grains (µg/kg) and wort (µg/L). LOQ was attributed to the values <LOQ. Spent grains: 3 µg/kg for DON, 6 µg/kg for DON-3-Glc, 1 µg/kg for ZEN, 0.5 µg/kg for 3-ADON, 15-ADON, α- and β-ZEL, 45 µg/kg for FB1.

Wort: 1 µg/L for DON, 2 µg/L for DON-3-Glc, 0.3 µg/L for ZEN, 0.17 µg/kg for 3-ADON, 15-ADON, α- and β-ZEL, 15 µg/L for FB1.

Figure(s)

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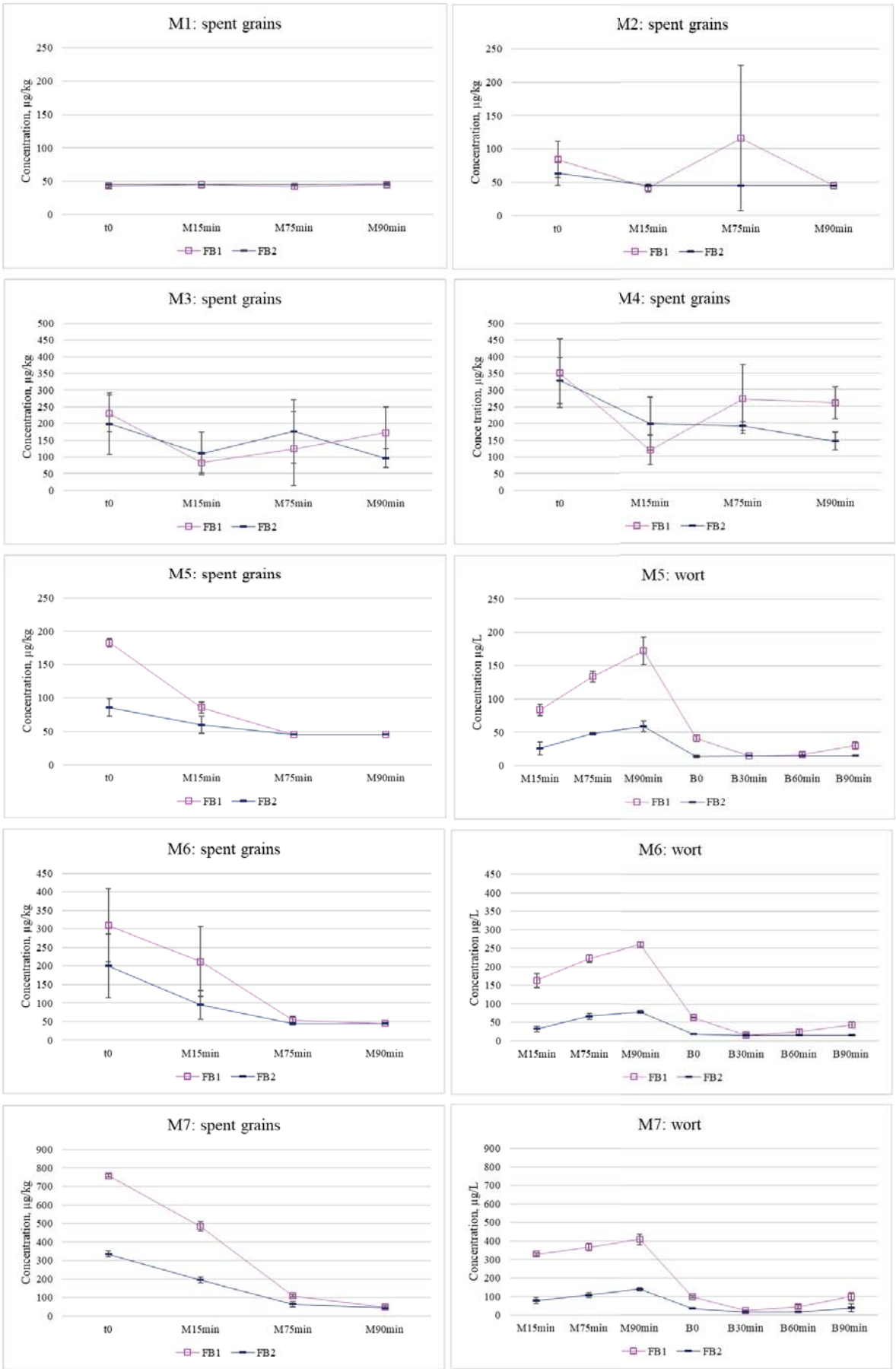

Figure 4: Evolution of fumonisins B_1 (FB1) and fumonisins B_2 (FB2) concentration originated from *F. proliferatum* contaminated maize added to the malt throughout mashing (M15min, M75min and M90min) and boiling processes (B0, B30min, B60min and B90min) in spent grains (µg/kg) and wort (µg/L). LOQ was attributed to the values <LOQ. Spent grains: 45 µg/kg for FB1 and FB2. Wort: 15 µg/L for FB1 and FB2.