Enzyme bread improvers affect the stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking.

Arnau Vidal, Asier Ambrosio, Vicente Sanchis, Antonio J. Ramos, Sonia Marín*

Food Technology Dept, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Spain.

* Corresponding author. Food Technology Dept, XaRTA-UTPV, Agrotecnio Center, University of Lleida, Rovira Roure 191, 25198 Lleida, Spain. Tel.: +34 973 702542; fax: +34 973 702596.

E-mail address: smarin@tecal.udl.cat (S. Marín).

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Abstract:

The stability of deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-glucoside) during the breadmaking process was studied. Some enzymes used in the bakery industry were added to determine the possible effect on these two mycotoxins. The level of DON in breads without added enzymes was reduced (17-21%). Similarly, cellulase, protease, lipase and glucose-oxidase addition did not modify in general this reducing trend. Regarding xylanase and α-amylase, their effect in DON content depended on the fermentation temperature, with 10-14% reduction at 45ºC, but a 13-23% increase at 30ºC. DON-3-glucoside had a reduction at the end of the fermentation, with a final reduction of 19-48% when no enzymes were used. However, the presence of xylanase, α-amylase, cellulase, and lipase resulted in a bread with a higher presence of DON-3-glucoside when the fermentation was at 30ºC. The results showed that wheat bran and flour may contain hidden DON that may be enzymatically released during the breadmaking process, when fermentation temperature is close to 30ºC.

1. Introduction

Deoxynivalenol (DON), also known as vomitoxin, is one of the most regular contaminants in cereals (Cano-Sancho et al., 2011a), and it can be found at relatively high concentrations in wheat and wheat containing products (like bread and pasta) (Cano-Sancho et al., 2011a). In addition, they are considered the major source of human intake for DON (Cano-Sancho, Gauchi, Sanchis, Marín, & Ramos, 2011b). Although DON is not classifiable as to its carcinogenicity to humans by the International Agency for Research on Cancer (IARC, 1993), it has been linked with human gastroenteritis (Pestka, 2010).

Contaminated wheat grains with DON may also contain deoxynivalenol-3-glucoside (DON-3-glucoside), a plant metabolite from DON (Berthiller et al., 2009). The reported levels of DON-3-glucoside are variable, however the ratio DON-3-glucoside/DON concentration is similar among the assays, from 10 to 30 % (Berthiller et al. 2009; Dall'Asta, Dall'Erta, Mantovani, Massi, & Galaverna, 2013). Moreover, Berthiller et al. (2011) showed that DON-3-glucoside can be hydrolysed to DON by several lactic acid bacteria that may be present in the intestine. Thus the FAO/WHO Expert Committee (JEFCA) considered DON-3-glucoside as an additional contributing factor of the total dietary exposure to DON (JEFCA, 2010).

Due to the high presence of DON and DON-3-glucoside in raw wheat, it is important to study their stability during breadmaking process. Contradictory reports exist regarding the fate of DON during this process. First of all, the results on the fermentation effect on DON are contradictory; while some authors pointed out important reductions (Neira, Pacin, Martínez, Moltó, & Resnik, 1997; Zachariasova, Vaclavikova, Lacina, Vaclavik, & Hajslova, 2012), other studies showed significant increase of DON after this step (Bergamini et al., 2010; Lancova et al., 2008; Vidal, Morales, Sanchis, Ramos, & Marín, 2014a). After baking, DON results are also contradictory and some authors observed DON reduction (Neira et al., 1997; Bergamini et al., 2010), whereas, other authors pointed out no changes or even an increase (Simsek, Burgess, Whitney, Gu, & Qian, 2012; Zachariasova et al., 2012). However, incoherencies may exist due to the fact that some studies were carried out at laboratory scale and others at industrial level (Bergamini et al., 2010). Moreover, Vidal, Sanchis, Ramos, & Marín (2015), using small size items, demonstrated that DON may be reduced only in the external part of loaves, due to the

reduced heat transmission, thus the size of the baked items may also explain the conflicting results reported for baking (Vidal et al. 2014a). Regarding DON-3-glucoside, the four existing publications show also contradictory results for the fermentation and the baking steps (Generotti et al., 2015; Suman, Manzitti, & Catellani, 2012; Vidal et al., 2014a; Vidal, Marín, Morales, Ramos, & Sanchis, 2014b; Zachariasova et al., 2012). Recently, Vidal et al. (2015) showed that DON-3-glucoside may be released under mild baking conditions of temperature/time (for instance 140 º for 35 minutes or 200 ºC for less than 10 minutes), but reduced under harsher baking conditions, i.e. longer time and higher temperatures.

The increases in DON and DON-3-glucoside reported during breadmaking might be due to enzyme activity (Simsek et al., 2012; Vidal et al., 2014a). Enzymes may hydrolyse mycotoxin bounds with carbohydrates or other components related to the ingredients of the recipe formulations causing an increase of mycotoxin concentration at the end of the breadmaking process. For example, Zhou, Schwarz, He, Gillespie, & Horsley (2008) detected higher DON levels in barley samples after the treatment with protease, xylanase and cellulase, and so did Simsek et al. (2012) in wheat samples after xylanase treatment. Finally, Zachariasova et al. (2012) found that α-amylase caused no changes in DON-3-glucoside when malt samples were treated for more than six hours.

Given that hydrolytic enzymes may affect DON release during breadmaking, the objective of the present work was to assess such effect for different enzymes (xylanase, α-amylase, cellulase, protease, lipase and glucose oxidase, usually used in breadmaking), on DON and DON-3-glucoside fate during bran bread production.

2. Materials and methods

2.1. DON and DON-3-glucoside initial levels in flour and bran

Flour and bran wheat were purchased in a flour mill in Lleida (Spain), and were analysed for natural DON and DON-3-glucoside contamination. The initial DON concentration in the flour was 251.51 ± 30.39 μg/kg (n = 3) and DON-3-glucoside was not detected, while in the bran,

DON and DON-3-glucoside concentrations were 2003.67 ± 72.39 and 578.57 ± 61.15 µg/kg (n = 3), respectively.

2.2. Dough preparation and baking

A flour + bran mix was prepared (200 g of bran/1000 g of flour) and used for the bread experiments. Therefore, the concentrations of DON and DON-3-glucoside were 650.63 ± 12.96 and 137.13 ± 23.22 µg/kg (n = 6), respectively, in the mix of flour + bran.

To each flour + bran mix (156 g), 2.3 g of salt, 4.7 g of sucrose, 4.7 g of lard and 6.2 g of commercial compressed yeast (*Saccharomyces cerevisiae*, Levanova, Lesaffre Ibérica, S.A., Spain) were added. The dough was obtained by adding 83 mL of water to the mixture. Different doughs were prepared, containing six different enzymes (xylanase, α-amylase, cellulase, protease, lipase and glucose oxidase) plus a control. The enzyme concentrations were adjusted following some breadmaking references: 1 U of xylanase/g flour (Oliveira, Telis-Romero, Da-Silva, & Franco, 2014), 10 U of α-amylase/g flour (Kim, Maeda, & Morita, 2006), 35 mU of cellulase/g flour (Haros, Rosell, & Benedito, 2002), 10 U of protease/g flour (Harada, Lysenko, & Preston, 2000), 1 U of lipase/g flour (Moayedallaie, Mirzaei, & Paterson, 2010) and 10 U of glucose oxidase/g flour (Hanft & Koehler, 2006). Enzymes were added in powder. Moreover, second fermentation was carried out separately at 30 or 45 ºC. Thus, fourteen treatments were tested in the study, and the experiment was repeated three times.

Dough was manually kneaded until held together with a non-sticky, smooth and satiny appearance and optimum handling properties. Rounded pieces weighing 250 g each were prepared. From this point, thermoprobes (Thermo Bouton, Proges Plus, France) were always used in the dough to record fermentation and baking temperatures; in particular, probes were placed in the centre of the loaf and close to the surface. Doughs were covered with a damp cloth and first fermentation was carried out at 30 ºC for 15 minutes. Then the pieces were placed in moulds, where the dough further fermented for 1 hour at 30 or 45 ºC. After the fermentation, a sample of 25 g was taken from each proofed dough; samples were lyophilized and stored at -20 ºC until mycotoxin analysis. The proofed doughs were then baked in an oven (Eurofred PE46SVR, Eurofred, Spain). Baking conditions were 180 ºC and 75 min. Such

conditions were established on the basis of previous experiments to obtain suitable bread. After baking, a representative sample was taken, lyophilized and stored at -20 ºC until analysis.

2.3. Chemicals, reagents and enzymes.

Mycotoxin (DON and DON-3-glucoside) standards were supplied by Sigma (Sigma-Aldrich, Alcobendas, Spain). Acetonitrile, methanol and ethanol were purchased from J.T. Baker (Deventer, The Netherlands). All solvents were LC grade. 3, 5-Dinitrosalicylic acid (DNS) (≥ 98 %), sodium azide (≥ 99.5 %), starch (from potato), *o*-dianisidine (peroxidase substrate), Triton X-100 (laboratory grade), copper (II) acetate (≥ 99.5 %), caseinate (from bovine milk) and trichloroacetic acid (≥ 99.0 %) were supplied by Sigma. Malic acid (≥ 99 %), 2,2,4-Trimethylpentane (≥ 99.5 %) and acetic acid (100 %) were supplied from VWR Prolabo (Llinars del Vallès, Spain). Sodium hydroxide (≥ 99.5 %), sulphuric acid (≥ 96 %) and sodium chloride (≥ 99.5 %) were supplied by Fisher Bioreagents (New Jersey, USA). Sodium and potassium tartrate (≥ 99 %) and Tris buffer (reagent grade) were supplied by Scharlau (Barcelona, Spain). Sodium carbonate (≥ 99.5 %) and Folin's reagent were supplied by Panreac (Castellar del Valles, Spain). Filter paper (Whatman No. 1) was purchased from Whatman (Maidstone, UK). Immunoaffinity chromatography columns (IAC) for DON (DONPREP®) extracts clean-up were purchased from R-Biopharm (Rhone LTD Glasgow, UK). Pure water was obtained from a milli-Q apparatus (Millipore, Billerica, MA, USA). The six enzymes used in the study, xylanase (from *Trichoderma longibrachiatum*), α-amylase (*Aspergillus oryzae*), cellulase (*Aspergillus niger*), protease (*Aspergillus oryzae*), lipase (*Aspergillus niger*) and glucose oxidase (*Aspergillus niger*) were purchased from Sigma.

2.4. Preparation of mycotoxin standard solutions

DON standard solution was dissolved in ethanol at a concentration of 10.0 µg/mL and stored at 4 ºC. The concentration in the stock solution was checked by UV spectrometry according to the AOAC Official methods of analysis. Working standards (2.5, 1.0, 0.5, 0.1 and 0.05 µg/mL) were prepared by appropriate dilution of known volumes of the stock solution with mobile phase and used to obtain calibration curves in the appropriated chromatographic system. DON-3-glucoside standard was dissolved in acetonitrile at a concentration of 10.0 µg/mL and stored at 4 ºC in a

sealed vial until use. Working standards (1.0, 0.5, 0.1, 0.05 and 0.01 µg/mL) were prepared as for DON, as well as calibration curves.

2.5. Enzyme activity quantification

To quantify the enzymatic activity in each sample, a calibration curve was required. Moreover, a blank reference was required to set the spectrophotometer to zero absorbance. For each enzyme protocol, the following common steps were followed:

- Preparation of the blank reagent (with neither substrate nor enzyme, only reagents). The blank reagent was used to set the spectrophotometer to zero absorbance.
- Preparation of calibration samples: substrate with known added enzyme concentrations plus a zero sample with no added enzyme. Calibration curves were built both for fermented dough and for bread.

2.5.1. Xylanase, α-amylase and cellulase activity

The activity of the three enzymes was analysed following the reduction of DNS to 3-amino,5-nitrosalicylic acid (ANS) by spectrophotometry at 540 nm (Miller, 1959). The extraction solution was the same for the three enzymes (250 mL of malic acid 0.2 M, 250 mL of sodium hydroxide 0.35 M, 250 mL of sodium chloride 0.2 M and 250 mL of sodium azide 0.003 M, all of that was a 1 L of extraction solution). DNS reagent was prepared with 1 g of DNS, 20 mL of sodium hydroxide 2 M, 10 mL of sodium and potassium tartrate 10.6 M and 70 mL of water.

2.5.1.1. Xylanase activity

Tubes with 3 g of fermented dough/bread samples were treated at 40 ºC for 20 minutes with 20 mL extraction solution without shacking. 0.5 mL of the supernatant was added to 1.5 mL of the substrate (5 g of wheat fibre in 80 mL of water, boiled for 15 min with agitation, followed by 15 minutes of agitation at room temperature; 10 mL of sodium acetate 1 M; brought to a volume of 100 mL with water). The tubes were heated for 8 minutes at 50 ºC and 3 mL of DNS reagent were added at the end of the heating step. Subsequently, the tubes were placed for 5 minutes in boiling water. Then, after the 5 minutes in boiling water the tubes were cooled to room

165 temperature in a water bath. Cooling to ambient temperature was made necessary by the effect
166 of temperature on the absorbance of the coloured reaction product. After this the absorbance at
167 540 nm was measured in the spectrometer. To build the calibration curves, xylanase calibration
168 samples (5, 2.5, 1, 0.5, 0.1 and 0.05 U/g) were prepared with fermented dough/bread samples
169 and used to obtain calibration curves (r^2 were 0.75 and 0.99, for fermented dough and bread,
170 respectively), following the same protocol for analysis as above.
171
172 *2.5.1.2. α-Amylase activity*

173 Tubes with 3 g of fermented dough/bread samples were treated at 40 ºC for 20 minutes with 20
174 mL extraction solution without shacking. 0.5 mL of the supernatant was added to 1.5 mL of the
175 substrate (1 g of starch in 20 mL of sodium hydroxide 1 M; 10 mL of sodium and potassium
176 tartrate 10.6 M, and 70 mL of water). The tubes were kept at 25 ºC for 3 minutes, and then 1 mL
177 of DNS reagent was added. The tubes were boiled for 5 minutes in boiling water. Then, after the
178 5 minutes in boiling water, the tubes were cooled to room temperature in a water bath, then 10
179 mL of water were added. Finally, the absorbance was measured in the spectrometer at 540 nm.
180 α-amylase calibration samples (50, 10, 5, 1, 0.5 and 0.05 U/g) were prepared with fermented
181 dough/bread samples and used to obtain calibration curves (r^2 were 0.86 and 0.96, for
182 fermented dough and bread, respectively), following the same protocol for analysis as above.

183 *2.5.1.3. Cellulase activity*

184 Tubes with 3 g of fermented dough/bread samples were treated at 40 ºC for 20 minutes with 20
185 mL extraction solution without shacking. 0.5 mL of the supernatant was added to 0.25 g of filter
186 paper (Whatman no 1). The tubes were kept at 50 ºC for 60 minutes, and then 3 mL of DNS
187 reagent was added. The tubes were boiled for 5 minutes in boiling water. Then, after the 5
188 minutes in boiling water, the tubes were cooled down to room temperature. The absorbance
189 was measured in the spectrometer at 540 nm. Cellulase calibration samples (50, 10, 5, 2.5, 1,
190 0.5 and 0.1 mU/g) were prepared with fermented dough/bread samples and used to obtain
191 calibration curves (r^2 were 0.81 and 0.94, for fermented dough and bread, respectively),
192 following the same protocol for analysis as above.

193 *2.5.2. Protease activity*

194 The assay followed the method described by Ladd and Butler (1971). Briefly, 2 g of fermented
195 dough/bread samples were weighted in a tube and blended with 5 mL of caseinate solution (10
196 mg/mL) in 0.1 M Tris-buffer pH 8.1. The tubes were heated for 60 minutes at 50 ºC without
197 shacking. Then, 1 mL of trichloroacetic acid 1.0 M was added. The mix was centrifuged and 2
198 mL of the supernatant were mixed with 3 mL of sodium carbonate 1.4 M and 1 mL Folin's
199 reagent 1.3 M. After waiting for 10 minutes at 25 ºC, the sample was measured
200 spectrophotometrically at 700 nm. Protease calibration samples (15, 10, 7.5, 5, 2.5 and 1 U/g)
201 were prepared with fermented dough/bread samples and used to obtain calibration curves (r^2
202 were 0.95 and 0.84, for fermented dough and bread, respectively), following the same protocol
203 for analysis as above.

204 *2.5.3. Lipase activity*

205 The method described by Duncombe (1963) was followed to measure the lipase activity in the
206 samples. 0.5 g of fermented dough/bread samples were weighted and mixed with 0.3 mL of
207 olive oil and 0.5 mL of Tris-HCl solution (100 mL of Tris-HCl 0.05 M with 1 mL of Triton-X, pH =
208 7.5). The samples were incubated for 60 minutes at 37 ºC without shacking. 0.1 mL of HCl 1 M
209 and 5 mL of isooctane were added to each sample. Then, the samples were placed in boiling
210 water for 5 minutes. 2.5 mL of copper reagent (copper acetate 0.28 M, pH = 6.1) were added. It
211 was then centrifuged for a few minutes to separate the phases, and the upper layer was taken
212 for measurement. Lipase activity was measured with the spectrophotometer at 540 nm. Lipase
213 calibration samples (2.5, 1.5, 1, 0.5 and 0.1 U/g) were prepared with fermented dough/bread
214 samples and used to obtain calibration curves (r^2 were 0.96 and 0.99, for fermented dough and
215 bread, respectively), following the same protocol for analysis as above

216 *2.5.4. Glucose-oxidase activity*

217 To measure the glucose-oxidase activity, we followed the method described by Bergmeyer,
218 Gawehn, & Grassl (1974). 3 g of fermented dough/bread samples were placed in a tube and 5
219 mL of extraction solution (20 mL of sodium acetate 8.3 M and 2.5 mL of acetic acid in water, up
220 to 1 L) plus 2.5 mL of glucose 1 M were added. The mix was kept at 30 ºC for 5 minutes
221 without shacking, then, 1 mL of *o*-dianisidine was added. The samples were kept at 30 ºC for 5
222 more minutes, and then 4 mL of the supernatant were mixed with 2 mL of sulphuric acid (9 M).

The samples were measured at 540 nm with a spectrophotometer. Glucose-oxidase calibration samples (15, 10, 5, 2.5,1 and 0.5 U/g) were prepared with fermented dough/bread samples and used to obtain calibration curves (r^2 were 0.74 and 0.79, for fermented dough and bread, respectively), following the same protocol for analysis as above.

2.6. Mycotoxins extraction, detection and quantification.

DON and DON-3-glucoside were extracted from 5 g of lyophilised ground sample (IKA® A11B basic analytical mill, IKA-Werke GmbH & Co. KG, Germany) with 30 mL of distilled water by magnetically stirring for 10 min. Then the sample was centrifuged for 8 min at 1780 *g*. The supernatant was filtered through a glass microfiber filter. Five milliliters of filtered sample were loaded on the DONPREP® IAC column and the column washed with 10 mL of distilled water. DON and DON-3-glucoside were eluted by applying 1.5 mL of methanol grade HPLC (with three backflushing steps) and 1.5 mL of milli-Q water, consecutively. Zachariasova et al. (2012) confirmed the good cross-reactivity of DON-3-glucoside with the IAC DONPREP® columns (99-102 % recovery for DON and DON-3-glucoside when less than 500 ng of these toxins were loaded). The purified extracts were dried under nitrogen stream at 40 ºC. Each dried sample was resuspended with 0.5 mL of the mobile phase solution (water:acetonitrile:methanol, 92:4:4). DON and DON-3-glucoside were determined in a HPLC Waters 2695® system, with an analytical column Waters Spherisorb® 5 µm ODS2, 4.6 x 250 mm, and coupled with a UV/Visible dual λ absorbance Detector Waters 2487. Absorption wavelength was set at 220 nm. The HPLC mobile phase flow rate was 0.6 mL/min, the injection volume was 100 µL, the column temperature was 40 ºC and the retention times for DON and DON-3-glucoside were 20 and 23 min, respectively.

2.6.1. Methods performance

The analytical methods for DON and DON-3-glucoside were assessed for linearity, precision and recovery. Standard curves were generated by linear regression of peak areas against concentrations (r^2 were 0.99 and 0.96, for DON and DON-3-glucoside, respectively). Precision was established by determining DON and DON-3-glucoside levels in flour and DON in bread samples at least by triplicate, in those samples fortified in order to calculate the recovery rates.

Recovery was not tested in dough, as it was considered to be similar in composition to both flour and bread. The limit of detection (LOD) was considered to be three fold the signal of blank noise, and the limit of quantification (LOQ) was calculated as 3 x LOD. Method performance characteristics for DON and DON-3-glucoside are summarized in Table 1.

2.7. Statistics

The results are given in dry weight basis. A Multifactorial ANOVA was applied to detect significant differences in enzyme activity due to the treatments. Also a Multifactorial ANOVA was applied to assess the significance of sample traits in the observed mycotoxin concentration levels as well as in the calculated percentages of increase/reduction.

3. Results and discussion

3.1. Impact of enzyme addition on DON presence

3.1.1. Fate of DON in bread with non-added enzymes

Wheat flour naturally contains several technologically important enzymes such as amylases, proteases, lipoxygenase, polyphenol oxidase and peroxidase. Although these enzymes are inactive during storage of grain and flour, when water is added they become active and play a significant role in determining the functional attributes of flour (Rani, Prasada Rao, Leelavathi, Haridas, & Rao, 2001).

The unkneaded mix of the ingredients contained 594.24 ± 11.84 μg/kg of DON while the mean concentration in the fermented doughs was 562.05 ± 16.91 and 460.43 ± 24.01 μg/kg, at 30 and 45 ºC of fermentation temperature, respectively (Table 2); both reductions (5 and 23 %) being significant, as well as the difference between them ($p < 0.05$). Similarly to what was observed here, the reduction of DON during fermentation has been observed among the existent literature on DON fate during breadmaking. For instance, Neira et al. (1997) observed a 21.6 % of reduction. Other studies reported lower DON concentration in fermented dough than in the

277 initial flour, but this may also be due to dilution by the recipe (Lancova et al., 2008). On the
278 other hand, an increase of DON at the end of fermentation has been observed in other studies
279 (Bergamini et al., 2010; Lancova et al, 2008; Vidal et al., 2014b). It can be noted that in some
280 cases when enzymes (especially α-amylase) were added in the recipes, DON increase was
281 detected. Simsek et al. (2012) detected up to 99 % DON increase after fermentation using α-
282 amylase, and Suman et al. (2012) had an increase up to 14 % using non specified enzymes.
283 Moreover, Vidal et al. (2014a) using flour improvers with non specified enzymes detected a
284 DON increase during the fermentation of 30 %. Moreover, sourdough use also led to increased
285 DON content during fermentation (Vidal et al. 2014b). In summary, those works in which either
286 malt flour or other enzymes were added reported a DON increase (Simsek et al., 2012, Suman
287 et al., 2012; Vidal et al., 2014a), while in the absence of added enzymes a reduction in DON
288 content seems to occur (Neira et la., 1997). Thus enzyme presence may be determinant for
289 DON fate during fermentation. The increase of DON during fermentation has been associated to
290 bound DON release from the wheat matrix catalysed by enzymes (Simsek et al., 2012; Vidal et
291 al., 2014a).
292
293 Moreover, in the present study, DON reduction was higher at 45 than at 30 ºC fermentation
294 temperature ($p < 0.05$). However, few authors have dealt with fermentation temperature. Samar
295 et al. (2001) assayed different temperatures of fermentation (from 30 to 50 ºC). They obtained
296 increasing DON reduction with increasing temperature (from 0 to 56 %), the highest reduction
297 being at the highest temperature (50 ºC) with the longest time tested (60 min), confirming our
298 results. Also, Generotti et al. (2015) assayed different fermentation temperatures (from 26 to 46
299 ºC) and they observed a reduction effect in DON stability due to fermentation temperature. Thus
300 fermenting at high temperature could be a good alternative to reduce DON content in bread, if
301 bread quality is not affected. Although proofing temperature can be as high as 54 ºC (Pyler,
302 1973), most authors agree in the range of 27-46 ºC for optimum bread production (Freilich,
303 1949; Hui, Corke, De Leyn, Nip, & Cross, 2007). Fermentation temperature around 30 ºC
304 results in good taste balance due to a good production of lactic acid. On the other hand,
305 fermentation above 40 ºC can cause a reduced lactic acid production and tasteless breads, but

306 the higher fermentation temperature can also lead to better baking volume (Dobraszyk,
307 Smewing, Albertini, Maesmans, & Schofield, 2003).

308 Regarding baking, the bread fermented at 30 ºC had a final concentration of 495.82 ± 27.10
309 μg/kg which meant a significant reduction of 12 % during the baking step ($p < 0.05$). The final
310 DON concentration of the bread fermented at 45 ºC was 466.58 ± 11.58 μg/kg (not significantly
311 different from fermented dough). Thus, DON reduction in baking depended on fermentation
312 temperature ($p < 0.05$). Considering the whole breadmaking process, the DON concentration in
313 bread was similar regardless of the fermentation temperature (mean reduction from beginning
314 to end ca. 19 %). The reduction in DON during baking was consistent with most previous
315 studies which reported reduction at temperature over 170 ºC, as long as baking time was longer
316 than 45 min (Vidal et al., 2014a). The present results agree with the response surface model for
317 DON reduction in bread baking made by Vidal et al. (2014a). No effect of baking was observed
318 in bread fermented at 45 ºC, this could be caused by the lower initial DON concentration in this
319 case; DON reduction during baking has been shown to be higher at higher initial toxin
320 concentration and not significant at low initial DON concentration (Vidal et al. 2014a, 2015).
321 DON reduction may result in thermodegradation products (norDONs A-F and DON lactones),
322 which are less toxic than DON itself. The losses that cannot be ascribed to the formation of
323 degradation products are most likely caused by pyrolysis or polymerization reactions (Bretz,
324 Beyer, Cramer, Knecht, & Humpf, 2006). Still, some existing studies reported in some cases no
325 DON reduction or even a slight increase during baking which could be attributed to extended
326 enzyme activity at the early stages of baking (Bergamini et al. 2010; Simsek et al., 2012; Suman
327 et al., 2012).

328

329 *3.1.2. Fate of DON in bread with added enzymes*

330 Xylanases are hydrolytic enzymes, which randomly cleave the β-1,4 backbone of plant cell wall
331 xylans. Xylanases are of great value in baking as they have been found to improve the bread
332 volume, crumb structure and reduce stickiness. In our case, xylanase activity increased during
333 fermentation with xylanase addition, however, the activity of the existing flour xylanase at 45 ºC
334 was still higher than the activity at 30 ºC with added xylanase ($p < 0.05$) (Table 3). Xylanase

addition had only a significant effect in DON variation during fermentation when it occurred at 45 ºC ($p < 0.05$, despite the increase in activity was not significant), where the final DON concentration in the fermented dough was 530.63 ± 8.15 μg/kg (a 15 % increase, Table 2); however, this level was still lower than before fermentation. The detected different behaviour of DON at 45 ºC may be linked to the optimum temperature for xylanase activity (45 ºC for xylanase produced by *T. longibrachiatum*; Chen, Chen, & Lin, 1997) was reached. The baking step produced a DON increase of 35 % in the xylanase -containing dough fermented at 30 ºC ($p < 0.05$) and the final concentration in the bread was 732.33 ± 28.28 μg/kg. The baking step did not produce any significant change in the DON concentration when the fermentation was at 45 ºC, but still the final bread with xylanase had a higher DON concentration (508.65 ± 16.26 μg/kg) than the control bread ($p < 0.05$), due to the increase during fermentation. In summary, DON increased with the presence of xylanase during the fermentation at 45 ºC and in the baking step when the fermentation was at 30 ºC. This may be linked with the moment when the optimum temperature for xylanase activity as we commented above. In the dough fermented at 30 ºC the major xylanase activity probably occurred in the early stages of baking, before enzyme inactivation over 55 ºC (Irfan, & Syed, 2012). Thus, xylanase added breads contained higher levels of DON at the end of breadmaking process than control ones regardless of the fermentation temperature ($p < 0.05$), however, only when fermenting at 30 ºC the concentration in the bread was higher than in the initial mix of ingredients. Similarly, Simsek et al. (2012) reported an increase of DON (13 %) in xylanase-treated wheat at 50 ºC for 18 hours. Zhou et al. (2008) found a trend to DON increase after treatment of barley grains with xylanase/cellulase (5 hours at 50 ºC). Xylanases cause the hydrolysis of cell wall material (arabinoxylan) in the dough resulting in a release of DON bound to the polysaccharides of cereal cell walls.

α-amylase is an enzyme that hydrolyses alpha bonds of large, alpha-linked polysaccharides, such as starch. In our case, α-amylase activity was not detected in the fermented doughs were the enzyme was not externally added, and the activity was similar in amylase-added doughs fermented at 30 than at 45 ºC, while some residual activity was still detected in the resulting breads (Table 3). Different studies showed the high thermo stability of α-amylase, for instance Raviyan, Tang, & Rasco (2003) showed the stability of α-amylase above 75 ºC; inside the bread

365 during baking the temperature is always below 100 ºC. The tested α-amylase produced an
366 increase of DON concentration during the fermentation at 30 ºC, with a final concentration in the
367 fermented dough of 656.26 ± 11.58 μg/kg (Table 2, 10 % increase compared to unkneaded mix,
368 $p < 0.05$). Baking produced no reduction of DON in breads previously fermented at 30 ºC
369 (670.35 ± 5.32 μg/kg). On the other hand, the baking step caused a DON increase (20 %) in
370 the breads fermented at 45 ºC, and the final DON concentration in bread was 534.56 ± 15.37
371 μg/kg. Thus breads with added α-amylase contained higher DON concentration than the
372 controls ($p < 0.05$), even in the bread fermented at 30 ºC the concentration was higher than in
373 the initial ingredient mix. The more marked effect on DON at 30 ºC was related with the activity
374 of the α-amylase from *A. oryzae* (Evstatieva, Nikolova, Ilieva, Getov, & Savov, 2010). α-amylase
375 is one of the most used enzymes in the breadmaking process, and our results at 30 ºC
376 demonstrated that the release of DON described in previous studies may be caused by its use.
377 Moreover, in the literature, the longer the fermentation time with α-amylase the higher the
378 increase in DON reported. For instance, Simsek et al. (2012) reported an increase in DON
379 concentration at the end of the fermentation (180 minutes at 30 ºC) near to 100 %, Bergamini et
380 al. (2010) about 38 % (85 minutes at 40 ºC) and Suman et al. (2012) about 10-14 % in biscuits
381 (4 minutes at 30 ºC). The results then suggest that α-amylase has an impact in DON balance
382 during breadmaking process, as it may be released from forms bound to starch.

383 Cellulase added to bread hydrolyses non-starch polysaccharides leading to an improvement of
384 the rheological properties of dough, bread loaf volume and crumb firmness. Very low activity
385 was detected in doughs without added cellulose, while the detected activity in cellulose added
386 doughs was higher when fermentation was at 45 than at 30 ºC. No cellulase activity was
387 detected in any of the breads (Table 3). No effect of cellulase addition was observed when
388 fermentation was carried out at 30 ºC (Table 2). Cellulase addition, however, caused changes in
389 the DON concentration when the fermentation was at 45 ºC ($p < 0.05$) with a DON
390 concentration of 752.17 ± 39.10 μg/kg (63 % higher than in the control) in the fermented dough,
391 and 559.61 ± 106.54 μg/kg in the final bread. In this case, the higher DON reduction during
392 baking found in breads fermented at 45 ºC could be caused by the higher DON concentration
393 found at the end of the fermentation, as commented before. Thus the final bread in this case
394 contained a level not significantly different from that in the unkneaded mix. The optimum

temperature of cellulase produced by *A. niger* is close to 45 ºC (Coral et al., 2002), this would explain the difference between temperatures. An increase of DON was observed as the result of the activity of a xylan/cellulase mix in barley samples (5 hours at 50 ºC) (Zhou et al. 2008). This indicates that DON may be bound to cell wall cellulose of cereals.

Too much protease activity would break up the gluten, destroying the network that forms during kneading. A little bit, however, softens the dough and makes it more workable. In addition proteases affect bread flavour. Protease activity results in single amino acids when the last peptide bond of the protein chain is broken. These amino acids can participate in the flavour and browning reactions that occur in the crust during baking. No protease activity was detected in our samples, except for fermented dough at 45 ºC (Table 3). This was consistent with DON results, where protease added samples did only differ from the controls in DON concentration in fermented doughs at 45ºC (Table 2). The optimum temperature for protease activity is at 60 ºC (Yin et al., 2013), and the highest protease activity was after fermentation at 45 ºC. No protease effect was detected by Simsek et al. (2012) when treating whole wheat grain; this was probably to the location of great part of proteins in the endosperm, which was poorly accessible in their experiment (Veraverbeke, & Delcour, 2002). As for the cellulase case, no differences were found in the final loaves compared to the control, as the increase observed in DON during the fermentation was compensated by the decrease occurring during baking.

Lipases are particularly effective in retarding bread staling. Lipase activity was quite similar in control and lipase added doughs, while no activity was detected in breads (Table 3). The use of lipase in the fermentation did not produce any effect on DON concentration. After baking, DON concentration slightly increased ($p < 0.05$). A low interaction of DON with the lipid fraction of bread ingredients can be hypothesised.

Glucose oxidase catalyses the oxidation of *β*-D-glucose to glucono-*δ*-lactone and the concomitant reduction of molecular oxygen to hydrogen peroxide. Its use results in stronger and more elastic doughs with a dry surface. Glucose oxidase activity was only detected in those doughs where the enzyme was intentionally added, and both fermented at 30 and 45 ºC (Table 3). However, no impact on DON was observed at 30 ºC (Table 2), while at 45 ºC it led to the highest DON concentration (821.49 ± 25.58 μg/kg, 38 % increase) in a fermented dough,

424 although it dropped to 551.30 ± 3.71 μg/kg due to baking, but still higher than the control ($p < 0.05$). This point could be linked with DON-3-glucoside presence, as discussed later. The optimum temperature of glucose oxidase is around 35-40 ºC (Bhatti, Madeeha, Asgher, & Batool, 2006) and certain activity was detected in the fermented dough at both 30 and 45 ºC.

428

429 It must be highlighted that the enzyme activities presented here for the fermented dough were
430 determined at the end of the fermentation step, thus the values presented in table 3 are not
431 indicative of the levels of activity which may have occurred previously during fermentation or
432 later in the early stages of baking.

433

434 In summary, the presence of some enzymes (α-amylase, cellulase, protease and glucose
435 oxidase) led to certain release of DON during fermentation, whereas xylanase produced a lower
436 DON reduction during fermentation. However, it depended on fermentation temperature. At 30
437 ºC, only presence of α-amylase caused a higher DON content after the kneading + fermentation
438 process comparing with the DON content in the unkneaded mix. The increase in DON content
439 detected in xylanase and α-amilase added breads fermented at 30 ºC after baking, represents
440 the main concern, because their DON content was higher than in the initial unkneaded mix. On
441 the other hand, fermentation at 45 ºC led to DON content increase respect to the initial
442 unkneaded mix when using cellulase, protease and glucose oxidase at the end of the
443 fermentation. The DON content in xylanase added fermented dough was higher than the control
444 fermented dough but lower than the initial unkneaded mix. For 45 ºC fermented doughs baking
445 led to lower levels of DON, thus resulting in general in breads which were not significantly
446 different from the controls, except for xylanase, α-amylase and glucose oxidase treated breads,
447 but the three enzyme additions did not cause a higher DON content in the final bread than the
448 DON content in the unkneaded mix.

449 To sum up, only xylanase and α-amylase added breads fermented at 30 ºC resulted in a higher
450 presence of DON in the final product than DON content in the flour mix. In these two particular
451 cases an additional risk should be considered. Thus the ability of some enzymes to release
452 bound DON from the flour during the breadmaking process was proven. Few studies have dealt

453 with food processing enzymes and mycotoxin behaviour, but they suggested this hypothesis
454 (Lancova, et al., 2008; Simsek et al., 2012; Zhou et al., 2006).

455

456 *3.2. Impact of enzyme addition in DON-3-glucoside presence*

457 DON-3-glucoside concentration (in dry basis) in the unkneaded mix was 125.18 ± 27.62 μg/kg,
458 and at the end of the fermentation without added enzymes had decreased to <LOQ (30 μg/kg),
459 regardless of the fermentation temperature (unlike what happened with DON). In the baking
460 step, DON-3-glucoside concentration was restored to 100.79 ± 16.98 μg/kg and 65.05 ± 38.88
461 μg/kg (Table 4). The detected reduction of DON-3-glucoside after dough proofing without
462 enzymes agrees with previous studies (Kostelanska et al., 2011).

463 When enzymes were added to the dough a clear decrease in DON-3-glucoside during
464 fermentation was also observed, regardless of the temperature level. Despite DON-3-glucoside
465 reduction at the end of fermentation, xylanase and protease at 30 ºC and cellulase and lipase at
466 45 ºC led to a lower reduction than control fermented doughs (Table 4). On the other hand, the
467 exception to DON-3-glucoside reduction at the end of fermentation was for glucose oxidase
468 activity, which led to a significant increase in DON-3-glucoside at both temperatures (359.71 ±
469 59.27 μg/kg at 30 ºC).The presence of glucose oxidase caused an important increase of DON-
470 3-glucoside in the fermented dough compared with the initial mix.

471 The enzyme effects during baking were more noticeable (Table 4), mostly in those doughs
472 which had been fermented at 30 ºC. Xylanase, α-amilase, cellulase and lipase led to significant
473 DON-3-glucoside increase, the outcome of these enzymes in the baking increase caused a
474 higher DON-3-glucoside content in final breads than in the initial mix. Glucose oxidase added
475 breads showed a significant decrease in concentration compared to fermented dough and
476 unkneaded mix, as well as compared to control breads. However, in the doughs fermented at
477 45 ºC baking had no significant effect ($p < 0.05$), and the resulting breads did not differ from the
478 control ones.

479 The use of enzymes as improvers in some past studies caused increase of DON-3-glucoside in
480 the fermentation step; however the exact enzymes were not described (Kostelanska et al. 2011;

Vidal et al., 2014a, 2014b). Simsek et al. (2012) used only α-amylase and they had a reduction of DON-3-glucoside at the end of the fermentation at 30 ºC (5 %) (as in the present case).

The increment of DON-3-glucoside after baking agrees with previous studies in wheat products (Vaclavikova et al., 2013; Vidal et al., 2014b, 2015), however some studies showed a reduction of DON-3-glucoside after baking (Kostelanska et al., 2011; Simsek et al., 2012). Vidal et al. (2015) showed DON-3-glucoside has a different behaviour in thermal treatments as a function of size of the product, temperature and time. The mild baking conditions tested in this assay (especially due to the big size of the product) caused an increase of DON-3-glucoside during the baking step, while harsher treatments would have led to DON-3-glucoside reduction. Our results showed the high impact of enzymes in the DON-3-glucoside release during baking in those breads fermented at 30 ºC with added xylanase, amylase, cellulase, protease and lipase. DON-3-glucoside could be bound to flour components and be released in the baking step. In general, no significant correlation was found between DON-3-glucoside increase and DON decrease or viceversa, only in those breads which had been fermented a 45 ºC there was a slight inverse relationship between both. Similarly, Kostelanska et al. (2011) considered the behaviour of the two mycotoxins was not linked because in their case the DON level remained intact; they pointed out a possible splitting of glycosidic bonds between DON-3-glucoside and cell polysaccharides could occur. This agrees with our observation of high increase of DON-3-glucoside in baking when especially xylanase and cellulase were present in the matrix. More studies to understand the relation between DON and DON-3-glucoside are necessary to obtain a full knowledge between the parent and conjugated mycotoxin in the food processes. Finally, the increase of DON-3-glucoside during the baking steps is of concern because, although DON-3-glucoside is far less active as protein biosynthesis inhibitor than DON (Poppenberger et al., 2003), DON-3-glucoside likely will be cleaved in the gastrointestinal tract due to chemical hydrolases or, more important, microbial activity in the intestine as shown *in vivo* in swine and *in vitro* using human intestinal microbiota (Berthiller et al., 2011), thus its presence is important for food safety.

In conclusion, DON concentration could be lower in the breadmaking process if no enzymes were added. For example, while non-enzyme added flour shows a certain decrease in DON concentration during breadmaking (fermentation at 30ºC), the presence of xylanase and α-

amylase can cause an increase of DON at the end of the breadmaking process compared with the initial DON concentration in the initial mix. Besides the different optimum temperature levels for the tested enzymes, this may also imply that DON is more likely to be bound to starch and arabinoxylans, than to other polysaccharides, fat and protein. Moreover, if the fermentation is made at 45 ºC the presence of glucose-oxidase may also cause an increase of DON in the final bread respect to the initial DON concentration. The presence of xylanase, α-amylase, cellulase and lipase resulted in a bread with a higher presence of DON-3-glucoside when the fermentation was at 30 ºC compared with the initial mycotoxin concentration.

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Table 1. Method performances for DON and DON-3-glucoside determination in flour and bread.

Mycotoxin	Product	LOD[a] (µg·kg^{-1})	LOQ[b] (µg·kg^{-1})	n	Spiking level (µg·kg^{-1})	Recovery[c] (%)	RSDr[d](%)
DON	Bread	60	180	5	100	100.01±16.27	16
				5	500	98.84±9.01	9
				5	1000	102.33±5.26	5
	Flour	60	180	3	300	123.26±30.29	41
				3	500	87.36±8.58	7
DON-3-glucoside	Bread	15	30				
	Flour	15	30	5	50	80.01±9.59	12
				5	250	79.71±4.84	6
				5	500	66.71±11.19	18

[a] LOD = Limit of detection.

[b] LOQ = Limit of quantification.

[c] Mean value ± standard deviation.

[d] RSDr = relative standard deviation.

Table 2. Mean DON concentration (µg/kg) ± SD in fermented doughs and breads, and percentage of reduction in the fermentation compared with the initial mix (594.24±11.84), reduction in the baking step (%) and complete increase (%) in the final product compared with the initial mix (594.24±11.84) for each type of treatment.

Temperature of fermentation (°C)	30					45				
	Fermented dough	Reduction in fermentation (%)	Bread	Reduction in baking (%)	Complete reduction (%)	Fermented dough	Reduction in fermentation (%)	Bread	Reduction in baking (%)	Complete reduction (%)
No enzymes	562.05 ± 16.91	5.41	495.82 ± 27.10	11.78	16.56	460.43 ± 24.01	22.52	466.58 ± 11.58	-1.33	21.48
Xylanase	542.37 ± 60.91	8.73	732.33 ± 28.28*	-35.02	-23.24	530.63 ± 8.15*	10.70	508.65 ± 16.26*	4.14	14.40
α-Amylase	656.26 ± 11.58*	-10.44	670.35 ± 5.32*	-2.14	-12.81	447.29 ± 75.40	24.73	534.56 ± 15.37*	-19.51	10.04
Cellulase	576.40 ± 91.84	3.00	483.93 ± 67.13	16.04	18.56	752.17 ± 39.10*	-26.57	559.61 ± 106.54	25.60	5.83
Protease	595.88 ± 7.38	0.27	430.82 ± 122.57	27.70	27.50	803.95 ± 23.89*	-35.29	410.75 ± 42.49	48.91	30.88
Lipase	486.61 ± 48.45	18.11	521.97 ± 49.41	-7.27	12.16	570.20 ± 120.84	4.04	645.65 ± 81.64	-13.23	-8.65
Glucose oxidase	525.25 ± 59.01	11.61	567.58 ± 159.60	-8.06	4.49	821.49 ± 25.58*	-38.24	551.30 ± 3.71*	32.89	7.23

* There are significant differences compared with the same matrix sample without enzymes and at the same fermentation temperature ($p < 0.05$).

Table 3. Mean enzyme activity (units) ± SD in fermented doughs and breads.

Temperature of fermentation (˚C)	30				45			
	Fermented dough		Bread		Fermented dough		Bread	
	No enzyme added	With enzyme added	No enzyme added	With enzyme added	No enzyme added	With enzyme added	No enzyme added	With enzyme added
Xylanase (U/g)	0.16 ± 0.06	0.35 ± 0.01*	<0.05	<0.05	0.51 ± 0.09	0.62 ± 0.03	<0.05	<0.05
α-Amylase (U/g)	<0.05	34.1 ± 22.17*	<0.05	3.89 ± 0.51*	<0.05	21.09 ± 17.18*	<0.05	2.97 ± 0.31*
Cellulase (mU/g)	<0.1	7.16 ± 0.63*	<0.1	<0.1	0.80 ± 0.75	9.01 ± 0.52*	<0.1	<0.1
Protease (U/g)	<1	<1	<1	<1	<1	3.94 ± 1.94*	<1	<1
Lipase (U/g)	0.25 ± 0.02	0.31 ± 0.03*	<0.1	<0.1	0.27 ± 0.03	0.35 ± 0.09	<0.1	<0.1
Glucose oxidase (U/g)	<0.5	8.76 ± 1.30*	<0.5	<0.5	<0.5	5.58 ± 3.10*	<0.5	<0.5

* There are significant differences compared with the sample without added enzymes at the same fermentation temperature level ($p < 0.05$).

Table 4. Mean DON-3-glucoside concentration (µg/kg) ± SD in fermented dough and breads. The DON-3-glucoside concentration in the initial mix was 125.18 ± 27.62 µg/kg.

Temperature of fermentation (˚C)	30		45	
	Fermented dough	Bread	Fermented dough	Bread
No enzymes	<LOQ	100.79 ± 16.98	<LOQ	65.05 ± 38.88
Xylanase	61.65 ± 28.26	562.80 ± 64.85*	<LOQ	<LOQ
α-Amylase	<LOQ	568.23 ± 131.14*	<LOQ	<LOQ
Cellulase	<LOQ	628.76 ± 289.55*	30.08 ± 0.13	35.97 ± 10.35
Protease	36.72 ± 5.16	1834.79 ± 1472.22	<LOQ	126.08 ± 40.96
Lipase	<LOQ	275.41 ± 18.94*	45.04 ± 8.70	<LOQ
Glucose oxidase	359.71 ± 59.27*	55.63 ± 11.57*	50.86 ± 0.49*	76.25 ± 9.99

* There are significant differences compared with the sample without enzymes submitted to the same temperature ($p<0.05$).