

**Universitat de Lleida**

Document downloaded from:

<http://hdl.handle.net/10459.1/60758>

The final publication is available at:

<https://doi.org/10.1016/j.foodres.2017.08.021>

Copyright

cc-by-nc-nd, (c) Elsevier, 2017



Està subjecte a una llicència de [Reconeixement-NoComercial-SenseObraDerivada 4.0 de Creative Commons](https://creativecommons.org/licenses/by-nc-nd/4.0/)

# Accepted Manuscript

Effect of xylanase and  $\alpha$ -amylase on DON and its conjugates during the breadmaking process

Arnau Vidal, Vicente Sanchis, Antonio J. Ramos, Sonia Marín

PII: S0963-9969(17)30457-X  
DOI: doi: [10.1016/j.foodres.2017.08.021](https://doi.org/10.1016/j.foodres.2017.08.021)  
Reference: FRIN 6900  
To appear in: *Food Research International*  
Received date: 22 May 2017  
Revised date: 8 August 2017  
Accepted date: 12 August 2017

Please cite this article as: Arnau Vidal, Vicente Sanchis, Antonio J. Ramos, Sonia Marín, Effect of xylanase and  $\alpha$ -amylase on DON and its conjugates during the breadmaking process, *Food Research International* (2017), doi: [10.1016/j.foodres.2017.08.021](https://doi.org/10.1016/j.foodres.2017.08.021)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



## Effect of xylanase and $\alpha$ -amylase on DON and its conjugates during the breadmaking process.

Arnau Vidal, Vicente Sanchis, Antonio J. Ramos and Sonia Marín\*

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

\*Corresponding author: Tel. +34-973-702542. Fax: +34-973-702596. E-mail address: smarín@tecal.udl.cat. Postal address: Avda. Rovira Roure 191, 25198 Lleida, Spain.

### Abstract

Deoxynivalenol (DON) is one of the most frequently occurring mycotoxins in wheat crops worldwide and poses a risk to human and animal health due to its wide range of adverse effects. DON-3-glucoside is a DON plant conjugate that is widely found in cereal products. As DON accumulation in the field seems unavoidable, it is important to investigate all of the conditions that affect its stability during food processing. One of the most consumed cereal product around the world is bread, however the published information about DON stability in bread shows a large variability of results because a huge amount of factors affect DON and its modified forms. So, the aim of this research was to study the fate of DON and its modified forms through the breadmaking process with the addition of xylanase and  $\alpha$ -amylase at different fermentation temperatures. Moreover, different  $\alpha$ -amylase and xylanase concentrations were added to the dough to be fermented. To quantify DON and its derived forms in the samples liquid chromatography with double mass spectrophotometer was used. DON was reduced during fermentation and baking; however, the reduction at each step was related to the fermentation temperature. The presence of  $\alpha$ -amylase and xylanase caused increases in DON during fermentation and during early baking. Deoxynivalenol-3-glucoside (DON-3-glucoside) was slightly reduced after fermentation and was widely increased (> 80 %) after baking. Deepoxy-deoxynivalenol (DOM-1) increased during the breadmaking process. Breadmaking process can reduce DON concentration, however xylanase and  $\alpha$ -amylase presence cause increases of DON.

**Keywords:** deoxynivalenol, deoxynivalenol-3-glucoside, breadmaking, enzymes.

ACCEPTED MANUSCRIPT

**Highlights:**

- DON concentration is reduced during the breadmaking process when enzymes are not added.
- Time and temperature of the process steps influence the reduction of DON.
- Xylanase and  $\alpha$ -amylase increase DON concentration, since DON is embedded in the wheat matrix.
- DON-3-glucoside widely increased after baking. Xylanase and  $\alpha$ -amylase could affect its stability.
- DOM-1 is increased after the breadmaking process.

## 1. Introduction

Wheat, such as the majority of cereals, is susceptible to contamination with mycotoxins (Samar, Fontán, Resnik, & Pacin, 2003; Pleadin et al., 2013). To date, over 300 mycotoxins have been identified; however, not all of them represent a risk in food. Mycotoxins are produced by fungi, the main mycotoxin-producing fungi in foods belonging to the genera *Aspergillus*, *Penicillium* and *Fusarium*. Different studies show the high presence of mycotoxins, especially deoxynivalenol (DON), in highly consumed products, such as beer and bakery products (Pacin, Resnik, Neira, Moltó, & Martínez, 1997; Cano-Sancho et al., 2011). DON is one of the most common contaminants in cereals (Jelinek, Pohland, & Wood, 1989). Although it is not classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC, 1993), it has been linked with human gastroenteritis (Pestka, 2010 a,b).

On the other hand, some DON modified forms have been identified in cereals and the co-occurrence of free and modified DON forms has been documented. Thus, deoxynivalenol-3-glucoside (DON-3-glucoside), a plant conjugate, is the most common DON modified form found in cereals and the ratio of DON-3-glucoside/DON concentrations in unprocessed cereals is similar among assays, ranging from 0.1 to 0.3 (Berthiller et al., 2009; Dall'Asta, Dall'Erta, Mantovani, Massi, & Galaverna, 2013; De Boevre et al., 2012). 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) fungal conjugates are also very common in cereals; however, the concentrations are usually lower than DON-3-glucoside (Amarasinghe, Simsek, Brûlé-Babel, & Fernando, 2016; De Boevre et al., 2012; Tibola, Fernandes, & Guarienti, 2015). The presence of DON modified forms in cereals is of concern because Berthiller et al. (2011) showed that some lactic acid bacteria hydrolyse DON-3-glucoside *in vitro* and 3-ADON and 15-ADON are rapidly converted to DON during digestion (Broekaert, 2015; Veršilovskis et al., 2012).

Due to the high presence of DON and its modified forms in raw wheat, it is important to study their stability during food processing. Processing of cereals at high temperatures may affect DON and its modified forms content (Vidal et al., 2016; Wu et al., 2017). However, the extent of DON and its reduction during the bread making process seems to be quite variable and dependent on the processing conditions applied: temperature, time, type of mycotoxin, enzymes, and size of cereal product (Vidal et al., 2016; Wu et al., 2017). For bakery products, some studies reported a significant decrease in DON levels during the bread baking process (Numanoglu, Gökmen, Uygun, & Koksel, 2012; Valle-Algarra, Mateo,

Medina, Mateo, Gimeno-Adelantado, & Jiménez, 2009). By contrast, the studies of De Angelis, Monaci, Pascale and Visconti (2013) and Zachariasova, Vaclavikova, Lacina, Vaclavik and Hajslova (2012) reported that DON is stable during processing steps. Moreover, a possible release of DON from the flour could occur resulting in an increase in DON after baking.

Similar to DON, DON-3-glucoside stability during baking is also affected by the different size of assayed products, causing variable results in past studies (Generotti et al., 2015; Suman, Manzitti, & Catellani, 2012; Vidal Morales, Sanchis, Ramos, & Marín, 2014; Vidal, Marín, Morales, Ramos, & Sanchis, 2014; Zachariasova et al., 2012). However, Vidal et al. (2015) showed that DON-3-glucoside may be released under mild baking conditions of temperature and time (for instance, 140 °C for 35 minutes or 200 °C for less than 10 minutes); however, DON-3-glucoside may be reduced under harsher baking conditions (i.e., longer periods of time and higher temperatures).

One important factor affecting DON and DON-3-glucoside stability during the bread making process may be enzyme presence. Vidal et al. (2016) showed that some enzymes (xylanase, cellulase,  $\alpha$ -amylase, etc.) could also produce a change in mycotoxin concentration. Moreover, the effect of enzyme presence is related to temperature fermentation.

The current study aimed to investigate the fate of DON and its modified forms during fermentation at different points of the bread making process at different fermentation temperature levels (30 and 45° C) and during the baking step of the bread making process. The effect of xylanase and  $\alpha$ -amylase was checked during the bread making process assaying two different initial concentrations of each enzyme.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Mycotoxin standard solution of DON, DON-3-glucoside, 3-ADON, 15-ADON, deepoxy-deoxynivalenol (DOM-1) and isotolabeled ( $^{13}\text{C}_{15}$ ) DON were supplied by Biopure (Tulln, Austria). ( $^{13}\text{C}_{15}$ ) DON was used as an internal standard for UPLC-MS/MS. Acetonitrile (99.9 %), methanol (99.9%) and ethanol (99.5 %) were purchased from J.T. Baker (Deventer, The Netherlands). Dichloromethane ( $\geq 99.8$  %) and ammonium acetate ( $\geq 98$  %)

were purchased from Sigma (Sigma–Aldrich, Alcobendas, Spain). All solvents were LC grade. Xylanase and  $\alpha$ -amylase were provided by Sigma (Sigma-Aldrich, Alcobendas, Spain).

## 2.2. Preparation of standard solutions

The standard solutions of DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 were dissolved in ethanol at a concentration of 10.0 mg/mL and stored at 4 °C in a sealed vial until use. DON concentration in the stock solution was checked by UV spectroscopy according to AOAC Official methods of analysis, chapter 49 (Horwitz & Latimer, 2006). Working standard solutions (50.0, 10.0, 5.0, 1.0, 0.5, 0.1 and 0.05  $\mu$ g/mL) were prepared by appropriate dilution of known volumes of the stock solution with the mobile phase and used to obtain calibration curves in the chromatographic system.

## 2.3. Initial levels of DON and DON-3-glucoside in flour and bran

Flour and bran wheat were purchased from a flour mill in Lleida (Spain) and were analysed for natural DON and DON-3-glucoside contaminations. Due to the high DON levels found in the purchased flour and bran (see section 3.1), spiking was not required. The mix of flour and bran (200 g of bran / 1000 g of flour) used for the experiments was  $402.6 \pm 25.7$  and  $117 \pm 21$   $\mu$ g/kg ( $n = 6$ ) for DON and DON-3-glucoside, respectively. So, the study was made with natural contamination.

## 2.4. Dough preparation and baking

A flour + bran mix was prepared (200 g of bran/1000 g of flour) and used for the bread experiments. 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 to each flour + bran mix (156 g). The dough was obtained by adding 83 mL of water to the mixture. Different doughs were prepared, containing two different enzymes (xylanase and  $\alpha$ -amylase) at two different concentrations plus a control. The enzyme concentrations were adjusted according to bread making standards: 1 and 2.5 U of xylanase/g flour (Oliveira, Telis-Romero, Da-Silva, 2014) and 10 and 20 U of  $\alpha$ -amylase/g flour (Kim, Maeda, Morita, 2006). The enzymes were added in powder form. Moreover, the fermentation was conducted separately at 30 or 45 °C. Thus, 10 treatments [(4 enzyme

treatments+control) x 2 temperature levels] were tested in the study, and the experiment was repeated three times.

Dough was manually kneaded until it was held together with a non-sticky, smooth and satiny appearance and had optimum handling properties. Rounded pieces weighing 250 g each were prepared. From this point, thermoprobes (Thermo Bouton, Proges Plus, France) were used in the dough to record fermentation and baking temperatures; specifically, probes were placed in the centre of the loaf and close to the surface. Next, the pieces were placed in moulds, where the dough further fermented for 1 hour at 30 or 45 °C. Samples of 25 g were taken from each dough before fermentation, and after 20, 40 and 60 minutes (final point). Samples were lyophilized and stored at -20 °C until mycotoxin analysis. The proofed doughs were then baked in an oven (Eurofred PE46SVR, Eurofred, Spain). Baking conditions were 180 °C and 75 min. Such conditions were used to obtain suitable bread based on previous experiments. A 25 g sample was taken after 15 minutes of baking, lyophilized and stored at -20 °C. After baking, a representative sample was taken, lyophilised and stored at -20 °C until the analyses were performed.

## 2.5. Sample preparation and analysis

### 2.5.1. Extraction of DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 for UPLC-MS/MS analysis

The method followed is described in Vidal et al. (2015) but is briefly summarised here: 1 g of ground lyophilised sample was extracted with 7 mL of extracting solution (54 % water, 45 % acetonitrile and 1 % acetic acid) by magnetically stirring it for 20 min. Then, the sample was centrifuged for 10 min at 1780 g. 2 mL of the supernatant was mixed with 2 mL of dichloromethane, and the mixture was stirred for 10 min and then centrifuged for 3 min at 500 g for the separation of the two phases. The upper layer was kept in a vial.

Chromatographic separation was performed using a 100 mm x 2.1 mm i.d., 1.8 µm, Acquity UPLC HSS T3 column (Waters, Milford, MA, USA). Column temperature was held at 40 °C. The mobile phases consisted of Acetonitrile (A) and 10 mM AcNH<sub>4</sub> in water (B). The gradient was as follows: we start with 20 % A and then from 0.5 min increase it linearly to 50 % for 2.5 min, and it reaches 50 % at 3 min. At min 3.01 the mobile phase changes to 100 % A until min 4.5; and at min 4.51 it switches to 20 % A, and then the column is equilibrated for

1.5 min before the next injection. The flow rate was 300  $\mu\text{L}/\text{min}$ , and an injection volume of 2.5  $\mu\text{L}$  was enabled.

All measurements were performed with the following settings: source temperature 150  $^{\circ}\text{C}$ , desolvation temperature 350  $^{\circ}\text{C}$ , cone gas flow was 2.5 L/min, desolvation gas flow was 16.7 L/min, collision gas flow 0.17 mL/min and the capillary was 3000 V. The analyte-dependent MS/MS parameters were optimized via direct infusion of reference standard solutions. Method performance characteristics for DON, DON-3-glucoside, 3-ADON, 15-ADON and DOM-1 are summarized in Table 1. . More detailed information about the analytical features are described in the Supporting material file.

### 2.5.2. 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. The following common steps were used in each enzyme protocol:

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

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

#### 2.5.2.1. Xylanase activity

For the xylanase determination the reduction of DNS was used. The suitability of such method for xylanase activity quantification was described by Jeffries et al. (1998). Tubes with 3 g of fermented dough/bread samples were treated at 40 °C for 20 minutes with 20 mL of extraction solution without shaking. A total of 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 1 M sodium acetate was then added; the solution then reached a volume of 100 mL with water). The tubes were heated for 8 minutes at 50 °C, and 3 mL of the DNS reagent was added at the end of the heating step. Subsequently, the tubes were placed in boiling water for 5 minutes. After 5 minutes in boiling water, the tubes were cooled to room temperature in a water bath. Cooling to ambient temperature was necessary because of the effect of temperature on the absorbance of the coloured reaction product. After cooling, the absorbance at 540 nm was measured using the spectrometer. To generate calibration curves, xylanase calibration samples (5, 2.5, 1, 0.5, 0.1 and 0.05 U/g) were prepared with fermented dough/bread samples and were used to obtain calibration curves ( $r^2$  values were 0.75 and 0.99 for fermented dough and bread, respectively) according to the same protocol for the analysis described above.

#### 2.5.2.2. $\alpha$ -Amylase activity

For the  $\alpha$ -amylase determination the reduction of DNS was used. The suitability of this method for  $\alpha$ -amylase activity quantification was described by Jeong et al. (2010). Tubes with 3 g of fermented dough/bread samples were treated at 40 °C for 20 minutes with 20 mL of extraction solution without shaking. Then, 0.5 mL of the supernatant was added to 1.5 mL of the substrate (1 g of starch in 20 mL of 1 M sodium hydroxide; 10 mL of 10.6 M sodium and potassium tartrate and 70 mL of water). The tubes were kept at 25 °C for 3 minutes, and then 1 mL of the DNS reagent was added. The tubes were placed for 5 minutes in boiling water. After 5 minutes in boiling water, the tubes were cooled to room temperature in a water bath, and 10 mL of water were then added. Finally, absorbance was measured in the spectrometer at 540 nm.  $\alpha$ -amylase calibration samples (50, 10, 5, 1, 0.5 and 0.05 U/g) were prepared with fermented dough/bread samples and were used to obtain calibration curves ( $r^2$  values were 0.86 and 0.96 for fermented dough and bread, respectively) according to the same protocol for the analysis described above.

## 2.6 Statistical analysis

All the results in dry basis were submitted to a statistical analysis. Multifactorial ANOVA was applied to assess the significance of sample traits in the observed mycotoxin levels. Moreover, multiple linear regressions were applied to assess the temperature/time effect of DON and its modified forms during the bread making process. The software used for statistical analysis was Microsoft Excel® (Microsoft® Corporation, California) and Statistics 20.0 (IBM SPSS® Statistics 20.0 Inc., Chicago).

### 3. Results and discussion

#### 3.1. Initial levels of DON and DON-3-glucoside in flour and bran

The initial DON concentration in the purchased flour was  $251 \pm 30$  µg/kg ( $n = 3$ ); DON-3-glucoside was not detected. In the purchased bran, DON and DON-3-glucoside concentrations were  $2004 \pm 72$  and  $579 \pm 61$  µg/kg ( $n = 3$ ), respectively.

DON presence in flour was similar to concentrations found in recent studies. For instance, Stanciu et al. (2017) found an average DON concentration of 190 µg/kg in wheat flour from Romania and Cunha et al. (2010) had 322 µg of DON/kg in wheat flour from Portugal. Although DON-3-glucoside was not detected over our LOD in flour, DON-3-glucoside is usually present when DON is present. Several studies showed the co-occurrence of the two mycotoxins in raw products and the concentration ratio DON-3-glucoside/DON is from 10 to 30 % (Berthiller et al., 2009; Dall'Asta et al., 2013).

The concentration of DON found in bran is of concern because it surpassed the maximum DON concentration (750 µg/kg) set up by the EC (EC 1881/2006) for bran for direct human consumption. Other studies showed DON concentrations in cereal based products over the maximum DON concentration set up by the EC (Cano-Sancho et al., 2013; SCOOP 2003; Vidal et al., 2013). DON-3-glucoside was detected in our bran and the ratio DON-3-glucoside/DON found (28 %) was within the common 10-30 % ratio concentration DON-3-glucoside/DON in raw cereal products (Berthiller et al., 2009; Dall'Asta et al., 2013). The major mycotoxin concentration in the bran than in the flour is expected because fungal development in intact grains always starts from the outside of the grain, so bran samples usually contain higher concentrations than the whole grains (Rios et al., 2008).

The mycotoxins levels found in flour and bran showed that despite all the effort to control mycotoxins presence through the food chain, DON and its modified forms still have a wide

incidence in wheat products. Moreover, the high levels of DON in cereals cause cereal based food to be the main source of exposure to this mycotoxin (Marin et al., 2013).

### 3.2. DON stability during the bread making process

DON concentration was reduced after fermentation without enzymes ( $p < 0.05$ ). Moreover the DON reduction was larger when the fermentation temperature was 45 °C ( $19.47 \pm 6.45$  %) relative to 30 °C ( $7.18 \pm 2.43$  %) ( $p < 0.05$ ). A gradual DON reduction through the fermentation was observed (Figure 1). DON was slightly reduced after 15 minutes of baking when enzymes were not present in the recipe ( $10.97 \pm 2.79$  %). At the end of the entire bread making process, the DON concentration was reduced  $25.67 \pm 7.15$  %. There was a tendency for a major reduction in baking after fermentation at 30 °C (18.87 %) compared to 45 °C (8.45 %).

$\alpha$ -amylase addition prevented DON reduction during fermentation. Moreover some DON increases were detected with  $\alpha$ -amylase presence. The DON increases were larger when the  $\alpha$ -amylase activity was higher (analysed activity = 56.5 U/g) and the fermentation temperature was 30 °C (Table 2).. Similarly, DON concentration was not reduced after 15 minutes of baking and a small reduction was observed after baking. So, DON concentration in the breads with  $\alpha$ -amylase activity ( $\geq 20$  U/g) was higher than in the controls (Figure 1). Xylanase caused the increase in DON during fermentation, especially when the fermentation was at 45 °C ( $p < 0.05$ ) (Figure 1). DON reduction after baking was similar with the presence of xylanase, however the increase of DON detected during fermentation caused a higher DON concentration in the final breads with xylanase than in the controls ( $p < 0.05$ ). The enzyme presence caused a lack of reduction of DON which could be caused by the release of DON from the wheat matrix. Although DON is reduced the final concentration of DON in the product does not show reduction because the amount of DON released may be higher than that destroyed. The release of DON from the matrix is evident during the first stage of baking.

DON was reduced during fermentation when no enzymes were added as it has been observed in several other studies (Neira et al., 1997) while other studies reported DON increases at the end of fermentation (Bergamini et al., 2010; Lancova et al., 2008; Vidal et al., 2014). It seems that enzyme presence affects DON stability during fermentation leading to an increase in this toxin. Simsek et al. (2012) detected an increase of up to 99 % in DON after fermentation using  $\alpha$ -amylase. Suman et al. (2012) detected an increase of up to 14 % in DON using non-specific enzymes. Moreover, Vidal et al. (2014a), using flour improvers

with non-specific enzymes, detected a 30 % increase in DON during fermentation. Moreover, sourdough use also led to increases in DON content during fermentation. Vidal et al. (2016) showed the importance of xylanase,  $\alpha$ -amylase and cellulase in the increase in DON during fermentation; the present results fully agree with those presented in this publication. The increase in DON due to enzyme activity could be associated with the release of DON from the wheat matrix through enzyme catalysis.

Vidal et al. (2016) also observed a bigger DON reduction at higher temperatures. Samar et al. (2001) assayed different temperatures of fermentation (from 30 to 50 °C). They found that reductions in DON concentrations were greater as the temperature increased (from 0 to 56 %). The highest reduction in DON levels was observed at the highest temperature (50 °C) and the longest time tested (60 min). Moreover, Generotti et al. (2015) assayed different fermentation temperatures (from 26 to 46 °C), and they found that the stability of DON was reduced at higher fermentation temperatures. Thus, fermenting at high temperatures may be a feasible alternative to reduce DON content in bread, as long as bread quality is not affected. Although proofing temperature can be as high as 54 °C (Pylar, 1973), most authors agree that a range from 27 to 46 °C is optimal for bread production (Hui, Corke, De Leyn, Nip, & Cross, 2007). A fermentation temperature of approximately 30 °C maximizes taste due to the high production of lactic acid. Conversely, fermentation above 40 °C can reduce the quantity of lactic acid and result in tasteless breads; however, higher fermentation temperatures can also lead to improved baking volumes (Dobraszyk, Smewing, Albertini, Maesmans, & Schofield, 2003). When enzymes are present, fermentation temperature is also important because differences have been detected in function of temperature and DON stability. Thus, DON increases have been detected due to  $\alpha$ -amylase presence when the fermentation temperature was at 30 °C. On the other hand, DON increases have been found with xylanase presence when the fermentation temperature was at 45 °C. These differences were related to the optimum enzyme activity temperature (Figure 1). The more marked effect on DON when  $\alpha$ -amylase was present at 30 °C was related to the activity of the  $\alpha$ -amylase from *Aspergillus oryzae* (Evstatieva, Nikolova, Ilieva, Getov, & Savov, 2010). Xylanase caused a bigger DON increase at 45 °C because the optimum temperature for xylanase activity is at 45 °C (xylanase produced by *Trichoderma longibrachiatum*; Chen, Chen, & Lin, 1997). The enzyme activity was also important because the breads with higher initial enzyme concentration (2.5 U/g initial concentration added, > 1.2 U/g activity measured) showed a higher DON increase. In summary, DON is reduced at the end of fermentation if there is no enzyme presence and the reduction depends on the fermentation temperature. On the other hand,  $\alpha$ -amylase and xylanase minimize DON

reduction and even a DON increase can be detected at certain temperatures and enzyme concentrations.

Fifteen minutes after baking, DON reduction was detected in breads without enzymes and DON concentration remained similar until the end of baking. Thus almost all DON reduction occurred in the first stage of baking. DON is probably only reduced in the external layers of bread because inside the bread, temperatures were lower than 100 °C and external layers reached high temperatures very quickly. On the other hand,  $\alpha$ -amylase and xylanase presence minimised this reduction and DON increases were detected at the end of this first baking stage. The DON increase was especially marked when xylanase was used after fermentation at 30 °C. Xylanase activity reached its optimal point during this first stage of baking.

DON reduction in the absence of added enzymes during baking critically depended on the fermentation temperature ( $p < 0.05$ ). However, considering the entire bread making process, the DON concentration in bread was similar regardless of the fermentation temperature (mean reduction from beginning to end was approximately 25 %). The reduction in DON content during baking is consistent with most previous studies, which have reported a reduction in DON content at temperatures over 170 °C as long as the baking time was longer than 45 min (Vidal et al., 2014a). The results of this study are consistent with the response surface model for DON reduction in bread baking formulated by Vidal et al. (2014a). A slight effect of baking was observed in bread fermented at 45 °C. This fact may be caused by the lower initial DON concentration in this case. Reduction of DON levels during baking has been shown to be higher at higher initial toxin concentrations and not significant at lower initial DON concentrations (Vidal et al., 2014a, 2015). Enzymes avoided the DON reduction due to the baking process; however, this was caused by the increase in the first stage of baking because a reduction was observed afterwards. The increase of DON due to enzyme presence could be caused by the release of 'hidden' DON from the matrix molecules; enzymes hydrolyse these compounds and they cause the mycotoxin release from the matrix. So, it is important to know the formulation of the recipe to know the final DON concentration in bread, because if enzymes are present the final bread concentration can be over the EU limit legislation in spite of legal DON levels in the initial flour. On the other hand, the increase of DON concentration compared with the initial DON concentration in flour means that ON analysis underestimates the DON content in the flour due to the presence of embedded DON in the raw matrix. The results obtained can be useful to calculate performance criteria (PC) and process criteria (PO) in order to achieve a desired food safety objective (FSO). Reduction of DON levels may result in thermodegradation products (norDONs A-F and DON lactones), which are less toxic than DON itself. The losses that

cannot be ascribed to the formation of degradation products are most likely caused by pyrolysis or polymerization reactions (Bretz, Beyer, Cramer, Knecht, & Humpf, 2006). Still, some existing studies have reported no reductions in DON levels or even slight increases during baking, which may be attributed to extended enzymatic activity at the early stages of baking (Bergamini et al. 2010; Simsek et al., 2012; Suman et al., 2012)..

### 3.3. DON-3-glucoside stability during the bread making process

DON-3-glucoside concentration was slightly reduced after fermentation, 7 % approximately without differences due to fermentation temperature (Figure 2). However, an extremely high increase in DON-3-glucoside was detected after baking (> 80 %), and the increase occurred in the final stage of baking. The presence of  $\alpha$ -amylase and xylanase did not affect the DON-3-glucoside concentration during fermentation although some slight increases were detected with  $\alpha$ -amylase presence ( $p > 0.05$ ). The baking step caused a similar DON-3-glucoside increase with the presence of enzymes with a DON-3-glucoside increase of 60 % in all treatments (Figure 2).

The detected reduction in DON-3-glucoside after dough-proofing without enzymes is consistent with previous studies (Kostelanska et al., 2011; Vidal et al., 2016). Despite a reduction in the DON-3-glucoside concentration at the end of fermentation,  $\alpha$ -amylase and xylanase at optimum temperatures of 30 and 45 °C, respectively, and at high enzyme concentrations led to slight increases in DON-3-glucoside concentrations (Figure 2).

Use of enzymes as improvers in past studies caused increases in DON-3-glucoside during fermentation; however, the exact enzymes responsible for this increase were not described (Kostelanska et al. 2011; Vidal et al., 2014a, 2014b). Simsek et al. (2012) used only  $\alpha$ -amylase and detected a reduction in DON-3-glucoside at the end of fermentation at 30 °C (5 %) (similar to the present study).

Changes in DON-3-glucoside after baking observed in this study are consistent with results from previous studies on wheat products (Vaclavikova et al., 2013; Vidal et al., 2014b, 2015); however, some studies have shown a reduction in DON-3-glucoside after baking (Kostelanska et al., 2011; Simsek et al., 2012). Vidal et al. (2015) showed that DON-3-glucoside exhibits different behaviours in thermal treatments as a function of the size of the product, temperature and time. The mild baking conditions tested in this assay (especially due to the large size of the product) promoted an increase in DON-3-glucoside during baking, while harsher treatments would have probably led to a reduction in DON-3-glucoside. Our results demonstrate that the increase occurs at the end of the baking process because

after 15 minutes of baking DON-3-glucoside concentration did not differ from that after fermentation. This fact could be linked to the temperature inside the bread, which increases slowly. DON-3-glucoside may be bound to flour components and released during baking. In general, no significant correlation was found between increases in DON-3-glucoside and decreases in DON or vice versa. Similarly, Kostelanska et al. (2011) concluded that the behaviours of the two mycotoxins were not linked because, in their study, the concentration of DON did not change. These authors suggested that a possible splitting of glycosidic bonds between DON-3-glucoside and cell polysaccharides may occur. More studies that examine the relationship between DON and DON-3-glucoside are necessary to fully understand the interactions between parent and modified mycotoxins in food processes. Finally, the increase in DON-3-glucoside during baking is of concern because, although DON-3-glucoside is far less active as an inhibitor of protein biosynthesis than DON (Poppenberger et al., 2003), DON-3-glucoside will likely be cleaved in the gastrointestinal tract due to chemical hydrolases or, more importantly, microbial activity in the intestines. The activity of chemical hydrolases and intestinal microbes has been demonstrated *in vivo* in swine and *in vitro* using human intestinal microbiota (Berthiller et al., 2011); thus, the presence of these enzymes and microbes is important for food safety.

#### **3.4. Stability of other DON conjugates during the bread making process**

Acetyldeoxynivalenol conjugates (3-ADON and 15-ADON) were not detected during the breadmaking process. On the other hand, DOM-1 was detected in all the analysed samples. DOM-1 was not reduced during fermentation and even increases of DOM-1 have been detected during this step. Fermentation temperature affects the DOM-1 stability because the DOM-1 increase was always higher at 30 °C than at 45 °C ( $p < 0.05$ ). Baking caused an important DOM-1 increase ( $> 20\%$ ) and it resulted in a higher DOM-1 concentration after the bread making process (Figure 3).

Acetyldeoxynivalenol conjugates are fungal conjugates and their concentration is usually very scarce in wheat flour. For instance, Yang et al. (2013) found only one sample with 3-ADON from four analysed wheat grain samples. The study of scientific cooperation (SCOOP, 2003) task 3.2.10 noted that only 8 % of the wheat samples contain 3-ADON. The information regarding 3-ADON stability during bread making is scarce; however, the levels of 3-ADON tend to decrease with increasing temperature and baking time (Vidal et al., 2015) and other studies indicated a fast transformation of 3-ADON to DON, norDON A, norDON B, and norDON C, when 3-ADON was submitted either to alkali or heat (Young et al., 1986).

Similar to 3-ADON, 15-ADON is another fungal metabolite which has been detected in wheat grain (Yang et al., 2013). The major presence of 3-ADON versus 15-ADON is a phenotypic difference within *Fusarium* species. In southern Europe, the predominant 15-ADON genotype is more predominant (Somma et al., 2014). No studies exist on 15-ADON thermal stability; however, De Boevre et al. (2013) detected 15-ADON in processed food products, which suggests a certain stability of this mycotoxin.

DOM-1 is known to be a conjugated product from mammalian metabolism, and it is used as a biomarker for DON exposure (Solfrizzo, Gambacorta, & Visconti, 2014). On the other hand, the detected increase in our results mainly after baking agrees with other studies which identified it as a degradation product of DON after a heat process (Mishra, Dixit, Dwivedi, Pandey, & Das, 2014 and Vidal et al., 2015). In other studies a significant negative correlation between DON and DOM-1 concentrations in those samples with quantifiable levels of both metabolites was found (Pearson correlation coefficient=-0.5884,  $p=0.0000$ ), which confirms DOM-1 as a degradation product of DON caused by heat. In an aqueous system, Mishra et al. (2014) observed the formation of DOM-1 as a degradation product of DON after heating at 125 – 200 °C. The effect on DOM-1 of xylanase and  $\alpha$ -amylase was not examined in other studies, but the behaviour of DOM-1 was the same regardless xylanase and  $\alpha$ -amylase addition. However, if DOM-1 is a DON degradation product and DON stability is linked to enzymes, DOM-1 could be affected indirectly by the presence of enzymes.

#### 4. Conclusion

In conclusion, DON can be reduced during the bread making process both during fermentation and baking, the DON reduction in each step will depend on the fermentation temperature. Notwithstanding the DON reduction in the final bread is > 20 % compared to the initial wheat flour. However, the presence of  $\alpha$ -amylase and xylanase could prevent the reduction of DON. It is important to know the effect of enzymes during the bread making process because the DON concentration could change in the final bread product, which could be over the legislation limit. So, it is important to know the features of the bread making process beforehand to select the wheat flour and anticipate the final DON concentration. DON-3-glucoside is slightly reduced after fermentation but a worrying increase is detected after baking (> 80 %) and enzymes have a poor effect on this behaviour. The obtained results confirmed the increase of DOM-1 during baking.

## 5. Acknowledgments

The authors are grateful to the Spanish government (project AGL2014-55379-P) for financial support. A. Vidal thanks the Spanish government (Ministry of Education) for the pre-doctoral grant.

## 6. References

- Bergamini, E., Catellani, D., Dall'Asta, C., Galaverna, G., Dossena, A., Marchelli, R., & Suman, M. (2010). Fate of *Fusarium* mycotoxins in the cereal product supply chain: The deoxynivalenol (DON) case within industrial bread-making technology. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, 27, 677-687.
- Bergmeyer, H.U., Gawehn, K., & Grassl, M. (1974). *Methods of Enzymatic Analysis*. (2nd ed.). New York: Academic Press, Inc.
- Berthiller, F., Dall'asta, C., Corradini, R., Marchelli, R., Sulyok, M., Krska, R., Adam, G., & Schuhmacher, R. (2009). Occurrence of deoxynivalenol and its 3- $\beta$ -D-glucoside in wheat and maize. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, 26, 507-511.
- Berthiller, F., Krska, R., Domig, K.J., Kneifel, W., Juge, N., Schuhmacher, R., & Adam, G. (2011). Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicology Letters*, 206, 264-267.
- Bhatti, H.N., Madeeha, M., Asgher, M., & Batool, N. (2006). Purification and thermodynamic characterization of glucose oxidase from a newly isolated strain of *Aspergillus niger*. *Canadian Journal of Microbiology*, 52, 519-524.
- Bretz, M., Beyer, M., Cramer, B., Knecht, A., & Humpf, H.U. (2006). Thermal degradation of the *Fusarium* mycotoxin deoxynivalenol. *Journal of Agricultural and Food Chemistry*, 54, 6445-6451.

- Cano-Sancho, G., Gauchi, J.-P., Sanchis, V., Marín, S., & Ramos, A.J., (2011b). Quantitative dietary exposure assessment of the catalonian population (Spain) to the mycotoxin deoxynivalenol. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, 28, 1098-1109.
- Cano-Sancho, G., Valle-Algarra, F.M., Jiménez, M., Burdaspal, P., Legarda, T.M., Ramos, A.J., Sanchis, V., & Marín, S. (2011a). Presence of trichothecenes and co-occurrence in cereal-based food from Catalonia (Spain). *Food Control*, 22, 490-495.
- Chen, C., Chen, J.-L., & Lin, T.-Y. (1997). Purification and characterization of a xylanase from *Trichoderma longibrachiatum* for xylooligosaccharide production. *Enzyme and Microbial Technology*, 21, 91-96.
- Dall'Asta, C., Dall'Erta, A., Mantovani, P., Massi, A., & Galaverna, G. (2013). Occurrence of deoxynivalenol and deoxynivalenol-3-glucoside in durum wheat. *World Mycotoxin Journal*, 6, 83-91.
- Dobraszczyk, B.J., Smewing, J., Albertini, M., Maesmans, G., & Schofield, J.D. (2003). Extensional rheology and stability of gas cell walls in bread doughs at elevated temperatures in relation to breadmaking performance. *Cereal Chemistry*, 80, 218-224.
- Evstatieva, Y., Nikolova, D., Ilieva, S., Getov, L., & Savov, V. (2010). Identification and characterization of  $\alpha$ -amylase and endoxylanase, produced by *Aspergillus* mutant strains. *Biotechnology & Biotechnological Equipment*, 24, 613-617.
- Freilich, J. (1949). Time, temperature and humidity: Factors in dough proofing. *Baker's Dig*, 33, 27-30.
- Generotti, S., Cirilini, M., Malachova, A, Sulyok, M, Berthiller, F, Dall'Asta, C., & Suman, M. (2015). Deoxynivalenol & deoxynivalenol-3-glucoside mitigation through bakery production strategies: effective experimental design within industrial rusk-making technology. *Toxins*, 7, 2773-2790.
- Harada, O., Lysenko, E.D., & Preston, K.R. (2000). Effects of commercial hydrolytic enzyme additives on Canadian short process bread properties and processing characteristics. *Cereal Chemistry*, 77, 70-76.
- Haros, M., Rosell, C.M., & Benedito, C. (2002). Effect of different carbohydrases on fresh bread texture and bread staling. *European Food Research and Technology*, 215, 425-430.

- Horwitz, W., & Latimer, G.W. (2006). *Official methods of analysis of AOAC International*. (18th ed.). AOAC Gaithersburg, Maryland.
- Hui Y.H., Corke, H., De Leyn, I., Nip, W.K., & Cross, N. (2006). *Bakery Products Science and Technology*. (eds). Ames, IA: Blackwell Publishing.
- IARC (International Agency for Research on Cancer). (1993). *Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins*. In *IARC monographs on the evaluation of carcinogenic risks to humans* (Vol. 56). Lyon, France.
- Irfan, M., & Syed, Q. (2012). Partial purification and characterization of Xylanase from *Trichoderma viride* produced under SSF. *International Journal of Applied Research in Natural Products*, 5, 7-11.
- JEFCA. 2010. WHO Technical Report Series 959: *JEFCA, 2010: Seventy-second Report of the Joint FAO/WHO Expert Committee on Food Additives: Evaluation of Certain Contaminants in Food*. WHO Technical Report Series. 959.
- Jeffries, T.W., Yang, V.W., Davis, M.W. 1998. Comparative study of xylanase kinetics using dinitrosalicylic, arsenomolybdate, and ion chromatographic assays. *Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology*, 70-72, 257-265.
- Jeong, W.-H., Harada, K., Yamada, T., Abe, J., Kitamura, K. 2010. Establishment of new method for analysis of starch contents and varietal differences in soybean seeds. *Breeding Science*, 60, 160-163.
- Kim, J.H., Maeda, T., & Morita, N. (2006). Effect of fungal  $\alpha$ -amylase on the dough properties and bread quality of wheat flour substituted with polished flours. *Food Research International*, 39, 117-126.
- Kostelanska, M., Dzuman, Z., Malachova, A., Capouchova, I., Prokinova, E., Skerikova, A., & Hajslova, J. (2011). Effects of milling and baking technologies on levels of deoxynivalenol and its masked form deoxynivalenol-3-glucoside. *Journal of Agricultural and Food Chemistry*, 59, 9303-9312.
- Lancova, K., Hajslova, J., Poustka, J., Krplova, A., Zachariasova, M., Dostalek, P., & Sachambula, L. (2008). Transfer of *Fusarium* mycotoxins and 'masked' deoxynivalenol deoxynivalenol-3-glucoside from field barley through malt to beer. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment*, 25, 732-744.

- Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry*, 31, 426-428.
- Neira, M.S., Pacin, A.M., Martínez, E.J., Moltó, G., & Resnik, S.L. (1997). The effects of bakery processing on natural deoxynivalenol contamination. *International Journal of Food Microbiology*, 37, 21-25.
- Oliveira, D.S., Telis-Romero, J., Da-Silva, R., & Franco, C.M.L. (2014). Effect of a *Thermoascus aurantiacus* thermostable enzyme cocktail on wheat bread quality. *Food Chemistry*, 143, 139-146.
- Pestka, J.J. (2010). Toxicological mechanisms and potential health effects of deoxynivalenol and nivalenol. *World Mycotoxin Journal*, 3, 323–347.
- Poppenberger, B., Berthiller, F., Lucyshyn, D., Siebrer, T., Schumacher, R., Krska, R., Kuchler, K., Glössl, J., Luschnig, C., & Adam, G. (2003). Detoxification of the *Fusarium* mycotoxin deoxynivalenol by a UDP-glucosyltransferase from *Arabidopsis thaliana*. *Journal of Biological Chemistry*, 278, 47905-47914.
- Pylar, E.J. (1973). *Baking Science & Technology*. (2nd ed.). Chicago: Siebel Publishing.
- Rani, K.U., Prasada Rao, U.J.S., Leelavathi, K., Haridas, & Rao, P. (2001). Distribution of enzymes in wheat flour mill streams. *Journal of Cereal Science*, 34, 233-242.
- Raviyan, P., Tang, J., & Rasco, B.A. (2003). Thermal stability of  $\alpha$ -amylase from *Aspergillus oryzae* entrapped in polyacrylamide gel. *Journal of Agricultural and Food Chemistry*, 51, 5462-5466.
- Samar, M.M., Neira, M.S., Resnik, S.L., & Pacin, A. (2001). Effect of fermentation on naturally occurring deoxynivalenol (DON) in Argentinean bread processing technology. *Food Additives and Contaminants*, 18, 1004-1010.
- Simsek, S., Burgess, K., Whitney, K.L., Gu, Y., & Qian, S.Y. (2012). Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in wheat. *Food Control*, 26, 287-292.
- Suman, M., Manzitti, A., & Catellani, D. (2012). A design of experiments approach to studying deoxynivalenol and deoxynivalenol-3-glucoside evolution throughout industrial production of whole grain crackers exploiting LC-MS/MS techniques. *World Mycotoxin Journal*, 5, 241-249.

- Vaclavikova, M., Malachova, A., Veprikova, Z., Dzuman, Z., Zachariasova, M., & Hajslova, J. (2013). "Emerging" mycotoxins in cereals processing chains: Changes of enniatins during beer and bread making. *Food Chemistry*, 136, 750-757.
- Veraverbeke, W.S., & Delcour, J.A. (2002). Wheat protein composition and properties of wheat glutenin in relation to breadmaking functionality. *Critical Reviews in Food Science and Nutrition*, 42, 179-208.
- Vidal, A., Ambrosio, A., Sanchis, V., Ramos, A.J., & Marín, S. (2016). Enzyme bread improvers affect the stability of deoxynivalenol and deoxynivalenol-3-glucoside during breadmaking. *Food Chemistry*, 208, 288-296.
- Vidal, A., Marín, S., Morales, H., Ramos, A.J., & Sanchis, V. (2014b). The fate of deoxynivalenol and ochratoxin A during the breadmaking process, effects of sourdough use and bran content. *Food and Chemical Toxicology*, 68, 53-60.
- Vidal, A., Morales, H., Sanchis, V., Ramos, A.J., & Marín, S. (2014a). Stability of DON and OTA during the breadmaking process and determination of process and performance criteria. *Food Control*, 40, 234-242.
- Vidal, A., Sanchis, V., Ramos, A.J., & Marín, S. (2015). Thermal stability and kinetics of degradation of deoxynivalenol, deoxynivalenol conjugates and ochratoxin A during baking of wheat bakery products. *Food Chemistry*, 178, 276-286.
- Vidal, A., Sanchis, V., Ramos, A.J., Marín, S. 2016. The fate of deoxynivalenol through wheat processing to food products. *Current Opinion in Food Science*, 11, 34-39.
- Wu, Q., Kuča, K., Humpf, H.-U., Klímová, B., Cramer, B. 2017. Fate of deoxynivalenol and deoxynivalenol-3-glucoside during cereal-based thermal food processing: a review study. *Mycotoxin Research*, 33, 79-91.
- Zachariasova, M., Vaclavikova, M., Lacina, O., Vaclavik, L., & Hajslova, J. (2012). Deoxynivalenol oligoglycosides: New "masked" *Fusarium* toxins occurring in malt, beer, and breadstuff. *Journal of Agricultural and Food Chemistry*, 60, 9280-9291.
- Zhou, B., Schwarz, P., He, G.-Q., Gillespie, J., & Horsley, R. (2008). Effect of enzyme pretreatments on the determination of deoxynivalenol in Barley. *Journal of the American Society of Brewing Chemists*, 66, 103-108.

Table 1. Method performances for DON, deoxynivalenol-3-glucoside, 3-ADON, 15-ADON and DOM-1 in bakery products.

Mycotoxin	LOD <sup>a</sup> (µg/kg)	LOQ <sup>b</sup> (µg/kg)	n	Spiking level (µg·kg <sup>-1</sup> )	Recovery <sup>c</sup> (%)	RSDr <sup>d</sup> (%)
DON	0.7	2.1	3	74.53	75.1±10.6	11.1
			3	501.35	88.5±8.7	8.8
			5	1002.70	72.9±8.8	8.9
DON-3-glucoside	1.6	4.8	3	5.61	104.1±6.1	5.9
			3	37.74	95.4±17.1	10.9
			5	75.48	87.4±7.2	3.3
3-ADON	1.5	4.5	3	5.61	87.4±4.7	5.1
			3	37.74	85.4±6.2	6.5
			5	75.48	88.2±8.6	8.9
15-ADON	1.5	4.5	3	5.56	89.0±7.2	7.5
			3	37.37	89.6±9.0	9.6
			5	74.74	78.1±2.3	2.9
DOM-1	0.5	1.5	3	5.56	82.8±4.6	5.8
			3	37.37	84.3±8.4	8.1
			5	74.74	78.4±10.5	10.8

<sup>a</sup> LOD = Limit of detection.

<sup>b</sup> LOQ = Limit of quantification.

<sup>c</sup> Mean value ± standard deviation.

<sup>d</sup> RSDr = relative standard deviation.

Table 2. Mean enzymatic activity (units)  $\pm$  SD for  $\alpha$ -amylase and xylanase in fermented doughs and breads when the fermentation was at 30 and 45 °C at three different enzymatic activities.

Enzyme added	Fermentation temperature (°C)		30		45	
	Added enzyme (U/g)	Fermented dough	Bread	Fermented dough	Bread	
$\alpha$ -Amylase (U/g)	0	<0.05	<0.05	<0.05	<0.05	
	10	29.2 $\pm$ 11.32*	3.25 $\pm$ 0.76*	22.97 $\pm$ 10.18*	2.68 $\pm$ 0.49*	
	20	56.5 $\pm$ 6.25*	5.26 $\pm$ 1.21*	50.6 $\pm$ 12.97*	4.81 $\pm$ 1.4	
Xylanase (U/g)	0	0.16 $\pm$ 0.06	<0.05	0.51 $\pm$ 0.09	<0.05	
	1	0.42 $\pm$ 0.12*	<0.05	0.58 $\pm$ 0.10	<0.05	
	2.5	1.2 $\pm$ 0.34*	<0.05	1.45 $\pm$ 0.12	<0.05	

\* Indicates significant differences compared to the sample without added enzymes at the same fermentation temperature ( $p < 0.05$ ).

Figure 1. Concentration of DON ( $\mu\text{g}/\text{kg}$ ) in dry basis in the different time points of the breadmaking process when the fermentation temperature was at 30 °C with high enzyme activity (A) and low enzyme activity (B) and at 45 °C with high enzyme activity (C) and low enzyme activity (D). Bars indicate standard deviation.

Figure 2. Concentration of DON-3-glucoside ( $\mu\text{g}/\text{kg}$ ) in dry basis in the different time points of the breadmaking process when the fermentation temperature was at 30 °C with high enzyme activity (A) and low enzyme activity (B) and at 45 °C with high enzyme activity (C) and low enzyme activity (D). Bars indicate standard deviation.

Figure 3. Concentration of DOM-1 ( $\mu\text{g}/\text{kg}$ ) in dry basis in the different time points of the breadmaking process when the fermentation temperature was at 30 °C with high enzyme activity (A) and low enzyme activity (B) and at 45 °C with high enzyme activity (C) and low enzyme activity (D). Bars indicate standard deviation.

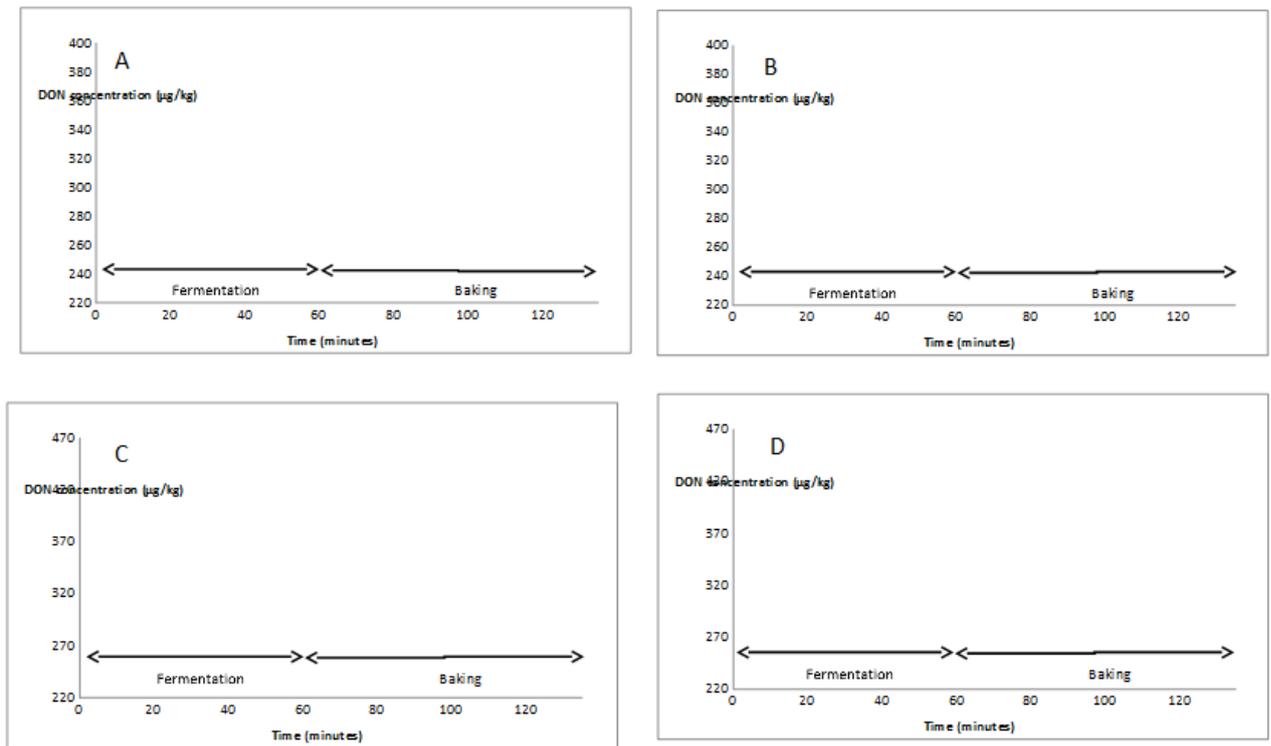


Fig. 1

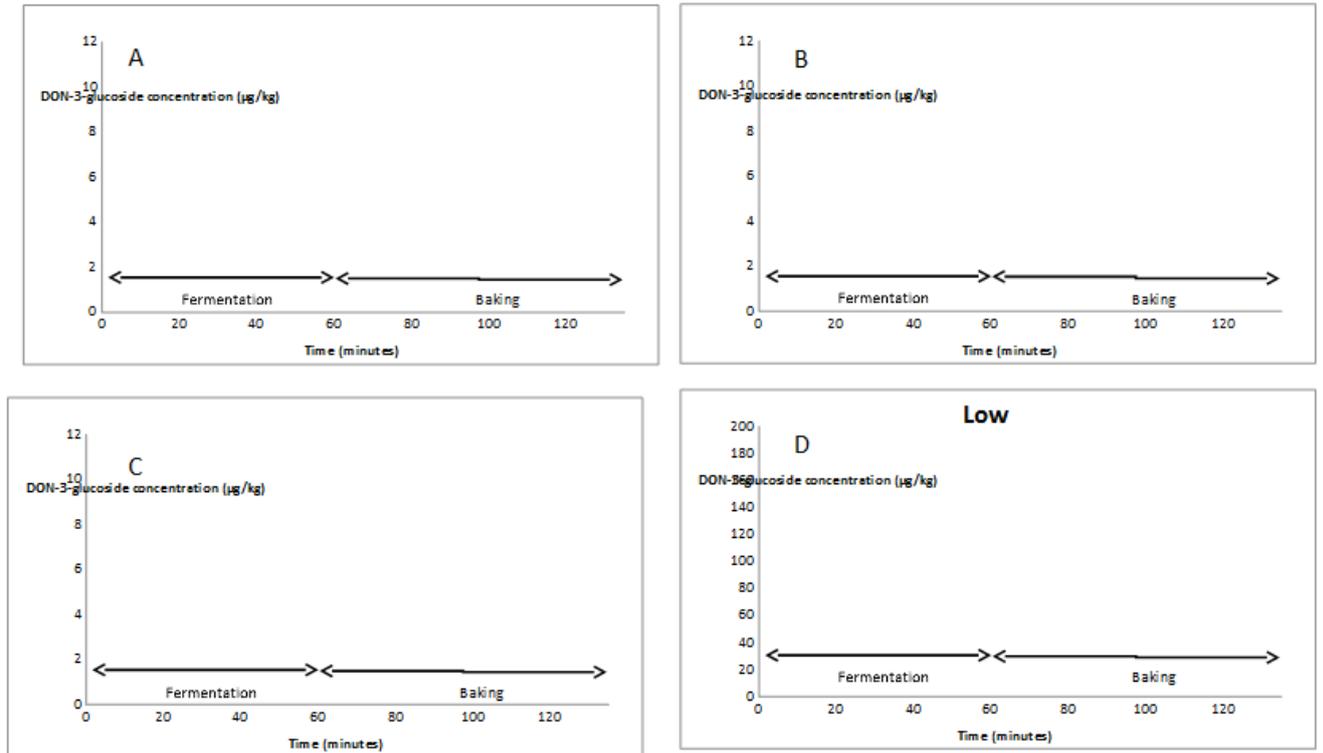


Fig. 2

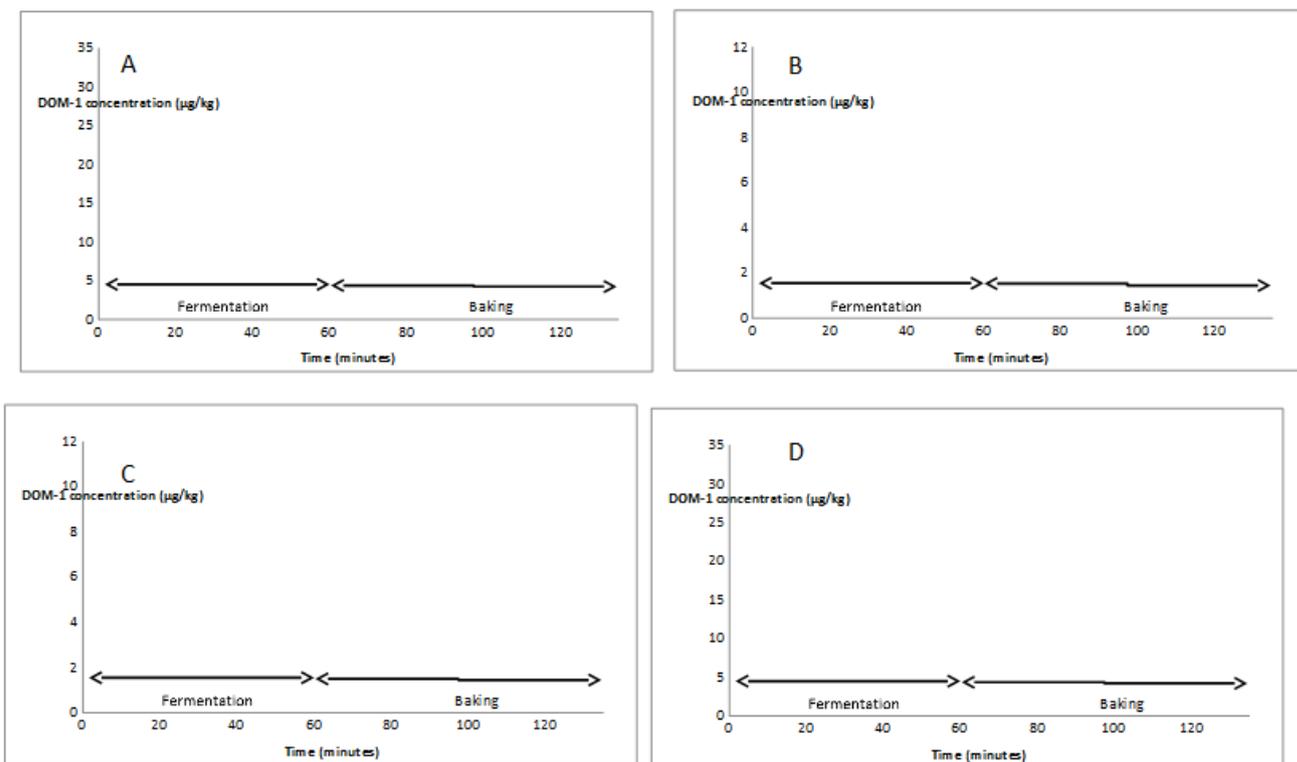


Fig. 3

Graphical Abstract

**DON and its conjugates are NOT totally REDUCED during bread making proces**



30 °C

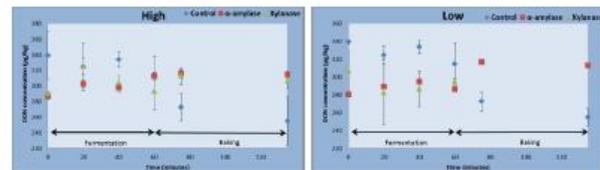
**Different conditions assayed:**

**Enzymes (2 concentrations):**

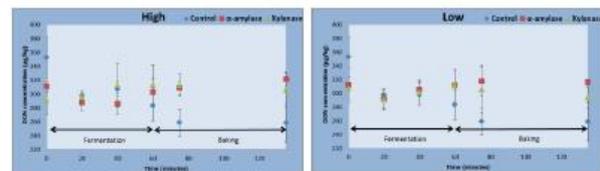
- Xylanase
- $\alpha$ -amylase

**Fermentation temperatura:**

- 30 °C
- 45 °C



45 °C



ACCEPTED MANUSCRIPT