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Stability of DON and OTA during the breadmaking process and determination of process and performance criteria

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ABSTRACT

The fate of deoxynivalenol (DON) and ochratoxin A (OTA) during the breadmaking process was studied. In particular, toxin content was analysed in mixed baking ingredients before kneading, after fermentation and proofing, and finally after baking. Fermentation and proofing were carried out at 30 °C for 1 h, while baking was performed at different temperature levels (from 170 to 210 °C) and baking times from 45 to 135 min, in a full factorial design. DON increased from unkneaded mix to fermented dough, and decreased due to baking; this trend depended on the initial concentration of DON in the flour. The level in the bread was significantly lower than in the initial mix of ingredients. In contrast, deoxynivalenol-3-glucoside (DON-3-G) content increased both during kneading and fermentation, and also during baking. Moreover, the results confirmed the high stability of OTA as no significant change in its content could be observed as a result of the breadmaking process. As conclusion, the design of bakery product processes may help to control DON in final products, because although quite stable, its levels can be reduced to some extent. However, high levels of DON-3-G were released during baking, and this point should be further investigated. Mycotoxins have been always considered as stable compounds; however, in depth knowledge of the processing steps that may lead to some reduction (although limited) and those which can stimulate their release from conjugated forms, will definitely help in their control in finished foodstuffs.

1. Introduction

Wheat, such as the majority of cereals, is susceptible to be contaminated with mycotoxins (Pleadin et al., 2013; Samar, Fontán, Resnik, & Pacin, 2003). 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) and ochratoxin A (OTA), in products of high consumption like beer and bakery products (Anli & Alkis, 2010; Cano-Sancho et al., 2011; Pacin, Resnik, Neira, Moltó, & Martínez, 1997; Vendl, Crews, Macdonald, Krska, & Berthiller, 2010). One of these products is bread. Wheat bread provides more nutrients to the world population than any other single food source (Peña, 2002). Bread is particularly important as a source of carbohydrates, proteins and vitamins B and E (Pomeranz, 1987). Moreover, the commonest cereal used for leavened bread is wheat (Betschart, 1982; Chaven & Kadam, 1989).

DON, also known as vomitoxin, is one of the most regular contaminants in cereals (Jelinek, Pohland, & Wood, 1989). 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, 2010a, 2010b). At the molecular level, DON disrupts normal cell function by inhibiting protein synthesis, affecting cell signalling, differentiation, and proliferation. An acute and high dose of DON can induce vomiting, whereas chronic dietary exposure to DON causes reduced food intake, decreased nutritional efficiency, reduced weight gain, and immune dysregulation (Pestka, 2010a, 2010b). OTA is a nephrotoxic mycotoxin which possesses carcinogenic, teratogenic, immunotoxic and possibly neurotoxic properties (SCF, 1998). This mycotoxin has been classified as a possible human carcinogen, in the group 2 B, by IARC (IARC, 2002). OTA has been reported in cereals, coffee, grape juice, wine, beer, spices, and meat based foodstuffs.

The European Commission has set maximum permitted levels in processed cereal products for direct human consumption in OTA of 3 μg kg^{-1}, and a maximum concentration of 500 μg kg^{-1} for DON in bread (European Commission, 2006, 2010).

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Flour processing into bread may affect DON and OTA content. Some studies reported a significant increase in DON levels during dough fermentation (Bergamini et al., 2010), while others showed a reduction of DON in the process (Samar, Neira, Resnik, & Pacin, 2001) (Table 1). Regarding baking, some studies reported that DON seems to be reduced and such reduction is affected by increasing time and temperature. However, other studies reported lower reduction of DON during baking which may have to do with the loaf size or to a mild heat treatment (Bergamini et al., 2010; Simsek, Burgess, Whitney, Gu, & Qian, 2012) (Table 1). On the other side, OTA is stable at high temperatures and its concentration does not decrease during baking (Scudamore, Banks, & MacDonald, 2003).

Although the published studies indicate that DON and OTA are largely stable and survive the breadmaking process, when concentrations are determined on an 'as is' basis as stipulated in the legislation, levels in finished products are usually lower than in the starting flour due to the dilution effect of other ingredients such as fat, sugar and water (Scudamore et al., 2009). Thus knowing the dynamics of reduction of DON and OTA during each stage in the process would allow adjusting the processing conditions and the quality of the incoming raw materials to obtain a safe product, setting then suitable performance criteria (PC) in order to achieve the desired Food Safety Objectives (FSO), which in the case of mycotoxins usually take the values of the legislated maximum permitted levels (García-Cela, Ramos, Sanchis, & Marin, 2012).

The aim of the study was to assess the stability of DON and OTA in two stages of bread making: dough fermentation and baking. 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).

2. Materials and methods

2.1. Obtaining DON and OTA contaminated flours

In order to obtain DON contaminated flour and OTA contaminated flour, two strains of either *Fusarium graminearum* or

Table 1
Effect of bakery processing on DON contamination in wheat products.

Reference	DON level in flour (μg/kg)	Loaf size	Fermentation + proofing conditions	Use of improvers	Baking conditions	%DON reduction referred to flour	Results referred to equal basis (in general dw basis)
Neira, Pacin, Martínez, Moltó, and Resnik (1997)	1370 (natural)	Industrial	5–11 h 25 °C	–	10–40 min 210 °C	21.6 (fermented dough) 44.3 (baked product) Both referred to kneaded dough	No
Samar et al. (2001)	150 (natural)	40 g	40–60 min, 30–50 °C	Malt flour	–	0–41% (fermented dough) Referred to kneaded dough	Yes
	150 (natural)	40 g	60–90 min, 30–50 °C	No	–	25–56% (fermented dough) Referred to kneaded dough	Yes
Lancova et al. (2008)	40/52/413/1223 (natural)	70 g	95 min, 30 °C	No	14 min 240 °C	54–62% (fermented dough) −32–(−45)% (proofed dough) 0–4% (baked product)	Yes
Valle-Algarra et al. (2009)	200/750/1500 (spiked)	80 g	1 h, 30 °C	No	30–50 min 190–240 °C	None (fermented dough) 47.9% (baked product)	No
Scudamore, Hazel, Patel, and Scriven (2009)	60–284 (natural)	Bread	60 min, 40–45 °C	Ascorbic acid, α-amylase	21 min 210 °C	4–57% (baked product)	No
	60–284 (natural)	Cakes		No	15–30 min 150–170 °C	41–79% (baked product)	No
	60–284 (natural)	Biscuits		No	3–5 min 245–280 °C	11–25% (baked product)	No
Bergamini et al. (2010)	100/160/967 (natural)	140 g (pilot scale)	45–85 min, 30–40 °C	Malt flour, other	8–16 min 180–210 °C	−77–5% (fermented dough) −88–3% (baked product)	Yes
	100/160/967 (natural)	140 g (industrial scale)	45–85 min, 30–40 °C	Malt flour, other	8–16 min 180–210 °C	−39% (fermented dough) −61% (baked product)	Yes
Pacin, Ciancio Bovier, Cano, Taglieri, and Hernandez Pezzani, (2010)	72 (natural)	35–65 g (industrial)	–	–	–	42–58% (baked product)	No
Kostelanska et al. (2011)	48–1049 (natural)	55 g	95 min, 30 °C	No	14 min 240 °C	0% (fermented dough) 3% (baked product) Referred to kneaded dough	No
Simsek et al. (2012)	48–182 (natural)	>100 g	180 min, 30 °C	α-amylase	25 min 220 °C	−99% (fermented dough) −69% (baked product) Referred to kneaded dough	Yes
Zachariasova, Vaclavikova, Lacina, Vaclavik, and Hajslova (2012)	729 (natural)	500 g	95 min, 30 °C	Yes	14 min 240 °C	28% (fermented dough) −4% (baked product)	No
Suman, Manzitti, and Catellani (2012)	238–633 (natural, from bran)	Biscuits	2–4 min, 20–30 °C	Flour malt, enzymes	1.5–5 min 225–270 °C	−14–10% (fermented dough) −17–42% (baked product) Referred to kneaded dough	Yes
De Angelis, Monaci, Pascale, and Visconti (2013)	816/954/1824 (natural)	500 g	60 min, ambient temp.	No	55 min, 200 °C	−18% (baked product)	Yes

'natural' refers both to contaminated flour from naturally contaminated and field inoculated wheat, while 'spiked' refers to directly spiked flour with toxin.

Aspergillus ochraceus were used. They were previously proved to be DON and OTA producers in wheat flour. The initial flour did not contain OTA and DON and DON-3-G levels were 250 ± 76.14 and 45.12 ± 15.34 μg kg^{-1} respectively.

The strains were inoculated and incubated in MEA at 25 °C until strong sporulation. A spore suspension of each strain was made in water and Tween 80 (0.005% v/v). Five millilitres of either *F. graminearum* or *A. ochraceus* spore suspension were inoculated in glass flasks containing 250 g of flour and 50 mL of water. In total, 3 kg of flour were inoculated with each strain. The flasks were stored at 25 °C for 19 days in the case of *F. graminearum* and 8 days in the case of *A. ochraceus* with periodic shaking. Then, each kind of flour (3 kg) was properly ground and homogenized and underwent either DON or OTA analysis. The content of DON and OTA was of 12,500 ± 1235 μg kg^{-1} and 75.5 ± 15.2 μg kg^{-1} respectively, in each contaminated flour, while DON-3G level remained unchanged.

2.2. Dough preparation and bread making

Each loaf bread was made with 156 g of wheat flour, 2.3 g of salt, 4.7 g of sucrose, 4.7 of lard, 6.2 of commercial compressed yeast (*Saccharomyces cerevisiae*), 83 mL of water, 3.1 g of flour improver (containing calcium carbonate, wheat flour, soya flour, lecithin, ascorbic acid and enzymes). The initial flour was prepared by mixing uninoculated flour, DON contaminated flour and OTA contaminated flour. Three different combinations of mycotoxin concentrations were assayed in the initial flour: a) high, b) medium and c) low. For each of the three initial concentrations and 20 baking conditions (see bellow) three different loaves were prepared (Table 2). A sample of the initial mix of solid ingredients was taken and stored at −20 °C until mycotoxin analysis.

Dough was manually kneaded until held together with a non-sticky, smooth and satiny appearance and optimum handling properties. Rounded pieces weighing 260 g each were prepared. From this point, thermoprobes 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 fermentation was carried out at 30 °C for 15 min. Then the pieces were placed in moulds, where the dough further fermented for 1 h at 30 °C. After the fermentation, a sample of 20 g was taken from every proofed dough which was stored at −20 °C until mycotoxin analysis (results for the finished bread were corrected accordingly). The proofed dough was then baked. Five temperature levels (210, 200, 190, 180 and 170 °C) and 4 baking times (45, 75, 105 and 135 min) were assayed in a full factorial design. Thus 3 initial toxin concentrations × 5 baking temperatures × 4 baking times × 3 replicates made 180 bread loaves. These conditions were established on the basis of previous experiments to obtain properly baked bread which was organoleptically acceptable.

After baking, bread pieces were stored at −20 °C until analysis. For this purpose bread was sliced. Additionally, some slices (those baked at 210 °C for 135 and 105 min and at 200 °C for 135 min) were separated in three portions, the crumb central part, the section 0.5 cm under the crust and the crust and all of them were stored at −20 °C until analysis.

Table 2
Toxin levels (mean ± SD) in the initial flours.

Mycotoxin	High concentration (μg kg^{-1})	Medium concentration (μg kg^{-1})	Low concentration (μg kg^{-1})
DON	2090 ± 525	1459 ± 444	1012 ± 269
DON-3-G	48 ± 27	43 ± 47	46 ± 41
OTA	9.5 ± 0.2	5.1 ± 0.1	0.8 ± 0.1

2.3. Chemicals and reagents

Mycotoxin 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. Filter paper (Whatman No. 1) was purchased from Whatman (Maidstone, UK). Immunoaffinity chromatography columns (IAC) for DON (DONPREP®) and OTA (OCHRAPREP®) 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) and was used when water was required. Phosphate buffer saline (PBS) was prepared with potassium chloride (0.2 g) (Panreac, Montcada i Reixach, Spain), potassium dihydrogen phosphate (0.2 g) (Sigma), disodium phosphate anhydrous (1.16 g) (Panreac) and sodium chloride (8.0 g) (J.T Baker) in 1 L of pure water; the pH was brought to 7.4 with hydrochloric acid 1 M.

2.4. Preparation of standard solutions

The standard of OTA was dissolved in methanol at a concentration of 5.0 mg·mL^{-1} and stored at 4 °C in a sealed vial until use. The 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 (0.5, 0.01, 0.005, 0.001 and 0.0005 μg mL^{-1}) were prepared by appropriate dilution of known volumes of the stock solution with the HPLC mobile phase and used to obtain calibration curves in the appropriated chromatographic system. The standard of DON was dissolved in ethanol at a concentration of 10.0 mg mL^{-1} and stored at 4 °C in a sealed vial until use. Working standards (50.0, 10.0, 5.0, 1.0, 0.5, 0.1 and 0.05 μg mL^{-1}) were prepared by appropriate dilution of known volumes of the stock solution with the HPLC mobile phase and used to obtain calibration curves in the appropriate chromatographic system. The standard of DON-3-G was dissolved in acetonitrile at a concentration of 10.0 mg mL^{-1} and stored at 4 °C in a sealed vial until use. Working standards (1.0, 0.5, 0.1, 0.05 and 0.01 μg mL^{-1}) were prepared by appropriate dilution of known volumes of the stock solution with the HPLC mobile phase and used to obtain calibration curves in the appropriate chromatographic system.

2.5. Mycotoxins extraction, detection and quantification

Prior to extraction, all samples were dried at 40 °C for 24 h and weight loss recorded in order to present the results in an 'as is' basis (μg kg^{-1}) or in total content of toxin (μg or ng).

2.5.1. OTA

Briefly, for OTA analysis, 5 g of ground sample were extracted with 30 mL of extractant solution (60% acetonitrile, 40% water) by magnetically stirring for 10 min and filtered with filter Whatman number 1. 4 mL of filtered solution was diluted with 44 mL of PBS solution and drained through the IAC column. After this, the column was washed with 20 mL of PBS and OTA was eluted by applying 1.5 mL of methanol grade HPLC (three times back flushing) and 1.5 mL of milli-Q water, consecutively. The purified extract was dried under nitrogen stream. Each dried sample was resuspended with acetonitrile:water:acetic acid (57:41:2).

OTA was determined by HPLC coupled with a Multi λ Fluorescence Detector Waters 2475®, an analytical column Water Spherisorb® 5 μm ODS2, 4.6 × 250 mm. Excitation and emission wavelengths were set, respectively, at 330 and 463 nm. Mobile phase consisted of acetonitrile, water and acetic acid (57:41:2). The mobile phase flow rate was 1 mL min^{-1}. The injection volume was 100 μL. The retention time was 15 min.

2.5.2. DON and DON-3-G

Regarding DON and DON-3-G, 5 g of ground sample were extracted with 30 mL of distilled water by magnetically stirring for 10 min. Then the sample was centrifuged for 8 min at 1780 g. Supernatant was filtered through a glass microfiber filter. Five millilitres of filtered sample was drained through the IAC column and the column washed with 10 mL of distilled water. DON was eluted by applying 1.5 mL of methanol grade HPLC (with three backflushing steps) and 1.5 mL of milli-Q water, consecutively. The purified extracts were dried under nitrogen stream. Each dried sample was resuspended with the mobile phase solution (water:-acetonitrile:methanol, 92:4:4). DON was determined by HPLC coupled with a UV/Visible dual λ absorbance Detector Waters 2487. Absorption wavelength was set at 220 nm. The mobile phase flow rate was 1.2 mL min^{-1}. The injection volume was 100 μL. The retention time for DON and DON-3-G were 20 and 23 min, respectively.

2.6. Methods performance

The analytical methods used were assessed for linearity, precision and recovery. Standard curves were generated by linear regression of peak areas against concentrations (r^2 were 0.97 and 0.99 for DON and OTA, respectively). Precision was established by determining OTA and DON levels in bread and DON and DON-3-G in flour samples at least by triplicate, in those samples fortified in order to calculate the recovery rates. 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 × LOD. Method performance characteristics for DON and OTA are summarized in Table 3.

2.7. Statistical analysis

The results obtained were processed in two different ways: a) DON or OTA content in each step (μg of DON or ng of OTA) and, b) concentration 'as is' (μg kg^{-1} of DON and OTA in wet basis). In the first case it was possible to assess the real impact of the processing steps in the mycotoxins, while in the second one it is possible to draw conclusions on either compliance of maximum levels or calculation of PC. For the 'unkneaded mix' results obtained for dry ingredients from the mycotoxin analysis were corrected for the amount of water to be added. Kruskal–Wallis test and multifactorial ANOVA were applied to assess the significance of sample traits in the observed mycotoxin levels. Finally, multiple linear regressions were applied to assess the temperature/time effect of DON reduction during baking.

Table 3
Method performance for ochratoxin A (OTA). Deoxynivalenol (DON) and deoxynivalenol-3-glucoside (DON-3-G) 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] (%)
OTA	Bread	0.14	0.42	5	0.5	103.90 ± 15.57	4
				5	1.5	110.13 ± 13.04	2
				5	3.0	99.65 ± 12.57	1
DON	Bread	60.00	180.00	5	100	100.01 ± 16.27	16
				5	500	98.84 ± 9.01	9
				5	1000	102.33 ± 5.26	5
	Flour	60.00	180.00	3	500	87.36 ± 8.58	7
				3	300	123.26 ± 30.29	41
DON-3-G	Flour	14.00	42.00	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.

3. Results

3.1. DON content

The level of DON increased from unkneaded mix to fermented dough, and decreased due to baking ($p < 0.05$); this trend depended on the initial concentration of DON in the flour. The level in the bread was significantly lower than in the initial mix of ingredients ($p < 0.05$).

DON content increased after dough proofing (Table 4). Average increase was of 16, 10 and 30% for high, medium and low DON content in the initial mixture, respectively (Fig. 1). However, according to Kruskal–Wallis test, this increase was only statistically significant ($p < 0.05$) for the low initial DON concentration in the mix. Regarding DON-3-G, as the experiment was designed taking only into account DON levels, but not DON-3-G levels, the initial concentration in flour of DON-3-G in the experiments with high/medium/low levels of DON was not significantly different, thus all results were pooled for a single initial concentration (mean 45.4 μg kg^{-1}, assuming LOD for the negative samples). According to Kruskal–Wallis test, fermentation led to a significant increase in DON-3-G content, from median = 2.5 μg, to median = 3.6 μg ($p < 0.05$).

Baking led to a significant reduction in DON content ($p < 0.05$) according to Kruskal–Wallis test, with mean percentages of 30, 17 and 33% for high, medium and low initial flour concentration, respectively, compared to fermented dough (Table 4). Baking for 75–135 min at 180–210 °C led to DON reduction ($p < 0.05$) for the three assayed initial concentrations in the mix (Table 5), while in some treatments for 45 min or at 170 °C some increase in DON could be observed (data not shown). The statistical analysis suggested that the assayed levels of baking time had a much important effect than temperature levels in DON stability. Slight differences were observed among temperature levels over 170 °C. While for the higher and medium initial concentration, both the effect of time and its interaction with temperature levels had a significant effect ($p < 0.05$) in the reduction of DON content, for the lower concentration the effects of temperature and time were not significant. The limited effect of temperature can be attributed to the similar temperatures reached inside the loaf bread even if oven temperatures were high. Fig. 2a shows the temperature profiles recorded in the centre of loaves when baked at 170, 190 and 210 °C for 105 min. The maximum temperature reached in the centre of the crumb was 98 °C, such temperature was independent of the oven temperature, then baking time becomes more relevant. For example, at 210 °C, for a baking time of 75 min, the crumb was 60 min over 90 °C, and 90 min and 120 min, for baking times of 105 and 135 min, respectively. Moreover, recording of temperature near to the surface of the loaves revealed temperatures in the crust up to 30 °C higher than in the crumb (Fig. 2b). DON analyses of the different parts of the bread (the crust, 0.5 cm below the crust and the crumb) showed a higher reduction in the crust compared to the other two fractions (data not shown), but the difference was not significant. Thus the major fraction of crumb compared to crust might determine the low effect of temperature on DON recorded in the whole bread. On the other hand, a significant increase of DON-3-G was observed in the bread compared to the fermented dough (from 8.8 to 25.4 μg). Although an increasing trend of DON-3G to increase with temperature levels was observed, temperature and baking time levels did not significantly affect the extent of such increase, which accounted for a mean value of 189% (data not shown).

Table 4
Evolution of DON mean content (μg) ±SD in the different breadmaking steps at the three initial concentration levels.

	High concentration	Medium concentration	Low concentration
Unkneaded mix	326 ± 82	217 ± 70	158 ± 50
Fermented dough	376 ± 121	239 ± 107	205 ± 80
Bread	239 ± 107	202 ± 103	130 ± 53

3.2. OTA content

Results confirmed the high stability of OTA as no significant change in its content could be observed as a result of the breadmaking process. While kneading and fermentation and baking had no significantly effect separately in OTA content, the final content in bread was significantly higher than in the initial mix of ingredients (41%). Although a mean increase of 19% was recorded during kneading and fermentation, it was not statistically significant. Unkneaded mix contained 1483, 801 and 123 ng for the three concentration levels, high, medium and low, respectively, and the fermented dough 1501, 1174 and 132 ng of OTA, respectively. Regarding baking, as no significant reduction was observed in the loaves baked at 210 °C for 105 and 135 min, and at 200 °C for 135 min, the remaining loaves were not analysed. The average result was an overall increase of OTA of 24%. Thus as a conclusion the total amount of OTA in the flour remained finally in the resulting bread.

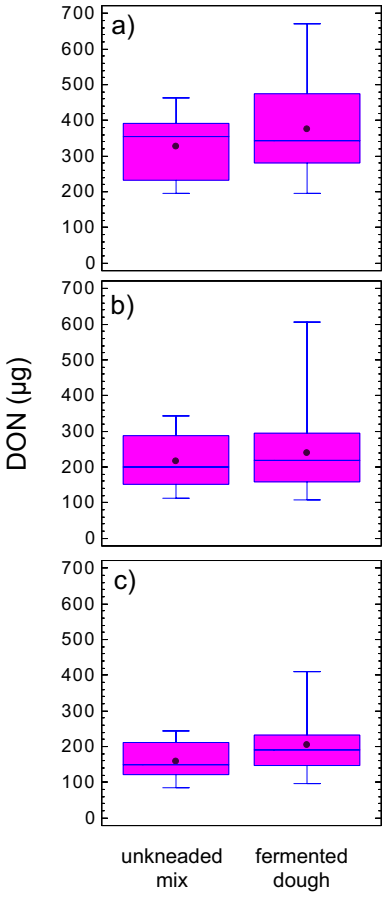


Fig. 1. Distribution ($n = 20$) of DON content (μg) in the initial mixture and fermented dough at the high (a), medium (b) and high (c) initial concentration.

Table 5
DON content reduction (mean% ± SD) as affected by baking time and temperature.

Temp (°C)		170	180	190	200	210
	Initial DON conc.					
75 min	High	10 ± 59	34 ± 21	24 ± 11	43 ± 26	33 ± 24
	Medium	−45 ± 57	34 ± 71	10 ± 23	47 ± 53	83 ± 31
	Low	35 ± 16	42 ± 31	34 ± 41	48 ± 29	40 ± 35
105 min	High	22 ± 6	34 ± 22	35 ± 23	38 ± 9	61 ± 2
	Medium	18 ± 45	−0.9 ± 18	14 ± 11	22 ± 12	26 ± 34
	Low	−2±62	73 ± 7	28 ± 11	32 ± 37	39 ± 32
135 min	High	46 ± 11	32 ± 24	38 ± 21	35 ± 9	48 ± 10
	Medium	26 ± 4	4 ± 71	8 ± 26	6 ± 28	16 ± 39
	Low	28 ± 35	31 ± 48	23 ± 18	24 ± 15	34 ± 1

3.3. DON concentration in flour and bread 'as is'

DON concentration in bread produced from a given flour batch will depend on the 'dilution' of the toxin with the addition of ingredients, on to a minor extent to water, carbon dioxide and ethanol balance, and finally to the stability of the toxin through the process, which was explained in the previous sections.

Combined 'dilution' plus increase in DON content due to fermentation led to significantly higher ($p < 0.05$) DON concentration in flour than in fermented dough. The reductions obtained in the three different concentrations were 31, 37 and 22% for the high, medium and low concentration respectively (Table 6).

Moreover, a significant reduction in DON concentration from proofed dough to bread was observed for baking times of 75 min and over. For the higher and medium initial concentration in the flour, the percentage of reduction depended on temperature and baking time levels assayed (Fig. 3a, $r^2 = 0.695$, %reduction $= -229.081 + 0.532 \cdot \text{temperature} + 2.850 \cdot \text{time} - 0.012 \cdot \text{time}^2$; Fig. 3b, $r^2 = 0.523$, %reduction $= -1180.62 + 5.130\ \text{temperature} + 11.986 \cdot \text{time} - 0.040\ \text{temperature} \cdot \text{time} - 0.021 \cdot \text{time}^2$), while at the lower concentrations the effect was not significant, thus a single value of reduction (26 ± 18%) could be considered in this case.

The lower percentages of reduction compared to those in DON content may be attributed to the evaporation of water and other dough components in the oven (mean weight loss of 8%), and consequent concentration of toxin.

A 25% decrease in DON-3-G was observed from flour to fermented dough, and a mean further increase of 224% from fermented dough to final bread (Table 6).

3.4. OTA concentration in flour and bread 'as is'

OTA concentration in bread produced from a given flour will depend only on the 'dilution' of the toxin with the addition of ingredients, and on a minor extent to water, carbon dioxide and ethanol balance due to OTA stability during the breadmaking process, as explained in the previous sections. Thus OTA concentration (wet basis) was reduced by average in a 36% from flour to fermented dough (theoretical decrease, assuming complete stability would be 40%).

On the other hand, the average increase from fermented dough to bread was 27% (wet basis), although not significant (theoretical increase, assuming complete thermostability of OTA and 8% weight loss in the bread would be 9%).

4. Discussion

Some studies dealt in the past with the fate of DON during breadmaking, and although some trends were pointed out, only a few reported statistically significant effects on equal basis for each studied stage (Scudamore et al., 2009). Similarly, in the present

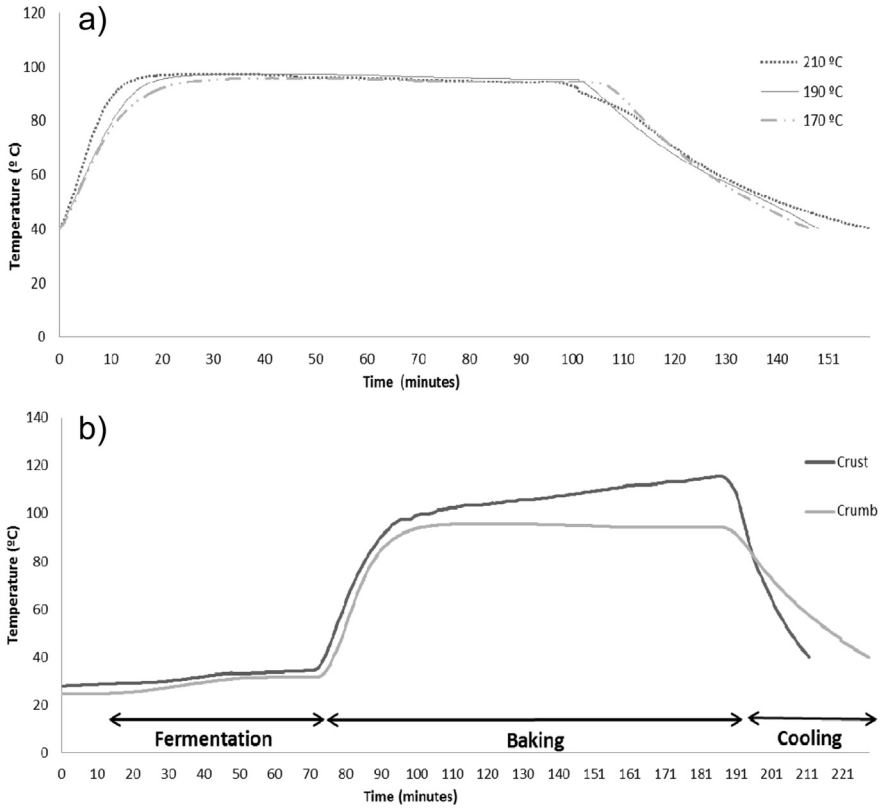
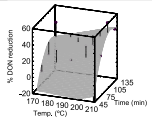
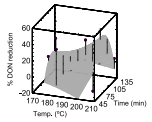


Fig. 2. Recorded temperatures a) in the centre of the loaves at 170, 190 and 210 °C for 105 min, and b) in the crumb and in the crust of the loaves after 105 min of baking.

study a great variability was observed among repeated experiments. Apart from known heterogeneous distribution of mycotoxins in foodstuffs, enzymatic or microbial processes that take place during breadmaking, could be the cause of additional variability.

In the present study, DON increased with fermentation; as the initial analysis was carried out before kneading, such increase was due to the joint contribution of kneading, fermentation and proofing. This increasing trend has been mainly previously observed in those studies in which the results were given by comparison of concentration in fermented dough to flour, thus including kneading. Lancova et al. (2008) reported an increase in DON concentration due to kneading, which could confirm this hypothesis. High increase in DON content was also reported in those studies in which enzymes (mainly α-amylase) were used as dough improvers (Table 1). Both circumstances coexisted in the present study. By contrast, studies at higher than 30 °C fermentation temperatures reported a reduction in DON concentration from kneaded dough to fermented dough (Samar et al., 2001), while no DON reduction was observed at 30 °C. Confirming our results, Young, Fulcher, Hayhoe, Scott, & Dexter (1984) and Bergamini et al. (2010) observed an increase in DON content during fermentation

Table 6
Evolution of mycotoxin concentration (μg kg^{-1}) in the different steps of breadmaking process (flour, fermented dough and bread).

	Flour (μg kg^{-1})	Fermented dough 'as is' (μg kg^{-1})	Bread 'as is' (μg kg^{-1})	% Reduction (kneading + fermentation + proofing) 30 °C 75 min	% Reduction baking
DON	2090	1445 ([a]1254)	Variable	31% ([a]40%)	
	1459	921 ([a]875)	Variable	37% ([a]40%)	
	1012	790 ([a]607)	585	22% ([a]40%)	26%
DON-3-G	45.4	34.03 ([a]27.3)	110.3	25% ([a]40%)	−224%
OTA	9.5	5.8 ([a]5.7)	8.9	39% ([a]40%)	−50%
	5.1	3.5 ([a]3.0)	5.0	31% ([a]40%)	−43%
	0.8	0.5 ([a]0.5)	0.6	37% ([a]40%)	−20%

[a] Calculated value taking into account only dilution by recipe.

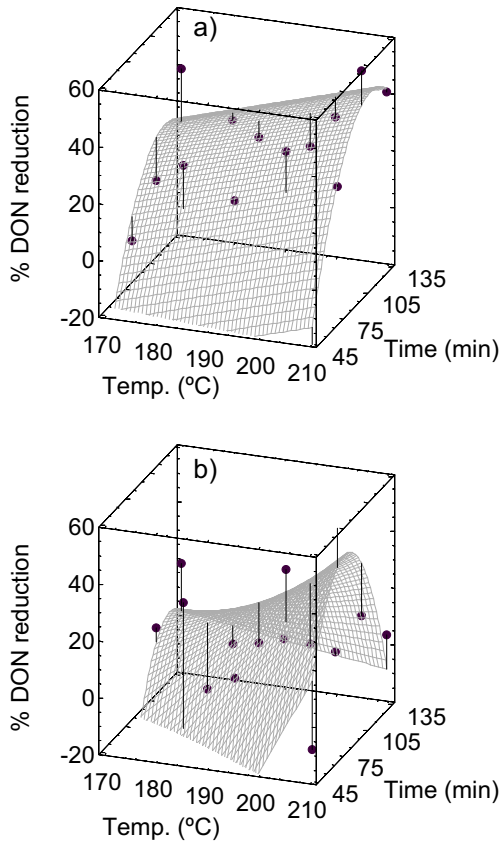


Fig. 3. Response surface model for %reduction of DON concentration from fermented dough to bread as a function of temperature and baking time. a) high b) medium initial DON concentration in the flour.

at 30 °C. The authors suggested that wheat flour contained DON precursors (3-acetyl-deoxyinivalenol and 15-acetyl-deoxynivalenol), which were converted to DON by the active yeast. In the present study, 3-ADON and 15-ADON were analysed in some samples at random (data not shown), and it was concluded that although the precursors disappeared during fermentation, their concentration in the flour was too low (4–5% of DON concentration for the sum of both precursors) to account for the increase in DON. Bergamini et al. (2010) hypothesised an enzymatic release of DON from its unknown bound forms. This hypothesis was confirmed by Kostelanska et al. (2011) and Zachariasova et al. (2012) who indeed reported a much significant increase in DON-3G than in DON due to the use of bread improvers, as was confirmed in the present study.

The level of DON-3-G in the uninoculated flour was similar or higher than those reported in naturally contaminated (or field inoculated) wheat flour intended for breadmaking (Kostelanska et al., 2011; Simsek et al., 2012). In the literature, DON-3-G has been attributed to DON conjugation at plant level or due to heat treatments (Bretz, Beyer, Cramer, Knecht, & Humpf, 2006). In our study, while both DON and DON-3-G increased during kneading and fermentation, DON decreased during baking but DON-3-G increased. By contrast, previous studies reported DON-3-G reduction during baking (Kostelanska et al., 2011; Simsek et al., 2012).

The reduction in DON during baking was consistent with previous studies which always reported reduction at temperature over than 170 °C as long as baking time was longer than 30 min (Table 1). Little difference was observed among the impact of the different temperature levels, thus for a target reduction level, several binomial temperature/time values could be used, satisfying at the same time the sensorial traits of the baked product. Few studies focused in the past on the combined effect of baking time and temperature in DON content in bakery products (Bergamini et al., 2010; Suman et al., 2012; Valle-Algarra et al., 2009), and in most cases no significant differences were observed or were not reported. Baking temperatures between 170 and 240 °C have been assayed with small differences in their impact. However, in general, oven temperatures were reported and, as presented in the present study, the temperature levels inside the baked loaves may not be significantly different. Placing temperature probes in the loaves allowed us to confirm that fermentation temperature in the dough was 3–4 °C under 30 °C (as also measured by Samar et al., 2001), and that maximum attained temperature in the crumb during baking was 98 °C, regardless of the oven temperature, similarly, Bergamini et al. (2010) reached a maximum of 86 °C in the loaf when baking at 225 °C. As a consequence of this, the size of the loaf may be determinant for the calculation of the extent of mycotoxin reduction during baking, as pointed out by Valle-Algarra et al. (2009). Looking at Table 1, this can be the reason for some contradictory presented results. Some studies reporting higher reduction, included small size loaves, cakes and biscuits (Samar et al., 2001; Suman et al., 2012), while, interestingly, Zachariasova et al. (2012) using a 500 g loaf weight did not report any reduction when sampling the whole bread for analysis. Unfortunately, from our results, although higher DON levels were in general found in the crumb, no significant difference could be confirmed between crust and crumb, while this point could be clearly confirmed by Valle-Algarra et al. (2009). DON thermodegradation products (norDONs A-F and DON lactones) occur in bread crust (Kostelanska et al., 2011); such degradation products 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 et al., 2006). In general, higher reductions were observed during baking with higher DON concentration, but the trend was not significant as concluded before (Bergamini et al., 2010; Neira et al., 1997). The increased DON levels observed sometimes at 45 min or 170 °C could be attributed to the prolongation of the enzymatic activity to the initial stages of baking, before enzyme inactivation.

The studies on OTA during the breadmaking process are scarce. In the present study little effect was observed, confirming the work of Scudamore et al. (2003). Baking led to an increase in OTA level that, although not significant, had never been reported before. Oppositely, baking of biscuits resulted in about two-thirds of the toxin being destroyed or immobilized (Subirade, 1996). The higher diminishing of OTA content in biscuits can be explained by the higher temperature reached when compared to bread and for the lower water content. On the other hand, Valle-Algarra et al. (2009) found an OTA reduction near to 30%, but they worked with OTA-spiked flour, which may be differently affected. In other fermented foods, reduction in OTA caused by the degradation of OTA or the adsorption in the yeast cell walls have been detected, especially in wine (Abrunhosa, Serra, & Venâncio, 2002).

This work was developed in an attempt to investigate the possible PC to be attributed to the breadmaking steps, and then to be able to calculate the toxin concentration in the final bread (performance objective) produced from a given flour with H_0 initial concentration of toxin. Looking at DON results and literature, it is clear that standard fermentation temperature, plus the widespread use of improvers leads to a minimising of the 'dilution' effect due to ingredients mixture to levels of 22–37% (PC). An alternative to increase this criterion would be to modify process criteria from 30 °C 1 h to higher fermentation temperature, if applicable, which may lead to DON degradation, as suggested by Samar et al. (2001). The baking stage led to a minimum reduction of 20% (PC) as long as time

was over 75 min and temperature over 180 °C (process criteria), thus temperature/time of baking can be adjusted for a desired process criterion. In general, a minimum 38% reduction can be expected for the breadmaking process from flour to bread (in a 260 g bread loaf, with improvers added), which is a bit higher than the 33% assumed by Commission Regulation 1881/2006 setting a maximum level of 750 μg/kg for flour and 500 μg/kg for bread (in our case the only dilution effect would account for 40% reduction, but different recipes for bakery products could lead to much lower reduction, as suggested by Scudamore et al. (2003)). On the other hand, DON-3G concentration greatly increased with baking, although its increase could not be linked to baking temperature/time levels. The reason could be the glycosidation of DON in the initial stages of baking, before enzyme inactivation. This hypothesis is reinforced by the fact that DON-3-G also increased during fermentation, and was previously suggested by Zachariasova et al. (2012). As a consequence, higher DON-3-G concentration could be found in bread than in the initial flour (opposite to what reported by De Angelis et al., 2013). This point is of outmost importance as part of DON can be *in vivo* released from its conjugate (Berthiller et al. 2013) and should be further investigated. Moreover, a joint regulation of DON + DON-3G maximum levels might be proposed. Finally, for OTA the PC for fermentation at 30 °C was near 40% which is the theoretical dilution factor by recipe, thus the fermentation process itself had no effect on OTA content. Although not significant, an increase in OTA during baking was observed, which resulted in final bread with similar OTA concentration to the initial flour. This confirms the suitability of an equal maximum level for both flour and bread (3 μg kg^{-1}), as set in the Commission Regulation 1881/2006.

As conclusion, the design of bakery products processes may help to control DON in final products, because although quite stable, its levels can be reduced to some extent; for this, higher fermentation temperature could be considered and longer baking times should be preferred. By contrast, OTA was highly stable. Finally, high levels of DON-3-G were released during baking, and this point should be further investigated. Mycotoxins have been always considered as thermostable compounds; however, in depth knowledge of the processing steps that may lead to some reduction (although limited) and those which can stimulate their release from conjugated forms will definitely help in their control in finished foodstuffs. Selection of flour lots with low toxin concentrations is still critical in food safety management.

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