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### ► **To cite this version:**

Elena Bergamini, Dante Catellani, Chiara Dall'Asta, Gianni Galaverna, Arnaldo Dossena, et al.. Fate of Fusarium mycotoxins in cereal product supply chain: the DON case within industrial bread-making technology. *Food Additives and Contaminants*, 2010, 27 (05), pp.677-687. 10.1080/19440041003660117. hal-00591171

**HAL Id: hal-00591171**

**<https://hal.science/hal-00591171>**

Submitted on 7 May 2011

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**Fate of Fusarium mycotoxins in cereal product supply chain: the DON case within industrial bread-making technology**

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2009-335.R1
Manuscript Type:	Special Issue
Date Submitted by the Author:	26-Jan-2010
Complete List of Authors:	Bergamini, Elena; Barilla SpA, Food Science & Research Labs Catellani, Dante; Barilla SpA, Food Science & Research Labs Dall'Asta, Chiara; University of Parma, Department of Organic and Industrial Chemistry Galaverna, Gianni; University of Parma, Department of Organic and Industrial Chemistry Dossena, Arnaldo; University of Parma, Department of Organic and Industrial Chemistry Marchelli, Rosangela; University of Parma, Department of Organic and Industrial Chemistry Suman, Michele; Barilla SpA, Food Science & Research Labs
Methods/Techniques:	Chemometrics, Chromatography - LC/MS, Statistical analysis
Additives/Contaminants:	Mycotoxins - fusarium, Mycotoxins - trichothecenes
Food Types:	Bread, Bakery products

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3 **Fate of Fusarium mycotoxins in the cereal product supply chain: the DON case**  
4 **within industrial bread-making technology.**  
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22  
23 **Abstract**  
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25 *Fusarium* mycotoxins are a relevant problem in the cereal supply chain at a worldwide level, being  
26 wheat, maize and barley the main contaminated crops; mould growth could happen in the pre-  
27 harvest phase and also during transport and storage due to ineffective drying conditions. Among  
28 *Fusarium* toxins, deoxynivalenol (DON) is considered the most important contaminant in wheat,  
29 due to its wide-spread occurrence. In the last years the European Food Safety Authority and the  
30 European Commission had frequently expressed opinions on *Fusarium* toxins, setting limits,  
31 regulations and guidelines in order to reduce their levels in raw materials and food commodities: in  
32 particular European legislation (Reg. 1881\2006) sets the maximum limit for deoxynivalenol in  
33 flour and bread as 750 µg/kg and 500 µg/kg respectively. Relatively few studies have taken into  
34 account the loss of trichothecenes during processing, focusing on how processing factors may  
35 influence their degradation. In particular, the description of DON behavior during bread-making is  
36 very difficult, since complex physic-chemical modifications occur during the transformation of raw  
37 ingredients into the final product. In the present study, we studied how DON concentration may be  
38 influenced by modifying bread-making parameters, with a special emphasis on the fermentation and  
39 baking stages, starting from a naturally contaminated flour at both pilot and industrial scales.  
40 Exploiting the power of a Design of Experiments (DoE) approach to consider the high complexity  
41 of the studied system, the obtained model shows satisfying goodness of fit and prediction,  
42 suggesting that the baking step (time/temperature ranges) is crucial for minimizing native DON  
43 level in bread.  
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3 **Keywords:-** bread; fermented dough; mycotoxins; industrial process; processed food; design of  
4 experiments; deoxynivalenol degradation; deoxynivalenol binding  
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## 8 **Introduction**

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10 Trichothecene mycotoxins, which frequently occur in cereals and cereal-based products, are  
11 secondary metabolites produced mainly by *Fusarium* ear blight pathogens, which are widely  
12 distributed in the temperate zone of all continents (D'Mello and Macdonald 1997; Creppy 2002).  
13 The incidence of these mycotoxins in food crops can vary considerably from year to year depending  
14 on many factors, such as weather conditions and agricultural practices (Edwards 2004). Since these  
15 toxins can never be completely removed by processing (Kuiper-Goodman 2004), preventive  
16 measures should be adopted at all stages of the production chain to prevent contamination of final  
17 food products.  
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26 Among trichothecenes, deoxynivalenol (DON) is the most important for wheat and derived  
27 products, on account of its widespread diffusion and of its implication in animal and human  
28 toxicoses (Hussein and Brasel, 2001), including feed refusal, decrease in body weight,  
29 immunomodulation and reduction of host resistance against infection. Natural occurrence of DON  
30 in wheat and in derived products has been reported world-wide.  
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37 To protect consumers from unacceptably high dietary intake of trichothecenes, maximum levels  
38 have been set in EU legislation (European Commission 2006). Currently, maximum levels for  
39 deoxynivalenol are regulated in cereals (1250 µg/kg), flour (750 µg/kg) and bread (500 µg/kg).  
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43 The fate of DON and other trichothecenes during processing has been documented in several  
44 studies. Experiments concerned with the distribution of DON among the milling fractions in  
45 naturally *Fusarium*-contaminated wheat showed that the highest concentration of this mycotoxin is  
46 in the bran layer; while in white flour, compared with the whole grain, it is reasonably lower (Abbas  
47 et al. 1985; Tanaka et al. 1986).  
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53 The first stage of cereals processing consists into removal of dust, broken grains and other foreign  
54 material. This action can significantly reduce mycotoxin concentrations, since most mycotoxins  
55 tend to concentrate in the bran fractions or outer layers. Whole grains are then milled to produce  
56 flour and other fractions, used to prepare bread, biscuits, cakes, etc. Most mycotoxins are quite  
57 stable at room temperature and under neutral conditions; however, many factors (e.g. temperature,  
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pH, enzymatic activities, food macro- and microconstituents) must be considered during processing, since mycotoxin modification and breakdown may be induced (Scott, 1998).

The occurrence of mycotoxins in the raw material, such as common wheat, should be therefore carefully studied, as well as the effect of processing and food manufacturing on their concentration in the correspondent retail products (Hazel and Patel, 2004).

Bakery processing has been reported to reduce DON contamination by some authors (Abbas et al. 1985, Seitz et al. 1986) while others suggested that DON is highly stable in this process (El-Banna et al. 1983, Scott et al. 1983, 1984). Moreover DON appeared to be very stable at 100°C at the pH of bread (Wolf and Bullerman, 1998).

The influence of various ingredients added to dough before baking on DON levels in bread was studied by Boyacioglu et al. (1993). In particular, a reduction of DON as high as 40% seemed to be due to the addition of several additives such as sodium bisulphite, L-cysteine and ammonium phosphate in dough. Other additives such as potassium bromate and L-ascorbic acid were ineffective.

Samar et al (2001) indicated that DON reduction during bread-making might be due not only to the thermal decomposition of the toxin, but also to the fermentation process exerted by yeasts. However, the effect of yeast on DON levels in dough during the baking fermentation step are rather contradictory. Neira et al. (1997) documented a 20% mean reduction of DON in fermented dough, Valle Algarra et al. (2009) indicate stability during the fermentation and reduction in the baking phase, whereas Young et al. (1984) observed an increase of DON in yeasted products. The latter authors suggested that perhaps the wheat used was also contaminated with a DON precursor which was converted in DON by active yeast. This fact is a crucial point to be considered and might explain somewhat the diversity of results found in the literature, as recently pointed out also by Lancova et al (2008). Technological processes, indeed, could also play an important role in the masking mechanism: mechanical or thermal energy during the transformation process (Scudamore, 2008) may cause modification, inducing reactions with macromolecular components such as sugars, proteins or lipids as well as release of native forms.

Published data on milling and bread-making processing appear thus conflicting. This may be in part due to the failure in understanding the complexity of milling technology or to very different

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3 approaches (handmade, pilot scale/lab trials), often far from achieving an edible and satisfying  
4 product for consumers. It is also evident that the behaviour of mycotoxins in laboratory\handmade  
5 studies could not adequately reflect what occurs in industrial scale operations such as in mills or  
6 bakery plants using naturally contaminated cereals (Scudamore et al., 2009). Last but not least, it is  
7 not always made clear whether values quoted are “as is” or corrected for moisture content (usually  
8 around 30-40%) and formulation (addition of other ingredients, additives etc.).  
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16 The aim of the present study was to verify how the DON concentration in final bread is influenced  
17 by modifications of technological parameters during the fermentation and baking steps, starting  
18 from naturally contaminated wheat flour and using firstly a pilot-scale bread-making plant and then  
19 a real industrial production line. The experiments were performed using statistical Design of  
20 Experiment (DoE) schemes which allow, when multiple parameters may influence the desired  
21 outputs, to explore the relationship between the analytical responses and independent variables  
22 conducting to an optimization of the process. In particular, in our case this approach was chosen in  
23 order to consider only those modifications which can really be applied to industrial scale to obtain a  
24 final product which can be considered appreciable by the consumer.  
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## 33 **Materials and methods**

### 34 **Chemicals**

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36 Deoxynivalenol (DON) as well as internal standard  $^{13}\text{C}_{15}$ -deoxynivalenol ( $^{13}\text{C}_{15}$ -DON) were  
37 purchased by Biopure GmbH (Tulln, Austria). Acetonitrile (HPLC grade), methanol (HPLC grade),  
38 ammonium acetate (puriss. p.a.) was obtained from Merck (Darmstadt, Germany). Acetic acid was  
39 also an HPLC gradient-grade reagent obtained from BDH VWR International Ltd (Poole, England).  
40 MycoSep® 226 Trichothecene columns (Romer Labs Inc., Union, MO) were purchased from Orsell  
41 Srl (Modena, Italy). Deionized water was used for all procedures.  
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### 50 **Samples**

51 For the baking experiments conducted within the first part of the study, 16 batches of wheat  
52 naturally infected with *Fusarium* spp. were analysed with a focus on DON contamination levels.  
53 Among the analysed batches, three flour batches were established, according to DON contamination  
54 level and technological characteristics for bread-making (e.g. quality and quantity of the gluten-  
55 forming proteins content, grain conformation etc.). In particular, three different levels of DON  
56 contamination were selected; the whole wheat batches were milled using a pilot-scale mill (BONA,  
57 B0430) to obtain three flour batches (212  $\mu\text{m}$  granulometry) with a different DON level  
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3 contamination (flour A  $160 \pm 15 \mu\text{g/kg}$ , flour B  $100 \pm 14 \mu\text{g/kg}$ , flour C  $967 \pm 49 \mu\text{g/kg}$ ), which  
4 was evaluated in triplicate on dry matter.  
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### 8 **Moisture content determination**

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10 The moisture contents of flour and bread were measured by taking 10g of ground sample and  
11 heating in a thermostatic oven at  $105^\circ\text{C}$  for more than 6 hours. The moisture content of fermented  
12 dough was measured by taking a 15g of sample and heating in an oven at  $105^\circ\text{C}$  for 3h; after a  
13 milling step, the sample was again heated at  $105^\circ\text{C}$  for 6 hours.  
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16 All the results were corrected and compared on dry matter basis.  
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### 20 **Experimental Design**

21 Design of Experiments (DoE) is the design of all information-gathering exercises where variation is  
22 present, whether under the full control of the analysis or not: the analyst is interested in  
23 investigating of phenomena and studying the synergistic effect of some interventions (the  
24 “treatments”) for a final process optimization.  
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31 The present study is organised on a Screening Design of Experiments (SDoE) with the aim to  
32 identify which predictors are most influential as well as what are their appropriate ranges for  
33 minimizing DON levels in bread-making. Among the bread-making parameters, several conditions  
34 were varied during the experiments according to the technological requirements: in particular, DON  
35 contamination level on wheat flour, fermentation rate, fermentation temperature, baking rate and  
36 baking temperature were included as treatments in the experimental design. Each selected treatment  
37 was varied within a range defined according to the technological requirements to obtain an  
38 organoleptically appreciable loaf for the consumer, as described in Table 1 for the pilot-scale plant  
39 and for the industrial-scale plant.  
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49 To describe in a synergic way all these variables, enabling a screening interaction model to be  
50 estimated taking into account of balancing and orthogonality for each factor, it was adopted a full  
51 factorial central composite design (CCD). Three replicates of the central points were performed to  
52 estimate the experimental error.  
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56 Experimental data were then analysed by a multivariate analysis approach based on the Partial Least  
57 Square technique, using a dedicated statistical-package (MODDE software, vers. 8.0, Umetrics;  
58 Umea, Sweden).  
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### Dough preparation and bread-making

Different doughs were prepared starting from selected flours in order to get a final loaf of about 120g; the ingredients were wheat flour (59%), water (30%), yeasts (*Saccharomyces cerevisiae*), bread-making promoting agents, malted barley flour, dextrose, sourdough powder, fractionated palm oil and salt (11% overall).

The process consists substantially of the following steps: firstly, flour is mixed with all solid powders ingredients (2 min) then moistened with salt suspended in water and oil (3 min). Dough is mixed with baking test kneader. Water optimal amount for each flour as well as reference baking conditions is established on the basis of previous experiments and internal technological knowledge. The overall bread-making process is summarized in Figure 1.

Since the experimental plan involved randomized changes in condition of bread fermentation and baking, 24 different baking tests for the pilot plant and 19 for the industrial plant were performed (see Tables 2 and 3). Two identical doughs were used for each single experiment: one was analysed for DON after the fermentation step and the other was analysed for DON after the baking process. Thus, rounded pieces of about 10 cm diameter (~140 gr) were obtained from each dough and let rest for 10 min at room temperature. Concerning the pilot plant, fermentation step was performed in a leaven cell (Fermalievita, Alaska) at a relative humidity (RU%) of 70%, while baking step was performed in a pilot scale oven (Pollin, Verona, Italy); concerning the industrial line, fermentation step was performed in a leaven cell at a relative humidity (RU%) of 85%, while baking step was performed in an indirect-radiating-cyclotherm-5 zones industrial oven (Imaforni, Verona, Italy). For each baking test, fermentation parameters (time and temperature) as well as baking parameters (time and temperature) were set according to the Experimental Pilot Design (DoE).

Prior to analysis, samples were stored at -18°C. Each sample was extracted in duplicate and each extract was measured in duplicate.

### Extraction and clean-up for DON

According to a previously published procedure (Suman et al., 2008), a total of 20g of bread or dough sample was extracted with 100ml of a mixture acetonitrile/water (84:16, v/v) by homogenization at medium-high speed for 2 min. The extract was filtered and 6ml of the filtrate was poured into a 10ml vial previously prepared in the following way: 100µl of an internal standard (<sup>13</sup>C<sub>15</sub>)-DON solution (1200µg/l in acetonitrile) was added and immediately evaporated to dryness



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3 under a nitrogen stream. This final solution was acidified by 30  $\mu$ l of acetic acid and then slowly  
4 pressed through a Mycosep® 226 column. A total of 2 ml of the eluate was evaporated using a gentle  
5 stream of nitrogen and the residue was reconstituted in 0.2 ml of starting mobile phase (phase B)  
6 and used for LC-MS/MS analysis (injection volume: 20  $\mu$ l aliquot).  
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### 10 11 **LC-MS/MS analysis**

12 The samples were analysed in duplicate in the Mass Spectrometry Laboratory of Barilla Food  
13 Research Labs and in the Food Chemistry Laboratory of Department of Organic and Industrial  
14 Chemistry, University of Parma.  
15

16 The pump used for reversed-phase high-performance liquid chromatography (RP-HPLC) was a  
17 Surveyor LC Pump (Thermo Finnigan, San Jose, CA, USA). The chromatographic separation was  
18 performed using a Supelcosil LC-18  $3\mu$ m 33x4.6 mm (Supelco, Bellefonte, PA, USA) column with  
19 a flow rate of 0.2 mL/min. Injections were made using a Surveyor Autosampler (Thermo  
20 Finnigan).  
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22 The electrospray ionization (ESI) experiments were carried out in a Linear-Ion-Trap LXQ (Thermo  
23 Scientific) mass spectrometer either in positive or negative scan mode.  
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25 Linear Ion Trap LC-MS/MS conditions were determined following the method of Suman et al.  
26 (2008). Briefly, a linear binary gradient was applied changing from 100% solvent A [20% methanol  
27 + 80% water (5 mM ammonium acetate)] to 100% solvent B [65% methanol + 35% water (5 mM  
28 ammonium acetate)] and to 100% solvent C [80% methanol + 20% water (5 mM ammonium  
29 acetate)] within a total run time of 25 minutes.  
30

31 The capillary temperature was set to 220 °C; the sheath gas set to 20 units; the spray voltage was  
32 kept at 4000 V; the capillary voltage was kept at -11 V; multiple Reaction Monitoring (MRM)  
33 experiments were performed according to parameters reported in Table 4. All parts of the  
34 equipment and data processing were performed by the computer software Xcalibur (Thermo  
35 Scientific).  
36

37 Concerning the experiments performed at the Food Chemistry laboratory, University of Parma, the  
38 instrumental conditions were reported below. The LC-MS/MS system consisted of a 2695 Alliance  
39 (Waters, Milford, MA, USA) equipped with a Quattro™ triple quadrupole mass spectrometer with  
40 an electrospray source (Micromass, Waters, Manchester, UK). Chromatographic conditions were  
41 the following: column, Phenomenex Synergi 2.5 $\mu$ m Fusion-RP 100A (50 x 2.00  $\mu$ m) equipped with  
42 a C18 precolumn cartridge (Phenomenex); flow rate, 0.25 mL/min; column temperature, 30°C;  
43 injection volume, 5  $\mu$ l. Gradient elution was performed using CH<sub>3</sub>COOH 5 mM (eluent A) and  
44 methanol (eluent B): 0–1 min, isocratic step 5% B; 1–9 min to 70% B; 9–20 min isocratic step 70%  
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3 B; 20-21 min to 5% B; finally, a re-equilibration step at 5% B (initial conditions) for 9 min was  
4 performed (total analysis time, 30 min). The MS parameters were the following: ESI- (negative ion  
5 mode); capillary voltage, 2.5 kV; cone voltage 10 V; extractor voltage, 3 V; source block  
6 temperature, 150°C; desolvation temperature, 350°C; desolvation and cone gas (nitrogen) 500 and  
7 50 L/h, respectively. Detection was performed using a multiple reaction monitoring (MRM) mode,  
8 by monitoring three transitions for each analyte, as reported in Table 4. The main transition was  
9 used for quantification, while two more transitions were chosen as qualifiers.

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11 All parts of the equipment and data processing were performed by the computer software MassLynx  
12 (Waters).  
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## 15 16 17 18 19 20 21 **Results and discussion**

22 Design of Experiments (DoE) is a statistical technique quite used by the industry today, mainly in  
23 the development and optimization of manufacturing processes. The main objectives of this  
24 approach are screening, optimization and robustness testing in the laboratory, in the pilot-plant or in  
25 the full-scale production plant. Therefore DoE involves making a set of experiments representative  
26 with regards to a given question in a problem-dependent way.  
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33 In particular, the objectives of the Screening Design of Experiment were: (i) to explore many  
34 factors in order to reveal whether they have an influence on the responses, and (ii) to identify their  
35 appropriate ranges.  
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40 In this study, the influence of four factors on the final DON level in bread was investigated on a  
41 pilot-plant scale. The four factors were fermentation rate, fermentation temperature, baking rate and  
42 baking temperature. For each rate/temperature parameters a well-defined variation range was  
43 assessed, as reported in Table 2: this variation range was established according to the technological  
44 requirements in order to get an acceptable final product. Moreover, three flour batches containing  
45 different initial DON amount were chosen, in order to simulate a medium-low contamination (level  
46 A and level B, 100 µg/kg and 160 µg/kg respectively) and a high contamination (level C, 967  
47 µg/kg) in raw material.  
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56 The statistical model required 24 single experiments; for each experiment DON level was measured  
57 by LC-MS/MS after the fermentation step and after the baking step.  
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59 At the end of the screening design, the software was able to extract an answer concerning the  
60 influence of a particular factor. This is done by fitting a polynomial model to the data and

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3 calculating the regression coefficient for each factor. These values, plotted in the Variable  
4 Importance Plot (VIP, Figure 2) indicated how the four factors influence the response and their  
5 relative interactions. Moreover, the construction of a response contour plot is also useful for  
6 decision making and result interpretation (Figure 3): information is thus gained about how to  
7 modify the settings of the important factors, to possible minimize DON content in ready-to-eat  
8 bread.  
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16 Afterwards, the validity of the established model was evaluated also on a full-scale plant, in order to  
17 check whether the observation obtained for pilot plant could be applied also for industrial bread.  
18 Finally, the prediction power of the model was tested by preparing and analysing a model loaf and  
19 comparing the expected and the found DON level in final bread.  
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### 24 **Bread-making pilot plant study: DON trend during processing.**

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26 Samples obtained from the application of the bread-making DoE were extracted in duplicate and  
27 then analysed using a linear ion trap LC/MS/MS in the Mass Spectrometry Laboratory of Barilla  
28 Food Research Labs, and using a triple quadrupole LC/MS/MS in the laboratory of Food Chemistry  
29 Labs, Department of Organic and Industrial Chemistry-University of Parma, as reported in the  
30 Material and Method Section. For each DoE experiment, two identical samples were prepared: the  
31 first was analysed after the fermentation step and the latter after baking, in order to check the effect  
32 exerted by the processing conditions during each step on the DON level.  
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38 The data were expressed on dry matter and corrected according to the recipe formulation and  
39 reported in Table 3.  
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44 As shown in Table 3, the DON level seems to be influenced by the processing and an increase  
45 (Tukey test,  $\alpha = 0.05$ ,  $p < 0.01$ ) is recorded after the fermentation step for the medium-low level  
46 contaminated samples, whereas negligible changes were recorded for high level contaminated  
47 samples. The DON contamination was averagely increased of 46% for group A samples (initial  
48 contamination: 100  $\mu\text{g}/\text{Kg}$ ) and of 31% for group B samples (initial contamination: 160  $\mu\text{g}/\text{Kg}$ );  
49 this trend could be due to the release of DON from some bound form occurring in the flour, which  
50 might be cleaved or solubilised during fermentation. The same phenomenon was not observed for  
51 highly contaminated flour (group C): in this case, the DON release from bound forms may be  
52 negligible when compared to high level of free DON occurring in the matrix (960  $\mu\text{g}/\text{Kg}$ ).  
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58 Since the aim of this study was the minimization of DON in industrial bread by means of statistical  
59 DoE, this phenomenon will be further studied by our group in the next future.  
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### **Bread-making pilot plant study: statistical evaluation of the prediction model**

Predictive modeling is the process by which a model is created to best predict the probability of an outcome: the model is chosen on the basis of detection theory to try to guess the probability of a signal, given a set amount of input data. In this work, the data sets obtained from the execution of the DoE planned trials, were treated to build a prediction model in order to describe the bread-making process. In particular, replicates values for DON concentration in each experiment were averaged and computed as initial dataset. Partial Least Square (PLS) based on three components was chosen as statistical regression treatment.

The statistical model efficacy was evaluated by means of two main parameters: fitting ( $R^2$ ) and prediction ( $Q^2$ ) values. In particular, “explained variation”  $R^2$  measures the proportion to which the statistical model accounts for the variation of a given dataset, while “predictive power”  $Q^2$  refers to its ability to generate testable predictions. The generated statistical model starting from the dataset gave a high fitting value ( $R^2 \gg 0.8$ ) and a correspondent good prediction value ( $Q^2 > 0.5$ ), referring to the MODDE software output settings.

Variable Importance Plot (VIP) explains consistently the importance of a variable within the measurements of a process\phenomena. It generates also information on the most important interactions and represents the most condensed way of expressing the PLS model output as a weighted summary of all loadings and across all responses.

This statistical algorithm elaboration applied to our bread-making DoE recorded dataset, indicates the different incidence, within the technological range, of the selected predictors on the possibility to control and influence the DON evolution during the industrial process: the baking phase seems to be particularly relevant with respect to the fermentation phase, as shown in Figure 2.

Although the most important variable indicated by the plot is the initial DON contamination of flour, this value can't be obviously regarded as a variable for our model, since it is already known at the beginning of the industrial process and can be out-line carefully controlled by raw material analysis.

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3 On the other hand, when only an acceptable technological range is considered, fermentation process  
4 cannot be changed in order to obtain a significant minimization of the DON level in final loaf, as  
5 deducible from the prediction model output.  
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8 Starting from this observation, a contour map can be obtained in order to outline the influence of the  
9 selected variables (baking rate and baking temperature) on the DON level in low-medium  
10 contaminated and in high DON-level containing flours, as reported in Figure 3a and 3b,  
11 respectively.  
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17 As shown by the contour maps, increasing the baking rate/temperature can determine a reduction of  
18 the free DON concentration in the final bread. The correspondence with the time/temperature  
19 domain that can really be applied in the industrial bread-making is illustrated in Figure 3: this is a  
20 crucial point, since many studies suggested that major DON decreases can be achieved by applying  
21 higher temperature and longer baking rate, but these conditions do not allow the production of  
22 acceptable bread.  
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29 In particular, temperature higher than 210°C may induce a significative decrease in DON  
30 concentration in bread, although this effect seemed to be remarkable only in case of medium-high  
31 contamination of the flour used as raw material. On the other hand, when low contaminated flour is  
32 used, negligible changes in DON level is predicted by the model.  
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### 38 **Bread-making pilot-plant study: evaluation of the DoE predictive power.**

39 Further pilot-plant bread-making trials were aimed to directly verify the predictive power showed  
40 by the statistical model by comparing the DON level predicted by the model and measured in final  
41 bread (obtained starting from a naturally contaminated flour) and applying the optimized bread-  
42 making conditions previously described. Since the minimization effect due to the baking stage  
43 seemed to be significative for highly contaminated flour, an initial DON level closed to the legal  
44 limit for raw material was chosen, namely  $717 \pm 32 \mu\text{g}/\text{kg}$ .  
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52 For the fermentation stage, the optimal processing parameters were set at 35°C for 65 min. The  
53 final moisture in bread required to reach a satisfying level was set at 35%. Baking step was  
54 performed at 215°C for 10 min: baking temperature was chosen above 210°C, as suggested by the  
55 statistical model, and the baking rate was chosen by monitoring bread browning and target  
56 moisture, in order to obtain an acceptable final product. The experiment was performed in triplicate  
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3 and each sample was analysed after the fermentation step and after the baking step, as previously  
4 reported.  
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8 Starting from set conditions (initial DON concentration, baking temperature and baking time), the  
9 statistical model returned a predicted final DON level in bread of 440 – 460  $\mu\text{g}/\text{kg}$  d.m. The DON  
10 concentration found in final bread and reported on dry matter was  $430\pm 25$   $\mu\text{g}/\text{kg}$ , showing thus a  
11 very good agreement with the DON predicted value.  
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### 15 16 17 **Industrial line trials: evaluation of the prediction model.**

18 The DoE model optimized for pilot plant was then tested also on the industrial line. As first, the  
19 predictor parameters range was adapted to the industrial plant requirements: for bread-making  
20 experiment a wheat flour containing  $333\pm 24$   $\mu\text{g}/\text{kg}$  of DON was chosen; also fermentation stage  
21 parameters were fixed (fermentation temperature:  $32^\circ\text{C}$ : fermentation rate: 56 min), in order to  
22 better investigate the influence of baking parameters on the DON concentration in final bread. In  
23 particular, the baking temperature was varied among  $170^\circ\text{C}$  and  $225^\circ\text{C}$  and baking time was varied  
24 among 0 and 13 min.  
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33 The comparison of the average DON levels in flour and in the final bread sample are reported in  
34 Figure 4.  
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38 The slight increase of deoxynivalenol concentration, related to the fermentation stage, during the  
39 processing already recorded in the pilot plant trials was confirmed also on the industrial line.  
40 Obviously, the industrial line experiments could be performed only using flour with a DON  
41 concentration significantly lower than the legally permitted limit, thus the data obtained on the pilot  
42 plant for high level of DON cannot be verified. Crumb temperature was checked carefully during  
43 the baking process and the collected values were related to the oven temperature, as reported in  
44 Figure 5.  
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52 As shown in Figure 5, the changes in DON concentration during the baking step is negligible: this  
53 can be explained taking into account the slight temperature changes recorded inside the loaf.  
54 Moreover, although the oven temperature was varied among  $170^\circ$  and  $225^\circ\text{C}$ , bread crumb  
55 experienced a maximum of  $86^\circ\text{C}$  at the final stage of the baking step: thus, relative low temperature  
56 inside the loaf combined with medium-low levels of the toxin in the starting flour may justify here  
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3 the lack of DON degradation or re-binding (reversing the process that happened during  
4 fermentation).  
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## 8 **Conclusions**

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10 In the present study, the influence exerted by bread-making parameters on the DON concentration  
11 in bread was investigated, starting from a naturally contaminated flour and using both pilot and  
12 industrial-scale equipments. The results were then used for the development of a statistical  
13 predictive model, which was finally tested on an industrial production line. Exploiting the power of  
14 a Design of Experiments (DoE) approach to consider the high complexity of the studied system, the  
15 obtained prediction model provided the following useful information: (i) the fermentation phase  
16 induce a DON increase, (ii) increasing time\Temperature in the baking phase, even staying in an  
17 acceptable technological range, could reduce the native DON content in bread only starting from  
18 high naturally contaminated flour.  
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28 The fermentation stage seemed to be a crucial step in the bread-making process, since a  
29 significative DON increase was recorded in fermented dough obtained on both pilot-scale and  
30 industrial-scale plants. This increase may be due to the enzymatic release of native DON from  
31 bound forms occurring in the raw material and will be further investigated in order to highlight the  
32 involved phenomena. On the other hand, a careful control of the time/temperature combination  
33 during the baking step may lead to a minimization of the DON level in the final bread, especially  
34 when the DON concentration in flour is closed to the EU legal limit.  
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42 In conclusion, this study represents a concrete example of how a planned sequence of production  
43 experiments combined with a reliable LC-MS/MS method, may generate potential strategies for  
44 minimizing consumer exposure to DON through appropriate management of the industrial cereals  
45 processing technologies.  
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## 51 **Acknowledgements**

52 Part of the present work has been performed within a project (SITEIA) partially financed by the  
53 Emilia-Romagna Region (Italy). The financial support from Spinner Consortium (Spinner 2013,  
54 Emilia-Romagna Region together with the European Social Fund and the Italian Ministry for  
55 Welfare) is also acknowledged. Finally, authors would like to thanks the following colleagues for  
56 time, help and fruitful discussions: C. Cauzzi, L. De Vito, A. Garulli, E. Grignafini, A. Leporati, U.  
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Table 1 – Bread-making conditions assumed during experiments  
in the pilot-scale plant (Levels: a,b,c) and in the industrial-scale plant.

Treatment	Bread-making conditions in pilot-scale plant			Bread-making conditions in industrial-scale plant	
	Fermentation temperature ( $T_f, ^\circ\text{C}$ )	30 (a)		40 (c)	32
Fermentation rate ( $t_f$ , min)	45 (a)	65 (b)	85 (c)	56	<i>Fixed value</i>
Baking temperature ( $T_b, ^\circ\text{C}$ )	180 (a)		210 (c)	170 (min)	225 (max)
Baking rate ( $t_b$ , min)	8 (a)		16 (c)	0 (min)	13 (max)

Table 2. Full factorial central composite design of experiments dataset and correspondent analytical results for screening variables effects on DON levels within the bread-making process steps  
- pilot scale plant -

Experiment Number	DON in flour ( $\mu\text{g}/\text{Kg d.m.}$ )	Fermentation stage			Baking stage		
		Rate (min)	Temperature ( $^{\circ}\text{C}$ )	DON in fermented dough ( $\mu\text{g}/\text{Kg d.m.}$ )	Rate (min)	Temperature ( $^{\circ}\text{C}$ )	DON in bread ( $\mu\text{g}/\text{Kg d.m.}$ )
1	160 $\pm$ 15	85	30	187 $\pm$ 11	8	180	211 $\pm$ 15
2	967 $\pm$ 49	45	30	851 $\pm$ 53	8	180	963 $\pm$ 40
3	160 $\pm$ 15	45	40	223 $\pm$ 23	8	180	216 $\pm$ 18
4	100 $\pm$ 14	65	40	152 $\pm$ 17	8	180	159 $\pm$ 14
5	967 $\pm$ 49	85	40	998 $\pm$ 40	8	180	931 $\pm$ 90
6	160 $\pm$ 15	65	30	186 $\pm$ 16	8	210	198 $\pm$ 31
7	100 $\pm$ 14	45	30	129 $\pm$ 26	8	210	145 $\pm$ 14
8	967 $\pm$ 49	85	30	927 $\pm$ 70	8	210	1004 $\pm$ 137
9	160 $\pm$ 15	85	40	221 $\pm$ 20	8	210	241 $\pm$ 23
10	967 $\pm$ 49	45	40	919 $\pm$ 33	8	210	960 $\pm$ 42
11	967 $\pm$ 49	65	40	820 $\pm$ 55	8	210	754 $\pm$ 57
12	160 $\pm$ 15	45	30	235 $\pm$ 22	16	180	243 $\pm$ 16
13	100 $\pm$ 14	85	30	167 $\pm$ 24	16	180	166 $\pm$ 15
14	967 $\pm$ 49	85	30	899 $\pm$ 62	16	180	1000 $\pm$ 121
15	160 $\pm$ 15	85	40	224 $\pm$ 31	16	180	246 $\pm$ 10
16	967 $\pm$ 49	45	40	875 $\pm$ 95	16	180	980 $\pm$ 111
17	160 $\pm$ 15	85	30	215 $\pm$ 20	16	210	227 $\pm$ 13
18	967 $\pm$ 49	45	30	940 $\pm$ 21	16	210	951 $\pm$ 69
19	160 $\pm$ 15	45	40	186 $\pm$ 14	16	210	208 $\pm$ 15
20	100 $\pm$ 14	65	40	167 $\pm$ 13	16	210	192 $\pm$ 22
21	967 $\pm$ 49	85	40	993 $\pm$ 47	16	210	981 $\pm$ 43
22	100 $\pm$ 14	65	40	128 $\pm$ 22	16	210	134 $\pm$ 12
23	100 $\pm$ 14	65	40	148 $\pm$ 12	16	210	159 $\pm$ 12
24	100 $\pm$ 14	65	40	136 $\pm$ 13	16	210	140 $\pm$ 13

Table 3. Design of experiments dataset and correspondent analytical results for screening variables effects on DON levels within the bread-making process steps  
- industrial scale plant -

Experiment Number	DON in flour (µg/Kg d.m.)	Fermentation stage		Baking stage		
		Rate (min)	Temperature (°C)	Rate (min)	Temperature (°C)	DON in bread (µg/Kg d.m.)
1	333 ± 24	56	32	0	30	275±13
2	333 ± 24	56	32	3	200	270±18
3	333 ± 24	56	32	3	225	302±19
4	333 ± 24	56	32	3	170	308±23
5	333 ± 24	56	32	6	200	299±18
6	333 ± 24	56	32	6	225	307±18
7	333 ± 24	56	32	6	170	318±17
8	333 ± 24	56	32	9	200	276±16
9	333 ± 24	56	32	9	225	287±18
10	333 ± 24	56	32	9	170	291±17
11	333 ± 24	56	32	11	200	274±10
12	333 ± 24	56	32	11	225	306±18
13	333 ± 24	56	32	11	170	313±17
14	333 ± 24	56	32	13	200	304±16
15	333 ± 24	56	32	13	225	314±18
16	333 ± 24	56	32	13	170	293±19
17	333 ± 24	56	32	9	225	303±15
18	333 ± 24	56	32	9	225	289±17
19	333 ± 24	56	32	9	225	312±22

**Table 4.** LC-MS/MS parameters for detection and quantification of DON and (<sup>13</sup>C<sub>15</sub>)-DON, adducts and internal standard in ESI-MRM mode.

Barilla Food Research Labs		<i>Retention time (min)</i>	<i>Parent ion m/z</i>	<i>Fragments monitored m/z</i>	<i>Normalized Collision Energy (%)</i>
		[DON+CH <sub>3</sub> COO] <sup>-</sup>	5.7	355	295, 265
	[( <sup>13</sup> C <sub>15</sub> )-DON +CH <sub>3</sub> COO] <sup>-</sup>	5.7	370	310, 290	24
Food Chemistry Labs University of Parma		<i>Retention time (min)</i>	<i>Parent ion m/z</i>	<i>Fragments monitored m/z</i>	<i>Collision Energy (eV)</i>
		[DON+CH <sub>3</sub> COO] <sup>-</sup>	6.4	355	295, 265, 59
	[( <sup>13</sup> C <sub>15</sub> )-DON +CH <sub>3</sub> COO] <sup>-</sup>	6.4	370	310, 279, 59	12, 15, 15

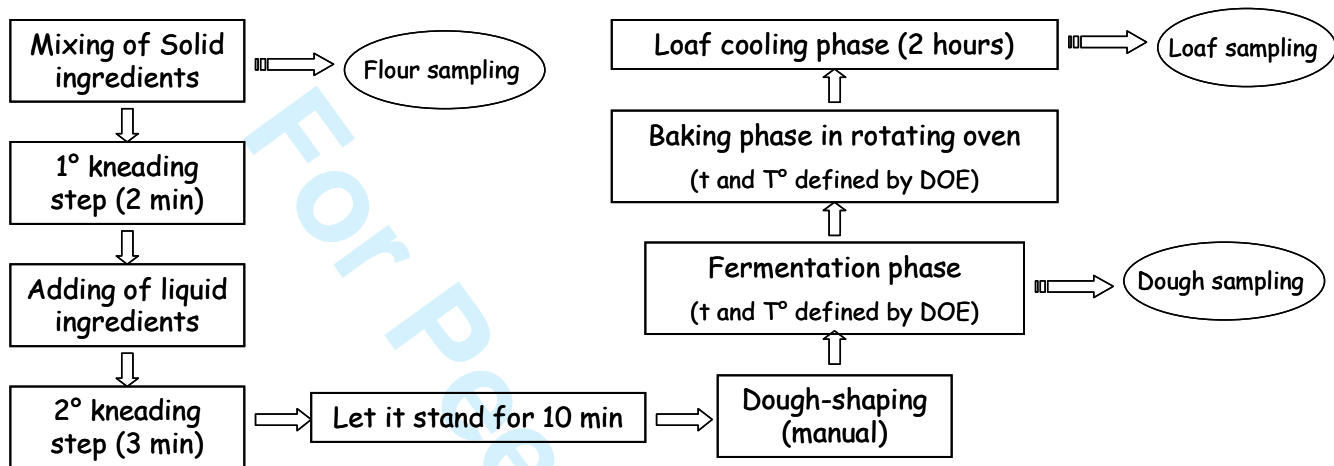
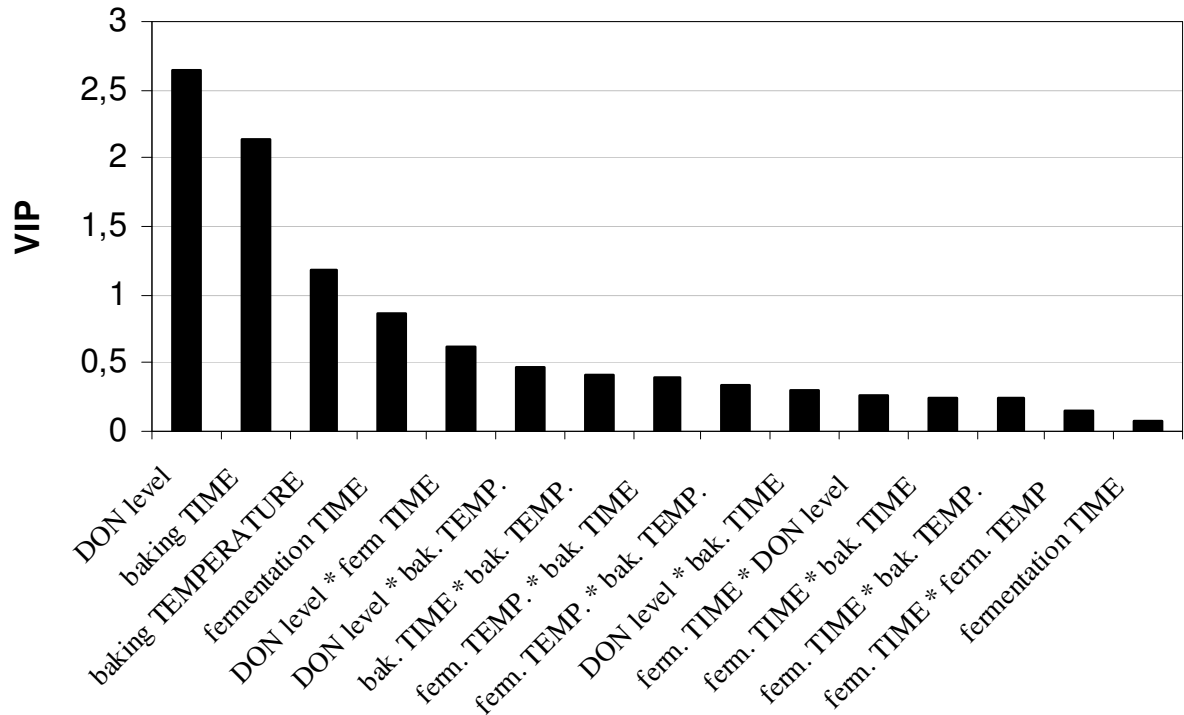


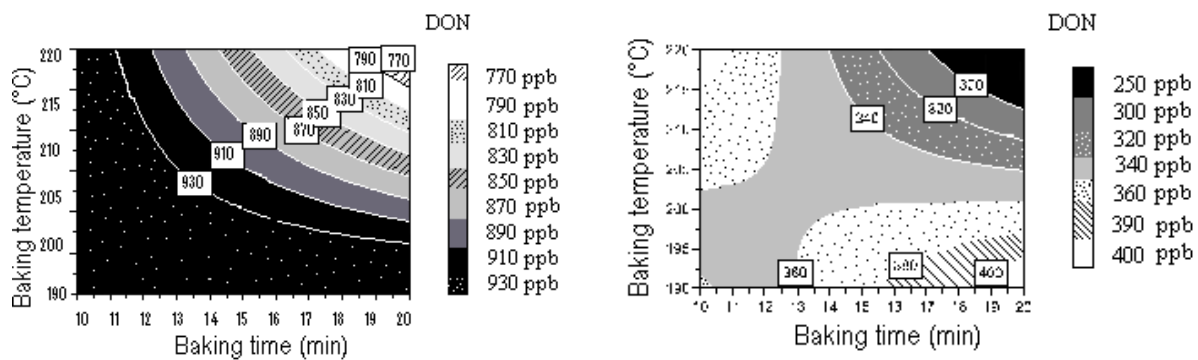
Figure 1: Scheme of the bread-making process.



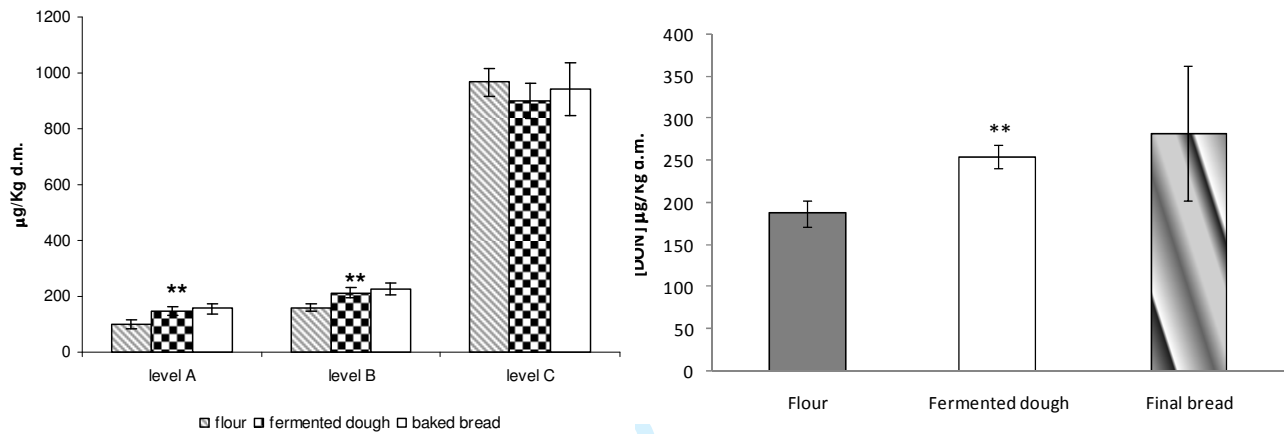


**Figure 2:** Variable Important Plot (VIP) obtained for the DoE dataset in pilot-scale bread-making experiments.

The labeling of the X axis reports abbreviations for the terms “baking” (bak), fermentation (ferm) and “Temperature” (Temp).

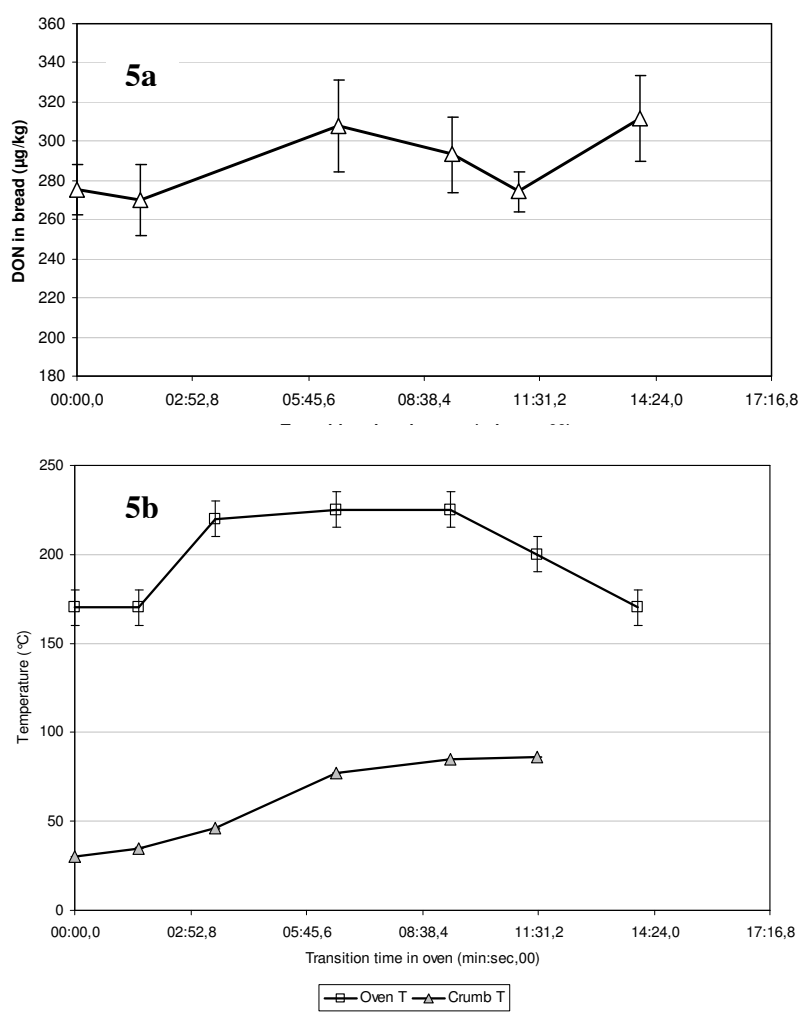


**Figure 3:** Contour maps obtained for pilot-scale bread-making experiments performed using a high level DON contaminated flour (a) or a medium-low DON contaminated flour (b).



**Figure 4:** Comparison of the average DON levels (standard deviation is represented by the error bars in the graph) found in the raw flour, in the fermented dough and in the final bread produced either in the pilot-scale (left side) or industrial-scale (right side) experiments: (\*\*) indicates a significant difference (Tukey test,  $\alpha = 0.05$ ,  $p < 0.01$ ).

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**Figure 5:** Changes in DON concentration during the industrial baking stage (a) and temperature variation recorded in the oven and in the loaf crumb during the baking process (b).