The Effect of Bread-making Process on Contents of Key Trichothecene Mycotoxins: Deoxynivalenol, T-2, and HT-2 Toxins

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Abstract

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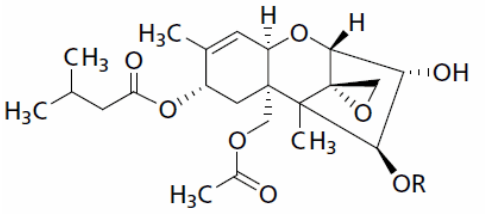
The fate of trichothecene mycotoxins deoxynivalenol (DON), T-2 and HT-2 toxins during the bread preparation and baking was investigated in order to obtain information about the influence of processing conditions on the levels of these toxins in final products. Highly artificially contaminated wheat was used to obtain flours with three contamination levels (DON 1615–398, T-2 toxin 927–160, and HT-2 toxin 258–38 µg/kg). Method for Test Baking of Wheat Flours (ICC Standard No.131) was used within the experiments for bread sample preparation. Various combinations of dough fermentation, proofing and baking times were used to prepare test breads. For determination of toxin levels in all tested matrices, HPLC–MS/MS method was employed. No substantial effect on DON levels was observed as a result of various conditions used for bread preparation and baking; maximum DON decrease 10%, compared to contaminated flour. On the other hand, significant changes in T-2/HT-2 toxin contents were found in experimental bread compared to contaminated flour; reduction of T-2 toxin concentration (30–50%) and increase of HT-2 toxin concentration (10–70%), respectively.

Keywords: HPLC–MS/MS; Fusarium toxins; baking

Agricultural crops, grown or stored under improper conditions, are often contaminated by filamentous fungi. Some strains of these fungi produce toxic secondary metabolites, typically low-molecular weight substances, referred to as mycotoxins. Among them, trichothecene mycotoxins produced by toxigenic strains of *Fusarium* genus are the most common natural contaminants in cereals and cereal-based foods. Thanks to their physico-chemical stability, these toxins can survive various processing practices, including heat treatment, thus they might be transferred into final products. Only occasionally, spontaneous outbreaks of Fusarium mycotoxicoses (i.e. acute poisoning cases) were recorded in Europe, Asia, New Zealand, and South America (FAO 1989; Bryden 2007), nevertheless, chronic exposure to trichothecene mycotoxins occurs on a regular and more widespread scale (Placinta 1999; Richard 2007).

The occurrence of trichothecenes in foodstuffs has been studied by many authors in recent decades. While DON, currently the only regulated toxin of this group (EC No. 1881/2006), has been involved in most studies, monitoring of T-2/HT-2 toxins (Figure 1) has been



T-2 toxin: R = Ac
HT-2 toxin: R = H

Figure 1. Structure of T-2/HT-2 toxins

Table 1. The reported occurrence of trichothecenes T-2/HT-2 in foodstuffs

Region	Commodity	Contamination level (mg/kg)/Incidence (%)	Reference
Turkey	cereals	0.5–3/23 (T-2)	Omurtag & Yazicioglu(2001)
Brazil	maize-based products	0.6–0.8/3 (DON, NIV, T-2, HT-2)	Milanez *et al.* (2006)
Germany	oat-based products	0.01–0.03/97 (T-2, HT-2)	Gottschalk *et al.* (2007)
Spain	cereal-based food	~ 0.2/10 (HT-2) ~ 0.3/1 (T-2)	Cano-Sancho *et al.* (2011)
Spain	barley	~ 0.02/24 (HT-2) ~ 0.04/10 (T-2)	Ibáñez-Vea *et al.* (2012)
Croatia	different grains (feed samples)	0.01–0.03/76 (HT-2) 0.002–0.03 (T-2)	Vulic *et al.* (2011)
Spain	bread pasta	0.04–0.07 (T-2); bread (3%) 0.03–0.3 (T-2); pasta (9%)	González-Osnaya *et al.* (2011)
South Korea	cereals	0.04–0.4/17 (T-2) 0.02–0.4/33 (HT-2)	Kassim *et al.* (2011)

initiated only recently, when the European Commission (EC) asked the European Food Safety Authority (EFSA) for a scientific opinion on the risk to human and animal health related to the presence of these trichothecenes A in food and feed. In Table 1, the outcome of the most extensive recent studies concerned with contamination of cereals and products thereof is summarised.

The conditions employed during foodstuff preparation, such as temperature, cooking time, water presence, and pH value, as well as the type of mycotoxin and its concentration in the food matrix can also play an important role for the reduction of mycotoxin content. The recent knowledge of mycotoxin fate in cereals and cereal-based products was summarised in a review by Pereira (2014). In Table 2, the overview of selected studies focused on the influence of single processing variables on trichothecene content is provided. As shown here, a certain reduction of these mycotoxins was observed in most studies, nevertheless, the observed changes varied to a large extent.

Currently, there is no clear evidence that the reduction of type A trichothecenes, such as T-2 and HT-2 toxins, can occur as a consequence of food processing. Existing data are very limited and rather controversial (Lancova *et al*. 2008; Monaci *et al.* 2011; De Angelis *et al.* 2013).

The objective of the present study was to contribute to the recent knowledge of type A trichothecene fate under baking conditions and evaluate the influence of individual processing steps on their concentration in the final product (bread). In addition to trichothecenes of group A, also DON levels in flour and bread were determined to enable a comparison with results of similar earlier studies.

MATERIAL AND METHODS

Experimental strain, growth conditions. In order to process the wheat flour highly contaminated with T-2 and HT-2 toxins, raw wheat kernels were artificially inoculated with *Fusarium* strain producing these toxins. *Fusarium tricinctum* toxicogenic strain obtained from the Institute for Biotechnology in Plant Production, University of Natural Resources and Life Sciences, Vienna (Austria), was used in our study. In line with literature data (Thrane *et al.* 2004) this strain was an efficient producer of T-2 and HT-2 toxins. Following inoculation on potato-dextrose agar, the fungus was incubated in dark at 25°C for one week.

Contamination of kernels. Potato-dextrose agar containing the mycelium and spores was resuspended in a small volume of distilled water. 10 ml of this suspension was applied onto 100 g of wheat kernels in Erlenmeyer flasks. Wheat kernels were disinfected prior to use by autoclaving in order to eliminate any possible natural microflora. The inoculated wheat kernels were incubated in dark at 25°C for two weeks. Erlenmeyer flasks were vigorously shaken every day to ensure aerobic conditions for fungal growth. After two-week cultivation, wheat kernels were dried in a forced draft oven at 40°C for 6 h and finely ground at an FQC 109 laboratory mill (Metefem, Budapest, Hungary). The content of T-2 and HT-2 toxins in artificially inoculated wheat flour was 1860 and 516 µg/kg, respectively.

Preparation of contaminated flour. To obtain flour contaminated with T-2/HT-2 toxins and DON, the inoculated flour was blended with flour that was naturally contaminated with DON at the

Table 2. Selected studies focused on effects of processing conditions on trichothecene content

Mycotoxin/food matrix	Food processing experiment	Aim/result of study	Reference
7 trichothecenes wheat grains	bread production (processing steps evaluated)	reduction of trichothecenes achieved by cleaning; no significant reduction occurred by bread-baking	Lancova *et al.* (2008)
DON (wheat flour)	bread-making process (Vienna, French bread)	DON reduction by 33–50%	Pacin *et al.* (2010)
DON (bread model)	bread-making parameters evaluated	concentration dependent DON changes occurred	Bergamini *et al.* (2010)
DON; T-2; HT-2 bread model food	bread processing evaluation (dough preparation, bread-baking)	dough preparation: T-2 hydrolysis to HT-2 bread-baking: 20–30% reduction of DON and HT-2	Monaci *et al.* (2011)
DON (Asian noodle)	yellow alkaline and instant noodles (cooking and frying evaluated)	DON reduction (43–66%) occurred by leaching of DON out of the noodles into a cooking medium	Farahany Moazami & Jinap (2011)
DON; DON-3-Glc bread model food	bread-making process (milling, baking)	DON/DON-3-Glc reduction (milling 40%; baking 10/13%)	Kostelanska *et al.* (2011)
DON; T-2; HT-2; glucoside derivatives (bread model food)	bread processing evaluation	DON increase (18%); T-2 reduction (63–74%); HT-2 reduction (18–25%); DON-3-Glc and HT-2-Glc reduction; T-2-Glc increase (glucoside derivatives were not quantified)	De Angelis *et al.* (2013)

concentration of 3260 µg/kg. The ratio of T-2/HT-2 and DON contaminated flours was 1 : 1. Finally, by adding a suitable amount of blank wheat flour, three trichothecene concentration levels were prepared, i.e. trichothecene concentration level one (Flour 1 – F1), level two (Flour 2 – F2), and level three (Flour 3 – F3). The contaminated flours prepared at three concentration levels were processed and analysed following the same procedures as described below (Table 3). Uninoculated wheat flour prepared and treated under identical conditions was used as a control.

Bread making. Method for Test Baking of Wheat Flours (ICC Standard No. 131:1980), performed in Agricultural Research Institute in Kroměříž (Czech Republic), was applied in the experiment. Different variants of dough preparation, i.e. various combinations of fermentation and proofing times, were used for bread making. For the preparation of loaves of experimental breads, 300 g of experimental flours F1/F2/F3 were taken and the following ingredients were added: malted flour, salt, yeast (*Saccharomyces cerevisiae*), sucrose, and ascorbic acid. Water was added according to results of farinograph instrument (Brabender, Duisburg, Germany). Baking was performed in an electric oven at 230°C, while two breads representing each variant were baked for 20 min; the third bread of the same variant was baked for 10 minutes. Test breads after baking were dried to constant weight in a forced draft oven at 40°C for 24 h and stored in a refrigerator until use. In total, 27 experimental breads were prepared in our study.

Extraction and clean-up procedure. Each dried test bread was finely milled and 12.5 g of the obtained material were extracted with 50 ml acetonitrile-water mixture (84 : 16, v/v) for 1 h using an automatic shaker (IKA Laboratortechnik, Staufen, Germany). Crude extracts were filtered then (Filtrak No.390; VEB Freiberger, Germany) and 8 ml aliquots transferred into sample tubes to which 80 µl of acetic acid (99%; Sigma-Aldrich, St. Louis, USA) were added. Purification was achieved by employing solid-phase extraction (SPE)

Table 3. Concentration levels of mycotoxins in contaminated experimental flours

Flour code	Contaminated flour (g)	Blank flour (g)	DON µg/kg)	T-2 (µg/kg)	HT-2 (µg/kg)
F1	1320	0	1615.2 ± 40.3	926.9 ± 88.6	258.0 ± 16.1
F2	440	880	578.0 ± 13.1	298.9 ± 21.0	82.5 ± 9.9
F3	220	1100	397.9 ± 49.2	160.3 ± 12.3	37.7 ± 7.1

MycoSep™ 226 cartridges (Romer, Tulln, Austria). A volume of 4 ml of purified extract was evaporated to dryness and residues were dissolved in 1 ml of a water-methanol mixture (80 : 20, v/v). The whole solution was passed through a 0.2 µm microfilter (Alltech, Vienna, USA) before further analysis.

Trichothecene analysis. High-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was used for the analysis of trichothecene mycotoxins in purified extracts.

Standards. Trichothecene standards (deoxynivalenol, HT-2 toxin, and T-2 toxin) were purchased from Sigma-Aldrich (Damstadt, Germany). Certified reference materials, DON in wheat flour (< 0.05 mg/kg, BCR 396; Geel, Belgium) and DON in naturally contaminated wheat (0.7 ± 0.1 mg/kg; R-Biopharm Rhone Ltd., Glasgow, UK), were used as controls to ensure the accuracy of measurements. The values obtained from DON determination were in the uncertainty range of the concentrations of reference materials used.

Chromatographic conditions. High-performance liquid chromatography (HP 1100 Binary Series LC system; Agilent Technologies, Santa Clara, USA) coupled with mass spectrometer (Finnigan LCQ Deca; Thermo Electron Corp., San Jose, USA) was used for the analysis of purified extracts. Chromatographic separation of sample components was carried out on a reverse-phase column with polar end-capping (Synergi Hydro RP, 150 × 3 mm × 4 µm; Phenomenex, Torrance, USA) heated at 40°C and operated under gradient conditions. The mobile phase was composed of 10mM ammonium acetate in purified water (A) and methanol (B). The flow rate of the mobile phase was set to 0.5 ml/min and the injection volume was 20 µl. Gradient elution was performed starting from A : B (80 : 20, v/v) and reaching A : B (30 : 70, v/v) in 8 minutes. From 8 to 15 min the ratio of A : B (30 : 70, v/v) was stable and then jumped to A : B (80 : 20, v/v). The time of post run lasted 7 minutes. Identification and quantification of analytes were performed using MS/MS with the following parameters: ion source type, APCI operated both in negative- and positive-ion modes; capillary temperature 150°C; temperature of vaporiser 450°C; nitrogen sheath gas flow 1.2 l/min; nitrogen auxiliary gas flow 3 l/min; source voltage 6 kV; collision gas helium; scan type selected reaction monitoring. APCI ionisation modes (±) and monitored fragments *m/z* (parent ion > daughter ion, confirmation ion) used for individual analytes were: DON^- (371 > 311, 281); $T\text{-}2^+$ (317 > 273, 299); $HT\text{-}2^+$ (442 > 425, 263).

Performance characteristics of analytical method. Limits of quantification were 5 µg/kg for DON and T-2 toxin, and 10 µg/kg for HT-2 toxin, respectively. Calibration curves for all analytes were linear within the working range from 5 µg/kg to 10 000 µg/kg Squared correlation coefficients (R^2) were in the range of 0.9991–0.9999 for all calibration curves. The analytical method used for sample examination was accredited (ISO 17025:1999) for cereals; as a part of external quality control the trueness of generated data was demonstrated through participation in the Food Analysis Performance Assessment Scheme (FAPAS) organised by the Central Science Laboratory (CSL, York, UK). The *z*-scores for all analyses (DON, HT-2, and T-2) were in the range of ± 2.

RESULTS AND DISCUSSION

In our experiments, three wheat flours containing DON, T-2 and HT-2 toxins at different concentration levels (Table 3) were used for dough preparation. Various processing conditions differing in fermentation and baking times were used for the production of experimental breads. The overview of results of mycotoxin analyses obtained for 27 experimental breads B1–B27 is summarised in Table 4, an example of chromatograms illustrating the used analytical method is shown in Figure 2.

Influence of fermentation and proofing. During dough fermentation, amylolytic enzymes contained in flour and malt flour cleavage starch producing small oligosaccharides that are consumed by yeasts during the process. In this set of experiments, we aimed to investigate whether such an enzymatic activity and accompanying pH value changes may influence concentration levels of target mycotoxins in final products. Fermentation of dough was performed using a temperature-controlled box (30 ± 1°C, relative humidity 85 ± 5%) for a defined time period, 30, 40, and 50 min, respectively. Thereafter, the loaves were formed and put into the temperature-controlled box (30 ± 1°C, relative humidity 85 ± 5 %) for a proofing time of 30 min to allow yeast to continue in gas production causing the continual dough rising.

A certain reduction of DON and T-2 toxin concentrations was observed as a result of the applied times of fermentation and proofing (in all experiments the baking time was 20 min). No significant differences in DON reduction were found between breads prepared from flour with contamination levels 1, 2, and 3; an example of obtained results is in Figure 3. Regardless of the fermentation time (30, 40, and 50 min), the content of DON in experimental breads was in the range of 82–96% of the original one (100% = content

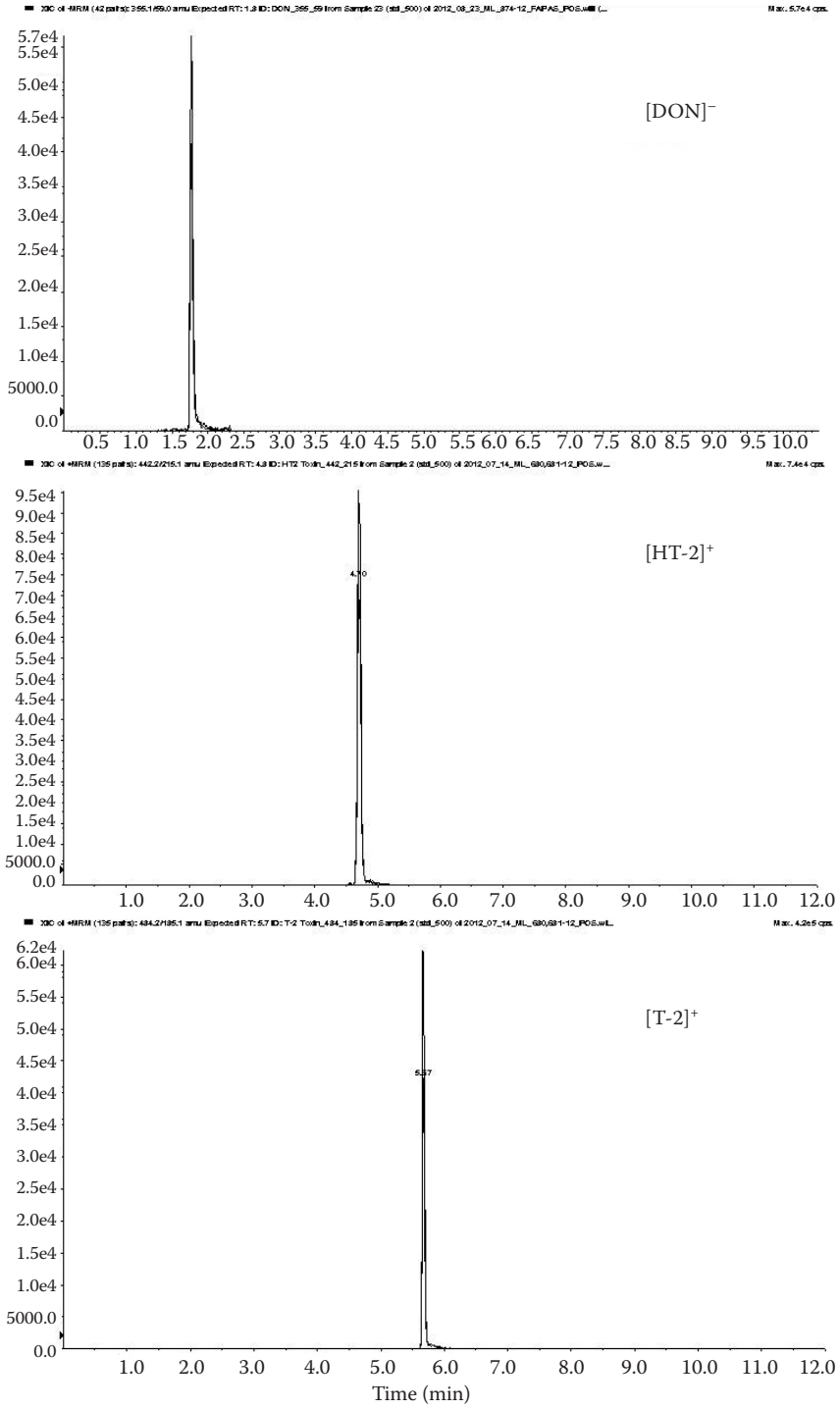


Figure 2. Overlay of chromatograms of DON, T-2, and HT-2 toxins obtained by the used LC-MS

in flour taken for the processing of a particular loaf). As reported by several authors, DON content in final bread samples depends on the conditions applied during dough preparation and bread baking. While some authors reported a DON decrease (Pacin *et al.* 2010; Monaci *et al.* 2011), others documented a moderate reduction (Kostalenska *et al.* 2011), dependence on DON concentration (Bergamini *et al.* 2010) or even an increase in DON content in final products (De Angelis *et al.* 2013).

A more significant reduction, however, was observed in the case of T-2 toxin. The mean toxin decreases in breads prepared from Flours 1, 2, and 3, were 54, 63, and 64%, respectively. No relationship with fermentation times was observed. Contrary to T-2, concentrations of HT-2 toxin in experimental breads were higher than in the original flour used for dough preparation. The mean HT-2 increase in breads from Flours 1, 2, and 3 was 18, 34, and 48% for the fermentation time of 30, 40, and 50 min, re-

Table 4. Concentration of monitored mycotoxins in experimental breads (baking times 10 and 20 min); percentage decrease/increase of the particular toxin is indicated in parentheses.

Bread code	DON	T-2 (μg/kg)	HT-2	Bread code	DON	T-2 (μg/kg)	HT-2	Bread code	DON	T-2 (μg/kg)	HT-2
B1	1496 (93)	423 (46)	318 (123)	B10	478 (83)	95 (32)	134 (161)	B19	375 (94)	57 (35)	62 (162)
B2	1510 (93)	422 (45)	316 (122)	B11	483 (84)	108 (36)	121 (146)	B20	348 (87)	51 (32)	65 (171)
B3*	1420 (88)	439 (47)	307 (119)	B12*	497 (86)	131 (44)	101 (122)	B21*	354 (89)	50 (31)	51 (134)
B4	1565 (97)	438 (47)	278 (108)	B13	499 (86)	116 (39)	106 (127)	B22	352 (88)	72 (45)	44 (115)
B5	1509 (93)	468 (50)	322 (125)	B14	512 (89)	129 (43)	98 (118)	B23	376 (95)	60 (37)	52 (137)
B6*	1434 (89)	434 (47)	322 (125)	B15*	507 (88)	115 (39)	136 (164)	B24*	389 (98)	52 (33)	55 (146)
B7	1510 (93)	468 (50)	278 (108)	B16	465 (81)	122 (41)	113 (136)	B25	383 (96)	56 (35)	55 (143)
B8	1434 (89)	393 (42)	322 (125)	B17	497 (86)	98 (33)	96 (115)	B26	311 (78)	47 (30)	60 (159)
B9*	1565 (97)	448 (48)	331 (128)	B18*	512 (89)	98 (33)	119 (144)	B27*	376 (94)	62 (39)	62 (164)

*baking time of experimental breads 10 minutes

spectively. The observed decrease of T-2 and, at the same time, increase of HT-2 toxin contents during bread preparation are supported by findings of partial conversion of T-2 to HT-2 due to selective deacetylation at the C-4 position of T-2 toxin catalysed by cereal carboxylesterases (Lattanzio *et al*. 2009).

Influence of baking time. During baking, some thermodegradation of mycotoxins may occur. To learn more about possible changes, not only finished breads baked for 20 min but also the loaf after 10 min of baking were analysed. Insignificant reduction of DON concentration in experimental breads was observed as a result of different baking time. In the case of Flour 1, the average DON reduction was 10% for 10 min baked breads and 8% for 20 min baked breads.

As regards T-2 toxin, its significant decrease occurred in the first phase of baking, after 10 min only 55% of the original content in flour was found; only a slow decrease of target toxin was observed in the next 10 min – 53% on average was left in bread baked for 20 minutes. These findings confirm the thermolabile behaviour of T-2 toxin as previously reported by De Angelis *et al*. (2013), who concluded a partial degradation of T-2 toxin as a consequence of thermal treatment.

An opposite trend was observed for HT-2 toxin: after 10 min of baking the content of toxin increased by approximately 24%, then a small decrease occurred with the final value of 118% for bread baked 20 min (compared to the original content in Flour 1 used for processing). An example of the influence of baking time on trichothecene concentration levels in

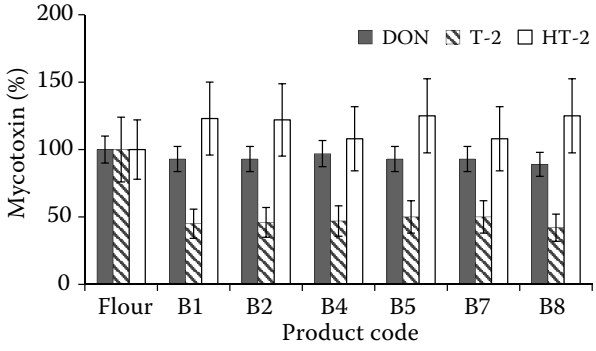


Figure 3. Influence of different fermentation time on trichothecenes concentration in experimental breads. Fermentation time 30 min (breads B1, B2); 40 min (breads B4, B5) and 50 min (breads B7, B8) (trichothecene concentration level 1 in original flour used)

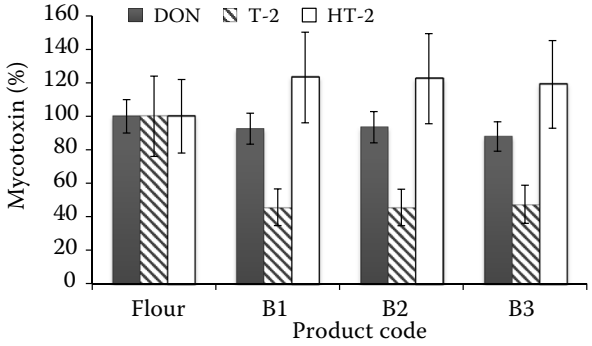


Figure 4. Influence of baking time on trichothecenes concentration in experimental breads baked for 20 min (B1, B2) and 10 min (B3), comparison with trichothecenes concentration in original flour (trichothecenes concentration level 1 in original flour used; all breads fermented for 30 min)

experimental breads for trichothecene concentration level 1 is shown in Figure 4.

Similar results were obtained for flour with trichothecene concentration level 2. The average DON reduction was 13% for 10 min baked breads and 16% for 20 min baked breads. Regarding T-2 toxin, its decrease as a result of 10 min baking was 61% and for 20 min 63%. HT-2 toxin concentrations, similarly like above, increased by 43% for 10 min and then slightly dropped to 134% for 20 min baked breads.

In principle, similar trends were confirmed in experimental bread baking for which flour with trichothecene concentration level 3 was used. DON decrease was 6% in 10 min and 11% in 20 min baked breads. T-2 toxin concentration decreased by 66% in 10 min and by 64% in 20 min baked breads, HT-2 toxin concentration increased again by 48% for both, 10 min and 20 min baked breads.

It is worth noticing that good compliance with trends reported in similar studies for DON changes during baking was obtained (e.g. Lancova *et al*. 2008; Berthiller *et al*. 2005; Kostelanska *et al*. 2011). On the other hand, in the recent study opposite results were reported by De Angelis *et al*. (2013), who observed a DON increase in the baked bread while HT-2 decreased. The only agreement between ours and this study was in a decrease of T-2 as the result of baking.

CONCLUSIONS

The results of this study concerned with the investigation of impacts of various conditions employed during dough preparation and bread baking on major trichothecenes contained in experimental wheat flour can be summarized as follows:

– Neither different dough preparation scheme nor different baking times used for bread making showed a substantial effect on DON levels in final products (maximum decrease was 10%). In other words, no reduction of dietary exposure to DON can be achieved supposing contaminated flour is used for household/industrial baking.
– Compared to DON, the stability of T-2 toxin under the same baking conditions was rather lower; depending on the time of dough fermentation and baking time, the decrease of this most toxic trichothecene A was in the range of 30–50% compared to the original amount. In this context, some detoxication occurred.
– At the same time, the content of another major trichothecene A, HT-2 toxin, contained in experimental flours, increased significantly (even by 60%) both during dough preparation and subsequent baking, the changes were process-dependent. It can be assumed that transformation (deacetylation) of T-2 into a related toxin occurred. Unfortunately, the direct proof of this conclusion was beyond the scope of the present study.

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