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Intersection of mycotoxins from grains to finished baking

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ABSTRACT

This work is focused on the evaluation of the content of deoxynivalenol and zearalenone in samples of winter wheat of the following varieties: Sultan, Cubus, Akteur, Seladon, Mulan, Chevalier, Evina, Hewitt, Bohemia, Baletka. The total amount of 10 samples harvested in 2011 and 2012 was evaluated and included variants both treated and untreated against fungal diseases. The samples were adjusted for mycotoxicological determination and subsequently measured by the ELISA method. The content of deoxynivalenol (DON) and zearalenone (ZEA) was measured in grain, flour and breadrolls in all samples. Out of all samples 43% were found to have positive content of DON and 75% of ZEA. In the treated variants, the average DON content was found to be 115 $\mu g.kg^{-1}$ in grain, 77 $\mu g.kg^{-1}$ in flour and 97 $\mu g.kg^{-1}$ in pastries. In the untreated variants, the average DON content was found to be 208 $\mu g.kg^{-1}$ in grain, 103 $\mu g.kg^{-1}$ in flour and 128 $\mu g.kg^{-1}$ in pastries. Moreover, the average ZEA content in the treated variant was 4.95 $\mu g.kg^{-1}$ in grain, 3.38 $\mu g.kg^{-1}$ in flour and 4.51 $\mu g.kg^{-1}$ in pastries, in the non-treated variant average ZEA content in grain was 3.07 $\mu g.kg^{-1}$, 4.97 $\mu g.kg^{-1}$ in flour and 2.81 $\mu g.kg^{-1}$ in pastries. The maximal acceptable limits given by the valid legislation were not exceeded in any analysed sample. It can be concluded wheat grain grown in the Czech Republic, whether it is treated or untreated by fungicides, is not dangerous for consumers. The content of both mycotoxins is not dependent on each other, and the untreated variant has a slightly higher dependency between DON and ZEA.

Keywords: mycotoxins; deoxynivalenol; zearalenone; grain; flour; finished product

INTRODUCTION

Microscopic fibrous micromycetes (moulds) create an important part of all organisms, especially in relation to humans and animals. They can cause skin, mucosal and internal organ diseases, collectively they are called mycoses **(Cempírková et al., 1997)**. Mycotoxicosis occurs after consumption, inhalation or after contact with toxic secondary metabolites of microscopic fungi. In favourable conditions, microscopis fungi may contaminate and destroy huge quantity of stock, food and feed by its adverse actions **(Tančinova et al., 2012)**. Every type of micromycet has different requirements for environment conditions, in which it grows, and this is also valid for types of the same species **(Diekman and Green, 1992)**. We can divide them into external factors (temperature, relative humidity, oxygen, period of stock storage), and internal factors (water activity, pH, texture, composition of stock, antimicrobial substances in stock) **(Tančinová et al., 2001, Mašková et al., 2012)**.

Danger of majority of mycotoxins lies rather in chronical effect of slight amounts than in actual toxicity **(Patočka et al., 2004)**. Deoxynivalenol (DON) is probably the most known and the most common mycotoxin contaminating feed and food made of grain crops. DON was for the first time isolated from corn infested by mould *Fusarium graminearum* in 1973. Further it is produced by toxicogenic species of *Fusarium culmorum*, *F. graminearum*, *F. sporotrichioidesaF. poae.* It occurs anywhere in the world where grains are grown. It has been found in a set of other food, e.g. in baby food made of grains, in corn, rice, millet, bran, ginger, garlic, beer, muesli, spaghetti and soybeans **(Malíř and Ostrý, 2003)**.

Zearalenone (ZEA, also F-2 toxin) was for the first time isolated in 1964 from the culture *Gibberellazeae* (anamorph of *F. graminearum*) of corn. It is produced by the genus *Fusarium*, especially *Fusarium graminearum*, *F. culmorum*, *F. equiseti*, *F. moniliformea* and *F. semitectum* belong to the most significant producers. The ideal temperatures for production of zearalenone are in the range of 12 – 14 °C, but also lower than 10 °C. *F. graminearum* is able to produce zearalenone even in the concentration 1900 µg/kg **(Magan and Olsen, 2004)**. First indirect evidence about masked mycotoxins appeared already in the first half of 80s in the 20th century. In animals, there were largely observed symptoms typical for mycotoxicosis in spite of the quantity of mycotoxins set in feed did not correspond with that. High toxicity of feed was apparently caused by conjugated forms of mycotoxins, which leaked from analytical determination **(Berthiller et al., 2013)**. While observing dynamics of mycotoxins in intentionally infected wheat, the increase of deoxynivalenol and subsequently its decrease was proved, which was probably caused by transformation of initial deoxynivalenol into its metabolites **(Hajšlová et al., 2009)**. Studies dealing with transformations of mycotoxins proved that conjugated forms of mycotoxins apparently occur during detoxification processes of grain crops. Until today, metabolites of deoxynivalenol, zearalenone, ochratoxin A and T-2 toxin have been identified **(Berthiller et al., 2013)**. Currently, only deoxynivalenol and zearalenone are observed as markers of grain contamination caused by fusarium mycotoxins. However, many studies proved that in a majority of cases, trichotecenenivalenol, T-2 toxin or HT-2 toxin are dominant. However, if deoxynivalenol is the only one analytically observed representative, the severity of contamination can be underestimated **(Nedělník et al., 2005)**. Commission Regulation (ES) No. 1126/2007 from 28th September 2007 determines maximum limits for deoxynivalenol, zearalenone (cereals - 100, flour - 75 and breadrolls - 50 µg per 1 kg) and tolerable daily intakes of these substances to 1 kg of body weight.

The objective of work was to compare and evaluate changes in the content of mycotoxins DON and ZEA in wheat grain, flour and breadrolls.

MATERIAL AND METHODOLOGY

Samples of winter wheat Sultan, Cubus, Akteur, Seladon, Mulan, Chevalier, Evina, Hewitt, Bohemia, Baletka were used for measuring, while they were obtained from experiments of the Central Controlling and Testing Institute for Agriculture, from its research station Hradec nad Svitavou. Grain was harvested from standardly treated crops (seed disinfected against bunt, dwarf bunt, morph regulator was used during vegetation, fungicide was applied against illnesses of haulm heels and against leaf and spikelet illnesses: T1 – treatment end of bracketing, T2 – beginning of heading until flowering). The test stations were located in the altitude 450 m, where long-term average temperature is 7.4 °C, long-term average rainfall is 616 mm. The soil type was typical brown soil and the soil class clay-loam (heavy soil). Content of DON and ZEA was observed in grain samples (especially whole grain groats, flour and breadrolls made of it were analysed).

Milled flour was let to ripen for a month. Breadrolls were made from the flour by the RMT method adjusted to laboratory conditions of MENDELU Brno. For quantitative evaluation of content of both mycotoxins, ELISA kits Veratox from the company Neogen were used. Kits contain plates with microtiter wells with bound antibody.

Results were evaluated through Statistika 8, the variable projection method into a factorial plane and pair test.

RESULTS AND DISCUSSION

All tested samples of wheat were positive for content of DON in case of the treated as well as non-treated variant. Measured values of DON were quite low in spite of the year 2013 was characteristic by considerable rainfall in the period of fungicide application and its efficiency did not have to be absolute. None of measured values exceeded the legislative limit. Concerning cereal supplementary food for infants and little children, the limit (200 $\mu g.kg^{-1}$) was exceeded in 5 samples of the non-treated variant. In the treated variant, DON in flour decreased on average by 33.04% in comparison to grain, in pastries it increased by 25.97% in comparison to flour, and in pastries it decreased by 15.65% in comparison to grain. In case of the non-treated variant, content of DON in flour in comparison to grain on average decreased by 50.48%, in pastries in comparison to grain it decreased by 38.46%. Decrease of values in both variants of flour in comparison to grain can be explained by low penetration of mycotoxins into endosperm, thus higher content was concentrated in outer covering layers, which were removed during milling. Increase of DON values in breadrolls in both variants can be explained by release of masked forms of mycotoxins (derivatives and conjugates of DON) and thus increase of their free forms, what was confirmed also by **Malachová et al., (2010)**. In addition to this, DON is relatively thermally stable, and therefore there are minimal losses during baking. Thus, passing of DON into finished products was confirmed. Moreover, yeast is added into recipe, enzymes of which are able to transform some of precursors of mycotoxins contained in flour into mycotoxins and thus to increase their quantity in pastries, what was also proved by **Young et al., (1984)**, that during fermentation processing of flour, the level of DON increased almost by 100%.

According to **Horáková (2013)** Cubus, Hewitt and Seladon belong to varieties very prone to content of DON, which can be also confirmed by measured results. The lowest content of DON was in the variety Akteur. Resistant varieties have not been bred yet.

In the Figure 1 and 2 we can see excessive quantity of DON and ZEA in both observed years.

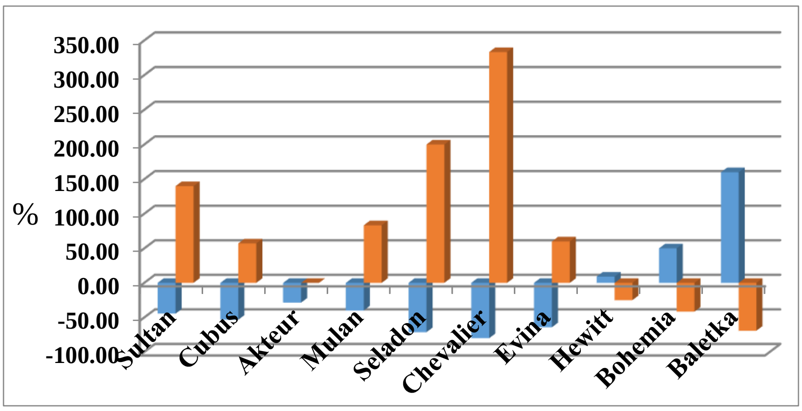

Figure 1 Change of DONcontent after milling and baking in the treated variant in %.

Note: blue-changing content DON grain / flour, red- changing content DON flour/pastries.

By comparison of the treated and non-treated variant, measured values of DON were clearly higher in the non-treated variant, on average by 80.87 % for grain, 33.77 % for flour and 31.96 % for pastries. Thus, we can say that positive effect of fungicide treatment was proved.
Statistical evaluation of measured values of DON is stated in the Table 1, where the two sided T-test for files of the same size was used. Decrease of DON in flour in comparison to grain seems to be statistically significant in case of the treated as well as the non-treated variant and further difference of DON quantity in grain in the treated in comparison to the non-treated variant. Many studies state that DON is thermally stable even during baking. **Scott et al., (1983)** and also **Lancová et al., (2007)** state that baking did not have any influence on DON content.

Zhang and Wang (2015) found**,** that concentrations of DON were verifiably higher in baked bread than in flour. On the contrary, according to **Young et al (1984)** baking has verifiable influence on decrease of DON concentration in bread, on average by 17 to 33% in comparison to the dough. **Boyacioglu et al., (1993)** state that content of DON decreased by 7% after baking bread, however the content of DON in bread with L-cystein as an additive in flour before baking decreased by 38 to 46 %. **Lešnik et al., (2008)** in his study baked 6 types of bread from 3 types of differently milled flour and in two different ovens (industrially used and classic ceramic). Average decrease of DON concentration in bread was 47. 2% in industrial ovens and 48.7% in ceramic ovens. The flour used was strongly contaminated by DON (average concentrations were 850–950 μg.kg^{-1}), however, breads after baking reached under 500 μg.kg^{-1}, and thus they met legislative limits. We can summarize that during technological processing, there occurs reduction of DON, but it is not completely removed from a finished product. **Hajšlová et al., (2008)** confirm that the most of waste fractions and bran tend to be the most contaminated and the lowest levels are in scouring flour. Further they stated that with increased time of dough ripening, there occurs increase of free DON and at the same time decrease of the conjugated form of D3G and that with increasing baking time, DON content decreases on average by 32% in comparison to shorter baking time.

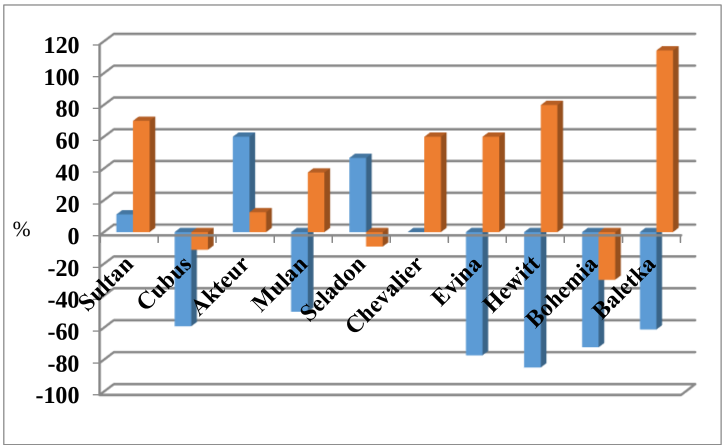

Figure 2 Change of DON content after milling and baking in the non-treated variant in %.

Note: blue-changing content DON grain / flour, red- changing content DON flour/pastries.

Table 1 Statistical significance (*P* <0.05) decrease/increase of DON values and comparison of the treated and the non-treated variant.

		p	Statistically significant difference
DON treated	grain / flour	0,028	+
	flour / pastries	0,188	-
	grain/ pastries	0,213	-
DON untreated	grain / flour	0,029	+
	flour / pastries	0,282	-
	grain/ pastries	0,079	-
DON treated/ untreated	grain T/ flour U	0,04	+
	flourT/ pastries U	0,232	-
	grain T/ pastries U	0,08	-

Note: *T-treated, U-untreated.

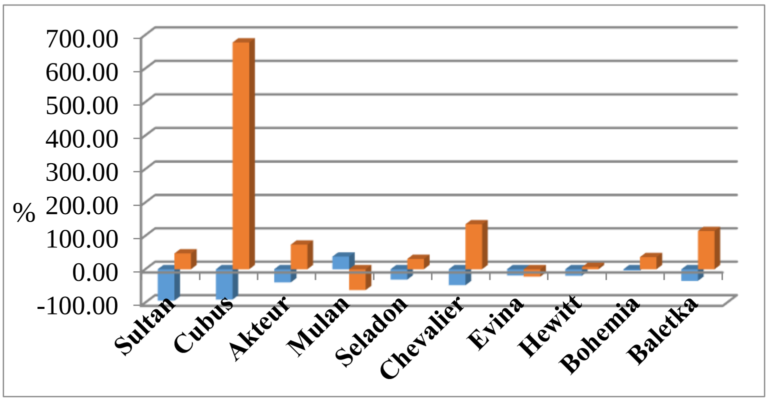

Figure 3 Change of ZEA content after milling and baking in the treated variant in %.

Note: blue-changing content ZEA grain / flour, red- changing content ZEA flour/pastries.

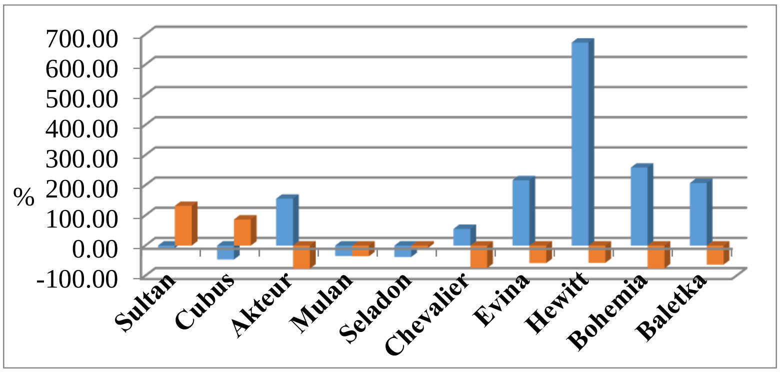

Figure 4 Change of ZEA content after milling and baking in the non-treated variant.

Note: blue-changing content ZEA grain / flour, red-changing content ZEA flour/pastries.

In the Figure 3 and 4 there are illustrated differences in the treated and non-treated variants and changes in ZEA content after milling and baking and in a finished product in comparison to grain.

All measured samples of wheat were positive for content of ZEA. None of the measured values of the treated and non-treated variant exceeded legislative limit. In total, values were very low and at so low level of contamination it is not possible to make clear conclusions.

When comparing the treated and the non-treated variant, higher ZEA values were measured in the treated variant, on average by 62.00% for grain and 62.23% for pastries. In flour, higher values were in the non-treated variant, on average by 46.85 %. Thus, the ZEA fungicide treatment did not have positive effect. This fact can also be caused by high rainfall at the time of fungicide application and its

Table 2 Evaluation of statistical significance (p <0.05) decrease/increase of ZEA values and comparison of the treated and non-treated variant

		p-value	Statistically significant difference
ZEA treated	grain / flour	0,07	-
	flour / pastries	0,273	-
	grain/ pastries	0,64	-
ZEA untreated	grain / flour	0,032	+
	flour / pastries	0,012	+
	grain/ pastries	0,713	-
ZEA treated/untreated	grain T/ flour U	0,019	+
	flour T/ pastries U	0,09	-

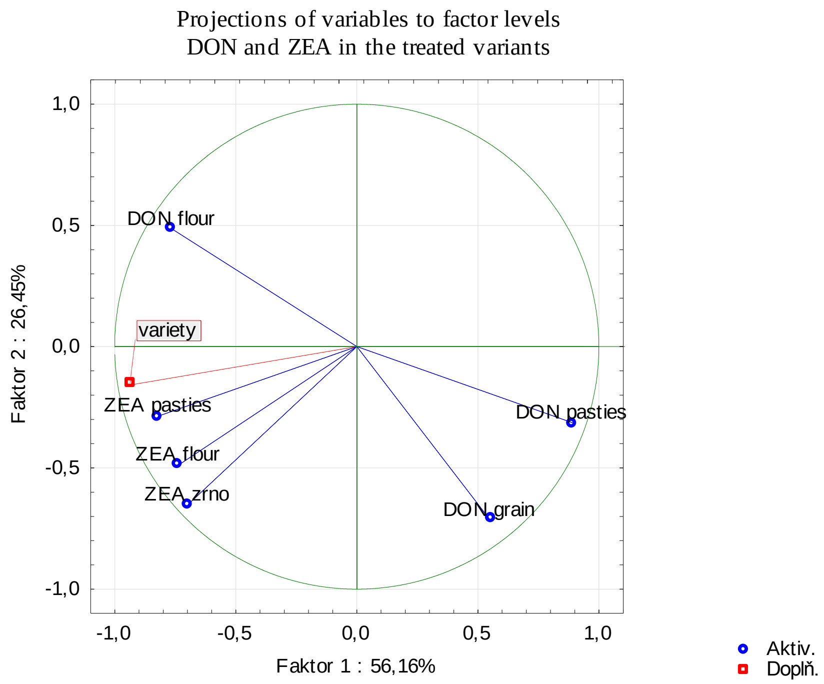

Figure 5 Projection of variables into factor plane – DON and ZEA in the treated variant.

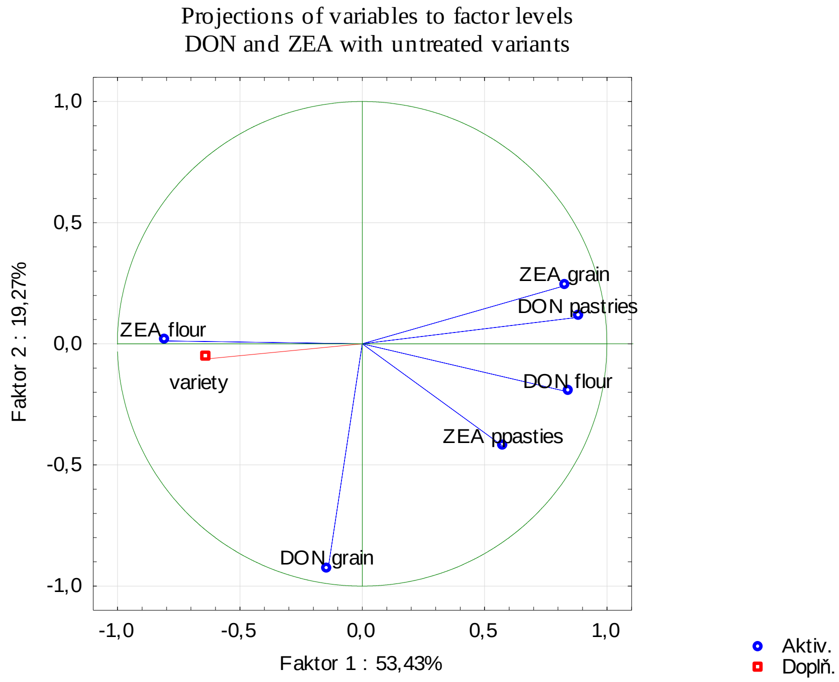

Figure 6 Projection of variables into factor plane – DON and ZEA in the non-treated variant.

efficiency thus did not have to be absolute. According to **Nedělník et al., (2005)**, it commonly happens that after fungicide treatment (especially of strobilurins) the increase of mycotoxins occurs because the stress factor affects fungus and it tries to save itself before extinction and thus it produces even higher quantity of mycotoxins, which is also confirmed by **Šafránková et al., (2010)** and **Malachová et al., (2010)**.

In the treated variant, ZEA in flour in comparison to grain decreased on average by 31.63%, in pastries in comparison to flour increased by 33.34% and in pastries in comparison to grain it decreased by 8.83%. In the non-treated variant ZEA content in flour in comparison to grain increased on average by 61.93% and in pastries in comparison to flour it decreased by 43.50% and in pastries in comparison to grain it decreased by 8.51%.

Decrease of values in flour in comparison to grain in the treated version can be explained by low penetration of mycotoxin into endosperm, and thus higher content was concentrated in outer covering layers, which were removed during milling. On contrary, in the non-treated variant, there is clear increase in flour in comparison to grain, which can be explained by opposite situation that penetration was high thus mycotoxins were concentrated in grain endosperm. Increase of ZEA values in pastries in the treated variant can be, similarly as for DON, explained by release of masked forms of mycotoxins. ZEA is also relatively thermally stable and losses in baking are small.

Statistical evaluation of measured ZEA values is stated in the Table 2. The two sided T-test was used for files of the same size. Increase of ZEA in flour in comparison to grain and decrease of ZEA in pastries in comparison to flour in the non-treated variant appears to be statistically significant. Furthermore, difference between ZEA quantity in grain of the treated version and non-treated version is statistically significant

Figure 5 and 6 are outcomes of processing of measured data in the program Statistica 8 and they illustrate projection of variables into the factor plane. There is the level of dependence of individual variables evaluated according to the angle size that they enclose. The smaller the angle, the stronger the dependence. At the same time, the angle cosine determines approximate value of correlation coefficient. If arrows are horizontally in the same direction, it means that variables are positively dependent on each other. If arrows are horizontal but in opposite direction, it means that variables are negatively dependent on each other. If arrows are perpendicular on each other, variables can be considered as independent. Length of an arrow indicates variability of measured data.

At the Figure 5 we can see that in the treated variant, species influence ZEA content in all products and at the same time, there is strong correlation dependency. DON content in individual products is independent on each other. DON and ZEA content are not dependent on each other. The highest variability of measured values was recorded in ZEA content in grain (the longest arrow).

In the Figure 6 in the non-treated variant, we can see strong dependency of species on ZEA content in flour. DON content (breadrolls, flour) and ZEA (grain, breadrolls) area closely related, especially ZEA content in grain and in breadrolls and DON in breadrolls and flour. The highest variability of measured values was also recorded in DON content in grain.

CONCLUSION

Average DON content in the treated variant was 115 µg.kg^{-1} in grain, 77 µg.kg^{-1} in flour and 97 µg.kg^{-1} in pastries, in the non-treated variant average DON content in grain was 208 µg.kg^{-1}, 103 µg.kg^{-1} in flour and 128 µg.kg^{-1} in pastries. Average ZEA content in the treated version was 4.95 µg.kg^{-1} in grain, 3.38 µg.kg^{-1} in flour and 4.51 µg.kg^{-1} in pastries, in the non-treated version average ZEA content in grain was 3.07 µg.kg^{-1}, 4.97 µg.kg^{-1} in flour and 2.81 µg.kg^{-1} in pastries. Measured ZEA contents are on a very low level of contamination. The maximal acceptable limits given by the valid legislation were not exceeded in any analysed sample. It implies that grain grown in the Czech Republic, whether it is treated or non-treated by fungicides, is not dangerous for consumers.

Content of both mycotoxins is not dependent on each other, in the non-treated variant there is slightly higher dependency of DON and ZEA. Species that have the highest influence on ZEA content, did not have any influence on DON content. In the treated variant ZEA contents in grain, flour and breadrolls contents closely related.

In the treated variant, DON content was decreased by milling on average by 33.04% in comparison to grain and on the other hand, its content in breadrolls was increased by 25.97%. In the non-treated variant, DON content decreased by 50.48% in comparison to grain and by baking, it again increased in comparison to flour by 24.27%. ZEA content in the treated version in flour decreased on average by 31.63% in comparison to grain, in pastries in comparison to flour, it increased by 33.34%. On the other hand, in the non-treated version, ZEA content was increased through milling by 61.93% in comparison to grain and during baking, its content decreased by 43.50% in comparison to flour. Decrease of mycotoxin content after milling means that mycotoxins were concentrated more in outer covering layers and less in endosperm. Covering layers are removed during milling and thus mycotoxin levels are decreased in flour. On the contrary, in the non-treated variant ZEA was more concentrated in endosperm and thus there was its increase in flour. Subsequently, increase of mycotoxins after baking in pastries can be explained by release of masked forms of mycotoxins or by activity of yeast during fermentation, what was confirmed by number of studies stated above. Both mycotoxins are quite thermally stable and thus they are not degraded during baking too much.

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