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# Transformation of ochratoxin A during bread-making processes

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ABSTRACT

Ochratoxin A (OTA) – secondary metabolite of fungi from genera *Aspergillus* and *Penicillium* may mainly contaminate cereal-based food. Thermal processing (including baking) may reduce the levels of OTA, but may also produce racemic mixtures with the 2′R-ochratoxin A (2′R-OTA) isomer. In this study, the stability of OTA during rye bread production processes and pizza wheat base production processes (including dough kneading, dough fermentation, and baking) was investigated. The bread was prepared using a rye flour naturally contaminated with OTA (concentration 6.41 ± 0.52 μg/kg) and yeast. The bread was baked for 40 min at 180 °C, or for 70 min at 240 °C. The pizza bases were prepared by using wheat flour artificially contaminated with OTA (concentration 8.0 or 18.0 μg/kg). The bases were baked for 8 min at 320 °C or for 6 min at 370 °C. No statistically significant changes in OTA concentration were found after the dough kneading and fermentation production process stages. OTA concentrations in the crumb of the baked breads and pizza bases were also similar to the concentrations in the fermented dough. However, OTA concentrations in the crust decreased as compared to the fermented dough by 25.6% for the bread baked at 180 °C, 23% for the bread baked at 240 °C, and 8.0%–25.4% for the pizza bases, depending on the OTA concentration in the dough and the baking temperature (lower concentrations were associated with higher temperatures). Partial degradation of OTA in the crust of the baked products was accompanied by slight OTA racemization. Approximately 3.5% of the OTA in the crust of the rye bread baked at 240 °C was transformed into 2′R-OTA, increasing its level to 0.18 μg/kg. Briefly, 2.2%–10.1% of the OTA in the crust of the pizza bases was transformed into 2′R-OTA, increasing its levels to 0.13–1.57 μg/kg, depending on the baking temperature (higher concentrations were associated with higher temperature). It is worth noting that so far, apart from a few studies on transformation of OTA during roasting of coffee beans, these mechanisms during baking of cereal-based products are practically unknown.

## 1. Introduction

Cereal-based food is an important source of nutrition and dietary staple for people worldwide. The main factor that may decrease the quality of such foods is that cereal plants are prone to infection by pathogenic fungi that produce toxic metabolites called mycotoxins. Besides, such fungi may develop in improperly stored grain. (Perincherry, Lalak-Kańczugowska, & Stępień, 2019). Fungi genera important from the perspective of cereal-based food safety include *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps*, and *Alternaria* (Karlovsky et al., 2016). Some species belonging to the first two families like as *Penicillium verrucosum*, *P. nordicum*, *Aspergillus carbonarius*, or *A. ochraceus* biosynthesise ochratoxin A (OTA). OTA is found in a wide variety of foodstuffs, mainly cereals and cereal-based products, but may also be present in coffee, grape juice, wine, herbs, spices, dried fruits, and some animal-origin raw materials (if the animals have been fed with OTA-contaminated feed) (Bhat, Rai, & Karim, 2010). The International Agency for Research on Cancer has classified OTA as "possibly carcinogenic to humans" (group 2B, IARC, 1993). Additionally, OTA's nephrotoxic, immunotoxic, mutagenic, and neurotoxic effects in humans have been discussed in numerous studies (EFSA 2020). Owing to such harmful effects, the EU and numerous countries have legally regulated OTA threshold concentrations in foodstuffs (EC, 2006a; USDA, 2018). EU-imposed OTA thresholds include: 5.0 μg/kg for unprocessed cereals; 3.0 μg/kg for products derived from unprocessed cereals, including processed cereal products and cereals intended for direct

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human consumption; 10 μg/kg for vine fruit (currants, raisins and sultanas); 5.0 μg/kg for roasted coffee beans and ground roasted coffee; 10.0 μg/kg for soluble (instant) coffee; 2.0 μg/kg for wine and grape juice; 0.5 μg/kg for processed cereal-based foods and baby foods for infants and young children; 0.5 μg/kg for dietary foods for special medical purposes (EC, 2006a).

Various procedures (clean-up, washing, grinding, fermentation, extrusion, baking, roasting, frying and so on) are performed during the processing of the majority of cereal products. In the course of these operations, mycotoxins may be removed or chemically transformed into modified mycotoxins (Karlovsky et al., 2016; Rychlik et al., 2014). These transformations may be induced either thermally (e.g. thermal degradation of DON into norDON derivatives during baking processes, Vidal, Sanchis, Ramos, & Marin, 2015) or enzymatically under the influence of either endogenous plant enzymes (e.g. DON-3G produced during grain malting, Ksieniewicz-Woźniak, Bryła, Waśkiewicz, Yoshinari, & Szymczyk, 2019) or microorganism-produced enzymes (e.g. DON-3G produced during dough fermentation, Vidal, Ambrosio, Sanchis, Ramos, & Marín, 2016). Various degrees to which OTA is degraded during such operations are reported in the literature. It is commonly believed that OTA concentration is reduced to a certain extent during thermal processing, even if the mycotoxin is quite resistant to moderate heating. The reduction is often enhanced by other factors, such as the presence of additives and other stabilizing substances, or fermentation (Ćolović et al., 2019). Cramer et al. reported that the concentration of OTA dry-heated to 150 °C for 1 h decreased by 30% and the decrease did not exceed 70% until the temperature was raised above 180 °C (Cramer, Königs, & Humpf, 2008). Some authors (Scudamore, Banks, & Macdonald, 2003) reported that operations of cleaning and milling OTA-contaminated grain reduced the OTA concentration; however, no significant decrease in OTA levels was observed after bread baking. Other authors (Bol, Araujo, Veras, & Welke, 2016; Valle-Algarra et al., 2009; Vidal et al., 2015) reported that bread baking significantly decreased OTA concentration in their samples.

So far, a little information is available about OTA transformation /degradation processes at high temperature. The most studies is about transformations of OTA in dry-roasted coffee beans, in which was shown that OTA might bind with polysaccharides present in the beans. At high processing temperatures (above 200 °C), OTA was decarboxylated, producing decarboxy-ochratoxin A (DC-OTA), 2′R-ochratoxin A (2′R-OTA), and α-amide ochratoxin A (OTamid) (Bittner, Cramer, Harrer, & Humpf, 2015; Bittner, Cramer, & Humpf, 2013; Cramer et al., 2008). OTamid was eventually identified as a less significant product of OTA thermal degradation (Bittner et al., 2015). Additionally, 2′R-OTA and DC-OTA were responsible for about half of the OTA losses that occurred during the operation, while the rest might be attributed to binding with polysaccharides in further reactions (Bittner et al., 2013). Cramer et al. found that 2′R-OTA was the new racemate component found most frequently, and was present in the highest concentrations after the roasting; its contribution in the racemate was estimated to be up to 25% (Cramer et al., 2008). The toxicological properties of the OTA-modified forms are not well known. The Cell Counting Kit-8 (CCK-8) test showed that 2′R-OTA exhibited about 10 times lower cytostatic effects in the IHKE (Immortalise Human Kidney Epithelia) cells than that exhibited by OTA (Cramer, Harrer, Nakamura, Uemura, & Humpf, 2010). Interestingly, 2′R-OTA was found in human blood at levels comparable to OTA's levels (Cramer et al., 2015).

Unlike the studied OTA degradation mechanisms active during the roasting of coffee beans, the mechanisms functional during baking of cereal-based products are practically unknown. In this study, we have studied OTA and 2′R-OTA in rye bread and pizza bases, products that represent all cereal-based products, the main source of exposure of human consumers to OTA. Despite the general decline in bread consumption, there is a growing demand for rye bread in Poland. Rye bread contains more nutrients than wheat bread, such as minerals, dietary fiber, protein and vitamins, so it should be included in the daily diet more often.

The processing stages of dough kneading, dough fermentation, and bread and pizza base baking (at various temperatures) were considered. Pizza bases were baked at temperatures higher than those used at home (above 300 °C, usually applied during industrial pizza production; dough baked for shorter periods at higher temperatures loses much less moisture than dough baked for longer periods at lower temperatures).

## 2. Materials and methods

### 2.1. Chemicals and reagents

Certified reference solutions of OTA in acetonitrile (Romer Labs, Tulln, Austria) and 2′R-OTA in acetonitrile (Aokin, Berlin, Germany) (10 μg/mL each) were the starting points for preparation of 0.25 μg/mL (OTA) and 62.5 ng/mL standard solutions necessary to generate calibration curves. HPLC-grade methanol, acetonitrile, and isopropyl alcohol, as well as 85% reagent-grade orthophosphoric acid, were purchased from Witko Sp. Z o. o., Łódź, Poland. Deionised water was purchased from Hydrolab, Straszyn, Polska. Phosphate-buffered saline (PBS) and immunoaffinity columns were purchased from Vicam (Watertown, MA, USA). According to some authors (Cramer et al., 2008; Sueck et al., 2019), Ochratest immunoaffinity columns show affinity not only to OTA, but also to 2′R-OTA, a diastereomer of OTA. So far this screening technique was used to simultaneously isolate both these compounds from coffee beans, cacao, cereal-based products including pumpernickel and expanded products, chicory coffee, and popcorn (Cramer et al., 2008; Sueck et al., 2019).

### 2.2. Research material

Rye flour naturally contaminated with OTA (concentration 6.41 ± 0.52 μg/kg) and wheat flour artificially contaminated with OTA at 8.0 μg/kg or 18.0 μg/kg were used (to two parts of 600 g flours, 480 and 1080 μL of OTA were added, respectively, from a concentration of 10 μg/mL in acetonitrile, and then allowed to evaporate at room temperature for 6 h). Flours contaminated with OTA to a relatively high degree were selected intentionally to facilitate identification of OTA degradation products. Pizza bases are usually produced from wheat flour with high gluten but low ash content. Thanks to high gluten content the dough is more elastic. As no naturally contaminated flour with high OTA levels was available, artificially contamination was required for the experiments.

The rye flour was characterized by ash content at level of 1.43% d. m., and a water absorption of 60.0%. The wheat flour was characterized by ash content at level of 0.56% d.m. Ash content and water absorption were evaluated according to the ISO 2171:2007 and ISO 17718:2013 standards, respectively.

### 2.3. Bread baking

Dough for bread was prepared using the direct method as follows: 700 g of rye flour was mixed with 10 g of salt and 20 g of yeast; water was added in the amount necessary to obtain 170.5% dough productivity. The used iodized food salt contained min. 99% sodium chloride NaCl. The used pressed baker's yeast produced by JSC Kharkiv Yeast Plant was characterized by dry matter content not less than 30% and driving force (expressed as the amount of carbon dioxide released in 2 h) more than 2000 mL of $CO_2$. The dough was kneaded in the KitchenAid Classic dough mixer (USA) operated for 8 min at its first speed, then fermented for 60 min, split into three pieces each of 350 g, and left in a growth chamber for 35 min. Loaves were baked in the Piccolo Wachtel Winkler shelf oven (Germany) at 180 °C or 240 °C. Shaped dough pieces were placed in a steamed-up baking chamber. The baking operation was determined when the temperature in the middle of the loaf crumb reached 98 °C. The baking time was 70 min and 40 min at 180 °C and

240 °C, respectively. To obtain crust and crumb samples, the baked loaves were cut into halves, and the crust was separated from the crumb using a knife along the colour change line. The samples were deep-frozen at −30 °C and then dried at 25 °C for 16 h in the Alpha 1–4 LSCplus lyophilizer unit (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The dried material was homogenised in the WŻ 1 Sadkiewicz laboratory mill (Bydgoszcz, Poland).

### 2.4. Pizza base baking

The pizza bases were prepared as follows: 600 g of wheat flour was mixed with 20 g of salt, 40 g of yeast, 80 mL of olive oil, and 300 mL of water. The same salt and yeast as reported for bread making were used to prepare the rye dough. The dough was kneaded in a KitchenAid Classic dough mixer (USA) operated for 15 min at its fourth speed and then fermented for 60 min. Four 0.5-cm thick 50-cm diameter pizza bases were prepared from the dough made of the flour contaminated with 8 μg/kg OTA, and four identical bases of the dough were prepared from flour contaminated with 18 μg/kg OTA. The bases were left in a growth chamber for 20 min (during which time each doubled its volume), then baked in the Piccolo Wachtel Winkler shelf oven (Germany) as follows: two of each batch at 320 °C for 8 min, and the other two at 370 °C for 6 min. Baked and cooled pizza bases were processed in a manner that was similar to that for the baked and cooled rye bread loaves (separation of the crust from the crumb and subsequent deep-freezing, drying, and homogenising).

### 2.5. Sample preparation

Two grams of the ground research material was weighed, placed in a centrifuge vial, supplemented with 5 mL of the methanol: 10% PBS (8:2) mixture, homogenised in the Unidrive X 1000 homogeniser (CAT Scientific, Inc., Pase Robles, CA, USA) for 2 min, and then centrifuged in the MPV-351R (Med. Instruments, Warsaw, Poland) laboratory centrifuge operated at 10,730×*g* for 10 min. Thereafter, 2.5 mL of the extract was dissolved with 10 mL of the 10% PBS buffer and filtered through a fine-grain filter. Eight millilitres of the thinned extract was allowed to pass through the Ochratest IAC column (Vicam, Watertown, MA, USA) at a rate between one and two drops per second. The column was then rinsed with 10 mL of PBS, followed by 10 mL of water. Analytes were washed out with 2 mL of methanol in a 5-mL reaction vial. The solvent was evaporated in a stream of nitrogen. The residues were redissolved in 0.2 mL of the acetonitrile: isopropyl alcohol mixture (1:1) and 0.3 mL of 0.25 M orthophosphoric acid. The solution was filtered through a 0.45-μm mesh syringe filter. Thereafter, 45 μL of the filtered solution was injected on the used chromatographic column via an autosampler (details mentioned below).

### 2.6. HPLC-FL analysis

Concentrations of OTA and 2′R-OTA in the tested samples of flour, dough, and the baked products (separate crust and crumb) were determined using the Knauer K 1001 high-performance liquid chromatograph (Knauer, Wissenschaftliche Geräte GmbH, Berlin, Germany) coupled with the RF-10AXL (Shimadzu, Kyoto, Japan) fluorescence detector. Analytes were separated on the Cosmosil 5C18-AR-II 4.6 μm × 250 mm (Nacalai Tesque, Kyoto, Japan) chromatographic column kept at a constant temperature of 45 °C. The separation was conducted in isocratic mode, in which the mobile phase consisted of a mixture of 0.25 M orthophosphoric acid: acetonitrile: isopropyl alcohol (600 : 200: 200, v/v/v) flowing at a rate of 1 mL/min. The total time of a single analysis was 35 min. The wavelength maxima corresponding to OTA and 2′R-OTA fluorescence emission were 330 and 460 nm, respectively. Typical obtained chromatograms are shown in Fig. 1.

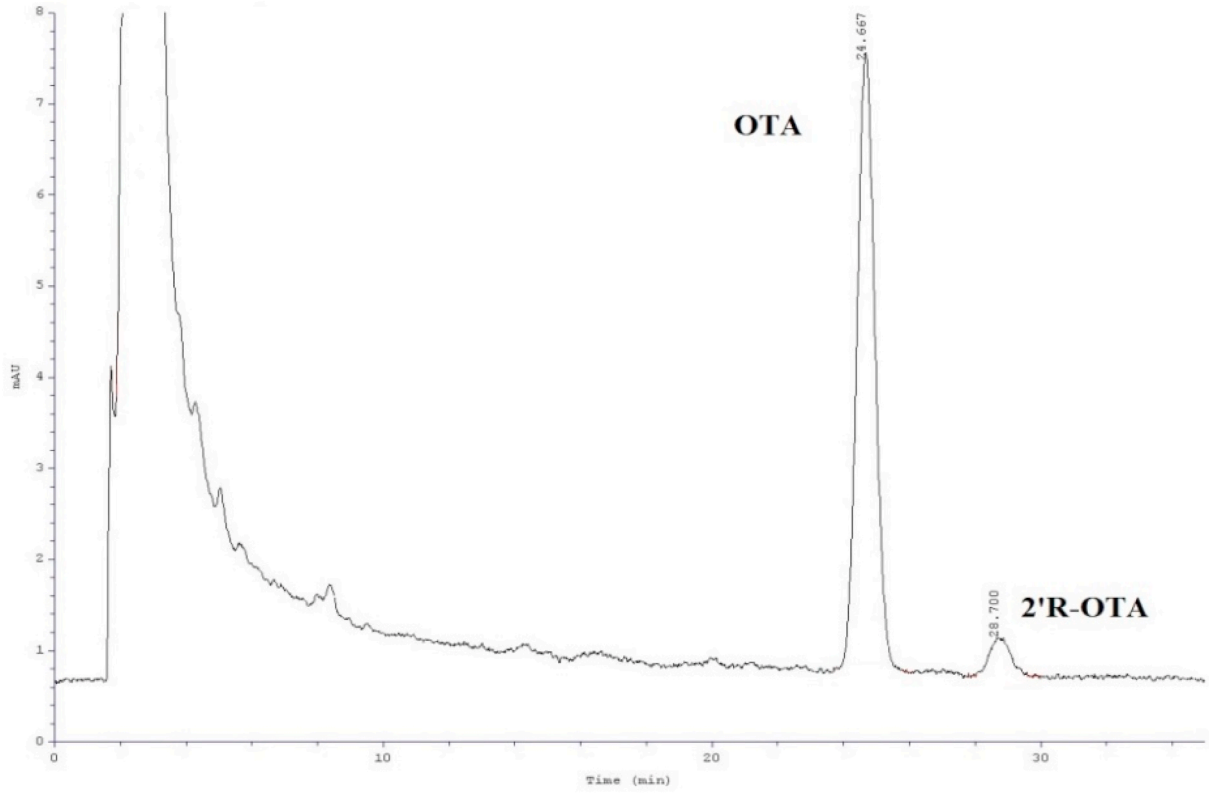


**Fig. 1.** Typical chromatogram of a sample prepared from the crust of pizza base baked at 370 °C.

### 2.7. Data normalization

To reveal actual changes of OTA and 2R′-OTA during bread and pizza-base baking technological processes, their concentrations measured in dough, crumb, and crust were normalised to the amount of flour dry mass. All samples (including flour) were lyophilized, hence the normalization was needed only to take care the added yeast and salt. The concentrations were recalculated according to the following formulae:

Flour: $x = a / b$, where

a is analyte concentration in flour (μg/kg)
b is flour dry mass fraction (%).

Dough, crumb, crust: $y = c / d$, where

c is analyte concentration in dough/crumb/crust dry mass (μg/kg)
d is flour fraction in dough/crumb/crust dry mass (%).

### 2.8. Method validation

To verify the applied analytical method, calibration curve linearity range, limit of quantification (LOQ, concentration of the given analyte at which its signal-to-noise ratio is 10:1), limit of determination (LOD, i.e. concentration at which the signal-to-noise ratio is 3:1), recovery rate (R), and precision (repeatability expressed as relative standard deviation, RSD) were measured for individual analytes and matrices (flour, crumb, and crust). Calibration curves were based on the results of analyses of six standard solutions made of appropriate volumes of the reference solution. Each volume was dried within the nitrogen stream and redissolved in 0.4 mL of the acetonitrile: isopropyl alcohol (1:1) mixture and 0.6 mL of 0.25 M orthophosphoric acid. In effect, the OTA standards covered the 0.5–18 μg/kg concentration range, and 2′R-OTA standards covered the 0.125–4.0 μg/kg range. The determination coefficients ($R^2$) for the obtained curves were 0.9926 (OTA) and 0.9937 (2′R-OTA). The LOQ and LOD for both investigated analytes were 0.3 μg/kg and 0.1 μg/kg, respectively.

Recovery rate and method repeatability were based on the results of analyses of three fortified samples for each of the three investigated matrices. Fortified samples consisted of 2 g of a given matrix material (crumb, crust, and flour) free of OTA and 2′R-OTA, mixed with the respective volume of the reference solution. Fortification levels were 4, 8, or 18 μg/kg for OTA and 1, 2, or 4 μg/kg for 2′R-OTA. The samples were analysed as per methods described above (identical to the real samples).

The European Commission established criteria for analytical methods for various mycotoxins, including OTA (EC, 2006b). No criteria for 2′R-OTA have been set; therefore, the parameters obtained in this work for the latter compound have been compared with the criteria established for OTA (2′R-OTA is OTA's only optical isomer). For concentrations of 1–10 μg/kg, the regulation recommends OTA recovery rates within the 70%–110% range, while method precision RSD may not be worse than 20%. For concentrations below 1 μg/kg, the parameters should be 50%–120% and not worse than 40%, respectively. The method developed in this work met the criteria of recovery rates within the 80%–102% range for all measured concentrations and tested matrices and RSD not exceeding 20%, as shown in Supporting information.

The developed method was also verified (at least in respect to OTA) in an interlaboratory proficiency test. The project focussed on OTA in ground wheat grain and was executed between April and June 2020 by Romer Labs, Tulln, Austria. The result obtained was 21.61 μg/kg versus the "true" (most probable) value of 21.88 μg/kg, which translated into z-score = −0.1. No similar interlaboratory proficiency test concerning 2′R-OTA has been performed thus far.

### 2.9. Statistical analysis of the experimental data

The results were statistically analysed using the Statgraphics 4.1 (Graphics Software System, STCC, Inc., Rockville, MD, USA) software package. One-way analysis of variance (ANOVA) was performed at a significance level of $\alpha = 0.05$. Homogenous groups were determined using the Fisher's (LSD) test. The samples were tested in three replicates. All three replicates of the crumb and crust were obtained from the same bread loaf and pizza base.

## 3. Results

To reveal OTA degradation at the subsequent stages of the bread production process, OTA and 2′R-OTA concentrations measured in semi-products and final products were normalised to flour content in the given sample. The latter content was calculated by considering all additives, except for water removed from all samples via sublimation.

Rye flour used in this work contained 6.41 ± 0.52 μg/kg OTA, which was more than twice the level stipulated in EU Regulation 1881/2006 as acceptable for OTA in rye flour (3 μg/kg). Flour contaminated to such a considerable extent was deliberately used to help identify and understand the degradation of OTA after individual stages of the bread production process. No statistically significant trend in OTA concentration was identified after the two first stages of the bread production process. The measured concentrations were 6.57 ± 0.64 μg/kg after dough kneading (an insignificant +2.5% more than that in the flour) and 6.77 ± 0.28 μg/kg after dough fermentation (an insignificant +3.0% more than that in the flour), as shown in Table 1.

**Table 1**
Top: Mean concentrations of OTA and 2′R-OTA found in the tested samples of rye bread and its semi-products. Data have been normalised to the amount of flour in each sample (with added yeast and salt). Values indicated by different letters are statistically different (α = 0.05). Bottom: Changes in OTA concentration at individual stages of the rye bread production process.

| Semi-/final product | | OTA (μg/kg) | 2′R-OTA (μg/kg) |
|---|---|---|---|
| Concentration (μg/kg) | Flour (n = 3) | $6.41^{b} \pm 0.52$ | n.d. |
| | Dough after mixing (n = 3) | $6.57^{b} \pm 0.64$ | n.d. |
| | Dough after fermentation (n = 3) | $6.77^{bc} \pm 0.28$ | n.d. |
| | Loaf crust (180 °C) (n = 9) | $5.04^{a} \pm 0.69$ | n.d. |
| | Loaf crust (240 °C) (n = 9) | $5.21^{a} \pm 0.22$ | 0.18 ± 0.05 (3.5%)* |
| | Loaf crumb (180 °C) (n = 9) | $7.46^{c} \pm 0.39$ | n.d. |
| | Loaf crumb (240 °C) (n = 9) | $7.11^{bc} \pm 0.245$ | n.d. |
| | **From stage to stage** | **Change from stage to stage (%)** | |
| Concentration (μg/kg) | Flour/Mixed dough | +2.5 | – |
| | Mixed dough/Fermented dough | +3.0 | – |
| | Fermented dough/Crust (180 °C) | ** −25.6 | – |
| | Fermented dough/Crust (240 °C) | ** −23.0 | – |
| | Fermented dough/Crumb (180 °C) | +10.2 | – |
| | Fermented dough/Crumb (240 °C) | +5.0 | – |
| | *After all processing stages* | | |
| | Flour/Crust (180 °C) | ** −21.4 | – |
| | Flour/Crust (240 °C) | ** −18.7 | – |
| | Flour/Crumb (180 °C) | ** +16.4 | – |
| | Flour/Crumb (240 °C) | +10.9 | – |

n.d. – not detected; *2′R-OTA/OTA ratio; ** Changes statistically significant.

However, OTA concentration statistically changed after bread baking, and was different in the loaf crust and in the loaf crumb. Rye breads were baked at 180 °C or 240 °C. Concentrations of OTA in the crust of the loaf baked at 180 °C and 240 °C were 5.04 ± 0.69 (25.6% less than that in the fermented dough) and 5.21 ± 0.22 μg/kg (23.0% less than that in the dough), respectively. OTA level reductions through all processing stages, such as the concentration in the loaf crust as compared to the concentration in the flour were somewhat lower (21.4% and 18.7% in loaves baked at 180 °C and 240 °C, respectively (see Table 1). As indicated in Table 1, OTA concentrations in the crust were statistically different from those in the flour and those in the crumb. The concentrations of OTA in the crumb of the loaves baked at 180 °C and 240 °C were 7.46 ± 0.39 μg/kg and 7.11 ± 0.24 μg/kg, respectively. On the other hand, the differences were insignificant or negligible from the viewpoint of two different baking temperatures. Even so, the 2′R-OTA isomer was detected at a relatively low level of 0.18 ± 0.05 μg/kg only in the crust of loaves baked at the higher temperature (240 °C), as shown in Table 1.

Pizza is a very popular food in numerous regions of the world. Normally, it is baked at higher temperatures than rye bread. If standard baking temperatures are applied, the baking time would have to be appropriately longer since excessive amounts of water would evaporate from the product during the operation, and the final product would be too dry in taste. Pizza bases were selected for this study because it was anticipated that high baking temperatures would enhance the transformation of OTA into 2′R-OTA. Pizza bases are normally prepared from wheat flour. Our flour samples were artificially contaminated with either 8.0 μg/kg or 18.0 μg/kg OTA. We baked our pizza base samples at either 320 °C for 8 min or 370 °C for 6 min. As shown in Table 2, no statistically significant changes in OTA concentration were identified after the following processing stages: dough kneading, dough fermentation, baking at 320 °C (crumb), and baking at 370 °C (crumb). In case of the flour contaminated with 8 μg/kg OTA, the respective concentrations were: 7.03 ± 0.49, 7.41 ± 0.61, 7.71 ± 0.39, and 7.56 ± 1.00 μg/kg. In case of the flour contaminated with 18 μg/kg OTA, the respective concentrations were: 18.42 ± 0.44, 18.69 ± 0.39, 18.69 ± 0.76, and 18.26 ± 2.34 μg/kg.

On the other hand, the concentration of OTA found in pizza base crust decreased significantly in relation to the flour. The decrease was more pronounced after baking at 370 °C (from 8 μg/kg in the flour to 5.53 ± 0.63 μg/kg, i.e. by 25.4%, and from 18 μg/kg in the flour to 15.55 ± 0.77 μg/kg, i.e. by 16.8%) compared to that after baking at 320 °C (from 8 μg/kg in the flour to 6.00 + 0.36 μg/kg, i.e. by 19.0%, and from 18 μg/kg in the flour to 17.20 ± 0.53 μg/kg, i.e. by 8.0%). Similar to the case of the rye bread, the 2′R-OTA isomer was found only in the crust of the baked pizza bases. In case of the less contaminated flour, its concentration was 0.13 ± 0.04 μg/kg (2.2% of OTA) after baking at 320 °C, and 0.52 ± 0.03 μg/kg (9.4% of OTA) after baking at 370 °C. In case of the more contaminated flour, its concentration was 0.25 ± 0.01 μg/kg (1.5% of OTA) after baking at 320 °C, and 1.57 ± 0.12 μg/kg (10.1% of OTA) after baking at 370 °C (see Table 2).

## 4. Discussion

Stability and variability of OTA during various food processing operations have been investigated in numerous studies. It is commonly accepted that thermal processing (baking, roasting, grilling, frying and so on) generally decreases the levels of mycotoxins, including OTA (Schaarschmidt and Fauhl-Hassek, 2018); in addition, other factors (such as food additives or fermentation process) may also affect their stability. However, data on OTA stability during dough kneading and fermentation as well as bread baking operations are not abundant. Milani and Heidari (2017) observed a decrease in the concentration of OTA after kneading and fermentation of wheat dough with added dried yeast, instant yeast, leavening, and pressed yeast (the lowest reduction by 9% was observed for the dried yeast and the highest reduction by 46% was observed for the pressed yeast), but no significant change in OTA concentration was observed after simple fermentation of wheat dough. Some authors suggest that OTA reduction during yeast-assisted dough fermentation may depend on the yeast strain used (Cecchini, Motassut, Garcia-Moruno, & Di-Stefano, 2006). Valle-Algarra et al. (2009) reported a 30%–34% decrease (depending on the OTA level in the flour) in OTA concentration after *Saccharomyces cerevisiae* yeast-assisted dough fermentation and different OTA reduction levels in bread crumb (7.3%–38.2%, depending on the 2–10 μg/kg OTA concentration in the flour) as compared to the crust (20.4%–51.3%). Bol et al. (2016) studied the baking process of wheat bread, crackers, and cakes; they observed an 80%–90% OTA reduction from flour to the baked products. Such high reduction levels might be caused by effects of water and other additives in the flour and a lack of normalization of the results to various amounts of flour in individual products (81%–95% of the dry mass). Besides, it should be pointed out that OTA degradation may depend on combination of the baked dough piece mass, baking temperature, and baking time. Bol et al. (2016) baked 70 g dough pieces at 220 °C for 35 min, while the combinations used in this work were 350 g/180 °C/70 min or 350 g/240 °C/40 min. In other studies, Milani and Heidari (2017) reported the highest reduction in the OTA level (by 56%) after baking dough fermented with pressed yeast (dough pieces 50 g, 200 °C, 20 min), but no significant OTA reduction was observed after baking dough fermented with leavening. They suggested that pressed yeast might be especially effective in reducing OTA since they produced more $CO_2$ than other yeast types, which in turn lowered the dough pH and produced more after-fermentation metabolites (Milani & Heidari, 2017). This reduction might also be assisted by the ability of yeast cell

**Table 2**
Top: Mean concentrations of OTA and 2′R-OTA found in pizza dough, baked pizza crumb, and baked pizza crust. Values indicated by different letters are statistically different (α = 0.05). Bottom: Changes of OTA concentration at individual stages of the pizza production process.

| Semi-/final product | | OTA level in flour = 8.0 μg/kg | | OTA level in flour = 18.0 μg/kg | |
|---|---|---|---|---|---|
| | | OTA | 2′R-OTA | OTA | 2′R-OTA |
| Concentration (μg/kg) | Mixed dough (n = 3) | $7.03^{bc}$±0.49 | n.d. | $18.42^{bc}$±0.44 | n.d. |
| | Fermented dough (n = 3) | $7.41^{bc}$±0.61 | n.d. | $18.69^{c}$±0.39 | n.d. |
| | Pizza crust (320 °C) (n = 6) | $6.00^{a}$±0.36 | 0.13 ± 0.04 (*2.2%) | $17.20^{b}$ ± 0.53 | 0.25 ± 0.01 (*1.5%) |
| | Pizza crust (370 °C) (n = 6) | $5.53^{b}$ ± 0.63 | 0.52 ± 0.03 (*9.4%) | $15.55^{a}$±0.77 | 1.57 ± 0.12 (*10.1%) |
| | Pizza crumb (320 °C) (n = 6) | $7.71^{c}$±0.39 | n.d. | $18.69^{c}$±0.76 | n.d. |
| | Pizza crumb (370 °C) (n = 6) | $7.56^{bc}$±1.00 | n.d. | $18.26^{abc}$±2.34 | n.d. |
| | Change from stage to stage | | | | |
| Concentration changes (%) | Mixed dough/Fermented dough | +5,4 | – | +1,5 | – |
| | Fermented dough/Pizza crust (320 °C) | ** −19.0 | – | **-8.0 | – |
| | Fermented dough/Pizza crust (370 °C) | ** −25.4 | – | **-16.8 | – |
| | Fermented dough/Pizza crumb (320 °C) | +4.0 | – | 0.0 | – |
| | Fermented dough/Pizza crumb (370 °C) | +2.0 | – | −2.3 | – |

n.d. - not detected; *2′R-OTA/OTA ratio; ** Changes statistically significant.

walls to bind OTA (Huwig, Freimund, Kappeli, & Dutler, 2001; Yiannikouris et al., 2006; Armando et al., 2012; Bzducha-Wróbel, Bryła, Gientka, Błażejak, & Janowicz, 2019). The number of factors possibly influencing degradation of OTA during the baking process may undoubtedly be higher, e.g. dough aeration, shape of dough pieces, any added baker's additives. However, diversity of the applied recipes is the main problem making difficult to compare results obtained by various authors.

Nevertheless, the results of other studies correlate well with ours, as evidenced by a lack of any statistically significant changes in OTA concentration after dough kneading, dough fermentation, and baking operations (Milani & Heidari, 2017; Scudamore et al., 2003; Vidal et al., 2014a, 2014b). For example, Scudamore et al. (2003) prepared bread from flour (contaminated with OTA at two concentration, 6.1 μg/kg and 42.2 μg/kg), and baked at 244 °C for 25 min; they reported no OTA degradation after baking. Similarly, Vidal et al. (2014b) prepared bread from flour (contaminated with OTA at two concentrations, 0.8 μg/kg and 8.6 μg/kg), and baked at 210 °C for 105 min or 135 min, and at 200 °C for 135 min; they also reported no OTA degradation after baking. In turn, Peng et al. (2015) reported that high levels of OTA (43 and 138 μg/kg) in wheat flour did not decrease after yeast-assisted fermentation of dough performed at 38 °C for 40 min. Similar results were published by Vidal et al. (2014a & 2014b). Regardless of the starting level in flour, the OTA concentration did not change after fermentation of dough (containing the same baker's agent) was performed at 30 °C for 75 min.

We did not observe any statistically significant changes in the OTA concentration after dough kneading and dough fermentation procedures, neither in our rye-based samples or in our wheat-based samples. We conclude that the addition of *Saccharomyces cerevisiae* baker's yeast did not affect the stability of OTA in our samples during fermentation. Thermal processing (180 °C or 240 °C for the rye-based samples, 320 °C or 370 °C for the wheat-based samples) induced a statistically significant reduction in the OTA concentration only in the loaf and pizza crust (8.0%–25.6%, depending on the product and baking temperature) but not in the loaf and pizza crumb. Such various effects might perhaps be explained by different temperatures within the crust and the crumb. During baking, a portion of the water from the dough surface evaporates, while a portion migrates toward the crumb. After the water is evaporated, the loaf crust temperature rises to approximately 180 °C, while the temperature inside the crumb usually does not exceed 100 °C (due to the water). The idea that the stability of OTA during thermal processing may depend on the amount of water in the processed product has already been previously suggested by Vidal et al. (2014b) and Bryła et al. (2020). Boudra, Le Bars, and Le Bars (1995) heated whole wheat flour with various amounts of added water and reported that the OTA concentration in flour containing 50% of water heated to 100 °C for 60 min, 150 °C for 64 min, and 200 °C for 24 min decreased by 18%, 56%, and 31%, respectively. For dry flour, the decrease amounted to 32%, 80%, and 88%, respectively, after heating at 150 °C for 64 min, at 200 °C for 24 min, and at 250 °C for 16 min.

Although we have not studied the kinetics of the OTA degradation process in our thermally processed dough samples, however, it can be naturally concluded that: (i) the degradation rate increases together with both the temperature and time of the processing and (ii) the process takes place mainly on the surface of the heated dough pieces. Another factor that possibly influences the OTA reduction rate is the mass of the dough pieces subjected to baking. Vidal et al. (2015) reported an OTA decrease of approximately 64% if dough pieces of 3 g each were baked at 200 °C for 40 min, while the decline was about 21% for baking at 140 °C for 40 min. The relatively low mass of the pieces used in the latter experiment indicates that OTA degrades faster in smaller dough pieces (of a larger surface area-to-mass ratio). The starting OTA level in flour might be another factor responsible for the variation in results. The flour used in our study was contaminated with OTA concentrations ranging from 6.42 ± 0.55 μg/kg to 18 μg/kg, while the contamination level in some of the studies discussed above did not exceed 0.22–0.24 μg/kg. Lastly, the separation of bread crust from bread crumb is not a precise operation, and some crumb left with the crust may impact the OTA reduction measured in the loaf crust.

The OTA reduction observed at the surface of the baked bread loaves and pizzas were accompanied by the detection of small amounts of 2′R-OTA in the loaf and pizza crust. Its concentrations ranged from 0.18 ± 0.05 μg/kg to 1.57 ± 0.12 μg/kg, depending on the product, baking temperature, and the OTA starting level in flour. These levels corresponded to approximately 3.5% of the OTA concentration found in the crust of bread baked at 240 °C, approximately 1.5% of the OTA concentration in the crust of pizza prepared from the less contaminated flour baked at 320 °C, and around 10.1% of the OTA concentration in the crust of pizza prepared from the more contaminated flour baked at 370 °C. Mechanisms that would explain the process the production of 2′R-OTA in food products have been studied by not many authors, mostly with respect to roasted coffee beans. Sueck et al. (2019) reported the start of racemization of pure heated OTA (10 μg) at 120 °C, while the process intensified at higher temperatures, thus increasing the 2′R-OTA share in the racemic mixture up to 25% after 30 min at 150 °C, 5 min at 180 °C, or 1 min at 240 °C. They tested OTA and 2′R-OTA in 51 samples of roasted coffee beans, and found them in 96% and 35% of the samples at the maximum concentrations of 28.4 μg/kg and 3.9 μg/kg, respectively. They also identified low levels of 2′R-OTA in instant malt coffee powder and in pumpernickel (≤0.22 μg/kg). As can be seen in Tables 1 and 2, even combined OTA and 2′R-OTA concentrations found in this work in bread crest may be too low to balance OTA concentration measured in dough. 2′R-OTA is commonly accepted as the main product of OTA thermal degradation. Other products (such as DC-OTA or ester-bound OTA-polysaccharides complexes reported by Bittner et al., 2013 in roasted coffee beans) may not be excluded. However, in case of the bread baking it is difficult to gain insight into these matters due to a relatively low degree to which OTA degrades during the baking.

## 5. Conclusions

In our studies, no significant degradation of OTA mycotoxin was identified after dough kneading, yeast-assisted dough fermentation, and baking of rye flour-based bread and wheat flour-based pizza bases, except for the loaf and pizza crust, where the degradation was statistically significant, i.e. 8.0%–25.6%, depending on the product and the baking temperature. The OTA reduction at the surface of the baked loaves and pizzas was accompanied by the detection of small amounts of 2′R-OTA and 1.5%–10.1% of OTA in the crust, depending on the product, the baking temperature, and the starting OTA concentration in the flour. Higher 2′R-OTA concentrations were detected after baking at higher temperatures. Studies on the toxicological effects of OTA-degradation products are underway. However, since cereal products are the basis of a daily human diet and as bread and pizza are consumed in much higher quantities than coffee and other roasted products, even presence of small amounts of 2′R-OTA may potentially be significant from a food safety perspective. It can be assumed that the risk of exposure to 2′R-OTA from bread is greater in the external part (crust) in the finished product. To develop a risk assessment related to the exposure of the consumers to 2′R-OTA, it is necessary to conduct further research on the presence of this degradation product in food generally recognised as a source of OTA, and subjected to thermal processes.

## CRediT authorship contribution statement

**Marcin Bryła:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration. **Edyta Ksieniewicz-Woźniak:** Investigation, Writing - original draft. **Sylwia Stępniewska:** Data curation, Formal analysis, Validation. **Marta Modrzewska:** Conceptualization, Methodology, Writing - review & editing. **Agnieszka Waśkiewicz:** Conceptualization, Methodology, Writing - review &

editing. **Krystyna Szymczyk:** Writing - review & editing. **Anna Szafrańska:** Data curation, Formal analysis.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2021.107950.

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