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Original research paper

STABILITY OF *Alternaria* TOXINS DURING BREAD – MAKING PROCESS

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ABSTRACT: During bread production, present mycotoxins can be reduced, transformed, and/or bound or released. In general, during bread production, mycotoxins might be affected by the presence of certain ingredients and/or additives and by the fermentation conditions, followed by the baking process. However, there are no available data on the stability and behavior of *Alternaria* toxins during bread-making process in real conditions. Therefore, the main objective of this study was to evaluate the effect of dough fermentation and baking processes on the behavior of tenuazonic acid (TeA), alternariol (AOH) and alternariol monomethyl ether (AME). For this purpose, spiked white wheat flour (100 µg/kg of each TeA, AOH, and AME in flour), 2.5% baker's yeast, 2.0% salt and 58% water calculated on flour basis were used as raw materials in a micro-scale baking test. Spiked wheat dough was fermented for 60 min at 37 °C, and then divided into 15 g pieces, molded by hand, and proofed for 50 min at 37 °C. Finally, the proofed dough was baked for 8 min at 250 °C. At each point (0, 60 and 110 min) the dough samples were taken, frozen, lyophilized, ground, and stored until further analysis. Bread samples were taken after cooling and the same procedure was applied to it as well. To study the fate of TeA, AOH and AME during bread production, validated method of high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used. The content of TeA and AME in bread was at the same level as in the raw material, although in the dough during fermentation, reductions of TeA by 29.2% and increase amount of AME by 13.8% were noted, compared to their content in dough after kneading (0 min). The content of AOH in dough after final proof was reduced by 31.8%, while in bread, its content was reduced by 34.8% compared to its content in dough after kneading (0 min).

Key words: *alternariol, alternariol monomethyl ether, tenuazonic acid, stability, bread production, LC-MS/MS*

INTRODUCTION

Among cereals, wheat is the second most produced grain worldwide (OECD-FAO, 2019) and represents the major part of the daily human diet. The major share of produced wheat is subjected to the milling process that converts it to flour which is processed into various foods (breads, pasta, noodles and cakes) (Pacin, et al., 2010). In the wheat production chain (from cultivation to processing), besides *Fusarium* species which remain a main source of mycotoxin contamination of wheat, other mycotoxigenic fungi have been recognized as important wheat contaminants, such as fungi of the genus *Alternaria* (EFSA, 2011). Its flexibility to different climate conditions is responsible for the apparent contradiction related to *Alter-*

naria diseases, which may develop both at high or low temperature, different humidity, and under multiple combinations of environmental factors (Lee et al., 2015). Various *Alternaria* species are capable of producing a variety of *Alternaria* toxins, which belong to several classes of chemical compounds (EFSA, 2011). Among approximately 70 toxic metabolites of the *Alternaria* spp., several *Alternaria* toxins (AOH, AME, TeA and tentoxin) frequently contaminate a wide range of agricultural and food products (grains and grain based products, tomato and tomato products, sunflower seeds and sunflower oil, fruits and fruit products including fruit juices, beer and wine) (EFSA, 2011). In recent years, several review papers have been published which include overviews of toxicity and toxicokinetics of *Alternaria* toxins (EFSA, 2011; Fraeyman, 2017; Lee et al., 2015; Solfrizzo, 2017). Some of *Alternaria* toxins show cytotoxic, fetotoxic and/or teratogenic activity, with proven mutagenic, clastogenic and oestrogenic effects in microbial and mammalian cell systems and they inhibit the cell proliferation. Published data around the world summarized by EFSA (2011) and published data in the period 2012–2018 from Norway (Uhlig, 2013), Germany (Müller and Korn, 2013), Serbia (Janić Hajnal et al., 2015), China (Zhao, 2015) and Albania (Topi et al., 2019) indicate frequent contamination of wheat with *Alternaria* toxins, so their presence is certainly expected in wheat-based products. However, only limited information on the stability and fate of *Alternaria toxins* in wheat processing chain is available. One of the few studies refers to investigation of the possibility of reduction of *Alternaria* toxins (AOH, AME and TeA) by extrusion processing of whole wheat using a simple pilot single screw extruder (Janić Hajnal et al., 2016). The highest reduction of AOH (87.9%), AME (94.5%) and TeA (65.6%) was achieved when high raw material moisture (w = 24 g/100 g), high feeding rate (q = 25 kg/h) and medium screw speed (v = 390 rpm) were applied. Further, in recently published study by Janić Hajnal et al. (2019) the effect of wheat milling process on the distribution of *Alternaria* toxins (AOH, AME and TeA) was investigated. According to the results obtained in this study, white flour intended for human consumption can be considered as relatively safe product, while low-grade flour may contain increased content of *Alternaria* toxins, since shorts, bran and flours from tail-end breaking and reducing runs had the highest *Alternaria* toxins content. The knowledge on the behavior of *Alternaria* toxins during bread baking is limited. Only one study refers to an investigation of the stability of AOH, AME and altenuene (ALT) by model experiments under various baking conditions (oven temperatures of 170 °C, 200 °C and 230 °C; at t = 30 min, 45 min and 60 min) in the presence (wet baking) or absence (dry baking) of water, by using a spiked wholegrain wheat flour. After dry baking at 230 °C for 1 h (i.e. exposing spiked wholegrain wheat flour to thermal process), a pronounced degradation was achieved (90% for ALT, 70% for AOH and 50% for AME), while after wet baking conditions by addition only water to wholegrain wheat flour (for 45–60 min at 200 °C or for 30–45 min at 230 °C), no degradation of examined *Alternaria* toxins was observed (Siegel et al., 2010). Until now, there is no available data on the behavior of *Alternaria toxins* during bread-making process under real conditions. Taking into consideration their possible harmful effects on human and animal health and the fact that scarce information is available worldwide about behavior of *Alternaria* toxins in wheat processing chain, the main objecttive of this study was to evaluate the effect of dough fermentation and baking processes on the behavior of TeA, AOH and AME.

MATERIALS AND METHODS

Materials

Three hundred grams of spiked white wheat flour (100 µg/kg of each TeA, AOH, and AME in flour), 2.5% baker's yeast, 2.0% salt and 58% water calculated on flour basis were used as raw materials in a micro-scale baking test.

Moisture content

Moisture content in white wheat flour sample, fermented dough and in bread samples were determined according to

ISO 712/2009 method (ISO, 2009). Moisture content was expressed on the dry matter basis.

Bread-making procedure

The procedure of bread - making is outlined in Figure 1. All ingredients were mixed with a low speed (at 85 rpm/min) mixer for 15 min. The obtained dough was fermented for 60 min at 37 °C and a relative humidity of 80%. After mixing, the dough was divided into 15 g pieces, molded by hand, and proofed for 50 min at 37 °C and a relative humidity of 80%. Finally, the proofed dough was baked in a furnace (Miwe Condo, Michael Wenz, D-97450, Arnstein, Germany) at 250 °C for 8 min. At each point (0, 60 and 110 min) the dough samples were taken, while bread samples were taken after cooling. The samples were frozen, lyophilized, ground, and stored at – 20 °C until further analysis.

Sample preparation

The method of sample preparation by Siegel et al. (2010) was slightly modified (Janić Hajnal et al., 2014; 2015) and used to prepare the extracts of the wheat flours, fermented dough and bread.

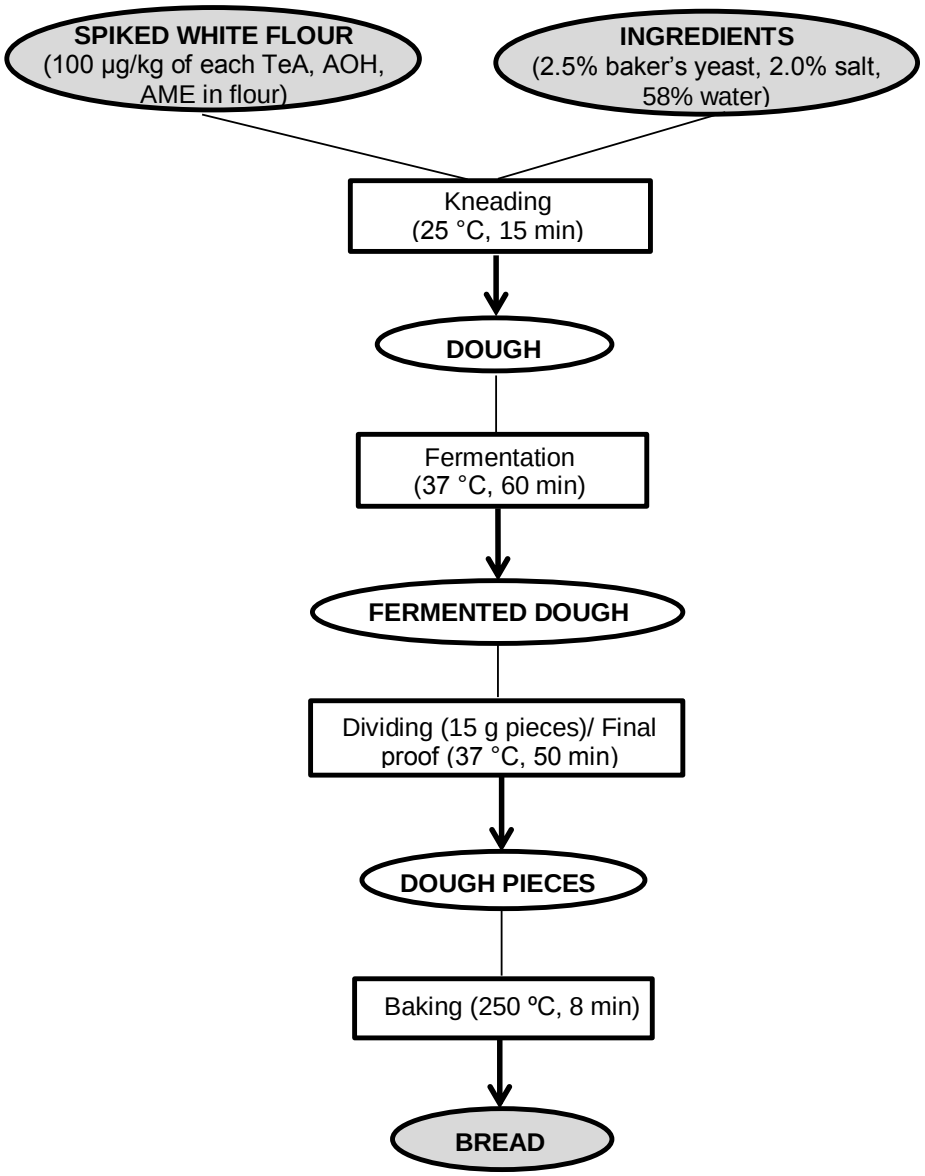

Figure 1. Scheme of the bread - making process

LC-MS/MS analysis

Alternaria toxins were quantified using our previously published method without any modifications (Janić Hajnal et al., 2015), including the equipment, chemicals and reagents.

Method validation

The developed LC-MS/MS method was validated according to an in-house quality control procedure following the guidelines of Commission Decision EC 657/2002 (European Commission, 2002). Method validation was performed in terms of matrix effects, linearity, trueness, precision (repeatability), limit of detection (LOD) and limit of quantification (LOQ). For linearity studies, the calibration curves for all of the compounds (TeA, AOH and AME) in pure solvent (solvent calibration, SC) and in matrix (matrix-matched calibration, MMC) were obtained by plotting the peak areas against the concentrations of the corresponding calibration standards at eight calibration levels in the ranges present in the Table 1. The linearity of calibration curves was expressed by the correlation coefficient (r^2). For overall method recovery assessment, the blank white wheat flour, fermented dough and bread samples were spiked in triplicate, over the ranges present in Table 1 for TeA, AOH and AME, respectively (eight -point R_A). Spiked samples were left overnight at room temperature to allow solvent evaporation and equilibration between analytes and matrix, and were analyzed using matrix-matched calibration curve. For the MMC curves, the blank white wheat flour, lyophilized fermented dough and bread samples were enriched with working standard solutions at the final reconstitution step, confirming linearity over the ranges present in the Table 1. To differentiate between extraction efficiency and matrix-induced signal suppression/enhancement, the slope ratios of the linear calibration functions were calculated to yield the apparent recovery (R_A), that is, the overall method recovery and the signal suppression/enhancement (SSE) due to matrix effects. The recovery of the extraction step (R_E), that is, sample preparation recovery, was calculated by dividing the overall recoverry by the matrix effect as follows:

$$R_A\ (\%) = 100 \times \text{slope}_{\text{spiked sample}} / \text{slope}_{\text{liquid standard}} \quad (1)$$

$$\text{SSE}\ (\%) = 100 \times \text{slope}_{\text{matix-matched standard}} / \text{slope}_{\text{liquid standard}} \quad (2)$$

$$R_E\ (\%) = 100 \times R_A/\text{SSE} \quad (3)$$

The precision of the method was expressed in terms of repeatability, i.e. as the relative standard deviation (%RSD) of 6 replicates at three concentration levels (25.0 µg/kg, 50.0 µg/kg and 100.0 µg/kg for TeA, AOH and AME) using the spiked blank white wheat flour, fermented dough and bread samples prior to analysis and the MMC curve.

The within-laboratory reproducibility was determined by preparing and analyzing the fortified white wheat flour, fermented dough and bread samples at the same concentration levels as for the repeatability, over the course of three days, using the same instrument and by the same operator.

RESULTS AND DISCUSSION

Evaluation of the LC-MS/MS method

The validation data of the analytical method for the determination of selected *Alternaria* toxins are presented in the Table 1. During the validation study, matrix-matched calibration (MMC) standards were used to compensate for the matrix effect, i.e., signal suppression or enhancement of the studied *Alternaria* toxins in the white wheat flour, fermented dough and bread matrix. TeA showed signal enhancement in the cases of white wheat flour and fermented dough, while slight signal suppression was observed in the case of bread. AOH and AME showed signal enhancement in case of all matrices. Method exhibited good linearity, with correlation coefficients (r^2) above 0.9946.

Trueness was evaluated through recovery studies. The overall method recoveries (R_A) and the sample preparation recoveries (R_E) for target analytes were calculated as described in subsection "Method validation". It can be seen that the overall method recoveries (R_A) and the sample preparation recoveries (R_E) for all target

analytes were above 70%, with the exception of R_A of TeA in bread. Precision for white wheat flour, fermented dough and bread samples, expressed as the repeatability and within-laboratory reproducibility, gave RSD values within the range of 3.8–10.9% and 5.1–13.8%, respectively, fulfilling the criteria of RSD ≤20% and indicating a good precision of the developed method (Table 2).

Table 1.
Recovery data of the employed analytical method based on matrix-matched (RE) calibration curves and matrix effect (SSE)

Analytes	Spiking level* (µg/kg)	R_A (%)	R_E (%)	SSE (%)	LOD/LOQ (µg/kg)
			wheat flour		
TeA	7.5 - 100	111.7	74.6	149.8	1.7/5.0
AOH	2.5 - 100	104.5	72.4	144.4	0.50/1.6
AME	1.0 - 100	101.9	81.7	124.7	0.30/0.90
			fermented dough		
TeA	7.5 - 100	89.7	75.7	118.6	2.0/6.0
AOH	2.5 - 100	78.7	71.0	110.9	0.62/1.8
AME	1.0 - 100	82.9	79.8	103.9	0.35/1.0
			bread		
TeA	7.5 - 100	61.9	71.8	86.2	2.5/7.5
AOH	2.5 - 100	93.3	75.6	123.4	0.57/1.7
AME	1.0 - 100	101.3	80.4	126.1	0.30/0.90

*TeA – tenuazonic acid; AOH – alternariol; AME – alternariol monomethyl ether; R_A -Overall method recovery (%); R_E-Sample preparation recovery (%); SSE-matrix effect (%); *Range of concentration of analytes for standard; matrix matched calibration curves and calibration curve of spiked samples (µg/kg)*

Table 2.
Precision parameters of selected *Alternaria* toxins

	TeA		**AOH**		**AME**	
Spiking level (µg/kg)	**RSD (%)* (n=6)**	**RSDs (%)** (n=3X6)**	**RSD (%)* (n=6)**	**RSDs (%)** (n=3X6)**	**RSD (%)* (n=6)**	**RSDs (%)** (n=3X6)**
			wheat flour			
25	10.9	13.8	9.0	9.3	7.6	9.1
50	8.5	9.4	5.6	6.9	5.2	6.8
100	7.9	8.7	5.2	6.1	3.8	5.2
			fermented dough			
25	9.1	10.4	9.4	10.8	7.2	8.1
50	7.7	9.8	6.9	7.6	6.7	7.4
100	6.5	6.8	5.3	6.1	4.6	5.5
			bread			
25	8.7	9.3	8.5	9.3	6.8	7.2
50	7.2	8.8	5.3	6.1	4.9	5.7
100	6.1	6.7	4.8	5.6	4.4	5.1

*TeA - tenuazonic acid; AOH – alternariol; AME - alternariol monomethyl ether; ***** Repeatability (%); ****** Within-laboratory reproducibility (%)*

Based on the obtained validation parameters, the developed method was successfully validated according to the criteria specified in Commission Decision EC/657/2002 (EC, 2002) for a quantitative confirmation method.

Determination of *Alternaria* toxins content

Alternaria toxins were quantified by external matrix - matched calibration procedure (separate calibrations were prepared for white wheat flour, fermented dough and bread samples). The obtained results were corrected for sample preparation recovery (R_E), and were expressed on a dry matter basis. All samples were prepared and analyzed in triplicates.

The changes in TeA, AOH and AME content during dough fermentation (fermented and final proofed dough) and in the final bread compared to its amount in the dough after kneading (0 min) are illustrated in Figure 2. As can be noticed, after fermentation of dough for 60 min TeA was reduced by 27.3%, while after proofing of dough pieces for 50 minutes its content was reduced by additional 2.0% in comparison to the TeA content in dough after kneading (0 min). Regarding the fate of AOH, this *Alternaria* toxin was reduced by 35.4% during fermentation process for 60 minutes, while after proofing of the dough pieces for 50 minutes the final reduction rate of AOH was 31.8%. Content of AME in dough after fermentation for 60 min was slightly increased (3.9%), while after proofing of dough pieces, the AME content increased to 13% compared to its amount in the dough after kneading (0 min).

After baking (Figure 2) the content of TeA and AME in bread was almost at the same level as in the raw material, while content of AOH was reduced by 34.8% compared with their concentrations in dough after kneading (0 min).

The obtained results in this study could not be completely compared to the published data, since to the best of authors' knowledge there is no previously published study regarding the fate of *Alternaria* toxins during bread-making process by real conditions on micro-scale level.

The findings of this study regarding the effect of baking process (thermal process) in terms of the behavior of AME is in agreement with the results published by Siegel et al. (2010). Namely, according to the authors, after wet baking conditions for 45–60 minutes at 200 °C, or for 30–45 minutes at 230 °C, no degradation of examined *Alternaria* toxins (AOH, AME and ALT) was observed, while the fate of TeA was not investigated in this study. However, unlike to the results obtained by Siegel et al, (2010), in our study during the bread-making process at real conditions there was a decrease (34.8%) of AOH content in the final bakery product.

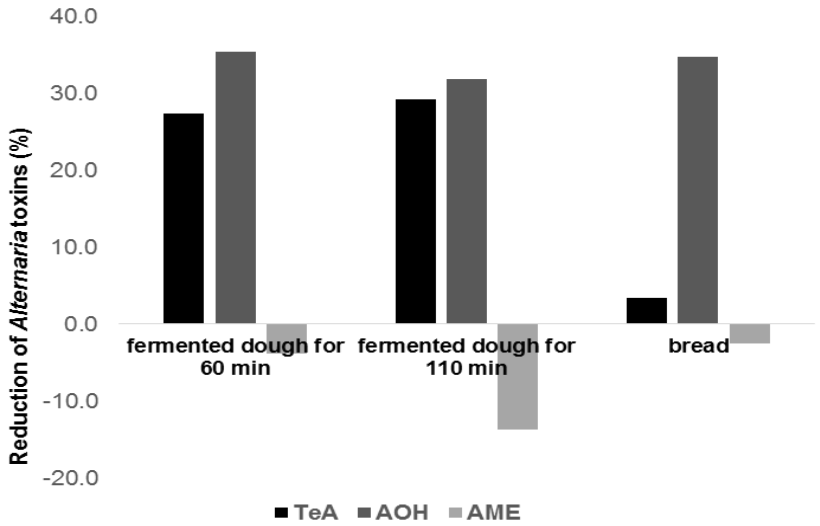

Figure 2. Reduction of *Alternaria* toxins during dough fermentation and baking process compared to their content in dough after kneading (0 min)

Based on the literature data, recently reviewed by Schaarschmidt and Fauhl-Hassek (2018) it can be noted, that the behaviors of so far studied mycotoxins (deoxynivalenol, deepoxy-deoxynivalenol, deoxynivalenol-3-glucoside, nivalenol, zearalenone, beauvericin, ochratoxin A, aflatoxin B1) during bread production in some cases are contradictory. According to this review, the high variation, which ranged from reduction to enhancement, is probably caused by different underlying processes, which are not fully understood so far. Furthermore, numerous of studies indicated, that the effect of fermentation on mycotoxins can strongly vary depending on several factors, including the present mycotoxins and their initial concentrations, the fermentation temperature and duration, the pH, the dough composition, and the microorganisms present in the dough. The effect of the baking step on the possible reduction rate of present mycotoxins is particularly dependent on the heat stability of the mycotoxins, as well as depends on the analyzed matrix (Schaarschmidt and Fauhl-Hassek 2018). If mycotoxins are incorporated into grain, effects of thermal process might be less pronounced compared to pure standards due to differences in the heat penetration and/or stabilization of the mycotoxins within the matrix (Meca et al., 2012; Yumbe-Guevara et al., 2003). On the other hand, Serrano et al. (2013) indicated that the matrix can also contribute to a more pronounced degradation of mycotoxins.

CONCLUSIONS

After dough fermentation, reduction of TeA and AOH by 29.2% and 31.8% were noted, while the AME content increased by 13.8% compared to their content in dough after kneading (0 min). After baking step (thermal process), the content of TeA and AME in bread was at the same level as in the raw material, while the content of AOH was reduced by 34.8%. Results obtained in this study represent a first report regarding to the fate of TeA, AOH, and AME during bread-making process in real conditions on micro-scale level. Future research should be related to the investigations of the fate of *Alternaria* toxins during bread-making process in the presence of lactic acid bacteria, various additives, enzymes and improvers in industrial-scale conditions using naturally contaminated wheat flour, as well as clarification of the mechanisms of the fate of these secondary metabolites during bread-making process.

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СТАБИЛНОСТ *Alternaria* ТОКСИНА ТОКОМ ПРОИЗВОДЊЕ ХЛЕБА

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Сажетак: Поступак производње хлеба може утицати на смањење садржаја микотоксина, као и на промену њихове структуре и форме (везана или слободна). Колики и какав ће утицај процеса производње хлеба бити на микотоксине, зависи и од присуства различитих компонената и/или адитива, услова ферментације, као и услова термичке обраде. Међутим, нема доступних података о стабилности и понашању *Alternaria* токсина током производње хлеба у реалним условима. Стога, главни циљ ове студије био је да се процени ефекат процеса ферментације теста и печења хлеба на понашање тенуазонске киселине (TeA), алтернариола (AOH) и алтернариол монометил етра (AME). У ту сврху, као сировина коришћено је бело пшенично брашно са додатком *Alternaria* токсина тј. „спајковано" брашно (са по 100 μg/kg TeA, AOH и AME у брашну), затим 2,5%, пекарског квасца, 2,0% соли и 58% воде, рачунато на количину брашна. „Спајковано" пшенично тесто ферментисано је 60 мин. на 37 °C, а затим је подељено у комаде од 15 g, ручно обликовано и завршно ферментисано 50 мин. на 37 °C. Након завршне ферментације тесто је печено 8 мин. на 250 °C. У свакој наведеној фази узети су узорци ферментисаног теста. Замрзнути узорци ферментисаног теста су лиофилизовани, уситњени и чувани до анализе. Исти поступак је примењен и за печени хлеб. За проучавање понашања TeA, AOH и AME током производње хлеба, коришћена је валидована метода течне хроматографије високих перформанси спрегнута са масеном спектрометријом (LC - MS/MS). Садржај TeA и AME у хлебу био је на истом нивоу као у сировини, иако је у тесту током ферментације забележено смањење садржаја TeA за 29,2% и повећање садржаја AME за 13,8%, у односу на њихов садржај у тесту након мешења (0 мин). Садржај AOH у тесту након завршне ферментације се смањио за 31,8%, док је у хлебу његов садржај био мањи за 34,8% у односу на садржај AOH у тесту након мешења (0 мин).

Кључне речи: *алтернариол, алтернариол монометил етар, тенуазонска киселина, стабилност, производња хлеба, LC-MS/MS*

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