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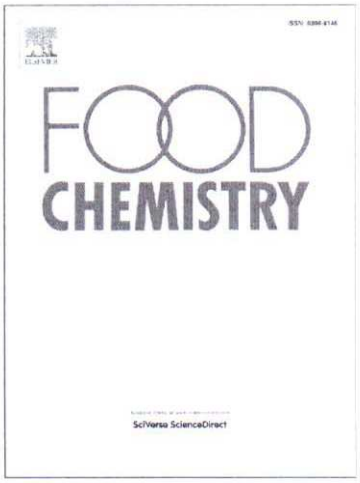

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Possibility of *Alternaria* toxins reduction by extrusion processing of whole wheat flour

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Abstract

This study represents the first report about possibility of reduction of *Alternaria* toxins in wheat using the extrusion process. Effects of extrusion processing parameters – moisture content (w=16, 20, 24 g/100g), feeding rate (q=15, 20, 25 kg/h), and screw speed (v=300, 390, 480 rpm), on reduction rate of tenuazonic acid (TeA), alternariol (AOH) and alternariol monomethyl ether (AME), in whole wheat flour. Temperature ranged between 111.1 and 160.8 °C, while the absolute pressure was from 0.17 to 0.23 MPa. The simultaneous influence of w and v was the most important for TeA reduction (p<0.05), while v and q were the most influential for AOH reduction (p<0.01). Level of AME reduction was mostly influenced by w and v (p<0.10). Optimal parameters for reduction of all three *Alternaria* toxins were as follows: w=24 g/100g, q=25 kg/h, v=390 rpm, with a reduction of 65.6% for TeA, 87.9% for AOH and 94.5% for AME.

Keywords: extrusion processing, whole wheat flour, LC-MS/MS; *Alternaria* toxins reduction

Chemical compounds studied in this article:

Alternariol (PubChem CID:5359485), Alternariol monomethyl ether (PubChem CID:5360741), Tenuazonic acid (PubChem CID:54737266).

1. Introduction

In addition to the fungi of the genus *Fusarium*, the fungi of the genus *Alternaria* are considered as another group of ubiquitous pathogens on wheat leaves and ears. Various *Alternaria* species, especially *Alternaria* (A.) *alternata* (Fr.) Keissler and *Alternaria tenuissima* ([Kunze ex Nees et T. Nees: Fries] Wiltshire), are frequently associated with several plant diseases of small grain cereals, for example, black point, kernel and leaf blight (Logrieco, Moretti, & Solfrizzo, 2009; Ostry, 2008; Patriarca, Azcarate, Terminiello, & Fernandez Pinto, 2007). Both species are capable to produce a variety of mycotoxins belonging to several classes of chemical compounds (Montemurro, & Visconti, 1992; Ostry, 2008; Visconti, & Sibilia, 1994). The most important *Alternaria* mycotoxins can be grouped into three different structural classes: dibenzopyrone derivatives – alternariol (AOH), alternariol monomethyl ether (AME) and altenuene (ALT); tenuazonic acid (TeA), a tetramic acid derivative, and altertoxins I (ALX-I), altertoxin II (ALX-II) and alertoxin III (ALX-III), which are perylene derivatives. Chemical characteristics and potential producers were reviewed by Montemurro, & Visconti (1992), Visconti, & Sibilia (1994), and Ostry (2008). These toxins show cytotoxic, fetotoxic and/or teratogenic activity, they are mutagenic, clastogenic and oestrogenic in microbial and mammalian cell systems and tumorigenic in rats, and they inhibit the cell proliferation (Brugger et al., 2006; Lehmann, Wagner, & Metzler, 2006; Logrieco, Moretti, & Solfrizzo, 2009; Tiemann et al., 2009). The occurrence of these mycotoxins in cereals as well as in fruits and vegetables has been reported repeatedly (Asam, Konitzer, & Rychlik, 2011; Asam, Lichtenegger, Liu, & Rychlik, 2012; EFSA, 2011; Janić Hajnal et al., 2015; Logrieco, Moretti, & Solfrizzo, 2009; Müller, & Korrn, 2013; Siegel, Rasenko, Koch & Nehls, 2009; Uhlig et al., 2013), but so far there are no specific international regulations nor any national regulation in the world for any of the

Alternaria toxins in food and feed. Quantitative and qualitative studies on degradation of various mycotoxins are frequently published and reviewed (Bullerman, & Bianchini, 2007; Kabak, 2009; Kushiro, 2008; Milani, & Maleki, 2014). However, only scarce information on the stability and fate of *Alternaria* toxins during processing of food and feed is available (summarized by EFSA, 2011). The stability of alternariol (AOH), alternariol monomethyl ether (AME) and altenuene was investigated under various baking conditions, using flour spiked with the toxins in the presence (wet baking) or absence (dry baking) of water (Siegel, Feist, Proske, Koch, & Nehls, 2010). Under the most realistic baking conditions (wet baking for 45-60 minutes at 200 °C, or for 30-45 minutes at 230 °C), no degradation was observed and the three toxins were recovered quantitatively. In contrast, a pronounced degradation was observed upon dry baking. Degradation after dry baking at 230 °C for 1 hour was about 90% for ALT, 70% for AOH, and 50% for AME. None of degradation products found was after refluxing in neutral solutions (Siegel, Feist, Proske, Koch, & Nehls, 2010). Combina et al. (1999) investigated the effect of heat treatment on the stability of AOH, AME and TeA at 100 °C, 115 °C and 121 °C in sunflower flour, which is used as a protein supplement to balance mixed feed rations for poultry and pigs. During humid heat treatment at 100 °C, the concentrations of AOH and AME remained constant for up to 90 minutes, while TeA level decreased with time to 50% of the initial concentration after 90 minutes. When humid heat and pressure treatment were employed, the concentrations of AOH, AME and TeA decreased. The most effective treatment in reducing AOH, AME and TeA levels was heating at 121 °C and 0.1 MPa for 60 minutes. Under these conditions a reduction of the toxin levels of 100%, 75% and 67% was observed for AME, AOH and TeA, respectively. Information on the chemical fate of the toxins was not given. Extrusion processing is used for producing a range of cereal products such as breakfast

foods, snacks and animal feed The food extruder is a device which expedites the shaping and restructuring process for food ingredients. The extrusion process utilizes a combination of high temperature, high pressure, and severe shear forces which results in chemical changes and modifications, including protein denaturaion, starch gelatinization, polymer cross-linking and Maillard reactions both for food components and contaminants. The type of extruder may affect chemical reactions within the processed material. Extruders with longer barrels have relatively longer residence times than short barrel extruders. On the other hand, reduction of barrel length may lead to decrease of operational and capital costs (Riaz, 2000). Extrusion processing can not only improve the quality by modifying the texture or increasing the digestibility of the processed products; it also has an effect on the levels of mycotoxins found in the final product (Wu, Lohrey, Cramer, Yuan, & Humpf, 2011). For instance, extrusion has been shown to promote the degradation of a range of *Fusarium* mycotoxins (Humpf, & Voss, 2004; Jackson et al., 2011; Scudamore, Guy, Kelleher, & MacDonald, 2008; Wu, Lohrey, Cramer, Yuan, & Humpf, 2011), and thus might also favor the degradation of *Alternaria* toxins. The reduction level of concentration of mycotoxins during extrusion processing is largely dependent on several factors, including the type of extruder, extruder temperature, screw speed, moisture content of the extrusion mixture and residence time in the extruder (Bullerman, & Bianchini, 2007), as well as the type of mycotoxin and its initial concentration in the raw material.

To the best of our knowledge, there are no data about the stability and fate of *Alternaria* toxins during extrusion processing. Thus, the main objective of this study was to investigate the influence of extrusion processing parameters – moisture content (w), feeding rate (q), and screw speed (v) – on reduction rate of TeA, AOH and AME, as well as on extrusion temperature (T), absolute pressure (p), mean residence time (t_r)

and specific energy consumption (E). Response surface methodology (RSM) was used as an effective tool for optimizing a variety of processes (Ahmad, Ahmad, Hamid, Abdin, & Javed, S., 2013; Zheng, Wei, Xu, & Fan, 2015).

Experimental results were subjected to analysis of variance (ANOVA) to show relations between applied assays. Principal Component Analysis (PCA) was applied to the experimental data (used as descriptors) to characterize and differentiate among the observed samples. In order to enable more comprehensive comparison between investigated samples, standard score analysis (SS) was introduced.

2. Material and methods

2.1 Material

The whole wheat flour sample (400 kg) was prepared by milling with a pilot scale mill stone from wheat (*Triticum aestivum* cv. Suba) with visible discoloration called black point, that indicate potential fungal infestation by *Alternaria* spp. The batches of whole meal flour were well mixed in twin shaft paddle mixer (Model SLHSJ0.2A, Muyang, China) before taking samples for analysis and before extrusion. Mixing homogeneity of whole meal flour was assured by Microtracer® method, using external tracers for mixing homogeneity testing (Clark, Behnke, & Poole, 2007), and also, eight subsamples were taken for analysis of investigated *Alternaria* toxins levels.

2.2 Extrusion conditions

Extrusion processing of the whole wheat flour was done in a simple pilot single screw extruder (OEE 8, Amandus Kahl, Germany), with short L:D (length: diamater) ratio (7.5:1) and with no jacket heating or cooling. Parameters that were varied during the extrusion processing were: moisture content of the whole wheat flour (w), feeding rate

of material into the extruder barrel (q), and extruder screw speed (v). Levels of extrusion parameters (Table 1) were set according to applied experimental design. For each of the treatments about 25 kg of the whole wheat flour was extruded. For measurement of extruder barrel temperature (T), Pt100 probe was used, which was positioned at the end section of the barrel. Specific energy consumption (E) was determined by reading out the energy consumption value of extruder display. The mean residence time of the extrusion process (t_r) was determined by introducing a tracer at the extruder inlet and measuring the tracer concentration at the die, according to the method of Altomare & Ghossi, (1986). Extruded products were cooled in the vibro cooler (FB 500x200, Amandus Kahl, Germany) for 10 minutes at the temperature of 20 °C and stored under room conditions.

2.3 Chemicals and reagents

Alternariol (AOH) (purity 99.0%), alternariol monomethyl ether (AME) (purity 99.5%) and tenuazonic acid (TeA) (purity 99.5%) standards were purchased from Sigma Aldrich (St. Louis, MO, USA). Stock solutions of AOH, AME and TeA were prepared in methanol and stored at −20°C. The following solvents were used: methanol (MeOH) (JT Baker, Deventer, Netherlands) and ethyl acetate (EtOAc) (Sigma-Aldrich), all LC-MS grade, formic acid (FA) (purity 99.9%) (Carlo Erba, Milan, Italy), and fuming HCl (37%), pa (Merck, Darmstadt, Germany). Deionised water was sourced from a Millipore Simplicity UV water purification system (Bedford, MA, USA).

2.4 Moisture content

Moisture content in whole wheat flour sample and in extruded product samples was determined according to ISO 712/2009, and was expressed on the dry basis.

2.5 Sample preparation

The method of sample preparation from Siegel Feist, Proske, Koch, & Nehls (2010) was slightly modified. The modified method used to prepare the extracts of the wheat flours was previously described by Janić Hajnal et al. (2015) for *Alternaria* toxins analysis by high performance liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and a same method of sample preparation was used for extruded product samples, also.

2.6 LC-MS/MS analysis

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

2.7 Method validation

The developed method was validated according to an in-house quality control procedure following the guidelines of Commission Decision EC 657/2002 (EC 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 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 ten calibration levels ranging from LOD to 100.0 µg/kg for TeA, AOH and AME, respectively. The linearity of calibration curves was expressed by the correlation coefficient (r^2). For overall method recovery assessment, the blank whole wheat flour and extruded product samples were spiked in triplicate, over the

range from 25.0 µg/kg to 100.0 µg/kg for TeA and from 2.5 µg/kg to 10.0 µg/kg for AOH and AME (four-point *R*A) (Table 2). 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 matrix-matched calibration curves (MMC), the blank whole wheat flour and extruded product samples were enriched with working standard solutions at the final reconstitution step, confirming linearity over the range from the 25.0 µg/kg to 100.0 µg/kg for TeA and from the 2.5 µg/kg to 10.0 µg/kg for AOH and AME (four-point MMC). The calibration curve in pure solvent (four-point SC) was prepared over the same range as well as for *R*A and MMC for all of the compounds, and was used for matrix effects evaluation. 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 recovery by the matrix effect as follows (modified after Matuszewski et al., 2003):

$$R_A(\%) = 100 \times \frac{a_{SP}}{a_{SC}} \quad (1)$$

$$SSE(\%) = 100 \times \frac{a_{MMC}}{a_{SC}} \quad (2)$$

$$R_E(\%) = 100 \times \frac{a_{SP}}{a_{MMC}} \quad (3)$$

where a_{MMC} is the slope of matrix-matched calibration, a_{SC} is the slope of solvent calibration, and a_{SP} is the slope of spiked sample-prepared curve.

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 and 2.5 µg/kg, 5 µg/kg and 10 µg/kg for AOH and AME) using the spiked blank whole wheat flour and extruded product samples prior to analysis using the MMC curve.

The within-laboratory reproducibility was determined by preparing and analyzing the fortified whole wheat flour and extruded product samples at the same concentration levels as for the repeatability, over the course of three days, using the same instrument and by the same operators.

The limit of detection (LOD) and limit of quantification (LOQ) were estimated by injecting decreasing concentrations of matrix-matched standards, and determining levels, resulting in and signal-to-noise ratio (S/N) of ≥3 and ≥10 for the LOD and LOQ, respectively (ICH, 2005).

2.8 Statistical analysis

The experimental data used for the analysis were derived using the Box and Behnken's fractional factorial (3 level-3 parameter) design, 1 block, according to RSM (Anonymous, 2015). The Box-Behnken's design was introduced in order to limit the sample size to a value of 15 with only one repetition (with 12 cases that describe the high and low factor levels and the 3 repetitions of central points) which is statistically correct for the estimation of the coefficients in a second degree least squares approximating polynomial. The main advantage of RSM is a reduced number of experimental runs that provide sufficient information for statistically valid results. The RSM equations describe the effects of the test variables on the observed responses, determine test variables interrelationships and represent the combined effect of all test variables in the observed responses, enabling the experimenter to make efficient exploration of the

process (Ahmad, Ahmad, Hamid, Abdin, & Javed, 2013; Anonymous, 2015; Zheng, Wei, Xu, Fan, 2015).

The following second order polynomial (SOP) model was fitted to the experimental data. Six models of the following form were developed to relate seven responses (Y) and three process variables (X), for each of the different mixtures:

$$Y_k^l = \beta_{k0}^l + \sum_{i=1}^{2} \beta_{ki}^l \cdot X_i + \sum_{i=1}^{2} \beta_{kii}^l \cdot X_i^2 + \beta_{k12}^l \cdot X_1 \cdot X_2, \; k=1\text{-}7, \; l=1\text{-}p, \qquad (4)$$

where: β_{k0}^l, β_{ki}^l, β_{kii}^l, β_{k12}^l are constant regression coefficients; Y_k^l, either: TeA, AOH, AME, as well as absolute pressure (p), temperature (T), mean residence time (t_r) and specific energy consumption (E); while X_1 - w; X_2 - q; X_3 - v.

Descriptive statistical analyses of all the obtained results were expressed by means, for each treatment. Collected data were subjected to ANOVA to explore the effects of process variables. Furthermore, pattern recognition technique PCA was applied successfully to classify and discriminate the different samples. The evaluation of RSM, ANOVA and PCA of the obtained results was performed using Statistica software version 12 (StatSoft Inc. 2013, USA)®.

2.7.1 Determination of normalized standard scores

In order to get a more complex observation of the ranking of observed mixtures, SS are evaluated using chemometric approach by experimentally measured TeA, AOH and AME. Min-max normalization is one of the most widely used technique to compare various characteristics of complex samples determined using multiple measurements, where samples are ranked based on the ratio of raw data and extreme values of the measurement used (Brlek et al., 2013). Since the units and the scale of the data from various physical and chemical characteristics are different, the data in each data set

should be transformed into normalized scores, dimensionless quantity, according to following equations:

$$\overline{x}_i = \frac{\max_i x_i - x_i}{\max_i x_i - \min_i x_i}, \quad \forall i \text{, where } x_i \text{ represents the raw data.}$$

The sum of normalized scores of a sample of different measurements when averaged give a single unitless value termed as SS, which is a specific combination of data from different measuring methods with no unit limitation. This approach also enables the ease of employing some others set of samples to this elaboration in the future comparisons.

3. Results and discussion

3.1 Evaluation of the LC-MS/MS method

The validation data of the analytical method for the determination of selected *Alternaria* toxins are given in Table 2. 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 whole wheat flour and extrusion products matrix. Tenuazonic acid showed signal enhancement, while slight signal suppression was observed for AME in both matrix. Alternariol showed slight signal suppression in whole wheat flour samples, while in extrusion product samples showed slight signal enhancement. Method exhibited good linearity, with correlation coefficients (r^2) above 0.9918.

Trueness was evaluated through recovery studies. The overall method recoveries and the sample preparation recoveries for target analytes were calculated as described in subsection *2.7*. It can be seen that the overall method recoveries and the sample preparation recoveries for all target analytes were above 70%.

Precision for whole wheat flour and extruded product samples, expressed as the repeatability and within-laboratory reproducibility, gave RSD values within the range of 5.5–16.0% and 8.0–18.1%, respectively, fulfilling the criteria of RSD ≤20% and indicating a good precision of the developed method (SupplementaryTable 1).

LODs and LOQs for whole wheat flour were 2.5 µg/kg and 7.5 µg/kg for TeA, 0.75 µg/kg and 2.5 µg/kg for AOH and 0.1 µg/kg and 0.3 µg/kg for AME, respectively. For the extruded products LODs and LOQs were 1 µg/kg and 5 µg/kg for TeA, 0.5 µg/kg and 2.0 µg/kg for AOH and 0.1 µg/kg and 0.3 µg/kg for AME, respectively.

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.

3.2 Determination of Alternaria *toxins content*

Alternaria toxins were quantified by external matrix-matched calibration procedure (separate calibrations were prepared for both whole wheat flour and extruded product samples). The obtained results were corrected for sample preparation recovery, and were expressed on a dry matter basis. Initial water content on a dry weight basis was 12.4 g/100g in naturally contaminated whole wheat flour, while initial concentrations (average values of eight measurements) of selected *Alternaria* toxins expressed on a dry matter basis were 133±11.7 µg/kg, 4.12±0.47 µg/kg and 3.62±0.40 µg/kg for TeA, AOH and AME, respectively. All extruded product samples were analyzed in triplicate. The water content of extruded product samples were ranged from 10.21 to 15.94 g/100g on a dry weight basis, while the final concentration expressed on dry matter basis of selected *Alternaria* toxins in extruded product samples were ranged from 45.75 to 79.42 µg/kg

for TeA, from < 0.50 to 1.13 µg/kg for AOH, and from 0.20 to 1.34 µg/kg for AME, respectively.

3.3 Reduction of Alternaria *toxins by extrusion processing*

The effect of extrusion process variables (*w*, *q*, and *v*) on observed responses (TeA, AOH, AME reduction rate, p, T, t_r and E) was determined (Table 3). Reduction of *Alternaria* toxins during the extrusion process is expressed as a percentage reduction in relation to its initial concentration in the whole wheat flour, and it is used as such in all the performed statistical analyses. Process variables (*w*, *q*, and *v*) were varied according to Box-Behnken experimental design (Table 3) and range of observed responses was: absolute pressure (p) from $1.7x10^5$ to $2.3 x10^5$ Pa, temperature (T) from 111.1 to 160.8 °C, specific energy consumption (E) from 63.7 to 108.4 kWh/t and mean residence time (t_r) in the range of 22.5 to 38.3 s. Reduction of all investigated *Alternaria* toxins was achieved in all trials (extruded product samples) (Table 3). Reduction of TeA ranged from 40.3% to 65.6%, while for AOH and AME ranged from 72.7% to 87.9%, and from 62.8% to 94.5%, respectively.

In order to characterize and differentiate among the observed samples pattern recognition technique (Principal Component Analysis - PCA) was applied to the experimental data (used as descriptors). The PCA allows a considerable reduction in a number of variables and the detection of structure in the relationship between measuring parameters, and selected responses. Prior to PCA, data were autoscaled (each value is subtracted from the mean value, and the quotient is divided by standard deviation). All samples (trials) were produced under various process parameters as shown by experimental design (Table 3) and predicted by PCA score plot (Figure 1). For visualizing the data trends and the discriminating efficiency of the used descriptors a

scatter plot of samples, using the first two principal components (PCs) issued from PCA of the data matrix, was obtained (Figure 1). The first two principal components explained 67.16% of the total variability, which can be considered sufficient for data representation (considering the proportion of the first three Eigenvalues: 3.21, 1.49 and 1.21, respectively) (Anonysmous, 2016). T (which contributed 27.6% of the total variability), E (14.0%), p (18.6%), t_r (25.1%) and TeA (11.3%) were the most influential for the first factor coordinate calculation, while the contribution of E (25.6%),AME (22.0%), and TeA (36.7%) were the most important variables for the second factor coordinate calculation. On the other hand, AOH was most influential for calculation of the third principal component (55.1%), while the influence of AME was also noticeable (34.7%). The influence of processing parameters can be observed in Figure 1, with higher specific energy consumption at the upper left side of graph and more AME, TeA and AOH reduction on the upper side of the graph. Reduction of TeA content was more influenced by initial moisture content (w) of whole wheat flour opposite to AOH which reduction rate negatively correlated with this input parameter. Reduction of AOH was more influenced by feeding rate (q) and screw speed (v). This means that at a lower moisture content of whole wheat flour (16%) and with an increasing feeding rate (q) and screw speed (v), a greater reduction in content of AOH would be achieved. Also, Figure 1 shows that reduction of AME was influenced mainly by screw speed (v). PCA graph showed good discrimination between all trials, which were found different due to variations in process responses (p, T, E, t_r), in the extruder barrel and reduction rate of TeA, AME and AOH in trials. Trial 3 showed to be very influenced by process parameters, showing the greatest reduction of AME (94.5%) and TeA (65.6%), which is also in accordance with SS results (Table 3).

Furthermore, to explore the effects of process variables the collected data were subjected to ANOVA. ANOVA shows the significant effects of independent variables to the responses (Table 4). The SOP models for all variables were found to be statistically significant and the response surfaces were fitted to these models. Linear terms of w and q were the most influential variables in T calculation (statistically significant, at $p<0.01$ level, 95% confidence limit), while the quadratic terms of w and v, as well as the linear term of v were also very important for T calculation (statistically significant, at $p<0.05$ level). Linear terms of w and v were the most important variables in E calculation ($p<0.01$), while the quadratic term of v was statistically significant at $p<0.10$ level. Linear terms of w and q were important in p calculation, while the interchange term of $w \times q$ was the most important term for p calculation ($p<0.10$). The linear term of w was the most important term for t_r calculation, but the influence of the linear terms of q and v was also noticed, statistically significant at $p<0.01$ level. It was observed that increase of moisture content (w) in raw material used for extrusion caused the decrease of temperature and specific energy consumption of extruder due to the lubricating effect of water which decreased friction in the extruder barrel. Increase of the feeding rate (q) increased the degree of fill, which subsequently caused pressure and temperature increase (Ding et al., 2005). Increase of the screw speed (v) intensified the forces of shearing, torsion and friction. Therefore, a screw speed increase caused significant increase of extruder barrel temperature ($p<0.05$) with significant increase in specific energy consumption of extruder ($p<0.01$).

Concerning the influence of processing parameters on the reduction rate of mycotoxins, interchange term of $w \times q$ was the most important for TeA reduction calculation (statistically significant, at $p<0.05$ level), while the linear terms of w, q and v were also important ($p<0.10$). The highest reduction rate of TeA (65.6%) was

obtained at the highest moisture content (24 g/100g), the highest feeding rate (25 kg/h), and medium screw speed (390 rpm). This suggests that heat transfer was better due to the higher moisture content of raw material which enhanced mechanism of TeA degradation even though the temperature was lower when moisture content was higher. Although moderate screw speed decreases barrel temperature in comparison with high screw speed, it also prolongs material mean residence time in the extruder barrel (Wu et al., 2008), as can be seen from Fig. 1, Table 3 and 4, which promoted degradation of TeA.

The linear terms of q and v, and the quadratic term of q were the most influential variables in AOH reduction calculation ($p<0.01$), while the quadratic term of w and interchange terms of $w \times q$ and $w \times v$ were also influential for AOH calculation (statistically significant, at $p<0.05$ level). According to Figure 1, AOH reduction was the highest under the most severe extrusion conditions, i.e. the highest extruder barrel temperature, pressure, and specific energy consumption of extruder, caused by low moisture content of extruded material and by high screw speed. Similarly to the results for TeA reduction, high level of AOH reduction was also obtained when raw material with high moisture content was extruded in combination with high feeding rate (q) and low or middle screw speed (v). This could also be related to more effective heat transfer through an extruded material due to higher moisture content, regardless of the lower temperature in the extruder barrel. Additionally, longer residence time of material in the extruder barrel caused by lower screw speed and increased degree of fill with higher feeding rate, contributed to AOH reduction. The calculation of AME reduction was influenced by the linear term of w and the quadratic term of v (statistically significant, at $p<0.10$ level). Level of AME reduction was generally higher comparing to TeA and AOH, ranging from 62.8 to 94.5%, and mostly influenced by moisture content of raw

material and screw speed of the extruder. Higher moisture content of raw material and higher screw speed of extruder results in higher reduction of AME.

The residual variance is also shown in Table 4. All SOP models had an insignificant lack of fit tests, which means that all the models represented the data satisfactorily. The r^2 values for p (0.804), T (0.969), E (0.936), t_r (0.989), TeA (0.840), AOH (0.964) and AME (0.606) were very good and show the good fit of the model to experimental results.

Using these models, due to the complex influence of extrusion process parameters on the level of reduction of investigated *Alternaria* toxins, the contour plots of TeA, AOH and AME reduction was plotted and superimposed to ascertain the optimum processing conditions (Figure 2). Optimization of the process is performed to ensure rapid processing conditions, with high TeA, AOH and AME reduction and a high throughput. The optimum operating area was derived with a few iterative steps in finding processing parameters that gave the highest reduction of TeA (60-65%), AOH (90%) and AME (95%). Optimal area is marked in yellow color, by approximating the optimum position in obtained area on the graph (Figure 2). Contour plots of TeA and AOH reduction showed that maximum values were obtained at the lower right side of the graph, while AME reduction tends to its maximum at the upper right side of the graphic. The optimum conditions are as follows: w=24 g/100g, q=25 kg/h, v=390 rpm.

Since the level of reduction of the examined *Alternaria* toxins was influenced by so many parameters and they were altered as the technological treatments change, standard score analysis (SS) was applied in order to enable more comprehensive comparison between investigated samples. Standard scores analysis showed that the optimum values for TeA, AOH, AME characteristics were experienced with trial 3, with

w=24 g/100g, q=25 kg/h, v=390 rpm (SS was 1.00). The SS analysis results coincide very well with the results gained by RSM.

4. Conclusions

It can be seen that similar results were obtained with all statistical analyses, pointing out that for trial 3 the best score (SS was 1.00) was gained. The highest reduction of all three *Alternaria* toxins was achieved when high raw material moisture (w=24 g/100g), high feeding rate (q=25 kg/h) and medium screw speed (v=390 rpm) were applied. Under these extrusion conditions, a reduction of 65.6%, 87.9% and 94.5%, for TeA, AOH and AME, respectively, was achieved. In final extruded product (trial 3) the concentration of TeA, AOH and AME was 45.75±1.37, <0.5 and 0.20±0.03 μg/kg on dry basis, respectively. The results of this study suggest the possibility of reduction of *Alternaria* toxins content in whole wheat flour by extrusion processing. Since the optimal conditions on the simple single-screw extruder used for this study were effective for *Alternaria* toxins reduction, it is most likely that the effectiveness of optimized extrusion conditions can be expected on more advanced types of extruders, such as polytropic single-screw or twin-screw extruder. Certainly, the effectiveness of other types of extruders should be confirmed in future studies. Furthermore, the functional properties of the extruded wheat flour were not the subject of this study and should be investigated in the future research. Certainly, the future research should be related to the monitoring of the reduction of *Alternaria* toxins in extruded products in a broad range of its concentrations in wheat, as well as monitoring of the possible degradation products of *Alternaria* toxins in wheat extruded products by optimal condition of the extrusion process. It should be emphasized that the best strategy would be not to have mycotoxins at all in the initial and final products. For that reason, the use of Good Agricultural and Manufacturing Practices (GAP/GMO) in the field is strongly

recommended as the first approach to control these contaminants in cereal products. Certainly, if they are present, the best technology and regulations should be placed into effect.

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Figure captions

Figure 1. Biplot graphic of reduction of *Alternaria* toxins by extrusion processing of whole wheat flour

Abbreviations: *w* - moisture content, *q* - feeding rate, *v* - screw speed, p – absolute pressure, T - temperature, tr – mean residence time, E - specific energy consumption, TeA - tenuazonic acid, AOH - alternariol, AME - alternariol monomethyl ether

Figure 2. Optimum region obtained after superimposing the contour plots of the system response

Abbreviations: *w* - moisture content, *q* - feeding rate, *v* - screw speed, TeA - tenuazonic acid, AOH - alternariol, AME - alternariol monomethyl ether

Fig. 1

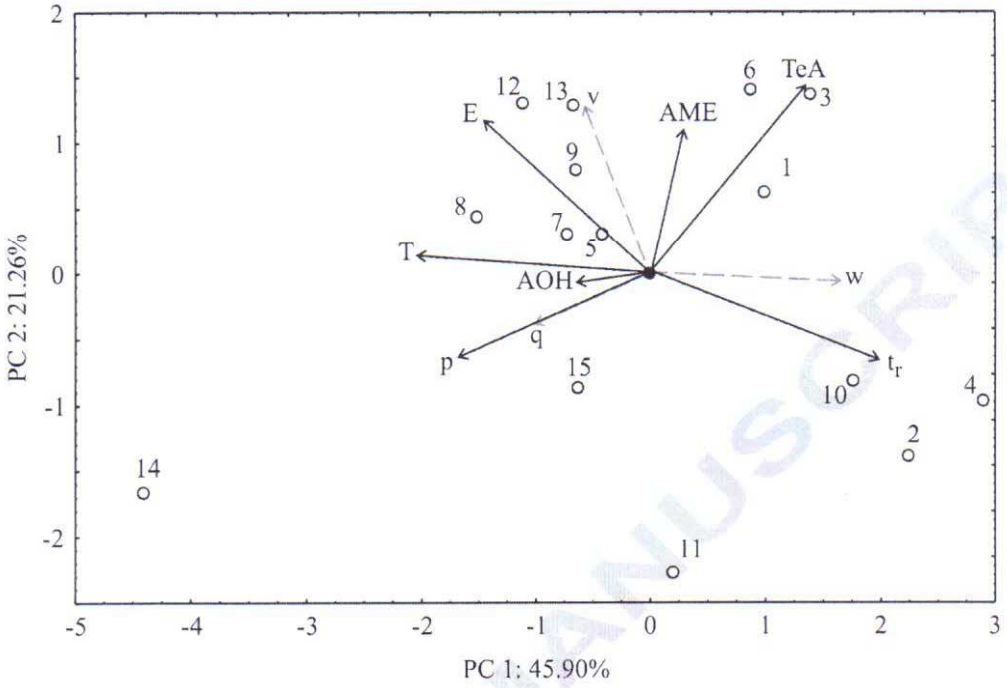

Fig. 2

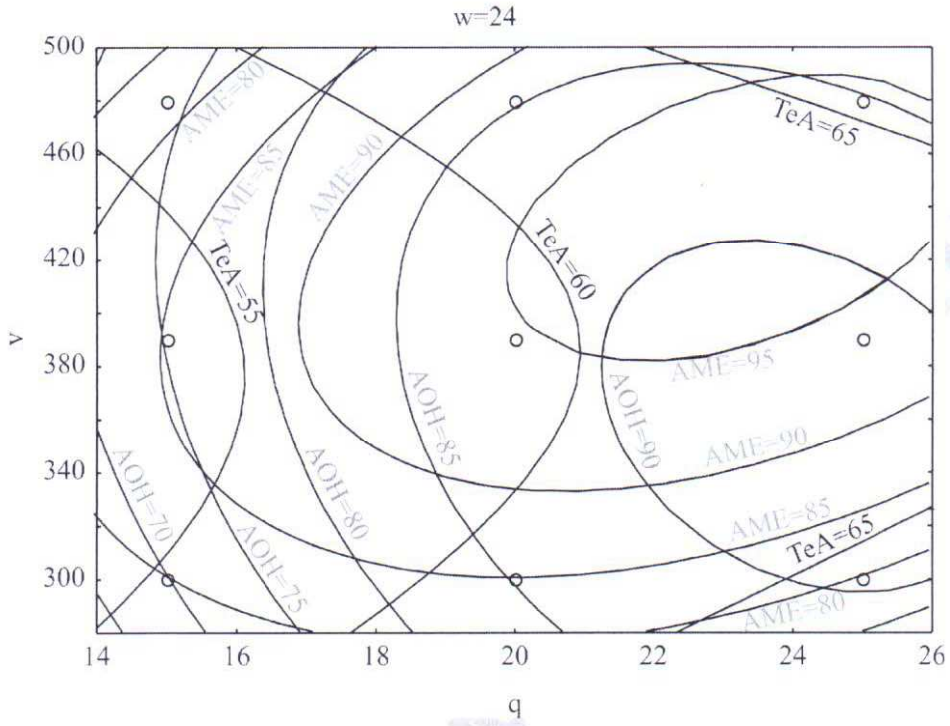

Table 1. Independent extrusion parameters and their levels

	Factor's level		
Experimental factor	(low)	(center)	(high)
Moisture content (%)	16	20	24
Feeding rate (kg/h)	15	20	25
Extruder screw speed (rpm)	300	390	480

Table 2. Recovery data of the employed analytical method based on solvent (R_A) and matrix-matched (R_E) calibration curves and matrix effect (SSE)

Analytes	Spiking level* (µg/kg)	Overall method recovery R_A (%)	Sample preparation recovery R_E (%)	Matrix effect SSE (%)
	whole wheat samples			
AOH	2.5-10	71.4	71.7	99.6
AME	2.5-10	70.7	76.2	92.7
TeA	25-100	87.6	70.0	125.3
	extruded products			
AOH	2.5-10	89.1	82.6	107.9
AME	2.5-10	70.6	75.3	93.8
TeA	25-100	114.4	84.2	136.0

Abbreviations: TeA - tenuazonic acid, AOH - alternariol, AME - alternariol monomethyl ether

* range of concentration of analytes for standard, matrix matched calibration curves and calibration curve of spiked samples (µg/kg).

Table 3. Descriptive statistics

Trial No.	Inputs			Responses							SS
	w (g/100g)	*q* (kg/h)	*v* (rpm)	p (Pa)	T (℃)	E (kwh/t)	t_r (s)	TeA (%)	AOH (%)	AME (%)	
1	24	20	480	1.7×10^5	113.0	92.0	30.0	59.3	87.9	91.4	0.88
2	24	15	390	1.7×10^5	111.1	81.2	37.0	55.3	72.7	77.1	0.35
3	24	25	390	1.7×10^5	113.9	92.3	30.3	65.6	87.9	94.5	1.00
4	24	20	300	1.7×10^5	111.6	63.7	38.3	60.1	87.9	92.9	0.91
5	20	20	390	1.7×10^5	142.7	89.8	27.3	55.1	87.7	89.6	0.81
6	20	15	480	1.7×10^5	125.3	99.8	29.2	64.4	74.4	87.7	0.62
7	20	20	390	1.7×10^5	143.3	92.1	26.8	51.9	87.9	93.0	0.80
8	20	25	480	1.9×10^5	150.6	95.1	23.3	56.8	78.5	85.6	0.58
9	20	20	390	1.8×10^5	142.7	99.9	29.0	58.0	86.4	90.3	0.82
10	20	15	300	1.7×10^5	116.0	82.6	34.3	55.7	74.6	82.4	0.45
11	20	25	300	1.7×10^5	129.0	80.7	30.8	50.4	87.9	62.8	0.47
12	16	20	480	1.7×10^5	146.0	108.4	23.0	62.5	80.1	74.7	0.58
13	16	15	390	1.7×10^5	139.5	106.6	27.4	59.2	87.9	87.3	0.84
14	16	25	390	2.3×10^5	160.8	101.8	22.5	40.3	87.9	82.9	0.54
15	16	20	300	1.8×10^5	136.8	87.3	27.5	52.7	87.9	78.5	0.66

Abbreviations: *w* - moisture content, *q* - feeding rate, *v* - screw speed, p - absolute pressure, T - temperature, E - specific energy consumption, t_r - mean residence time, TeA - tenuazonic acid, AOH - alternariol, AME - alternariolmonomethyl ether, SS - standard score

Table 4. ANOVA calculation for temperature, specific energy consumption, absolute pressure, mean residence time and TeA, AOH and AME reduction during extrusion process

Term	dF	p	T	E	t_r	TeA	AOH	AME
w	1	0.06**	2227.78^{+}	698.07^{+}	154.22^{+}	81.92**	79.38	132.03**
w^2	1	0.01	206.31*	0.05	3.50**	9.40	260.79*	0.54
q	1	0.08**	486.72^{+}	0.01	55.34^{+}	57.78**	1055.70^{+}	9.46
q^2	1	0.02	62.07	9.56	1.52	0.19	341.76^{+}	97.30
v	1	0.00	215.28*	819.17^{+}	80.22^{+}	72.60**	371.28^{+}	64.98
v^2	1	0.01	271.50*	132.40**	4.05**	40.72	55.56	142.314**
w × q	1	0.09**	85.56	63.74	0. 88	213.16*	285.61*	118.81
w ×v	1	0.00	15.21*	12.96	3.51**	28.09	156.25*	1.32
q ×v	1	0.01	37.82	2.05	1.46	1.32	107.12	76.56
Error	5	0.07	113.51	119.37	3.42	95.75	101.59	406.66
r^2		0.804	0.969	0.936	0.989	0.840	0.964	0.606

Abbreviations:w - moisture content, q - feeding rate, v - screw speed, p – absolute pressure, T - temperature, E - specific energy consumption, t_r - mean residence time, TeA - tenuazonic acid, AOH - alternariol, AME - alternariolmonomethyl ether, dF - degrees of freedom. $^{+}$Significant at $p<0.01$ level, *Significant at $p<0.05$ level, **Significant at $p<0.10$ level, 95% confidence limit, error terms have been found statistically insignificant.

Highlights

- **Reduction of *Alternaria* toxins by extrusion processing of wheat was investigated**
- **The Box and Behnken's design was used for obtaining the experimental data**
- ***Alternaria* toxins were analysed by the developed and validated LC-ESI-MS/MS method**
- **The PCA, RSM and SS were used for assessing the effect of process parameters**
- **Maximum reduction under optimal conditions was: TeA=65.6%, AOH=87.9% and AME=94.5%.**