Fate of Free and Conjugated Mycotoxins within the Production of Distiller’s Dried Grains with Solubles (DDGS)

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ABSTRACT: Contamination of feed with mycotoxins represents a serious worldwide problem concerning animal health and related economic losses. The present paper provides comprehensive knowledge about the fate of mycotoxins during the production of distiller’s dried grains with solubles (DDGS). The study was carried out using naturally infected maize material in five repetitions. For mycotoxin analysis, a QuEChERS-like (“Quick, Easy, Cheap, Effective, Rugged, and Safe”) isolation approach and ultrahigh-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC−MS/MS) was used. A significant increase of deoxynivalenol (DON) and its glycosylated form, DON-3-glucoside (DON-3-Glc), was observed during the first part of fermentation, when hydrolytic enzymes were added. After yeast addition, the total DON content rapidly decreased. An opposite trend was observed for fumonisin B1 (FB1), in which yeast addition contributed to increase of its content. Further considerable change in mycotoxin content occurred during the drying step, in which approximately two-thirds of the original content was lost.

KEYWORDS: feed, distiller’s dried grains with solubles, mycotoxins, bioethanol technology, ultrahigh-performance liquid chromatography, tandem mass spectrometry

INTRODUCTION

The expanding use of oxygenated biofuels is steadily leading to an extensive growth of the ethanol industry worldwide. Currently, the primary commodity used as a raw material for bioethanol production is maize, in addition to wheat or sorghum, which are utilized to a lesser extent depending on the country of production. The increasing demand for bioethanol subsequently generates a growing amount of byproducts. Distiller’s dried grains with solubles (DDGS) together with wet distiller’s grains (WDG or wet cake) represent two main byproducts, being often exploited in the animal feed industry because of quite rich nutrient values.1,2 On average, maize byproducts, being often exploited in the animal feed industry

As in the case of many other grain-based feed ingredients, mycotoxin contamination of DDGS also represents a significant toxicological problem.6,8,9 Adverse effects on animal health resulting from poor economic conditions of livestock production are factors that require knowledge-based solu-

† Supporting Information

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after the evaporation of fermented maize centrifugate supernatant after ethanol distillation). The main outcome of this study was that the total amount of original DON contained in maize decreased to approximately 87% in DDGS despite the fact that the DON concentration in DDGS increased approximately 2.9 times in comparison to its concentration in original maize. The absolute amount of DDGS biomass decreased approximately 3.4 times due to the depletion of starch (and possibly other polysaccharides). With regards to DON-3-Glc and ZEA, their concentrations in DDGS were lower than expected. However, ADONs and NIV, which were not originally determined in maize, were detected in the DDGS product. Authors have speculated that this phenomenon could be connected with a metabolic action of yeast. However, because of the absence of the intermediates of fermentation and the WDG (sediment originating after the centrifugation of the fermented maize after ethanol distillation), it was difficult to unequivocally support this hypothesis. The fermentation process and its influence on several mycotoxins was described in detail by Khatibi et al. (2011).

In this study, the conversion process and its influence on several mycotoxins was described in detail by Khatibi et al. (2011). In this study, the conversion of DON to 3-acetyldeoxynivalenol (3-ADON) by genetically modified yeasts to produce increased amounts of 3-O-acetyltransferase was characterized within a laboratory-scale fermentation experiment, providing a basis for evaluating novel detoxification enzymes, such as DON de-epoxide hydrolases, which have the potential to further reduce DON in DDGS.

It is worth noting that, because of the considerable complexity of the DDGS matrix posing possible difficulties within analysis of mycotoxins, some of the studies were also focused on the development of suitable and valid analytical methods. In this context, validation of the method based on QuEChERS (“Quick, Easy, Cheap, Effective, Rugged, and Safe”) isolation and ultrahigh-performance liquid chromatography separation and tandem mass spectrometry detection (UHPLC-MS/MS) for the analysis of 55 mycotoxins was also a part of research undertaken within this study. However, the main aim was to implement the strategy for the assessment of the fate of mycotoxins within the ethanol–DDGS production and identify the technological steps contributing to their possible reduction. All of the analyzed material (the input maize material, major intermediates, bioethanol, and final DDGS) resulted from natural contamination and was collected during real-life industrial scale processes in several repetitions. In addition to DON and fumonisin B1 (FB1), unique results on the behavior of glycosylated DON (DON-3-Glc) are discussed here in detail.

### MATERIALS AND METHODS

Analytical strategy including analysis of target mycotoxins in samples originating from the ethanol–DDGS production process is described in the following paragraphs. The comprehensive description of the method development, parameters of the UHPLC–MS/MS method, sample preparation, method validation, and determination of all performance characteristics for a range of mycotoxins and their metabolites in plant-based matrices is described in detail in previously published papers by Dzuman et al. (2014) and Veprikova et al. (2015).

#### Analytical Standards

In total, 55 mycotoxins and mycotoxin metabolites were included in this study. The majority of analytical standards were purchased from Biopure (Tulln, Austria) and Sigma-Aldrich (Prague, Czech Republic) and contained 22 *Fusarium* mycotoxins: nivalenol (NIV), deoxynivalenol (DON), deoxynivalenol-3-glucoside (DON-3-Glc), fusarenon X (FUS-X), neosolaniol (NEO), 3- and 15-acetyldeoxynivalenol (3-ADON, 15-ADON), diacetoxyscirpenol (DAS), HT-2 and T-2 toxins (HT2 and T2), verrucarol (VER), fumonisins B1, B2, and B3 (FB1, FB2, and FB3), zearalenone (ZEA), α- and β-zearalenol (α-ZOL and β-ZOL), enniatins A, A1, B, and B1 (ENN-A, ENN-A1, ENN-B, and ENN-B1), and beauvericin (BEA); 17 *Aspergillus* and *Penicillium* toxins: aflatoxins B1, B2, G1, and G2 (AFB1, AFB2, AFG1, and AFG2), ochratoxin A (OTA), citrinin (CIT), cyclopiazonic acid (CPA), sterigmatocystin (STE), patulin (PAT), gliotoxin (GLIO), meleagrin (MEL), mycophenolic acid (MPA), paclitaxel (PAX), penicillic acid (PEN), penitrem A (PEN-A), roquefortine C (ROQ-C), and verruculogen (Verruc); 12 ergot alkaloids produced by *Claviceps*: ergocornine (E-cornine), ergocristine (E-cristine), ergocorninine (E-corninine), ergocristine (E-cristine), ergotamine (E-tamine), ergotamine (E-tamine), and ergometrine (E-metrine); one *Stachybotrys* toxin: stachybotryactlam (STACH); and three *Alternaria* mycotoxins: alternariol (AOH), alternariol–monomethyl ether (AME), and

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**Figure 1.** Ethanol–DDGS production flowchart. Materials analyzed for mycotoxins analysis are highlighted in yellow. The abbreviation related to fermentation \( (n = 1, 2) \) refers to the fermentation step and the particular sampling within the fermentation step \( (x = 1–3) \); SAC, saccharification; LIQ, liquefaction.
tentoxin (TEN). The declared purity of all analytical standards was in the range of 96.0–99.8%. The working solution of all mycotoxins was prepared at a concentration of 1000 ng mL$^{-1}$.$^{12}$ Stock solutions and working solutions of mycotoxins were stored in a freezer (−20 °C) and brought to room temperature before use.

**Chemicals and Reagents.** HPLC-grade acetonitrile (MeCN) and methanol (MeOH) were supplied by Merck (Darmstadt, Germany). Deionized water was prepared from a Millip-Q System (Millipore, Bedford, MA). Anhydrous magnesium sulfate (≥99.5%), sodium chloride (299%), ammonium formate (299%), and ammonium acetate (≥99%) were purchased from Sigma-Aldrich (Prague, Czech Republic).

**Analyzed Samples.** Altogether, samples originating from five different batches of the ethanol–DDGS production process were collected in cooperation with a commercial producer. Each of batches contained 14 examined samples (input maize, 11 intermediates, bioethanol, and final DDGS), which were sampled across the production process and analyzed for mycotoxins. The production flowchart and analyzed materials are shown in Figure 1 (highlighted in yellow). In the first step of the ethanol–DDGS production process, input maize was thoroughly homogenized (dry-grinding technology), and obtained maize meal was mixed with water and an enzymatic preparation containing α-amylase (liquefaction; 82 °C, 3 h) and then β-glucanase (saccharification; 62 °C, 4 h). After the addition of Ethanol Red yeasts (Sacchomyces cerevisiae), five fermentation steps under slightly different conditions in each step followed, ensuring large-scale production continuity (tens of tons per day) are commonly processed by the DDGS producer, with total volume fermentation vats exceeding 5 000 m$^3$. Ethanol was then distilled from the fermented material (14% of ethanol) followed by the centrifugation of whole stillage from which wet distiller’s grains (WDG) and CDS acquired by preconcentration of thin stillage were obtained. WDG and CDS were then dried together in rotary drum dryers under very high temperatures around 500 °C to produce DDGS. During drying, the airflow enters the drum dryer in the same direction (along its axis) as the mixture of WDG and CDS and gradually removes its moisture. Details of the mass balance of the technological process are listed in Table S1.

Tested samples were collected in amounts about 1 kg for solid samples (maize, maize meal, WDG, and DDGS). This aggregate sample consisted of five subsamples (0.2 kg). Reduction of the aggregate sample followed to approximately 50 g according to the current European legislation (Commission Regulation no. 401/2006/EC) provided for the analysis of mycotoxins.$^7$ Regarding the liquid samples (intermediates after liquefaction (LIQ), saccharification (SAC), the first fermentation step (F1−1 and F1−2), second fermentation step (F2−1, F2−2, and F2−3), whole stillage, CDS, and bioethanol), a representative sample was obtained by sampling of each material from six different places of each vat (combination of three different places and sampling depths of 25 cm and 1 m below the level) to give an aggregate sample of 1 L, which was then provided for analysis. The sampling was performed after finishing the respective procedure (liquefaction, saccharification, fermentation, etc.) because the whole process is continuous. The abbreviation (Fr − x) refers to the fermentation stage ([n = 1, 2] and particular fermentation step ([x = 1−3]; five different fermentation vats were utilized, as shown in Figure 1). After the sampling, samples were stored in a freezer (−20 °C) until the analysis. Prior to mycotoxin analysis, samples were tempered to laboratory temperature. Solid samples, i.e. maize, maize meal, WDG, and DDGS, were thoroughly homogenized; with regards to the WDG with the higher moisture content, they were dried in a laboratory oven at 40 °C for 12 h to standardize moisture content before analysis. Liquid samples were shaken thoroughly. All of the examined samples were analyzed in three replicates to compensate for possible nonhomogeneity.

**QuEChERS Extraction Procedure.** For solid samples, the method previously published by Dzuman et al. (2014) and Veprikova et al. (2015) was followed: 2 g of representative sample was weighed into a 50 mL polytetrafluorethylene (PTEF) centrifugation tube, and 10 mL of 0.2% formic acid in deionized water were added. Next, the sample was mixed and left to soak for 30 min. Afterward, 10 mL of MeCN were added, and the final mixture was shaken for 30 min using a laboratory shaker (240 rpm).$^{16,20}$

Regarding the liquid samples, 10 mL of a sample was directly mixed with 10 mL of MeCN (0.2% formic acid) and the tube vigorously shaken by hand for 1 min. Extraction was followed by addition of 4 g NaCl and shaking additionally for 10 min. After the extraction was completed, samples were centrifuged for 5 min at 15800 g. Then, a 1 mL aliquot from the upper acetonitrile phase was filtered through a 0.2 μm microfilter (Ciro, Boca Raton, FL) prior to injection into the UHPLC–MS/MS system.

**Ultrahigh-Performance Liquid Chromatography Separation and Tandem Mass Spectrometry Detection.** The UHPLC–MS/MS analyses were performed according to the previous method published by Dzuman et al. (2014) and Veprikova et al. (2015).$^{15,20}$ UHPLC was realized by using an Acquity UPLC System (Waters) equipped with a C18 reverse-phase Acquity UPLC HSS T3 analytical column (100 × 2.1 mm i.d., 1.8 μm particle size; Waters) maintained at 40 °C, and a 10 μL sample loop operating in partial-loop injection was used. The temperature of autosampler was kept at 10 °C. The prepared sample (2 μL) was injected into the system. The mobile phases were different for ESI(+) and ESI(−) (electrospray) analyses: 5 mM ammonium formate and 0.2% formic acid in both Milli-Q water (A1) and MeOH (B) were used in ESI(+). In ESI(−), 5 mM ammonium acetate in Milli-Q water (A2) and pure MeOH (B) were used. The run time of the method was 12 and 10.5 min in ESI(+ and −), respectively. In ESI(+), the starting mobile-phase composition was 10% of B with a flow rate of 0.35 mL min$^{-1}$, which was linearly changed to 50% of B in 1 min. A slower linear gradient from 50% of B to 100% of B in 10 min followed, simultaneously with the flow-rate change from 0.35 to 0.55 mL min$^{-1}$. The column was then washed for 2 min at 0.7 mL min$^{-1}$ with 100% of B and reconditioned for 2 min in the starting composition of mobile phases at 0.45 mL min$^{-1}$. In ESI(−), the gradient started at 10% of B at 0.35 mL min$^{-1}$, there was a rapid change of mobile-phase composition to 50% of B in 1 min (0.35 mL min$^{-1}$), and another gradual change to 100% of B in 6.5 min followed along with simultaneous flow-rate change to 0.5 mL min$^{-1}$. The column was then washed and reconditioned using the same conditions as in ESI(+).

The Acquity UPLC System was connected with a tandem mass spectrometer QTRAP 5500 (AB SCIEX; Toronto, ON, Canada) equipped with a TurboIon electrospray ESI ion source operated in both positive and negative mode, as mentioned above. The ESI(+) ion source parameters were as follows: needle voltage 4.5, kV; curtain gas, 35 psi; nebulizer and turbo gas (gas 1 and gas 2, respectively), 55 psi; turbo gas temperature, 500 °C. In ESI(−), parameters were set as follows: needle voltage, −4.5 kV; curtain gas, 35 psi; gas 1 and gas 2, 55 psi; and turbo gas temperature, 450 °C. Declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were optimized during the infusion of mixture of analytes (10−100 ng mL$^{-1}$) by employing an automatic function of the Analyst software 1.5 (AB SCIEX; Toronto, ON, Canada), which was also used for control of the LC–MS system and data evaluation.

**QA and QC Samples and Calibration Strategy.** The applied method was validated for both maize and maize-based DDGS matrices. Matrix-matched calibration standards of matrices were made by (i) fermenting maize intermediate (F2−3), and (iii) DDGS were prepared at concentration range of 1−1000 μg kg$^{-1}$ using processed mycotoxin-free samples (blanks) of maize and maize-based DDGS (10 standards). Recoveries of mycotoxins were determined using artifically contaminated blanks at concentration of 500 and 100 μg kg$^{-1}$ ($n = 6$). Matrix effects were calculated as signal suppression or enhancement (SSE) after a comparison of calibration curves of analytes in the presence of matrix extract and solvent (acetonitrile) as a reference. Limits of quantification (LOQs) were determined as the lowest concentration levels from matrix-matched calibration standards that provided signal-to-noise (S/N) ratios higher than 10 and 3 for quantifier and qualifier ions, respectively. For quantification, the external matrix-matched calibration was utilized. Mycotoxins in raw maize; maize meal; intermediates after liquefaction, saccharification,
and fermentation; the whole stillage, and WDG were quantified by using the maize-based matrix-matched standards. Mycotoxins in DDGS were quantified by using the DDGS-based matrix-matched standards. All of the results were corrected for recovery.

As a part of an external quality control procedure, the trueness of the generated data was tested by periodical participation in the Food Analysis Performance Assessment Scheme (FAPAS) organized by the Food and Environment Research Agency (FERA, York, UK).

**Identification of DON Degradation Products.** The analysis and identification of degradation products of the mycotoxin DON was performed by the UHPLC system Dionex UltiMate 3000 RSLC equipped with a C18 reverse-phase core–shell analytical column (HESI II). Sample preparation, chromatographic separation, use of 5 mM ammonium acetate as a mobile-phase buffer, and negative ESI ionization (ESI(−)) conditions were used according to the study of Kostelanska et al. (2011); the only exception was the use of 5 mM ammonium acetate as a mobile-phase buffer. The mass spectrometer was operated in fullMS-data-dependent MS/MS (fullMS–ddMS/MS) mode. Scan range parameters of 100–1 250 m/z at a resolution of 140 000 full width at half-maximum; 1.5 Hz, defined for m/z 200) were set in fullMS mode. Further conditions in fullMS were as follows: capillary temperature, 250 °C; AGC target, 3e6; and maximum inject time, 100 ms; and in ddMS/MS: scan range, 50–fragmented mass m/z (m/z > 25) resolution 17 500 full width (12 Hz; defined for m/z 200); normalized collision energy, 35%; with stepped option of ±20%; AGC target, Se5; and maximum inject time, 50 ms. For data evaluation, Xcalibur 3.0 software (Thermo Scientific, Bremen, Germany) equipped with a heated ESI probe (HESI II). Sample preparation, chromatographic separation, and negative ESI ionization (ESI(−)) conditions were used according to the study of Kostelanska et al. (2011); the only exception was the use of 5 mM ammonium acetate as a mobile-phase buffer. The mass spectrometer was operated in fullMS-data-dependent MS/MS (fullMS–ddMS/MS) mode. Scan range parameters of 100–1 250 m/z at a resolution of 140 000 full width at half-maximum; 1.5 Hz, defined for m/z 200) were set in fullMS mode. Further conditions in fullMS were as follows: capillary temperature, 250 °C; AGC target, 3e6; and maximum inject time, 100 ms; and in ddMS/MS: scan range, 50–fragmented mass m/z (m/z > 25) resolution 17 500 full width (12 Hz; defined for m/z 200); normalized collision energy, 35%; with stepped option of ±20%; AGC target, Se5; and maximum inject time, 50 ms. For data evaluation, Xcalibur 3.0 software (Thermo Scientific, Bremen, Germany) was used.

**RESULTS AND DISCUSSION**

**Validation of the UHPLC–MS/MS Method.** In the first phase of our study, the focus was on the validation of the analytical method. Despite our long-term experience in mycotoxins analysis, it was unsure whether the conventional approach based on QuEChERS-like isolation approach was applicable for some rather uncommon matrices, such as the DDGS process intermediates with higher content of water (intermediates after liquefaction, saccharification, fermentation, etc.). The overview of the obtained validation parameters is summarized in Table S2. Generally, low LOQs were obtained for all analytes in tested maize matrices, which was an important assumption for the analysis of mycotoxins in samples with a higher degree of matrix dilution, as in the DDGS production intermediates. Acceptable recoveries in the range of 71–111%, 76–110, and 71–105%, were obtained for maize, maize fermentation products, and DDGS, respectively. In line with our previous experience, lower transfer into acetonitrile when the QuEChERS-like method was employed was observed for DON-3-Glc for the tested matrices (44, 54, and 48% for maize, maize fermentation intermediates, and DDGS, respectively), because of the higher polarity of this conjugate compared to the parent DON. The repeatability of measurements expressed as relative standard deviations (RSDs) were calculated for all of the examined analyte–matrix combinations at two spiking concentration levels: 500 and 100 μg kg−1. Lower RSDs were observed at higher spiking levels (0.4–12.2%, 0.9–7.5%, and 1.4–11.6% for maize, maize fermentation intermediates, and DDGS, respectively). Slightly higher RSDs (0.9–14.2%, 0.9–13.1, and 1.9–15.4% for maize, fermentation intermediates, and DDGS, respectively) were obtained for lower spiking level. Matrix effects calculated as SSE ranged between 65 and 156% for maize and 71 and 161% for fermentation intermediates. It is worth noting that the difference between the SSE values for the particular analyte–matrix combinations did not differ too much. As can be seen in Table S2, the ∆SSE did not exceed 7%. This refers to the effectiveness of the purification assured by the QuEChERS-like extraction method and justified the use of the maize-based matrix standards for quantification of all maize fermentation intermediates. With regards to the matrix effects for DDGS, the range of SSE values was slightly broader, i.e. 37–146%; also, the ∆SSE values expressing the difference in matrix effects between DDGS and maize were considerably higher than in the previous case. On the basis of the results, we used separate DDGS-based matrix calibration for the accurate mycotoxin quantification. The regression curves for the matrix-matched standards were linear within the working range 0.2–200 ng mL−1 (1–1 000 μg kg−1) for all analytes in all of the examined matrices.

**Fate of Mycotoxins within the Bioethanol–DDGS Production Process.** As mentioned earlier, until now, only a limited number of studies concerned with mycotoxin contamination throughout the entire ethanol-production process have been conducted. Many of the existing studies primarily focused on monitoring mycotoxin levels in the final DDGS product; occasionally, the information about contamination of input raw materials was available. However, under these conditions, the critical assessment of the impact of individual processing phases on mycotoxins is practically impossible because the process parameters are not sufficiently described; moreover, the information on the total amount of mycotoxins in processed grain batches and in respective output products is not available. In this context, we took the opportunity to establish an efficient collaboration with an industrial partner, producing not only bioethanol but also large quantities of DDGS. It is important to notice that organization of such industrial-scale experiments under real-life conditions is not an easy task, also because, fortunately, not all of the batches...
of processed maize are contaminated at high levels suitable for achieving the objective: describing the fate of mycotoxins within the particular DDGS production process and identifying the particular steps possibly leading to the reduction of mycotoxin content in the final product.

The processing flow-chart is illustrated in Figure 1; sampling points are shown here, too. Altogether, 14 samples (i.e., the input maize, 11 intermediates, bioethanol, and the final DDGS) were analyzed for mycotoxins within each of five processing cycles. It should be noted that bioethanol samples were “mycotoxin-free” for all batches, and positive findings were not expected. Its contamination is therefore not discussed in the following paragraphs. The contamination pattern of input maize varied largely. In the first batch of grains, a wide spectrum of mycotoxins (DON, DON-3-Glc, ADONs, FB1, ZEA, enniatins, BEA, AOH, and AME) were detected; however, only DON, DON-3-Glc, and FB1 occurred at sufficiently high concentrations (686, 114, and 43 μg kg⁻¹, respectively), allowing the accurate measurement of their transfer across the production process. With regards to the other Fusarium toxins and both altertoxins, their concentrations in the intermediates due to production dilution of original matrix either dropped below their LOQs or the uncertainty of measurement was too high to allow the accurate balancing of their fate (see Tables S3–S7). Rather high uncertainty values associated with the increased RSDs at such low concentration levels prevented the qualified balance calculation during the DDGS production. In the second batch of examined samples, the input maize contained DON, AME, BEA, and enniatins; however, again, only DON occurred at sufficiently high concentration (410 μg kg⁻¹), which was necessary for relevant evaluation of its mass balance during the DDGS production. The other mycotoxins originally detected in maize (ENN-B, AME, BEA, and enniatins) were not further detected in any of process intermediates except for ENN-B1 at levels close to their LOQs. Similarly, in the case of batches 3, 4, and 5, the contamination of input maize, as well as process intermediates, was too close to LOQs of the respective mycotoxin or below them; therefore, it was impossible to calculate the mass balance.

With regards to the facts stated above, only the fate of DON, DON-3-Glc, and FB1 is discussed in this study. The concentration levels of these three major mycotoxins contained in processed maize and quantified in ethanol–DDGS production intermediates and DDGS samples are summarized in Table 1. Due to the known mass balance of the ethanol–
DDGS process provided by the producer (see Table S1), it was possible to assess the fate of these three mycotoxins within the DDGS production technology (Figures 2 and 3).

As documented in Figure 2, a significant increase of DON and DON-3-Glc content was observed during the liquefaction, saccharification, and first phase of fermentation, when the hydrolytical enzymes were used to support the fermentable saccharides production. A similar phenomenon was observed in our earlier studies.24,25 It might be assumed that the apparent increase of the amount of mycotoxins (up to 147 and 207% for DON and DON-3-Glc, respectively, when considering the total measurable amount in input maize batch as 100%) is due to their release from their bonds on the maize starch matrix. The mass balance of DON within the second batch of the DDGS process was very similar to the first one, and an increase up to 204% of DON content was observed (see Figure 3). In the second phase of fermentation, a successive decrease of the DON/DON-3-Glc content occurred (down to 91 and 119% for DON and DON-3-Glc, respectively, for the first batch and down to 98% of DON for the second batch). Considering the published studies, it might be assumed that the drop of DON and its conjugate in this phase was most likely caused by their adsorption on yeasts cells26,27 or, to some extent, by biotransformation.16,17

With regards to the behavior of FB1 during the liquefaction, saccharification, and first part of fermentation, its mass balance was more or less the same, oscillating within the range 83−124%. However, during the second part of fermentation, the amount of FB1 significantly increased, up to 166%. This trend was somehow different from the results published by Bothast et al. (1992), who reported partial degradation of FB1 during the fermentation of naturally contaminated maize.23 Theoretically, yeast extracellular enzymes might be responsible for FB1 release from covalent bonds with maize constituents such as proteins or starch, thereby increasing its content in the system. In spite of the fact that such FB1 covalent bonds were previously described only as the results of thermal treatment of processed maize,28 the occurrence of bound fumonisins has been recently reported also in unprocessed grains treated by mild drying after harvesting (to prevent the fungal growth during storage).29

In the follow-up steps involved in the investigated processing technology, the fermented maize mash was distilled to recover ethanol produced from starch by fermentation, and the remaining stillage was centrifuged to obtain the solid fraction of the thin stillage and WDG suspension. As the results from this study show, the main part of the mycotoxins was transferred into the CDS obtained by evaporation of the thin stillage (58, 66, and 77% of DON, DON-3-Glc, and FB1, respectively, for the first batch and 59% of DON for the second batch). These results are similar to those obtained by Schaafsma et al. (2009), who reported approximately 57% of DON in the same intermediate.16 The remaining content of toxins was associated with sediment after the centrifugation of WDG.

In the last phase of the ethanol–DDGS production process, the CDS and WDG are mixed together and dried using high temperatures reaching up to 500 °C. Contrary to the data published by Schaafsma et al. (2009), the assumption of the detection of higher mycotoxin content in the preconcentrated final product (DDGS) was not confirmed, and a significant decrease in the content of the discussed mycotoxins during the final drying step of DDGS production was observed.16 The mycotoxin concentrations found in the dry weight of DDGS were, in spite of a triple matrix preconcentration, comparable to those detected in input corn. The only exception was represented by FB1; its concentration in DDGS was approximately two times higher than the concentration determined in the initial corn. The mycotoxin content comparatively decreased according to their content in the

Figure 4. Chromatograms and HRMS/MS spectra of thermal degradation products of DON, norDON B, and norDON C found in DDGS.
input corn to 32%, 31%, and 56% for DON, DON-3-Glc, and FB1, respectively.

**Identification of Thermal Degradation Products of Mycotoxins.** The generated data clearly showed that the drying step had a significant influence on the final DON and DON-3-Glc content in DDGs. As discussed above, approximately 60% of their original content was eliminated. To confirm the hypothesis regarding the thermal degradation of these mycotoxins, we performed additional examination of the DDGS extract employing the UHPLC–HRMS/MS system. On the basis of the studies by Bretz et al. (2006) and Kostelanska et al. (2011), a wide range of breakdown products that may originate from DON/DON-3-Glc under high temperatures was targeted (Table S8). In the study by Kostelanska et al. (2011), the DON degradation products were identified as norDON A, norDON B, norDON C, norDON D, and DON-lactone or norDON F. Regarding the main degradation products of DON-3-Glc, the presence of DON-3-Glc lactone, norDON-3-Glc C, and norDON-3-Glc D was confirmed. Similar experimental conditions, e.g., reverse-phase system and ionization settings, were also used in our study for the identification of potentially occurring degradation products in DDGS. From the list of potential degradation products, norDON B and norDON C (both of the elemental formula C₉₁H₅₄O₂₉) were detected (Figure 4). Contrary to the study by Kostelanska et al. (2011), ammonium acetate buffered mobile phases were used. When we tested ammonium formate buffered mobile phases, only one peak of norDON C was detected, with approximately 2-fold lower intensity as compared to its ammonium analogue. Confirmation of its presence was achieved by the detection of three fragment ions identical for both norDON B and norDON C, as shown in Figure 4. Other degradation products of both DON and DON-3-Glc were not detected, probably due to their lower concentration levels; initial contamination of the examined DDGS samples was relatively low compared to the tested materials in the study of Kostelanska et al. (2011). We also tried to find the thermal degradation for FB1 (e.g., fumonisin-reducing sugar browning reaction products). Unfortunately, due to very low relative concentrations of the parent FB1 in maize, no degradation products were confirmed.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b00304.

Tables S1–S8: (S1) mass balance of the bioethanol–DDGS production process; (S2) validation parameters of the used QuEChERS-like method; (S3–S7) mycotoxin concentration levels found in the analyzed samples within the five analyzed batches of bioethanol–DDGS production process; and (S8) overview of the monitored DON and DON-3-Glc degradation products exact masses in DDGS (batch no. 1). (PDF)

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**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS USED**

15-ADON, 15-acetylenivalenol; 3-ADON, 3-acetylenivalenol; A-clavine, agroclavine; ADONs, sum of 3- and 15-acetylenivalenol; AFBI, aflatoxin B1; AFB2, aflatoxin B2; AFG1, aflatoxin G1; AFG2, aflatoxin G2; AME, alternariol–methyl ether; AOH, alternariol; BEA, beauvericin; CDS, condensed distiller’s solubles; CE, collision energy; CIT, citrinin; CPA, cyclopiazonic acid; CXP, collision cell exit potential; DAS, diacetoxyscirpenol; DDGS, distiller’s dried grains with solubles; dDMS/MS, data-dependent MS/MS; DON, deoxynivalenol; DON-3-Glc, deoxynivalenol-3-glucoside; DP, declustering potential; Enn-A, enniatin A; Enn-A1, enniatin A1; Enn-B, enniatin B; Enn-B1, enniatin B1; ESI, electrospray; E-cornine, ergocornine; E-corninine, ergocornine; E-cristine, ergocristine; E-cristinine, ergocristinine; E-cryptine, ergocryptine; E-cryptinine, ergocryptinine; E-metrine, ergometrine; E-sine, ergosine; E-sinine, ergosinine; E-tamine, ergotamine; E-taminine, ergotaminine; EP, entrance potential; fullMS, full spectral acquisition; FB1, fumonisin B1; FB2, fumonisin B2; FB3, fumonisin B3; FUS-X, fusarone X; fwhm, full width at half-maximum; Glio, gliotoxin; HRMS/MS, high-resolution tandem mass spectrometry; HT-2, HT-2 toxin; LC–MS, liquid chromatography–mass spectrometry; LIQ, liquefaction; LOQ, limit of quantification; McCN, acetonitrile; MEL, meleagrin; MeOH, methanol; MPA, mycophenolic acid; MRM, multiple-reaction monitoring; MS/MS, tandem mass spectrometry; NEO, neosolaniol; NIV, nivalenol; OTA, ochratoxin A; OTE, ochratoxin B; OTs, ochratoxins; PAT, patulin; PAX, paclixine; PEN, penicillic acid; PEN-A, penitrem A; PTFE, polytetrafluoroethylene; QuEChERS, quick, easy, simple, effective, rugged, safe; ROQ-C, roquefortine C; RPM, revelation per minute; RSD, relative standard deviation; S/N, signal-to-noise ratio; SAC, saccharification; SSE, signal suppression and enhancement; STACH, stachybotryalactam; STE, sterigmatocystin; T-2, T-2 toxin; TDI, tolerable daily intake; TEN, tentoxin; UHPLC, ultrahigh-performance liquid chromatography; VER, verrucarol; Verruc, verruculogen; WDG, wet distiller’s grains; ZEA, zearalenone; α-ZOL, α-zearalenol; β-ZOL, β-zearalenol

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