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The Safety of Distillers Dried Grains with Solubles (DDGS)

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1. Abstract

There are more than 300 potential mycotoxins that can contaminate food and feed and cause adverse effects in humans and animals. The data on the co-occurrence of mycotoxins in novel animal feed materials, such as distiller's dried grain with solubles (DDGS), are limited. Thus, a UHPLC−MS/MS method for the quantitation of 77 mycotoxins and other fungal metabolites was used to analyze 169 DDGS samples produced from wheat, maize, and barley and 61 grain samples. All DDGS samples analyzed were contaminated with 13−34 different mycotoxins. Fumonisins were present in all 52 maize DDGS samples (81.0−6890 μg/kg for fumonisin B1), and deoxynivalenol was present in all 99 wheat DDGS samples (39.3−1120 μg/kg). A number of co-occurring mycotoxins were also identified. Due to the high co-occurrence of mycotoxins, routine screening of the animal feed ingredients is highly recommended to allow the highlighted risks to be effectively managed. Commercially available test kits were evaluated as rapid screening analysis for the most commonly found mycotoxins in DDGS. Elemental analysis of the DDGS was also carried out. Results were compared to known literature values and low and high elevations of certain essential and toxic elements have been highlighted for the DDGS used and produced in the UK.

(The work on mycotoxins in DDGS has been published (Oplatowska-Stachowiak et al, 2015))

2. Introduction

Distillers Dried Grains with Solubles (DDGS) are playing an increasing role in the world feed market because fermentation residues of cereal crops are characterised by their high protein content and are, therefore, very valuable in feeding livestock at a low price. The cereal crops used in the process are subjected to the usual applications of fertilizers during cultivation, inhibitors (e.g. antibiotics) or other substances added within the process to optimise the total fermentation yield and could result in contaminated residues being used for the production of feeding stuffs. Other safety issues with the final DDGS product, associated with the cereal crop and the biofuel process, are (i) mycotoxins (including emerging and masked), (ii) heavy metal concentrations (iii) Sulphur concentrations which at levels >0.4% are associated with neurological disorders in cattle and unpalatability with other animals and (iv) antibiotic residues (although this should not be an issue in the UK as the use of antibiotics in the process is not permitted for use but may be an issue with imported DDGS). In the present proposal, which has been designed using a risk analysis approach, mycotoxins and elemental composition (including heavy metals and sulphur content) of DDGS will be investigated with regards to safety aspects of this feed ingredient. The main objective was to appraise the safety of DDGS from the bio ethanol, distilling and brewing industries, in terms of their contaminants content, including mycotoxins and heavy metals, and the impact of processing factors, such drying treatments and storage conditions, based on a risk analysis approach.

3. Materials and methods

3.1. Mycotoxin Analysis of DDGS by UHPLC-MS/MS

3.1.1. Chemicals

Magnesium sulfate (>99.5%), dimethyl sulfoxide (DMSO, >99.9%) formic acid for mass spectrometry (98%), ammonium hydroxide solution (≥25% in water), LC−MS grade methanol (MeOH), and LC−MS grade acetonitrile (MeCN) were obtained from Sigma-Aldrich (Gillingham, UK). Sodium chloride (>99.5%) was obtained from Fisher Scientific (Loughborough, UK). Bondesil C18 40 µm was supplied from Agilent (Wokingham, UK). A Milli-Q system (Millipore, Molsheim, France) was used as a source of deionized water. Start typing here.

3.1.2. Analytical Standards

Aflatoxins B2, G1, G2, and M1; deepoxydeoxynivalenol; fumonisins B1 and B2; and verrucarol were purchased from Sigma-Aldrich (Gillingham, UK). 3-Acetyldeoxynivalnol, 15-acetyldeoxynivalenol, aflatoxin B1, beauvericin, deoxynivalenol, deoxynivalenol-3-glucoside, 13 ergot alkaloid toxins (agroclavine, ergocornine, ergocorninine, ergocristine, ergocristinine, ergocryptine, ergocryptinine, ergometrine, ergometrinine, ergosine, ergosinine, ergotamine, ergotaminine), fumonisin B3, fusarenon X, neosolaniol, nivalenol, ochratoxin A, roquefortine C, T-2 toxin, verruculogen, and

zearalenone were obtained from Romer Labs (Tulln, Austria). Alternariol, alternariol monomethyl ether, aurofusarin, curvularin, equisetin, ochratoxin B, and stachybotrylactam were obtained from Insight Biotechnology (Middlesex, UK). Citrinin; cytochalasin B; enniatins A, A1, B, and B1; gliotoxin; meleagrin; moniliformin; mycophenolic acid; penitrem A; α-zearalanol; β-zearalanol; zearalanone; α-zearalenol; and β-zearalenol were obtained from tebu-bio (Peterborough, UK). Cyclopiazonic acid was obtained from Abcam (Cambridge, UK). Altenuene was purchased from Analyticon Discovery (Potsdam, Germany). Diacetoxyscripenol was obtained from Discovery Fine Chemicals (Wimborne, UK). Emodin and penicilic acid were purchased from Cambridge Bioscience (Cambridge, UK). Fusaric acid and patulin were obtained from Fisher Scientific (Loughborough, UK). Apicidin, HT-2 toxin, macrosporin, skyrin, sterigmatocystin, and tentoxin were purchased from Enzo Life Sciences (Exeter, UK). The masked mycotoxins zearalenone-14-glucoside, zearalenone-16-glucoside, zearalenone-14-sulfate, α-zearalenol-14-glucoside, and β-zearalenol-14-glucoside were obtained from the Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna, Austria. The standards that were obtained in a powder forms were prepared at the concentration of 1 mg/mL in the appropriate amount of solvent (MeCN or MeOH) according to the manufacturer instructions. The standards were stored at −20 or 4 °C, depending on the recommended storage condition.

For the spiking experiments, the concentrated stock solutions were prepared in volumetric flasks by mixing appropriate amounts of single mycotoxins. The concentrated stock solution of all the mycotoxins included in the method- except for fumonisins B1, B2 and B3; the five masked zearalenone metabolites; deoxynivalenol-3- glucoside; and moniliformin; was defined as ALPHA. The mycotoxins were assigned to seven different calibration groups (Table 1), depending on the requirements and/or sensitivity, and appropriate amounts of single mycotoxins were mixed together in such a way that after 50 times dilution of the ALPHA stock in solvent the first calibration level was obtained for each mycotoxin. Similarly, fumonisins B1, B2, and B3 were mixed together in MeCN:H2O (50:50), and this solution was defined as FUM stock. The third calibration stock, defined as MM stock, contained the five masked zearalenone metabolites, deoxynivalenol-3-glucoside, and monilifomin. FUM and MM concentrated stock solutions were prepared in such a way that after 50 times dilution in solvent the first calibration level was obtained for each mycotoxin. The further intermediate stock solutions of ALPHA and FUM were obtained by diluting the concentrated stocks 10 times. These five solutions were used for preparing calibrants in matrix.

Concentration (µg/kg)							
Level	A	B	C	D	E	F	G
1	5000	1000	250	50	25	25000	10000
2	2500	500	125	25	12.5	12500	5000
3	1250	250	62.5	12.5	6.25	6250	2500
4	500	100	25	5	2.5	2500	1000
5	250	50	12.5	2.5	1.25	1250	500
6	125	25	6.25	1.25	0.625	625	250
7	50	10	2.5	0.5	0.25	250	100
8	10	2	0.5	0.1	0.05	50	20
[a]The mycotoxins were classified to the calibration groups according to the required quantitation range and/or sensitivity							

Table 1: Seven Calibration Ranges (Groups A, B, C, D, E, F, and G) and the Mycotoxin Concentration at Each Calibration Level[a].

3.1.3. DDGS Samples

In total, 230 samples of wheat, maize, barley, and mixed DDGS; wheat grain; and barley grain were obtained from the biofuel and feed industries (Table 2). One biofuel plant provided a set of 52 wheat DDGS samples and a set of 52 wheat grain samples used to produce these DDGS samples. Similarly, two sets of nine barley DDGS and barley grain samples were also received from the same plant.

3.1.4. Sample Preparation for Mycotoxin Analysis

The samples weighing between 1-2 kg were homogenized to obtain a fine powder using a laboratory blender. The sample extraction procedure was based on the QuEChERS (Qu ick E asy Ch eap E ffective R ugged S afe) method with a few modifications. A 1.00 ± 0.01 g sample was weighted into a 50 mL polypropylene tube, 5.00 mL of 2% formic acid in water (v/v) was added, and the sample was left to soak for 30 min. Then 5.00 mL of acetonitrile was added and the sample was vortexed for 30 min on multitube vortexer. A 2.00 g portion of magnesium sulfate and 0.05 g of sodium chloride were added, and the tube was immediately shaken for 30 s. After centrifugation for 5 min at 4000g to induce separation of the aqueous phase from the organic phase, 2.00 mL of the upper organic phase was collected and added to a 15 mL centrifuge tube containing 0.10 g of C18 silica and 0.30 g of magnesium sulfate. The tube was shaken immediately for 30 s and then centrifuged at 4000g for 1 min. An aliquot of 1.00 mL of the sample was collected into a HPLC glass vial and 200 µL of DMSO was added. The extract was concentrated at room temperature under nitrogen on MiniVap blowdown evaporator for 45 min to evaporate the remaining acetonitrile, and then 800 µL of MeOH was added to each sample. The resulting solution was filtered through a 0.2 µm PTFE syringe filter (GE Healthcare, Chalfont St Giles, UK) and transferred to a new vial.

sample type	number analysed	origin, year (number)
wheat DDGS	47	UK, Dec 2010−Jan 2011 (36) UK, Sep –Nov 2014 (6) unknown , 2013 (2) Austria, 2013 (2) Sweden, 2013 (1)
maize DDGS	52	USA, 2013 (16) The Netherlands, Oct 2013 (15) Austria, 2013 (4) Hungary, Aug-Sep 2013 (4) unknown, 2013 (13)
mixed DDGS	9	Austria, 2013 (8) Germany, 2013 (1)
barley/barley DDGS	2×9	Europe, June 2014
wheat/wheat DDGS	2×52	Europe, Sep-Nov 2014
total	230	

Table 2: Summary of the samples collected

3.1.5. UHPLC-MS/MS Analysis

Chromatographic separation was carried out using an Acquity UPLC I-class system equipped with a 100 mm × 2.1 mm i.d., 1.6 µm, CORTECS UPLC C18 column (Waters, Milford, MA). The column temperature was maintained at 40 °C and the injection volume was 1 µL. Two sets of mobile phases were used: “acidic”, which is (A) 0.1% formic acid in water and (B) in MeOH:MeCN (1:1, v/v), and “neutral”, which is (A) 1 mM ammonium formate in water (pH adjusted to 6.5) and (B) in MEOH:MeCN (1:1, v/v). The gradient was the same for both sets of mobile phases: the starting composition was 1% organic phase B with linear change to 99% with a flow rate of 0.4 mL/min over 10 min, followed by a cleaning step consisting of maintaining the final composition of 1:99 A:B with an increased flow rate to 0.6 mL/min over 3.5 min before returning to original conditions (99:1, A:B, flow rate 0.4 mL/min). Intotal, 34 mycotoxins were analyzed in neutral mobile phase and 43 in acidic.

The Aquity UPLC system was coupled to a triple quadrupole tandem mass spectrometer (Xevo TQ-S, Waters) with electrospray ion source (ESI) operating with polarity switching in a single injection. The ion source parameters were as follows for both ES+ and ES−: capillary voltage, 1.5 kV; source temperature, 150 °C; desolvation temperature, 500 °C; desolvation gas flow, 1000 L/h; and cone gas flow, 150 L/h. Cone voltage and collision energy were optimized by infusion of each individual analyte. The optimization was performed using the automatic IntelliStart function.

3.1.6. Calibration Curve Generation

The quantitation was achieved by preparing a standard curve consisting of samples spiked before extraction to correct for the recovery losses. The blank matrix of the same type as the set of samples to be analyzed was selected. As it was impossible to find a single sample completely free from all mycotoxins, the matrix with the lowest amount of mycotoxins and at lowest levels was used to prepare eight calibration levels by spiking with appropriate amounts of mycotoxin stock ALPHA and FUM solutions. Two non-spiked samples were also prepared. The calibration curve in matrix was extracted together with each batch of samples of the same matrix type. Additionally, each run contained also four recovery controls: matrix spiked after extraction, two samples at level 5 and two samples at level 2. Depending on the sensitivity and method requirements, the mycotoxins were allocated to different concentration groups (Table 1). Due to limitations in the amount of standards available for the masked zearalenone mycotoxins, deoxynivalenol-3-glucoside, and moniliformin, it was not possible to prepare calibrants spiked before extraction for these mycotoxins. MM mycotoxins stock solution containing these seven analytes was used to prepare an additional calibration curve in matrix spiked after extraction. If these toxins were present in the analyzed sample, the concentrations obtained were corrected for recovery losses using the recovery data obtained during validation. The data were analyzed using TargetLynx processing software (Waters, Wilmslow, UK). Linear 1/x weighted calibration curves were calculated. When the blank matrix used for calibration was contaminated with mycotoxin, the area of the analyte peak in the blank was subtracted from the area of each standard peak and a new calibration was constructed that was used to calculate the concentration of the mycotoxins in unknown samples.

3.1.7. Method Validation

Matrix-induced suppression/enhancement (SSE) was determined by comparing the response for the matrix spiked with standards after extraction (at level 2) to a solvent standard at the same concentration. This experiment was performed for six different samples for each of six matrices: wheat DDGS, maize DDGS, barley DDGS, wheat, maize, and barley. SSE was calculated as the ratio of the peak area of the analyte in the matrix and solvent multiplied by 100.

Method performance parameters, limits of detection (LOD), limits of quantitation (LOQ), and recoveries, were determined for six matrices. LOD was estimated as the lowest matrix-matched calibration standard corresponding to a signal-to-noise ratio of at least 3:1 and LOQ to at least 10:1. Extraction recovery was determined by analyzing the samples spiked before extraction and spiked after extraction and calculating the ratio of the peak areas for each analyte. Two blank samples of each of six matrices were spiked before extraction at levels 2 and 5 by adding the appropriate amount of solvent standards directly to the samples. Spiking concentrations for each group of mycotoxins at each level are presented in Table 1. Two blank samples of each matrix were also extracted at the same time, and the extracts were spiked after extraction with the appropriate amount of solvent

standards to obtain concentration levels 2 and 5 (taking into account a dilution factor for the sample preparation method, which was 5). Two replicates at each level were prepared. The extraction recovery (%) was calculated as the ratio of the peak area in the sample spiked before extraction to the peak area in the sample spiked after extraction at the same level multiplied by 100. From each experiment, a set of two recovery data were obtained: one for spiking level 2 and one for level 5. The extraction recovery data were collected from three different experiments for all DDGS matrices and maize grain and from two experiments for wheat and barley grains. They were used to calculate the mean extraction recovery ± SD.

A within-laboratory reproducibility study was performed for DDGS wheat, maize, and barley matrices for 70 mycotoxins. Seven mycotoxins (MM stock) were not included due to the limited amount of standards available. As it was impossible to select samples that were completely free from mycotoxins, three representative samples that contained the lowest levels and smallest number of mycotoxins were selected and used as blanks for validation. The samples were spiked at 1.0, 1.5, and 2.0 × level 5 (n = 6 at each level). A calibration curve consisting of samples spiked before extraction was prepared at the same time. The validation was performed as within-laboratory reproducibility with three different analysts performing the experiments on three different days (separately for each matrix). The concentrations of each mycotoxin in 18 spiked samples were calculated from the standard curve that was prepared and extracted with each set of samples. The data were used to calculate within-laboratory accuracy and precision.

3.2. Mycotoxin Screening of DDGS using LFDs

3.2.1. Kits

Neogen

Reveal Q+ for DON and Reveal Q+ for fumonisin lateral flow devices (LFDs) were obtained from Neogen (Ayr, Scotland). Each test box contains 25 LFD strips, dilution buffer, cups for sample dilution and QR code for lot-specific calibration. Both tests have competitive immunoassay format. The conjugate release pad contains analyte-specific antibodies conjugated to colloidal gold. The strip is inserted into a sample to be analysed and it is wicked through the release pad. If the contaminant is present in a sample it is captured by the antibody and the immunocomplex is formed. Then it is wicked onto the membrane that contains analyte-protein conjugate immobilized onto the test line. This test line captures any uncomplexed antibody forming a visible line. Therefore, the more analyte present in the samples, the less visible the test line. Control line contains another antibody that captures the immune complex regardless of the presence of the analyte. The results are read using calibrated AccuScan Pro Reader than can convert the intensity of the test and control line into a quantitative result in µg/kg (ppb). The range of quantification of Reveal Q+ DON in DDGS matrix is 300-6000 ppb. The range of quantification of Reveal Q+ FUM is 600-12000 ppb in DDGS matrix.

Tecna

Smart Strip FUMO lateral flow devices (LFDs) were obtained from Tecna (Trieste, Italy). Each test box contains 20 LFD strips, dilution buffer and business card barcode for lot-specific calibration. The tests have competitive immunoassay format. The results are read using Tecna Reader than can convert the intensity of the test and control line into a quantitative result in µg/kg (ppb). The range of quantification of Smart Strip FUMO is 150−4000 ppb and 750−20000 ppb after sample dilution.

3.2.2. Samples

Wheat DDGS sample containing low level of DON was used as a "blank" matrix in spiking experiments and 10 naturally contaminated samples containing different concentrations of DON were used in determining the correlation between LFD result and UHPLC-MS/MS analysis. Similarly, one maize DDGS sample containing low level of fumonisins and 10 naturally contaminated maize DDGS samples containing different levels of fumonisins were selected. The samples were previously analysed by a validated multi-mycotoxin UHPLC-MS/MS method, the results of the analysis of each sample are presented in Table 3. Each naturally contaminated maize DDGS sample showed similar contamination profile - approximately 70% of total fumonisins was FB1, 20 % FB2 and 10% FB3.

Table 3: UHPLC-MS/MS analysis of wheat DDGS samples for deoxynivalenol and maize DDGS samples for fumonisins content.

wheat DDGS sample code	DON [µg/kg]	maize DDGS sample code	fumonisin [µg/kg]				% of total fumonisin content		
			FB_1	FB_2	FB_3	total fum	FB_1	FB_2	FB_3
W_blank	108	M_blank	201	49	23	273	74	18	8
W1	1122	M1	3999	1203	514	5716	70	21	9
W2	994	M2	4214	1104	486	5803	73	19	8
W3	1030	M3	2355	617	303	3276	72	19	9
W4	358	M4	2174	511	253	2938	74	17	9
W5	517	M5	2397	601	275	3273	73	18	8
W6	505	M6	1257	338	157	1752	72	19	9
W7	430	M7	1363	360	171	1894	72	19	9
W8	424	M8	1247	380	158	1785	70	21	9
W9	394	M9	1062	288	124	1474	72	20	8
W10	378	M10	943	291	134	1368	69	21	10

3.2.3. Sample Preparation

Neogen Kits

According to the manufacturer instruction both Reveal Q+ DON and Reveal Q+ fumonisin are suitable for analysis of DDGS, therefore, the recommended procedure was followed.

In the case of Reveal Q+ DON 10 g of homogenized wheat DDGS was mixed with 100 mL of deionized water and shaken for 3 min and then filtered using a filter syringe filled with cotton (Neogen, cat#9420). The pH of the samples was between 4−5, therefore the pH was adjusted to be in the range 6−8, as recommended using 1 M NaOH solution. The pH was measured using the pH test strips (4.5-10 pH, resolution 0.5 pH unit, Sigma, Dorset). The sample was then diluted in the dilution buffer provided with the kit by adding 100 µL of the extract to 1 mL of buffer. The test strip was inserted into a cup containing 100 µL of the diluted sample, allowed to develop for 3 min and then read using AccuScan Pro reader. This extraction procedure was the same as for other commodities such as wheat or maize, with only one additional pH adjustment step due to the acidity of DDGS samples.

In the case of Reveal Q+ fumonisin, 10 g of maize DDGS was mixed with 40 mL of 65% ethanol (Sigma, Dorset) and shaken for 3 minutes. After filtration of the extract using syringe filled with cotton, the pH of the sample was adjusted to 6−8 using 1 M NaOH. 100 µL of this extract was added to 500 µL of the dilution buffer provided with the kit. The test strip was inserted into a cup containing 100 µL of the diluted sample, allowed to develop for 6 min and then read using AccuScan Pro reader. When comparing to the extraction procedure of other commodities such as corn, the extraction method for DDGS involved 1:4 ratio of the sample to the extraction solvent, while it was 1:5 for corn. The final dilution factor in dilution buffer was 6, while it was 3 for corn. As a result the reader result had to be multiplied by a factor of 2 and the range of quantification was 600-12000 ppb, while it was 300-6000 ppb for corn.

Tecna Kits

The sample preparation method provided by the manufacturer was followed with a small modification. 10 g of maize DDGS was mixed with 30 mL of 70% methanol (Sigma, Dorset) and shaken for 3 minutes. After centrifugation of the extract at 5000×g for 1 min, the pH of approximately 1 mL aliquot was adjusted to 7 using 1 M NaOH. DDGS samples have much higher acidity with typical pH from 4 to 5, therefore, it was necessary to adjust this pH as it could interfere with antibody-antigen interaction. After pH adjustment the sample was centrifuged again at 5000×g for 1 min. 100 µL of the supernatant was added to 200 µL of the dilution buffer provided with the kit. 100 µL of the diluted sample was applied onto the cartridge, allowed to develop for 5 min and the read using LFD reader. For sample containing >4000 ppb of fumonisins, the sample was further diluted 1:5 in buffer before adding to the LFD strip.

3.2.4. Study design and Data Analysis

The repeatability and recovery were determined in spiked samples. For each matrix type − wheat and maize DDGS, low contaminated material was selected (as determined by UHPLC-MS/MS analysis) and spiked with 3 different concentrations of mycotoxins within the working range of the test. The spiking levels were 500, 1500 and 5000 µg/kg for DON in wheat DDGS and 600, 2500 and 10000 for FB1 in maize DDGS. The samples were prepared in triplicate on two different days (that gives 6 replicates in total). Similarly, repeatability and recovery were determined in naturally contaminated samples and the results were be correlated to that obtained by UHPLC-MS/MS method. 10 different samples for each matrix in triplicates were analysed on 2 different days (6 replicates in total). For each data set (n=6) including both spiked and naturally contaminated samples recovery and repeatability (expressed as relative standard deviation) were calculated. The agreement between the results obtained using the kits and UHPLC-MS/MS method was evaluated by regression analysis (ordinary and through the origin).

3.3. Elemental Analysis

DDGS and the grain material from which they derived were analysed by either ICP-MS (Inductively coupled plasma mass spectrometry) or XRF (X-Ray fluorescence) for full-scans and for lead isotopes. A rice flour CRM (NCS ZC73009) was run in each batch to test recoveries of each element for each analysis type. All samples were freeze dried and ball-milled before analysis.

3.3.1. Full Scan/Total As determination by ICP-MS

Samples were digested and analysed in batches of 10 samples plus 1 blanks and 1 CRM. Sample sequence was chosen in a randomised order within each batch. Sub-samples (100mg) of powdered sample was weighed out using Discovery OHAUS digital weighing scales into Teflon microwave digestion tubes and the precise weights recorded. BDH Prolabo Aristar 69% nitric acid, 2ml, was added to each digestion tube. The same volume of nitric was added to 1 tube designated as a blanks and 1 tube designated for wheat certified reference material in each sub batch of 12 digests. Tubes were vortexed briefly and 2mls of BDH Prolabo Analar Normapur 30% hydrogen peroxide was added to each tube via pipette. Tubes were then left open for 15 minutes to outgas. Tubes were then placed into the carrousel for the CEM Mars 6 1800W microwave digestor and the appropriate digestion programme selected. The programme chosen heated the samples up to 180^{o}C gradually through a 3 stage process. Ramp from ambient to 95^{o}Cin 15 minutes, hold for 10 minutes; ramp to 135^{o}C in 10minutes, hold for 10 minutes; ramp to 180 ^{o}C in 10 minutes hold for 30 minutes. Total time 85 minutes plus cooling. After cooling the digestate was transferred, with rinsing, to a pre-weighed 50ml polypropylene centrifuge tube, internal standard (Fluka Analytical Rhodium internal ICP-MS standard) was added at 10ppb to each sample and the tubes were made up to their final weights (~30g) with deionised water and these precise weights recorded.

Eight standards were made up, including one blank and also, including internal standard. Multi-Element 2 (SPEX CLMS-2 Multi-Element Solution 2, matrix: 5% HNO3) and Multi-Element 4 (SPEX CLMS-4 Multi-Element Solution 4, matrix: water/Tr-HF) were used to make up all standards in a range of 0-100 ppb. The standard tubes were then made up to final weight (50g) with 1% HNO3 aq. The Rhodium internal standard (Fluka Analytical Rhodium internal ICP-MS standard) was added to all standards also at 10ppb. All internal standard additions and final weights for samples and standards were precisely recorded. An aliquot, 10ml, from the final digestate was poured into 15ml polypropylene tubes to be placed into the auto-sampler (Cetak ASX-520 Auto Sampler) in a predetermined random run order.

Analysis of the samples was carried out for a full scan of elements using ICP-MS (Thermo Scientific iCap Q ICP-MS) which was connected to the auto-sampler (Cetak ASX-520 Auto Sampler). The ICP/MS operating conditions were: Forward RF power- 1550W; Nebuliser gas flow- 1L/min, Nebuliser sample flow rate- 0.35ml/min. Helium was used as a collision gas at a flow rate of 5 ml/min. Samples were analysed by comparison to the standards previously mentioned.

3.3.2. Lead isotopes

An aliquot, 10ml, from the final digestate (above) was poured into 15ml polypropylene tubes to be placed into the auto-sampler (Cetak ASX-520 Auto Sampler) in a predetermined random run order.

Analysis of the samples was carried out for lead isotopes [206Pb; 207Pb and 208Pb] using ICP-MS (Thermo Scientific iCap Q ICP-MS) which was connected to the auto-sampler (Cetak ASX-520 Auto Sampler). The ICP/MS operating conditions were: Forward RF power- 1550W; Nebuliser gas flow- 1L/min, Nebuliser sample flow rate- 0.35ml/min. No collision gas was used.

3.3.3. Arsenic speciation

Samples were digested and analysed in batches of 48 samples plus 3 blanks and 3 CRMs. Sample sequence was chosen in a randomised order within each batch. Sub-samples (100mg) of powdered sample was weighed out using Discovery OHAUS digital weighing scales into labelled 50ml polypropylene (pp) centrifuge tubes and the precise weights recorded. 10ml of Nitric Acid 1% aq by volume (BDH Prolabo Aristar 69%) was added to each centrifuge tube. The same volume of 1% nitric acid was added to 3 tubes designated as blanks and 3 tubes designated for wheat certified reference material. Tubes were vortexed briefly and left overnight to soak. Tubes were then placed into the carrousel for the CEM Mars 6 1800W microwave digestor and the appropriate digestion programme selected. The programme chosen heated the samples up to 95^{o}C gradually through a 3 stage process over a period of 50 minutes. Ramp from ambient to 55^{o}Cin 5 minutes, hold for 10 minutes; ramp to 75^{o}C in 5minutes, hold for 10 minutes; ramp to 95 ^{o}C in 5 minutes hold for 30

minutes. Total time 65 minutes plus cooling. After cooling, the tubes were made up to their final weights (~10g) with deionised water and these precise weights recorded.

Five calibration standards, including one blank, were made up using Dimethylarsenic acid (DMA- 99.5%, Supelco) in a range of 0-5 ppb. The standard tubes were made up to final weight (10g) with 1% HNO3 aq and the precise weights recorded.

Qualitative solutions, ~5ppb, of other arsenic species were also made: Arsenobetaine (AB- BCR 626, ECC Bureau of Reference); Tetramethylarsonium Iodide (Tetra-, in house supply); Mono sodium acid methane arsonate sesquilhydrate (MMA- 95%, Chem Service); Arsenite (As III-, Trace Cert, Aldrich); Sodium arsenate dibasic heptahydrate (AsV-, 99.5% Aldrich). A mixed As species solution, including DMA plus all of the preceding As species, was made at approx. 1ppb for qualification of each individual species retention time.

An aliquot, 1ml, from each final extract and standard and mixed species, were pipetted into 1.5 ml polypropylene autosampler vials, to be placed into the auto-sampler of an ion-exchange chromatography separation system (Thermo Scientific IC 5000) in a predetermined random run order. Each vial was treated with 10µl of hydrogen peroxide (BDH Prolabo Analar Normapur 30%) to convert any arsenite, As III, to arsenate, As V, to suit optimal chromatographic separation.

Chromatographic flow was set at 0.3ml/min and optimal separation was achieved using a gradient programme on an IonPac AS7 Specialty Anion-Exchange Column (Thermo Scientific): Mobile phase A [20mM Ammonium Carbonate] Mobile phase B [200mM Ammonium Carbonate]: Starting with: 100% Mobile phase A, 0% Mobile phase B; increasing to 27% Mobile B after 4 minutes; increasing to 100% Mobile B after 10 minutes; returning to 100% Mobile A after 10.5 minutes; up to a total time of 12.5 minutes. Total time 12.5 minutes, 750 seconds.

Analysis of the samples was carried out for arsenic only [As75] using ICP-MS (Thermo Scientific iCap Q ICP-MS) which was connected to the IC 5000 The ICP/MS operating conditions were: Forward RF power- 1550W; Nebuliser gas flow- 1L/min, Nebuliser sample flow rate- 0.35ml/min. Helium was used as a collision gas at a flow rate of 5 ml/min. Samples were analysed by comparison to the standards previously mentioned.

3.3.4. XRF analysis

Between 3 and 5g of each freeze dried, milled sample was weighed into an XRF cup and the precise weights recorded. Each XRF cup was then hand compressed to ~300psi, and the resultant sample thickness recorded. XRF cups were analysed in sub batches of 9, 8 sample cups in a predetermined random order, plus one cup pressed from the wheat CRM (NCS ZC73009).

4. Results and Discussion

4.1 Mycotoxin Analysis by UPLC-MS/MS

4.1.1. Method Performance Characteristics and Validation

The LODs ranged from 0.005 to 250 µg/kg. LOD and LOQ could only be determined in matrices that were completely free from the analytes. No single matrix was free from beauvericin. Enniatins A, A1, B, and B1 were found in every samples analyzed apart from maize. There was no blank matrix available for some ergot alkaloids in wheat and barley DDGS. Fumonisins were present in all maize, maize DDGS, and barley DDGS samples. Several other mycotoxins were also found in some of the matrices, limiting the possibility of determining the LOD and LOQ for each mycotoxin in each matrix. In general, the LOD and LOQ were higher in DDGS samples than in grain samples due to the greater matrix effect. SSE for most of the analytes in grain samples (wheat, maize, and barley) was within the range 80−120%. Matrix-induced suppression below 80% was observed for two analytes, zearalenone- 16-glucoside and moniliformin, in all three matrices and for a further three mycotoxins, zearalenone-14-sulfate, deoxynivalenol,and deoxynivalenol-3-glucoside, in barley only. Matrix-induced enhancement between 152 and 171% was observed for fumonisins in all three matrices, and a further seven and six analytes showed more than 120% SSE in maize and barley, respectively. In general, these three matrices presented similar SSE patterns, with wheat causing the least matrix effect. The matrix effect in DDGS was greater, and SSE was below 80% for 17, 32, and 17 analytes in wheat, maize, and barley DDGS, respectively. Matrix-induced signal enhancement was observed for fumonisins, apicidin, and aurofusarin in all DDGS matrices and for a further two and four analytes in wheat and maize DDGS, respectively. All three DDGS matrices showed a similar profile of SSE. The highest variation among these three matrices was observed for aflatoxin G1, citrinin, and moniliformin. The extraction recovery for most of the analytes was in the range 70−110% for all the matrices tested. Incomplete extraction was observed for deoxynivalenol-3-glucoside (32−48%). The extraction recovery for moniliformin was only 13−21%, therefore, the method was not suitable for quantitative analysis of this mycotoxin. Additionally, the recovery was determined to be slightly lower than 70% for citrinin, aurofusarin, fusaric acid, nivalenol, and penitrem A in some matrices. Ergocornine, ergocristine, ergocryptine, ergosine, and ergotamine but not ergometrine gave recoveries higher than 110%, and this effect was more significant in grain than in DDGS. Analysis of the grain samples spiked before extraction with ergotaminine only confirmed that some of the analyte was converted to ergotamine during the extraction procedure, but such conversion did not happen in the matrix samples spiked after extraction. The method accuracy was tested in three types of DDGS samples for 70 mycotoxins. Seven toxins were not included in this study due to the limited amount of standards available. The accuracy was typically within the range 80−120%, with results for citrinin, ergocryptine, ergotamine, ergocornine, and fusaric acid in wheat DDGS and for citrinin and beauvericin in maize DDGS outside this range. The coefficients of variation were below 20% for

most of the analytes, with only a few analytes, such as beauvericin, citrinin, some ergot alkaloids, fusaric acid, penicillic acid, and patulin, giving higher values.

4.1.2. Sample Analysis.

The developed UHPLC−MS/MS method was applied to screen 169 DDGS samples and 61 grain samples for 77 mycotoxins.

Wheat DDGS (n = 47), Maize DDGS (n = 52), and Mixed DDGS (n = 9).

The number of mycotoxins detected in wheat DDGS samples ranged from 14 to 27 (Table 4). All of the samples contained between 39.3 and 1120 μg/kg of the regulated Fusarium toxin, deoxynivalenol. Other emerging Fusarium toxins, enniatins A, A1, B, and B1 and beauvericin, were also detected in every sample, and the enniatin B level had the highest concentration, ranging from 174 to 1490 μg/kg. Almost half (47%) of the samples contained zearalenone. Other mycotoxins produced by Fusarium, including fumonisins, apicidin, aurofusarin, equisetin, and fusaric acid, were found in a small number of samples. All the samples contained between 6 and 12 different ergot alkaloids that are produced mainly by *Claviceps purpurea* fungi. The range of contamination was very broad, with total ergot alkaloids content ranging from 4.7 to 1230 μg/kg with a median value of 7.7 μg/kg. Low levels (up to 3.5 μg/kg) of regulated Aspergillus/Penicillium toxin ochratoxin A were found in 81% of the samples. Penicillium toxins, namely mycophenolic acid and meleagrin, were found in 94% and 21% of the samples, respectively.

Maize DDGS samples contained between 13 and 28 mycotoxins. All samples were contaminated with regulated Fusarium toxins fumonisins, with fumonisin B1 having the highest concentration, which ranged from 81.0 to 6890 μg/kg. All samples also contained other Fusarium toxins, namely, enniatins A, A1, B, and B1; beauvericin; and fusaric acid. Beauvericin concentration was higher compared to enniatins and it ranged from 46.1 to 561 μg/kg. Equisetin was found in 98% of the samples. Other Fusarium toxins found were T-2 toxin (81% of the samples), deoxynivalenol (62%), zearalenone (42%), β-zearalenol (10%), aurofusarin (42%), and neosolaniol (15%). The masked mycotoxin deoxynivalenol-3-glucoside was found in 10% of the samples, which were also highly contaminated with deoxynivalenol. With regard to Aspergillus toxins, aflatoxin B1 was present in 75% of the samples, aflatoxin B2 in 63%, aflatoxin G1 in 37%, and aflatoxin G2 in 2%. Traces of aflatoxin M1 were also found in 50% of the samples. Aflatoxin M1 is a metabolite of aflatoxin B1 that is excreted into milk; however, it has also been reported to be present at low levels in such commodities as corn (Huang et al, 2010) and peanuts (Shotwell et al, 1976). Aspergillus/Penicillium toxins ochratoxin A, ochratoxin B, and cyclopiazonic acid were detected in 67%, 23%, and 73% of the samples, respectively. Other Penicillium metabolites, i.e., mycophenolic acid, meleagrin, roquefortine C, were also found. The Alternaria toxins alternariol, alternariol monomethyl ether, and tentoxin were found in 33%, 21%, and 27% of the samples, respectively.

Table 4: The Mycotoxin Profiles in DDGS Samples

Analyte	wheat DDGS (n=47)					maize DDGS (n=52)					mixed DDGS (n=9)				
	Freq. [number]	Median [µg/kg]	25th Percentile [µg/kg]	75th Percentile [µg/kg]	Range. [µg/kg]	Freq. [number]	Median [µg/kg]	25th Percentile [µg/kg]	75th Percentile [µg/kg]	Range. [µg/kg]	Freq. [number]	Median [µg/kg]	25th Percentile [µg/kg]	75th Percentile [µg/kg]	Range. [µg/kg]
3- and 15-Acetyldeoxynivalenol						8	1570	254	1820	<LOQ−2430	2				<LOQ−269
3-Acetyldeoxynivalenol						12	60.9	28.0	320	<LOQ−478	2	49.0			48.1−49.9
Aflatoxin B_1						39	9.4	4.4	11.2	<LOQ−16.0					
Aflatoxin B_2						33	1.2	1.0	2.0	<LOQ−4.1					
Aflatoxin G_1						19	2.5	2.3	2.7	<LOQ−3.4					
Aflatoxin G_2						1	<LOQ								
Aflatoxin M_1						26	0.45	0.40	0.60	<LOQ−1.1					
Alternariol						17	16.2	12.9	32.3	<LOQ−47.0	7	21.9	18.0	27.9	<LOQ−52.5
Alternariol monomethylether						11	24.8	12.8	28.1	<LOQ−30.9	2				<LOQ−32.0
Apicidin	3	80.0			33.8−81.6	1	30.7				1	<LOQ			
Aurofusarin	6	38.5	26.3	62.3	2.0−83.8	22	5.1	3.1	17.9	2.2−129	2	16.8			14.1−19.6
Beauvericin	47	20.7	19.8	22.7	3.1−108	52	139	123	177	46.1−561	9	60.6	33.9	74.8	20.2−195
Curvularin	1	<LOQ									4	32.9	26.7	35.8	<LOQ−36.4
Cyclopiazonic acid						38	8.8	6.0	11.0	<LOQ−16.8					
Deoxynivalenol	47	155	114	188	39.3−1120	32	612	242	1390	144−16000	9	452	356	786	303−2560
Deoxynivalenol-3-glucoside						5	733	598	991	560−1360					
Enniatin A	47	5.7	5.1	6.2	1.6−30.7	19	0.30	0.18	0.45	0.13−2.7	9	3.8	3.5	4.8	3.2−15.0
Enniatin A1	47	33.5	29.6	36.5	9.3−172	52	0.70	0.61	0.81	0.07−16.1	9	21.1	17.2	26.5	14.1−75.8
Enniatin B	47	384	271	460	174−1490	52	5.0	4.2	10.1	1.2−183	9	247	242	313	39.0−330
Enniatin B1	47	158	130	181	42.5−533	52	1.7	1.5	2.7	0.10−49.5	9	114	95.9	114	52.8−231
Emodin	2	<LOQ													
Ergocornine	13	9.3	3.9	15.9	<LOQ−120						7	1.5	1.1	4.7	<LOQ−9.1
Ergocorninine	28	0.24	0.13	2.7	0.10−9.8						9	0.78	0.28	1.5	0.15−4.2
Ergocristine	47	2.4	2.1	3.2	1.5−431						9	2.5	1.3	8.5	<LOQ−13.9
Ergocristinine	47	0.46	0.38	0.68	0.25−18.7						8	0.73	0.21	2.4	0.19−3.6
Ergocryptine	34	0.39	0.32	5.8	<LOQ−56.5						7	1.5	0.97	4.8	0.68−8.4
Ergocryptinine	47	0.31	0.24	1.3	0.15−5.4	2	0.14			0.12−0.16	9	1.3	0.58	2.3	0.27−9.1
Ergometrine	9	1.4	0.70	1.7	<LOQ−24.2						3	<LOQ			
Ergometrininie	10	0.68	0.48	0.86	0.29−2.0	2	<LOQ				3	0.53			0.43−0.59

Ergosine	47	0.99	0.74	1.6	<LOQ−405						7	1.2	0.76	3.7	<LOQ−6.7
Ergosinine	24	0.19	0.11	3.8	<LOQ−25.6						5	0.33	0.12	0.78	<LOQ−0.96
Ergotamine	47	2.5	2.2	3.3	0.60−124						7	0.93	0.70	3.4	0.40−5.5
Ergotaminine	46	0.31	0.25	0.41	0.18−9.2						3	0.54			0.39−0.90
Equisetin	3	3.0			2.9−9.5	51	15.3	11.1	17.3	2.4−37.8	8	3.2	2.0	6.0	<LOQ−8.8
Fumonisin B_1	5	14.3	4.7	41.3	1.2−125	52	1278	944	1420	81.0−6890	9	52.8	22.4	165	5.1−511
Fumonisin B_2	2	15.9			9.5−22.4	52	294	273	392	22.5−2060	7	21.6	6.9	54.8	1.9−124
Fumonisin B_3	2	7.2			2.9−11.5	52	169	125	181	10.9−667	7	8.6	2.1	21.1	<LOQ−62.5
Fusaric acid	3	225			155−271	52	939	668	1010	384−3450	8	389	195	617	116−849
Meleagrin	10	0.22	0.15	0.27	<LOQ−0.35	42	2.0	0.50	2.9	<LOQ−3.3					
Mycophenolic acid	44	22.2	14.4	28.4	1.2−49.5	50	13.1	10.8	14.3	3.2−67.1	7	5.0	3.0	6.8	1.9−18.7
Neosolaniol						8	7.2	3.8	7.9	<LOQ−8.4					
Ochratoxin A	38	1.5	1.2	1.9	<LOQ−3.5	35	27.1	3.9	29.3	<LOQ−45.8	2				<LOQ−1.9
Ochratoxin B						12	<LOQ								
Roquefortine C						27	1.6	1.1	1.9	<LOQ−17.6	2	0.73			0.64−0.83
Skyrin						1	33.4								
T-2 toxin						42	16.7	12.9	23.2	<LOQ−102	2				<LOQ−13.8
Tentoxin	3	8.4			7.4−12.4	14	2.1	1.7	3.2	<LOQ−9.9	8	6.0	4.0	6.8	3.3−11.9
beta-Zearalenol						5	345	306	457	305−465					
Zearalenone	22	21.8	20.1	28.4	<LOQ−72.7	22	54.2	40.9	111	31.2−743	9	45.6	39.8	60.3	17.0−224

Table 5: Comparison of the Wheat Grain and Wheat DDGS Samples Obtained from Bioethanol Plant

Analyte	wheat grain (n=52)					wheat DDGS (n=52)					F[a]
	freq. [number]	median [µg/kg]	25th percentile [µg/kg]	75th percentile [µg/kg]	range [µg/kg]	freq. [number]	median [µg/kg]	25th percentile [µg/kg]	75th percentile [µg/kg]	range [µg/kg]	
Apicidin	32	38.6	27.4	73.3	17.0−187						
Aurofusarin	52	13.2	6.0	24.5	1.4−400	9	2.1	1.3	2.3	<LOQ−3.3	
Beauvericin	52	3.8	2.4	4.7	0.50−10.3	52	11.0	10.2	12.0	8.3−109	2.9
Curvularin	2	<LOQ									
Deepoxy-deoxynivalenol	1	25.0									
Deoxynivalenol	44	110	56.7	175	<LOQ−2540	52	232	212	269	177−394	2.1
Enniatin A	39	0.33	0.19	0.61	0.10−1.3	52	2.1	1.9	2.4	1.5−3.2	6.5
Enniatin A1	49	1.7	0.84	2.7	0.14−7.4	52	11.7	10.6	13.2	8.5−17.3	7.0
Enniatin B	52	37.3	17.5	66.3	3.5−127	52	244	215	291	164−379	6.5
Enniatin B1	52	10.2	7.9	16.7	3.4−28.4	52	51.0	46.2	57.0	38.5−78.2	5.0
Ergocornine	2	24.0			8.6−39.4	51	5.1	4.0	8.0	2.2−16.3	
Ergocorninine	2	2.6			0.25−4.9	52	1.8	1.6	3.1	0.15−6.1	
Ergocristine	6	4.3	1.0	62.3	<LOQ−347	52	21.0	14.4	30.0	5.1−61.3	
Ergocristinine	4	2.3	0.74	5.0	0.15−9.1	52	4.7	3.7	7.1	1.2−14.5	
Ergocryptine	4	6.1	2.7	9.9	<LOQ−13.5	52	5.9	4.8	8.1	1.5−16.4	
Ergocryptinine	2	1.4			0.54−2.3	52	2.0	1.8	2.8	0.49−5.5	
Ergometrine	7	0.70	0.25	3.1	<LOQ−86.1	35	0.55	0.50	0.75	<LOQ−1.0	
Ergometrininie	1	11.6				44	0.50	0.35	0.60	<LOQ−1.1	
Ergosine	6	8.7	2.0	14.4	0.87−267	52	12.4	10.2	18.9	3.4−39.8	
Ergosinine	5	0.71	0.55	2.0	0.18−17.4	52	2.1	1.9	3.4	0.61−7.5	
Ergotamine	8	1.0	0.78	12.2	0.45−41.6	52	6.2	5.2	8.1	3.7−15.9	
Ergotaminine	3	2.3			0.19−3.7	52	1.3	1.1	1.8	0.84−3.9	
Equisetin	20	6.1	3.4	8.2	<LOQ−30.3						
Fumonisin B_1						6	155	73.9	170	27.2−200	
Fumonisin B_3						6	15.4	7.0	18.0	2.9−19.7	
Fusaric acid						5	175	169	196	63.7−238	
Meleagrin	8	0.15	0.11	0.16	<LOQ−0.25						
Mycophenolic acid	22	8.2	5.8	11.9	3.8−70.2	52	22.3	11.2	30.4	7.5−44.8	
Ochratoxin A	3	2.2			1.4−3.1	27	1.1	0.96	1.2	<LOQ−1.9	
Sterigmatocystin	1	4.6									
T-2 toxin	1	19.6				3	<LOQ				
Zearalenone	3	12.0			<LOQ−21.7	10	13.7	9.5	15.6	<LOQ−18.1	

[a]The increase factor ''F'' was calculated as the ratio of the median concentration in DDGS to the median concentration in grain. The factor estimates the average increase of mycotoxin concentration when comparing starting material (grain) and product (DDGS). It was calculated only for mycotoxins that were present in at least 75% of both grain and DDGS samples.

Table 6: Comparison of the Barley Grain and Barley DDGS Samples Obtained from Bioethanol Plant

analyte	barley grain (n=9)					barley DDGS (n=9)				
	freq. [number]	median [µg/kg]	25th percentile [µg/kg]	75th percentile [µg/kg]	range [µg/kg]	freq. [number]	median [µg/kg]	25th percentile [µg/kg]	75th percentile [µg/kg]	range [µg/kg]
3-and 15-Acetyldeoxynivalenol						9	453	402	540	293−623
3-Acetyldeoxynivalenol						9	56.6	48.9	58.2	34.4−73.4
Aurofusarin	9	16.4	6.9	44.8	4.8−90.9	9	14.4	13.4	17.1	9.0−21.8
Beauvericin	6	2.5	2.2	5.1	1.3−7.0	9	360	329	402	260−438
Deoxynivalenol	5	88.7	83.8	93.2	81.0−102	9	2390	1730	2430	1370−2550
Deoxynivalenol-3-glucoside						1	<LOQ			
Enniatin A	9	0.97	0.50	1.5	0.41−4.4	9	2.3	1.9	2.4	1.6−2.7
Enniatin A1	9	7.3	2.0	11.5	1.4−30.8	9	11.7	9.3	13.0	8.8−13.5
Enniatin B	9	296	88.4	469	24.3−1200	9	344	331	376	239−419
Enniatin B1	9	49.5	12.1	58.3	6.5−149	9	55.2	45.0	58.6	40.9−66.6
Ergocornine						7	1.2	0.88	1.4	<LOQ−2.4
Ergocorninine						8	0.35	0.29	0.45	0.10−0.60
Ergocristine	1	2.0				9	5.5	4.6	6.7	2.2−9.0
Ergocristinine	1	0.19				9	1.4	1.2	1.7	0.45−2.1
Ergocryptine						9	1.2	0.93	1.2	<LOQ−2.2
Ergocryptinine						9	0.39	0.34	0.52	0.13−0.66
Ergosine	1	1.1				9	3.5	3.0	4.0	1.5−5.2
Ergosinine	1	0.18				9	0.69	0.64	0.85	0.28−1.0
Ergotamine	1	0.62				9	1.4	1.1	1.6	0.68−2.1
Ergotaminine						9	0.38	0.23	0.41	0.13−0.47
Equisetin	3	3.2			1.5−8.2	9	6.0	5.3	7.6	4.2−10.6
Fumonisin B1	1	37.5				9	742	597	915	551−1084
Fumonisin B2	2	24.6			21.0−28.2	9	161	153	202	131−210
Fumonisin B3	1	5.6				9	71.5	58.4	87.0	54.0−92.1
Fusaric acid						9	1290	1127	1328	893−1560
HT-2 toxin						6	<LOQ			
Meleagrin	9	0.31	0.20	0.57	0.15−0.76	9	0.19	0.17	0.21	0.12−0.31
Mycophenolic acid	3	1.4			<LOQ−2.7	9	17.7	12.1	36.1	10.3−42.8
Ochratoxin A	2	9.5			5.9−13.1	9	2.1	1.8	2.4	1.6−3.1
T-2 toxin	1	3.2				9	48.8	34.5	58.1	32.3−62.0

Mixed DDGS samples contained mixtures of mycotoxins that were present in both wheat and maize DDGS. The number of mycotoxins in each sample was from 16 to 31, and 100% of samples contained the Fusarium toxins deoxynivalenol, fumonisins, zearalenone, and enniatins. Other Fusarium metabolites found were fusaric acid, equisetin, T-2 toxin, apicidin, and aurofusarin. From 3 to 12 different ergot alkaloids were found in each sample. The Penicillium toxins mycophenolic acid and roquefortine C were detected in 78% and 22% of the samples. Ochratoxin A was found in 22% of the samples. The Alternaria toxins alternariol, alternariol monomethyl, and tentoxin were also present. Mycotoxin contamination is a result of infection with toxin producing fungi belonging to Fusarium, Aspergillus, Penicillium, Alternaria, and Claviceps genera during the growth of the cereals in the field and/or postharvest during grain storage and processing. EU Commission Directive 2003/10/EC3 sets the maximum limits for aflatoxin B1 (5−20 μg/kg), and Commission Recommendation 2006/576/EC5 gives guidance values for deoxynivalenol (900−12 000 μg/kg), zearalenone (100−3000 μg/kg), ochratoxin A (50−250 μg/kg), and fumonsins B1 + B2 (5000−60000 μg/kg) in animal feed. The exact value depends on animal species and feed type. From a regulatory perspective, the most prevalent mycotoxins identified in this study were Fusarium toxins: deoxynivalenol in wheat DDGS and fumonisins in maize DDGS. These toxins appear in DDGS at high levels, and as guidance limits are established for feed, the monitoring of DDGS quality in terms of mycotoxin contamination should focus on them. Two analyzed maize DDGS samples exceeded the guidance limit for deoxynivalenol in cereal by-products to be used as feed ingredients (12 000 μg/kg). The rest of the samples were contaminated with mycotoxins below the guidance or indicative limits. However, the co-occurrence of mycotoxins in every sample analyzed raises the question about possible synergistic and/or additive effects of the cocktails of these contaminants on animal health and performance.

Data on the contamination of DDGS with mycotoxins are limited to a few publications focusing mainly on regulated mycotoxins. The analysis of 67 maize DDGS samples for aflatoxins, deoxynivalenol, fumonisins, T-2 toxin, and zearalenone collected from US bioethanol industry between 2009 and 2011 revealed the presence of deoxynivalenol at a level higher than 2 mg/kg in 12% of the samples, fumonisins at a level higher than 5 mg/kg in 6% of the samples, and zearalenone in most of the samples at a concentration between 0.1 and 0.3 mg/kg (Zhang & Caupert, 2012). Rodrigues and Chin (2012) analyzed 409 maize DDGS samples for aflatoxins, zearalenone, deoxynivalenol, fumonisins, and ochratoxin A and found 92% of the samples contaminated with more than two mycotoxins. Khatibi et al. (2014) analyzed 141 corn DDGS samples collected from 78 bioethanol plants in the United States in 2011 for the presence of deoxynivalenol, 3- and 15-acetyldeoxynivalenol, nivalenol, and zearalenone. The authors found >1 mg/kg of deoxynivalenol, 15-acetyldeoxynivalenol, and zearalenone in 30%, 15%, and 3% of the analyzed samples, respectively. The analysis of 59 maize DDGS samples

from Thailand for fumonisins B1 and B2, deoxynivalenol, zearalenone, and beauvericin showed co-occurrence of these five mycotoxins in 50.8% of the samples (Tansakul et al, 2013). Li et al. (2014) analyzed 17 DDGS samples from China and found all of them contaminated with deoxynivalenol, zearalenone, and ochratoxin A and 16 of them with aflatoxin B1. A more comprehensive study on DDGS was published by Zachariasova et al (2014). Among other samples, 71 maize DDGS and 16 wheat DDGS were analyzed for 56 mycotoxins. The authors found that DDGS samples contained a broad spectrum of mycotoxins, including deoxynivalenol and its metabolites, T-2/HT-2 toxins, zearalenone and its metabolites, fumonisins, enniatins, beauvericin, Alternaria toxins, ergot alkaloids, sterigmatocystin, ochratoxin A, and mycophenolic acid. Similar mycotoxin profiles in DDGS samples were identified in this study.

Regulated mycotoxins, such as deoxynivalenol and fumonisins, have well-known adverse effects in humans and animals. This study identified common co-occurrence of other mycotoxins for which toxicity data are limited. Enniatins and beauvericin were found in all samples analyzed. They have ionophoric properties and can promote transport of ions through biological membranes, leading to changes in normal physiological concentrations of these ions (Jestoi, 2008). Enniatins and beauvericin may bioaccumulate due to their lipophilic nature. Their influence on the toxicity of other mycotoxins is unknown. Another important toxin, fusaric acid, acts synergistically with deoxynivalenol when given to immature pigs, causing depression in weight gain and feed intake (Smith et al, 1997). Fusaric acid acts also synergistically with fumonisins, as enhanced toxicity was observed when fusaric acid and fumonisin B1 were administered together to developing chicken embryo (Bacon et al, 1995). Mycophenolic acid has immunosuppressive properties; it inhibits antibody formation and the production of cytotoxic T cells (Schneweiss et al., 2000). 100% of wheat DDGS was contaminated with ergot alkaloids. Ergot alkaloids can act on a number of neurotransmitter receptors and interfere with reproductive processes (EFSA, 2012). The maximum content of rye ergot sclerotia in feedstuff is 1000 mg/kg in the European Union (EC Directive, 2002/32/EC). While there are currently no other regulatory limits for ergot alkaloids in feed in the European Union, the European Food Safety Authority (EFSA) established a no observed adverse effect level (NOAEL) for ergot alkaloids at 0.15 mg/kg of feed for pigs and 1.4 mg/kg of feed for poultry. Six wheat DDGS samples analyzed in this study exceeded or were close to the NOAEL value for pig feed. The inclusion rate of DDGS in animal diet is generally in the range of 10−20%; therefore, if these wheat DDGS batches were used as a pig feed ingredient, the ergot alkaloid content would be diluted below the NOAEL level, although it is illegal in the European Union to knowingly dilute contaminated materials.

Comparison of Wheat Grain and Wheat DDGS Samples.

Fifty-two samples of wheat grain and wheat DDGS were obtained from an EU bioethanol plant between September and November 2014. Due to the manufacturing practice, one wheat grain batch could not be matched directly with another DDGS batch, as different batches can be mixed together during the production process. As a result, the data were analyzed as two separate sets: grain wheat and wheat DDGS (Table 5). A total of between 6 and 17 mycotoxins were found in grain wheat samples and between 17 and 24 in wheat DDGS samples. Deoxynivalenol and ergot alkaloids were not detected in every grain sample, but they were found in all DDGS samples. This could be a result of their presence below the LOD values in grain. After concentration during ethanol production, they were detectable in DDGS samples. The variation in the level of contamination for these two groups of mycotoxins in wheat grain was very high, ranging from traces to heavy contamination, while DDGS samples were more uniformly contaminated with these mycotoxins. Deoxynivalenol was found in all wheat DDGS samples, and the concentration ranged from 177 to 394 µg/kg. The concentration factor for deoxynivalenol calculated on the basis of the median values for DDGS and grain was 2.1. This is in agreement with the work of Hanschmann and Krieg (2006), who found deoxynivalenol and zearalenone to increase 2−4 times during production of bioethanol from triticale. Also, Schaafsma et al. (2009) reported deoxynivalenol concentration 3 times higher in DDGS than in the maize used for its production.

Traces of ergot alkaloids were found only in 10 grain samples, and high levels of contamination were found in 3 samples (total ergot alkaloids content: 78, 228, and 688 µg/kg). All DDGS samples were contaminated with at least 10 ergot alkaloids, and the sum of ergot alkaloids content ranged from 17.6 to 189 µg/kg. The median concentrations of enniatins A, A1, B, and B1 andbeauvericin were higher in DDGS samples by factors of 6.5, 7, 6.5, 5, and 2.9, respectively, when compared with grain. This indicates the presence of the concentration effect on these mycotoxins during bioethanol production. Low levels of the Fusarium toxins fumonisins, fusaric acid, and zearalenone; the Penicillium toxin mycophenolic acid; and Aspergillus/Penicillium toxin ochratoxin A were found in a higher number of wheat DDGS samples than wheat grain samples, again indicating the concentration of these toxins during bioethanol production. All grain samples were contaminated with aurofusarin from 1.4 to 400 µg/kg. Aurofusarin was found in only nine DDGS samples, indicating that this toxin does not accumulate in DDGS or is transformed during bioethanol production to other metabolites. DDGS was less contaminated with apicidin and equisetin comparing to grain samples, suggesting that these toxins do not accumulate in DDGS or are converted to other metabolites.

Comparison of Barley Grain and Barley DDGS Samples.

Nine samples of barley grain and nine samples of barley DDGS were obtained from the same EU bioethanol plant. A total between 31 and 34 mycotoxins were found in each DDGS sample and between 7 and 16 in barley grain (Table 6). All barley DDGS samples were contaminated with the regulated the Fusarium toxins deoxynivalenol, fumonisins, and zearalenone. The masked mycotoxin deoxynivalenol-3-glucoside was found at a level <LOQ in one sample and zearlaenone-14-sulfate in eight samples. Other Fusarium toxins found in each sample were enniatins, beauvericin, aurofusarin, equisetin, and T-2/HT-2 toxins. Low levels of ergot alkaloids, the Penicillium metabolites mycophenolic acid and meleagrin, and Aspergillus/Penicillium toxin ochratoxin A were also found in every sample.

Ergot alkaloids, fumonisins, deoxynivalenol and its metabolites,T-2/HT-2 toxins, zearalenone, beauvericin, equisetin, and mycophenolic acid were detected in all barley DDGS samples, while they were absent or present only at low concentrations in a limited number of grain samples. Large differences were observed for deoxynivalenol and beauvericin, which were present in the barley grain at the median concentrations 88.7 and 2.5 µg/kg, respectively, and at 2390 and 360 µg/kg in barley DDGS samples. The median concentrations of enniatins A, A1, B, and B1 were similar in grain and DDGS; however, grain samples showed a much higher variation in the level of contamination. Other Fusarium toxins, i.e., fumonisins B1, B2, and B3; fusaric acid; T-2 toxin; and zearalenone, were detected in a limited number of barley grain samples at low concentrations, but they were present in all barley DDGS samples at median concentrations of 742, 161, 71.5, 1290, 48.8, and 243 µg/kg, respectively.

The frequency and levels of contamination with a number of Fusarium toxins, such as beauvericin, deoxynivalenol, fumonisins, fusaric acid, T-2 toxin, and zearalenone, were much higher in barley DDGS than in barley grain samples. High contamination of barley DDGS but not of grain is probably attributed to other factors apart from mycotoxin accumulation during bioethanol roduction. The available sample set was limited to only nine samples of barley grain and nine of barley DDGS and all of them were collected within a short (up to one month) time period. Due to the manufacturing practice, it was not possible to match a single grain sample with a single DDGS product. All the before-mentioned mycotoxins are produced by Fusarium ssp., which can also contaminate different agricultural crops during storage. In the work of Sherwood and Peberdy (1974), the growth of Fusarium graminearum on stored wheat, maize, barley, and oat and the production of mycotoxin zearalenone were examined under different conditions. The authors found that at a moisture content of 18% the grains were susceptible to invasion, even at a temperature lower than 7 °C. At moisture content 15−18%, the infection was possible at higher temperatures. The highest fungus growth was observed at 25 °C, and maximum toxin production was between 12 and 18 °C. Therefore, grain moisture content above 14% can make it more susceptible to fungal growth during storage (Richard, 2007).

More sample analysis is needed to determine if the observed effect was an isolated incident due to the contamination of the storage facility for barley DDGS, if barley DDGS is more susceptible to mould growth during storage, or if there are any other factors contributing to these findings.

Conclusions

Overall, the results of this research support the hypothesis stated by other studies that co-occurrence of mycotoxins in animal feed ingredients is a fact and that the toxicological potential of such mixtures can be an underestimated hazard for the productivity of farm animals. DDGS is at even greater risk of containing higher levels of mycotoxins, as it is three times more concentrated than the original grain. Due to the higher risk of mycotoxins contamination in DDGS, it is recommended to routinely screen DDGS for mycotoxin content, especially for regulated mycotoxins such as deoxynivalenol, fumonisins, and zearalenone, to avoid introduction of highly contaminated batches into animal diets. Another solution for limiting exposure to mycotoxins is addition of special mycotoxin detoxifying agents to animal feed. There are a number of such products available on the market, and they are composed of adsorbing (mineral, organic, or biological) and/or biotransforming agents (microbes and enzymes) that limit the absorption of mycotoxins in the gastrointestinal tract and/or transform them into less toxic metabolites (Wielogorska et al, 2015). As the efficacy of most of them has been tested only for regulated mycotoxins, more research is needed to determine if mycotoxin binders can be also the solution for limiting animal exposure to the mycotoxins cocktails identified in this study.

4.2. Mycotoxin Screening using LFDs

4.2.1. Neogen Kits

Reveal Q+ DON

The samples that contained lowest amount of DON was used as "blank" in spiking experiment and it contained 108 µg/kg of DON according to the UHPLC-MS/MS method. The analysis of this sample using Reveal Q+ DON gave a value of 138 µg/kg. The "blank" samples were spiked at 3 different levels with 3 replicates at each level. The experiment was performed on 2 different days, therefore 6 replicates were performed at each level. Each data set data was analysed in two ways: with and without subtraction of the "blank" concentration determined by UHPLC-MS/MS analysis (Table 7). When the blank was not subtracted, the mean recoveries were 95−115%. With blank subtraction, the recoveries were 92−95%. The coefficients of variation of the six readings were 12 or 14% for lowest spiking level and 4−6% for higher levels, showing very good precision of the test.

Table 7: Results for determining DON concentration in wheat DDGS spiked at 3 levels. On each level 3 extractions were performed on day 1 and day 2, which gives 6 replicates at each level.

Spiking level [µg/kg]	Reveal Q DON			Reveal Q DON (blank subtracted)		
	Mean [µg/kg]	Recovery [%]	CV [%]	Mean [µg/kg]	Recovery [%]	CV [%]
500	575	115	12	467	93	14
1500	1540	103	4	1432	95	4
5000	4731	95	6	4623	92	6

The results for 10 naturally contaminated samples are presented in Table 8 and Fig.1. Reveal Q+ DON had a high agreement with UHPLC-MS/MS method - the square of the correlation coefficient was 0.9278 and 0.9699 for ordinary regression and regression through the origin, respectively.

Table 8: Results for the analysis of the naturally contaminated wheat DDGS samples (n=6) using Reveal Q+ DON and correlation to UHPLC-MS/MS results.

	Reveal Q+ DON	
Wheat DDGS code	**Recovery [%]**	**CV [%]**
W1	93	6
W2	99	10
W3	122	7
W4	98	15
W5	113	15
W6	70	12
W7	73	20
W8	75	29
W9	59	27
W10	80	38
Mean	99	15

	Ordinary regression	Regression through the origin
R^2	0.9278	0.9699
Slope ± standard error	1.191 ± 0.1175	0.980±0.058

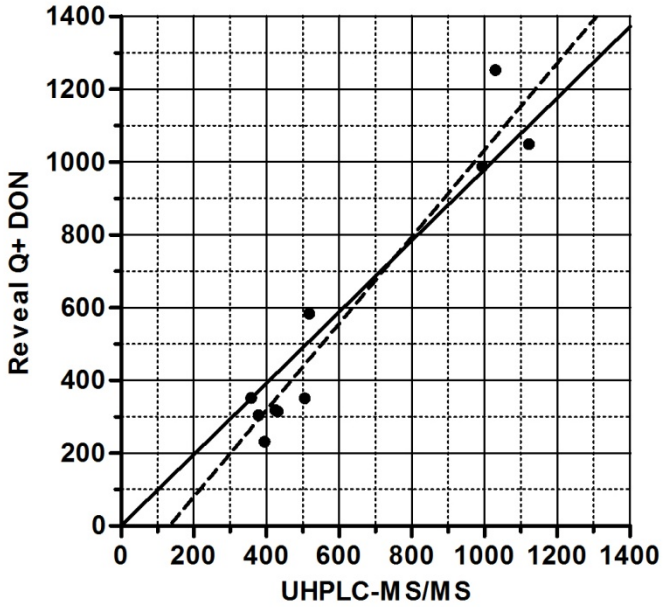

Fig.1. Correlation between DON concentrations in naturally contaminated wheat DDGS sample as determined by UHPLC-MS/MS method (x-axis) and Reveal Q+DON LFD (y-axis). Results are in µg/kg. Dashed line presents ordinary regression and solid line regression through the origin.

Reveal Q+ fumonisin

As maize DDGS matrix completely free from fumonisins was not available, the sample with lowest concentration containing 273 µg/kg total fumonisins (sum of FB_1, FB_2, FB_3) as analysed by UHPLC-MS/MS was used as "blank" matrix in spiking experiments. Reveal Q+ fumonisin reading for this sample was <600 µg/kg. The mean recoveries for samples spiked at three different levels were 89−123% and 84−106% when "blank" value was subtracted from each reading (Table 9). The coefficients of variations were lower than 20%.

Table 9: Results for determining FB_1 concentration in maize DDGS spiked at three levels. On each level 3 extractions were performed on day 1 and day 2, which gives 6 replicates at each level.

	Reveal Q+ fumonisin			**Reveal Q+ fumonisin (blank subtracted)**		
Spiking level [µg/kg]	**Mean [µg/kg]**	**Recovery [%]**	**CV [%]**	**Mean [µg/kg]**	**Recovery [%]**	**CV [%]**
600	738	123	7	501	84	5
2500	2881	115	16	2644	106	19
10000	8865	89	16	8628	86	16

Due to the high cross-reactivity of the antibody used in the LFD with FB_1 (100%), FB2 (80%) and FB3 (80%) the Reveal Q+ fumonisin results in naturally contaminated samples were correlated to total fumonisins content (sum of FB_1, FB_2 and FB_3) determined by UHPLC-MS/MS, not the content of FB_1 only (Table 10, Fig. 2.). A clear pattern was observed: the two least contaminated samples − M9 and M10 gave lower recoveries: 60 and 67%, middle contaminated samples M4−M8 showed very good recoveries between 91 and 104%, while three highest contaminated samples M1−M3 gave slightly overestimated readings when comparing to UHPLC-MS/MS results. Nevertheless, the correlation of Reveal Q+ fumonisin and UHPLC-MS/MS results was very good and the square of the correlation coefficient was 0.9849 for the ordinary regression and 0.9816 for regression through the origin.

Table 10: Results of the analysis of the naturally contaminated maize DDGS samples (n=6) using Reveal Q+ fumonisin and correlation to UHPLC-MS/MS results (sum of FB_1, FB_2 and FB_3).

	Reveal Q+ fumonisin	
Maize DDGS code	**Recovery [%]**	**CV [%]**
M1	129	16
M2	122	13
M3	122	18
M4	103	9
M5	95	27
M6	104	16
M7	102	18
M8	91	18
M9	60	6
M10	67	10
Mean	99	15

	Ordinary regression	**Regression through the origin**
R^2	0.9849	0.9816
Slope ± standard error	1.406 ± 0.062	1.155 ± 0.053

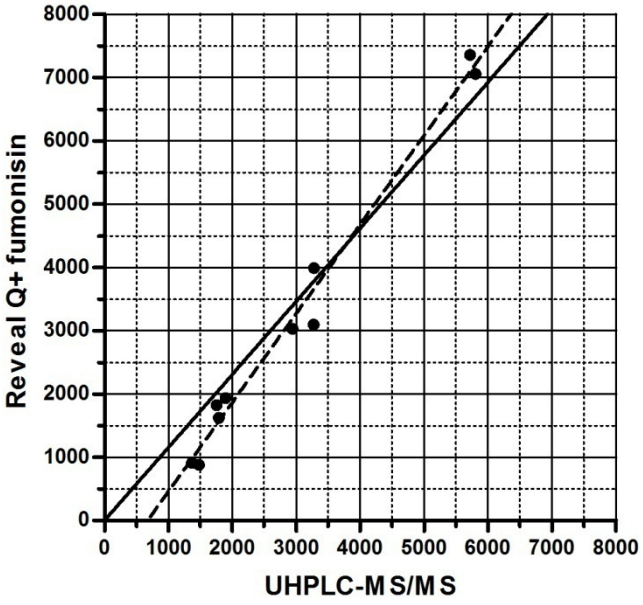

Fig.2. Correlation between total fumonisin concentrations in naturally contaminated maize DDGS sample as determined by UHPLC-MS/MS method (x-axis) and Reveal Q+ fumonisin LFD (y-axis). Results are in µg/kg. Dashed line presents ordinary regression and solid line regression through the origin.

DDGS samples can contain a broad spectrum of mycotoxins and at higher levels, due to the mycotoxin concentration during ethanol production. It is important to routinely screen these animal feed ingredients to assure highly contaminated batched are not introduced into animals diets. Ideally, the method for such screening should be fast, robust and easy to use. In the recent years a number of food safety companies have developed LFDs for a detection of mycotoxins in cereal and cereal products. They are especially applicable for on-site screening at food and feed manufacturing plant to quickly identify the contaminated materials. The study on the evaluation of commercial LFDs to detect mycotoxins in DDGS has not been done thus far. Recently, van der Fels-Klerx and de Rijk (2014) evaluated the performance of four LFDs for the detection of deoxynivalenol in wheat matrix. In another study Aamot *et al.* (2012) evaluated the performance of two ELISA test kits and two LFDs for the detection of deoxynivalenol in wheat and oat.

In this study, the application of the commercially available LFDs produced by Neogen for the detection of DON and fumonisin in DDGS matrix was assessed. While both LFDs have been validated by the manufacturer to use for DDGS samples analysis, it has not been specified if they can be used for any type of DDGS that can be produced from different types of starting grain such as maize, wheat, barley or triticale. In this study the application of Reveal Q+ DON was tested in wheat DDGS matrix and Reveal Q+ fumonisin in maize DDGS. Our

previous survey of 169 DDGS samples demonstrated that, while every maize DDGS sample analysed (n=52) was contaminated with fumonisin, the occurrence of DON in these type of samples was also common (62% of the analysed samples) and the levels could even be as high as 16000 µg/kg. While in the USA maize DDGS is the main type produced, in the EU other types of DDGS originating from wheat, barley, rye, triticale or sugar beet are also produced (ePURE, 2014). Therefore, testing of these commodities will become more and more important for the feed manufacturing industry.

The most important point to consider while analysing DDGS samples is a higher acidity of the DDGS sample extracts what can interfere with the antigen-antibody interaction. Therefore, it is necessary to correct the pH of each sample to neutral pH before analysis and dilution in the assay buffer. When comparing nutrient content of DDGS and grain, DDGS is 3 times more concentrated, so it contains 3 times more matrix components such as protein, fibre and oils. While Reveal+ DON sample preparation method already includes high dilution (factor 110 for the whole procedure), it was not necessary to include any additional step for the DDGS samples analysis comparing to grain samples to overcome the matrix effect. The analysis of both spiked and naturally contaminated samples gave accurate results.

In case of Reveal Q+ fumonisin, the manufacturer recommended the use of different ratio of matrix to extraction solvent (ratio 1:4) to correct for lower recoveries from DDGS matrix comparing to grain (ratio 1:5). This way the same calibration for both grain and DDGS samples could be used. They also recommended to increase the dilution factor in the assay buffer from 3 to 6 to overcome higher matrix effect caused by DDGS, so the dilution factor for the whole procedure was 24, while it was 15 for the grain samples preparation. This extraction procedure was shown to give accurate results for spiked samples. Reveal Q+ fumonisin gave slightly overestimated results when comparing with UHPLC-MS/MS analysis of three highest naturally contaminated maize DDGS samples - M1, M2 and M3. The recoveries for these samples were 122−129%.This effect was not observed in spiked samples at high concentration. This suggests that this effect in naturally contaminated samples can be caused by presence of masked forms of fumonisins that covalently (bound forms) and non-covalently (hidden forms) bind to proteins or carbohydrates and escape LC-MS/MS analysis; however, they might be detected by the antibodies in LFD format. It is possible that these forms are released in gastrointestinal tracts; therefore, in recent years a number of studies have been published to highlight the risk of underestimation of total fumonisin content in samples analysed by chromatographic methods. Bryla *et al.* (2016) analysed maize based products for free and total (free+hidden) fumonisins and found mean hidden to free fumonisin ratio in different products to be between 1.47 and 5.95. Latorre *et al.* (2015) found that hidden FB_1 and FB_2 accounted for 65% and 39%, respectively of total FB_1 and total FB_2 in corn silage samples.

Free fumonisins and also masked fumonisins can be degraded in alkaline media releasing a side chain (hydrolyzed fumonisin) that can be quantified using LC-MS/MS.

Conclusions

Overall, both DON and fumonisin LFDs are suitable for screening of DDGS.

4.2.2. Tecna Kits

As maize DDGS matrix completely free from fumonisins was not available, the sample with lowest concentration containing 273 µg/kg total fumonisins (sum of FB_1, FB_2, FB_3) as analysed by UHPLC-MS/MS was used as "blank" matrix in spiking experiments. The reading for this sample was <250 µg/kg by Smart Strip FUMO. The mean recoveries for samples spiked at three different levels were 101−147% for the Smart Strip FUMO (Table 11). When "blank" value was subtracted from each reading the recoveries were 98−101%. The coefficients of variations were lower than 21%.

Table 11: Results for determining FB_1 concentration in maize DDGS spiked at three levels. On each level 3 extractions were performed on day 1 and day 2, which gives 6 replicates at each level.

Spiking level [µg/kg]	Mean [µg/kg]	Recovery [%]	CV [%]	Mean [µg/kg]	Recovery [%]	CV [%]
	Smart Strip FUMO			Smart Strip FUMO (blank subtracted)		
600	880	147	15	608	101	21
2500	2727	109	8	2454	98	9
10000	10083	101	5	9811	98	5

According to the manufacturer's information, the cross-reactivity of the antibody used in Smart Strip FUMO LFD is 100% with FB_1, 70% with FB_2 and 106% with FB_3. Therefore, results in naturally contaminated samples were correlated to total fumonisins content (sum of FB_1, FB_2 and FB_3) determined by UHPLC-MS/MS, not the content of FB_1 only (Table 12, Fig. 3). The correlation of LFD and UHPLC-MS/MS results was very good and the squares of the correlation coefficients were 0.9849 for ordinary regression and 0.9706 for the regression through the origin. The slight overestimation was observed for samples M1-M7 compared to the UHPLC-MS/MS results. This effect was not observed in spiked samples at high concentration. This suggests that this effect in naturally contaminated samples can be caused by presence of masked forms of fumonisins that covalently (bound forms) and non-covalently (hidden forms) bind to proteins or carbohydrates and escape LC-MS/MS analysis; however,

they might be detected by the antibodies in the LFD format. It is possible that these forms are released in gastrointestinal tracts; therefore, in recent years a number of studies have been published to highlight the risk of underestimation of total fumonisin content in samples analysed by chromatographic methods. For example Bryla *et al.,* 2016 analysed maize based products for free and total (free+hidden) fumonisins and found mean hidden to free fumonisin ratio in different products to be between 1.47 and 5.95. Latorre *et al.,* 2015 found that hidden FB_1 and FB_2 accounted for 65% and 39%, respectively of total FB_1 and total FB_2 in corn silage samples.

Table 12: Results of the analysis of the naturally contaminated maize DDGS samples (n=6) using Smart Strip FUMO and correlation to UHPLC-MS/MS results (sum of FB_1, FB_2 and FB_3).

	Smart Strip FUMO	
Maize DDGS code	**Recovery [%]**	**CV [%]**
M1	161	11
M2	153	20
M3	145	13
M4	117	13
M5	125	14
M6	115	14
M7	127	13
M8	99	13
M9	42	20
M10	61	9
Mean	115	14

	Ordinary regression	**Regression through the origin**
R^2	0.9869	0.9706
Slope ± standard error	1.735 ± 0.07058	1.339 ± 0.07738

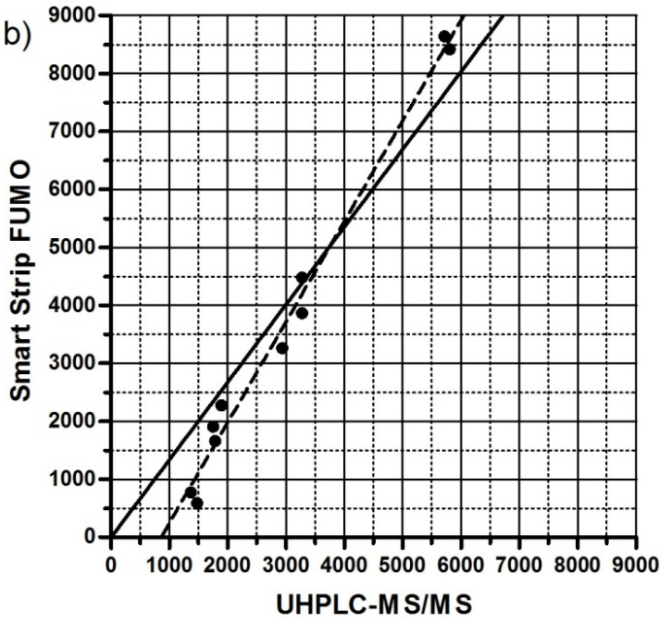

Fig.3. Correlation between total fumonisins concentration in naturally contaminated maize DDGS sample as determined by UHPLC-MS/MS method (x-axis) and Smart Strip FUMO (y-axis). Results are in µg/kg. Dashed line presents ordinary regression and solid line regression through the origin.

Conclusions

Overall, the fumonsisin LFD is suitable for the analysis of DDGS for a quick identification of highly contaminated batches.

4.3. Elements

4.3.1. Archived DDGS

Archived wheat, corn and tri-mix, from Austria, Hungary or the USA, DDGS were analysed for 23 elements, for inorganic arsenic content, and for their lead isotope ratios (Fig. 4). The salient features of this dataset is that inorganic arsenic is low, <0.025 mg/kg, and typical of levels previously reported in wheat, barley and corn (Adomako et al., 2011; Williams et al. 2007). US and Hungarian corn are particularly low in inorganic arsenic, <0.01 mg/kg. Austrian DDGS is elevated in wheat, corn and trimix inorganic arsenic compared to other geographic regions. Total arsenic, which consists of inorganic and organic species, is more evenly distributed across the regions and DDGS grain type. Cadmium is around double the levels in UK wheat, at 0.12 mg/kg, compared to other regions, with elevation of cadmium in arable/horticultural crops known to be problematic in specific regions due to the relatively high availability of

cadmium in soils (Meharg et al., 2014; Norton et al., 2015). Lead is not specifically elevated for any grain or for any region for the DDGS data presented here. Lead isotopes were not particularly discriminatory between grain type or region. Selenium in US corn was elevated at 0.25 mg/kg, double other grains and regions, with US soils known to be selenium rich leading to high selenium in grain (Zhu et al., 2009). Other elements where at concentrations expected in grains (Norton et al., 2014) and DDGS (Liu, 2011) where not particularly noteworthy.

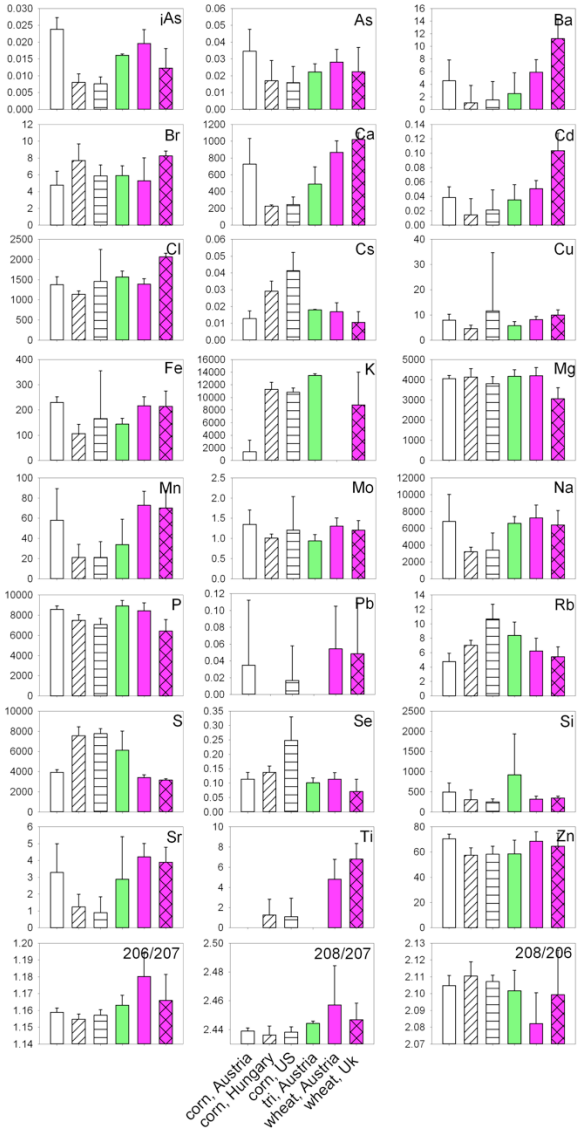

Fig. 4. Elemental content, inorganic arsenic concentration and lead isotope ratios of DDGSs derived from different grains and from different regions. The y-axis concentrations are in mg/kg for elements, while the lead isotope axis are ratios.

Further DDGS from UK sources were analysed, bringing the total amount of DDGS samples to 162. The multi-elemental analysis of these samples, broken down by grain type, are shown in Fig. 5. Boxplots show that wheat, barley and corn have similar levels of most elements, and that these elemental concentrations are typical of grains (Norton et al. 2014, Williams et al. 2007) and corn DDGS (Liu, 2011). The exceptions are cadmium which are elevated in wheat at ~0.1 mg/kg, almost an order of magnitude higher than corn, with barley being intermediate.

This is consistent with higher levels of cadmium reported in UK arable produce (Norton et al., 2015). Selenium was very low in UK wheat, also an observation that has been made before (Zhu et al., 2009).

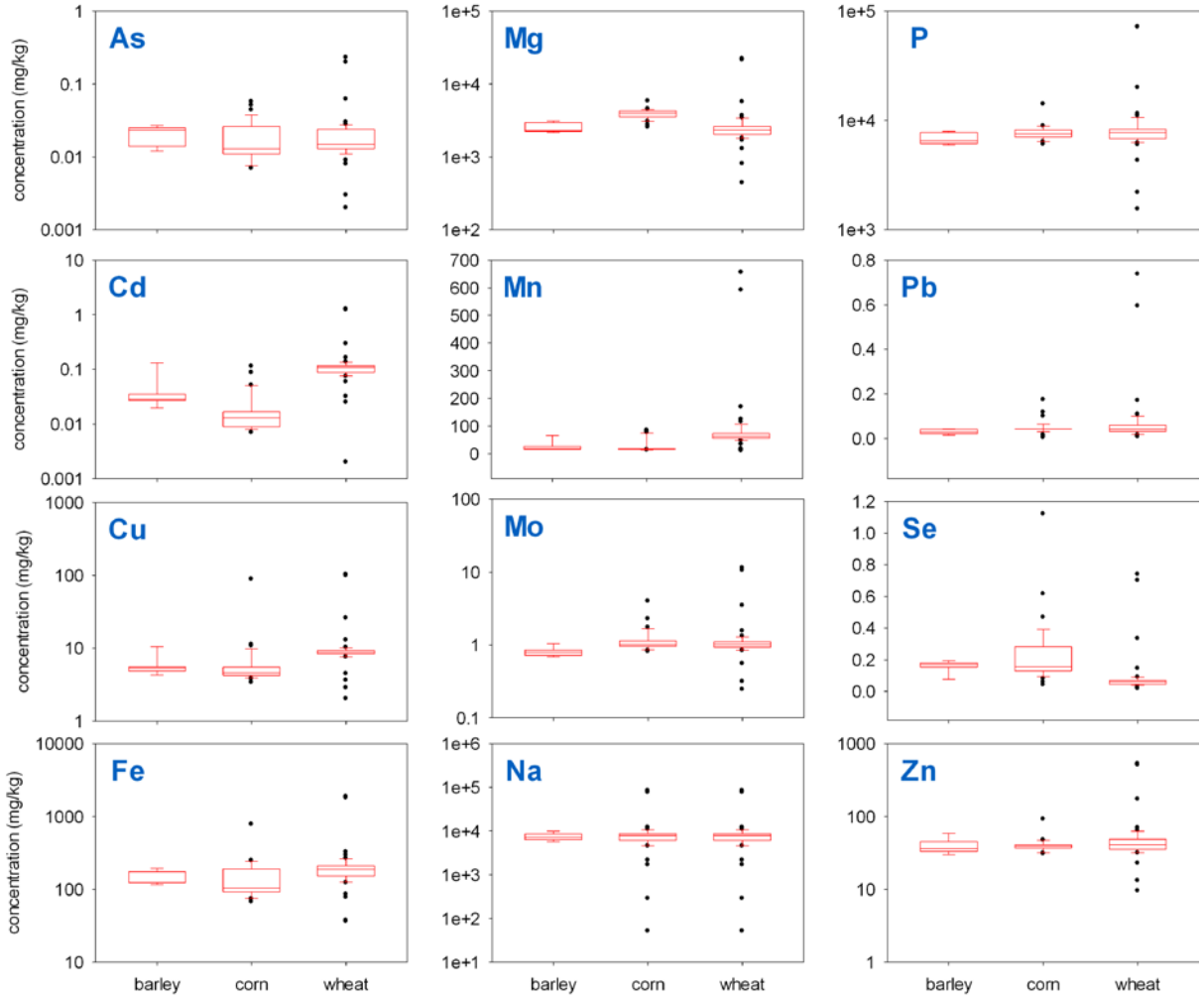

Figure 5. Boxplots which show median, 25th and 75th, 10th and 90th, and outliers for multi-elements in wheat barley and corn DDGS.

There were 52 grain-DDGS pairs which enabled the change in element content on DDGS preparation to be ascertained. The ratio of DDGS/grain is given in Fig.6. This plot shows that elemental concentration increases ~3-fold on DDGS preparation across all elements. The major exception to this is sodium where there was a 300-fold increase in concentration. This very strongly implies that sodium is added to the grains during DDGS preparation. A 3-fold increase for other elements is to be expected as fermentation will lead to a concentration of recalcitrant components of the biomass. The average sodium content of UK DDGS is 0.85%. This compares to a literature value of 0.25 – 0.48%, so is on the high-side (Batal and Dale, 2003). The sodium content is also variable in our wheat DDGS analysis at the low

concentration end, with the 10th percentile at 0.09%. Sodium being high and variable in content was raised as a major issue for DDGS use in chicken feed (Batal and Dale, 2003). Liu (2011) surveyed the concentrations of a sodium from the corn DDGS literature. From the 6-studies, sodium ranged on average from 0.0001-0.51%. This means that the UK wheat DDGS wheat sodium content is higher than in comparable corn studies.

The concentrations of phosphorus, magnesium, manganese, iron, copper and zinc reported here were very similar for the average of 12 US corn DDGS samples origin (Batal and Dale, 2003). The 3-fold elevation in most element contents reported here for wheat was also observed in corn derived DDGS, with sodium, sulphur, calcium and iron being higher, which the authors attributed as to being exogenously added during manufacture (Liu and Han, 2011). We only recorded sodium and iron from this list with our iron also being elevated, 6-fold, between grain and DDGS. Iron content can vary ~10-fold between corn DDGS studies (Liu, 2011). Liu (2011) surveyed the concentrations of a range of elements reported here (phosphorus, magnesium, iron, zinc, manganese and copper) from literature studies and these are within the ranges reported here.

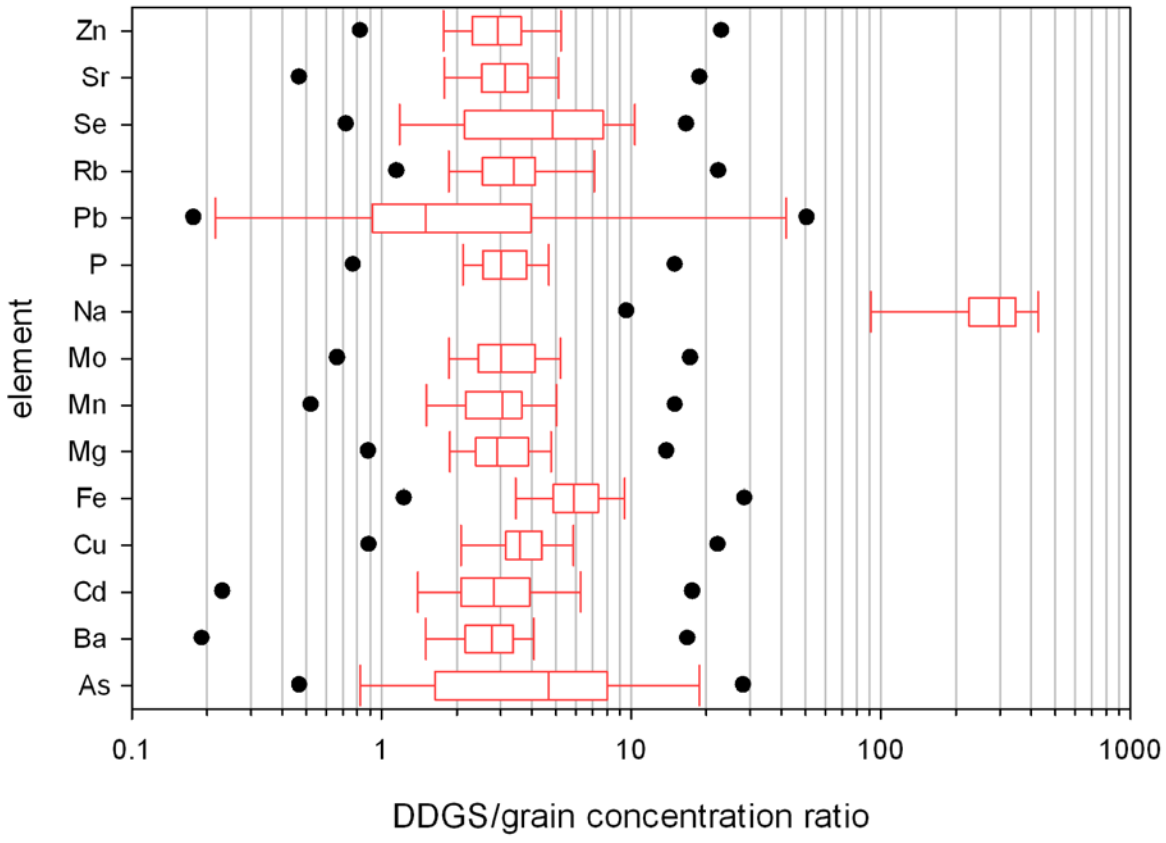

Figure 6. Boxplots which show median, 25th and 75th, 10th and 90th, and outliers for multi-elements' DDGS/grain ratio.

5. References

Aamot, H.U., Hofgaard, I.S., Brodal, G., Elen, O., Jestoi, M., Klemsdal, S.S. (2012) Evaluation of rapid test kits for quantification of deoxynivalenol in naturally contaminated oats and wheat. World Mycotoxin Journal, 5(4), 339-350.

Adomako EE, Williams PN, Deacon C, Meharg AA (2011) Inorganic arsenic and trace elements in Ghanaian grain staples. *Environmental Pollution* 159, 2435-2442.

Bacon, C. W.; Porter, J. K.; Norred, W. P. (1995) Toxic interaction of fumonisin B1 and fusaric acid measured by injection into fertile chicken eggs. *Mycopathologia*, 129, 29−35.

Batal A and Dale. (2003) Mineral composition of distillers dried grains with solubles. J. Applied Poultry Research, 12, 400-403.

Bryła, M., Roszko, M., Szymczyk, K., Jedrzejczak, R., Obiedziński, M.W. (2016) Fumonisins and their masked forms in maize products. Food Control, 59, 619-627.

Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. *Off. J. Eur. Communities*, *L 140*, 10−21.

ePURE European Renewable Ethanol. Renewable ethanol: driving jobs, growth and innovation, throughout Europe state of the Industry Report 2014 (http://www.epure.org/media/1137/state-of-the-industry-report-2014.pdf).

European Food Safety Authority (2012) Scientific opinion on ergot alkaloids in food and feed. *EFSA Journal*, 10(7):2798.

Hanschmann, G; Krieg, D. (2006) The fate of fusarium toxins in the course of the synthesis of bioethanol from contaminated grain [Verhalten von Mykotoxinen bei der Ethanolerzeugung aus Getreide. *Mycotoxin Res*., *22*, 174−177.

Huang, B.; Han, Z.; Cai, Z.; Wu, Y.; Ren, Y. (2012) Simultaneous determination of aflatoxins B1, B2, G1, G2, M1 and M2 in peanuts and their derivative products by ultra-high-performance liquid chromatography-tandem mass spectrometry. *Anal. Chim. Acta***,** *662*, 62−68.

Jestoi, M. (2008) Emerging fusarium-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin - a review. *Crit. Rev. Food Sci. Nutr.*, *48*, 21−49.

Khatibi, P. A.; McMaster, N. J.; Musser, R.; Schmale, D. G. (2014) Survey of mycotoxins in corn distillers' dried grains with solubles from seventy-eight ethanol plants in twelve states in the U.S. in 2011. *Toxins*, *6*, 1155−1168.

Li, X.; Zhao, L.; Fan, Y.; Jia, Y.; Sun, L.; Ma, S.; Ji, C.; Ma, Q.; Zhang, J. (2014) Occurrence of mycotoxins in feed ingredients and complete feeds obtained from the Beijing region of China. *J Anim. Sci. Biotechnol.*, *5*, 1−8.

Liu, KS, Han, JC (2011) Changes in metal concentrations and phosphorus profile during dry-grind processing into ethanol. *Bioresource Technology* 102, 3110-3118.

Liu, KS (2011) Chemical composition of distillers grains' a review. *Journal Agriculture Food Chemistry*, 59, 1508-1526.

Meharg AA, Norton G, Deacon C, Williams PN, Adamako E, Price A, Zhu YG, Zhao FJ, Villada A, Sommella A, De Silva MSS, Bremmer H, Dasgupta T, Islam R (2013) Variation in cadmium in rice related to human exposure. *Environmental Science Technology* 47, 5613-5618.

Monbaliu, S.; Van Poucke, C.; Detavernier, C.; Dumoultn, F.; Van Velde, M. D. E.; Schoeters, E.; Van Dyck, S.; Averkieva, O.; Van Peteghem, C.; De Saeger, S. (2010) Occurrence of mycotoxins in feed as analyzed by a multi-mycotoxin LC-MS/MS method. *J. Agric. Food Chem.*, *58*, 66−71.

Norton GJ, Douglas A, Lahner B, Yakubova E, Guerinot ML, S Pinson SRM, Tarpley L, Eizenga GC, McGrath SP, Zhao FJ, Islam MR, Islam S, Duan G, Zhu YG, Salt DE, Meharg AA, Price AH (2014) Genome wide association mapping of grain arsenic, copper, molybdenum and zinc in rice (Oryza sativa L.) grown at four international field sites. *PLoS ONE* 9, e89685.

Norton G, Deacon C, Mestrot A, Feldmann J, Jenkins P, Baskaran C, Meharg AA (2015) Cadmium and lead in vegetable and fruit produce. *Science Total Environment* 533, 520-527.

Oplatowska-Stachowiak, M., Haughey, S.A., Chevallier, O.P. Galvin-King, P., Campbell, K., Magowan, E. Adam, G., Berthiller, F., Krska, R. and Elliott, C.T., (2015) Determination of the Mycotoxin Content in Distiller's Dried Grain with Solubles Using a Multianalyte UHPLC–MS/MS Method *J. Agric. Food Chem*., 63 (43), 9441–9451.

Richard, J. L.(2007) Some major mycotoxins and their mycotoxicoses - an overview. *Int. J. Food Microbiol.*, *119*, 3−10.

Rodrigues, I.; Chin, L. J. (2012) A comprehensive survey on the occurrence of mycotoxins in maize dried distillers' grain and solubles sourced worldwide. *World Mycotoxin J.*, *5*, 83−88.

Schaafsma, A. W.; Limay-Rios, V.; Paul, D. E.; Miller, J. D. (2009) Mycotoxins in fuel ethanol co-products derived from maize: a mass balance for deoxynivalenol. *J. Sci. Food Agric.*, *89*, 1574−1580.

Schneweis, I.; Meyer, K.; Hormansdorfer, S.; Bauer, J. (2000) Mycophenolic acid in silage. *Appl. Environ. Microbiol.*, *66*, 3639−3641.

Sherwood, R. F.; Peberdy, J. F. (1974) Production of the mycotoxin, zearalenone, by *Fusarium graminearum* growing on stored grain. I. Grain storage at reduced temperatures. *J. Sci. Food Agric.*, *25*, 1081−1087.

Shotwell, O. L.; Goulden, M. L.; Hesseltine, C. W. Aflatoxin M1. (1976) Occurrence in stored and freshly harvested corn. *J. Agric. Food Chem*., *24*, 683−684.

Smith, T. K.; McMillan, E. G.; Castillo, J. B. (1997) Effect of feeding blends of *Fusarium* mycotoxin-contaminated grains containing deoxynivalenol and fusaric acid on growth and feed consumption of immature swine. *J. Anim. Sci.*, *75*, 2184−2191.

Streit, E.; Schatzmayr, G.; Tassis, P.; Tzika, E.; Marin, D.; Taranu, I.; Tabuc, C.; Nicolau, A.; Aprodu, I.; Puel, O.; Oswald, I. P. (2012) Current situation of mycotoxin contamination and co-occurrence in animal feed focus on Europe. *Toxins*, *4*, 788−809.

Streit, E.; Schwab, C.; Sulyok, M.; Naehrer, K.; Krska, R.; Schatzmayr, G. (2013) Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. *Toxins*, *5*, 504−523.

Tansakul, N.; Jala, P.; Laopiem, S.; Tangmunkhong, P.; Limsuwan, S. (2013) Co-occurrence of five *Fusarium* toxins in corn dried distiller's grains with solubles in Thailand and comparison of ELISA and LC-MS/MS for fumonisin analysis. *Mycotoxin Res.*, *29*, 255−260.

Van der Fels-Klerx J. & de Rijk T.C. (2014) Performance evaluation of lateral flow immune assay test kits for quantification of deoxynivalenol in wheat. *Food Control*, 46, 390-396.

Wielogorska, E.; MacDonald S.; Elliott, C. T. (2015) A review of the efficacy of mycotoxin detoxifying agents used in feed in light of changing global environment and legislation. *World Mycotoxin J.*, in press.

Williams PN, Villada A, Deacon C, Raab A, Figuerola J, Green AJ, Feldmann J, Meharg AA (2007) Greatly enhanced arsenic shoot assimilation in rice leads to elevated grain levels compared to wheat & barley. *Environmental Science & Technology,* 41, 6854-6859.

Zachariasova, M.; Dzuman, Z.; Veprikova, Z.; Hajkova, K.; Jiru, M.; Vaclavikova, M.; Zachariasova, A.; Pospichalova, M.; Florian, M.; Hajslova, J. (2014) Occurrence of multiple mycotoxins in European feedingstuffs, assessment of dietary intake by farm animals. *Anim. Feed Sci. Technol.*, *193*, 124−140.

Zhang, Y.; Caupert, J. (2012) Survey of mycotoxins in U.S. distiller's dried grains with solubles from 2009 to 2011. *J. Agric. Food Chem.*, *60*, 539−543.

Zhu YG, Pilon-Smits EAH, Zhao FJ, Williams PN, Meharg AA (2009) Selenium in higher plants: understanding mechanisms of uptake and metabolism for biofortification and phytoremediation. Trends Plant Sci., 14(8), 436-42.