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Sampling of Wheat Dust and Subsequent Analysis of Deoxynivalenol by LC-MS/MS

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Supporting Information

ABSTRACT: An LC-MS/MS method was developed and validated for the determination of deoxynivalenol in wheat dust. Extraction was carried out with acetonitrile/water/acetic acid (79/20/1, v/v/v) followed by a hexane defatting step. Analysis was performed using a Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer. The method was validated according to the criteria mentioned in Commission Decision 2002/657/EC. Due to a high contamination level of wheat dust compared to wheat, limit of detection and limit of quantitation levels of 358 ng/g and 717 ng/g, respectively, were obtained. A small survey was executed on raw wheat materials and their corresponding dust samples (n = 12). The samples were analyzed according to the developed procedure. A linear correlation (R^2 = 0.941) was found for the deoxynivalenol concentration in dust versus the deoxynivalenol concentration in wheat. Therefore, it would be possible to estimate the cereal contamination through dust contamination.

KEYWORDS: *LC-MS/MS, deoxynivalenol, wheat dust, validation, correlation*

INTRODUCTION

Trichothecene mycotoxins are the most frequently found mycotoxin group in Europe and are particularly produced by *Fusarium* (*F.*) spp. Deoxynivalenol, which is illustrated in Figure 1, is one of the most abundant trichothecenes in wheat,

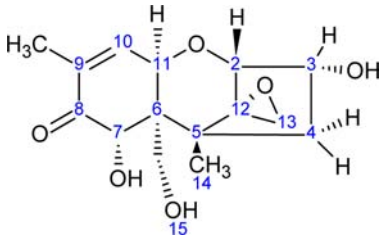

Figure 1. Chemical structure of deoxynivalenol.

mainly produced by *F. graminearum* and *F. culmorum*. Through ingestion of contaminated food and feed, diverse effects can be initiated such as reduced food and feed intake, anorexia, skin irritation, emesis, diarrhea, and hemorrhage. Trichothecenes exert their effects by acting as inhibitors of protein and DNA synthesis due to the binding at the 60 S ribosomal subunit and the subsequent prevention of polypeptide chain initiation and elongation. Furthermore, they affect the immune system and have either immunosuppressive or immunostimulative effects.[1−5]

Regarding food and feed safety, it is necessary to determine the mycotoxin content in different matrices. However, analyzing grain samples for the presence of mycotoxins is not easy. A good sampling plan, which consists of sampling, sample preparation, and analysis, is a prerequisite for correct classification of cereal lots. Because of the inevitable errors associated with each analysis step, the results should always be reported with an estimate of uncertainty. Precision and accuracy are the most important parameters related to the uncertainty. The variance (V), standard deviation (SD), and coefficient of variation (CV) are used as a measure to determine precision. Accuracy is associated with a bias, which is an influence that causes the deviation of the measured value from the true value. The final sample and analysis procedure needs to be selected to obtain high precision and high accuracy.[6−8]

A first step in the sampling plan is the sample selection. The collection of laboratory lots out of a bulk lot can be performed in two different ways: dynamic or static collection. Dynamic sampling is the movement of the lot on a conveyor belt; sampling is performed by collecting the increments at different places at a fixed time schedule. Static storage in containers, silos, load docks, and cargo ships is more frequently used. Sampling is more difficult, as several increments of all the layers of the bulk need to be charged. The mycotoxin concentration of the sample is determined as an estimation of the true mycotoxin concentration in the bulk lot or as a comparison to a defined accept/reject limit (ARL) that is usually equal to a maximum level or regulatory legal limit. The regulatory limits for deoxynivalenol in food and feed are defined in European

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Commission Regulation (EC) No. 1881/2006 and European Commission Recommendation 2006/576/EC, respectively.

To obtain a homogeneous test sample, a lot of cereals needs to be blended thoroughly. According to IUPAC, the degree of heterogeneity (i.e., opposite of homogeneity) is the determining factor of sampling error. Other sources of error and consequently uncertainty during the sampling step can be, for example, cross-contamination and the instability of the samples. Yet heterogeneity remains the largest problem of sampling and is generally the most significant source of uncertainty. To overcome the problem of heterogeneous distribution of mycotoxins in food and feed, the sample taken for analysis should be an accumulation of many small portions (i.e., incremental samples). By increasing the size of the sample and reducing the particle size followed by efficient mixing, the bias can also be reduced. Other measures to reduce the problem of heterogeneity together with sampling protocols are included in European Commission Regulation (EC) No. 401/2006.[7−11]

The relationship between the probability of accepting a lot with a specific mycotoxin concentration and the mycotoxin concentration of the bulk lot is illustrated by the operating characteristic (OC) curve.[11] The areas under the curve (AUC) limited by the ARL describe the economic risk (seller's risk) and the consumer risk (buyer's risk). The seller's risk is the risk of rejecting good lots (false positives), and the buyer's risk is the risk of accepting bad lots (false negatives). The slope of the OC curve has a high economic and health relevance. When designing a sampling plan, it is crucial to maximize the slope of the OC in order to reduce consumer and producer risks and minimize the risk of lot misclassification. The shape of the OC curve is defined by the sample size, degree of grinding, subsample size, the number of analyses, and the ARL. Therefore these parameters can be used to reduce the buyer's and seller's risks associated with a sampling plan.[9,11]

However, sample selection can be made more easy to perform, less labor-intensive, and less costly with a lower error of uncertainty by sampling dust instead of parts of the cereal bulk lot. Previously, the accumulation of mycotoxins on dust particles was confirmed.[12,13] Dust is created when grain is transported through closed systems such as the unloading of a container. Also, accumulation in mills and storage facilities is described. Because of the small size of the dust particles and the accumulation of deoxynivalenol in the dust fraction, the proposed sampling technique is promising for the determination of deoxynivalenol in wheat. In the future, this technique can be extrapolated for the determination of other mycotoxins in wheat and different matrices.

The aim of this study was to develop and validate a liquid chromatography−tandem mass spectrometric (LC-MS/MS) method for the determination of deoxynivalenol in wheat dust.

MATERIALS AND METHODS

Reagents and Chemicals. LC-MS grade methanol was obtained from BioSolve BV (Valkenswaard, The Netherlands). Acetonitrile (Analar Normapur), *n*-hexane (Hipersolv Chromanorm), and ammonium acetate (analytical reagent) were purchased from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100%) was obtained from Merck (Darmstadt, Germany). Ethyl acetate and dichloromethane were purchased from Acros Organics (Geel, Belgium). Water was obtained from a Milli-Q SP Reagent water system from Millipore Corp. (Brussels, Belgium).

Deoxynivalenol (Figure 1) (10 mg) and de-epoxy deoxynivalenol (50 ng/μL) standards were purchased from Fermentek (Jerusalem, Israel) and Coring System Diagnostics (Gernsheim, Germany), respectively. The deoxynivalenol standard was dissolved in methanol in a concentration of 1 μg/μL and stored at 4 °C. Working solutions of deoxynivalenol (10 ng/μL) and de-epoxy deoxynivalenol (2.5 ng/μL) were prepared in methanol and stored at −18 °C.

Determination of Dust Particle Size and Quantity. Dust fractions were laboratory fractionated by the use of a vibratory sieve shaker (Retsh, Aartselaar, Belgium). Freshly harvested wheat samples were cleaned by sieving, separating all grain particles smaller than 2.5 mm. A particle size distribution was built up with particles smaller than 2.5 mm using different sieve pore diameters on the sieve shaker. The bottom fraction, which can be considered as dust, was further divided in >100 μm, 50−100 μm, and <50 μm. The quantity of dust of each of these fractions was determined.

Dust Collection. Dust was produced out of wheat samples by the use of a dust collection facility. Wheat samples were transferred through the dumping pit to the vertical elevator system via the Archimedes screw. At the highest point of the system the grains fall in the vertical bin, on top of which the dust collection device (i.e., vacuum cleaner) was mounted. The dust was collected by removing the paper bag of the vacuum cleaner and, after weighing the dust, transferred in closed containers.

Sample Preparation. One gram of dust sample was weighed in a Gosselin extraction tube (50 mL). A stock solution (20 μL) of the internal standard de-epoxy deoxynivalenol (2.5 ng/μL) was added. The dust samples were vortexed (Labinco BV, Breda, The Netherlands) and soaked for 15 min. The extraction was performed with 10 mL of acetonitrile/water/acetic acid (79/20/1, v/v/v) for 60 min using an agitator decanter overhead shaker (Agitelec, J. Toulemonde and Cie, Paris, France) followed by centrifugation at 3000*g* for 15 min. The extraction residue was filtered using a Whatman No. 4 filter (VWR International, Zaventem, Belgium). To the filtered residue was added 10 mL of hexane for defatting, followed by shaking for 15 min using the agitator decanter overhead shaker. After centrifugation at 3000*g* for 15 min, the hexane layer was removed and the filtrate was evaporated until dryness under a stream of nitrogen at 40 °C. Then, the dry residue was redissolved in a 100 μL injection solvent of methanol/water/acetic acid (41.8/57.2/1, v/v/v) with 5 mM ammonium acetate. The redissolved sample was centrifuged for 10 min at 10000*g* prior to LC-MS/MS analysis.

LC-MS/MS Analysis. LC-MS/MS analysis was performed using a Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray interface (ESI) by injecting a volume of 10 μL. Chromatographic separation was performed using an Acquity UPLC BEH C_{18} column (1.7 μm, 100 mm × 2.1 mm i.d.) equipped with a guard column of the same material (5 mm × 2.1 mm i.d.) supplied by Waters (Milford, MA, USA). The column was kept at 60 °C, and the temperature of the autosampler was set at 10 °C. A mobile phase consisting of water/methanol/acetic acid (94/5/1, v/v/v) containing 5 mM ammonium acetate (A) and methanol/water/acetic acid (97/2/1, v/v/v) containing 5 mM ammonium acetate (B) was used at a flow rate of 0.3 mL/min. The gradient elution program started at 99% mobile phase A with a linear decrease to 50% mobile phase A in 3 min. A linear increase to 99% mobile phase B was established in 0.5 min. An isocratic gradient of 99% mobile phase B initiated at 3.5 min for 2 min. Re-equilibration to 99% mobile phase was established after an additional 3 min. The duration of each UPLC run was 8.5 min. The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode. MS parameters for the analysis were as follows: ESI source block and desolvation temperatures, 120 and 400 °C, respectively; capillary voltage, 3.2 kV; argon collision gas, 1.15×10^{-2} mbar; cone nitrogen and gas flow, 50 and 800 l/h, respectively. The data acquisition was performed using selected reaction monitoring (SRM). The optimized MS/MS parameters for the precursor ion and product ions of deoxynivalenol and de-epoxy deoxynivalenol were determined. Deoxynivalenol was set at m/z 297.10 ($[M + H]^+$) with product ions of m/z 231.20 (quantifier ion) and m/z 249.20, while de-epoxy deoxynivalenol was determined at m/z 281.00 ($[M + H]^+$) with product ions of m/z 109.10 (quantifier ion) and m/z 137.00. The mycotoxins eluted at 2.54 and 3.10 min, respectively. Masslynx version

Table 1. Size Distribution for Wheat Particles Smaller than 2.5 mm

sieve pore diameter	residual amount 1 (g)	residual amount 2 (g)	residual amount 3 (g)	mean mass (g)	SD (g)	percentage (%)	cumulative sieve residue (%)
3.15 mm	17.02	11.25	9.82	12.70	3.81	6.35	6.35
2.0 mm	164.00	170.25	172.04	168.76	4.22	84.38	90.73
1.4 mm	14.09	12.89	13.28	13.42	0.61	6.71	97.44
1.0 mm	2.04	2.15	1.88	2.02	0.14	1.01	98.45
800 μm	0.78	1.03	0.75	0.85	0.15	0.43	98.88
710 μm	0.33	0.42	0.42	0.39	0.05	0.20	99.07
500 μm	0.51	0.64	0.76	0.64	0.13	0.32	99.39
400 μm	0.20	0.30	0.32	0.27	0.06	0.14	99.53
355 μm	0.08	0.12	0.13	0.11	0.03	0.06	99.58
bottom	0.43	0.58	0.53	0.51	0.08	0.26	99.84

4.1 and Quanlynx version 4.1 software (Micromass, Manchester, UK) were used for data acquisition and processing.

Method Validation. The method was validated in terms of linearity, apparent recovery, limit of detection (LOD), limit of quantitation (LOQ), precision, and accuracy based on the Commission Decision 2002/657/EC of August 12, 2002, concerning the performance of analytical methods and the interpretation of results. Although this EU legislation is not particularly established for dust analysis, it was chosen as a reference because of its clear criteria for LC-MS/MS contaminant analysis.

Blank wheat dust was spiked during three consecutive days at six different concentration levels of 250, 500, 1000, 3000, 9000, and 18 000 ng/g with deoxynivalenol. De-epoxy deoxynivalenol was added as internal standard, and the quantitation was performed by the use of the relative standard peak area. A linear regression was applied.

The apparent recovery was calculated by the use of a matrix-matched calibration curve. In detail, dust samples were spiked with deoxynivalenol at different concentration levels and were analyzed using the optimized LC-MS/MS method. The observed signal was plotted against the actual concentration. The measured concentration was determined using the calibration curve, and the apparent recovery was calculated by the following equation (eq 1):

$$\%\text{apparent recovery} = \text{measured concentration (ng/g)} / \text{actual spiked concentration (ng/g)} \times 100 \quad (1)$$

LOD and LOQ were determined as respectively 3 and 6 times the standard error of the intercept, divided by the slope of the standard curve. Also, the LOD and LOQ values were verified according to the IUPAC guidelines, stating that the signal-to-noise ratio (S/N) should be more than 3 and 10 times, respectively.

The intraday precision or repeatability (RSD_r) of the method was determined by repeated analysis on the same day of dust samples spiked at different concentration levels (1000, 3000, 7500, 9000, 17 000 ng/g). The same experiments were performed on three consecutive days for the determination of the interday precision or within-laboratory reproducibility (RSD_R) and accuracy. From the accuracy data, biases were evaluated and a conclusion was taken according to the acceptability of the method. All calculations were performed and processed using Microsoft Office Excel 2010 and IBM SPSS 20.

Collection of Wheat and Corresponding Dust Samples. Wheat samples (n = 12) were randomly collected in Belgium. From one part of every wheat sample, dust was aspirated by the use of the dust collection facility, while the other part of the wheat sample was ground.

Dust Correlation Study. The deoxynivalenol content of the wheat samples (n = 12) was determined with the LC-MS/MS method described previously.[18] From each wheat specimen, dust was produced and collected with the vacuum cleaner. The dust samples were analyzed using the developed LC-MS/MS method. Verifying a possible correlation, the Pearson correlation coefficient was determined for the batch of samples. Matrix effects of dust were evaluated in terms of peak response of wheat dust compared to wheat at deoxynivalenol levels between 300 and 9600 ng/g.

RESULTS AND DISCUSSION

Determination of Dust Particle Size and Quantity. The particle size distribution of wheat particles smaller than 2.5 mm collected after cleaning of different wheat samples obtained by a vibratory sieve shaker is presented in Table 1. Only 1.5% of the wheat sample was smaller than 2.5 mm. When sieved on the vibratory system, 1.5% of this fraction was found to be smaller than 1 mm. The bottom fraction (dust), which was divided into >100 μm, 50−100 μm, and <50 μm, was weighed, and it was found that the <50 μm fraction was most abundant (Figure 2). Therefore, it was decided to use this fraction for LC-MS/MS analysis.

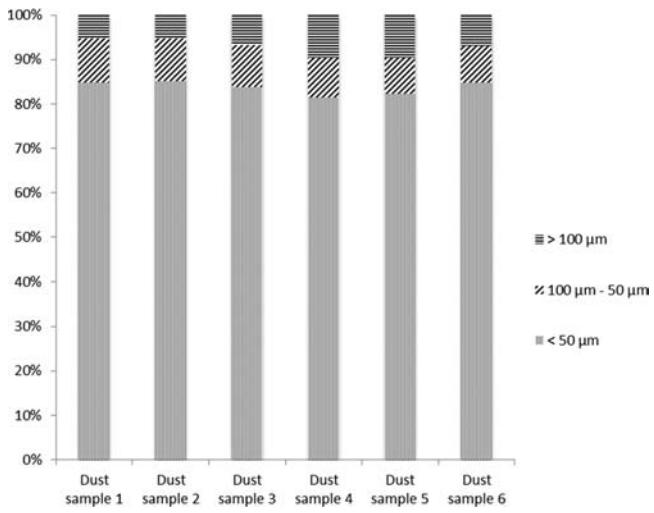

Figure 2. Cumulative distribution of the dust fractions in which three fractions can be distinguished: <50 μm, 50−100 μm, and >100 μm.

Screening of Deoxynivalenol Content in Wheat Dust. To estimate the deoxynivalenol content in wheat dust, the dust fraction <50 μm and nonsieved wheat grains were first analyzed using the Veratox Neogen (Scotland, United Kingdom) enzyme-linked immunosorbent assay (ELISA) test kit.[14] According to the screening method, the deoxynivalenol content in the dust was 10-fold more than the deoxynivalenol content in the wheat grain.

Optimization of Sample Preparation and Cleanup. De Boevre et al.[18] described the use of liquid liquid extraction (LLE) with acetonitrile/water/acetic acid (79/20/1, v/v/v) as

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extraction solvent and a hexane defatting step, as sample preparation for wheat matrix. A supplemental cleanup step with C_{18} columns of 500 mg/6 mL (Achrom, Zulte, Belgium) was investigated. The solid-phase extraction (SPE) was performed under acidic conditions by the use of acetonitrile/water/acetic acid (79/20/1, v/v/v) as extraction solvent. After the hexane defatting step, one part of the extract was filtered through a Whatman glass filter. Another part of the defatted extract was further purified by the use of a MultiSep 226 AflaZon+ column and acetonitrile/acetic acid (99/1, v/v) as washing solvent. Both purified extracts were combined and evaporated to dryness under a stream of nitrogen at 40 °C.[15,16] Additional sample preparation experiments were performed under neutral conditions. For these experiments, the extraction efficiency was tested by the use of methanol/water (90/10, v/v) and 100% ethyl acetate followed by 100% dichloromethane.[17] All cleaned samples were first visually evaluated, as the color was a measure of the dirtiness of the sample. Dust samples extracted under neutral conditions were eliminated due to the obtained dirty extract, and it was decided not to proceed with these extracts to protect the LC-MS/MS equipment.

Optimization of the LC-MS/MS Method. For the chromatographic separation two different columns were compared: the ZORBAX Eclipse XDB C_{18} column and the Symmetry C_{18} with a Symmetry C_{18} guard column. For the Zorbax Eclipse XDB C_{18} column, a mobile phase consisting of water/methanol (95/5, v/v) and methanol/water (95/5, v/v) buffered with 10 mM ammonium acetate and adjusted to pH 3 with glacial acetic acid was used. [18] The Symmetry C_{18} column was used in combination with a mobile phase consisting of water/methanol/acetic acid (94/5/1, v/v/v) containing 5 mM ammonium acetate and methanol/water/acetic acid (97/2/1, v/v/v) containing 5 mM ammonium acetate.[16] Both columns were kept at room temperature, and the flow rate was 0.2 and 0.3 mL/min, respectively. For the detection, MS/MS conditions were optimized for deoxynivalenol and de-epoxy deoxynivalenol via direct injection into the mass spectrometer with a syringe pump at a flow rate of 10 μL/min. Cone voltages of 26 and 37 V were applied and compared. In total, two different sample preparation methods were combined with two different LC methods and two different MS/MS conditions. For the choice of the most optimal combination of sample preparation, LC, and MS/MS conditions, the following factors were taken into account: the resulting relative peak area and the costs and time for sample preparation. According to the observed signal (expressed as relative peak area) for both extraction methods and LC methods, a 4.4-fold higher signal was observed using no cleanup, a cone voltage of 26 V, and the Symmetry C_{18} column. Adding a cleanup step increased the signal by a factor of 4.7; however it was decided not to add the SPE cleanup in the final sample preparation method for wheat dust. In conclusion, the most optimal results were obtained using the combination of the Symmetry C_{18} column for chromatographic separation and the MS/MS conditions with 26 V as cone voltage.

Another parameter for optimization was the total run-time for each LC. By using the Symmetry C_{18} column, it was possible to have a chromatographic separation of deoxynivalenol and de-epoxy deoxynivalenol in only 18 min. Therefore, UPLC conditions were preferred. The Acquity UPLC BEH C_{18} column was suitable due to similar characteristics to the LC column. The same LC-MS/MS conditions were used, but the temperature of the column was set at 60 °C. Using these UPLC conditions, it was possible to reduce the time of each run to 8.5 min. The deoxynivalenol and internal standard de-epoxy deoxynivalenol showed a retention time of 2.54 and 3.10 min, respectively. As a consequence, a sufficient separation of deoxynivalenol and de-epoxy deoxynivalenol with a 60 s difference was obtained.

Method Validation. The LC-MS/MS method was successfully validated for deoxynivalenol in wheat dust based on the Commission Decision 2002/657/EC. A deoxynivalenol spiking range from 250 to 18 000 ng/g was taken for the calibration curve, because of the high deoxynivalenol concentration in dust observed by ELISA and LC-MS/MS. The linearity was evaluated by the correlation coefficient (R^2) and a lack of fit test. An R^2 of 0.996 and a p-value of 0.551 conclude that the calibration curve revealed good linearity. The apparent recovery ((RSD, %), n = 3) determined at medium level (1000 and 3000 ng/g) was 97% (SD = 13) and 109% (SD = 4). The LOD and LOQ were 358 ng/g and 717 ng/g, respectively. During the precision study, RSDs were measured at the five different concentration levels; however, only the data obtained at the medium and high level (7500 and 17 000 ng/g) were recorded. For the intraday precision an RSD_r of 6.87% and 2.09%, respectively, for the medium level and high level was observed. For the interday precision an RSD_R of 9.02% and 2.25%, respectively, was determined. The RSD_r and RSD_R data obtained are in agreement with the acceptable RSD values for repeatability according to Commission Decision 2002/657/EC. A maximum deviation of the deoxynivalenol concentration (accuracy) of 26% was observed for all spiking levels. Therefore the method can be concluded as acceptable.

Analysis of Collected Wheat and Dust Samples. The wheat and dust samples (n = 12) were determined according to the described LC-MS/MS method. Each wheat and corresponding dust test sample was determined once. All wheat samples were contaminated with deoxynivalenol in a range of 241 to 1251 ng/g with a mean contamination level of 640 ± 326 ng/g (median = 606 ng/g). Dust samples clearly showed higher levels in a range from 3756 to 17 621 ng/g with a mean contamination of 9713 ± 4434 ng/g (median = 9286 ng/g). According to the Commission Regulation (EC) No. 1881/2006 of December 19, 2006, which set maximum levels for certain contaminants in foodstuffs, deoxynivalenol in unprocessed cereals other than durum wheat, oats, and maize, was set at 1250 ng/g. Taking the measurement uncertainty into account, sample 11 exceeded the maximum permitted value. The overall deoxynivalenol level in dust exceeded the maximum level of 1250 ng/g; however the dust is never used as such in the food chain. The results of the analysis are detailed in Table 2.

Dust Correlation Study. The LC-MS/MS results for each wheat test sample and the corresponding dust sample are presented in a scatterplot (Figure 3). A correlation between the deoxynivalenol concentration in dust versus the deoxynivalenol concentration in wheat was proved according to the correlation coefficient, R^2 (0.941). Via trend analysis (i.e., residuals) and a linear regression model, the calibration curve revealed good linearity. The slope of the trend line described a value of 13.192, which corresponds to a 13-fold accumulation of mycotoxins on small particles. The statements previously made can be confirmed by this correlation study. The determined matrix effects of wheat dust at different deoxynivalenol contamination levels had values between 0.814 and 1.038, which corresponds to a minor ion suppression in the dust matrix compared to the wheat matrix.

Table 2. Deoxynivalenol Concentrations of Analyzed Wheat and Dust Samples (ng/g)

sample	wheat	dust
1	316	3756
2	241	4026
3	282	5458
4	362	6548
5	533	8933
6	520	9638
7	678	8468
8	740	10 959
9	734	12 494
10	959	12 954
11	1251	17 621
12	1063	15 701

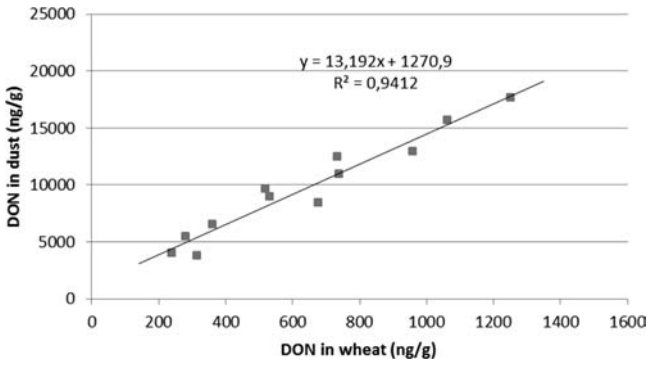

Figure 3. Scatterplot of the deoxynivalenol concentration in dust (*y*-axis) versus wheat (*x*-axis).

The sampling of dust can be considered as an easy-to-use technique that can be performed on-site prior to LC-MS/MS analysis. When analyzing wheat and corresponding wheat dust samples, a linear correlation was found between the amount of deoxynivalenol in dust versus the amount of deoxynivalenol in wheat. The results show the possibility of estimating the deoxynivalenol content in cereals through the determination of deoxynivalenol in dust.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

ARL,accept/reject limit; AUC,area under the curve; CV,coefficient of variation; EC,European Commission; ELISA,enzyme-linked immunosorbent assay; ESI,electrospray interface; ESI +,positive electrospray ionization; LC-MS/MS,liquid chromatography−tandem mass spectrometry; LLE,liquid liquid extraction; LOD,limit of detection; LOQ,limit of quantitation; OC,operating characteristic; RSD,intraday precision or repeatability; RSD_R,interday precision or within-laboratory reproducibility; SD,standard deviation; S/N,signal-to-noise ratio; SPE,solid-phase extraction; SRM,selected reaction monitoring; V,variance

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