

Determination of the amount of bioaccessible fumonisin B₁ in different matrices after *in vitro* digestion

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RESEARCH ARTICLE

Abstract

Conventional analytical methods used for the analysis of fumonisin content in animal feeds fail to take into account the fumonisin content bound to the matrix, which is otherwise bioaccessible and can be absorbed from the gastrointestinal tract. Moreover, underestimation of fumonisin content using routine analytical methods can affect animal experiments using cereals contaminated by fungal culture. In the present study the hidden fumonisin B₁ was analysed in two cereal substrates (maize and wheat), which were inoculated with *Fusarium verticillioides* (MRC 826). The study compared a routine extraction procedure with *in vitro* digestion sample pre-treatment. We found that all samples showed a higher content of fumonisin B₁ after digestion, compared to the free fumonisin obtained only by extraction. The percentage of the hidden form was 38.6% (± 18.5) in maize and 28.3% (± 17.8) in wheat, expressed as the proportion of total fumonisin B₁. These results indicate that the toxin exposure of the experimental animals determined by the routine fumonisin analysis was underestimated, generally by 40%, as bioaccessibility was not taken into consideration. This is crucial in interpretation (and maybe in re-evaluation) of the results obtained from animal experiments.

Keywords: hidden fumonisin B₁, bioaccessibility, *Fusarium verticillioides*

1. Introduction

Fumonisin which are natural contaminants of [maize](#) and [maize](#) based products are mycotoxins produced mainly by *Fusarium verticillioides* (*F. moniliforme*) and *Fusarium proliferatum* (Nelson *et al.*, 1993). Fumonisin are responsible for several toxic effects in animals and it has been associated with oesophageal cancer in humans (Voss *et al.*, 2002). According to a survey performed in 2011, 50% of tested agricultural samples were found to be contaminated with fumonisin (Schatzmayer and Streit, 2013).

Exposure assessments are based on the chemical analyses of foods and feeds to detect the exact quantity of mycotoxin contamination. From a food safety point of view it is especially important to know not only the true initial amount of toxin entering the organism but the possible changes occurring during the digestion process (e.g. enzymatic hydrolysis, microbial metabolism) has also be taken into consideration.

Mycotoxins that are undetectable by conventional, extraction-based analytical methods are known as masked mycotoxins (Berthiller *et al.*, 2013). While extractable mycotoxins can be easily detected, bound mycotoxins are not directly detectable, they have to be liberated from the matrix by chemical or enzymatic pre-treatment prior to chemical analysis. Dall'Asta *et al.* (2010), who reported the occurrence of non-covalently bound fumonisin in raw maize, suggested an *in vitro* digestion model to evaluate their levels. With this method after an enzymatic pre-treatment significantly more (30-40%) fumonisin was detected, compared to that measured after the conventional extraction method.

In the past decade more studies have been published concerning the formation and role of [matrix-associated \(hidden\)](#) mycotoxins in naturally infected and contaminated foods and feeds. In risk assessment processes animal experiments are needed to get appropriate toxicological data on certain toxic substances.

It is economically not viable to use pure toxins in such long-term animal experiments (especially in livestock); therefore, it is a general practice to introduce fumonisin into the experimental diets by fungal culture materials, containing the toxin in high concentrations. For this purpose, MRC 826, a strain of *Fusarium verticillioides* (accession number FRC M-1325, Fusarium Research Center, Pennsylvania State University, PA.) has been used to produce fumonisin in numerous studies all over the world. [Culture material of the *F. verticillioides* isolate known as MRC 826 contain predominantly fumonisin of the B series. Therefore, the results of studies with these materials corroborate those in which pure fumonisin B₁ was used \(JECFA, 2001\).](#)

This was also the strain fumonisin were first isolated from (Gelderblom *et al.*, 1988). So far, the highest yield of fumonisin B₁ (FB₁) was obtained from whole maize kernels (17,900 mg/kg) as culture material, with *F. verticillioides* MRC 826 as inoculum, incubated at 20°C in the dark for 13 weeks (Alberts *et al.*, 1990).

According to the literature of the last ca. 30 years, the techniques of formulating contaminated experimental animal diets are very similar. The experimental diet is prepared by using some ten, but occasionally some hundred kilograms of basic (standard, control, toxin free, commercial) feed, and only 0.1-1% fungal culture is mixed into the commercial, toxin free diet.

Gelderblom *et al.* (2001) published results of a prolonged feeding study in which velvet monkeys were exposed for 13.5 years, using autoclaved [maize](#) inoculated with the strain MRC 826. This publication was the basis in the declaration of the chronic effects of long-term fumonisin feeding. Fazekas *et al.* (1998) also published determinative results concerning the relationship between porcine pulmonary oedema and fumonisin administration, using *Fusarium verticillioides* strain (designated 14/A) in a maize-based experimental feed.

A vast amount of literature is available about laboratory-scale fumonisin production (ratio of fumonisin series, time-dependent rate of production, occurrence of derivatives, etc. Le Bars *et al.*, 1994; Nelson *et al.*, 1994; Marín *et al.*, 1995), however, the proportion of hidden fumonisins has not yet been investigated and there is no information available whether the ratio of hidden mycotoxins produced in artificially infected plants is similar to that produced naturally. Therefore, the aim of this study was to investigate the amount of hidden fumonisin B₁ present in maize and wheat culture material inoculated with MRC 826 using *in vitro* gastrointestinal (GI) model. The GI model simulated human digestive conditions in order to determine the bioaccessibility of FB₁. The amount of fumonisin obtained this way was compared to the amount of fumonisin B₁ obtained by a routine LC-MS analytical method.

2. Materials and methods

Chemicals

FB₁ standard (50 µg/ml in acetonitrile/water) and U-[¹³C]-labelled FB₁ (25 µg/ml in acetonitrile/water) were obtained from Romer Labs GmbH (Tulln, Austria). LC-MS grade methanol and acetonitrile were purchased from J.T. Baker (Mallinckrodt Baker, Phillipsburg, NJ, USA). Double distilled water was produced in our laboratory using Milli-Q system (Millipore, Marlborough, MA, USA). Every inorganic chemical (37% hydrochloric acid, potassium hydroxide, potassium thiocyanate, potassium dihydrogen phosphate, potassium chloride, ammonium chloride, sodium sulfate, sodium dihydrogen phosphate monohydrate, sodium chloride, sodium hydrogen carbonate, calcium chloride, magnesium chloride hexahydrate) was supplied by VWR International (Debrecen, Hungary).

Urea (98%), D-(+)-glucose (99.5%), D-glucuronic acid, D-(+)-glucosamine hydrochloride (99%), type III mucin from porcine stomach, uric acid, type VIII A alfa-amylase from barley malt, bovine serum albumin (BSA), pepsin from porcine gastric mucosa, pancreatin from porcine pancreas, type III lipase from porcine pancreas, and bovine and ovine bile which were used for the preparation of the digestive juices were purchased from Sigma (Schnelldorf, Germany).

Fumonisin production, samplings

Fusarium verticillioides (NRRL 20960 (=MRC 826) Syn. *F. moniliforme*) fungal culture (7 days old) was grown on 0.5 strength potato dextrose agar (PDA; Chemika-Biochemica, Basil, Switzerland). Agar discs (5 mm) were prepared with cork borer (Boekel Scientifica, Pennsylvania, USA), which were then stored at 10°C in darkness in test tubes containing sterile distilled water (10 discs/10 ml distilled water).

For toxin production, maize or wheat (40 g) was soaked in distilled water (40 ml) at room temperature for 1 hour in Erlenmeyer flasks (500 ml), which were closed with cotton wool plugs. This was followed by the addition of the inoculated agar discs (10 agar discs per flask) to the two-times autoclaved (20 min.) matrix. The cultures (10-10 maize and wheat containing jars) were then stored and incubated at 24°C for 1 or 3 weeks, respectively, leading to an ultimate sample number of 40. The flasks were shaken twice every day during the first week of incubation. When the incubation time was complete the fungus-infected cereal was dried at room temperature and ground.

Sample preparation for conventional FB₁ analysis

Ground and homogenized cereal samples (maize and wheat) (1.00 ± 0.01 g) were weighed into 50 ml polypropylene tubes (VWR International, Bruchsal, Germany). Samples were extracted with 20 ml water/methanol (25:75 v/v) and blended for 3 minutes at 5000 rpm in an Edmund Bühler GmbH SM30 rotary shaker (Hechingen, Germany) and then centrifuged (Model Janetzki T23 VEB MLW Zentrifugenbau Engeldorf, Germany). Supernatant (1 ml) was diluted (100 or 1000-fold) with water/acetonitrile (1:1 v/v), and these samples were then analyzed by LC-MS.

In vitro digestion assay

The preparation of artificial digestion juices (saliva, gastric juice, duodenal juice and bile) were carried out according to the protocol of Versantvoort *et al.* (2005). Before digestion, all digestion juices were heated to $37 \pm 2^\circ\text{C}$. The digestion started by adding 3 ml saliva to 1 g of ground sample, followed by an incubation step of 5 minutes. Then, 6 ml of gastric juice was added, and the mixture was incubated for 2 hours. Finally, 6 ml of duodenal juice, 3 ml of bile and 1 ml of 1 M NaHCO_3 solutions were added simultaneously to the mixture. The final incubation step lasted for 2 hours. During the *in vitro* digestion, the mixture was stirred by a multiple (4) heating magnetic stirrer (Velp Scientifica, Usmate (MB) – Italy) to obtain a gentle mixing of the matrix with the digestive juices. This was followed by the addition of distilled water (1 ml) to the final chyme (19 ml). The samples were then centrifuged for 20 minutes at 4000 rpm, yielding the chyme as the supernatant and the digested matrix as the pellet. Raw chyme (200 μl) was diluted 10-fold with distilled water. This was followed by desalting step through Sep-Pak C18 cartridges (Waters Co., Milford, MA, USA). Briefly, after preconditioning the columns with 2 ml of methanol followed by 2 ml of water, 2 ml of the diluted chyme was loaded on the column, which was then washed again with 2 ml of water. Fumonisin B₁ was eluted using 2 ml of water/acetonitrile, 1:1 v/v. The stability of FB₁ during the digestion and recovery (100%) was checked in an earlier experiment (Dall' Asta *et al.*, 2010). Prior to analysis the eluent was diluted again 10- or 100-fold with water/acetonitrile (1:1 v/v). Sample preparation methods for the analysis of the free and total FB₁ are summarized in Figure 1.

Figure 1. The two schemes of sample preparation for the analysis of fumonisin B₁ in the experiment

LC-MS analysis

LC-MS analysis was performed by a Shimadzu Prominence UFLC separation system equipped with a LC-MS-2020 single quadrupole (ultra-fast) liquid chromatograph mass spectrometer (Shimadzu, Kyoto, Japan) with electrospray source. Samples were analyzed on a Phenomenex Kinetex 2.6 μ X- C18 column (100 mm \times 2.1 mm). The column temperature was set to 50°C , the flow rate was 0.3 ml/minute and the injection volume was 1 μl . The gradient elution was performed using double distilled water (eluent A) and methanol (eluent B), both acidified with 0.2% formic acid; initial condition at 60% A, 0-2 minutes isocratic step, 2-6 minutes linear gradient to 70% B, 6-13 minutes linear gradient to 100% B, 13-15 minutes isocratic step at 40% B. Total analysis was 15 minutes. MS parameters: source block temperature 90°C ; desolvation temperature 250°C ; heat block temperature 200°C ; drying gas flow 15.0 l/minute. Detection was performed using SIM mode.

The mass spectrometer was operating in the selective ion monitoring mode, at m/z 722.4 for FB₁ and 756.5 for U-[¹³C]-labelled FB₁.

Calibration curves using FB₁ and U-[¹³C]-labelled FB₁ standard in the range of 10-500 µg/kg were prepared. U-[¹³C]-labelled FB₁ (50 µl, 100 µg/kg) was used as internal standard. The internal standard was added to the analyte in case of the *in vitro* digestion after the clean-up procedure; while by the conventional extraction it was added before the final dilution of the analyte. A further reason of the application of the internal standard was to overcome possible different matrix effects (e.g. ion suppression).

The limit of detection (LOD) for FB₁ was 3 µg/kg, while the limit of quantification was (LOQ) 10 µg/kg.

Statistical analysis

Statistical analyses were performed using IBM SPSS 20.0 (2012). Data processing and the mathematical-statistical calculations were performed using the Compare Means (Independent-Samples-t-Test, oneway ANOVA with Tukey *post-hoc* test), Correlate and Descriptive Statistics modules.

3. Results

By means of the two different incubation time intervals of *maize* and *maize* with the MRC 826 strain of *F. verticillioides* and with the application of two matrices it was possible to ascertain, whether any of these two factors have an influence on the binding extent of fumonisin B₁ to the matrix. [Table 1](#) shows that in the *maize* matrix after one week of incubation the mean total FB₁ concentration is lower (about 30%), as compared to the wheat. After three weeks the increase in FB₁ concentration was nearly tenfold in *maize*, while only a minor (by 3%) increase for the total FB₁ concentration was found in wheat. *Maize seems to be a better substrate for FB production as it was underscored as well by the literature (Leslie, 1996; Munkvold, 2003).*

The proportion of hidden FB₁ was likewise constant in both matrices, independently of the length of incubation.

As a general observation, total FB₁ levels measured after digestion were significantly higher than those measured by the extraction procedure in 60% of all cases ([Table 1](#)). All data were statistically compared using independent samples t-test ($p \leq 0.05$). These results confirmed that the GI enzymes are able to destroy the matrix-fumonisin interaction, and are capable to release hidden fumonisin. The percentage of the hidden form expressed as the proportion to total fumonisin FB₁ was 38.6% (± 18.5) and 28.3% (± 17.8) in *maize* and wheat, respectively.

[Table 1](#). Comparison of extractable to total fumonisin B₁, level and ratio of the hidden fumonisin B₁ in the fungal cultures^a

Samples	Extractable FB ₁ (mg/kg) ^b	±SD ^f	Total FB ₁ (mg/kg) ^c	±SD	Hidden FB ₁ (mg/kg) ^d	Hidden (%)	T-test ^e
C1	114.5	0.7	315.5	1.4	201	63.7	**
C2	492.1	3.2	858.7	0.9	366.6	42.7	**
C3	33.6	0.3	41.4	7.3	7.8	18.8	>0.05
C4	122.2	1.1	148.1	2.6	25.9	17.5	*
C5	47.4	2.4	51.3	0.9	3.9	7.6	>0.05
C6	38.8	8.7	83.6	37	44.8	53.6	>0.05
C7	438.9	10.5	603.2	59.3	164.3	27.2	*
C8	326.6	50.1	526.9	0.6	200.3	38	**
C9	108.1	2.7	377.3	1.1	269.2	71.3	**
C10	223.1	13.7	514.3	22.2	291.2	56.6	**
mean C1-C10	194.5		352		157.6	39.7	
W1	1268.8	183.1	1508.7	124.3	239.9	15.9	>0.05
W2	1098.9	32	1183.9	35.2	54.2	4.7	>0.05
W3	138.3	5.7	157.3	3.8	19	12.1	*
W4	465	25.7	700.8	34	235.8	33.6	**
W5	641.4	37.3	1143.8	111.9	502.4	43.9	**
W6	441.9	26	953.2	18.1	511.3	53.6	**
W7	667.3	15.8	1316.3	66.8	649	49.3	**
W8	548.7	154.9	788.1	128.7	239.4	30.4	>0.05
W9	1086.8	254.9	1132.6	17.7	45.8	4	>0.05
W10	242.8	20.6	291.3	13.5	48.5	16.6	>0.05
mean W1-W10	660		917.6		254.5	26.4	
CH1	1678.2	10.6	2827.1	95.3	1148.9	40.6	**
CH2	722.1	19.2	1416.1	16.8	694	49	**
CH3	423.9	19.6	977.1	74.5	553.2	56.6	**
CH4	2993.7	24.6	4817.8	362.3	1824.1	37.9	**
CH5	1919.1	138.2	3041.6	557.1	1122.5	36.9	>0.05
CH6	3563.6	271.5	6241.5	240.6	2677.9	42.9	**
CH7	872	23.6	1688.7	127.8	816.7	48.4	**
CH8	746.8	99.4	1300.8	57.8	554	42.6	**
CH9	2641.5	275.2	2869.4	50.8	227.9	7.9	>0.05
CH10	688	54.2	787.8	58.3	99.8	12.7	>0.05
mean CH1-CH10	1624.9		2596.5		971.9	37.5	
WII1	653.1	76.7	916.7	94.5	263.6	28.8	>0.05
WII2	182.6	24.6	490.9	15.3	308.3	62.8	**
WII3	383.8	24.1	785.8	70.6	402	51.2	*
WII4	737.3	25.4	1126	165.8	388.7	34.5	*
WII5	669	42.4	852.5	26.6	183.5	21.5	>0.05
WII6	356.2	29.3	409.2	32.7	53	13	*
WII7	569.7	8.4	833.1	173.6	263.4	31.6	>0.05
WII8	1377.5	239.7	1512.3	71.4	134.8	8.9	>0.05
WII9	1986.7	109.3	2514.8	204.2	528.1	21	>0.05
mean WII1-WII9	768.4		1049		280.6	30.4	

^a n=2; * p<0.05; ** p<0.01. ^b Extractable FB₁: fumonisin obtained by LC-MS analysis. ^c total FB₁: fumonisin obtained after digestion assay and analyzed by LC-MS. ^d hidden FB₁: calculated difference between total and extractable fumonisin. ^e T-test between total and extractable fumonisin
^f Standard deviation

‘C’ stands for **maize** and ‘W’ stands for wheat samples incubated for 1 week, while ‘CH’ and ‘WII’ stands for samples incubated for 3 weeks, **maize** and wheat, respectively.

In a study carried out by Dall' Asta *et al.* (2010) using raw maize, results are presented as total, extractable and hidden FB, expressed in $\mu\text{g}/\text{kg}$. From the published 31 results the hidden % can be calculated (“hidden % = (hidden FB conc./total FB conc. meaning FB conc. after digestion)*100”); this calculated percentage of hidden fumonisins (FB₁, FB₂, FB₃) was $35.6\pm 22.3\%$.

In our study, calculating for the total dataset (pooling week 1 and 3), the proportion was $38.6\pm 18.5\%$, while in the samples taken after 3 weeks of production this was $37.5\pm 15.5\%$ in maize. Since the ratio of FB₁, FB₂ and FB₃ is strongly similar in fungal cultures and naturally infected cereals (Marín *et al.*, 1995), it can be stated that the ratio of the hidden FB₁ is as well analogous in the two studies. Moreover, the datasets (Dall' Asta *et al.*, 2010 vs. ours) are statistically not different, as compared with t-test.

Albeit the standard deviation within all groups was relative high, the data of the maize samples after the 3 week-incubation period were significantly different from the others, applying Tukey test. Moreover, independently from the incubation period, there were significant differences between the percentage of the hidden fumonisin in maize and wheat.

Figure 2 shows close linear relationship between extractable and total FB₁ concentration values, in both matrices.

These results indicate that there is a need for a new risk prediction to be developed for mycotoxin contamination of animal feeds. These findings are in close agreement with those already reported for raw maize (Dall' Asta *et al.*, 2010).

Figure 2 a and b. Correlation between extractable and total FB₁ concentration in maize and wheat during the toxin production (1st and 3rd week together)

In the light of these results (156 individual analyses) it is likely that in animal experimental diets the fumonisin content is underestimated by approximately 40%, as most of these experiments are based on a maize matrix inoculated with fungal culture.

4. Discussion and conclusion

The *in vitro* digestion model mimics all of the most important physicochemical conditions until the end of the ileum (where the absorption occurs) in humans, apart from the fermentation by the intestinal microbiota and permeation or transport across the intestinal barrier.

The bioaccessible fraction is considered to represent the maximum amount of contaminant available for absorption from the gastrointestinal tract. Therefore, the *in vitro* digestion model can make exposure assessment more accurate by taking the bioaccessibility of contaminants into consideration (Versantvoort *et al.*, 2005). Since the linear relationship between extractable and total FB₁ concentrations shows a marked similarity with that of Dall' Asta *et al.* (2010) found in naturally infected maize, our results underline the fact that fungal culture is applicable in animal toxicological studies and for investigations of other toxicological purposes. The hidden proportion of fumonisin is identical in case of naturally contaminated (field derived) crop samples ($35.6\pm 22.3\%$, Dall' Asta *et al.*, 2010) and in laboratory-scale produced ($38.6\pm 18.5\%$), inoculated crop cultures.

The aim of comparing our data to those of Dall' Asta *et al.* (2010) was to see, if the ratio of free and total FB₁ is different or similar in artificially or naturally contaminated maize and wheat. It is obvious that the production of fumonisins by inoculation of kernels is evidently different from

the “on field “ situation, however, the original question of the study was to determine the really bioavailable toxin amount possibly and theoretically taking up in an animal experiment. It is important to take into consideration that because of the hidden (and not detected) fumonisins even 1.5 or 2-fold more toxin can be absorbed, as compared to the estimated amount. This is crucial in interpretation (and maybe in re-evaluation) of the results coming from animal experiments. In this respect we draw the conclusion that with the *in vitro* simulated digestion model a similar proportion of hidden FB₁ was present in the two cases.

However, the toxin exposure of the experimental animals determined by the routine fumonisin analysis was underestimated, generally by 40%, as bioaccessibility was not taken into consideration.

This can explain the paradox experienced in investigations with purified FB₁, which failed to reproduce earlier observations obtained by using fungal culture of *F. moniliforme* (Shier, 2000). Next to the several plausible explanations (including the possible effect of not analyzed or yet not known new fumonisin derivatives) results could be affected by the higher intestinal bioavailability of the fumonisin in the fungal culture.

Moreover, it is worth to mention that both humans and animals may be exposed to markedly higher toxin load, as surveys are based purely on conventional chemical analyses. Thus the routine mycotoxin analysis results should be handled carefully, only as informational data, and not as the true mycotoxin contamination of foods and feeds.

From an analytical point of view it has to be emphasized that digestive approach is able to enzymatically destroy the complexation or interaction occurring between fumonisins and maize macromolecules (i.e. amylose, amylopectin and proteins). In addition, when food processing is involved, other covalent or non-covalent interactions may arise, thus leading to a more complex system. It is proven that the hidden moiety is not a result of a less effective methanolic extraction, since water:methanol:acetonitrile (50:25:25 v/v/v) and water:methanol (30:70 v/v) extraction mixtures were also evaluated and compared successfully, complemented with a comparative study using 5 different analytical methods involving two different labs (Dall’Asta *et al.* 2009).

Possible derivative formation during methanolic extraction (methyl-esters) is less realistic, since carboxyl group modification to methyl-esters needs lower pH, total absence of water, higher temperature and longer reaction time (Christie, 2003).

Based on the linear relationship found, the conclusion may be drawn that the entire digestion and the subsequent analysis of the hidden moiety is not always essential; instead these in the applied matrices a simple correction factor can as well be useful for the estimation of the total FB₁ concentration.

Acknowledgements

The research was supported by the Hungarian Academy of Sciences (within the frame of the MTA-KE “Mycotoxins in the food chain” Research Group), and by the Bolyai János research grant by the Hungarian Academy of Sciences (BO_499_13 to J. Sz-F.; BO_26/11/4 to A.Sz.). Brigitta Bóta and Anna Konyári-Takács (University of Kaposvár, Hungary) provided practical help in the laboratory.

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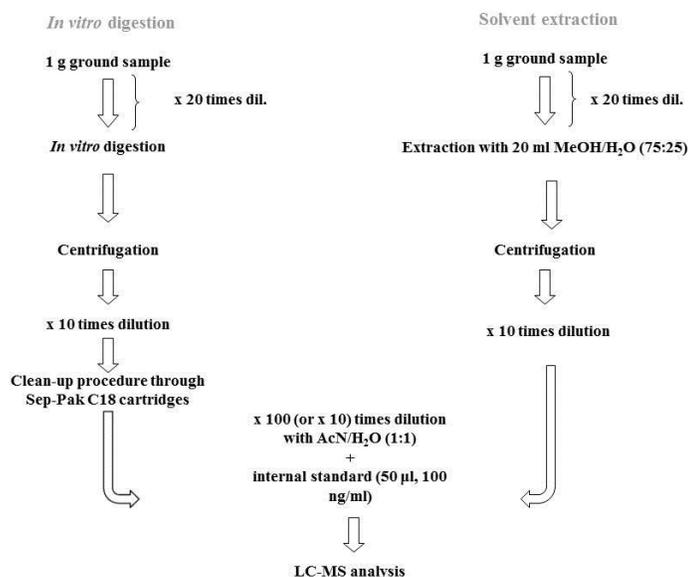
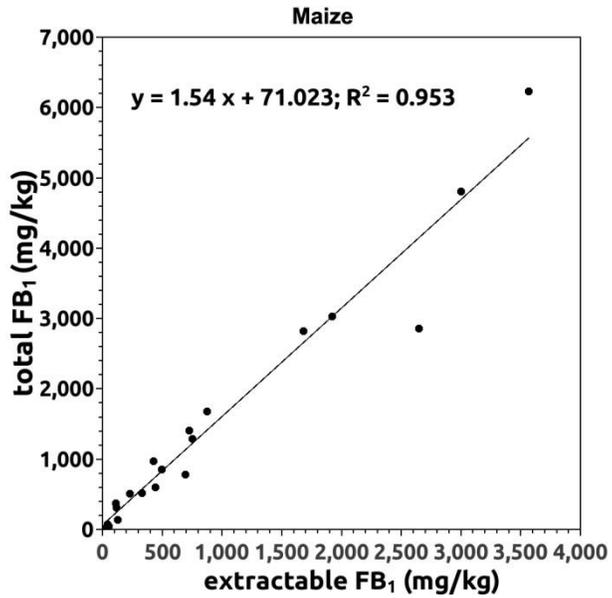
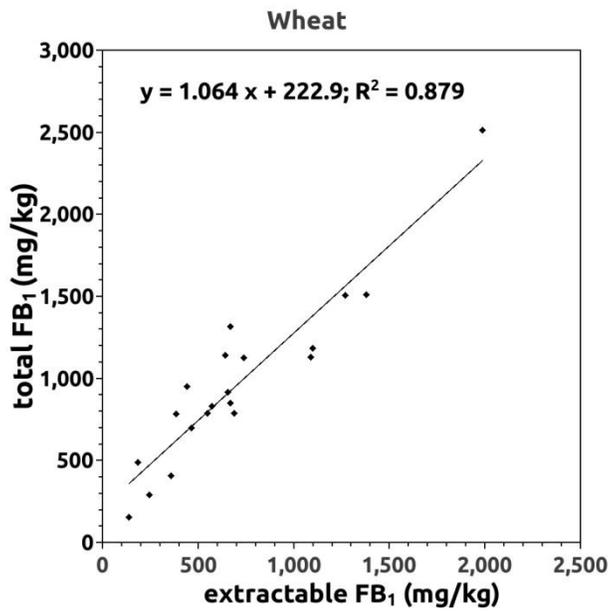


Figure 1. Sample preparation for the analysis of fumonisin B₁ in the experiment



2a)



2b)

Figure 2. Correlation between extractable and total FB₁ concentration in **maize** and wheat during the toxin production (1st and 3rd week together)

FW: World Mycotoxin Journal - Decision on Manuscript ID wmj-2014-04-1771.R1 - Message (Plain Text)

From: Dr. Szabó-Fodor Judit
To: Prof. Dr. Kovács Melinda; Chiara Dall'Asta (chiara.dallasta@gmail.com); chiara.dallasta@univr.it; claudia.falavigna@univr.it; szcsi.arpad@agrar.mta.hu
Cc: Mariam Kachlek; Dr. Szabó András
Subject: FW: World Mycotoxin Journal - Decision on Manuscript ID wmj-2014-04-1771.R1

Message: CTA-World-Mycotoxin-Journal.doc (131 KB)

-----Original Message-----
From: onbehalfofwmj@wageningenacademic.com [mailto:onbehalfofwmj@wageningenacademic.com] On Behalf Of wmj@wageningenacademic.com
Sent: Saturday, July 26, 2014 8:58 PM
To: Dr. Szabó-Fodor Judit
Cc: Hans.vanEgmond@wur.nl
Subject: World Mycotoxin Journal - Decision on Manuscript ID wmj-2014-04-1771.R1

26-Jul-2014

Dear Dr. Szabó-Fodor,

I am pleased to inform you that your revised manuscript entitled "Determination of the amount of bioaccessible fumonisin B1 in different matrices after in vitro digestion" is accepted for publication in World Mycotoxin Journal, provided that no problems arise during the editing stage at the publisher. The comments of the reviewer who reviewed your manuscript are included at the foot of this letter. I noticed that the word "corn" still appears in figure 2 and in its legend. Could you provide the publisher (Marijn van der Gaag [VanderGaag@WageningenAcademic.com] with a revised figure 2 please?

Note that the publisher needs a signed Copyright Transfer Agreement (CTA) from the first or the corresponding author before the manuscript can be published. Please fax or send the CTA by mail to the publisher as soon as possible if you have not done already (+31 317453417 or wmj@wageningenacademic.com). For your convenience, I have attached a blank CTA form to this email.

I also would like to draw your attention to the possibility of publishing your manuscript as 'open access' for 1800 Euro in World Mycotoxin Journal. With open access, your article will be free available online for everyone. Open access publishing significantly increases the exposure and citation of your work. Often research grants have funding available for dissemination of the results of the research.

Thank you for your fine contribution. On behalf of the Editors of the World Mycotoxin Journal, we look forward to your continued contributions to the Journal. World Mycotoxin Journal has now an IF of 2.541 (2012 Journal Citation Report, Thomson Reuters 2013).

Sincerely,

Hans P. van Egmond
Editor-in-Chief, World Mycotoxin Journal Hans.vanEgmond@wur.nl

Reviewers' Comments to Author:
Reviewer: 1
Comments to the Author

See more about: Dr. Szabó-Fodor Judit.