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Immunochemical approach for zearalenone-4-glucoside determination

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ABSTRACT

Zearalenone-4-β-D-glucopyranoside (zearalenone-4-glucoside) detection techniques, based on a combination of acidic or enzymatic hydrolysis of the masked mycotoxin to the parent form (i.e. zearalenone), and immunochemical determination of zearalenone-4-glucoside as a difference between the zearalenone concentration after and before cleavage of the glycosidic bond were developed. The limit of detection for zearalenone-4-glucoside, achieved for the enzyme linked immunosorbent assay, was 3 μg kg^{-1}; the cut-off level for the sum of zearalenone and zearalenone-4-glucoside determination by a qualitative gel-based immunoassay was 50 μg kg^{-1}.

Trifluoromethanesulfonic acid was checked for acidic hydrolysis and resulted in approximately 70% of glycosidic bond cleavage in optimal conditions. Seven different glycoside hydrolases were tested during the design of the enzymatic hydrolysis technique. Enzymatic hydrolysis combined with enzyme linked immunosorbent assay and gel-based immunoassay determinations was applied for the determination of zearalenone-4-glucoside or the sum of zearalenone and zearalenone-4-glucoside in cereal samples. The chosen enzyme (glucosidase from *Aspergillus niger*) allowed to cleave 102% of zearalenone-4-glucoside in standard solutions and 85% in cereal samples. Liquid chromatography coupled to tandem mass spectrometry was used as confirmatory method. As a result, good correlations between immunochemical techniques and the chromatographic data were obtained. The developed technique is suitable for simultaneous immunochemical determination of zearalenone and its masked form, zearalenone-4-glucoside.

1. Introduction

Zearalenone (ZEN) is a non-steroidal, estrogenic mycotoxin, produced by *Fusarium* fungi, mainly *F. graminearum*, *F. culmorum*, *F. crookwellense* and *F. sporotrichioides*. ZEN can cause infertility with decreased serum testosterone levels, sperm counts, reduced incidence of pregnancy and altered progesterone levels. Hepatotoxic, hematotoxic, cytotoxic and genotoxic activity is also described [1]. Barley, wheat, rice, sorghum, maize and other cereal-based commodities are highly susceptible for contamination with ZEN. Weather conditions such as high humidity and low temperature during the cereal growth and storage affect mold development and ZEN formation [2]. The European Union has recently established maximum limits for ZEN as 100 μg kg^{-1} for unprocessed cereals and 350 μg kg^{-1} for unprocessed maize [3].

Plants are very flexible systems: they have the ability to minimize risks and dangers of external environments by their mutation. The degree of modification depends on the characteristics of plants and external xenobiotics, and is regulated by the cellular homeostasis [4]. There are two mechanisms of plant protection against external chemical impurities, such as mycotoxins: physical cleavage resulting in their separation in different organs (tissues, cells, organelles), and chemical deactivation resulting in derived forms or conjugates [5]. Plants convert weak polar mycotoxins, such as ZEN, in more polar forms via conjugation to sugars, amino acids, acetic or sulfate groups by the involvement of enzymes [6–10]. The term "masked mycotoxin" was introduced by Gareis et al. for such forms [11]. Likewise, these toxins can appear during technological processes such as cereal milling, particularly in cereal products. These mycotoxin derivatives are characterized by altered chemical properties and analytical effects, but the most threatening is the possible hydrolysis of these compounds into their parent form in the digestive tract of humans and animals [12]. As a consequence, the total exposure to parent mycotoxins might be underestimated and an unexpected high toxicity of food and feed might cause severe problems. Natural occurrence, metabolism and bioavailability data of masked mycotoxins are weakly investigated [6,13–20].

There are two strategies for masked mycotoxin determination: the *direct* and *indirect* approach. Direct techniques correspond with chromatographic separation with fluorescence [21] or more common with mass spectrometric detection [6,22–24], and presume individual detection of each form (at least each known form).

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The indirect approach is based on the acidic, basic or enzymatic hydrolysis of the conjugate resulting in the conversion of "masked" mycotoxins into their parent mycotoxins. The total amount of the parent form is quantified before and after conversion. The amount of the masked form is estimated as the difference. Such procedures (enzymatic and acidic hydrolysis) are described for deoxynivalenol-3-glucoside [25,26]. For ZEN masked forms only the direct approach (LC–MS/MS) was described [22,27].

Zearalenone-4-β-D-glucopyranoside (zearalenone-4-glucoside, Z4G) was discovered in the 80s, formed by *Rhizopus spp.*, and identified as a glucose derivative [28]. Berthiller et al. proved Z4G as a phase II-metabolization product [27]. There are no data available on the immunochemical determination of Z4G, but a large variety of different immunochemical techniques for ZEN determination was developed. ELISA (LOD 0.1 $\mu g\,kg^{-1}$) [29], immunochromatographic assay (LOD 6 $ng\,mL^{-1}$) [30], gel- and membrane-based immunoassays (the cut-off level for both techniques was 100 $\mu g\,kg^{-1}$) [31], immunosensors (LOD 0.77 $ng\,mL^{-1}$ [32] and 0.4 $ng\,mL^{-1}$ [33]) were published. Sensitivities of these assays depend on format, procedure, kind of matrix and immunoreagents properties.

Therefore, the aim of this work was the development of an immunochemical technique for the Z4G determination based on its destruction to the parent ZEN. This is the first report in which an enzymatic hydrolysis technique of "masked" mycotoxins was tested for the analysis of real samples.

2. Material and methods

2.1. Reagents and materials

Zearalenone (ZEN) was supplied by Fermentek (Jerusalem, Israel). Glucosidase from *A. niger*, cellobiase from *A. niger*, cellulase from *Trichoderma viride*, cellulase from *A. niger*, β-glucuronidase from *Helix pomatia*, β-glucuronidase from *Escherichia coli*, glucosidase from almonds, trifluoromethanesulfonic acid (TFMSA), O-(carboxymethyl)hydroxylamine hemihydrochloride (CMO), peroxidase from horseradish (HRP, type VI, 250–330 units mg^{-1} solid), bovine serum albumin (BSA), N-hydroxysuccinimide (NHS), N,N′-dicyclohexylcarbodiimide (DCC), α-zearalenol, β-zearalenol, zearalanone, Tween 20 (Tween, polyoxyethylenesorbitan monolaurate), glycine, casein sodium salt from bovine milk, skim milk powder, phosphate buffered saline (PBS) tablets, carbonate bicarbonate buffered saline tablets and sealing film for 96-well multiwell plates were purchased from Sigma-Aldrich (Bornem, Belgium).

The monoclonal anti-ZEN antibodies (MAb1, MAb2, MAb3, MAb4) were received from D.V. Volkov (Impact, Moscow). The anti-ZEN monoclonal antibody (MAb 5) was produced from a stable hybridoma cell line 2D8 [31] by culturing in a two-compartment bioreactor CELLine CL 350 (INTEGRA Biosciences AG, Zizers, Switzerland). The collected supernatant, containing a high density of anti-ZEN MAb, was used for the subsequent experiments. Polyclonal rabbit anti-mouse immunoglobulins (2.5 $g\,L^{-1}$) were obtained from Dako Denmark A/S (Glostrup, Denmark).

3,3′,5,5′-tetramethylbenzidine (TMB) substrate was purchased from ALerCHEK Inc. (Maine, USA). Methanol (LC–MS grade) was supplied by BioSolve BV (Valkenswaard, The Netherlands); acetonitrile (Analar Normapur), n-hexane (Hipersolv Chromanorm) and ammonium acetate were obtained from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100%) and formic acid were supplied by Merck (Darmstadt, Germany). All other chemicals and solvents were of analytical grade. Water obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium) was used throughout.

CNBr-activated sepharose 4B (sepharose) and PD-10 desalting columns were purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Plastic tubes (Bond Elut reservoir, 1 and 3 mL) and polyethylene frits (1/4 and 3/8 in. diameter) were supplied by Varian Belgium NV/SA (Sint-Katelijne-Waver, Belgium). Microtiter plates (96 flat-bottom wells with high binding capacity; clear and black Maxisorp) were purchased from Nunc A/S (Roskilde, Denmark). Protein concentrators (9 K, 20 mL) were purchased from Thermo Scientific (Rockford, USA).

The mycotoxin standard solution (1 $mg\,mL^{-1}$) was prepared in methanol and storable for a minimum of 1 year at −18 °C [34]. The working solutions in methanol were renewed monthly, while the working solutions in PBS were renewed daily. Z4G was synthesized in-house based on the procedures of Grabley et al. [12] and Schneweis et al. [22]

PBS 0.01 M, pH 7.4 was used as working assay buffer for the enzyme linked immunosorbent assay and column gel-based immunoassay.

2.2. Preparation of ZEN–HRP conjugates

ZEN does not contain active groups suitable for conjugation with enzymes or proteins, as a consequence, a carboxymethyloxime derivative of ZEN (ZEN–CMO) was used for the synthesis.

To synthesize ZEN–CMO, a modified technique, described by D. Thouvenot [35] was applied. ZEN (10 mg) and O-(carboxymethyl)hydroxylamine hemihydrochloride (20 mg) were dissolved in 1 mL of pyridine. The reaction mixture was stirred for 24 h at RT, and then evaporated to dryness in a rotor evaporator at 50 °C. The residue was mixed with 5 mL of distilled water with NaOH to adjust the pH at 8, sonicated for 3 min to suspend the white residue, and the unreacted ZEN was extracted with 10 mL of chloroform (5 times × 2 mL portions). The chloroforme fractions were removed. The hapten was precipitated in the aqueous phase by the addition of HCl (pH 3) and extracted four times with 10 mL of ethyl acetate. The extract was dried over anhydrous sodium sulfate, filtered and evaporated under vacuum at 50 °C.

For gaining horseradish peroxidase-labeled ZEN (ZEN–HRP), firstly, an activation of the carboxyl group by NHS and DCC was executed. Achieving this, 46 mg (approximately 400 μmol) NHS and 125 mg (approximately 600 μmol) DCC were dissolved in 2.5 ml DMFA. Five hundred μl of this solution was added dropwisely to 19 μmol of ZEN–CMO solution in DMFA at constant mixing rate. The reaction mixture was stirred with an orbital shaker for 4 h at RT and incubated overnight at 4 °C. The formed precipitate was removed by centrifugation (10 min, 10,000 g).

For the synthesis of ZEN–HRP, the molar ratio of the activated hapten and enzyme was 5/1. The activated hapten (59.2 μL, 1.25 μmol) was added dropwisely to cooled HRP solution (11 mg (0.25 μmol) in 2 mL carbonate buffer, pH 9.6). The reaction mixture was stirred for 2 h at RT, after which it was incubated overnight at 4 °C. The protein concentrator tube (20 mL/9 K) was applied for the removal of unbound low-molecular weight substances (procedure according to manufacturer's recommendation). PBS was chosen as washing buffer. The concentration of the obtained conjugate (2.4 $mg\,mL^{-1}$) was determined spectrophotometrically. ZEN–HRP was dissolved in 50% glycerol and stored at −18 °C.

2.3. Enzyme-linked immunosorbent assay (ELISA) procedure

The 96-well transparent microtiter plates were coated with the rabbit anti-mouse antibody (5 $\mu g\,mL^{-1}$, 100 $\mu L\,well^{-1}$) in coating buffer (0.05 M sodium carbonate buffer, pH 9.6) during 2 h at 37 °C or overnight at 4 °C. The plates were covered with an adhesive plate sealing film to prevent evaporation. Further, the

plates were washed three times with PBS–Tween 20 (0.05%, v/v) and blocked with a blocking buffer (PBS, containing 1% casein, w/v; 300 μL well^{-1}) for 1 h at 37 °C. After blocking, the plates were washed twice with PBS–Tween 20 (0.05%, v/v). Firstly, an aliquot of anti-ZEN MAb 5 diluted with PBS (1/10 000, 100 μL well^{-1}) was added, and the plates were incubated for 2 h at 37 °C. Afterwards, the plates were washed three times with PBS-Tween 20 (0.05%, v/v). Standard ZEN solutions (diluted in PBS) or the diluted sample extract (50 μL well^{-1}) and the ZEN–HRP (diluted 1/300 in PBS, 50 μL well^{-1}) were added and incubated at RT for 1 h by agitation on a horizontal shaker. Then, the plates were washed four times and 100 μL well^{-1} of the substrate solution (TMB/H_2O_2) was added. The plates were shaken approximately 20 min to obtain color development. Finally, the enzyme reaction was stopped with sulfuric acid (5%, 50 μL well^{-1}) and absorbance was read at 450 nm with a microplate reader (Safire, Tecan). All measurements were performed in triplicate.

The standard ELISA sigmoidal curves were plotted in semi logarithmic scale: absorbance or relative absorbance (ratio of values of absorbance measured at standard concentrations and zero concentration) at 450 nm, and logarithm of ZEN concentration. These curves were described by Rodbard's four-parameter function

$$y = (A-D)/[1+(x/C)^b]+D$$

A: maximum optical density value, D: minimum optical density value, b: slope of the curve in the IC_{50} plot, C: IC_{50} concentration of ZEN. The limit of detection (LOD) was defined as three times the signal-to-noise ratio.

2.4. Gels preparation

2.4.1. Gel coupled with rabbit anti-mouse antibody (anti-mouse antibody coupled gel, AM-CG)

0.5 g of sepharose was washed on a glass filter with 100 mL of 1 mM HCl. 150 μL of the rabbit anti-mouse antibody (2.5 g L^{-1}) and 450 μL of the coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) were added to sepharose, and the mixture was shaken for 2 h at RT with the horizontal shaker. The gel was washed with the 0.1 M $NaHCO_3$ (containing 0.5 M NaCl, pH ~ 8.3) (5 mL) to remove the excess of uncoupled antibody. Afterwards, the unbound surface active groups were blocked with 6 mL of the blocking buffer (0.2 M glycine, 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0) for 2 h at RT under constant shaking. The obtained gel was washed with 3 cycles of alternating pH: each cycle consisted of a wash solution with 10 mL of 0.1 M acetate buffer (pH 4.0, containing 0.5 M NaCl) followed by a wash solution with 10 mL of PBS. The prepared gel was suspended in PBS (1/3, v/v) and stored at 4 °C.

2.4.2. Blocked gel (BG)

Preparation of the gel with blocked active groups was similar as described above with the exception of the antibody coupling step. Thus, BG was used for dilution of AM-CG and its consumption was larger, concluding a higher amount was prepared (2 g of sepharose were used with proportional increase of all reagents).

2.4.3. Gel coupled with anti-ZEN antibody (ZEN-CG)

0.5 mL of AM-CG was placed in a 3 mL tube with a bottom polyethylene frit. Twenty five μL of the primary mouse anti-ZEN antibody (MAb 5) was added and carefully mixed for 7 minutes. The liquid was flowed away using a plunger and the obtained gel was washed twice with 1.5 mL of PBS. 0.5 mL of PBS was added for dilution of the gel.

2.4.4. Gel-based immunoassay procedure

ZEN determination by gel-based immunoassay was executed according to the modified standard procedure of this method [36,37]. Preparing the test column, ZEN-CG was diluted with BG (1/75, v/v) in a 3-mL tube, followed by washing with a threefold volume of PBS. Afterwards, the gel was suspended in PBS (1/3, v/v). 200 μL of this mixture were placed on the bottom frit of an empty 1-mL Bond Elut cartridge and then covered with a second frit. The column was washed with the PBS solution and kept at 4 °C until use.

The ZEN standard solution prepared in PBS (10 mL), the mixture of ZEN and Z4G in the different concentration range (10 mL) or the diluted sample extract (10 mL) was treated by the glucosidase from *A. niger* (15 min, 37 °C) and passed through the prepared test column, followed by a washing step with 5 mL of PBS-Tween (0.05%, v/v). Then, 50 μL of ZEN–HRP (dilution 1/150 in PBS) was added and incubated for 6 min. Excess of the conjugate was removed by passing 4 mL of PBS through the test column. 50 μL of chromogenic substrate was added, and removed by the plunger after 20 s of incubation. A visual detection was pointed out within 6 min after chromogenic substrate application.

2.4.5. Analytical performance of the tests

The cut-off level of the qualitative test was defined as the lowest analyte concentration, which is characterized as no color development at a fixed detection time. Absence of color was considered as a positive result (ZEN concentration equal or above the cut-off level), the appearance of a blue color, independent on its brightness, was interpreted as an indication of a negative result (ZEN concentration below the cut-off level). Samples with ZEN concentration higher than the cut-off level, which resulted in negative results, were considered to be false negative. Respectively, samples with ZEN concentration lower than the cut-off level, which resulted in positive results, were designated as false positive.

Performance parameters were calculated based on summarized data of repeated experiments according to Trullols et al. [38]. An intra-laboratory test validation was performed with different blank (absence of ZEN was confirmed by LC–MS/MS) and spiked maize samples (at concentrations of ZEN less, equal and more than the cut-off level). The numbers of the total amount of positive, negative, false positive and false negative results were calculated. The false negative rate was determined as the ratio of the number of false negative results and total of positive results. The false positive rate was calculated as the ratio of the number of false positive results and total of negative results. The specificity of the test was determined as the ratio of the number of reliable negative results and the total of numbers of negatives. The sensitivity was found as the ratio of the amount of reliable positive results and total amount of positive samples.

2.4.6. Sample preparation for immunoassay

Cereal samples were ground with a household coffee grinder (M-20-grinder IKA-WERKE, Staufen, Germany). A portion of sample (0.5 g) was extracted with 2.5 mL of methanol/water (80/20, v/v) for 45 min on a horizontal shaker at RT. After centrifugation (4000 g, 14 °C, 15 min), the supernatant was transferred and filtered through a paper filter (Whatman, 1). The cereal extracts were diluted with PBS and used for hydrolysis or directly to ELISA or column immunoassay.

Naturally contaminated wheat and maize samples from different fields in Flanders, Belgium were kindly provided by Prof. Dr. Geert Haesaert and Dr. Kris Audenaert (University College Ghent, Department of Plant Production, Belgium). Feed samples

and breakfast cereals were obtained in the framework of a regular Belgian monitoring program.

2.4.7. Enzymatic hydrolysis procedure

Aliquots of Z4G and ZEN standard solutions (60 ng mL^{-1} in methanol), sample extract, methanol and methanol/water mixture (80/20, v/v), all of them 200 μL, were placed into the flasks and evaporated to dryness. The same volumes of enzymatic solutions were added, followed by shaking of the mixtures during 3 minutes, and incubation at optimal temperature under constant shaking.

As enzymes glucosidase (*A. niger*) (GAn), glucosidase (*Almonds*) (GAl), cellulase (*T. viride*) (CTv), cellulase (*A. niger*) (CAn), β-glucuronidase (*H. pomatia*) (GHp), β-glucuronidase (*E. coli*) (GEc) and cellobiase (*A. niger*) (CbAn) were used. Medium-temperature conditions of the enzymes applications were used according to the producer's protocol and are presented in Table 1. For the neutralization of the enzyme and reaction stoppage, aliquots of the incubated solutions were diluted with 200 μL methanol/water (50/50, v/v). Finally, probes were diluted proportionally with PBS and applied to ELISA for the determination of the ZEN concentration.

2.4.8. Acidic hydrolysis procedure

Aliquots of Z4G and ZEN standard solution (60 ng mL^{-1} in methanol), methanol and methanol/water (80/20, v/v), all 200 μL, were evaporated to dryness. The same volume of TFMSA solution was added in each tube; mixtures were shaken on vortex during 3 minutes, and incubated at different temperatures (25, 40 and 60 °C). Afterwards, sodium hydrocarbonate solution (1 M) was added to obtain acid neutralization and reaction stoppage. Probes were diluted proportionally with PBS and applied to ELISA.

2.4.9. LC–MS/MS procedure

The method described by De Boevre et al. [39] was used for the LC–MS/MS determination of ZEN and Z4G. Concisely, wheat samples (2.5 g) were ground, vigorously homogenized with a spatula and extracted with 10 mL acetonitrile/water/acetic acid (79/20/1, v/v/v), combined with a hexane defatting (5 mL), using the overhead shaker. Sample extracts were centrifuged at 3000 g for 15 min; afterwards, the hexane layer was removed. The aqueous layer was filtered through the filter paper and evaporated to dryness under a gentle N_2 stream at 40 °C. The residue was redissolved in 100 μL injection solvent, consisting of methanol/water (50/50, v/v) and 10 mM ammonium acetate, adjusted to pH 3 with glacial acetic acid. Finally, the redissolved sample was vigorously vortexed for 3 min, collected in an Ultrafree-MC centrifugal device (Millipore, Bedford, MA, USA) and centrifuged for 10 min at 10 000 g.

A Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) equipped with an electrospray interface (ESI) was used. Chromatographic separation was performed, applying a ZORBAX Eclipse XDB C18 column (3.5 mm, 100 mm × 4.6 mm) (Agilent Technologies, Diegem, Belgium). The column was kept at RT, while the autosampler was set at 10 °C. A mobile phase consisting of water/methanol (95/5, v/v (A)) and methanol/water (95/5, v/v (B)), buffered with 10 mM ammonium acetate and adjusted to pH 3, was used at a flow rate of 0.2 mL min^{-1}. The gradient elution program started at 50% mobile phase A for 2 min with a linear increase to 100% mobile phase B in 8 min. An isocratic gradient of 100% mobile phase B was initiated at 10 min for 5 min. The duration of each HPLC run was 20 min, including re-equilibration. The interface was used in positive electrospray ionization mode. The MS parameters included the following settings: ESI source block temperature 150 °C; desolvation temperature 300 °C; capillary voltage 4 kV; argon collision gas 9×10^{-6} bar; cone gas flow 50 L h^{-1} and desolvation gas flow 800 L h^{-1}. The acquisition of data was performed by applying selected reaction monitoring (SRM). The transitions followed for ZEN were 319.2 < 283.3; 319.2 < 301.4, and for Z4G 481.2 < 283.4; 481.2 < 319.2. The cone voltage and collision energy were optimized and selected for the most suitable parent ion of each analyte, implemented with two product ions. MassLynx™ version 4.1. and QuanLynx® version 4.1 software (Micromass, Manchester, UK) were used for data acquisition and processing.

Matrix matched calibration plots were constructed for the determination of ZEN and Z4G. ZAN, a structural analog of the myco-estrogen ZEN, was used as internal standard. The LODs varied in a range of 7 and 7 μg kg^{-1} for ZEN and Z4G respectively for maize and feed, 6 and 8 μg kg^{-1} for wheat and 9 and 10 μg kg^{-1} for breakfast cereals. Quantification was guaranteed at a low ppb-level with LOQs in a range of 14 and 14 μg kg^{-1} for maize, 12 and 16 μg kg^{-1} for wheat, 18 and 20 μg kg^{-1} for breakfast cereals for ZEN and Z4G respectively.

3. Results and discussion

3.1. Development of ELISA technique

Direct competitive ELISA was applied for the determination of ZEN. As the concentration of ZEN was simultaneously determined in duplo (one portion was measured after enzymatic or acidic hydrolysis, and the other portion was not treated); the difference between ZEN concentrations in these two portions allowed to estimate the Z4G content. It was assumed that after hydrolysis each Z4G molecule produced one molecule of ZEN. Taking the molecular weight difference into account (318 g mol^{-1}, ZEN and 480 g mol^{-1}, Z4G), the corresponding values for Z4G (LOD and

Table 1
Optimal conditions and percentages of zearalenone-4-glucoside enzymatic hydrolysis with different glycoside hydrolases ($n=5$).

Enzyme	Buffer solution	T (°C)	Percentage of cleavage (%)	
			Standard solution in methanol	Standard solution in blank maize extract
Glucosidase (*A. niger*)	0.1 M SAB[a] (pH 4.0)	37	102 ± 11	89 ± 13
Glucosidase (*Almonds*)	0.1 M SAB (pH 5.0)	37	6 ± 3	5 ± 2
Cellobiase (*A. niger*)	0.1 M SAB (pH 5.0)	40	91 ± 5	64 ± 9
Cellulase (*T. viride*)	0.1 M SAB (pH 5.0)	37	113 ± 14	141 ± 23
Cellulase (*A. niger*)	0.1 M SAB (pH 5.0)	37	21 ± 7	26 ± 6
β-Glucuronidase (*H. pomatia*)	0.1 M SAB (pH 5.0)	37	< 1	< 1
β-Glucuronidase (*E. coli*)	0.05 M SPB[b] (pH 6.8)	37	< 1	< 1

[a] sodium acetate buffer.
[b] sodium phosphate buffer.

IC_{50}) were calculated with factor 1.51, on the basis of the LOD and IC_{50} values obtained for ZEN.

The sensitivity and selectivity of the immunoassay are dependent on the specific antibody characteristics. Therefore, each immunoassay technique development is optimized with the choice of the immunoreagents. Five different monoclonal anti-ZEN antibodies were compared. The optimal conditions of the ELISA procedure were found respectively: the secondary antibody was diluted till 5 μg mL^{-1} in carbonate buffer solution; as a blocking buffer PBS-casein (1%) and PBS-skim milk (1, 2 and 3%) were compared, and PBS with 1% casein was taken as optimal blocking agent. MAb 4 did not show specificity to ZEN. To compare the other four different MAbs, calibration curves for ZEN determination were constructed and analytical performances were calculated (Fig. 1, Table 2). For checking the specificity of the antibodies, the cross-reactivity (CR, %) for several compounds structurally related to ZEN, like α-zearalenol, β-zearalenol and zearalanone (Fig. 2) were determined as the ratio of the IC_{50} value of the target analyte (ZEN) to the IC_{50} value of cross-reactive substances (Table 3). MAb 5 was characterized with the highest sensitivity (IC_{50} value, 0.4 ng mL^{-1}) and the best specificity; the antibody was characterized with high cross-reactivities for α-zearalenol (62%) and zearalanone (36%). So, this antibody was chosen for further experiments.

The key feature of the tested antibodies was their cross-reactivity to the "masked" form, Z4G. If the antibody was specific to both ZEN and Z4G, this antibody could be used to determine the sum of ZEN and Z4G. If the antibody was only specific for ZEN and not specific for Z4G this antibody could be applied for the specific detection of ZEN, thereby, for determination of Z4G.

Cross-reactivity of an antibody depends on the hapten-protein conjugate used for immunization. Regarding the MAb 5 antibody production, a ZEN–BSA-conjugate was used. Coupling ZEN with the protein was obtained via ZEN–CMO synthesis and realized in the opposite part of the glycosidic bond in the Z4G molecule (Fig. 2). MAb 5 was, as expected, characterized with insignificant cross-reactivity to Z4G (< 10%). Therefore, this antibody could be applied for Z4G screening.

The robustness test estimates the ability of the developed technique to withstand changes in the different parameters. It was found that the small deviation from optimal conditions (± 2 °C for incubation temperature, ± 7 min for incubation time and samples extraction time) does not affect on the analysis data. Intraday and interday repeatability precisions were calculated for three samples, analyzed during one and three consecutive days, and were 6.6 and 10.2% respectively.

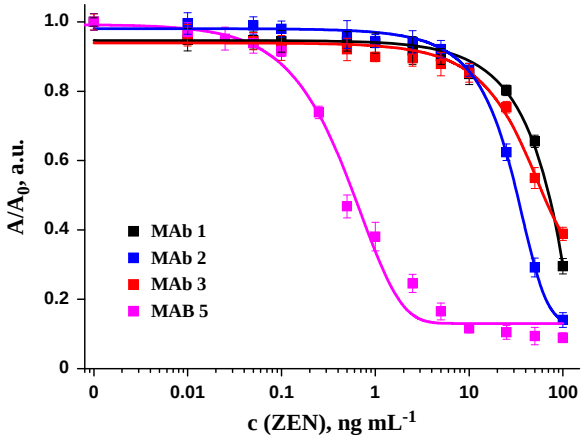


Fig. 1. Calibration curves for zearalenone determination by competitive ELISA ($n=5$).

Table 2
Analytical parameters of zearalenone determination in standard solutions, ($n=5$).

	MAb 1	MAb 2	MAb 3	MAb 5
LOD, ng mL^{-1}	3	5	1	0.08
IC_{50}, ng mL^{-1}	49 ± 5	28 ± 2	27 ± 3	0.4 ± 0.1
Linear range, ng mL^{-1}	10–75	11–50	8–60	0.15–2

3.2. Enzymatic hydrolysis of Z4G standard solutions

There are many different glycoside hydrolases, enzymes which catalyze the hydrolysis of the glycosidic bond in all kinds of glycosides, glycans and glycoconjugates with release of carbohydrates. They cleave the linkage between two carbohydrates and/or a carbohydrate and another molecule. The physiological functions of glycoside hydrolases depend on the origin (plants, fungi, animals or bacteria) and the substrate specificity.

For the enzymatic cleavage of Z4G, seven different glycoside hydrolases were compared as described above. They have different origins: fungi (*A. niger*, *T. viride*), bacterium (*E. coli*), mollusk (*H. pomatia*) and plant (almond). All of these enzymes breakdown the molecules containing the glycosidic bond, nevertheless they

Table 3
Cross reactivity values for zearalenone structurally related compounds.

Analyte	Cross-reactivity (%)			
	MAb 1	MAb 2	MAb 3	MAb 5
Zearalenone	100	100	100	100
α-zearalenol	> 100	> 100	> 100	62
β-zearalenol	> 100	> 100	> 100	8
Zearalanone	> 100	> 100	< 10	36
Zearalenone-4-glucoside	< 10	< 10	< 10	< 10

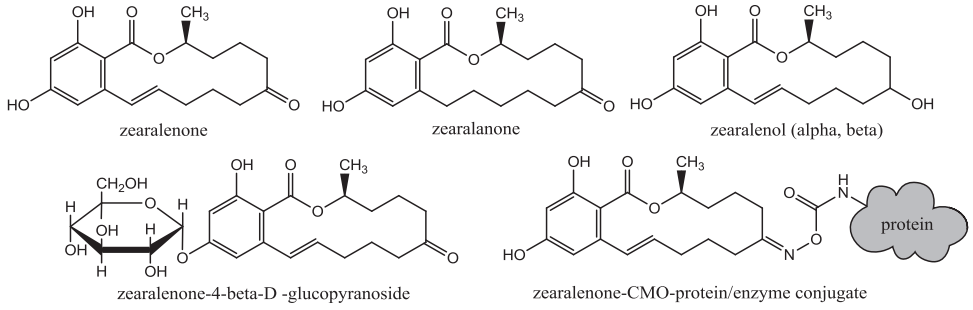


Fig. 2. Chemical structures of zearalenone, its structurally related derivatives: α-zearalenol, β-zearalenol, zearalanone, zearalenone-4-glucoside and zearalenone–protein/enzyme conjugates.

are specific to different substrates and altered activity in glycosidic bond cleavage. Thus, different kinds of glycoside hydrolases were compared, and hydrolysis conditions were optimized.

Optimization involved the choice of the time-temperature-medium conditions and the optimal amount of each enzyme. The manufacturer's enzyme description contained information about the optimal conditions for glycosidic linkage cleavage; the enzyme amount was calculated as a rate of units per μmol bound. However, in the case of real sample screening even the approximate concentration of glycosidic derivatives was unknown.

Ten different enzyme amounts in the range of 0.02–3.0 U were taken. Working temperatures and pH values were chosen according to the manufacturer's protocols and are presented in Table 1.

Firstly, the technique was optimized for standard solutions. ZEN, Z4G and mixtures of ZEN and Z4G were prepared in different concentrations and ratios in methanol. Small aliquots (200 μL) were placed in glass tubes, evaporated to dryness under a gentle N_2-stream at 40 °C and treated with the enzymes. Aliquots, treated with working buffer solution, were simultaneously placed at the required temperatures to control their stability under experimental conditions. It was observed that an enzyme concentration less than 0.6 U in standard solutions gave no good results: the percentage of cleavage was insignificant. An amount of 0.6 U showed good cleavage ability, but an increase of enzyme up to 2 U allowed to improve the extent of hydrolysis. As optimal amount 2 U was chosen. Three enzymes (GAn, CAn and CTv) were selected for further experiments, because they gave satisfying results in standard solution (Table 1).

For the determination of the optimal hydrolysis time, kinetic experiments were executed (Fig. 3). GAn-hydrolysis showed a good percentage of cleavage in 15 min, while the adequate extent of hydrolysis with CTv and CAn was reached only overnight.

3.3. Acidic hydrolysis of Z4G standard solutions

TFMSA was checked to hydrolize the glycoside bond in Z4G. TFMSA is one of the strongest known Brønsted acids (pKa = −13), characterized with extreme thermal stability and a high resistance towards reductive and oxidative cleavage.

The procedure of acidic Z4G destruction included several steps. The standard solutions of ZEN, Z4G and their mixture in methanol or sample extracts were evaporated to dryness, treated with acid and incubated under different conditions. After incubation the acid was neutralized with a solution of sodium carbonate (1 M). Then, the samples were dissolved with PBS and used for analysis.

Different temperatures (25, 40 and 60 °C) and TFMSA concentrations (in the range of 0.01–0.1 M) were checked. Simultaneously, the stability of ZEN was checked: aliquots of ZEN in different concentrations were treated with TFMSA and placed under the same temperature conditions as the Z4G treated probes. After TFMSA application in the concentration range of 0.01 M–0.025 M, a constant increase of ZEN concentration was observed. However, further increase of the acid concentration resulted in a decrease of the ZEN amount (Fig. 4). This fact could be explained by the destruction of ZEN at high TFMSA concentration, and this was confirmed by LC–MS/MS analysis of the treated ZEN probes in a concentration range of 50–200 μg kg^{-1}.

Also, the hydrolysis kinetics were studied: the use of 0.025 M TMFSA at 40 and 60 °C allowed to reach the same extent of Z4G destruction in 1 h as in 3 h at 25 °C with the same TFMSA concentration.

Hence, the selected optimal conditions were 40 °C, 0.025 M TMFSA and 1 h-incubation. However, because the results obtained with enzymatic hydrolysis of Z4G were better, only enzymatic cleavage was tested for Z4G determination in real samples.

3.4. Method validation using naturally contaminated cereal samples

A set of different cereals samples (maize, wheat, feed and breakfast cereals) selected according to the LC–MS/MS results were analyzed by ELISA. This set included 33 maize samples (21 "positive" samples (Z4G > LOQ) and 12 "negative" samples (Z4G < LOQ)), 17 negative wheat samples, 2 positive breakfast cereals samples and 1 positive feed sample. All chosen samples were characterized with different Z4G and ZEN contents ranging from < LOQ to > 350 μg kg^{-1}.

Fig. 5 shows the analytical scheme of the required procedure. Owing to the high sensitivity of the developed ELISA procedure (IC_{50} value for ZEN determination—0.4 ng mL^{-1}, Z4G – 0.6 ng mL^{-1}), the treated probes were diluted with a large volume of PBS, and as a result matrix effects decreased. Simultaneously, aliquots without enzymatic treatment were diluted with the same amount of PBS and analyzed on the same ELISA plate.

Investigating matrix effects, calibration curves were constructed using ZEN reference standards, prepared in blank wheat, maize and breakfast cereals extracts (absence of ZEN was confirmed by LC–MS/MS) (Fig. 6). To determine the analytical performances of Z4G a recalculation was executed using molecular weight differences (the factor was 1.51). The IC_{50} value for the Z4G determination changed from 0.6 ng mL^{-1} for the PBS standard solution to 1.2 ng mL^{-1} for maize and wheat extracts and 2.1 ng mL^{-1} for the breakfast cereals extract. Taking into account extraction and dilution, analytical parameters for Z4G determination in cereals by the developed ELISA procedure were defined: the LOD was 3 μg kg^{-1}, IC_{50} was 15 μg kg^{-1} and the linear range was 6–76 μg kg^{-1} (PBS calibration curve was used).

The three selected hydrolytic enzymes (GAn, CAn and CTv) were compared on real samples. However, the amount of enzyme

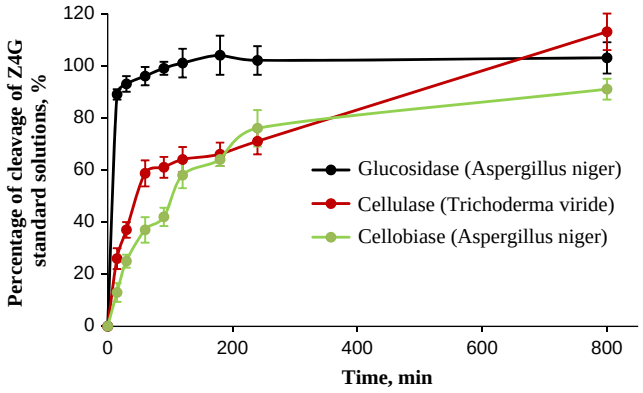


Fig. 3. Kinetics of the enzymatic hydrolysis of zearalenone-4-glucoside in standard solutions (n=5).

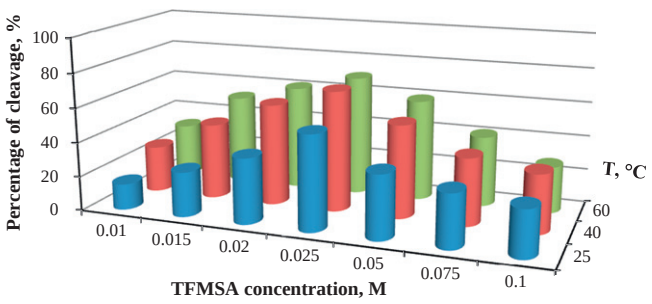


Fig. 4. Dependence of the acidic cleavage of zearalenone-4-glucoside in standard solutions vs. temperature and trifluoromethanesulfonic acid concentration (n=5).

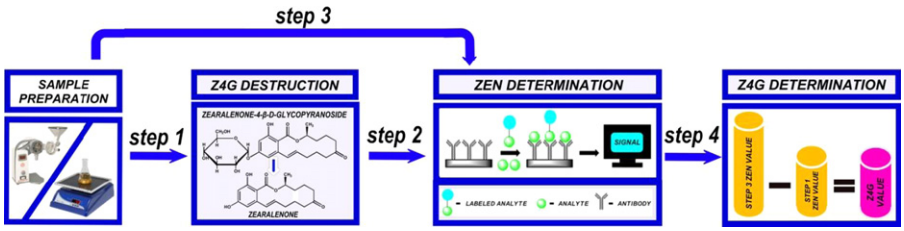
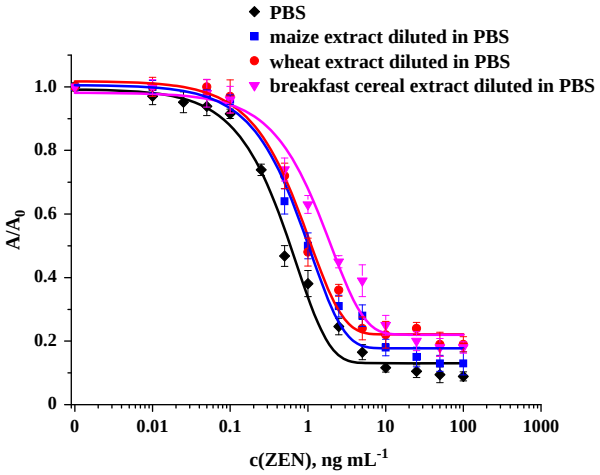


Fig. 5. Scheme of zearalenone-4-glucoside determination in real samples using immunoassay.



IC50, ng mL^{-1}	ZEN	Z4G
PBS	0.4	0.6
Maize extract	0.8	1.2
Wheat extract	0.8	1.2
Breakfast cereals extract	1.4	2.1

Fig. 6. Calibration curve for the determination of zearalenone using standard solutions in PBS and in wheat, maize and breakfast cereals extracts diluted with PBS (1/5) ($n=5$). Obtained IC_{50} data for zearalenone and calculated IC_{50} data for zearalenone-4-glucoside are also presented (ng mL^{-1}).

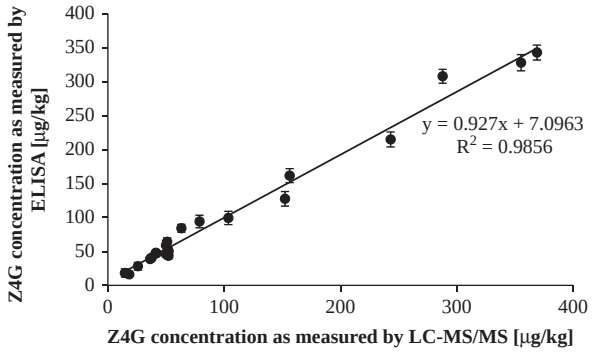


Fig. 7. Linear regression equation derived using ELISA and LC–MS/MS data for zearalenone-4-glucoside screening in maize samples ($n=5$).

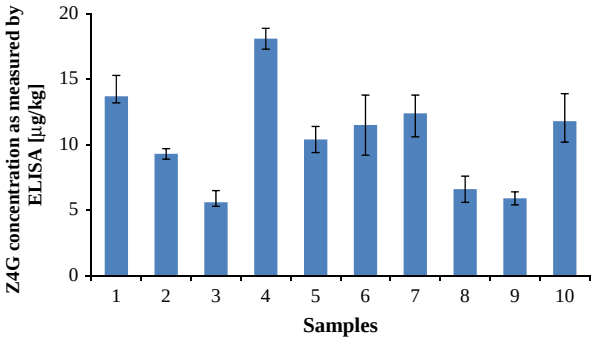


Fig. 8. Zearalenone-4-glucoside determination in wheat samples by ELISA ($n=5$).

selected for the standard solutions (2 U), resulted in an increase of matrix effects when applied to real samples. The matrix effect appeared as an uncontrolled increase of the ELISA optical density for both non-spiked and spiked sample extracts. So, the enzyme amount was decreased to 1 U.

The next important goal of the hydrolysis technique development in real samples was the choice of the optimal reaction time. The percentage of hydrolysis increased in 5, 10, 20 and 30 min. However, in already 40 min an uncontrolled increase of analytical signal was observed for several maize samples. A reaction time equal to 1 h (optimal for standard solutions destruction) led to the acquisition of unaccountable out-of-order results. Therefore, a 30 min-reaction time was chosen, because satisfactory results were obtained as more than 85% of Z4G dissolved in blank maize extracts were hydrolyzed (Table 1).

By comparing three enzymes, it is necessary to mention, that CTv led to an overestimation of the defined Z4G concentrations in maize samples. Probably, this overestimation was related to incomplete reduction of matrix effect. GAn and CAn did not hydrolyze Z4G completely, but the application of GAn resulted in a very high cleavage percentage of Z4G (Table 1).

As mentioned before, different cereal samples were analyzed by the developed technique and results were compared with direct method (LC–MS/MS). Maize samples which had a Z4G concentration range of 5–14 µg kg^{-1} with ELISA, were "negative" according to the LC–MS/MS-procedure. Z4G immunochemical and chromatographic analysis was compared for 21 "positive" maize samples by displaying as a regression curve (Fig. 7). Error bars are represented for the five measurements. The linear coefficient can be used to estimate the convergence of the methods. The obtained graph demonstrates a good agreement of both techniques.

10 from 17 wheat samples gave a Z4G concentration in the range of 5–12 µg kg^{-1} (Fig. 8), seven wheat samples were negative. According to the LC–MS/MS data, none of them were contaminated (LOQ=14 µg kg^{-1}). For the breakfast cereals samples with 90 and 21 µg kg^{-1} of Z4G (according to LC–MS/MS data), 73 and 32 µg kg^{-1}, respectively, were found by ELISA. Also, the feed sample did not demonstrate a large deviation (32 and 38 µg kg^{-1} were determined by LC–MS/MS and ELISA, respectively).

3.5. Gel-based immunoassay (GBA) for Z4G determination

The developed enzymatic hydrolysis procedure was also tested for determination of the sum of free and the masked zearalenone (ZEN+Z4G) with a non-instrumental test. The principle of this test is analogous to ELISA, but antibodies are chemically attached

Table 4
Zearalenone-4-glucoside determination in maize samples by gel-based immunoassay and LC–MS/MS, ($n=3$).

Maize samples	C(ZEN) (μg kg^{-1})		
	Gel-based immunoassay		LC–MS/MS
	Non-spiked	Spiked, 50 μg kg^{-1}	
Sample 1	+ ± +	+ + +	50.0
Sample 2	− − −	+ + +	41.2
Sample 3	− − −	+ + +	< LOQ[a]
Sample 4	− − −	+ + +	< LOQ
Sample 5	+ + ±	+ + +	50.0
Sample 6	− − −	+ + +	36.3
Sample 7	+ + +	+ + +	51.0
Sample 8	− − −	+ + +	< LOQ

+ positive result, absence of blue color (C (Z4G) ≥ 50 μg kg^{-1}).
− negative result, presence of blue color (C (Z4G) < 100 μg kg^{-1}).
[a] limit of quantification (LOQ) for LC–MS/MS was 14 μg kg^{-1}.

onto the gel inside a flow-through column. In the present study GBA with an HRP-label was used as a qualitative method.

There are no established EU maximum limits for Z4G in cereals, therefore, taking the maximum levels for ZEN as 100 μg kg^{-1} for unprocessed cereals and 350 μg kg^{-1} for unprocessed maize [3] in to account, it was the objective to develop test methods for the sum of ZEN and Z4G determination with a cut-off level in the concentration range of 50–100 μg kg^{-1}. A variety of ZEN–CG:BG ratios was tested, and the optimal mixture was 1/75. Synchronously with the ZEN–CG dilution, different ZEN–HRP conjugate dilutions were compared. Minimizing the non-specific sorption of ZEN–HRP onto the sepharose gel surface, the conjugate was dissolved 1/150 in PBS containing 0.05% Tween-20. By observation with the naked eye, in the absence of ZEN or its presence in concentrations less than 10 ng mL^{-1}, an intense blue color was developed. ZEN concentrations equal or more than 10 ng mL^{-1} resulted in a reproducible absence of blue color. The detection time was set at 6 min.

An intra-laboratory validation was performed with blank maize extracts (absence of ZEN and Z4G was confirmed by LC–MS/MS) spiked with ZEN and Z4G at concentrations less, equal and more than the cut-off level. Performance parameters were determined for the cut-off level at 10 ng mL^{-1} based on the summarized data of repeated experiments. The false negative rate was 3.2%, the false positive rate 3.6%, the specificity rate 96.4% and the sensitivity rate 96.8%. Concluding, the obtained rates were in conformity with all parameters set by the Commission Decision 2002/657/EC [40]

The sample extracts were diluted ten times in PBS in order to reduce the methanol content, to increase their compatibility with the immunoassay.

The extraction and recalculation factor bearing in mind, the cut-off for ZEN determination was 50 μg kg^{-1} with a visible and clear color contrast between positive and negative samples. The hydrolysis technique was applied for Z4G determination by GBA by using the spiked maize samples. No false negative results were obtained.

Developed test was applied for Z4G determination in eight maize samples. To prevent false negative results due to matrix interferences, for each sample non-spiked and spiked (Z4G 50 μg kg^{-1}) portions were analyzed. Results are presented in Table 4. No false negative results were obtained for spiked samples. Comparison of the immunoassays and chromatography results showed good agreement both for positive and negative samples.

4. Conclusions

Enzymatic and acidic hydrolysis of Z4G was investigated and immunochemical techniques for its determination were developed. Trifluoromethanesulfonic acid and different glycoside hydrolases were compared for the glycosidic bond cleavage. Glucosidase from *A. niger* was chosen as an optimal agent for the breaking down of Z4G to the parent ZEN. ZEN concentration was determined before and after conversion; by subtraction the amount of Z4G was calculated.

The developed ELISA was applied to the determination of Z4G in cereal samples, and gel-based immunoassay was applied for the determination of the sum of free and masked ZEN. Liquid chromatography coupled to tandem mass spectrometry was used as a confirmatory method. As a result, good correlations were obtained between immunochemical techniques and the chromatographic data. The method allowed to hydrolyze more than 85% of zearalenone-4-glucoside.

Summarizing, for the first time an immunochemical approach was used for the determination of Z4G in real samples, and an enzymatic hydrolysis procedure was successfully applied for cereal screening. The developed ELISA technique has a useful advantage: screening of large numbers of real samples can be executed in a relatively short time. Conformation of results must be carried out using a chromatography method to accurately quantify the content of the positive samples.

Acknowledgments

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References

[1] Ch. Zaied, N. Zouaoui, H. Bacha, S. Abid, Food Control 25 (2012) 773–777.
[2] R. Manova, R. Mladenova, Incidence of Zearalenone and Fumonisins in Bulgarian cereal production, Food Control 20 (2009) 362–365.
[3] EC Commission Regulation 1126/2007 of 28 September 2007 amending Regulation (EC) no. 1881/2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs as Regards Fusarium Toxins in Maize and Maize Products. Off. J. Eur. Union L255/14, 2007.
[4] D. Bowles, E.K. Lim, B. Poppenberger, F.E. Vaistij, Annu. Rev. Plant Biol. 57 (2006) 567–59.
[5] G. Engelhardt, G. Zill, B. Wohner, P.R. Wallnöfer, Naturwissenschaften 75 (1988) 309–310.
[6] F Berthiller, R. Schuhmacher, G. Adam, R. Krska, Anal. Bioanal. Chem. 395 (2009) 1243–1252.
[7] O. Vendl, F. Berthiller, C. Crews, R. Krska, Anal. Bioanal. Chem. 395 (2009) 1347–1354.
[8] J. Plasencia, C. Mirocha, Appl. Environ. Microbiol. 57 (1991) 146–150.
[9] B. Poppenberger, F. Berthiller, D. Lucyshyn, T. Sieberer, R. Schuhmacher, R. Krska, K. Kuchler, J. Glossl, C. Luschnig, G. Adam, J. Biol. Chem. 278 (2003) 47905–47914.
[10] I. Schneweis, K. Meyer, G. Engelhardt, J. Bauer, J. Agric. Food Chem. 50 (2002) 1736–1738.
[11] M. Gareis, J. Bauer, J. Thiem, G. Plank, S. Grabley, B. Gedek, J. Vet. Med. 37 (1990) 236–240.
[12] S. Grabley, M. Gareis, W. Bockers, J. Thiem, Synth. Stuttgart 11 (1992) 1078–1080.
[13] K. Lancova, J. Hajslova, J. Poustka, A. Krplova, M. Zachariasova, P. Dostalek, L. Sachambula, Food Addit. Contam. 25 (2008) 732–744.
[14] M. Kostelanska, J. Hajslova, M. Zachariasova, A. Malachova, K. Kalachova, J. Poustka, J. Fiala, P.M. Scott, F. Berthiller, R. Krska, J. Agric. Food Chem. 57 (2009) 3187–3194.

[15] O. Vendl, C. Crews, S. MaCdonald, R. Krska, F Berthiller, Food Addit. Contam. 27 (2010) 1148–1152.
[16] S. Monbaliu, C. Van Poucke, C. Detavernier, F. Dumoulin, M. Van De Velde, E. Schoeters, S. Van Dyck, O. Averkieva, C Van Peteghem, S. De Saeger, J. Agric. Food Chem. 58 (2010) 66–71.
[17] C.M. Placinta, Anim. Feed Sci. Technol. 78 (1999) 21–37.
[18] J.W. He, T. Zhou, J.C. Young, G.J. Boland, P.A. Scott, Trends Food Sci. Technol. 21 (2010) 967–976.
[19] A. Desmarchelier, W. Seefelder, World Mycotoxin J. 4 (2011) 29–35.
[20] M. De Boevre, J.D. Di Mavungu, S. Landschoot, K. Audenaert, M. Eeckhout, P. Maene, G. Haesaert, S. De Saeger, World Mycotoxin J., In press.
[21] G. Zill, W. Ziegler, G. Engelhardt, P.R Wallnöfer, Chemosphere 21 (1990) 435–442.
[22] I. Schneweis, K. Meyer, G. Engelhardt, J. Bauer, J. Agric. Food Chem. 50 (2002) 1736–1738.
[23] J.D. Di Mavungu, S. De Saeger, in: S De Saeger (Ed.), Determining Mycotoxins and Mycotoxingenic Fungi in Food and Feed, Woodhead publishing limited, Cambridge, UK, 2011, pp. 385–400.
[24] F. Berthiller, R. Schuhmacher, G. Buttinger, R. Krska, J. Chromatogr. 1062 (2005) 209–216.
[25] F. Berthiller, R. Krska, K.J. Domig, W. Kneifel, N. Juge, R. Schuhmacher, G. Adam, Toxicol. Lett. 206 (2011) 264–267.
[26] S.T. Tran, T.K. Smith, Anim. Feed Sci. Technol. 163 (2011) 84–92.
[27] F. Berthiller, U. Werner, M. Sulyok, R. Krska, M.T. Hauser, R. Schuhmacher, Food Addit. Contam. 23 (2006) 1194–1200.
[28] H. Kamimura, Appl. Environ. Microbiol. 52 (1986) 515–519.
[29] S.-C. Pei, W.-J. Lee, G.-P. Zhang, X.-F. Hu, S.A. Eremin, L.-J. Zhang, Food Control 31 (2013) 65–70.
[30] Y.-K. Wang, Y.-B. Shi, Q. Zou, J.-H. Sun, Z.-F. Chen, H.-A. Wang, S.-Q. Li, Y.-X. Yan, Food Control 31 (2013) 180–188.
[31] N.A. Burmistrova, I.Y. Goryacheva, E.Y. Basova, A.S. Franki, D. Elewaut, K. Van Beneden, D. Deforce, C. Van Peteghem, S. De Saeger, Anal. Bioanal. Chem. 39 (39) (2009) 1301–1307.
[32] N.V. Panini, F.A. Bertolino, E. Salinas, G.A. Messina, J. Raba, Biochem. Eng. J. 51 (2010) 7–13.
[33] M. Hervas, M.A. Lopez, A. Escarpa, Analyst 136 (2011) 2131–2138.
[34] M.C. Spanjer, P.M. Rensen, J.M. Scholten, Food Addit. Contam. 25 (2008) 472–489.
[35] D. Thouvenot, R.F. Morfin, Appl. Environ. Microbiol. 45 (1983) 16–23.
[36] I.Y Goryacheva, N.V. Beloglazova, D.A. Mikhirev, R. Niessner, D. Knopp, Talanta 75 (2008) 517–522.
[37] N.V. Beloglazova, I.Y Goryacheva, S. De Saeger, M.L. Scippo, R. Niessner, D. Knopp, Talanta 85 (2011) 151–156.
[38] E. Trullols, I. Ruisanchez, F. Xavier Rius, Trends Anal. Chem. 23 (2004) 137–145.
[39] M. De Boevre, J.D. Di Mavungu, P. Maene, K. Audenaert, D. Deforce, G. Haesaert, M. Eeckhout, A. Callebaut, F. Berthiller, C. Van Peteghem, S.De Saeger, Food Addit. Contam. 29 (2012) 819–835.
[40] EC Commission Decision 657/2002, Implementing Council Directive 96/23/EC Concerning the Performance of Analytical Methods and the Interpretation of Results. Off. J. Eur. Commun. L 221/8, 12 August 2002.