Strategy for masked mycotoxin screening

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1. Introduction and objectives

Together with mycotoxins, which are secondary fungal toxic metabolites, many of their conjugated forms, so-called 'masked mycotoxins', generated by plant metabolism or by food processing, were found. During these processes mycotoxins (for example, zearalenone (ZEN), deoxynivalenol (DON)) are converted into more polar forms via conjugation with sugars, amino acids or sulphate groups. These mycotoxin derivatives are characterized by different chemical properties, but the most threatening is that these compounds can be hydrolyzed to their precursors in the digestive tracts of human and animals and cause unexpected high toxicity of food and feed. Natural occurrence, metabolism and bioavailability data of masked mycotoxins are weakly investigated.

There are two strategies for masked mycotoxin determination: the *direct* and *indirect* approach. Direct techniques correspond with chromatographic separation with fluorescence (Zill et al., 1990) or more common with mass spectrometric detection (Schneweis et al.,2002, Berthiller et al., 2005, Berthiller et al., 2009), and presume individual detection of each form (at least each known form). 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 DON-3-glucoside (Berthiller et al., 2011, Tran et al., 2011). For ZEN masked forms only the direct approach was described (Berthiller et al., 2006, Schneweis et al., 2002)

In this project, enzymatic and acidic techniques for zearalenone-4-β-D-glucopyranoside (zearalenone-4-glucoside, Z4G) hydrolysis were evaluated. There are no data available on the immunochemical determination of Z4G. Therefore, the aim of this project was the development of an immunochemical technique for the Z4G determination based on its destruction to the parent ZEN.

Two immunoassay formats for the detection of ZEN in cereal samples were employed: enzyme-linked immunosorbent assay (ELISA) and gel-based column test-method.

2. Methodologies

2.1. Development of ELISA for ZEN and Z4G determination

Direct competitive ELISA was applied for the determination of ZEN. Because 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 ZEN-HRP synthesis. To synthesize ZEN-CMO, a modified technique, described by D. Thouvenot (Thouvenot et al., 1983) was applied.

The sensitivity and selectivity of the immunoassay are dependent on the specific antibody characteristics. Therefore, each immunoassay development is optimized with the choice of the immunoreagents. Five different monoclonal anti-ZEN antibodies were compared.

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 (Burmistrova, 2009) 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.

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 1).

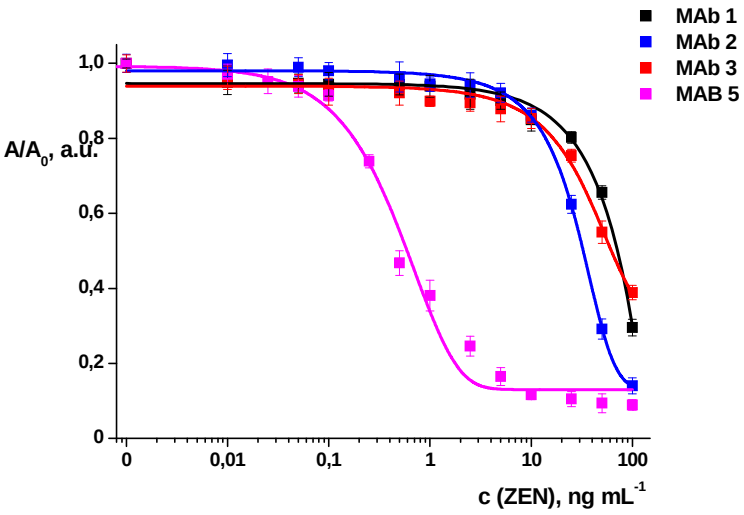

Fig. 1. Calibration curves for zearalenone determination by competitive ELISA (n = 5)

Table 1. Analytical parameters of zearalenone determination in standard solutions

	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

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 2). 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.

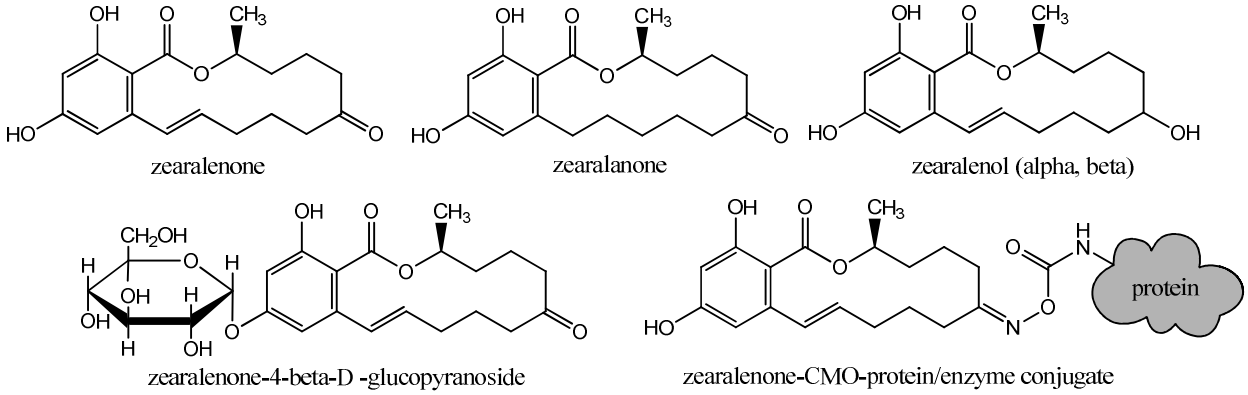

Fig. 2. Chemical structures of zearalenone, its structurally related derivatives: α-zearalenol, β-zearalenol, zearalanone, zearalenone-4-glucoside and zearalenone - protein/enzyme conjugates.

Table 2. 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

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 indirect 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 indirect Z4G screening.

2.2. 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. 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. They have different origins: fungi (*Aspergillus niger, Trichoderma viride*), bacterium (*Escherichia coli*), mollusk (*Helix pomatia*) and plant (almond). All of these enzymes breakdown the molecules containing the glycosidic bond, nevertheless they 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 to 3.0 U were taken. Working temperatures and pH values were chosen according to the manufacturer's protocols and are presented in Table 3. 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 (Glucosidase from *Aspergillus niger* (GAn), cellulase from *Aspergillus niger* (CAn) and cellulase from *Trichoderma viride* (CTv)) were selected for further experiments, because they gave satisfying results in standard solution (Table 3).

Table 3. 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

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.

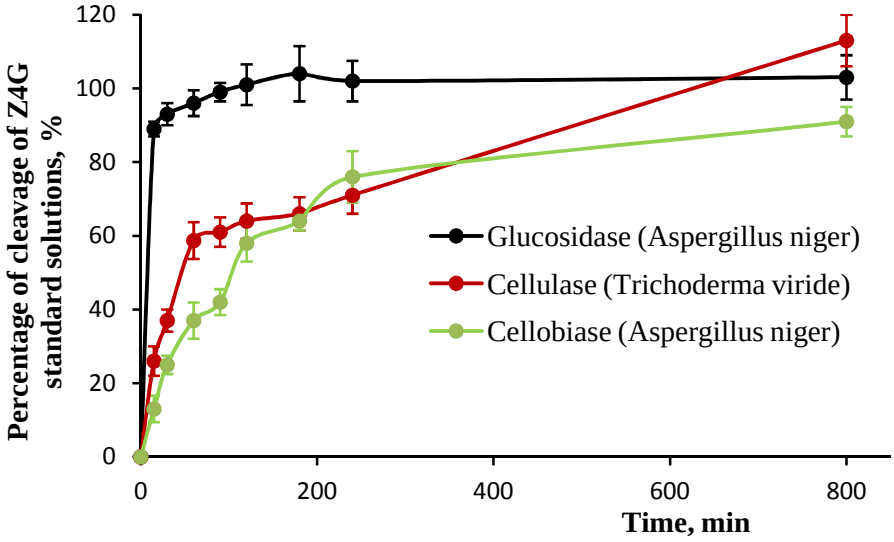

Fig. 3. Kinetics of the enzymatic hydrolysis of Z4G in standard solutions (n = 5)

2.3. Acidic hydrolysis of Z4G standard solutions

Trifluoromethanesulfonic acid (TFMSA) was checked to hydrolyze 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 0.01M to 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 $\mu g\ kg^{-1}$.

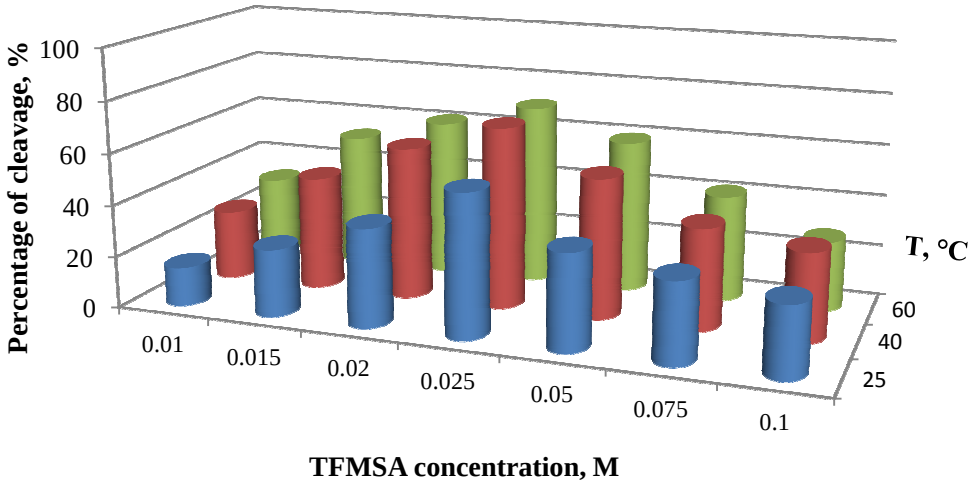

Fig. 4. Temperature - acid concentration dependence of the acidic hydrolysis of Z4G in standard solutions (*n = 5*)

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.

2.4. ELISA 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}. LOQs were 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.

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). 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 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 1U.

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 3).

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 3).

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

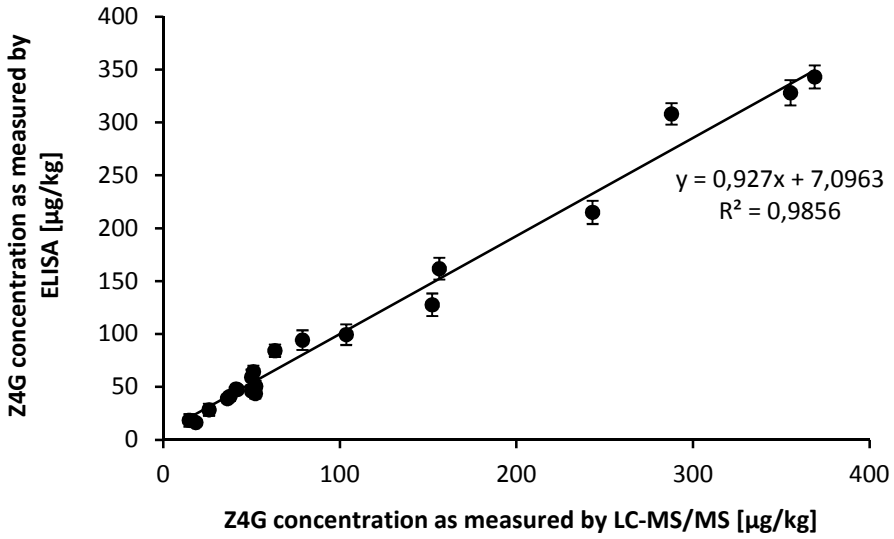

Fig. 5. Linear regression equation derived using ELISA and LC–MS/MS data for zearalenone-4-glucoside screening in maize samples (*n = 5*)

The screening of the 17 wheat samples was also characterized with a good correlation between the data obtained by the developed technique and the LC-MS/MS: low concentrations of Z4G (≤ LOQ) were determined by LC-MS/MS, and the ELISA results were in a concentration range of 5 to 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).

2.5. Gel-based immunoassay (GBA) for ZEN and 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 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 (Commission Regulation 1126/2007) 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 to 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 (Commission Decision 657/2002).

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.

3. 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 *Aspergillus 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.

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1. Beloglazova, N.V.; Speranskaya, E.S.; De Saeger, S.; Hens, Z.; Abé, S.; Goryacheva, I.Y. Quantum dot based rapid test for zearalenone detection. *Anal Bioanal Chem.* **2012,** *403(10),* 3013-3024.
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Contribution to Scientific Meetings

1. Beloglazova, N.V.; Goryacheva, I.Y., Speranskaya, E.S.; De Boevre, M.; De Saeger, S. Immunochemical approach for masked mycotoxin determination. 34th Mycotoxin Workshop. 14th-16th May 2012, Braunschweig, Germany.
2. Beloglazova, N.V.; Shmelin, P.S.; Speranskaya, E.S.; Grebennikov, E.P.; Goryacheva, I.Yu.; De Saeger, S. Quantum dots and quantum dots loaded liposomes as the labels for immunoassay. XIth International Conference on AgriFood Antibodies. 3^{d} - 5th September 2012, Vienna, Austria.