HPLC and ELISA Methods for Detection and Quantification of Fumonisins in Naturally Contaminated Maize

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Research Article

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Abstract

Fumonisins are naturally occurring secondary metabolites of pathogenic fungi that frequently contaminate maize and other cereals in the field and/or during storage. Although the mass spectrometry multi-mycotoxin detection methods are considered as the state-of-the-art in this area of research, the lack of necessary equipment and experience in many laboratories as well as difficulties to analyze fumonisins in multi-toxin methods are the main reasons for use of ELISA and/or HPLC methods for routine testing.

The aim of this study was to evaluate the use of HPLC method with post column derivatisation method for confirmation of the results of competitive ELISA assay for detection and quantification of fumonisins (FB1, FB2 and FB3) in maize intended for use in feed industry. Calculated correlation parameters showed strong linear relationship of tested methods indicating that both methods are sensitive, reliable, reproducible, cost-effective and applicable for quantitative routine determination of fumonisins in maize.

1. Introduction

Fumonisins are toxic secondary metabolites produced by Fusarium verticillioides (formerly F. moniliforme), F. proliferatum, F. fujikuroi and other less common Fusarium species, Alternaria alternata f. sp. Lycopersici, Aspergillus awamori and Aspergillus niger. A great number of researches have confirmed a significant relationship between FB1 contamination and risk of liver and oesophageal cancer, neural tube defects and a number of cases of acute mycotoxicosis in humans. In animals, it is associated with Porcine Pulmonary Edema, Equine Leukoencephalomalacia, hepatotoxicity and high toxicity to a variety of experimental animals (EFSA, 2018). After initial discovery, a great number of outbreaks of diseases in horses and pigs, availability of research data on fumonisin toxicity in humans and animals as well as data of their occurrence, resulted in evaluation of fumonisins by the IARC Monographs programme of the carcinogenic hazard to humans. Fumonisin B1 and B2 are classified as possibly carcinogenic to humans (Group 2B) (IARC, 1993, 2002; Yazar & Omurtag, 2008; JECFA 2011; FAO/WHO, 2017).

Fumonisins are long-chained poly-hydroxyl-amines that differ in chemical structure (variably hydroxylated and esterified with organic acids) that is considered responsible for their potential toxic effects. These highly polar compounds are easily soluble in water and other polar solvents. They can be present in samples as ‘hidden forms’ and/or “modified forms”. Their various reactive groups are capable of interaction with matrix components (i.e. matrix-associated and modified form). Potential presence of all these forms make detection of total fumonisin content in samples, as well as evaluation of their potential harmful effects on human and/or animal health, quite challenging and laborious task (Rychlik et al., 2014; Braun & Wink, 2018; EFSA, 2018). Fumonisin B (FB) series include the most toxic and most abundant naturally occurring mycotoxins: FB1, FB2, FB3 and FB4. They are structural analogs of sphingosine and sphinganine that inhibit ceramide synthases, disrupt sphingolipid metabolism causing diseases in plants and animals. The chemical structures of fumonisin B1, fumonisin B2, fumonisin B3 and fumonisin B4 are given in Fig. 1 (JECFA, 2001; Rheeder et al., 2002; Bartók et al., 2006).
Fumonisins are common contaminants of cereals and cereal products (up to 100% of tested samples with the highest concentrations of 2.339 µg/kg). They are also found in rice, sorghum, beans, soya, tea, eggs, milk, meat and other food and feedstuffs worldwide, in various concentration ranges. It has been estimated that FB1 accounts for up to 80% of the total fumonisins produced (Griessler et al., 2010; Varga et al., 2010; Rodrigues & Naehrer, 2012; Scott, 2012; Streit et al., 2012; Logrieco et al., 2014; Li et al., 2015; Deepa & Sreenivasa, 2017; FAO/WHO, 2017; Braun & Wink, 2018; Gruber-Dorninger et al., 2019). In analysing the occurrence data of fumonisins by EFSA (2018), additional contribution of 60% of hidden forms has been documented. Among analysed samples, the majority data were on cereal grains, their products and by products (47%) of which the highest number of reported samples were for maize in the concentration range from 0.3 to 1.678.1 µg/kg (EFSA, 2018). It is important to point out that until recently, data on occurrence of fumonisins contained only information about FB1 and FB2 (90% of the samples analyzed). The actual contamination of all three FB series toxins is usually made by estimation. Updated Opinion of the Scientific Committee on Food on fumonisin B2, B2 and B3 has concluded that all three fumonisins have similar toxicological profiles and potencies. Therefore, new studies on occurrence are needed that monitor all three fumonisin analogues at the same time (EFSA, 2005; EFSA, 2018).

There are several screening and confirmatory, qualitative and quantitative analytical methods for detection of fumonisins including thin layer chromatography, gas-liquid chromatography (GLC), high performance liquid chromatography (HPLC), mass spectrometry, various enzyme-linked immunosorbent assays (ELISA) and molecular methods. The mass spectrometry methods are considered as the state-of-the-art in this area of research due to their high sensitivity and specificity for detection and quantification with limit as low as 0,001 µg/g (González-Jartín et al., 2021). Most methods are focused on detection of FB1. Use of liquid chromatography-mass spectrometry with electrospray ionization and ESI with tandem mass spectrometry enables detection of lesser-known fumonisin analogs that are not detected with other analytical techniques due to the necessary derivatization processes (Arranz et al., 2004; Bartók et al., 2006; Musser and Plattner, 1997; Deepa and Sreenivasa, 2017; FAO/WH0, 2017; Janik et al., 2021). However, expensive equipment and high costs of its maintenance as well as specialized operators are still lacking in a great number of laboratories worldwide. Therefore, ELISA and HPLC methods are still often used for determination of fumonisins in food and feed. Since a great number of published data did not include mentioned three analogs, there is a need for evaluation of different methods for detection of three B series fumonisin analogs. Therefore, this research on fumonisins detection will provide new data on applicability and comparison of methods for determination of FB1, FB2 and FB3 and thus providing valuable data for comparison of similar research of other research groups.

The aim of this research was to evaluate the performance and applicability of both, the modified high-performance liquid chromatography method with post column derivatisation and fluorescence detection (HPLC-FLD) described by Sydenham et al. (1996) and commercial enzyme-linked immunosorbent assay (AgraQuant® Total fumonisin Assay 0.25/5.0, RomerLabs, Austria) for simultaneous determination of three B series fumonisin analogs (FB1, FB2 and FB3) in maize samples intended for use as animal feed in a wide range of concentrations.
2. Materials And Methods

Evaluation included testing of 30 naturally contaminated maize samples in accordance with guidance and performance criteria set in European Decisions (EC, 2002; 2006a). According to available data in literature, modified forms occur in rather low numbers (< 10%) and were not included in this study. Fumonisin B4 has not been evaluated in this study, since it occurs mainly in grapes, it is still not considered toxic and there is a lack of data on toxicity and toxicokinetics (EFSA, 2005, 2018).

2.1. Sampling and spiking procedure

The sample containing FB1 + FB2 + FB3 at < 25 µg/kg was selected and sub-sampled for validation studies with ELISA and HPLC methods. Test material (maize grain) from the Proficiency Testing Programmes MPZ UKZUZ for the Analytical Laboratories has been acquired with calculated values (robust mean) of 1.721,36 µg/kg (range of z-score ± 2 = 1.157,59–2.133,20 µg/kg) for FB1 and 287,97 µg/kg (range of z-score ± 2 = 173,11–391,69 µg/kg) for FB2.

Thirty samples of maize have been collected from storage facilities in Croatia (from March to May 2021) according to criteria established for the sampling for the control of the levels of mycotoxins described in Commission Regulation (EC) No. 401/2006. All the samples were handled following procedures for size reduction and treatment to achieve complete homogenization. All samples were analyzed by both, commercial direct competitive enzyme-linked immunosorbent assay (ELISA), AgraQuant® Total fumonisin Assay 0.25/5.0 (Romer Labs® Singapore Pte. Ltd.) and by HPLC-FLD method described by Sydenham et al. (1996) with slight modifications. Both methods were characterised by all the performance criteria (EC, 2017). All maize samples were ground in the ultra centrifugal mill ZM200 (Retsch®, Germany), thoroughly mixed and twenty grams of samples were used for both analysis.

Spiking experiment has been performed by artificial contamination of ground maize (sample containing FB1 + FB2 + FB3 at < 25 µg/kg) with fumonisin standard solution at the three levels: combined contamination level of 240 µg/kg, 1,600 µg/kg and 60,000 µg/kg (FB1, FB2, FB3 ratio was 5:2.1). Spiking levels were selected to meet the current legislative decision limits for food and feed. Blank sample (20.00 g) was weighted in the clean jar and standard solution aliquots were added and left for one hour in a sealed jar in dark. All chemicals were of analytical grade and purchased from Sigma Aldrich Chemie GmbH (Steinheim, Germany). Corresponding standard solutions for analytical testing and spiking purposes were prepared in methanol by appropriate dilution of the stock standard solution.

2.2. ELISA method

Commercial direct competitive enzyme-linked immunosorbent assay (ELISA), AgraQuant® Total fumonisin Assay 0.25/5.0 (RomerLabs, Austria) has been used for testing of samples according to manufacturer instructions (Romer Labs, 2021). Fumonisin standard solutions included in the ELISA test were at concentrations of 0, 250, 500, 1,000 and 5,000 µg/kg. Sample preparation: the representative sample was ground so that 95% pass through a 20-mesh sieve (sieve opening 0.84 µm) and thoroughly
mixed. Twenty grams of final sample was weighed out in a clean jar and 100 mL of 70/30 (v/v) methanol/water extraction solvent was added. Sealed jar was shaked for three minutes. After five minutes (when the sample has settled) the top layer of extract was filtered through a cellulose filter with particle retention of 11 µm and ash content ≤ 0.06%. The sample extract was further diluted in ration 1:20 with de-ionized water. In a case of higher concentrations of toxins in samples (higher than the highest selected concentration of the standard), the filtered extract has been diluted with 70/30 (v/v) methanol/water before further dilution with deionized water so that the results were in the range of 250–5,000 µg/kg and reanalyzed to obtain accurate results. All samples were tested in three-replicates.

**Assay Procedure:** Conjugate (200 µL) was dispensed in Dilution Strip in a microwell strip holder for each standard and sample. Each standard/sample (100 µL) has been added in dilution well and mixed carefully (pipetting it up and down three times) and 100 µL was transferred immediately into Antibody Coated Microwell strip. After ten minutes of incubation in a dark environment, the content of the microwell strips was emptied into a waste container. Washing has been done with repeated filling of each microwell with deionized water and dumping the water from the strips. The residual water after the fifth wash was removed with several layers of absorbent paper towels. Reaction of standards and sample extracts with enzyme-conjugated toxin was made visible with addition of 100 µL enzyme substrate in each microwell and incubation in dark for five minutes. The reaction was stopped by addition of 100 µL of Stop Solution. The colour of the solution in the strip changed from blue to yellow. Optical densities were measured with an absorbance filter of 450 nm (differential filter of 630 nm).

**Interpretation of the Results:** Results of tested samples were calculated from the dose-response curve of five standards using the Log/Logit regression model with the linearity coefficient of calibration curve greater than 0.990. The dilution factor was included in calculation of the final results. The Optical Density values higher than 0.5 absorbance units for the first standard (zero mg/kg) were used to assure that there was no deterioration of reagents. According to manufacture instructions, the limit of detection for the Assay is 200 µg/kg while the limit of quantification is 250 µg/kg. Limit of detection in this study was determined by calculation of the results of the average values of ten fumonisin free samples plus two standard deviation values.

### 2.3. HPLC method

HPLC-FLD method described by Sydenham et al. (1996) has been optimised using individual fumonisin standards before further testing. Modification of the method included combination of specific extraction columns and mobile-phase mixture that has shown the best results in recovery study. In short, twenty grams of samples were homogenized in methanol-water (3:1, v/v; 100 ml) for three minutes and centrifuged. An aliquot was used for clean-up step with Extract Clean™ C-18 columns using a vacuum manifold (Grace, USA) and derivatized with Phthaldialdehyde (OPA). The HPLC Agilent 1100 series (Agilent, Waldbronn, Germany) equipped with a quaternary pump, autosampler, column thermostat, diode array detector (DAD) and fluorescence detector (FLD) and Zorbax Eclipse plus C 18 analytical column. Quantification of fumonisin(s) was performed by measurement of the peak areas at FB1, FB2 and FB3 retention time and comparison with the relevant calibration curve in Agilent ChemStation software. The mobile phase was a mixture of methanol and 0.1 M sodium phosphate (80/20, v/v, at pH 3.3) and the flow rate was 1 ml/min. In this combination, retention time of FB1, FB2 and FB3 were at around 7, 21 and
24 min, respectively. Limit of detection (LOD) and limit of quantification (LOQ) were calculated as three-times and ten-times the signal-to-noise ration. All samples were tested in three-replicates.

2.4. Evaluation of the Methods Performance

Evaluation of both methods were made according to the Commission Regulation (EC) No. 401/2006 and characterisation of methods of analysis defined in Annex III of Regulation (EU) 2017/625. To assure that methods of analysis are appropriate for food control purposes we have tested that both methods comply with general requirements of performance criteria (listed below). In addition, specific requirements of the EC 401/2006 for method for determination of fumonisins were included, even though the criteria are set only for fumonisin B1 and B2 (Table 1). Statistical analysis of data was made with Statistica software at significance level of 95% (P = 0.05). Comparison has followed the European Decisions (EC, 2002, 2006A) for performance criteria for fumonisins for screening and confirmatory methods.

Table 1

<table>
<thead>
<tr>
<th>Level (µg/kg)</th>
<th>Fumonisin B1 or B2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RSD&lt;sub&gt;r&lt;/sub&gt;</td>
</tr>
<tr>
<td>≤ 500</td>
<td>≤ 30</td>
</tr>
<tr>
<td>≥ 500</td>
<td>≤ 20</td>
</tr>
</tbody>
</table>

RSD<sub>r</sub> – standard deviation of measurements under repeatability conditions, RSD<sub>r</sub> – standard deviation of measurements under reproducibility conditions.

Performance characteristics that need to be determined for both methods were Detection limit, Precision, Selectivity & Specificity and Applicability & Ruggedness & Stability. Although Decision limit and Trueness & Recovery are additional criteria for HPLC method, we have determined these parameters for both methods. **Applicability/Ruggedness** is susceptibility of an analytical method to variations in experimental conditions, which could affect the analytical result (i.e. stability of reagents, composition of the sample, pH, etc.). This factor has been considered as less important in this analysis since the stability of reagents, pH of samples as well as other quality control procedures were regularly monitored in the laboratory. **Stability** can be important in respect to stability of the analyte or matrix constituents in the sample during storage or analyses. To overcome potential variations, samples were analysed as soon as they were acquired in the laboratory. All stock standard solutions and other reagents have been prepared fresh prior to analysis. **Detection limit (CCβ)** was calculated by analysing the spiked blank samples at and above the decision limit. In addition, we have determined the detection limit at and below 200 µg/kg to check if the methods are applicable for testing of maize and maize-based food intended for human consumption (EC 1881/2006). **Decision limit (CCα)** has been calculated by analysing 20 blank samples spiked with the analyte(s) at the permitted limit. For maize and maize products (with a moisture content of 12%) intended for animal feed, (maximal) guidance value for fumonisin (FB1 + FB2) is 60.000 µg/kg (EC 576/2006). **Precision** is expressed in terms of imprecision. It was computed as standard deviation of the test results.
under repeatability and reproducibility conditions. We have tested precision of the methods in 
**repeatability** conditions by repetition of the same methods on identical sample in the same laboratory by 
the same operator using the same equipment. **Reproducibility**, precision under reproducibility conditions, 
was tested by application of the same method by different operators. All the results, if not stated 
differently, were expressed as the mean value with corresponding standard deviation. **Selectivity** and 
**specificity** were tested on positive (spiked and reference material) and negative samples by 20 repetitions 
and the results were expressed as the percentage of correct results. **Trueness** has been calculated by 
testing of reference material taking into account that it does not contain information about fumonisin B3. 
Additionally, it was calculated by testing of the spiked samples and evaluated as the mean value. The 
**recovery** was calculated for both methods by dividing the amount obtained with the concentration spiked 
in a feed sample (or the value of reference material) and multiplied by 100 to express it in percentage.

### 2.5. Determination of the correlation of ELISA and HPLC method

Fumonisin (FB1 + FB2 + FB3) concentrations in 30 maize samples analysed by both, ELISA and HPLC 
methods have been compared using a correlation test (Statistica software).

### 3. Results And Discussion

Considering the importance of maize in the food and feed industry, control of mycotoxin contamination is 
important for health and wellbeing of humans and animals, but also for prevention of significant 
economical losses. Therefore, regulatory limits for fumonisins in food and feed are in force worldwide. 
The limits for maize intended for human consumption is set at the level of 1.000 µg/kg, and for some 
processed maize-based foods for babies and young children as low as 200 µg/kg. Guidance maximal 
level for fumonisins in maize intended for use as animal feed is 60.000 µg/kg (EC, 2006b; EC, 2006c; EC, 

ELISA methods are used for long time in mycotoxin testing and they are generally considered as rapid, 
easy-to-use, accurate, sensitive, specific and reproducible. However, considering the principle of the 
method where antibiotides (of high specificity and sensitivity) target mycotoxins (and not the antigens), 
there is a potential of interaction with other compounds that have similar chemical structure. As a result 
of this “matrix effect”, overestimation in mycotoxin content has been encountered in routine testing. 
Uncertainty associated with fumonisin testing is specifically challenging because they exhibit a strong 
interaction with matrix components and there is a possibility of determination of hidden and/or modified 
forms. Therefore, extraction protocol might have significant influence on the final result (Sutkino et al., 
1996; Ono et al., 2000; EFSA; 2018). On the other hand, HPLC methods for fumonisin testing are 
considered more specific. They differ in extraction step and chromatographic separation. Since 
fumonisins lack a strong chromophore or fluorophore, HPLC detection can be made after pre-column or 
post-column derivatization. Post-column derivatisation is preferred because formed derivatives are more 
stable (Stroka et al., 2008). Taking into account the lack on data for all three fumonisins, importance of
accurate and reproducible testing as well as aforementioned challenges in fumonisin testing, evaluation and comparison of different methodologies on spiked and naturally contaminated samples are important for comparison studies of different research groups in this area.

Specificity of the matrix and variability of commodity contamination with different undesirable substances highlights the importance of convincing demonstration that methods are reliable, reproducible and applicable for testing of food/feed samples. Achievement of reliable and accurate results of each analytical method is assured by calibration and use of positive and negative control samples. Even though ELISA and HPLC techniques have been used for a long period of time, data on comparison of the techniques on naturally and spiked feed samples are important.

### 3.1. Fumonisin contamination levels by ELISA and HPLC method

In this research, all tested samples were positive for fumonisin(s) by ELISA method. Chromatographic methods confirmed presence of fumonisin B1 in all tested samples. Only five samples contained fumonisin B2, while none of the samples contained fumonisin B3 at detectable level. Since the results of ELISA test are evaluated as the Total fumonisin content and there is no possibility to differentiate the quantity of each analog, results of HPLC tests are here also presented here as the total fumonisin content. Results of contamination levels by ELISA and HPLC method are presented in Table 2. Results of this study are in agreement with the results of other authors confirming high contamination of maize. Namely, maize and maize-based products are considered as one of the most commonly contaminated cereal grains in Europe (Scudamore & Patel, 2009; Griessler et al., 2010; Rodrigues & Naehrer, 2012; Streit et al., 2012; Deepa & Sreenivasa, 2017; FAO/WHO, 2017; Braun & Wink, 2018; EFSA, 2018; Gruber-Dorninger et al., 2019). Fumonisins B2 and B3 are included in testing since they might also have hazardous effects, described for fumonisin B1, although their contribution is still under evaluation. Potential smaller contribution of FB2 and FB3 can be explained with the absence of the hydroxyl group in FB2 and FB3 and lower water solubility than FB1 (Thiel et al., 1996; Henry & Wyatt, 2001). We have detected a wide range of concentrations with the highest quantities of 11,590,88 µg/kg by ELISA test and 11,869,94 µg/kg by HPLC method. Since the limit of detection of ELISA test is set by manufacturer at concentration of 200 µg/kg and quantitation at 250 µg/kg, all data below this level are under routine conditions evaluated as bellow limit of detection. Samples that had the quantity of FBs greater than 5,000 µg/kg (N = 7) were re-tested after appropriate dilution. Considering the Recommendation (EC, 2006b) where the guidance value of fumonisin B1 and B2 in maize and maize products is 60,000 µg/kg, all tested samples were below this level and would therefore be used for preparation of animal feedingstuffs. Results of contamination level, detected in our study, show increased concentrations of fumonisins in respect to previous studies of fumonisins in Croatia (Pleadin et al., 2012), but is in agreement with the results of other authors (Šegvić-Klarić et al., 2009; Berardo et al., 2011, EFSA, 2018; Gromadzka et al., 2019; Carbas et al., 2021). These differences confirm variations in the contamination levels of mycotoxins and the need to continuous monitoring. Such variations were also noticed in our
fifteen-year study and are influenced by different factors. It has been documented that climate change effects had an important role in these variations (Miraglia et al., EFSA, 2018; Gromadzka et al., 2019; Leslie et al., 2021; Sokolovic et al., 2022).

Table 2

Data analysis of determination of total fumonisin (B1, B2 and B3) content in maize samples by commercial ELISA and HPLC method.

<table>
<thead>
<tr>
<th>Maize Samples (N = 30)</th>
<th>ELISA</th>
<th>HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean value (µg/kg)</td>
<td>3.147,05</td>
<td>3.133,73</td>
</tr>
<tr>
<td>Median value (µg/kg)</td>
<td>2.411,80</td>
<td>1.666,58</td>
</tr>
<tr>
<td>Min (µg/kg)</td>
<td>11,28</td>
<td>10,55</td>
</tr>
<tr>
<td>Max (µg/kg)</td>
<td>11.590,88</td>
<td>11.869,94</td>
</tr>
</tbody>
</table>

3.2. Results of the method performance testing

Results of the performance testing of ELISA and HPLC method in respect to proposed parameters on spiked and reference materials are presented in Tables 3 and 4.
Table 3
Results of the performance testing of ELISA method in respect to proposed parameters on spiked and reference materials.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Controls</th>
<th>Spiked sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Detection limit (µg/kg)</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>Decision limit (µg/kg)</td>
<td>&lt; 250</td>
<td>≥ 250</td>
</tr>
<tr>
<td>Trueness*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (µg/kg)</td>
<td>&lt;LOD</td>
<td>2.001,74</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>-</td>
<td>13,45</td>
</tr>
<tr>
<td>Precision**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repeatability</td>
<td>&lt;LOD</td>
<td>2.001,74 ± 13,45</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>&lt;LOD</td>
<td>2.003,71 ± 12,29</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive sample</td>
<td>-</td>
<td>100%</td>
</tr>
<tr>
<td>Blank sample</td>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>-</td>
<td>99,62</td>
</tr>
</tbody>
</table>

Notes: *Trueness = expressed as average value of 20 repetitions in repeatability conditions; **Precision = expressed as SD value of 20 repetitions for repeatability and reproducibility. Specificity = % of positive samples in positive/spiked sample and % of negative in blank sample. Decision limit is expressed here only for corn. Level 1: 240 µg/kg; Level 2 = 1600 µg/kg; Level 3: 60 mg/kg (FB1, FB2, FB3 ratio was 5:2.1). Negative control: blank sample; Positive control: reference material.
Table 4
Results of the performance testing of HPLC method in respect to proposed parameters on spiked and reference materials.

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Controls</th>
<th>Spiked sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Level 1</td>
</tr>
<tr>
<td>Detection limit (µg/kg)</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Decision limit (µg/kg)</td>
<td>&lt; 250</td>
<td>≥ 250</td>
</tr>
<tr>
<td>Trueness*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (µg/kg)</td>
<td>&lt;LOD</td>
<td>2014,12</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>-</td>
<td>14,25</td>
</tr>
<tr>
<td>Precision**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Repeatability</td>
<td>&lt;LOD</td>
<td>2.014,12 ± 14,25</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>&lt;LOD</td>
<td>2.008,72 ± 17,34</td>
</tr>
<tr>
<td>Specificity (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive sample</td>
<td>-</td>
<td>100%</td>
</tr>
<tr>
<td>Blank sample</td>
<td>100%</td>
<td>-</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>-</td>
<td>100,24</td>
</tr>
</tbody>
</table>

Notes: *Trueness = expressed as average value of 20 repetitions in repeatability conditions; **Precision = expressed as SD value of 20 repetitions for repeatability and reproducibility. Specificity = % of positive samples in positive/spiked sample and % of negative in blank sample. Decision limit is expressed here only for corn. Level 1: 240 µg/kg; Level 2 = 1600 µg/kg; Level 3: 60 mg/kg (FB1, FB2, FB3 ratio was 5:2.1). Negative control: blank sample; Positive control: reference material.

According to available information for Reference material, the sum of B1 + B2 in the sample is 1.948,61 µg/kg with standard deviation value of 639,43. According to the set performance criteria for fumonisins B1 and B2 (Table 1, Regulation (EU) 2017/625, both methods are fit for the purpose (Tables 3 and 4).

3.3. Comparison of ELISA and HPLC-FLD methods

Calibration of each method with five different standards showed adequate calibration curves ($r^2 > 0.99$) and a correlation coefficient value of 0.98. For tested methods, all the performance criteria were achieved to assure that both methods are fit for purpose. Limit of quantitation of the HPLC method for maize was 25 µg/kg. Therefore, method is also appropriate for testing of maize and maize-based intended for human consumption.

The results of other authors indicate that ELISA test for determination of fumonisins overestimate the actual quantities of this mycotoxins and it has been reported that correlation between the results of HPLC
and ELISA methods vary from 0.51 to 0.97 (Pestka et al., 1994; Sydenham et al., 1996; JECFA, 2011). In our research, we have determined that the correlation between these two methods (testing of naturally contaminated samples) was 0.98 while significant overestimation has not been detected. However, we noticed greater sensitivity and specificity for HPLC method (Fig. 2). In addition, HPLC method has the advantage of measuring the individual concentration of each fumonisin analog, while ELISA test detects only the total concentration of mycotoxins (FB1, FB2 and FB3). On the other hand, ELISA test is easy to use and less expensive making it adequate method for screening purposes. Finally, it is generally recommended that all the positive results and especially suspected non-compliant result should be confirmed by confirmatory methods such as HPLC and/or LC-MS method, if available. The results of this research indicate that both methods are sensitive, valid, cost-effective and easily transferable for quantitative routine determination of fumonisins in maize and maize-based products and that HPLC method can be used as confirmatory method.

4. Conclusions

Mycotoxins are ubiquitous all over the world in many foodstuffs and feedstuffs. fumonisin(s) incidence and level of contamination is high and depends upon different factors. They can be present in samples as 'hidden forms' and/or “modified forms”. Majority of research on occurrence of fumonisins contain information about FB1 and FB2 (90% of the samples analyzed). Their presence can be correlated with a number of human and animal diseases symptoms and syndromes. Current evaluation of fumonisin B2, B2 and B3 states that all three forms have similar toxicological profiles and potencies. Therefore, new studies on occurrence are necessary with information about all three fumonisin analogs, and if possible data on hidden and modified fumonisins.

This comparison study of ELISA and HPLC-fluorescence methods for detection of fumonisins in maize showed that both methods are accurate (determined by determining trueness and precision) for determination of fumonisins and that HPLC method can be used as confirmatory method. All the performance parameters including recovery and repeatability data in our study were in accordance with the EU criteria for the acceptability of the analytical methods for detection and determination of fumonisins. Therefore, both methods can be used in valuable research on occurrence of fumonisins in food and feed.

Abbreviations

TLC, thin layer chromatography, GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; ELISA, enzyme-linked immunosorbent assays; FB1, fumonisin B1, FB2, fumonisin B2, FB3, fumonisin B3.

Declarations
Competing interests: I, as author, declare no competing interests for the preprint submission article: "HPLC and ELISA Methods for Detection and Quantification of Fumonisins in Naturally Contaminated Maize".

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**References**


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**Figures**

![The chemical structures of fumonisin B1 (R₁:OH; R₂:OH), fumonisin B2 (R₁:OH; R₂:H), fumonisin B3 (R₁:H; R₂:OH) and fumonisin B4 (R₁:H; R₂:H). (JECFA, 2001)](image-url)
Figure 2

Correlation of analysis data for detection of total fumonisin content (B1, B2 and B3) in naturally contaminated maize samples by ELISA and HPLC method (x-axis: sample, y-axis: concentration in µg/kg).