ANALYSIS OF AFLATOXINS B1 AND G1 IN MAIZE
BY QUECHERS

ABSTRACT: A reliable and easy method has been developed for the determination of aflatoxins B1 and G2 in maize samples. High performance liquid chromatography coupled with FLD (HPLC-FLD) with photochemical derivatization was used. Mycotoxins were extracted from maize using a QuEChERS-based extraction procedure. The optimized analytical conditions were evaluated in terms of recoveries, reproducibility, LOD, LOQ and linearity for aflatoxin B1 and aflatoxin G1 in maize. Extraction, chromatographic and detection conditions were optimized in order to increase sample sensitivity. The linearity was analyzed in the range of 0.4-20 μg/kg and the correlation coefficients \( R^2 \) were higher than 0.99 for aflatoxin B1 and G1. Blank samples were spiked at 1.0, 2.0 and 4.0 μg/kg, and the average recovery for aflatoxin G1 was 96.96±1.72% and for aflatoxin B1 it was 86.80±1.24%. RSDs were lower than 25% for both mycotoxins. LOD for both aflatoxins was 0.5 μg/kg and LOQ was 1.0 μg/kg, respectively.

KEY WORDS: aflatoxin B1, aflatoxin G1, QuEChERS, HPLC

INTRODUCTION

Aflatoxins, a group of chemicals produced by *Aspergillus flavus*, *Aspergillus parasiticus* and the less common *Aspergillus nomius* (Sîrhan et al., 2011), can be recognized by olive green or grey green color on maize kernels in the field or in storage. Although aflatoxins are not automatically produced whenever grains become moldy, the risk of aflatoxin contamination is greater in damaged, moldy maize than in maize with little mold. Aflatoxins are harmful or fatal to livestock and are considered carcinogenic to humans and animals.

The current mycotoxin extractions have comprised a liquid-liquid extraction (LLE) which requires great amounts of organic solvents. The extraction takes long and depends on the matrix as well as on the studied mycotoxins.
while the main disadvantage of LLE is the possibility of mycotoxins loss due to their absorption on the walls of glass containers (Turner et al., 2009); supercritical fluid extraction (SFE) (Huoatalhti et al., 1997; Huoatalhti and Jarvenpaa, 2000) which greatest advantage is high extraction selectivity from the obtained relatively pure extracts and carbon-dioxide is used as an extraction reagent; solid phase extraction (SPE) which has proved to be an excellent extraction for mycotoxins from various matrices based on the columns filled with silica gel and joint phases in a stationery phase (Bursic et al., 2012); pressure liquid extraction (PLE), matrix solid phase dispersion (MSPD), ultrasound and homogenizing extraction with various mixtures of organic solvents (Pastorini, 2006) are only some of the techniques in the stream of those which can be applied to the extraction of mycotoxins from various matrices.

After extraction, it was necessary to remove the substances which, by their presence, could hinder the detection and determination of mycotoxins such as lipids, carbo-hydrates or proteins from raw extracts. For the purification of raw extracts, the columns used were those filled with active charcoal and aluminium-oxide, with or without ion exchanger and the columns filled with silica-gel or florisil. In recent years, immuno-affinity columns (Mycosep columns, AlfaOta clean etc.) have been used and QuEChERS has been recognized as the most modern procedure for extraction and extract purification.

In recent years, the trends have been directed towards the decrease in the sample amount for analyses with the approach which is safer and less damaging to the environment, such as QuEChERS, and which, at the same time, implies a quicker and simpler way of sample preparation, ensuring high yields and good precision. Anastides et al. (2003) developed quick, easy, cheap, efficient, rugged and safe method (QuEChERS) in order to overcome the limitations of the existing preparation methods, which was successfully used in the analyses of pesticides from various matrices. With certain modifications, QuEChERS has become a method of interest for the extraction and purification of samples and for the mycotoxin determination. Only 5-10 g of a sample was taken during the analysis, and the extraction in PTT tubes was performed with water and acidified acetonitril. On addition of anhydrous magnesium sulfate and sodium acetate (or sodium chloride), a sample is centrifuged. Supernatant can immediately be injected into a liquid chromatograph (Romero-Gonzalez et al., 2011; Garrido-Frenich et al., 2011) or its purification can be done by adding primary-secondary amines (PSA) and anhydrous magnesium sulfate and after the repeated centrifugation, supernatant is analyzed by liquid chromatography (Wu et al., 2010).

The aim of this experimental work was the validation of QuEChERS method used for the detection of the content of aflatoxin B1 and aflatoxin G1 in maize.
MATERIAL AND METHOD

**Chemicals and materials:** Aflatoxin standards were purchased from Sigma Aldrich (Germany). Standard stock solutions were prepared in methanol. Working solutions were prepared by diluting the stock standards with mobile phase to the final mass concentration of 0.45 μg of aflatoxin B1/ml and 1.0 μg of aflatoxin G1/ml. The QuEChERS extraction comprised sodium chloride (J.T. Baker, Holland), trisodium citrate 5.5-hydrate (Zorka pharma, Šabac, Srbija), anhydrous magnesium sulfate (Hemrad, Belgrade, Serbia), anhydrous sodium acetate (Sigma–Aldrich, Germany), methanol, acetonitrile and cc acetic acid – 99.5% (J.T. Baker, Holland).

**HPLC analysis:** The HPLC analyses were carried out with Agilent 1100 system, consisting of a degasser, binary pump, auto sampler, column oven and a fluorescence detector. The chromatographic separation was performed with the Zorbax EclipsePlus C18 (3.5 μm, 3.6 mm x 150 mm) chromatographic column. The mobile phase was (A) water and (B) MeCN. The gradient conditions were 0-15 min 35% B, 15-18 min 35% B, 18-20 min 60% B and 20-25 min 70% B. The column temperature was 30 °C at the flow rate of 1.5 ml/min to achieve the optimum resolution of the aflatoxins. The injection volume was maintained at 50 μl for both standard and sample solutions.

**Validation parameters:** The linearity was checked by preparing the mycotoxins mixture standard in mass concentrations of 0.4, 1.0, 5.0, 10.0, 15.0 and 20 ng/ml.

The recovery: Untreated samples of maize were enriched with 23.45 and 89 μl working solution of aflatoxin B1 and with 10, 20 and 40 μl of working solution of aflatoxin G1 in three replicates. The final mass concentrations of aflatoxins B1 and G1 in the spiked samples were 1, 2 and 4 μg/kg. After thirty minutes, the QuEChERS extraction was carried out and presented in Scheme 1.

Scheme 1 – Sample preparation (QuEChERS method)

<table>
<thead>
<tr>
<th>10 g sample</th>
<th>+ 10 ml 1% CH₃COOH in MeCN + 5 ml H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>↓</td>
<td>Shake vigorously for 1 min</td>
</tr>
<tr>
<td>↓</td>
<td>Add 1.67 g CH₃COONa</td>
</tr>
<tr>
<td>↓</td>
<td>Shake tube directly after the salt addition for 2 min</td>
</tr>
<tr>
<td>↓</td>
<td>Add 4 g MgSO₄</td>
</tr>
<tr>
<td>↓</td>
<td>Centrifuge for 20 min at 3000 g</td>
</tr>
<tr>
<td>↓</td>
<td>Upper layer analyzed by HPLC</td>
</tr>
</tbody>
</table>
Reproducibility of the method was determined by analyzing the sample of the same concentration level (5.0 μg/kg) in six replications and it was expressed as relative standard deviation – RSD (%).

The limits of detection (LOD) were calculated by means of “Calculate Signal-to-Noise” calculator within the Qualitative MussHunter B.03.01 program (Agilent Technologies, 2010) based on the relation of standard deviation of the peak height and noise height in the chromatograms for the mycotoxin mixture standard at concentration of 0.05 μg/kg.

The limits of quantification (LOQ) were determined by adding 100 μl of mycotoxin mixture standard in the concentration of 1.0 μg/ml.

RESULTS AND DISCUSSION

Validation parameters: LOD for aflatoxin B1 was 0.16 μg/kg and for aflatoxin G1 it was 0.08 μg/kg. LOQ was 0.4 μg/kg for both mycotoxins. The linearity range for both aflatoxins was from 0.4 to 20.0 μg/kg. Reproducibility of the method expressed as a RSD (%) was 9% for aflatoxin B1 and 27% for aflatoxin G1 and they were in accordance with the Commission Regulation (EC) No 401/2006, which permitted RSD to be up to 30%.

Table 1 shows the recovery values obtained by QuEChERS method.

<table>
<thead>
<tr>
<th>Aflatoxin concentration in samples (μg/kg)</th>
<th>Replicates</th>
<th>Average of recovery (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>G1</td>
<td>1</td>
<td>78.42</td>
<td>79.50</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>80.50</td>
<td>81.40</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>83.40</td>
<td>85.20</td>
</tr>
<tr>
<td>B1</td>
<td>1</td>
<td>98.42</td>
<td>95.17</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>95.14</td>
<td>92.17</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>100.66</td>
<td>100.10</td>
</tr>
</tbody>
</table>

By employing QuEChERS techniques, high extraction recoveries were obtained with average recoveries for aflatoxin G1 being 96.96±1.72% and for aflatoxin B1 being 86.80±1.24%.

Figures 1, 2 and 3 show chromatograms of analyzed samples.
Fig. 1 – Control maize sample

Fig. 2 – Standard of aflatoxins G1 and B1

Fig. 3 – Chromagram of spiked samples with aflatoxins B1 and G1
CONCLUSION

A new method based on QuEChERS extraction procedure and HPLC-FLD was developed for the simultaneous determination of aflatoxin B1 and aflatoxin G1. The extraction procedure, using water/acetonitril acidified with acetic acid, was simple and it required no further clean-up steps. Therefore, it can be recommended as an alternative to the time-consuming extraction as well to the more expensive imunoaffinity columns and multifunction column for aflatoxins determination in maize.

REFERENCES


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QUECHERS ANALIZA АФЛАТОКСИНА B1 И G1 У КУКУРУЗУ

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Резиме

Развијен је поуздан и лак метод одређивања афлатоксина B1 и G1 у узорцима кукуруза. Коришћене су високо перформантна течна хроматографија уз FLD (HPLC-FLD) и фотохемијска дериватизација. Микотоксини су екстраховани из кукуруза употребом QuEChERS екстракционе процедуре. Оптимизација аналитичких услова је процењена на нивоу приноса екстракције, репродуктивности, LOD, LOQ и линеарности афлатоксина B1 и афлатоксина G1 у кукурузу. Екстракција, хроматографија и услови одређивања су оптимизовани у циљу повећања осетљивости. Линеарност је одређена за опсег концентрација од 0.4-20 μg/kg са коефицијентом корелације (R²) вишим од 0.99 за афлатоксине B1 и G1. Контролнi узорци су обогаћени на нивоу 1.0, 2.0 и 4.0 μg/kg, са просечним вредностима прноса екстракције од 96.96±1.72% за афлатоксин G1 и 86.80±1.24% за афлатоксин B1. RSD вредности су биле ниже од 25% за оба микотоксина. LOD оба афлатоксина је износио 0.5 μg/kg са LOQ од 1.0 μg/kg.

КЉУЧНЕ РЕЧИ: афлатоксин B1, афлатоксин G1, QuEChERS, HPLC

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