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OPTIMIZATION AND EVALUATION OF ELISA IMMUNOASSAY FOR MYCOTOXIN DETECTION OF BREAKFAST CEREALS

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Abstract

Sixteen samples of breakfast cereals purchased from the Romanian market were studied in order to evaluate the possible occurrence of mycotoxins. The influence of microbiological and physical-chemical attributes that may determine the incidence of the toxins was studied. A BIOLOG system was used for the identification of the fungal strains. A sandwich-type enzyme –linked immunosorbent assay (ELISA) method was optimized and validated in-house for the quantification of two mycotoxins: deoxinivalenol (DON) and zearalenone (ZEA). The validation of the method was based on following performance parameters: accuracy (measured as percent error), precision (measured as coefficient of variance), reproducibility and repeatability (precision within- and between-day and analyst variability), limit of detection (LOD) and limit of quantification (LOQ). Recovery of the method was tested at low, medium and high levels of the working range (three concentration levels) for each mycotoxin in spiked breakfast cereal samples. According to the results, all samples presented levels far below their legal limits.

Key words*: breakfast cereals, filamentous fungi, mycotoxins.*

INTRODUCTION

Cereals are very susceptible to fungal attacks, both in the field and during storage. Depending on environmental conditions, a fungal infection, mainly produced by species of *Aspergillus*, *Fusarium* and *Penicillium*, may result in a mycotoxin contamination of the crop. Mycotoxins are a group of toxic compounds produced by spore-forming fungi. Mycotoxin contamination of cereals is a major issue in regard to public health, food safety and food security. Consequently, a regular contamination can be expected for cereal-based commodities (Montes et al., 2012).

Deoxinivalenol and zearalenone are two mycotoxins produced by members of the *Fusarium graminearum* species complex (Tralmazza et al., 2016). Both mycotoxins are the most found over the world and at acute doses they cause a wide range of toxic effects, while a constant intake of small amounts of mycotoxins leads to a weakened immune system. A wide range of food products could be contaminated with mycotoxins, both pre- and post-harvest (Zain, 2011). In this context, the purpose of this study was to investigate the incidence of deoxynivalenol and zearalenone mycotoxins within breakfast cereals samples purchased from the Romanian market. The influence of microbiological and physical-chemical attributes that may determine the incidence of the toxins was studied, while a BIOLOG system was used for the identification of the fungal strains.

MATERIALS AND METHODS

Food samples

Sixteen samples of breakfast cereals were purchased from different supermarkets in Bucharest, Romania. Thus, there were selected four corn flakes samples, with (n=2) and without (n=2) added sugar, one of these last samples labelled as grains from ecologic farming. Müsli breakfast cereals with added dry fruits and sugar (n=2) and without sugar (n=1) were also the subject of these tests. Furthermore, whole grains breakfast cereals (n=6) were selected, as well as breakfast cereals with added sugar, vitamins and preservatives (n=3). All samples were kept in their original packages and stored at room temperature in a dark and dry place until analysis.

Physico-chemical and microbiological analysis

Humidity measurement was performed using a Mettler LJ16 infrared dryer (Mettler-Toledo Ltd., UK). The working method consists in drying 5 g of grounded and well homogenized sample at 130 °C. Water activity was measured using the Aquaspector AQS-31 (NAGY Messsysteme GmbH, Germany) at 24 °C. Grinded and ungrounded breakfast cereal samples were places in the measurement cup and readings were noted. The pH of all samples was determined using an Inolab 730 WTW pH meter (WTW Wissenschaftlich-Technische Werkstätten GmbH, Germany). After grinding, 10 g of each sample were homogenized for 30 s in 90 mL distilled water using a stomacher (Seward Limited, UK) and then pH was determined. The acidity of each sample was determined. Thus, in a conical container 5 g sample were loaded and 50 mL water were added. The mix was stirred for 5-10 minutes. After homogenization, 3 drops of phenolphthalein solution were added to the aqueous extract and titrated with sodium hydroxide solution until the appearance of pink colour that persists for 1 minute.

Yeasts and moulds and *Enterobacteriaceae* were monitored. An amount of 10 g breakfast cereals was aseptically removed from the package using a sterile spatula and transferred to a sterile filter stomacher bag (Seward Limited, UK), containing 90 mL sterile homogenate solution (0.85% NaCl and 0.1% neutralized bacteriological peptone). The samples were homogenized using a stomacher (Seward Limited, UK) for 30 s at room temperature. Tenfold dilution series were made in sterile peptone saline solution as needed for plating. Dichloran Glycerol (DG 18) agar (Oxoid, UK) was spread-plated with 100 µL of the appropriate sample dilution and incubated at 25 °C for 7 days for yeasts and moulds. For members of *Enterobacteriaceae* family, 1 mL of the appropriate sample dilution was inoculated into 10 mL molten (45 °C) violet rose bile glucose agar (Oxoid, UK). After setting, 8 mL overlay of molten medium was added and incubated at 37 °C for 24 h. All plates were examined visually for typical colony types and morphological characteristics associated with each growth medium. Microbial counts were expressed as log cfu g^{-1}.

Biolog OmniLog Identification system

Each isolated fungi was first grown on two plates of 2% malt extract agar, MEA (Scharlab SL, Spain), at 25 °C. After an incubation period of 5 days, conidia were collected with sterile cotton swabs. The swabs were dipped into screw-top culture tubes containing 16 mL Biolog FF inoculating fluid. The conidial suspension was adjusted at 75% transmittance by a turbidimeter, as recommended by the manufacturer. A volume of 100 µL was pipetted into each of the 96 wells of a single Biolog FF plate. The resulting plates were incubated at 25 °C and the biochemical reactions were recorded using a microplate reader with a 590 nm wavelength filter at 1, 2, 3, 4 and 7 days, as suggested in the Biolog product information.

ELISA analysis

ELISA type immunoenzymatic test was selected for mycotoxin detection, using Max Signal® ELISA test kits (Bioo Scientific Corporation, USA). All samples were first finely ground using a laboratory mill (MRC Ltd., Israel) and mixed thoroughly to achieve complete homogenization. Furthermore, for DON and ZEA detection, 5 grams of grinded sample were homogenized in 100 mL distilled water and 25 mL 70% methanol (v/v), respectively. Samples were homogenized vigorously using an orbital shaker (GFL Gesellschaft für Labortechnik mbH, Germany) and then centrifuged for 10 minutes at 4000 rpm and room temperature, using a high speed centrifuge, model 5804R (Eppendorf AG, Germany). Further, samples were diluted according to the test kit manual, using distilled water and sample extraction buffer, respectively. 100 µL (DON) or 50 µL (ZEA) standards and samples, in duplicate, were added into different wells. DON-HRP conjugate and zearalenone antibody mix were added to each well and further, the plated were incubated at room temperature for 45 minutes and 30 minutes, respectively. After washing the plates, 100 µL TMB substrate were added and

after incubating for 15 minutes at room temperature, 100 µL stop buffer were added. The plates were read on a Sunrise™ plate reader (Tecan Group Ltd., Switzerland). For each detection two repetitive samples have been used. The media of these samples has been employed in data analysis.

Method performance

Validation experiments established the performance characteristics of the ELISA method. The following performance parameters were investigated: accuracy (measured as percent error), precision (measured as coefficient of variance), reproducibility and repeatability (precision within- and between-day and analyst variability), limit of detection (LOD) and limit of quantification (LOQ). Recovery of the method was tested at low, medium and high levels of the working range (three concentration levels) for each mycotoxin in spiked breakfast cereal samples.

Data analysis

Microbiological and physicochemical experiments were replicated twice and analysis were run in triplicate for each replicate (n = 2 x 3). Microbiological data were expressed as logarithms of the number of colony forming units (cfu g^{-1}). Means and standard deviations were calculated. Significance was defined at P<0.05. LSD test which was applied only to microbiological data. Data were analysed with IBM® SPSS® Statistics 20 (IBM Corp., USA). Data analysis for metabolic profiling of fungi was conducted using BioTek Gen5 software (Biolog, Inc., USA).

RESULTS AND DISCUSSIONS

Effects of physico-chemical and microbiological attributes

Humidity and water activity are two physico-chemical attributes that have a great impact on the microbiological contamination of food products. These parameters were measured in order to assess the impact of the breakfast cereals type when samples were compared with each other.

According to figure 1, which indicates the humidity values, müsli and whole grains breakfast cereals noted the highest humidity values.

The pH measurement noted, as expected, the lowest levels for the müsli breakfast cereals with dry fruits added (values ranged from 4.77 to 5.76), while the highest values were registered by the breakfast cereals with added sugar and synthetic preservatives (values between 6.40 and 7.19).

When referring to the water activity measurement, the results registered between grinded and ungrounded samples were not significant different (P>0.05).

However, the whole grains breakfast cereals noted the highest water activity values, while the corn flakes registered the lowest levels (data not shown).

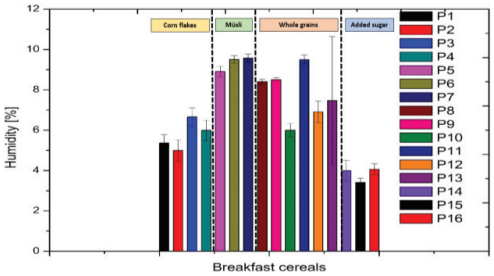

Figure 1. Humidity values for breakfast cereals

Furthermore, the microbiological results showed no *Enterobacteriaceae* contamination for any of the analysed samples.

Four samples, of which two müsli (P5 and P6) and two whole grains (P12 and P13) breakfast cereals samples noted yeasts and moulds levels of ca. 3 logs.

These microbiological results are closely related to the results on the incidence of mycotoxins, as showed further.

Biolog OmniLog identification system

The isolated fungi were confirmed using the Biolog OmniLog identification system.

The acceptable instrument readings (fig. 2) at the end of the incubation period noted a similarity index of 0.50-0.75 (good identification) for genus and species identification of *Aspergillus niger* (Singh, 2009; Wang et al., 2016).

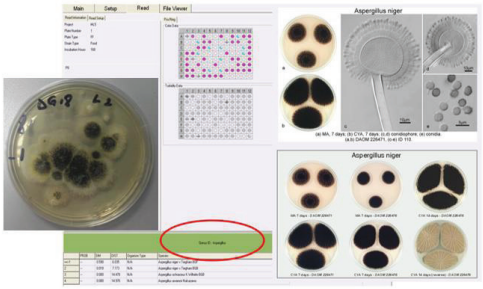

Figure 2. Identification of *Aspergillus niger* using Biolog OmniLog system

Aspergillus niger was identified in two samples, both müsli breakfast cereals with added dry fruits. The presence of added sugar in these food products did not influenced the presence of microorganisms, as *A. niger* was identified in both samples with and without sugar added commodities.

Occurrence of mycotoxins and analytical quality control

Concerning the occurrence of mycotoxins in cereal products, 4 samples noted no presence of deoxinivalenol or zearalenone, which represents a percentage of 25% of the analysed samples. Within these samples, most of them were whole grains breakfast cereals. Of all tested samples, only three registered levels of DON. The highest level of DON was 131.70 µg/kg, being noted by a breakfast cereal sample with a great percent of added sugar and synthetic preservatives (fig. 3). The corn flakes cereals noted no detectable level of DON. Regarding the ZEA concentrations, with the exception of the four samples mentioned before, all the breakfast cereals noted small levels of ZEA. The highest ZEA level was of 6,45 µg/kg and it was noted by a müsli breakfast cereal sample, with added dry fruits, results also noted by Mahnine et al. (2011). The same sample registered a DON level of 110.70 µg/kg and *A. niger* contamination, as this microorganism was isolated and further identified using the Biolog OmniLog system.
Commercially available ELISA kits for detection of mycotoxins are normally based on a competitive assay format that uses either a primary antibody specific for the target molecule or a conjugate of an enzyme and the required target.

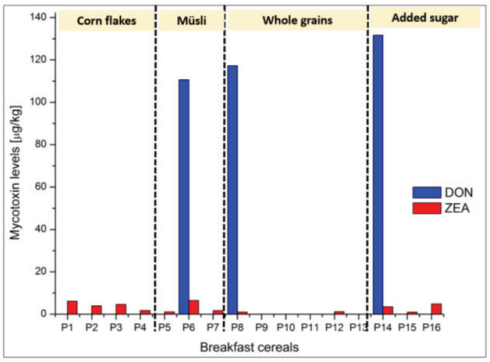

Figure 3. The occurrence of mycotoxins depending of the sample type

The recoveries and standard deviation of mycotoxins in the selected food matrix at different spiking levels are summarised in table 1.
Recovery rates at the lower spiking levels were between 117.67% for ZEA and 147.67% for DON, while for the highest spiking levels they were noted to be between 70.97% for ZEA and 147.67% for DON.
The lowest recovery corresponded to zearalenone at the 100 ppb spiking level (highest level).

Table 1. Recoveries and relative standard deviations (%) of mycotoxins in spiked breakfast cereals

Mycotoxin	Spiking levels (µg/kg)	Recoveries ± SD (%)
Deoxynivalenol	100	147.67 (±25.80)
	200	109.55 (±1.18)
	500	125.34 (±13.35)
Zearalenone	20	117.67 (±2.24)
	50	83.74(±4.20)
	100	70.97(±1.42)

The validation of the method for the selected food matrix was based on following performance parameters: accuracy (measured as percent error), precision (measured as coefficient of variance), reproducibility and repeatability (precision within- and between-day and analyst variability), limit of detection (LOD) and limit of quantification (LOQ).
The obtained results are given in table 2, along with other information regarding mycotoxin detection.

Table 2. Performance parameters for validation of mycotoxins for breakfast cereals food matrix

	Deoxynivalenol	Zearalenone
Maximum levels, µg/kg (EC Commission Regulation No. 1881/2006)	500	50 (maize based breakfast cereals) 100 (breakfast cereals excluding maize based breakfast cereals)
Precision, µg/kg	0.05	0.02
Reproducibility, %	4.98	1.83
Repeatability, %	3.31	4.92
Limit of detection, µg/kg	142.26	29.09
Limit of quantification, µg/kg	167.05	35.19

The accuracy of both methods was high. The difference between the taken value and the obtained results was very small, of 8,969 µg/kg for DON (-1.79%) and 4,735 µg/kg for ZEA (-4.74%), respectively. According to EU Commission Decision 2002/657/EC, the minimum accuracy of quantitave methods is between the range of -20% to +10%. Mean recoveries were respectively 98.21% for DON and 95.27% for ZEA. The stability was assessed during four weeks (n = 3 replicates) and the variation values were 446.48 µg/kg – 519.68 µg/kg for DON and 88.49 µg/kg – 105.64 µg/kg for ZEA. The LOD and LOQ of DON are 142.26 µg/kg and 167.05 µg/kg, while for ZEA they are 29.09 µg/kg and 35.19 µg/kg, respectively. Samples with a concentration higher than the LOQs were considered positive.

The advantage of the selected method is represented by the rapid sample preparation method and the short time requirement. The disadvantage of the method lies in the fact that they are for single use, which can increase costs of bulk screening. Additionally, competitive ELISA suffers from having a limited detection range due to the narrow sensitivity of the antibodies.

CONCLUSIONS

The occurrence of filamentous fungi and deoxinivalenol and zearalenone mycotoxins was determined in samples of breakfast cereals. *Aspergillus niger* was isolated from two müsli breakfast cereals samples and one of this samples registered also the highest DON level, of 110.70 µg/kg and a ZEA concentration of 6.45 µg/kg.

It could be concluded from this study that müsli breakfast cereals seem to be more contaminated than the other cereal products that were analysed.

The registered mycotoxins level are consistent with the European legislation regarding these commodities, but the noted results show that a special attention to contamination levels in foods is necessary.

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