CO-OCCURRENCE OF AFLATOXINS, OCHRATOXIN A AND ZEARALENONE IN BREAKFAST CEREALS FROM SPANISH MARKET

María Ibáñez-Vea [1], Rosa Martínez [1], Elena González-Peñas [1]*, Elena Lizarraga [1], Adela López de Cerain [2]

[1] Department of Organic and Pharmaceutical Chemistry

[2] Department of Nutrition, Food Sciences, Physiology and Toxicology

Faculty of Pharmacy, C.I.F.A., University of Navarra

Irunlarrea 1, 31008, Pamplona, Navarra, Spain

Corresponding author: Elena González-Peñas PhD: Tel.: +34 948 425653. Fax: +34 948 425652. E-mail: mgpenas@unav.es

E-mail addresses of all coauthors: mivea@alumni.unav.es (María Ibáñez-Vea PhD); rmartinez.7@alumni.unav.es (Rosa Martínez); elizarraga@unav.es (Elena Lizarraga PhD); acerain@unav.es (Adela López de Cerain PhD).

ABSTRACT

Forty-six breakfast cereal samples from the Spanish market have been analyzed for the occurrence of aflatoxins (AFB1, AFG1, AFB2 and AFG2), ochratoxin A (OTA) and zearalenone (ZEA). According to the results, 9% of the samples were contaminated with AFB1 although no sample exceeded the LOQ (0.2 μg kg^{-1}), and no sample presented detectable levels of the other aflatoxins (AFB2, AFG1 and AFG2). Zearalenone and OTA contaminated 48 and 39% of the samples, respectively, with mean values of the samples having quantification levels of 25.40 and 0.37 μg kg^{-1}, respectively. The co-occurrence of OTA and ZEA was observed in 28% of the samples. Aflatoxin B1 appeared only in the corn-based breakfast cereals, whereas ZEA and OTA showed the highest contamination rates in the samples containing wheat and wheat and rice, respectively. No sample of high-fiber content was contaminated with AFB1, whereas OTA and ZEA occurred with higher incidence in high-fiber content samples. Moreover, the daily exposure to AFB1, OTA and ZEA is discussed.

Keywords: Mycotoxin, aflatoxins, ochratoxin A, zearalenone, co-occurrence, breakfast cereals.

1. Introduction

Cereals and other crops are very susceptible to fungal attacks while in the field and during storage. Depending on environmental conditions and other factors, a fungal attack may result in mycotoxin contamination of the crop. These toxins are stable compounds and do not completely degrade at high temperatures (Kabak, 2009a); therefore, mycotoxins can contaminate processed foods and enter the human food chain through cereal-derived foods. The main mycotoxins, due to their toxicity and prevalence, are aflatoxins (AFs) and ochratoxin A (OTA) and to a lesser extent, zearalenone (ZEA), among others.

Mycotoxins can cause a great variety of toxicological effects in both animals and humans. Aflatoxins are among the most potent mutagenic and carcinogenic substances known, and AFB1 is consistently genotoxic *in vitro* and *in vivo* (EFSA, 2007). Ochratoxin A is a nephrotoxic mycotoxin which possesses carcinogenic, teratogenic, immunotoxic and possibly neurotoxic properties (SCF, 1998). ZEA is a non-steroidal estrogenic toxin which has been involved in incidents of precocious pubertal changes. With regard to their carcinogenicity, aflatoxin B1 and naturally-occurring mixtures of aflatoxins have been classified as human carcinogens (group 1), OTA has been classified as a possible carcinogen to humans (group 2B) and ZEA is not classifiable with regard to its carcinogenicity to humans (group 3) (IARC, 1993, IARC, 2002).

Due to the health hazards of these compounds for humans and animals and the occurrence in foodstuffs for human consumption, the European Commission has set maximum permitted levels in breakfast cereals: 2 $\mu g\ kg^{-1}$ for AFB1 and 4 $\mu g\ kg^{-1}$ for the sum of AFB1, AFG1, AFB2 and AFG2; 3 $\mu g\ kg^{-1}$ for OTA and for ZEA, 50 $\mu g\ kg^{-1}$ in the case of breakfast cereals, excluding maize-based products, and 100 $\mu g\ kg^{-1}$ for

maize-based breakfast cereals (European Commission, 2006a, European Commission, 2007).

Data reported on these toxins in cereal grains in the reference literature is quite extensive. It has been observed that AFB1 and ZEA contaminate cereal grains, especially corn (Gareis et al., 2003, Rustom, 1997). OTA is present, with higher incidence and contamination levels in wheat and rye (Miraglia and Brera, 2002).

The presence of mycotoxins in breakfast cereals has been reported in different works of research: OTA in Spain (Araguás, González-Peñas and López de Cerain, 2005), ZEA in Portugal (Gareis et al., 2003), OTA, ZEA, deoxynivalenol, citrinin and fumonisins in France (Molinié, Faucet, Castegnaro and Pfohl-Leszkowicz, 2005, Leblanc, Tard, Volatier and Verger, 2005), ZEA and trichothecenes in Germany (Schollenberger, Müller, Rüfle, Suchy, Planck and Drochner, 2005), ZEA in UK (Gareis et al., 2003), AFB1 and OTA in Greece (Villa and Markaki, 2009), OTA in Turkey (Kabak, 2009b) and AFs or OTA and ZEA in Canada (Tam et al., 2006, Roscoe et al., 2008). However, to the best of the author's knowledge, the simultaneous presence of AFs, OTA and/or ZEA in breakfast cereal samples has only been studied by Roscoe *et al*. (2008) in Canada and by Villa and Markaki in Greece (2009).

Grains may often contain more than one mycotoxin as well as other components that enhance the toxicity properties of toxins (Prelusky, Rotter and Rotter, 1994). In fact, it is apparent that co-occurrence of zearalenone with aflatoxins, trichothecenes and fumonisins in individual samples of corn is common (EFSA, 2004). Therefore, the toxicity of fungal metabolites cannot be estimated by only determining the concentration of a single mycotoxin (Prelusky, Rotter and Rotter, 1994).

The aim of this research work was to evaluate the possible co-occurrence of aflatoxins, ochratoxin A and zearalenone in breakfast cereal samples from the Spanish market and to assess consumer risk. In addition, the influence of other parameters that may determine the occurrence of the toxins in breakfast cereals was studied; some examples of the parameters are the type of cereal used in manufacturing, fiber content, and name-brand products vs. generic brand products.

2. Materials and methods

2.1. Chemicals and reagents

Aflatoxins, ochratoxin A and zearalenone standards dissolved in acetonitrile were purchased from Fluka (Schnelldorf, Germany) as certified reference materials. Potassium chloride, potassium phosphate dibasic and formic acid were obtained from Panreac (Barcelona, Spain) and sodium chloride, sodium phosphate dibasic and Tween 20 from Merck (Darmstadt, Germany). These reagents were of pro-analysis grade. Acetonitrile and methanol HPLC grade were supplied by Sigma-Aldrich (St. Quentin Fallavier, France). Millipore type I water was obtained daily from a Milli-Q water-purifying system. Immunoaffinity columns AOZ were purchased from Vicam (Watertown, MA, USA).

Phosphate buffered saline (PSB) was prepared by dissolving potassium chloride (0.2 g), potassium phosphate dibasic (0.2 g), sodium phosphate dibasic (1.16 g) and sodium chloride (8 g) in 900 mL water type II. Next, the pH of the solution was adjusted to 7.4 with HCl or NaOH and two drops of Tween 20 were added. Finally, the volume was adjusted to 1 L.

2.2. Standard solutions

A stock standard solution containing 500 μg L^{-1} of AFB1, AFG1 and OTA, 125 μg L^{-1} of AFB2 and AFG2 and 20 mg L^{-1} of ZEA was prepared by diluting different standard solution volumes of each mycotoxin in a mixture of acetonitrile and methanol (50:50; v/v). Working standard solutions of 100, 10 and 1 μg L^{-1} of AFB1, AFG1 and OTA, 25, 2.5 and 0.25 μg L^{-1} of AFB2 and AFG2 and 4000, 400 and 40 μg L^{-1} of ZEA, respectively, were prepared from this stock standard solution. These solutions were stored at -20ºC and maintained at room temperature and in darkness for 30 min before each use. Calibration samples were prepared by evaporating different volumes of the working standard solution under vacuum at 40ºC, and then residues were dissolved in 150 μL mobile phase (aqueous and organic (60:40)). The acetonitrile extract from cereal samples was processed in the same way.

2.3. Breakfast cereal samples

Forty-six breakfast cereal samples (commercialized packages of 500 g) were obtained from different hypermarkets and supermarkets in a northern region of Spain (Navarra). Different brand names were selected in order to have a market-representative sampling. Samples were classified into different groups, based on their label information, with regard to the type of cereal (corn, wheat or wheat and rice based cereals), their fiber content (whole grain or normal cereals) and the type of product (name-brand or generic brand). Twenty-one samples were collected from corn, 14 from wheat, 8 from wheat and rice, 2 from wheat and corn, and 1 from rice. Thirty-three samples were from whole grain cereals, and 13 from normal cereals. Of the 46 samples, all of the corn samples were normal fiber content products, 7 of the 8 samples of wheat and rice were from whole grain cereals, and of the wheat samples, 8 were normal fiber content products and

6 were from whole grain cereals. In addition, thirty-two samples were generic brand products and fourteen were name-brand products. Samples were stored in their original packets at 4ºC until analysis.

2.4. Mycotoxin analysis

The method used for mycotoxin analysis in breakfast cereal samples is based on that described by Ibáñez-Vea, Corcuera, Remiro, Murillo-Arbizu, González-Peñas and Lizarraga (2011) for the analysis of AFs, OTA and ZEA in barley, with some changes regarding sample treatment, and then revalidated for the breakfast-cereal matrix.

Mycotoxin extraction was as follows: The cereal from each package was ground in a Restch ZM100 mill, using a sieve measuring 0.75 mm. Next, mycotoxins were extracted from ten grams of milled sample with 50 mL of acetonitrile-aqueous 0.166% KCl (pH = 1.5) (70:30, v/v) in a horizontal shaker SSL1 (Stuart®) for 30 min. The extract was filtered by gravity, and then 10 mL of the filtrate were mixed with 40 mL of PBS. The mixture was kept in refrigeration for 15 minutes in order to promote fat precipitation, and then it was centrifuged at 6249 x g and 4ºC for 20 minutes. Finally, 15 mL of the supernatant were subjected to a purification process. The clean-up was carried out with immunoaffinity columns AOZ (Vicam), pre-conditioned with 10 mL of PBS. After the sample had passed through the column, the column was washed with 10 mL of PBS and 20 mL of water. Finally, the column was dried with air and the mycotoxins were eluted with 3 mL of acetonitrile, after having maintained acetonitrile and antibodies in contact with each other for 5 min. The extract was evaporated to dryness at 40ºC in an evaporator (GeneVac) and the residue was redissolved in 150 µL of a mixture (40:60) of acetonitrile-methanol (50:50) and water, both acidified with 0.5% formic acid. The sample was maintained at 4ºC in the chromatograph tray until its

analysis. Extraction and analytical processes should be carried out avoiding exposure of the samples to light, in order to protect aflatoxins from their degradation.

Samples were analyzed in a 1200 rapid resolution liquid chromatographic system equipped with a fluorescence detector (Agilent Technologies). Separation was achieved on an Ascentis Express (fused-core technology) (Supelco) C18 column (150 mm x 2.1 mm; 2.7 μm) with a flow rate of 0.9 mL min^{-1} and a column temperature of 60ºC. The injection volume was 30 μL. The mycotoxin analysis was performed with a linear gradient of a mixture of acetonitrile and methanol (50:50; v/v) (A) and water (B), both acidified with 0.5% formic acid. The initial gradient condition was 16% A and 84% B, changing linearly to 53% A and 47% B in 12 min. A post-column photochemical derivatization was used to enhance the AFB1 and AFG1 response, using a PHRED photochemical reactor (AURA Industries, NY, USA) with a mercury lamp (λ = 254 nm) and a knitted reactor coil of 0.25 mL (5 m x 0.25 mm). Wavelengths of excitation and emission were fixed at 365 and 440 nm for aflatoxins, 234 and 458 nm for ZEA and 225 and 469 nm for OTA, respectively.

2.5. Validation of the analytical method

Prior to this research work, a method was validated in-house for the quantification of AFB1, AFB2, AFG1, AFG2, OTA and ZEA in barley samples (Ibáñez-Vea, Corcuera, Remiro, Murillo-Arbizu, González-Peñas and Lizarraga, 2011). The validation of the method was based on the following criteria: selectivity, linearity, precision (within- and between-day and analyst variability), and accuracy, limit of detection and limit of quantification, recovery and robustness. The linearity study was developed using calibration samples in the following ranges: 0.6 – 4 μg L^{-1} and 4 - 40 μg L^{-1} for AFB1, AFG1 and OTA; 0.15 - 1 μg L^{-1} and 1 – 10 μg L^{-1} for AFB2 and AFG2; and 24 -

160 μg L^{-1} and 160 - 1600 μg L^{-1} for ZEA, respectively, and showed an adequate relation between the instrumental response (area of toxin peak) and the respective toxin concentration (x). In breakfast cereal samples, the equivalent concentrations were obtained using the following expression:

$$C_{samples} = \left(\frac{C_{STD}}{CF}\right) \cdot \left(\frac{100}{Rec}\right)$$

in which, C_{STD} is the measured vial concentration, CF is the concentration factor (4) and Rec is the recovery percentage for each toxin. Therefore, the ranges in the breakfast cereal samples were 0.15 - 1 μg kg^{-1} and 1 - 10 μg kg^{-1} for AFB1, AFG1 and OTA; 0.04 - 0.25 μg kg^{-1} and 0.25 - 2.5 μg kg^{-1} for AFB2 and AFG2; and 6 - 40 μg kg^{-1} and 40 - 400 μg kg^{-1} for ZEA, respectively.

Considering that the matrix depends on some of the validation parameters such as selectivity, limits of detection and quantification and recovery, the analytical method has been revalidated for breakfast cereal samples. Selectivity has been tested in this matrix by adding mycotoxins to positive samples, and then by observing the increase of each toxin peak without observing broadening or distortion of peak shapes.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by analyzing spiked breakfast cereal samples in triplicate, at three different concentrations (0.2, 0.3 and 0.5 μg kg^{-1} for AFB1, AFG1 and OTA; 0.05, 0.075 and 0.125 μg kg^{-1} for AFB2 and AFG2; and 8, 12 and 20 μg kg^{-1} for ZEA, respectively). LOD was calculated using a method based on the calibration curve extrapolation at zero concentration (A.E.F.I, 2001). The limit of quantification (LOQ) corresponds to the minimum concentration assayed in samples with adequate precision (RSD ≤ 40% for AFs, OTA

and ZEA) and recovery (between 50 and 120% for AFs and OTA; between 60 and 120% for ZEA) values (European Commission, 2006b).

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 (0.2, 1 and 10 μg kg^{-1} for AFB1, AFG1 and OTA, 0.05, 0.25 and 2.5 μg kg^{-1} for AFB2 and AFG2 and 8, 40 and 400 μg kg^{-1} for ZEA, respectively). Ten grams of milled breakfast cereals were spiked with adequate volumes of stock and working standard solutions for the purpose of obtaining the desired mycotoxin concentration. They were processed after 24 hours in order to ensure evaporation of the solvent. Recovery was determined extrapolating the absolute responses (area of toxin peak) obtained from the spiked samples in the calibration curve; the calculated concentration was compared to the expected concentration for a 100% recovery. The repeatability and reproducibility of this process were tested carrying out the complete sample process and recovery experiment in triplicate on one day and on three different days, respectively.

2.6. Statistical analysis

The statistical analysis was performed with the SPSS 15.0 program. The study has taken into account the levels between the LOD and LOQ; for samples having no detectable levels of mycotoxins, half of LOD value was assigned. This makes the use of nonparametric statistical methods mandatory.

Differences between sample groups were evaluated by nonparametric Mann-Whitney U test or Median test for two independent samples; Kruskal-Wallis test or Median test was used for k independent samples, after having evaluated the homogeneity of variances with the Levene's test. Correlation between the levels of ZEA and OTA has been assayed with Spearman's Rank Correlation test, due to the lack of univariate and

bivariate normality. A probability value of 0.05 was used to determinate the statistical significance.

3. Results

3.1. Method validation

When samples with nondetectable levels of mycotoxins were analyzed, no interferences were observed with the mycotoxin peaks at the retention times of each of the compounds. The retention time of each toxin in the samples corresponded to the retention time in the calibration samples with a tolerance of ± 2.5%. Moreover, when mycotoxins were added to positive samples, an increase of the toxin peaks was observed without the appearance of shoulders or interferences.

In figure 1, chromatograms obtained from a calibration sample and from a breakfast cereal sample are shown.

[Insert figure 1 about here]

The LOD and LOQ values and recovery percentages for each mycotoxin in breakfast cereal samples are summarized in table 1. Recoveries were homogeneous (RSD < 13.5%) at the levels assayed, demonstrating the precision of the method (see table 1).

[Insert table 1 about here]

For all mycotoxins and levels assayed, the recovery values obtained are adequate (between 50 and 120% for AFs and OTA; between 60 and 120% for ZEA, and with RSD ≤ 40% for AFs, OTA and ZEA) (European Commission, 2006b). However, the tendency for recovery to diminish at the highest levels of the assayed mycotoxins has

been observed. Moreover, a recovery lower than that observed in the previous study in barley samples for some mycotoxins, such as OTA, has been obtained. This behavior demonstrates the need to re-validate the method for this type of matrix.

All the results have been corrected with recovery. Due to the fact that no levels higher than 1 μg kg^{-1} for AFB1 or OTA, or 40 μg kg^{-1} for ZEA have been found in the samples, the correction factor for each mycotoxin was calculated as the mean value obtained for recovery at the two lower levels assayed for each one of them.

3.2. AFs, OTA and ZEA in breakfast cereal samples

In the study of the occurrence of mycotoxins, samples with mycotoxin levels above the LOD have been considered positive. Very low levels of mycotoxins have been observed for all of the samples. According to the results, 9% of the samples were positive (levels > LOD) for AFB1 although no sample exceeded the LOQ (0.2 μg kg^{-1}), with median and maximum levels being 0.03 and 0.13 μg kg^{-1}, respectively. No detectable levels were observed for the other aflatoxins (AFB2, AFG1 and AFG2). Zearalenone and OTA were in 48 and 39% of the samples, respectively. However, the levels of these toxins were below the maximum permitted limit established by EU, with 38.61 and 1.12 μg kg^{-1} being the maximum levels found for ZEA and OTA, respectively, and with a mean value of the positive samples being 12.49 and 0.29 μg kg^{-1}, respectively.

Thirty-three percent of the samples have no detectable levels for any of the mycotoxins, whereas 39% of the samples presented > LOD levels for one mycotoxin and 28% of the samples presented > LOD levels for two toxins. When two mycotoxins were detected, 92% of the samples showed the co-occurrence of ZEA and OTA, and only one sample presented detectable levels of AFB1 and ZEA simultaneously. In fact, a good positive correlation was observed between the presence of ZEA and OTA ($r_s = 0.537$).

The samples were classified using the label information of each sample, based on *type of cereal* (see table 2). Aflatoxin B1 only appeared in the corn-based breakfast cereal samples, and at low levels (median value = 0.03 μg kg^{-1}). Zearalenone and OTA showed the highest contamination rates in the samples containing wheat and wheat and rice, respectively, with the maximum level found for both mycotoxins in wheat-based products. In the case of ZEA, no significant differences have been observed between the contamination levels based on the composition of the samples. However, in the case of OTA, these differences have been significant between corn-based and other cereal-based products, but not between the wheat- and rice-based and the wheat-based samples.

[Insert table 2 about here]

Taking into account the *fiber content*, AFB1 was only present in the normal-fiber samples (see table 3). Zearalenone and OTA showed the highest occurrence (69 and 92%, respectively) in the whole grain breakfast cereals, although the maximum levels of contamination for both mycotoxins were found in normal-fiber samples. These results are conditioned by the type of cereal, due to the fact that nearly all of the samples of normal-fiber content are corn-based breakfast cereals and the samples of high-fiber content are mainly wheat and rice-based products. After carrying out a statistical comparison using only wheat-based samples of high- and normal-fiber content, a higher incidence of OTA and ZEA in whole grain cereals (83 and 50% for OTA and 100 and 38% for ZEA in high- and normal-fiber content samples, respectively) was observed, although the maximum levels of ZEA and OTA (38.6 and 1.1 μg kg^{-1}, respectively) were found in wheat-based breakfast cereals of normal-fiber content. The Mann-

Whitney U test has shown significant differences between the OTA levels in the two groups of samples, although no differences have been found between the ZEA levels.

[Insert table 3 about here]

Finally, the samples were classified according to the *type of brand* (see table 4). A higher occurrence was observed for AFB1, ZEA and OTA in name-brand samples. The AFB1 maximum level was found in generic brand products, which are mainly corn-based cereal; for ZEA and OTA, maximum level was found in name-brand products. However, significant differences have not been found between the two groups of samples.

[Insert table 4 about here]

4. Discussion

4.1. Method validation

This paper has described a fast and simple method for the simultaneous analysis of six mycotoxins from three different families in breakfast cereal samples. The procedure is based on a previously in-house validated method for the analysis of AFB1, AFG1, AFB2, AFG2, OTA and ZEA in barley that has been adapted to and revalidated for this new matrix. The validation of the method in any matrix is of great interest because it is well known that the type of matrix involved may condition the extraction and purification processes, affecting recovery of the toxins and consequently, their quantification, as has been confirmed in this research work.

Commission Regulation (EC) Nº 401/2006 states that recovery between 50 and 120% in the < 1 μg kg^{-1} levels, and between 70 and 110% in the 1 – 10 μg kg^{-1} levels is

acceptable for AFs and OTA; recovery between 60 and 120% in the ≤ 50 μg kg^{-1} levels, and between 70 and 120% in the > 50 μg kg^{-1} levels is considered acceptable for ZEA. In the method developed, the recovery values were valid for the analysis for all of the mycotoxins. On the other hand, the limits of detection and quantification of this method are lower or similar to those given in the reference literature (Araguás, González-Peñas and López de Cerain, 2005, Molinié, Faucet, Castegnaro and Pfohl-Leszkowicz, 2005, Schollenberger, Müller, Rüfle, Suchy, Planck and Drochner, 2005, Kabak, 2009b, Roscoe et al., 2008).

4.2. AFs, OTA and ZEA in breakfast cereal samples

Co-occurrence of mycotoxins in cereal and cereal-derived products has been reported in several research studies. Different fungi can grow and produce mycotoxins in the same environmental conditions and, in addition, the different phases which foodstuff goes through, from the cereal grain growing in the field to being processed, favors the production of several mycotoxins. However, this is the first time that possible co-occurrence of AFs, OTA and ZEA in breakfast cereal samples has been studied. Roscoe *et al*. (2008) reported regular occurrence of low levels of multiple toxins, including OTA and ZEA, in breakfast cereal samples, although they did not present data of co-occurrence.

In this study, it has been observed that 67% of the samples presented detectable levels of at least one mycotoxin (AFB1, OTA or ZEA), although, in every case, the levels found were far below the established legal limits. Of the four aflatoxins studied, AFB1 was detected in only 9% of the samples analyzed, always with levels below the LOQ (0.2 μg kg^{-1}). However, in Canada, 50% of the breakfast cereal samples analyzed contained AFB1, although the levels were low (between 0.002 and 1 μg kg^{-1}) (Roscoe et

al., 2008). The samples analyzed from the Athens markets presented a high incidence (56%), with levels between 0.05 and 4.3 μg kg^{-1} for AFB1 (Villa and Markaki, 2009). These differences could be due to origin and year of harvest of the raw materials, both of which can affect the content of mycotoxins (Kuiper-Goodman, 1999).

With regard to ochratoxin A, the incidence found in this survey is lower than in other studies conducted in Europe, where occurrence between 60 and 90% was found (Araguás, González-Peñas and López de Cerain, 2005, Molinié, Faucet, Castegnaro and Pfohl-Leszkowicz, 2005, Villa and Markaki, 2009), although it is similar to other research studies carried out in Canada and Turkey (Kabak, 2009b, Roscoe et al., 2008). However, the contamination levels are similar to those found by other researches that observed mean OTA levels of 0.27 μg kg^{-1} (Araguás, González-Peñas and López de Cerain, 2005), 0.11 μg kg^{-1} (Villa and Markaki, 2009) and mean levels between 0.01 and 0.38 μg kg^{-1}, depending on the type of cereal (Roscoe et al., 2008). In this study, no sample presented levels higher than the maximum permitted limit (3 μg kg^{-1}), whereas a French study reported 16% of samples with OTA levels higher than this limit (Molinié, Faucet, Castegnaro and Pfohl-Leszkowicz, 2005).

There are very few studies regarding the occurrence of ZEA in breakfast cereals. The results found in this study have shown a high contamination rate (48%), but levels are far below the maximum permitted limit (50 μg kg^{-1}). These results are quite similar to those observed in Portugal, where the incidence was 64%, with mean (of positive samples) and maximum values of 7.3 and 11 μg kg^{-1}, respectively (Gareis, 2003). In the UK and Canada, the occurrence rates were lower, 18 and 22%, respectively, although the maximum levels found were above the legal limit established by the EU (232 and 100 μg kg^{-1}) (Roscoe et al., 2008, Gareis, 2003).

This study has also demonstrated the co-occurrence of mycotoxins in breakfast cereals. More than one quarter of the samples (28%) showed the co-occurrence of two toxins, mainly the combination of OTA and ZEA. No relationship was observed between the presence of AFB1 and ZEA, despite the fact that both toxins have a high probability of contaminating corn (EFSA, 2004). A co-occurrence of mycotoxins should be taking into account when carrying out risk assessment.

When classifying the samples according to the type of cereal, AFB1 only appears in corn-based breakfast cereals. These results coincide with those found by Tam *et al*. (2006), who reported higher coincidence and levels in corn-based breakfast than in wheat-based samples. However, OTA and ZEA were mainly found in wheat-based and wheat and rice-based samples, with the maximum levels found in wheat-based products. This could be explained by the fact that aflatoxins contaminate cereal grains, and particularly, corn (Pittet, 2001), while OTA is often present in higher incidence and contamination in wheat than in corn (Miraglia and Brera, 2002). In the case of ZEA, these results contrast with the SCOOP report (2003), where higher occurrence was reported in corn and corn-based products with respect to wheat and wheat-based products (Gareis, 2003). However, they coincide with results reported by Roscoe *et al*. (2008), who found higher ZEA incidence in wheat-based products.

Another parameter to take into account is the fiber content. According to different researches, mycotoxins are found on and directly beneath the epidermis of the grain (Rafai, Bata, Jakab and Vanyi, 2000). Therefore, the foodstuffs with high-fiber content would be more prone to have mycotoxins. However, in this survey it has been observed that contamination with mycotoxins depends not only on the fiber content but also on the type of cereal. In the case of AFB1, no samples of whole grain cereals presented this

mycotoxin; however, 12% of the normal-fiber content samples, which were corn-based breakfast cereals, showed the presence of AFB1. Ochratoxin A and ZEA occurred with higher incidence in high-fiber content. This coincides with the findings in the reference literature (Araguás, González-Peñas and López de Cerain, 2005, Osborne et al., 1996). However, the maximum levels for OTA and ZEA have been found in normal-fiber content samples made of wheat. The explanation underlying these results may not necessarily be due to the fact that OTA and ZEA accumulate on and directly beneath the epidermis of grain seeds, but rather related to the type of cereal, as these toxins contaminate wheat and rice grains with great frequency. On the other hand, by comparing the results of the samples of high and normal-fiber content in wheat-based cereals, a high incidence in the whole grain cereals was observed, although with less contamination.

Aflatoxin B1 is considered to be a genotoxic and carcinogenic compound. For this toxin, the FAO/WHO Joint Expert Committee on Food Additives (JECFA) and EC Scientific Committee on Food (SCF) recommended that the level of the contaminant in food be reduced so as to be As Low As Reasonably Achievable (ALARA), because it is not possible to identify an intake without risk (EFSA, 2007). For this reason, most agencies have not set a TDI for AFB1. However, Kuiper-Goodman established a Provisional Maximum Tolerable Daily intake (PMTDI) of 1 ng kg^{-1} bw for adults and children without hepatitis B (Kuiper-Goodman, 1998). Considering this value, and a daily consumption of breakfast cereals of 30 g, the AFB1 calculated intake is very low even for children (< 20% in the worst case) (see table 5). For OTA and ZEA, a tolerable daily intake (TDI) and a temporary-TDI of 5 ng kg^{-1} bw and 0.2 µg kg^{-1} bw, respectively, were fixed by the SCF, whereas the provisional maximum tolerable daily intake (PMTDI) established by JECFA was 14 ng kg^{-1} bw and 0.5 µg kg^{-1} bw,

respectively. The calculated values of intake for the mean and the maximum levels of OTA and ZEA are very low and therefore, the risk for consumers can be considered low.

[Insert table 5 about here]

5. Conclusions

In this paper, co-occurrence of mycotoxins AFB1, AFG1, AFB2, AFG2, OTA and ZEA in breakfast cereals has been studied for the first time using a validated UHPLC-FLD method.

No sample presented detectable levels of AFB2, AFG1 or AFG2. Sixty-seven percent of the samples presented detectable levels of AFB1, OTA or ZEA, although with levels far below their legal limits. Twenty-eight percent of the samples showed co-occurrence of two toxins, mainly the combination of OTA and ZEA.

AFB1 appears only in corn-based breakfast cereals. On the other hand, OTA and ZEA were mainly found in wheat-based and wheat and rice-based samples, with the maximum levels being found in the wheat-based products.

According to the fiber content, no sample of high-fiber content was contaminated with AFB1, whereas OTA and ZEA occurred with higher incidence in high-fiber content samples. Finally, and regarding the type of product (name-brand or generic brand products) significant differences have not been found between the two groups of samples.

Considering a daily consumption of 30 g of breakfast cereals, the values of calculated daily intake found for the mean and the maximum levels of AFB1, OTA and ZEA are very low and therefore, the risk for consumers can be considered insignificant.

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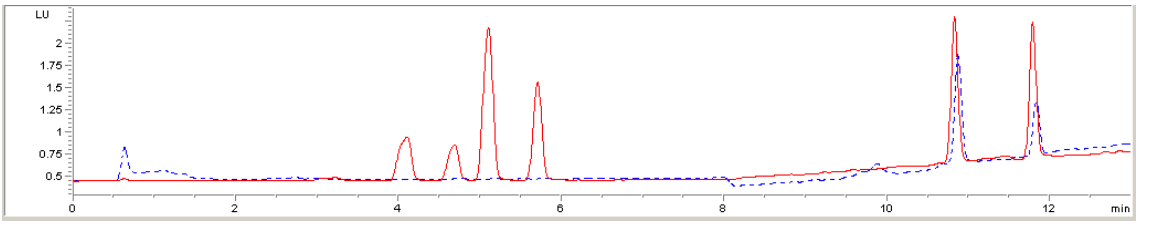
Figure captions

Figure 1. Chromatograms obtained from a calibration sample (—) and a breakfast cereal sample (- - -).

Figures

Figure 1



Tables

Table 1. LOD, LOQ and recovery in breakfast cereal samples.

Mycotoxin	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)	Toxin added ($\mu g\ kg^{-1}$)	Within-day recovery (RSD; %) (n = 3)	Between-day recovery (RSD; %) (n = 9)	Global recovery* (n = 18)
AFG2	0.013	0.05	0.05	68.6 (7.2)	66.5 (7.5)	68.6 (8.1)
			0.25	70.5 (7.8)	70.8 (7.1)	
			2.5	60.6 (1.8)	63.0 (3.7)	
AFG1	0.03	0.20	0.2	95.5 (10.5)	94.9 (6.4)	96.8 (6.4)
			1	95.0 (6.7)	98.8 (5.4)	
			10	73.9 (1.9)	77.5 (5.1)	
AFB2	0.014	0.05	0.05	102.9 (7.1)	100.8 (4.8)	102.7 (6.0)
			0.25	100.2 (7.7)	104.5 (6.1)	
			2.5	92.1 (1.4)	94.7 (2.8)	
AFB1	0.051	0.20	0.2	103.7 (6.1)	101.1 (5.7)	100.5 (5.9)
			1	95.7 (5.9)	99.9 (5.8)	
			10	83.4 (2.0)	86.7 (3.8)	
ZEA	1.91	8.00	8	104.5 (8.5)	103.3 (5.6)	104.6 (6.0)
			40	101.6 (4.8)	105.9 (5.7)	
			400	95.5 (1.6)	96.4 (2.5)	
OTA	0.062	0.20	0.2	71.2 (8.0)	71.3 (6.4)	69.3 (9.2)
			1	68.0 (5.8)	67.2 (10.3)	
			10	56.4 (9.5)	53.4 (13.3)	

* Recovery correction factor calculated as recovery mean value at the low and medium levels assayed for each mycotoxin.

Table 2. Occurrence of AFs, OTA and ZEA in breakfast cereal samples, based on the type of cereal.

Type of cereal	Parameter	AFB1	ZEA	OTA
Corn (n = 21)	% positives samples *	19	43	5
	Mean value of positive samples (μg kg^{-1})	0.08	5.42	0.10
	Mean value (μg kg^{-1})	0.04	2.87	0.03
	Median value (μg kg^{-1})	0.03	0.95	0.03
	Maximum level found (μg kg^{-1})	0.13	17.93	0.10
Wheat (n = 14)	% positives samples *	0	64	64
	Mean value of positive samples (μg kg^{-1})	< LOD	22.80	0.43
	Mean value (μg kg^{-1})	0.03	15.00	0.29
	Median value (μg kg^{-1})	0.03	11.51	0.20
	Maximum level found (μg kg^{-1})	< LOD	38.61	1.12
Wheat and rice (n = 8)	% positives samples *	0	50	88
	Mean value of positive samples (μg kg^{-1})	< LOD	5.22	0.16
	Mean value (μg kg^{-1})	0.03	3.09	0.15
	Median value (μg kg^{-1})	0.03	1.58	0.13
	Maximum level found (μg kg^{-1})	< LOD	12.67	0.24
Median test	Statistic	---	2.311	21.909
	Significance	---	0.315	< 0.001

* Positive samples: mycotoxin level > LOD.

Table 3. Occurrence of AFs, OTA and ZEA in breakfast cereal samples, based on the fiber content.

Fiber content	Parameter	AFB1	ZEA	OTA
Whole grain cereals (n = 13)	% positives samples	0	69	92
	Mean value of positive samples (μg kg^{-1})	< LOD	17.41	0.23
	Mean value (μg kg^{-1})	0.03	12.35	0.21
	Median value (μg kg^{-1})	0.03	3.53	0.20
	Maximum level found (μg kg^{-1})	< LOD	32.12	0.59
Normal cereals (n = 33)	% positives samples	12	39	18
	Mean value of positive samples (μg kg^{-1})	0.08	9.09	0.42
	Mean value (μg kg^{-1})	0.03	4.16	0.10
	Median value (μg kg^{-1})	0.03	0.95	0.03
	Maximum level found (μg kg^{-1})	0.13	38.61	1.12
Mann-Whitney U Test	Statistic	---	3.327*	59.500
	Significance	---	0.068	< 0.001

* Median Test.

Table 4. Occurrence of AFs, OTA and ZEA in breakfast cereal samples, based on the type of product.

Type of product	Parameter	AFB1	ZEA	OTA
Generic brand products (n = 32)	% positives samples	6	41	31
	Mean value of positive samples (μg kg^{-1})	0.09	12.11	0.22
	Mean value (μg kg^{-1})	0.03	5.49	0.09
	Median value (μg kg^{-1})	0.03	0.95	0.03
	Maximum level found (μg kg^{-1})	0.13	32.12	0.59
Name-brand products (n = 14)	% positives samples	14	64	57
	Mean value of positive samples (μg kg^{-1})	0.07	13.05	0.38
	Mean value (μg kg^{-1})	0.03	8.73	0.23
	Median value (μg kg^{-1})	0.03	2.53	0.11
	Maximum level found (μg kg^{-1})	0.07	38.61	1.12
Mann-Whitney U Test	Statistic	206.000	175.500	2.741*
	Significance	0.379	0.211	0.098

* Median Test.

Table 5. TDI of AFs, OTA and ZEA in breakfast cereal samples.

		AFB1	OTA	ZEA
Mean (μg kg^{-1})		0.03	0.13	6.47
Maximum (μg kg^{-1})		0.13	1.12	38.61
Daily intake for an adult (ng kg^{-1} b.w.)*	Mean	0.01	0.06	2.84
	Maximum	0.06	0.48	16.55
Daily intake for an adolescent (ng kg^{-1} b.w.)**	Mean	0.02	0.08	3.88
	Maximum	0.08	0.67	23.17
Daily intake for children (ng kg^{-1} b.w)***	Mean	0.04	0.20	9.71
	Maximum	0.20	1.68	57.92

* Body weight of 70 kg; ** Body weight of 50 kg; *** Body weight of 20 kg.