CO-OCCURRENCE OF AFLATOXINS, OCHRATOXIN A AND ZEARALENONE IN BARLEY FROM A NORTHERN REGION OF SPAIN

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ABSTRACT

123 barley samples from a region of Spain (Navarra) were analyzed in order to evaluate the possible co-occurrence of aflatoxins (AFB1, AFG1, AFB2 and AFG2), ochratoxin A (OTA) and zearalenone (ZEA). The results indicated that 80% of the samples presented detectable, although very low levels, of two or more mycotoxins. The most frequent combinations were AFB1 and OTA; AFB1, ZEA and OTA; and AFB1 and ZEA. In general, the statistical study did not show significant differences between levels or incidence for the mycotoxins in different years of harvest, variety of barley, farming or origin. The calculated values for daily intake were low and the risk to consumers could be assumed to be very low. However, the co-occurrence of several mycotoxins, and therefore synergic or additive effects, should be taken into account when determining permitted levels or risk assessment.

Keywords

Mycotoxin, aflatoxins, ochratoxin A, zearalenone, co-occurrence, barley.

1. Introduction

Mycotoxins are of great worldwide concern due to their toxic effects on human and animal health. The main agriculturally-important fungal toxins are aflatoxins (AFs), ochratoxin A (OTA), trichothecenes (TRs), fumonisins (FMs) and zearalenone (ZEA) (Miller, 1995). These toxins are mainly produced by fungal species belonging to the genus *Aspergillus*, *Penicillium* and *Fusarium*. While *Fusarium* species are destructive plant pathogens producing mycotoxins before, or immediately post harvesting, *Penicillium* species are more commonly found as contaminants of commodities and foods during drying and subsequent storage. *Aspergillus* species are commensal organisms that can produce mycotoxins in the field or during drying and storage. Aflatoxins are produced mainly by *Aspergillus flavus* in tropical and subtropical areas, whereas OTA is produced by *Penicillium verrucosum*, in temperate climates, and by *Aspergillus ochraceus* and related species in warmer climates (Pitt, 2006). Zearalenone is produced by *F. graminearum* in moist-warm continental climates, and by *F. culmorum*, in maritime and cooler European areas (Pitt, 2006).

Aflatoxins are the most toxic compounds produced by fungi, considered to be both genotoxic and carcinogenic (EFSA, 2007). Aflatoxins were found to cause carcinomas in human liver and therefore, have been classified in group 1 (carcinogenic to humans) by the International Agency ~~on~~ for Research on Cancer (IARC) (International Agency for Research on Cancer (IARC), 1993, International Agency for Research on Cancer (IARC), 2002). Ochratoxin A possesses carcinogenic, nephrotoxic, teratogenic, immunotoxic and possibly neurotoxic properties, and has been related with BEN (Balkan Endemic Nephropathy) and urinary tract tumors (UTT) in humans (SCF, 1998). However, there is no evidence regarding its carcinogenicity in humans; therefore, it has

been classified in group 2B (possibly carcinogenic to humans) by the IARC (International Agency for Research on Cancer (IARC), 1993). Zearalenone is a non-steroidal estrogenic compound which has been associated with problems of early menarche. It has been classified in group 3 (non classifiable as to its carcinogenicity to humans) by the IARC (International Agency for Research on Cancer (IARC), 1993).

Cereals represent the main OTA and ZEA sources of human intake (Gareis et al., 2003, Miraglia & Brera, 2002). Among cereal grains, AFs and ZEA mainly appear in corn (EFSA, 2004; EFSA, 2007), whereas barley has a particularly high likelihood of OTA contamination (Bennett & Klich, 2003). Over the past few years, there has been emerging evidence of potential aflatoxin contamination of feed materials grown in areas of southern Europe, where a subtropical climate and extensive agricultural practice favor fungal growth and the subsequent formation of aflatoxins (EFSA, 2007).

Due to the serious effects that mycotoxins can have on humans and animals, many countries have implemented regulations on mycotoxins in food and feed to protect human and animal health as well as the economical interest of producers and traders. The European Commission has established maximum permitted levels for mycotoxins of major concern in unprocessed cereals other than maize, with 2 μg kg^{-1} being the maximum permitted level for AFB1 and 4 μg kg^{-1} for the sum of AFB1, AFG1, AFB2 and AFG2; 5 μg kg^{-1} for OTA and 100 μg kg^{-1} for ZEA (European Commission, 2006).

Although these maximum levels have been established taking into account only the presence of individual mycotoxins, some surveys have demonstrated that multi-mycotoxin contamination can occur in foodstuffs and feed. The co-occurrence of several mycotoxins in the same sample is of great importance because the combination of mycotoxins could lead to antagonistic, additive or synergistic effects. The following

multi-mycotoxin combinations have been observed: AFs, trichothecenes, fumonisins and/or zearalenone in maize (EFSA, 2004); TRs, ZEA and/or OTA (Jaimez, Fente, Franco, Cepeda & Vázquez, 2004, Rafai, Bata, Jakab & Vanyi, 2000) or AFB1, FB1, ZEA and OTA (Sangare-Tigori, Moukha, Kouadio, Betbeder, Dano & Creppy, 2006) in cereal grains (wheat, rice,…).

In Spain, very few studies have been carried out which assess the simultaneous presence of mycotoxins in cereal grains, and particularly in barley, despite being the crop with the largest percentage of arable land (52% in 2007). In 2001, a survey was carried out to study the presence of OTA in cereals (wheat, barley and corn) from Navarra (Spain) (Araguás, González-Peñas, López de Cerain & Bello, 2003). It was observed that 65% of barley was contaminated with OTA, although at low levels. On the other hand, the presence of *Aspergillus, Penicillium* and *Fusarium* species has been observed in malting barley from Spain (Medina, Valle-Algarra, Mateo, Gimeno-Adelantado, Mateo & Jiménez, 2006). Therefore, the simultaneous occurrence of AFs, OTA and ZEA could be possible in cereals from this country.

In this research work, the simultaneous occurrence of AFs, OTA and ZEA in 123 barley samples collected in Navarra (a northern region of Spain) was analyzed. Different factors which could affect the production of these toxins, such as year and place of harvest, type of farming (organic or traditional), and variety of barley, were studied.

2. Materials and methods

2.1. Chemicals and reagents

Aflatoxins, ochratoxin A and zearalenone 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 were obtained 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 (PBS) 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 of type II water. 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 with water 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 by diluting this stock standard solution with acetonitrile-

methanol (50:50). All prepared solutions were stored at -20ºC and maintained at room temperature and in darkness for 30 minutes before use. Calibration samples were prepared by evaporating a given volume of the working standard solution under vacuum at 40ºC in an evaporator (GeneVac). The residue was then dissolved in 150 µL of a mixture (40:60) of organic solvent (acetonitrile-methanol (50:50)) and water, both acidified with 0.5% formic acid. The acetonitrile extract from each barley sample was evaporated and dissolved in the same way.

2.3. Barley samples

123 barley samples were collected from the 2007 and 2008 harvests in Navarra, a northern region of Spain. Most of the samples were provided by national factories dedicated to the production of foodstuffs and feed, after applying their own sampling procedures for cereal quality control. Other samples have been collected from agricultural cooperatives. In all of the cases, barley had not been processed before sampling. Navarra's climate is characterized by its diversity. From north to south, it can be divided into four climatic areas and seven zones (see figure 1). The climate in the northwestern area is temperate maritime warm, with an annual rainfall of 1100 – 2500 L/m^2 and an average annual temperature of 8.5 - 14.5ºC. The annual mean rainfall in the Pyrenees area ranges from 2200 to 700 L/m^2, and the mean temperature is between 7 and 15ºC. The central area of Navarra, formed by Pamplona, Tierra Estella and Navarra Media zones, is characterized by a Mediterranean climate (annual mean rainfall: 1100 – 450 L/m^2; annual mean temperature: 11 – 14ºC). The southern area of Navarra (Ribera Alta and Tudela zones) is the driest area of Navarra (annual mean rainfall lower than 500 L/m^2) and the average annual temperature is 14ºC.

Barley samples were collected from six climatic zones during two years of harvest (2007 and 2008) and from more than a dozen varieties. In addition, some of the samples

were from organic farms. Due to the climatic conditions in Navarra, the moisture content of the samples was ≤ 15% upon arrival at the laboratory. The samples were then stored at 4ºC until analysis.

2.4. Analysis of mycotoxins

Extraction, purification and analysis of the samples were carried out as previously described in Ibáñez-Vea, Corcuera, Remiro, Murillo-Arbizu, González-Peñas & Lizarraga (2011). In brief, ten grams of milled sample were extracted with a mixture of acetonitrile-water (60:40, v/v) 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 centrifuged and 15 mL of the supernatant were passed through an immunoaffinity column AOZ (Vicam) pre-conditioned with water and PBS. After the sample had passed through the column, the column was washed with PBS and water. The column was dried with air. After having maintained acetonitrile and the antibodies in contact with each other for 5 min, the mycotoxins were eluted with 3 mL of acetonitrile. The extract was evaporated to dryness in an evaporator (GeneVac) and the residue was redissolved in 150 µL of mobile phase. The sample was maintained at 4ºC in the chromatograph tray until its analysis.

The samples were analyzed in a 1200 rapid resolution liquid chromatographic system equipped with a fluorescence detector (Agilent Technologies). Separation was achieved using an Ascentis Express C18 column (150 mm x 2.1 mm; 2.7 µm). The injection volume was 30 µL and the flow rate was 0.9 mL min^{-1}. Chromatography was performed at 60ºC 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 in order to enhance the AFB1 and AFG1 response, using a PHRED photochemical reactor with a mercury lamp (λ = 254 nm) and a knitted reactor coil of 0.25 mL (5 m x 0.25 mm). The 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.

The method was previously validated in-house in barley samples in the ranges 0.15 – 1 μg kg^{-1} and 1 - 10 μg kg^{-1} for AFB1, AFG1 and OTA, 0.0375 - 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. The limit of detection ranged from 0.5 to 15 ng kg^{-1} for aflatoxins, and was 13 ng kg^{-1} for OTA and 340 ng kg^{-1} for ZEA. The limit of quantification ranged from 37.5 to 150 ng kg^{-1} for aflatoxins and OTA and 6000 ng kg^{-1} for ZEA. Recovery percentages (applied as correction factor in the quantification of the samples) were between 78.2 and 109.2% for all the mycotoxins, with a relative standard deviation (in intermediate precision conditions) lower than 15% in all cases. The method was valid for the analysis of AFB1, AFB2, AFG1 and AFG2, OTA and ZEA, and fulfilled the validation requirements established by the European Commission.

2.5. Confirmation

The presence of mycotoxins was confirmed in 10% of the samples using an Agilent Technologies 1200 liquid chromatographic system coupled to a MSD Trap XCT Plus mass spectrometry (G2447A model) equipped with an electrospray ionization interface (ESI). Mycotoxin analysis was performed using an Ascentis Express C18 column (150 mm x 2.1 mm; 2.7 μm) from Supelco, at 55ºC and with a linear gradient of methanol (A) and water (B), both of which contained 0.1% formic acid and 5 mM ammonium formate. The initial gradient condition was 40% A and 60% B, changing

linearly to 80% A and 20% B in 11 min. The column was re-equilibrated for 4 minutes. The injection volume was 20 μL and the flow rate was 0.3 mL min^{-1}.

The mass spectrometer was operated in positive ion mode. Ionization and spectrometric settings were optimized infusing the separate mycotoxin solutions (2 - 0.5 μg mL^{-1}) at a flow rate of 5 μL min^{-1} via a syringe pump. Data acquisition was performed working in multiple reaction monitoring (MRM) mode using the $[M+H]^+$ ions.

2.6. Statistical analysis

The SPSS 15.0 program was used for statistical analysis. The study took into account the levels between the LOD and LOQ; in the case of obtaining a value lower than the LOD, half of the LOD value was used. This forced us to use non-parametric statistical methods.

Nonparametric Mann-Whitney U test or Median test for two independent samples, and Kruskal-Wallis test or Median test for k independent samples, were used to evaluate possible level differences among groups of samples, after having evaluated the homogeneity of variances with the Levene's test. In addition, contingency test was used for evaluating the possible differences between the incidences of each mycotoxin within the different groups of samples. The correlation between the levels of two toxins was verified using Spearman's Rank Correlation test. A probability value of 0.05 was used to determine statistical significance.

3. Results

3.1. Incidence of mycotoxins

Results obtained from the analysis of AFB1, AFB2, AFG1, AFG2, ZEA and OTA in 123 barley samples are summarized in table 1. One hundred percent of the samples

presented detectable levels of AFB1, although both the mean and the median values obtained were less than the limit of quantification of the method. For the other aflatoxins, the incidence and the contamination rates were very low. Zearalenone and OTA occurred in a 39 and 58% of the samples, respectively, with mean values of the positive samples being very low. In fact, the maximum levels found for all the mycotoxins that were analyzed were far below the maximum permitted limits established by the European Union (European Commission, 2006). Between 0.8 and 3.3% of the samples showed mycotoxin values higher than the limits of quantification. The presence of AFB1 was the only exception as this mycotoxin was found in 32% of the samples, with levels ranging between 0.15 and 0.34 $\mu g\ kg^{-1}$.

3.2. Co-occurrence of mycotoxins

The results indicated that 80% of the samples presented two or more mycotoxins. Thirty-seven percent of the samples presented detectable levels of two mycotoxins and 29 and 13% of the samples presented three and four mycotoxins, respectively. Twenty seven per cent of the samples showed a combination of AFB1, ZEA and OTA; 31% of the samples showed a combination of AFB1 and OTA; and 12% of the samples showed a combination of AFB1 and ZEA. A low positive significant correlation was found between AFB2 and AFG2 (r_s = 0.189), AFG1 and ZEA (r_s = 0.216), AFG2 and ZEA (r_s = 0.213), and ZEA and OTA (r_s = 0.199). A low negative significant correlation was observed between AFB2 and OTA (r_s = -0.186).

3.3. Influence of different factors on the presence of mycotoxins

For a more detailed study, barley samples were divided according to year of harvest, climatic zone and variety. In addition, samples from organic and traditional farming were compared.

With regard to the samples of traditional farming classified by year of harvest (2007 or 2008), the samples cultivated during the 2008 harvest generally showed a higher occurrence of aflatoxins and ZEA, whereas OTA had a higher incidence in the samples from the 2007 harvest (see table 2). The mean values obtained from positive samples for all of the toxins were higher in the samples from the 2008 harvest, with the exceptions of AFB2 and AFG2. However, a statistical study indicated that there were no significant differences between incidence and levels obtained for the mycotoxins between 2007 and 2008 harvest years, with the exception of significant differences observed between harvest years 2007 and 2008 with regard to AFB1 and AFG2 levels.

Traditional samples were also classified according to their geographic origin. Only samples from the zones of Tudela, Ribera Alta, Navarra Media and Tierra Estella were included in the statistical study because those collected from Pamplona and the Pyrenees were too few in number. With regard to the AFs, the differences found between the zones were minimal (see table 3), except in the case of AFG1, for which significant differences were observed between Tudela and the rest of the zones. Tudela was the area with the highest incidence (57%) and levels of ZEA. Tierra Estella had the highest level and incidence (81%) of OTA, showing significant differences when compared to the other zones.

In 2007 and 2008, the most cultivated varieties of barley in Navarra were Hispanic, Pewter and Naturel. The Kruskal-Wallis test showed no significant differences between varieties. However, the results of the traditional samples showed that the greatest differences in incidence were for AFG1, AFG2 and OTA (see table 4), while incidence for AFB1, AFB2 and ZEA were similar. Hispanic was the variety with the highest mean levels of AFB1 and Naturel presented the highest mean levels of AFB2, AFG1 and AFG2. Zearalenone presented similar occurrence in the three varieties studied, although

Pewter was the variety with the highest levels of ZEA. On the other hand, OTA contaminated the Pewter variety with a higher incidence than in the Hispanic and Naturel varieties, with Pewter having the highest mean and maximum levels.

In this survey, almost all of the samples were from traditional farming (112), and the organic samples (11) came entirely from the Tudela zone. Upon comparing the Tudela organically-farmed samples with the Tudela traditionally-farmed samples, it was observed that the contamination levels of both AFG1 and ZEA were highest in the traditional samples while the OTA and AFB1 contamination levels were highest in the organic samples. Aflatoxin B2 showed higher prevalence in the traditional samples, although the mean value was less in this type of samples. On the other hand, AFG2 showed a similar mean of positive samples in both types of farming (see table 5). The statistical study did not show significant differences between toxin incidence and concentrations between both types of farming, except for the OTA levels.

4. Discussion

The technical limitations and the chemical diversity of toxins have hindered the development of the study of co-occurrence of mycotoxins in foodstuffs. In fact, there are few studies in the literature, and to the best of author's knowledge, no one evaluate the simultaneous presence of AFs, OTA and/or ZEA in barley. In this survey, the results found for 6 mycotoxins in 123 barley samples from Navarra have been presented. Eighty percent of the samples presented detectable levels of two or more mycotoxins, and a weak relationship has been observed between AFB2 and AFG2, AFG1 and ZEA, AFG2 and ZEA, and OTA and ZEA. This could indicate a link between the producing fungi or between the factors involved in mycotoxin production. In addition, the results have shown a weak negative correlation between AFB2 and OTA, which may indicate a

competitive relationship between the fungi producing these toxins or the prevalence of a fungus in determinate environmental conditions.

Few studies have revealed the contamination of barley with aflatoxins, although the data regarding the presence of aflatoxigenic fungi belonging to the *A. flavus/A. parasiticus* group in barley from Spain (Medina *et al*., 2006), and the presence of AFs in beer from several countries, including Spain (Nakajima, Tsubouchi & Miyabe, 1999), suggests the possibility of aflatoxin contamination in this type of cereal. In this survey, the contamination level was very low. In fact, only 32% of the samples presented quantifiable levels of AFB1, with 0.34 μg kg^{-1} being the maximum value found. In 37% of the samples, levels higher than the quantification limit have been found for one or more aflatoxins. These results are lower than those found in barley samples from Czech Republic, where 87% of the samples presented detectable levels of AFB1 (LOD = 0.3 μg kg^{-1}), with 2.1 and 4.0 μg kg^{-1} being the mean and the maximum values found, respectively (Sedmikova, Reisnerova, Dufkova, Bárta & Jílek, 2001).

The levels and incidence of OTA in this study are lower than in another survey carried out in Navarra during 2001 harvest (Araguás *et al*., 2003). In the latter survey, 65% of the barley samples analyzed were contaminated with OTA, with a mean concentration of 0.20 μg kg^{-1}. However, in this study, 58% of the samples were contaminated and the mean level found was 0.06 μg kg^{-1}. This finding suggests that good agricultural practices or climatologically conditions have helped reduce OTA contamination. Just as in the 2001 study, no sample was above the maximum permitted limit set by the EU. In both cases, the mean values obtained are below the mean value (0.30 μg kg^{-1}) reported for barley from five countries (Finland, France, Germany, Italy, UK) in the SCOOP (2002) report (Miraglia & Brera, 2002). In addition, studies from Europe showed higher levels of OTA, although its incidence was less. In Poland, the average frequency of

contamination in conventional samples was 4%, with mean levels of 0.3 $\mu g\,kg^{-1}$ (Czerwiecki, Czajkowska & Witkowska-Gwiazdowska, 2002). In the UK, in one survey, 27% of barley samples were positive with a mean level of 0.69 $\mu g\,kg^{-1}$ (Scudamore, Patel & Breeze, 1999), whereas in another survey, OTA was present in 18% of the barley samples with levels between 0.3 and 117 $\mu g\ kg^{-1}$ (MacDonald, Prickett, Wildey & Chan, 2004). In Denmark, 11 of 41 traditional samples presented values higher than 0.05 $\mu g\,kg^{-1}$ and there the mean level was 0.9 $\mu g\,kg^{-1}$ (Jørgensen, Rasmussen & Thorup, 1996). On the other hand, in the USA, among 103 barley samples tested, only 11 were positive, with levels ranging from 0.1 to 17.0 $\mu g\,kg^{-1}$ (Trucksess, Young, White, Page & Giler, 1999). Higher levels were found in barley samples from Tunisia, where 40% of the samples were contaminated and the mean content was of 96 $\mu g\,kg^{-1}$ (Zaied *et al*., 2009).

With regard to ZEA, the mean levels observed in this survey were similar to or less than the results founds by other researches, although the occurrence was higher. According to the SCOOP (2003) report, the mean level found in barley samples from six countries was 0.83 $\mu g\,kg^{-1}$ and the contamination frequency was 5% (Gareis et al., 2003). In a survey performed in the UK, 10% of the barley samples were above 3 $\mu g\,kg^{-1}$ and only 2% exceeded 10 $\mu g\,kg^{-1}$, with 44 $\mu g\,kg^{-1}$ being the maximum value detected (Edwards, 2009). The levels found in Lithuanian barley from the 2004 and 2005 harvests ranged from traces to 194.3 $\mu g\,kg^{-1}$, and the incidence rates varied between 25 and 65%, depending on the year of harvest (Mankeviciene, Butkuté, Dabkevicius & Suproniene, 2007). The occurrence of ZEA in a southwestern area of Germany was 22%, with mean contents between 2.6 and 36.5 $\mu g\,kg^{-1}$, depending on the year of harvest (Müller, Reimann, Schumacher & Schwadorf, 1997).

The production of mycotoxins might be expected to vary from year to year depending on climatic and storage conditions. In this survey, it was observed that mycotoxins produced by pathogens or commensal organisms, which contaminate cereal grains mainly in the field, occur with higher incidence and contamination in the 2008 harvest. However, OTA produced by fungi during storage appeared in higher occurrence in the samples of 2007 harvest, although their contamination levels were lower than those corresponding to samples of 2008. This could be explained by the fact that accumulated precipitation during May of 2007 was similar to the mean annual precipitation while the accumulated rainfall during May 2008 was more than twice the average. In addition, in 2008, the rainfall was concentrated during the flowering period of crops (May), leading to the emergence of weeds and diseases such as septoria and fusaria (Lafarga, Goñi, Armesto, Carro, Eslava & Segura, 2008), which could have propitiated the occurrence of mycotoxins such as AFs and ZEA.

With regard to the origin of the samples, the results have shown a tendency of higher AFs and ZEA contamination in southern Navarra (Tudela and Ribera Alta zones), whereas OTA was mainly found in Tierra Estella. These findings suggest that the warm climate, characteristic of the region of Tudela, could favor the infection of the crops with fungi *Fusarium*, principally responsible for ZEA production, and with *Aspergillus* species, responsible for AFs production. On the other hand, *Aspergillus* and *Penicillium* species producing OTA may be more prone to attack the cereal grains in the region of Tierra Estella, coinciding with a previous survey carried out in 2001 (Araguás *et al*., 2003).

Among all of the parameters influencing mycotoxin production, the variety of barley may be a factor to consider in the prevention of mycotoxins. In fact, there are some assays which have been carried out on corn and wheat for evaluating the resistance of

the crops to the *Fusarium* fungi infections. However, in this case significant differences were not found.

There is limited and conflicting evidence in the reference literature regarding fungicide efficacy for controlling *Aspergillus* and *Fusarium* mycotoxin production (European Commission. Scientific Committee on plants, 1999). Some researchers have shown that fungicide treatments do not always reduce *Fusarium* and *Aspergillus* contamination (D'Mello, Macdonald, Postel, Dijksma, Dujardin & Placinta, 1998, Magan, Hope, Colleate & Baxter, 2002) whereas on other occasions, lower contamination has been found for mycotoxins in organic farming samples. With regard to OTA, several reports indicate that this mycotoxin occurs more frequently in samples from organic farming (Czerwiecki *et al*., 1996). In this study, no significant differences were found between the samples of traditional and organic farming, except for the OTA levels that showed a higher occurrence in organic samples.

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 these reasons, 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 cereals in Spain of 239 g (Varela, Moreiras, Carbajal & Campo, 1995), the AFB1 mean intake is very low; however, the maximum ingestion value obtained was higher than the PMTDI proposed by Kuiper-Goodman (see table 1). 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}, respectively, were established by the SCF (SCF, 1998; SCF, 2000), 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 (JECFA, 2001). The values of intake found for the mean levels of OTA and ZEA are low and the risk for the consumers could be assumed as insignificant (see table 1). However, if the maximum values found are considered, in the case of OTA, the daily intake is close to the PMTDI proposed by JECFA and higher than the TDI established by SCF, and in the case of ZEA, the calculated intake is below the values set by these two organizations. In any case, these values are overestimated because it has been assumed that the 239 g of cereals consumed every day are from barley.

5. Conclusions

The analysis of 123 barley samples from Navarra has demonstrated the co-occurrence of aflatoxins, zearalenone and ochratoxin A in this type of matrix, due to the fact that 80% of the samples were contaminated with more than one mycotoxin. In all of the samples, the maximum levels found for the different mycotoxins were far below the maximum permitted levels established by the EU.

In general, the statistical study has not shown significant differences of mycotoxin incidence in different years of harvest, varieties of barley, types of farming or zones; the same occurs with regard to mycotoxin levels. The absence of statistical differences could be due to the low levels encountered for all mycotoxins in all of the samples.

The calculated values for daily intake found using the mean levels of mycotoxins were low and the risk for the consumers could be assumed to be very low also. However, the co-occurrence of several mycotoxins, and therefore synergic or additive effects, should be taken into account when determining permitted levels or risk assessment.

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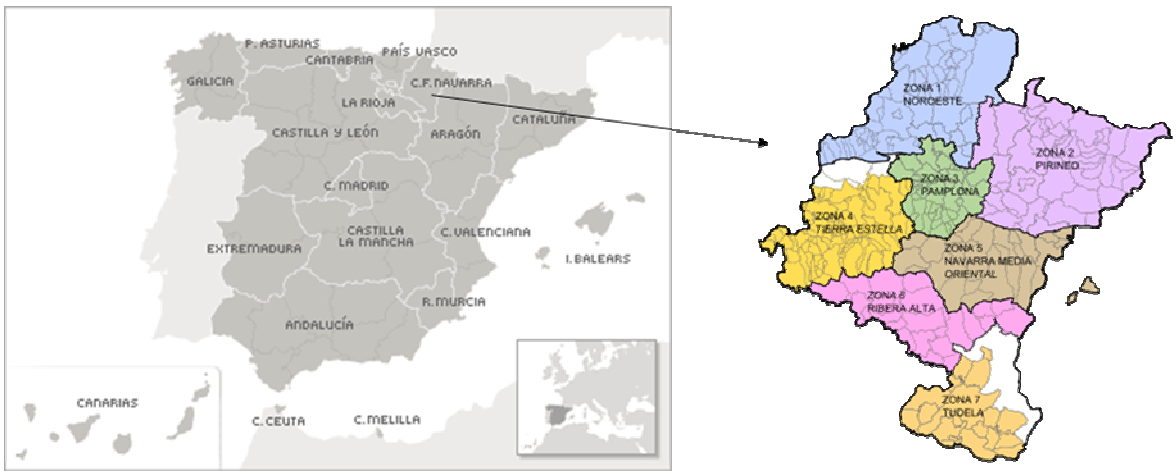
Figure captions

Figure 1. Geographic regions of Navarra (based on “Zonificación 2000”).

Figure 1.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C. VALENCIANA
EXTREMADURA
I. BALEARS
R. MURCIA
ANDALUCÍA
CANARIAS
C. CEUTA
C. MELILLA
ZONA 1 NOROESTE
ZONA 2 PIRINEO
ZONA 3 PAMPLONA
ZONA 4 TIERRA ESTELLA
ZONA 5 NAVARRA MEDIA ORIENTAL
ZONA 6 RIBERA ALTA
ZONA 7 TUDELA

Table 1. Summary of mycotoxin levels found in barley samples and daily intake for AFB1, ZEA and OTA.

Parameter		Sum of AFs	AFG2	AFG1	AFB2	AFB1	ZEA	OTA
% positive samples		100	18	5	17	100	39	58
Mean value of positive samples (μg kg^{-1})		0.14	0.02	0.13	0.01	0.13	1.89	0.10
Mean value (μg kg^{-1})		0.15	$3.97\ 10^{-3}$	0.01	$1.89\ 10^{-3}$	0.13	0.84	0.06
Median value (μg kg^{-1})		0.14	$1.50\ 10^{-3}$	0.01	$2.50\ 10^{-4}$	0.12	0.17	0.04
Maximum level found (μg kg^{-1})		0.75	0.10	0.61	0.04	0.34	18.53	3.53
Daily intake (ng kg^{-1} b.w.)*	Mean					0.44	2.87	0.21
	Maximum					1.16	63.27	12.05

* b.w. = 70 kg for an adult.

Table 2. Summary of mycotoxin levels found in traditional barley samples according to the year of harvest.

Year of harvest	Parameter	Sum of AFs	AFG2	AFG1	AFB2	AFB1	ZEA	OTA
2007 (n = 44)	% positive samples	100	2	7	11	100	36	64
	Mean value of positive samples ($\mu g\ kg^{-1}$)	0.12	0.04	0.05	0.01	0.11	0.76	0.04
	Mean value ($\mu g\ kg^{-1}$)	0.13	$2.30\ 10^{-3}$	0.01	$1.38\ 10^{-3}$	0.11	0.38	0.03
	Median value ($\mu g\ kg^{-1}$)	0.11	$1.50\ 10^{-3}$	0.01	$2.50\ 10^{-4}$	0.10	0.17	0.04
	Maximum level found ($\mu g\ kg^{-1}$)	0.33	0.04	0.06	0.04	0.33	1.03	0.06
2008 (n = 68)	% positive samples	100	26	4	22	100	41	53
	Mean value of positive samples ($\mu g\ kg^{-1}$)	0.15	0.02	0.22	$4.09\ 10^{-3}$	0.14	2.65	0.05
	Mean value ($\mu g\ kg^{-1}$)	0.16	$5.10\ 10^{-3}$	0.02	$1.10\ 10^{-3}$	0.14	1.19	0.03
	Median value ($\mu g\ kg^{-1}$)	0.15	$1.50\ 10^{-3}$	0.01	$2.50\ 10^{-4}$	0.13	0.17	0.03
	Maximum level found ($\mu g\ kg^{-1}$)	0.75	0.10	0.61	0.02	0.34	18.53	0.17
Mann-Whitney U Test	Statistic	931.000	1141.000	1458.500	1326.000	1028.000	1340.000	0.599*
	Significance	0.001	0.001	0.567	0.129	0.005	0.291	0.439
Contingency Test	Statistic	---	11.104	0.015	2.083	---	0.259	1.248
	Significance	---	0.001	0.902	0.208	---	0.694	0.329

* Median Test.

Table 3. Summary of mycotoxin levels found in barley according to sample origin.

Zone	Parameter	Sum of AFs	AFG2	AFG1	AFB2	AFB1	ZEA	OTA
Tudela (n = 14)	% positive samples	100	14	14	21	100	57	43
	Mean value of positive samples (μg kg^{-1})	0.14	0.01	0.03	$2.47\ 10^{-3}$	0.13	4.71	0.05
	Mean value (μg kg^{-1})	0.15	$2.77\ 10^{-3}$	$1.10\ 10^{-2}$	$7.20\ 10^{-4}$	0.13	2.78	0.03
	Median value (μg kg^{-1})	0.12	$1.50\ 10^{-3}$	$7.50\ 10^{-3}$	$2.50\ 10^{-4}$	0.09	0.66	0.01
	Maximum level found (μg kg^{-1})	0.36	0.01	0.04	$2.66\ 10^{-3}$	0.34	18.53	0.11
Ribera Alta (n = 30)	% positive samples	100	27	0	17	100	33	50
	Mean value of positive samples (μg kg^{-1})	0.14	0.01	$7.50\ 10^{-3}$	0.01	0.14	2.29	0.04
	Mean value (μg kg^{-1})	0.15	$2.66\ 10^{-3}$	$7.50\ 10^{-3}$	$2.00\ 10^{-3}$	0.14	0.88	0.03
	Median value (μg kg^{-1})	0.14	$1.50\ 10^{-3}$	$7.50\ 10^{-3}$	$2.50\ 10^{-4}$	0.13	0.17	0.02
	Maximum level found (μg kg^{-1})	0.29	0.01	< LOD *	0.04	0.28	11.14	0.06
Navarra Media (n = 40)	% positive samples	100	15	3	18	100	35	55
	Mean value of positive samples (μg kg^{-1})	0.13	0.03	0.02	$2.74\ 10^{-3}$	0.13	1.06	0.05
	Mean value (μg kg^{-1})	0.14	$5.17\ 10^{-3}$	$7.80\ 10^{-3}$	$6.85\ 10^{-4}$	0.13	0.48	0.03
	Median value (μg kg^{-1})	0.14	$1.50\ 10^{-3}$	$7.50\ 10^{-3}$	$2.50\ 10^{-4}$	0.13	0.17	0.03
	Maximum level found (μg kg^{-1})	0.26	0.10	0.02	$3.29\ 10^{-3}$	0.22	3.56	0.16
Tierra Estella (n = 21)	% positive samples	100	5	0	10	100	29	81
	Mean value of positive samples (μg kg^{-1})	0.12	0.07	$7.50\ 10^{-3}$	$2.33\ 10^{-3}$	0.11	0.65	0.05
	Mean value (μg kg^{-1})	0.12	$4.56\ 10^{-3}$	$7.50\ 10^{-3}$	$4.48\ 10^{-4}$	0.11	0.31	0.04
	Median value (μg kg^{-1})	0.11	$1.50\ 10^{-3}$	$7.50\ 10^{-3}$	$2.50\ 10^{-4}$	0.10	0.17	0.04
	Maximum level found (μg kg^{-1})	0.25	0.07	< LOD *	$2.40\ 10^{-3}$	0.24	0.72	0.17
Kruskal-Wallis Test	Statistic	3.551 **	3.666	8.189	1.136	5.067 **	3.320 **	4.374
	Significance	0.314	0.300	0.042	0.768	0.167	0.345	0.036
Contingency Test	Statistic	---	4.856	5.003	1.102	---	3.320	6.728
	Significance	---	0.223	0.069	0.819	---	0.363	0.079

* Below the limit of detection.

** Median Test.

Table 4. Summary of mycotoxin levels found in barley samples according to the variety of barley.

Variety	Parameter	Sum of AFs	AFG2	AFG1	AFB2	AFB1	ZEA	OTA
Hispanic (n = 20)	% positive samples	100	35	5	20	100	35	50
	Mean value of positive samples (µg kg^{-1})	0.16	0.01	0.04	$2.62\ 10^{-3}$	0.15	0.89	0.04
	Mean value (µg kg^{-1})	0.17	$4.76\ 10^{-3}$	0.01	$7.23\ 10^{-4}$	0.15	0.42	0.03
	Median value (µg kg^{-1})	0.16	$1.50\ 10^{-3}$	0.01	$2.50\ 10^{-4}$	0.14	0.17	0.02
	Maximum level found (µg kg^{-1})	0.33	0.04	0.04	$3.19\ 10^{-3}$	0.33	1.23	0.06
Pewter (n = 37)	% positive samples	100	8	11	22	100	38	65
	Mean value of positive samples (µg kg^{-1})	0.13	0.03	0.03	$2.59\ 10^{-3}$	0.13	2.25	0.05
	Mean value (µg kg^{-1})	0.14	$3.52\ 10^{-3}$	0.01	$7.56\ 10^{-4}$	0.13	0.96	0.04
	Median value (µg kg^{-1})	0.13	$1.50\ 10^{-3}$	0.01	$2.50\ 10^{-4}$	0.12	0.17	0.04
	Maximum level found (µg kg^{-1})	0.36	0.07	0.06	$3.29\ 10^{-3}$	0.34	18.53	0.17
Naturel (n = 15)	% positive samples	100	20	7	20	100	40	40
	Mean value of positive samples (µg kg^{-1})	0.15	0.04	0.61	0.01	0.10	1.05	0.04
	Mean value (µg kg^{-1})	0.16	$9.25\ 10^{-3}$	0.05	$2.03\ 10^{-3}$	0.10	0.52	0.02
	Median value (µg kg^{-1})	0.10	$1.50\ 10^{-3}$	0.01	$2.50\ 10^{-4}$	0.09	0.17	0.01
	Maximum level found (µg kg^{-1})	0.75	0.10	0.61	0.02	0.18	2.88	0.05
Kruskal-Wallis Test	Statistic	4.122	5.684	0.544	0.015	5.882	0.009	4.195
	Significance	0.127	0.058	0.762	0.992	0.053	0.996	0.123
Contingency Test	Statistic	---	6.268	0.668	0.029	---	0.095	3.019
	Significance	---	0.068	0.858	1.000	---	1.000	0.218

Table 5. Summary of mycotoxin levels founds in traditional and organic barley samples.

Type of farming	Parameter	Sum of AFs	AFG2	AFG1	AFB2	AFB1	ZEA	OTA
Traditional (n = 13)	% positive samples	100	15	15	23	100	54	38
	Mean value of positive samples (µg kg^{-1})	0.13	0.01	0.03	$2.47\ 10^{-3}$	0.12	5.24	0.05
	Mean value (µg kg^{-1})	0.13	$2.87\ 10^{-3}$	0.01	$7.61\ 10^{-4}$	0.12	2.92	0.02
	Median value (µg kg^{-1})	0.10	$1.50\ 10^{-3}$	0.01	$2.50\ 10^{-4}$	0.09	0.66	0.01
	Maximum level found (µg kg^{-1})	0.36	$1.37\ 10^{-2}$	0.04	$2.66\ 10^{-3}$	0.34	18.53	0.11
Organic (n = 11)	% positive samples	100	27	0	9	100	36	64
	Mean value of positive samples (µg kg^{-1})	0.14	0.01	0.01	0.09	0.13	1.06	0.57
	Mean value (µg kg^{-1})	0.15	$3.36\ 10^{-3}$	0.01	$8.83\ 10^{-3}$	0.13	0.50	0.37
	Median value (µg kg^{-1})	0.15	$1.50\ 10^{-3}$	0.01	$2.50\ 10^{-4}$	0.15	0.17	0.06
	Maximum level found (µg kg^{-1})	0.23	$9.51\ 10^{-3}$	< LOD*	0.09	0.22	1.49	3.53
Mann-Whitney U Test	Statistic	53.000	64.000	---	63.000	58.000	59.000	39.000
	Significance	0.284	0.541	---	0.448	0.434	0.430	0.040
Contingency Test	Statistic	---	0.044	1.846	0.134	---	0.734	2.593
	Significance	---	0.834	0.482	0.714	---	0.444	0.217

* Below the limit of detection.