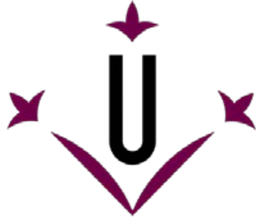

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Determination of Aflatoxin B1, Ochratoxin A and Zearalenone on Sorghum Grains Marketed in Tunisia Using High-Performance Liquid Chromatography Coupled with Fluorescence Detection

Running title: Mycotoxin detection in sorghum by HPLC

Food Additives & Contaminants: Part B Surveillance

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Abstract

A total of 64 samples of sorghum (37 Tunisian sorghum samples and 27 Egyptian sorghum samples) were collected during 2011-2012 from markets in Tunisia. Samples were analysed for contamination with aflatoxin B1, ochratoxin A and zearalenone by HPLC-FD. Aflatoxin B1 was found in 38 samples in the range from 0.03 to 31.7 µg kg^{-1}. Ochratoxin A was detected in 24 samples with concentrations ranging from 1.04 to 27.8 µg kg^{-1}. Zearalenone was detected in 21 samples and the concentration varies between 3.75 and 64.52 µg kg^{-1}. The ANOVA analysis of the influence of the country of origin on the incidence and concentration of mycotoxins in the samples studied showed that there was no significant difference ($P > 0.05$) between the two batches of

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samples for each of the three mycotoxins studied. The studied mycotoxins contaminate sorghum, and may also co-exist because of the diversity of the mycobiota in this cereal.

Keywords: aflatoxin B1, ochratoxin A, zearalenone, sorghum, incidence

Introduction

Sorghum (*Sorghum bicolor*) is an Old World grass originating from the African and Asian continents and is now distributed worldwide across temperate and tropical regions. It is ranked as the fifth most important cereal crop in the world, after wheat, rice, maize and barley and the second most important crop (after maize) in sub-Saharan Africa (FAO, 1994). It is mainly cultivated in semi-arid and subtropical regions because of its resistance to harsh weather conditions and its efficient use of water makes it the crop of choice to boost food security in drought stricken regions. For this reason, sorghum cultures increased during past years. In 1997, there was a 23% increase in the world's production of sorghum with respect to that of the previous year; the United States and India were the main producers, contributing 30 and 15% of the total yield, respectively. In Brazil, the 1997 annual sorghum crop totaled 385.2 thousand tons harvested from 487.28 acres of cultivated land (Olivetti and Camargo, 1997). The total 2006-2007 sorghum crop was 1624.2×10^3 t harvested from 733.8×10^3 ha of cultivated land in Brazil (Conab, 2009). As of 2007, sorghum production in Africa increases significantly even to the detriment of rice and wheat production (FAOSTAT, 2012) and Nigeria become the world's largest producer of sorghum, followed by India, USA, Ethiopia and Argentina (FAOSTAT, 2012). In 2009 the world's production of sorghum was 61.69×10^6 t, with Nigeria (11.50×10^6 t) and the USA (9.65×10^6 t) standing out as major producers (USDA, 2009). The total yield in Africa was estimated to be more than 25.7 million metric tons (FAOSTAT, 2015). Sorghum grains are used as feedstock for poultry, pigs and cattle feed, but also for human beings as staple foods in some African and Asian countries (Veiga, 1986). It constitutes the main grain food for over 750 million people who live in semi-arid tropics of Africa, Asia and Latin America (Codex Alimentarius Commission, 2012). In fact, a large proportion of African populations, especially in rural communities, consume mainly cereal-based foods and including sorghum (Oniang'o et al., 2003).

However, sorghum grains are susceptible to fungal colonisation during panicle and grain developmental stages (Waliyar et al., 2007), which constitutes a major constraint to an increase in sorghum production worldwide. It is estimated that annual economic losses in Asia and Africa due to mould infestation are in excess of US $130 million (Chandrashekar, Bandyopadhyay, & Hall, 2000). Several species of *Aspergillus*, *Alternaria*, *Fusarium*, *Cladosporium*, *Curvularia* and *Penicillium* are among the prevalent grain mould pathogens in sorghum (Bandopadyay et al., 2000; Lahouar et al., 2015). Mycotoxigenic strains of these

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61 fungal species have been isolated from sorghum samples (Chandrashekar et al., 2000; Lahouar et al., 2015).
62 Toxigenic species of *Aspergillus flavus*, *Aspergillus niger* aggregates, *Aspergillus flocculosus* and *Fusarium*
63 *incarnatum* has been associated with sorghum seeds in Tunisia (Lahouar et al., 2015).Therefore, sorghum may
64 contain different types of mycotoxins such as zearalenone, fumonisins, aflatoxins and ochratoxin A
65 characterized by their severe adverse effects on human and animal health. Aflatoxin is the most intensively
66 studied of all of the mycotoxins that are known to occur on sorghum. Nonetheless, sorghum is usually less
67 heavily contaminated with aflatoxins and fumonisins than is maize (da Silva et al., 2006; Bandyopadhyay et al.,
68 2007). However, Chala et al. (2015) reported that zearalenone was the most typical mycotoxin found in
69 sorghum, followed by fumonisins and aflatoxins. In Tunisia, preliminary surveys of mycotoxins showed
70 relatively high levels of aflatoxins contaminations in sorghum (Boutrif et al., 1977). Since 1977 several studies
71 have confirmed mycotoxin contamination in Tunisian sorghum (Ghali et al., 2008; Ghali et al., 2010; Serrano et
72 al., 2012; Oueslati et al., 2012; Oueslati et al., 2014). In Nigeria, the first reported mycotoxin contamination was
73 in sorghum samples (Elegbede, 1978; Salifu, 1978), In Asia, the first study on sorghum was reported in India
74 (Sashidhar et al., 1992). Because of the lack of data on the occurrence of mycotoxin in sorghum and the growing
75 importance of this cereal as food and feed in a high number of countries, the Codex Alimentarius Commission
76 opened a discussion paper on ''mycotoxin in sorghum grain'' to ask for studies to obtain more data on the
77 occurrence of mycotoxins in sorghum (Codex Alimentarius Commision 2012).

78 The aim of this study was to provide information on the possible incidence and co-occurrence of
79 aflatoxin B1(AFB1), ochratoxin A (OTA) and zearalenone (ZEN), using HPLC with fluorescence detection, in
80 sorghum grains commercialised in Tunisia and to compare the levels of contamination to the European limits
81 and other scientific papers.

82 **Materials and methods**

83 ***Samples***

84 From August 2011 to January 2012, sixty four (64) samples of sorghum seeds were purchased in a
85 random manner from retail outlets (shops, markets, small groceries and specialised food stores) located in the
86 region of Sahel in Tunisia. These included Tunisian (37) and Egyptian (27) samples. The weight of the retail
87 packs ranged between 500 g and 1000 g. All samples were kept at 4 °C in polyethylene bags while awaiting
88 analysis. Before analysis, the samples were mixed and an aliquot of 200 g was taken from each original
89 packaging and putted into a new plastic bag. From this sample, an aliquot was taken after thorough mixing,
90 extracted and analysed as described below.

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91 ***Solvents and reagents***

92 All reagents (potassium chloride, sodium chloride, phosphoric acid, hydrochloric acid, citric acid,
93 acetic acid) were of analytical grade. All solvents (methanol, acetonitrile, propanol-2-ol, hexane, chloroform,
94 dichloromethane, trifluoroacetic acid) were of HPLC grade. Deionised water was used for the preparation of all
95 aqueous solutions and for HPLC. Standard toxins of AFB1, OTA and ZEN were supplied by Sigma-Aldrich
96 (Saint-Louis, Missouri, USA). HPLC grade methanol, acetonitrile, hexane, chloroform, dichloromethane, citric
97 acid, acetic acid and trifluoroacetic acid (TFA) were obtained from Loba chemie (Mumbai, Maharashtra, India).
98 Potassium chloride, sodium chloride and orthophosphoric acid were provided by Scharlau (Sentmenat,
99 Barcelone, Spain). Hydrochloric acid, diatomaceous earth sorbent and NaOH were obtained from Prolabo
100 (Fontenay-sous-Bois, France).

101 ***Preparation of standard solutions***

102 Standard solutions AFB1, OTA and ZEN were prepared by dissolving 1 mg of AFB1, OTA and ZEN
103 in 1 ml of methanol. The concentrations of AFB1, OTA and ZEN stock solutions were determined by measuring
104 the UV absorbance at 360, 333 and 274 nm, respectively and calculating by using the molar extinction
105 coefficient ε of 21800, 5440 and 13900 mol^{-1} cm^{-1}, respectively (AOAC, 2000). The stability of the stock
106 solution was checked spectrophotometerically and all working standard solutions were prepared immediately
107 before use by diluting the stock solution with methanol. A series of working standards ranging from 0.5 to 150
108 $ng\ ml^{-1}$ of methanol for AFB1 and OTA and from 50 to 400 $ng\ ml^{-1}$ for ZEN were prepared by dilution. They
109 were used to calibrate the LC detector responses.

110 ***Confirmation of the presence of OTA***

111 The confirmation of the presence of OTA in some sorghum samples was achieved by the following
112 technique: an aliquot, taken from the purified extract of a sample where OTA was detected by the HPLC
113 analysis, was dried. The pellet was dissolved in 975 µl of a buffer solution of 0.04 M Tris-HCl, 1 M NaCl, pH
114 7.5. Then, 25 µl of carboxypeptidase (100 U ml^{-1} H_2O) was added and the mixture was incubated at room
115 temperature overnight. The sample was analysed under the same HPLC chromatographic conditions as used
116 above. The OTA peak disappeared whereas the peak of α-OT appeared.

117 ***Confirmation of the presence of AFB1***

118 The confirmation of AFB1 presence in samples was performed by derivatisation using TFA
119 (trifluoroacetic acid) which convert AFB1 to their respective highly fluorescent hemiacetal AFB2a. Briefly, 200
120 µl of hexane and 50 µl of TFA were added to the sample and mixed for 30 seconds. The mixture was allowed to

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121 stand 5 min for the phases to separate. A volume of 1.950 ml of water/acetonitrile (10:90) was added. The
122 mixture was shacked 30 seconds and allowed to stand for 10 min the two phases to separate. Finally, the lower
123 phase was injected under the same HPLC conditions.

124 ***Simultaneous method of AFB1 and OTA analysis***

125 *Sample extraction and cleanup*

126 AFB_1 and OTA were analysed by the method of Nguyen et al. (2007). To this end, 20 g of the sample
127 was mixed with 100 ml of acetonitrile-4% aqueous solution of potassium chloride (9:1). The mixture was
128 adjusted to pH 1.5 with undiluted hydrochloric acid and shaken for 20 min on an orbital shaker and filtered
129 through a Whatman paper no 4. The filtrate was cleaned with 100 ml of hexane and the mixture was shaken for
130 10 min. After separating, the upper phase (hexane) was discarded. This step was repeated with 50 ml of hexane.
131 The lower phase was extracted with 50 ml of chloroform and 50 ml of deionised water and the solution was
132 shaken for 10 min. After separation, the lower phase (chloroform) was collected. The upper phase was re-
133 extracted, twice, with 25 ml of chloroform and 25 ml of deionised water, using the above conditions. The
134 chloroform extracts were pooled and evaporated to near dryness under vacuum by using a rotary evaporator in a
135 40 °C water bath at low speed. Mycotoxins were resuspended in two milliliters of methanol and the solution was
136 filtered through a 0.45 µm filter and evaporated to dryness under nitrogen. For analysis in the HPLC, 500 µl of
137 methanol were added.

138 *HPLC conditions*

139 The determination of AFB1 and OTA was done using a Waters (Milford, MA, USA) chromatograph
140 with a reverse phase C_{18} silica gel column (Water Spherisorb 3 µm ODS2 4.6 x 150 mm, Milford, MA, USA),
141 followed by fluorescence detection (Waters 2475 florescence detector, Waters, Milford, MA, USA). Different
142 excitation and emission fluorescence parameters (AFB1 λexc 365 nm; λem 440 nm; OTA λexc 335 nm; λem
143 465 nm) were used to achieve the optimal conditions of detection for each toxin. The system was run
144 isocratically. The mobile phase was constituted with (0.33 M) H_3PO_4/acetonitrile/propanol-2-ol (650/400/50)
145 with a flow rate of 0.5 ml min^{-1}. The injection volume was 50 µl and the retention times of AFB1 and OTA were
146 about 14 and 56 min, respectively.

147 ***ZEN analysis***

148 *Sample extraction and cleanup*

149 To determine the ZEN levels, the samples were analysed following the AOAC official method 985.18.
150 To this end, 50 g of the sample was mixed with 25 g of diatomaceous earth sorbent and 20 ml of water and

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151 rotated until mixed. The sample was extracted with 250 ml of chloroform for 30 min in an orbital shaker. After
152 filtration through a Whatman paper no 4, 50 ml of the extract was transferred to a separatory funnel and mixed
153 with 10 ml of a saturated NaCl solution and 50 ml of NaOH solution (2%w/v), shaking vigorously for 5 min. the
154 lower layer and the sediment were discarded and the aqueous layer was re-extracted with 50 ml of chloroform
155 and shaken for 1 min. After discarding the organic (lower) layer, the aqueous phase was treated with 50 ml of a
156 citric acid solution (106 g l^{-1}) and mixed with 50 ml of dihloromethane, shaking for 5 min. The lower phase was
157 drained through 40 g of anhydrous Na_2SO_4 with glass wool ball and the aqueous phase was re-extracted with 50
158 ml of dichloromethane. The organic layer was drained through the anhydrous Na_2SO_4 that was washed with 10-
159 15 ml of dichloromethane to avoid analyte losses. The collected sample solution was evaporated to dryness in
160 rotary evaporator and redissolved in 4 ml of dichloromethane. The extract was evaporated to dryness under
161 nitrogen and dissolved in 500 µl of mobile phase for HPLC analysis.

162 *HPLC conditions*

163 The determination of ZEN was done using a Waters (Milford, MA, USA) chromatograph with a
164 reverse phase C18 silica gel column (Water Spherisorb 5 µm ODS2 4.6 x 150 mm, Milford, MA, USA),
165 followed by fluorescence detection (λexc 274 nm; λem 455 nm) (Waters 2475 fluorescence detector, Waters,
166 Milford, MA, USA). The mobile phase was constituted with acetonitirle/MilliQ water (60:40) and the pH was
167 adjusted at 3.2 with acetic acid. The injection volume was 100 µl and a flow rate of 1 ml min^{-1}. The elution time
168 was 5.1 min and the column temperature was 40 °C.

169 ***Method of validation***

170 The method of validation involves the determination of different parameters which are: linearity, limit
171 of detection (LOD), limit of quantification (LOQ), repeatability, reproducibility, retention time and extraction
172 recoveries (Thompson et al., 2002). Linearity was done by injecting triplicate AFB1 and OTA standard
173 solutions at 0.5, 1, 5, 10, 50, 100 and 150 ng ml^{-1} and ZEN standard solutions at 50, 100, 150, 200, 250, 300,
174 350 and 400 ng ml^{-1}. The limit of detection (LOD) and the limit of quantification (LOQ) were determined by
175 taking 3.3 and 10 times the standard deviation, respectively, divided by the slope of the curve calibration of each
176 toxin. The slope and the standard deviation were calculated from the linearity line of the standard solutions.
177 Samples analysed previously by HPLC method, which shown no mycotoxins contamination, were used for
178 extraction recoveries and precision calculation. Extraction efficiency was tested by extracting blank samples
179 spiked at 0.5, 5 and 10 µg kg^{-1} of AFB1 and OTA and at 25, 50 and 100 µg kg^{-1} of ZEN concentrations.
180 Recoveries were calculated by comparison between the final concentration of mycotoxin in a spiked sample

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after quantification by HPLC and the known initial concentration. Precision was calculated in terms of intra-day repeatability RSDr (n=3) and inter-day reproducibility RSDR (5 different days) at 0.5, 5 and 10 µg kg^{-1} spiking levels for AFB1 and OTA and 25, 50 and 100 µg kg^{-1} spiking levels for ZEN. Retention time varies depending on pH, mobile phase composition, flow rate, pressure and temperature. The standard deviation of the 5 measurements carried out for each spiked sample makes it possible to evaluate the dispersion of the measurements around the mean concentration (Zinedine, 2004). The coefficient of variation is used to check the reproducibility of the method for the spiked samples (Zinedine, 2004):

$$Cv = \frac{\sigma}{Cm} \times 100$$

Peak identification was achieved with the retention times obtained after injection of each mycotoxin standard solution separately under the same conditions. Quantitative determination was accomplished by applying the calibration plots equations (y = ax + b).

Statistical analysis

Normal distribution of toxin contents, means, standard error and validation data were analysed by Statgraphics Centurion (XVII.I French). The calibration curve used for quantification was calculated by the least-squares method (least-124 squares method) and means comparison was made by ANOVA test. The differences in mycotoxins levels between the two countries were analysed by ANNOVA test ($P < 0.05$).

Results and discussions

Assay validation

The proposed HPLC methods enabled the AFB1, OTA and ZEN detection in analysed sorghum samples with high selectivity and sensibility. The methods proved to be rapid and reproducible. In-house performance characteristics were established; calibration curves were linear from 10 to 150 ng ml^{-1} for AFB1, from 25 to 150 ng ml^{-1} for OTA and from 200 to 400 ng ml^{-1} for ZEN. The limits of detection (LOD) and quantification (LOQ) of the three mycotoxins are presented in Table 1. Using the same method of simultaneous analysis of AFB1 and OTA in cereals, different LOD and LOQ values were found. Molinié et al. (2005) obtained LOD and LOQ OTA values equal to 0.05 and 0.2 µg kg^{-1}. While Nguyen et al. (2007) reported LOD and LOQ values of AFB1 equal to 0.07 and 0.22 µg kg^{-1}.

The use of H_3PO_4/ acetonitrile/ propanol-2-ol as a mobile phase allowed a good separation of AFB1 and OTA from the matrix compound with retention times of 14 and 56 min, respectively. The performance parameters of the two methods used are summarised in Table 2. The recovery rates (Rc) vary between 78.1% and 90.5% and within acceptable limits as set by the AOAC (70-125% at the 10 µg kg^{-1} level and 70-110% for a

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210 level of 10-100 µg kg^{-1}) (AOAC, 2002). The precision parameters RSDr and RSDR were relatively low and
211 between 0.07 and 7.39 and measurement uncertainty was in the range 0.09-9.60 µg kg^{-1}.

212 ***Incidence of AFB1, OTA and ZEN in sorghum samples***

213 The ANOVA analysis of the influence of the country of origin on the incidence and concentration of
214 mycotoxins in the samples studied showed that there is no significant difference ($P > 0.05$) between the two
215 batches of samples for each of the three mycotoxins studied. The values of the incidence of each mycotoxin, the
216 sample frequencies exceeding the maximum limits set by the EC, the concentration ranges and the mean values
217 of the mycotoxin contents are summarised in Table 3.

218 Among sixty four (64) samples, thirty eight (38) sorghum samples (59.4%) were found contaminated
219 with AFB1 in the range from 0.03 to 31.7 µg kg^{-1} and an average value of 1.71±0.57 µg kg^{-1}. Among them, only
220 9 samples (14.1%) contained AFB1 above the maximum limit set by the European Commission (2 µg kg^{-1})
221 (European Commission, 2006). Our results were in agreement with those found by other researchers. Indeed,
222 Ghali et al. (2010) reported that 73.4% of sorghum samples were contaminated with AFB1 with levels ranging
223 from 0.4 to 25.1 µg kg^{-1}. In Ethiopia, Ayalew et al. (2006) showed that 6.1% of sorghum samples are AFB1
224 positive up to 26 µg kg^{-1} with an average concentration of 5.9 µg kg^{-1}. Da Silva et al. (2000) found that 12.8% of
225 sorghum grain samples from Brazil were contaminated with AFB1 at levels ranging from 7 to 33 µg kg^{-1} and
226 only 4.5% of the total samples exceeded the maximum level for aflatoxins in Brazil (20 µg kg^{-1}). Very high
227 concentrations of AFB1 were observed in freshly harvested sorghum samples from West Africa ranging from
228 1.6 to 90 µg kg (Bandyopadhyay et al., 2007). Similarly, Ghali et al. (2009a) observed high levels of AFB1 up
229 to 52.9 µg kg^{-1} in Tunisian sorghum. However, relatively much lower levels of AFB1, ranged between 0.02 and
230 8 µg kg^{-1}, have been observed in sorghum from several regions in Sudan (Elbashir & Ali, 2013). Similarly,
231 Oueslati et al. (2014) reported a range of 0.07- 8.23 µg kg^{-1} AFB1 in sorghum grain samples purchased from
232 different markets in Tunisia. Ayejuyo et al. (2011) also reported a low range of 5.20 -6.25 µg kg^{-1} in sorghum
233 grains. Ratnavathi et al. (2012) reported that 73% of the samples are positive for AFB1, but only 0.75% of the
234 samples contain toxin above the maximum limit set by the Codex Alimentarus (20 µg kg^{-1}) (Codex Alimentarus
235 Commission, 1989). Other researchers have not detected AFB1, despite the isolation of *A. flavus* in the 46
236 samples of sorghum malt studied (Nkwe et al., 2005). Indeed, the incidence and the content of AFB1 in
237 sorghum samples vary from one year to year and from one region to another depending on climatic conditions
238 (Ratnavathi et al. (2012). Furthermore, aflatoxin B1 content increase in stored sorghum when compared to

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freshly harvested grain (Taye et al., 2016) especially when storage conditions are generally poor and lead to damage and predisposition of mould growth and mycotoxin production (Alborch et al., 2011).

Our results showed that OTA was detected in 37.5% of samples (24 of 64 samples) with concentrations ranging from 1.04 to 27.8 µg kg^{-1} and an average concentration of 1.85±0.55 µg kg^{-1}. Only 11 samples (17.2%) contain an OTA level above the maximum limit (3 µg kg^{-1}) (European Commission, 2006). Similarly, Ghali et al. (2009b) showed that 51% of the sorghum samples were contaminated with OTA with levels between 0.11 and 33.8 µg kg^{-1} and an average level of 5.4 µg kg^{-1}. The EU allows a maximum level of OTA of 3 µg kg^{-1} in cereals intended for human consumption (CEC, 2006). Nevertheless, much higher levels were found in some sorghum samples from Ethiopia with an average content of 174.8 µg kg^{-1} (Ayalew et al., 2006). Similar results were obtained by Zaied et al. (2009), who showed that 38% of the Tunisian sorghum samples are contaminated with OTA with an average concentration of 117 µg kg^{-1}. Maaroufi et al. (1995) showed the contamination of several food products frequently consumed in Tunisia by OTA. Whereas, Elbashir and Ali (2013) found a low incidence of OTA (3.3%) in sorghum and derived products from different parts of Sudan.

Our results showed that ZEN was detected in 32.8% (21 of 64 samples). The concentration of ZEN varies between 3.75 and 64.52 µg kg^{-1} and the mean value is 7.74±1.37 µg kg^{-1}. No sample was contaminated with a ZEN level above the maximum limit of 75 µg kg^{-1} as set by the EC (European Commission, 2006). There is very little published work on sorghum contamination by ZEN around the world. Shotwell et al. (1980) detected ZEN in 28% of the sorghum samples harvested in the USA at concentrations ranging from 200 to 6900 µg kg^{-1}. Bagneris et al. (1986) reported the presence of ZEN in the five samples analysed at concentrations of 47 to 1280 µg kg^{-1}. In Japan, the incidence of ZEN in sorghum is about 52.5% with grades between 60 and 7260 µg kg^{-1} (Aoyama et al., 2009). However, ZEN was not detected in sorghum samples from Sudan (Elbashir & Ali, 2013).

In some African countries, the consumption of sorghum-based alcoholic beverages is very common. Indeed, high ZEN frequencies have been reported in beer, must and malt produced from sorghum grains from Botswana (David et al., 2005). It seems that the fermentation process does not affect the contamination of sorghum products by the mycotoxins. In Brazil, AFB1 was detected in pre and post-fermented sorghum used for feeding beef cattle. The level of contamination increased significantly in sorghum samples after silage (Keller et al., 2012).

In this study, the most frequent mycotoxin was AFB1. However, Chala et al. (2015) and Ayalew et al. (2006) reported that ZEN was the most typical mycotoxin found in sorghum, followed by fumonisins and

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1
2
3 269 aflatoxins. Our results do not agree with those found by other researchers. Furthermore, mycotoxin content
4
5 270 varies with country of origin due to variable climatic conditions, mycobiota composition and agricultural
6
7 271 practices and genetic diversity between different varieties of sorghum grown around the world.
8
9 272 In this work, we have shown that AFB1, OTA and ZEN contaminate sorghum, and may also co-exist
10
11 273 because of the diversity of the fungal flora of this cereal (Table 4). Thus, 12.5% of the samples analysed are
12
13 274 contaminated by the three mycotoxins. Moreover, previous work has also shown the co-occurrence of AFB1,
14
15 275 OTA and ZEN in sorghum grain samples. Ghali et al. (2008) found that 58.8% of sorghum samples contained
16
17 276 AFB1 at 1.5 to 41.4 $\mu g\ kg^{-1}$ and an average of 11.6 $\mu g\ kg^{-1}$, 52.9% of samples of sorghum contain OTA with
18
19 277 levels ranging from 2.5 to 36.4 $\mu g\ kg^{-1}$ and an average concentration of 14.4 $\mu g\ kg^{-1}$. These same researchers
20
21 278 showed that only 23.5% of sorghum samples are contaminated with ZEN in a range of 7.3 to 14 $\mu g\ kg^{-1}$ and an
22
23 279 average of 10.9 $\mu g\ kg^{-1}$. They also reported that the frequency of occurrence of AFB1, OTA and ZEN in
24
25 280 sorghum is 23.5%. The co-occurrence of AFB1, OTA and ZEN can occur in other food commodities such as
26
27 281 barley, spices and wheat (Ghali et al., 2008). Sangare-Tigori et al. (2006) showed that 86% of Côte d'Ivoire's
28
29 282 rice, maize and groundnut samples are contaminated with AFB1, OTA, ZEN and fumonisin B1.
30
31 283 **Conclusion**
32
33 284 The two methods used for HPLC extraction and analysis of AFB1, OTA and ZEN have been shown to
34
35 285 be sensitive and reproducible for the detection and determination of these mycotoxins in sorghum grains. The
36
37 286 determination of the validation parameters showed good recovery rates for the three mycotoxins. The results
38
39 287 obtained showed that sorghum marketed in the Tunisian market is contaminated with AFB1, OTA and ZEN. We
40
41 288 noticed the co-occurrence of these three mycotoxins in some samples. Comparing our results with those
42
43 289 obtained by other researchers showed that the incidence and levels of mycotoxins in sorghum vary from country
44
45 290 to country and from year to year. This could be explained by variation in the composition of mycobiota
46
47 291 depending on climatic conditions, use of pesticides or genetic differences between varieties of sorghum. The
48
49 292 determination of the composition of the mycobiota of these sorghum samples makes it possible to identify the
50
51 293 species responsible for the production of the mycotoxins detected as well as their incidence and their capacity to
52
53 294 produce the toxins studied.
54
55 295 **Acknowledgments**
56
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58
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Table 1. LOD and LOQ of AFB1, OTA and ZEN

	AFB1	OTA	ZEN
LOD (μg kg^{-1})	0.008	0.03	3.1
LOQ (μg kg^{-1})	0.02	0.09	9.39

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Table 2. The values of the average recoveries, the precision parameters (RSDr and RSDR), the coefficient of variation and the measurement uncertainty of the analysis methods of AFB1, OTA and ZEN

Mycotoxin	Spiking level (μg kg^{-1})	Average recovery (%)	RSDr	RSDR	Cv	Measurement uncertainty (μg kg^{-1})
AFB1	0.5	79.7	0.08	0.06	20.01	0.11
	5	84.2	0.51	0.38	12.11	0.64
	10	87.0	0.62	0.62	7.12	0.74
OTA	0.5	84	0.07	0.07	16.66	0.09
	5	79.0	0.67	0.58	16.95	0.84
	10	90.5	0.52	0.5	5.74	0.65
ZEN	25	78.1	1.83	1.95	9.36	2.56
	50	77.9	2.77	2.52	7.11	3.22
	100	82.7	7.39	6.96	8.92	9.60

RSDR: inter-day reproducibility

RSDr: repeatability intra-day

Cv: coefficient of variation

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Table 3. Analytical results of sorghum samples of the region of Sahel in Tunisia

Mycotoxin	Origin	Incidence (%)	Range	Mean concentration	>limit (%)
AFB1	Egypt (27)	59.3 (16)	0.05-31.7	2.32±1.29	11.1 (3)
	Tunisia (37)	59.5 (22)	0.03-9.48	1.24±0.34	16.2 (6)
	Total (64)	59.4 (38)	0.03-31.7	1.71±0.57	14.1 (9)
OTA	Egypt (27)	40.7 (11)	0.14-16.44	2.06±0.79	22.2 (6)
	Tunisia (37)	35.1 (13)	1.04-27.8	1.71±0.76	13.5 (5)
	Total (64)	37.5 (24)	1.04-27.8	1.85±0.55	17.9 (11)
ZEN	Egypt (27)	29.6 (8)	6.08-31.18	6.62±1.42	0
	Tunisia (37)	35.1 (13)	3.75-64.52	8.56±2.15	0
	Total (64)	32.8 (21)	3.75-64.52	7.74±1.37	0

>limit: Incidence of positive samples exceeding the EC limit

Range and Mean concentration: µg kg^{-1}

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Table 4. Co-occurrence of AFB1, OTA and ZEN in sorghum samples

	AFB1-OTA (%)	AFB1-ZEN (%)	OTA-ZEN (%)	AFB1-OTA-ZEN (%)
Egypt	37.0	18.5	11.1	11.1
Tunisia	34.3	27.0	16.2	13.5
Total	29.7	23.4	14.1	12.5

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