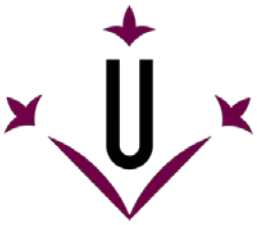

Hydrolysers of modified mycotoxins in maize: α-amylase and cellulase induce an underestimation of the total aflatoxin content.

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Abstract:

Aflatoxins are the most potent genotoxic and carcinogenic mycotoxins. To date, research only focused on the presence of free aflatoxins in agricultural commodities. Therefore, the main objective of this study was to investigate the occurrence of possible modified aflatoxins in maize. Different hydrolysis methods were applied to convert modified mycotoxins into their free aflatoxins. Eighteen aflatoxin-contaminated maize samples were incubated with potassium hydroxide, trifluoromethanesulfonic acid and several enzymes to induce hydrolysis. Potassium hydroxide caused a total reduction of aflatoxins, while trifluoromethanesulfonic acid did not lead to an increase of free aflatoxins, neither treatment with protease. However, α-amylase and cellulase incubation caused significant increases of the total free aflatoxin content, 15±8% and 13±5%, respectively. These results show that a small proportion of aflatoxins could be associated to matrix substances in plants. Consequently, hydrolysis could occur during food processing and during mammalian digestion, leading to an underestimation of the total aflatoxin content.

Highlights

- Treatment under alkaline conditions caused a total reduction of aflatoxins.
- Trifluoromethanesulfonic acid conditions did not cause an increase of aflatoxins.
- Protease treatment did not change the aflatoxins content.
- Treatment with α-amylase and cellulase increased the aflatoxins content.
- Matrix-associated aflatoxins are embedded *in planta*.

Keywords

Aflatoxins, modified aflatoxins, cereals, matrix, modified mycotoxins

1. Introduction

Mycotoxins are toxic secondary metabolites produced by a variety of filamentous fungi and the most important mycotoxins are produced by species of the genera *Aspergillus*, *Fusarium*, *Alternaria* and *Penicillium* (Pitt, Basilico, Abarca, & Lopez, 2000). Fungal contamination and consequent mycotoxin production in agricultural commodities may occur under pre- or postharvest conditions (*e.g.* storage) (Moss, 1992). Worldwide, cereal-based crops are spoiled by toxigenic moulds and the mycotoxins they produce (Bennet & Klich, 2003). This kind of food spoilage not only reduces the amount of the available food for consumers, but also adversely affects the ability of countries to trade with the rest of the world (Otsuki, Wilson, & Sewadeh, 2001).

Aflatoxins (AF) show many harmful effects on human health, and are the most potent genotoxic and carcinogenic mycotoxins (Creppy, 2002). AF cause hepatocellular cancer being together with fumonisins related to stunting in African children (Peraica, Radic, Lucic, & Pavlovic, 1999; Reports, 2015). Acute AF mycotoxicosis, leading to human death, has repeatedly occurred in Sub-Saharan Africa(Probst, Njapau, & Cotty, 2007), however is rather rare in other parts of the world. Acute aflatoxicosis results in direct hepatocellular damage and subsequent death, nevertheless, chronic sub-symptomatic exposure is of more concern. The four main AF, namely aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2), are therefore classified in Group 1 by the International Agency for Research on Cancer (IARC, 2010).

AF occur in a wide range of staple crops and raw food commodities including cereals, nuts, spices, figs and dried fruits. The presence of AF in cereals such as maize has been extensively described (Andrade & Caldas, 2015; Hove, Van Poucke, Njumbe-Ediage, Nyanga, & De Saeger, 2016; Huong, Tuyen, Do, Madsen, Brimer, & Dalsgaard, 2016; Jager, Tedesco, Souto, & Oliveira, 2013). Weather extremes associated with climate change further deteridorate and complicate the situation on AF occurrence(Medina, Rodriguez, & Magan, 2014; Miraglia, Marvin, Kleter, Battilani, Brera, Coni, et al., 2009). As a result of changing weather conditions, shifts in the fungal population and mycotoxin profile have been observed in Southern Europe(Battilani, Toscano, van der Fels-Klerx, Moretti, Leggieri, Brera, et al., 2016; Miraglia, et al., 2009; van der Fels-Klerx & Stratakou, 2010). *Aspergillus* spp. are typically seen in (sub)tropical regions. Until a decennia ago, these species were not observed in Europe or in more temperate areas. However, since the 2000s, an increased occurrence of *Aspergillus flavus* and consequent AF incidence was observed(Medina, Rodriguez, & Magan, 2014). In 2003 and 2008, AF outbreaks have been repeatedly reported in Italy, and in 2013 a serious outbreak of AF contamination in maize occurred in Balkan regions(de Rijk, van Egmond, van der Fels-Klerx, Herbes, de Nijs, Samson, et al., 2015).

AF are the most investigated mycotoxins worldwide because of their impact on human and animal health, therefore, monitoring programs especially focus on the occurrence of these free mycotoxins, AFB1, AFB2, AFG1 and AFG2. Nonetheless, these free mycotoxins might not be the only hazard for consumers' health. Modified mycotoxin forms are present in foods, and are

not detected during routine mycotoxin analysis. Contrary to the wealth of information on free AF, no data are available on modified AF. Moreover, a major concern and potential risk for consumers is the possible hydrolysis of modified AF into their toxic free forms during food processing and during mammalian digestion(Broekaert, Devreese, De Mil, Fraeyman, Antonissen, De Baere, et al., 2015; Gareis, Bauer, Thiem, Plank, Grabley, & Gedek, 1990; Nagl, Wöchtel, Schwartz-Zimmerman, Hennig-Pauka, Moll, Adam, et al., 2014).

Some of these modified forms are called matrix-associated mycotoxins (Rychlik, Humpf, Marko, Danicke, Mally, Berthiller, et al., 2014). This term includes forms either complexes with matrix compounds, are physically dissolved or trapped, or are covalently bound to matrix components.

To date, these modified mycotoxins are indirectly determined through hydrolysis in which derivatives are converted to their free mycotoxins(C. Dall'Asta, Galaverna, Aureli, Dossena, & Marchelli, 2008; C. Dall'Asta, Mangia, Berthiller, Molinelli, Sulyok, Schuhmacher, et al., 2009). Alkaline, acid or enzymatic treatments have been successfully used as a hydrolytic step to determine modified mycotoxins (C. Dall'Asta, et al., 2009); (Beloglazova, De Boevre, Goryacheva, Werbrouck, Guo, & De Saeger, 2013; Vidal, Ambrosio, Sanchis, Ramos, & Marin, 2016).

Mycotoxins can be present in modified forms, and could be hydrolysed to free mycotoxins, therefore, the objective of this study was to explore the presence of modified AF in foods through the application of several hydrolytic steps. Acid, alkaline and hydrolytic enzymes (protease, α-amylase and cellulase) treatments were applied to explore the total and modified AF content.

2. Materials & methods

2.1. Reagents and chemicals

The individual mycotoxin solid calibration standards (1 mg) of AFB1, AFB2, AFG1, AFG2 and zearalanone (ZAN) (internal standard) were obtained from Sigma Aldrich (Bornem, Belgium). All mycotoxin solid standards were dissolved in methanol (1 mg/mL), and were storable for a minimum of 1 year at - 18 °C. The working solutions of AFB1, AFB2, AFG1, AFG2 and ZAN (10 ng/µl) were prepared in methanol, stored at -18 °C, and renewed monthly. Water was obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). Disinfectol® (denaturated ethanol with 5 % ether) was supplied by Chem-Lab (Zedelgem, Belgium). Methanol (LC-MS grade) was purchased from BioSolve (Valkenswaard, the Netherlands), while acetonitrile (Analar Normapur) and ammonium acetate were obtained from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100 %) was supplied by Merck (Darmstadt, Germany). MultiSep® 228 AflaPat columns were purchased from Romer Labs (Tulln, Austria). Protease (*Aspergillus oryzae*, 500 U/g), α-amylase (*Aspergillus oryzae,* 30 U/mg) and cellulase (*Aspergillus niger*, 0.3 U/mg) were purchased from Sigma Aldrich (Bornem, Belgium), as TFMSA (≥ 99 %) and KOH (≥ 99 %). Sodium acetate buffer (SAB) was prepared with sodium acetate (27.21 g, ≥ 99 % from Sigma Aldrich), glacial acetic acid (6 mL) and Milli-Q water (994 mL), and the pH was adjusted to 5.0 with NaOH (10 M).

2.2. Collection of the cereal samples

A total of 18 AF-contaminated maize samples from different Nigerian fields were analysed. 3 kg of each sample was collected from Nigeria, the samples were hand-mixed, coarse grounded and allowed to pass through a No. 14 mesh screen. Sub-samples of 500 g were taken from each sample, ground with a milling machine (Greiffenberger, Germany) and sieved with 1-mm mesh. Sub-samples of 50 g were further taken from the lots into zip-lock envelopes and stored at freezing conditions (-20℃) until analysis to prevent moulding of the matrix.

2.3. Sample preparation and extraction

Each sample was ground (IKA® A11B basic analytical mill, IKA-Werke GmbH & Co. KG, Germany). After each milling step, cleaning and decontamination of the equipment was performed using water and bleach. The ground material was vigorously homogenised with a spatula before weighing. Then, the sample preparation process changed in function of the treatment (control, enzymatic, acid and basic).

Briefly, 2.5 g of the ground control samples were soaked with 7 mL of water. The acid treatment was performed according to Beloglazova et al. (2013)(Beloglazova, De Boevre, Goryacheva, Werbrouck, Guo, & De Saeger, 2013). Two and a half g of the ground sample were left for equilibration with 7 mL of TFMSA (0.025 M) for 13 h at 40 ºC in an incubation bath. The alkaline treatment was performed according to Dall'Asta et al. (2009)(C. Dall'Asta, et al., 2009). Shortly, 2.5 g of the ground sample were blended in an Ultraturrax T25 high-speed blender (IKA, Stauffen, Germany) with 7 mL of 5 M KOH for 10 min at 6000 rpm. The enzymatic treatments were performed according to Beloglazova et al. (2013) (Beloglazova, De Boevre, Goryacheva, Werbrouck, Guo, & De Saeger, 2013). Two and a half g of the ground sample were dipped with 7 mL of SAB, and the enzyme of interest (2 U/g of α-amylase from *Aspergillus oryzae*, 50 U/g of cellulose from *A. oryzae* and 3 U/g of protease from *A. niger*) was accurately added to the sample. The ground sample with buffer and enzyme were incubated overnight at optimum enzyme temperature: 37 °C for all of them.

After each treatment, 28 mL of acetonitrile/acetic acid (99/1, v/v) was added to all samples, resulting in a total volume of 35 mL. The samples were vigorously shaken for 60 min using the Agitator decanter overhead shaker (Agitelec; J. Toulemonde & Cie., Paris, France). After centrifugation (3,000 *g*, 15 min), the supernatant (32 mL) was evaporated to dryness (N_2, 40 ºC). Then, the residue was redissolved in 30 mL of acetonitrile/formic acid (99/1, v/v), and loaded on the MultiSep® 228 AflaPat column, and washed with 2 mL of acetonitrile/formic acid (99/1, v/v). The purified extracts were dried under a stream of nitrogen (N_2, 40 ºC). Finally, the dry residue was redissolved in 100 µL of injection solvent, consisting of water/methanol/acetic acid (57/42/1, v/v) and 5 mM ammonium acetate.

2.4. LC-MS/MS methodology

A Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA) was used to detect the mycotoxins in the samples. Data acquisition and processing was performed with MassLynx™ version 4.1 and QuanLynx® version 4.1 software (Waters, Manchester, UK). A ZORBAX Eclipse XDB C18-column (1.8 µm, 100×2.1 mm) was applied (Agilent Technologies, Diegem, Belgium). The mobile phase consisted of water/methanol/acetic acid (94/5/1, v/v (A)) and methanol/water/acetic acid (97/2/1, v/v (B)), both buffered with 5 mM ammonium acetate, at a flow rate of 0.3 mL/min. The gradient elution programme started at 70 % mobile phase A for 4.25 min. Then, the mobile phase B increased linearly to 99 % in 8 min. Mobile phase B was kept at 99 % for 2 min. The mobile phase linearly decreased till 30 % for 0.5 min. Mobile phase A (70 %) and mobile phase B (30 %) isocratically ran until 12 min. The duration of each HPLC run was 12 min, including reequilibration. The mass spectrometer was operated in the positive electrospray ionisation mode (ESI^+). The capillary voltage was 20 kV, and nitrogen was applied as spray gas. Source and desolvation temperatures were set at 120 °C and 400 °C, respectively. The argon collision gas pressure was 9×10^{-6} bar, the cone gas flow 50 L/h and the desolvation gas flow 800 L/h. Two selected reaction monitoring (SRM) transitions with a specific dwell time were chosen for each analyte, in order to increase the sensitivity and the selectivity of the mass spectrometric conditions. The SRM-transitions for each analyte are described in Table 1. The developed LC-MS/MS method was successfully validated based on Commission Regulation (EC) No. 401/2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs(401/2006/EC, 2006). Matrix-matched calibration plots were constructed for the determination of the analytes. ZAN was used as internal standard in the multi-mycotoxin analysis. Evaluating the linearity, the homogeneity of variance was checked before fitting the linear model. The linearity was interpreted graphically using a scatter plot. The obtained values were in conformity with the ranges set(401/2006/EC, 2006). The precision was calculated in terms of the relative standard deviation (RSD, %). Limit of detection (LOD, µg/kg) was calculated as three times the standard error of the intercept, divided by the slope of the standard curve; the limit of quantification (LOQ, µg/kg) was similar, differing by six times the standard error. The calculated LOD and LOQ were verified by the signal-to-noise ratio (s/n), according to the IUPAC guidelines(IUPAC, 1995). The results of the performance characteristics of the LC-MS/MS method were in good agreement with the criteria mentioned in Commission Regulation (EC) No 401/2006(401/2006/EC, 2006) (Table 2).

2.5. Statistical analysis

Data processing and calculations were performed using Microsoft Office Excel 2007 (Redmond, WA, USA) and IBM SPSS 19 (Armonk, NY, USA).

3. Results & Discussion

3.1. Aflatoxin content of maize samples

All selected maize samples (n=18) were positive for AFB1. The concentrations of AFB1 varied from 74 to 1820 µg/kg with an average concentration of 615 ± 563 µg/kg (Table 3). These concentrations are representative values for both low-risk (*e.g.* Northern Europe) and high-risk regions (*e.g.* Sub-Sahara Africa). All AFB2-contaminated samples contained AFB1, however, AFB2 concentrations were significantly lower (157 ± 165 µg/kg, max = 602 µg/kg). This phenomenon was also observed in other studies, and is explained by the fact that AFB2 is the dihydro-derivative of AFB1(Hove, Van Poucke, Njumbe-Ediage, Nyanga, & De Saeger, 2016; Rodrigues & Chin, 2012). The analysed samples in this study showed AFG1 and AFG2 were less common (< 40 %), nonetheless, they could be present in large concentrations as we found in one analysed sample (max. AFG1 = 3817 µg/kg). Similar to AFB2, the average concentration of AFG2 (49 ± 57 µg/kg) was lower than AFG1 (1037 ± 1557 µg/kg), because AFG2 is the dihydro derivative of AFG1. AFB1 was more present than AFG1 (89 %), however, larger concentrations of AFG1 than AFB1 were observed. Contrary, the concentration of AFB1 is commonly larger than AFG1 (EFSA, 2007). The ratio [AFB1]/[AFG1] varies depending on the temperature as AFG1 is produced at a lower growth temperature(Lin, Ayres, & Koehler, 1980). The higher incidence of AFG1 could be attributed to the probable use of different storage conditions (Matumba, Sulyok, Njoroge, Ediage, Van Poucke, De Saeger, et al., 2015).

All the analysed cereals (100 %) contained AF levels higher than the maximum level according to the European Commission Regulation (1881/2006/EC, 2006). The European Commission has set maximum permitted levels in AF in cereals are 2 µg/kg for AFB1, and 4 µg/kg for the total sum of AF, and in maize without processing 5 µg/kg for AFB1 and 10 µg/kg for the total sum of AF.

Although most of the studies reported contamination levels below 10 µg/kg in African countries (Fandohan, Zoumenou, Hounhouigan, Marasas, Wingfield, & Hell, 2005; Mukanga, Derera, Tongoona, & Laing, 2010; Nyagui et al., 2016; Rodrigues & Chin, 2012; Rodrigues, Handl, & Binder, 2011), many reports indicate levels higher than the European maximum limit. Matumba et al. (2013) investigated Malawian maize samples, and detected levels over 592 µg/kg of AFB1 (Matumba, et al., 2015). Rodrigues et al. (2011) detected high levels of AF in maize from different countries, with a maximum detected level of 556 µg/kg of AF (Rodrigues & Chin, 2012). The high levels of AF detected in the selected control samples were ideal to explore the incidence of modified AF.

3.2. Alkaline treatment

AF concentrations were drastically reduced after treatment with KOH (pH = 12) and all the AF concentrations were < LOD (Fig. 1) thus the final concentration was different to the other treatments ($p<0.05$). AF are proven sensitive to pH variations(Lee, Her, & Lee, 2015)'(Saalia & Phillips, 2010). The food industry benefits from this phenomena, and alkalinisation is therefore widely used in food processing. For instance, an alkaline environment is generated in tortilla-processing during nixtamalization, when pH values over 10 are generated. Some studies showed the total reduction of AF (100 %) during tortilla processing (Moreno-Pedraza, Valdes-

Santiago, Hernandez-Valadez, Higuera, Winkler, & Guzman-de Pena, 2015). The results in this study (pH = 12) agree with the AF reduction in comparison to the nixtamalization pH (pH = 10). The reduction of AF after KOH treatment should result in AF degradation products. Although we did not investigate for degradation products after the alkaline treatment, two unknown degradation products from AFB1 have been detected after nixtamalization (Moreno-Pedraza, Valdes-Santiago, Hernandez-Valadez, Higuera, Winkler, & Guzman-de Pena, 2015). Up to now, no studies were performed to investigate on the toxicity and possible carcinogenicity of these degradation products.

KOH treatment is a valid method to detect and quantify modified mycotoxins from certain free mycotoxins, especially fumonisins and DON, however this is not the case for AF. Although the alkaline treatment did not result in the detection of possible conjugated AF, KOH treatment permitted to detect hidden fumonisins in foods as in corn flour, snacks, bread, pasta and extruded products and they can represent more than 25 % of the total fumonisins (Chiara Dall'Asta, Galaverna, Mangia, Sforza, Dossena, & Marchelli, 2009; C. Dall'Asta, et al., 2009; Oliveira, Diel, Rauber, Fontoura, Mallmann, Dilkin, et al., 2015; Bryla, Roszko, Szymczyk, Jedrzejczak, & Obiedzinski, 2016). Furthermore, several food products with acceptable EU limits, were found contaminated above the limit when also the hidden forms were considered(C. Dall'Asta, et al., 2009). Some authors pointed out that hidden fumonisins could be embedded to starch or proteins of the matrix. Respecting DON, alkaline conditions cause a transformation of 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON) to DON(Malachova, Stockova, Wakker, Varga, Krska, Michlmayr, et al., 2015). These authors showed that 32 % of 3-ADON and 47 % of 15-ADON in flour matrix were hydrolysed to DON when samples were submitted to KOH treatment. This was caused by the presence of additional (unknown) sources of DON in the sample(Malachova, et al., 2015). Some DON could be hidden in starch or proteins of the matrix like fumonisins.

3.3. Acid treatment

TFMSA is one of the strongest known Brønsted acids (pKa = 13), characterized with high thermal stability and resistance towards reductive and oxidative cleavage. TFMSA has shown a better efficiency in the release of free mycotoxins than other similar acids such as trichloroacetic acid (TCA) and trifluoroacetic acid (TFA)(Tran & Smith, 2011). Similar AF concentrations in the analysed samples were obtained after TFMSA treatment (Table 3), so TFMSA did not cause any change in the AF content ($p>0.05$). The final average concentrations (average increase ± standard deviation compared to control) of the different AF analysed were: 607 µg/kg (-1.4 ± 1.9 %) for AFB1, 165 µg/kg (+4.6 ± 8.4 %) for AFB2, 1053 µg/kg (+1.5 ± 3.8 %) for AFG1 and 50 µg/kg (+2.7 ± 5.6 %) for AFG2. While, an alkaline environment caused AF reduction, an acid environment did not reveal changes in the AF stability. AF are reported to be stable at low pH(Rastegar, Shoeibi, Yazdanpanah, Amirahmadi, Khaneghah, Campagnollo, et al., 2017), however, acid conditions gave rise to the incidence of modified mycotoxins.

An increase of ZEN was detected after acid hydrolysis with TFMSA (0.025 M). The acidic environment hydrolysed (60 %) the glycosidic bond in ZEN-14-glucoside(Beloglazova, De Boevre, Goryacheva, Werbrouck, Guo, & De Saeger, 2013). In the same way, DON stability was studied, revealing a 70 % increase (Malachova, Dzuman, Veprikova, Vaclavikova, & Zachariaso, 2011; Tran & Smith, 2011)Tran et al. (2011)) also observed that the sum of acetylated DON was lower than the overall DON increase after TFMSA treatment. This increase could be caused by hidden DON in the food matrix, probably linked to starch or proteins(Tran & Smith, 2011). On the contrary, some mycotoxins are sensitive to low pH, and are reduced in acidic environments, such as nivalenol(Humer, Lucke, Harder, Metzler-Zebeli, Bohm, & Zebeli, 2016), beauvericin(Luciano, Meca, Manyes, & Manes, 2014) and enniatin(Garcia-Moraleja, Font, Manes, & Ferrer, 2015; Serrano, Font, Manes, & Ferrer, 2016). Although acid treatment can produce an increase of free mycotoxins due to hydrolysis of modified mycotoxins, AF were not affected by TFMSA incubation.

3.4. Enzymatic treatment

The total AF concentration after treatment with different common enzymes as protease, α-amylase and cellulase was investigated and the following sub sections describe the obtained results.

3.4.1 Protease

Proteases (also called proteinases) are enzymes that perform proteolysis, *i.e.* the hydrolysis of the peptide bonds that link amino acids together in a polypeptide chain. The AF concentration did not change after treatment with protease ($p>0.05$) (Table 3). The final concentrations for each AF were: 560 µg/kg (-8.9 ± 13 %) for AFB1, 150 µg/kg (-4.8 ± 5.3 %) for AFB2, 1104 µg/kg (+6.4 ± 7.8 %) for AFG1 and 53 µg/kg (+7.5 ± 11 %) for AFG2. Proteases are barely used in mycotoxin analysis. There are some studies revealing that proteases affect DON accumulation. Unlike the obtained results for AF, proteases caused an increase of the free mycotoxin concentration in DON-contaminated cereal samples(Simsek, Burgess, Whitney, Gu, & Qian, 2012; Vidal, Bendicho, Sanchis, Ramos, & Marin, 2016; Zhou, Schwarz, & He, 2008) with a maximum DON increase of 35 %(Vidal, Bendicho, Sanchis, Ramos, & Marin, 2016). These results showed that DON is probably linked to some proteins in the cell wall. During some food processing techniques these types of enzymes are used, leading to an increase of DON (*e.g.* in the bread making process to improve the bread quality). The absence of an AF increase after protease treatment proves that AF are not associated to proteins. Unfortunately, other free mycotoxins were not studied yet.

3.4.2. α-Amylase

α-Amylase is an enzyme that hydrolyses α-bonds of large, α-linked polysaccharides, such as starch, and hydrolyses them into polymers composed of glucose units. α-Amylase is present in the human saliva, and has large applications in food industry. Significant increases for all

analysed AF were detected after α-amylase treatment. An increase of the total AFB1 content was estimated at 13 ± 9.5 %, for the total AFB2 17 ± 7.3 %, for the total AFG1 19 ± 9.6 %, and for the total AFG2 11 ± 6.9 % (Fig. 1). The standard deviations are quite high compared to the final increase. But the initial AF concentrations were very different and the initial mycotoxin concentration in hydrolysis may affect the percentage of increase, as initial mycotoxin concentrations affects the mycotoxin fate during food processing (Bergamini, Catellani, Dall'asta, Galaverna, Dossena, Marchelli, et al., 2010). The percentage of increase obtained during α-amylase was higher in AFB1, AFB2 and AFG1 compared with acid treatment ($p<0.05$) (Fig. 1). Thus, when a raw cereal sample is monitored for the AFB1-concentration, and an α-amylase-based treatment is followed, a false negative result will be obtained: *e.g.* AFB1-result raw cereal sample 1.9 µg/kg – AFB1-result α-amylase-processed cereal sample 2.2 µg/kg (AFB1 maximum limit 2.0 µg/kg). This sample should be withdrawn from the market, as it does not comply with the European Regulation(1126/2007/EC, 2007), however, based on the acquired result of the raw cereal sample, the food will remain in the food supply.

The release of AF after α-amylase treatment agrees with other studies that α-amylase activity causes increases of the total mycotoxin amount (*e.g.* ZEN and DON). Regarding ZEN, α-amylase revealed a transformation of ZEN-14-glucoside to ZEN(Beloglazova, De Boevre, Goryacheva, Werbrouck, Guo, & De Saeger, 2013). AF glucoside forms have not been detected, but the slight increase of total AF could be caused by conjugation. In the same way, the total DON amount increased by the incubation with α-amylase, and although DON glucoside forms have been identified, the DON variation was not be linked(Kostelanska, Hajslova, Zachariasova, Malachova, Kalachova, Poustka, et al., 2009; Zachariasova, Vaclavikova, Lacina, Vaclavik, & Hajslova, 2012). α-Amylase caused an increases in the total DON concentration (> 20 %)(Simsek, Burgess, Whitney, Gu, & Qian, 2012; Vidal, Ambrosio, Sanchis, Ramos, & Marin, 2016). Contrary to ZEN-14-glucoside, the DON-3-glucoside increased by the presence of α-amylase, and larger amounts of DON (up to 500 %) were observed(Vidal, Ambrosio, Sanchis, Ramos, & Marin, 2016). The increase of DON during the α-amylase treatment could be attributed to the cleavage of glycosidic bonds between mycotoxins and cell polysaccharides(Kostelanska, Zachariasova, Lacina, Fenclova, Kollos, & Hajslova, 2011). This may also imply that mycotoxins could be more likely bound to starch and polysaccharides than other molecules. In conclusion, AF could be embedded to polysaccharides from the matrix, and could be released during enzyme treatment. This means that embedded AF could be released during food processing or mammalian digestion, and could produce a significant increase of AF exposure.

3.4.3. Cellulase

Cellulase is an enzyme which decomposes cellulose and some related polysaccharides. This enzyme is widely used in the food industry and is added to bread mainly for the improvement of the rheological properties of dough, bread loaf volume and crumb firmness. In our work, cellulase treatment caused increases of the total AF level. In detail, an increase of 15 ± 1.9 %

was obtained for AFB1, 17 ± 9.2 % for AFB2, 11 ± 5.4 % for AFG1 and 7.6 ± 3.5 % for AFG2 (Fig. 1). The percentage of increase obtained during cellulase treatment was higher in AFB1, AFB2 and AFG1 compared with acid treatment ($p<0.05$) (Fig. 1). Thus –as stated with α-amylase-, when a raw cereal sample is monitored for the AFB1-concentration, and a cellulase-based treatment is not followed, a false negative result will be obtained in the samples with a concentration close to maximum limit: *e.g.* AFB1-result raw cereal sample 1.9 µg/kg – AFB1-result cellulase-processed cereal sample 2.2 µg/kg (AFB1 maximum limit 2.0 µg/kg). This sample should be withdrawn from the market, as it does not comply with the European Regulation (1126/2007/EC, 2007), however, based on the acquired result of the raw cereal sample, the food will remain in the food supply.

The relation among cellulase and free mycotoxins has been investigated in few studies, and analogous results to α-amylase were obtained. Firstly, cellulase is able to cleave all ZEN-14-glucoside after 10 hours of treatment, and transform ZEN-14-glucoside to ZEN(Beloglazova, De Boevre, Goryacheva, Werbrouck, Guo, & De Saeger, 2013). Regarding DON, cellulase also releases DON from wheat during the bread making process. Although the detected increases of total DON due to cellulase use were similar among them (26 % (Simsek, Burgess, Whitney, Gu, & Qian, 2012; Vidal, Ambrosio, Sanchis, Ramos, & Marin, 2016), the AF increases in this study were slightly lower (12 %) than the increases detected in DON. As in α-amylase, DON-3-glucoside increases occurred during the bread making processes, and the DON concentration was not affected by this increase, confirming that DON and DON-3-glucoside are not linked, and are both embedded to the carbohydrates of the cereal matrix(Vidal, Ambrosio, Sanchis, Ramos, & Marin, 2016). Similar to DON, AF could be embedded in cellulose from the cell wall. This phenomenon is a problematic situation as cellulase is a common enzyme used in the food industry. The presence of cellulase could increase the total AF at the end of the food processing steps due to their ability of hydrolysation, and as a consequence worsening AF exposure.

The presence of modified mycotoxins in raw cereals is lower for AF compared to other mycotoxins. In raw cereals, free fumonisins could represent only 37 % of the total concentration of fumonisin(C. Dall'Asta, et al., 2009), or DON could represent 50 % of the total DON(Berthiller, Schuhmacher, Adam, & Krska, 2009; De Boevre, Vanheule, Audenaert, Bekaert, Diana Di Mavungu, Werbrouck, et al., 2014). The lower level of modified AF is probably attributed to the chemical structure of AF, and their formation during storage contrary to other pre-harvest mycotoxins *i.e.* DON, ZEN and fumonisins. Some of the most common conjugates found in foods originate from plants, mostly glucoside conjugates (DON-3-glucoside, ZEN-14-glucoside, T-2 and HT-2 glucoside …). On the other hand, matrix associations with mycotoxins could also occur in the field. Other mycotoxins produced during storage also have less conjugated mycotoxins such as OTA. Although, the lower incidence, modified AF represent an important issue as an underestimation of the total AF content could be present. Furthermore, the presence of modified mycotoxins in foods is of concern because they can be transformed to their free mycotoxins during food processing or during mammalian digestion (Nagl, et al., 2014). It is of crucial importance that more in-depth studies are performed to investigate modified AF.

4. Conclusions

KOH treatment is not a useful method to detect embedded AF in matrix, also TFMSA incubation did not cause an increase of total AF. Conversely, proteases did not produce any change in the AF concentration proving that AF are not associated to proteins from the cell wall. This study reports that AF could be associated to carbohydrates from the matrix as α-amylase and cellulase caused significant increases of the total AF content. The control of the (modified) AF content in foods is imperative due to its carcinogenic property, but also due to the expected increase of AF in the coming years in terms of climate change, especially in temperate zones as Europe(Medina, Rodriguez, & Magan, 2014). Although low levels of modified AF were found in our samples, more in-depth research is necessary to protect consumers' exposure to these carcinogens. This report involves the presence of possible matrix-associated AF in highly contaminated samples. This is the first study describing the problematic issue of modified AF, and more research is needed to confirm the statements made in this report, and to identify possible modified AF.

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Table 1. The optimized LC-ESI-MS/MS parameters for the confirmation and quantification of aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), aflatoxin B1 (AFB1), aflatoxin B2 (AFB2) and zearalanone (ZAN).

Mycotoxin	Precursor ion (m/z)	Product ions [a](m/z)	CE[a,b] (eV)	CV[c] (v)	Retention time (min)
AFG1	329.0	243.0/311.2	25/20	40	7.09
AFG2	331.0	313.1/245.2	25/30	53	6.73
AFB1	313.0	285.1/241.2	24/36	51	7.70
AFB2	315.0	287.2/259.2	27/30	51	7.42
ZAN	321.2	189.1/303.3	22/14	12	7.59

[a] Values are given in the order: quantifier ion/ qualifier ion
[b] CE: Collision energy
[c] CV: Cone Voltage
AFG1 = aflatoxin G1, AFG2 = aflatoxin G2, AFB1 = aflatoxin B1, AFB2 = aflatoxin B2 and ZAN = zearalanone.

611 Table 2. Validation parameters for the analyzed mycotoxins in maize.

Mycotoxin	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	Calibration Range (µg/kg)	R[c](mean)	SE[d]	Apparent recovery (%)	RSD_r[e] (%)	RSD_R[f] (%)	U[g] (%)
AFB1	10	20	25-400	0.97	0.005	95	9	18	19
AFB2	13	26	25-400	0.97	0.011	97	8	11	19
AFG1	10	20	25-400	0.97	0.005	95	12	20	20
AFG2	10	20	25-400	0.97	0.008	98	10	16	28

612 [a] LOD = Limit of detection.
613 [b] LOQ = Limit of quantification.
614 [c] R = Pearson's correlation coefficient.
615 [d] SE = Standard error of mean.
616 [e] RSD_r = relative standard deviation intra-day precision.
617 [f] RSD_R = relative standard deviation inter-day precision.
618 [g] U = measurement uncertainty.
619 AFB1 = aflatoxin B1, AFB2 = aflatoxin B2, AFG1 = aflatoxin G1 and AFG2 = aflatoxin G2.

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Table 3. Number of positive samples (n), average concentration ± standard deviation (SD) (ng/g) and range (ng/g) of aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) for all different treatments assayed (control, alkaline, acid, protease, α-amylase and cellulase).

Treatment	n (%)	Control		Alkaline		Acid		Protease		α-amylase		Cellulase	
		Average±SD (ng/g)	Range (ng/g)	Average±SD (ng/g)	Range (ng/g)	Average±SD (ng/g)	Range (ng/g)	Average±SD (ng/g)	Range (ng/g)	Average±SD (ng/g)	Range (ng/g)	Average±SD (ng/g)	Range (ng/g)
AFB1	19 (100.0)	615 ± 563	73-1820	< LOD	< LOD	606 ± 575	68-1861	560 ± 574	56-1791	695 ± 551	92-2204	706 ± 590	95-2248
AFB2	19 (100.0)	158 ± 165	9-602	< LOD	< LOD	165 ± 149	9-58	150 ± 160	9-582	185 ± 176	15-784	184 ± 173	17-819
AFG1	7 (36.8)	1037 ± 1557	7-3817	< LOD	< LOD	1052 ± 1594	6-3854	1104 ± 1548	7-3842	1229 ± 1594	9-4627	1150 ± 1581	12-4780
AFG2	6 (31.6)	49 ± 57	2-138	< LOD	< LOD	50 ± 61	2-161	53 ± 55	2-154	54 ± 47	3-176	53 ± 51	4-188

AFB1 = aflatoxin B1, AFB2 = aflatoxin B2, AFG1 = aflatoxin G1 and AFG2 = aflatoxin G2, n = number of samples; LOD = limit of detection and SD = standard deviation.

Figure 1. Average increase (%) of aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) for all different treatments assayed (control, alkaline, acid, protease, α-amylase and cellulase).

Bars mean standard deviation.

Different letters mean significant statistical difference ($p<0.05$) compared with the other AF for the same treatment .