In vitro models to evaluate the capacity of different sequestering agents to adsorb aflatoxins

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Abstract

Eight potential aflatoxin-sequestering agents (SAs) were tested for their ability to adsorb aflatoxin B₁ (AFB₁) and aflatoxin G₁ (AFG₁) in vitro. They belong to main SA classes: silicate minerals (calcium, magnesium and sodium bentonites, kaolinite, zeolite and clinoptilolite), activated carbon and yeast cell wall-derived. The AFB₁ and AFG₁ used in present work were extracted from a contaminated corn meal (82.21 mg/kg of AFB₁ and 97.20 mg/kg of AFG₁). Three single-concentration adsorption tests, consisting of a simply-water (W), a gastro-intestinal simulating monoagastric model (MM) and a ruminant model (RM) were used. The methods differed for dilution media, incubation steps and pH condition in which they were conducted. In particular, one step (2h at 39°C) at pH 7 for W; two steps (4h at 39°C) at pH 2 and 7 for MM; and a pre-incubation in rumen fluid (pH 7 for 2h at 39°C) + two steps (4h at 39°C) at pH 2 and 7 for RM, characterized each method. The AFB₁:SA ratio (g/g) and dilution factor (ng of incubated AFB₁:m L of volume) were chosen (1:500,000 and 4.1, respectively) to reflect field conditions. The AFB₁ and AFG₁ recovered in controls were 92.3% and 104.9% in W and 89.5% and 101.5% in MM; while in RM were 65.2% and 81.9%; respectively. This supported the idea of intrinsic rumen fluid factors could be involved in sequestering of aflatoxins. In the present study, three SAs (activated carbon, Mg bentonite and Na bentonite) were very efficient to sequester the available AFB₁, with a sequestering activity of over 99.0% with each method. The Ca bentonite and clinoptilolite were able to bind available AFB₁ in MM and RM methods, while they appeared inefficient (available AFB₁ sequestered less than 80%) when W was used. The adsorption ability of zeolite was confirmed only with the W method. Ineffective or limited sequestering activity were obtained with kaolinite and yeast cell wall-derived products with each method. The AFB₁ and AFG₁ sequestering efficiencies observed in the present work resulted very similar showing strong and positive correlation (P<0.001) within methods (r=0.79, r=0.96 and r=0.99, respectively for W, MM and RM methods). The two simulated gastrointestinal methods (MM and RM, respectively) gave similar results and could be considered useful for in vitro pre-screening of potential sequestering agents. However, the major practical and analytical implications related to rumen fluid method suggested that MM method should be used.

Introduction

Aflatoxins are hepatocarcinogenic molecules (IARC, 2002) produced mainly by Aspergillus flavus and A. parasiticus either in field, storage or transport of many crops (Scheidegger and Payne, 2003). The primary aflatoxins produced by these fungi are aflatoxin B₁ (AFB₁), aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), and aflatoxin G₂ (AFG₂).

The AFB₁, the most toxic and carcinogenic aflatoxin (Roebeck and Maxuitenko, 1994), once ingested by dairy animals is rapidly absorbed in the gastro-intestinal tract, and rapidly appears in blood (Gallo et al., 2008) and in milk as aflatoxin M₁ (AFM₁), the principal AFB₁ milk metabolite (Veldman et al., 1992; Battacone et al., 2003; Masoero et al., 2007). The AFB₁ carry over (CO) rate into milk as AFM₁ has been determined to range from 1% to 3% in lactating dairy cows and to be principally affected by milk yield (Diaz et al., 2004; Van Eijkeren et al., 2006; Masoero et al., 2007), with a reported maximum value of about 6% (Veldman et al., 1992).

These findings supported the European Union (EU) decision to fix limits for AFB₁ in animal feeds at 20 µg/kg and in complete feedstuffs for dairy animals at 5 µg/kg (European Commission, 2003). In milk, the EU set the AFM₁ maximum permitted level at 0.050 µg/kg (European Commission, 2006).

A practical approach to control aflatoxicosis in livestock is the addition of aflatoxin-sequestering agents (SA) to diet (CAST, 2003). These compounds are effective to sequester toxin molecules, such as aflatoxins, forming a complex in the gastrointestinal tract (Jouany, 2007; Masoero et al., 2009) through electronic charges, hydrogen bonds, Van der Waals bonds and physical capture (Phillips et al., 1990; Diaz and Smith, 2005; Yiannikouris et al., 2005; Phillips et al., 2008). Besides, they are added to animal diet in small amount and are impurities-, flavour- and odours-free (Diaz and Smith, 2005; Jouany, 2007).

A variety of SAs has been tested and currently traded. Some of the most commonly used SAs belong to three different groups: silicate materials or clay minerals (Phillips et al., 1991; Ramos and Hernandez, 1996; Rao and Chopra, 2001; Diaz et al., 2003; Jouany, 2007), yeast cell wall-based products (Karaman et al., 2005; Yiannikouris et al., 2005) and activated carbons (Galvano et al., 1996; Rao and Chopra, 2001; Diaz et al., 2003; Diaz and Smith, 2005).

Several in vitro adsorption tests have been proposed to pre-screen SAs before their use in vivo. Differences in the findings of in vitro studies are based upon the tested aflatoxins (crystalline pure AFB₁, or extracted from natural contaminated feeds), the dilution factor (AFB₁ concentration, w/v), the AFB₁:SA ratio (ww), the pH conditions and the media in which adsorption test was conducted (Ramos and Hernandez, 1996; Grant and Phillips, 1998; Ledoux and Rottinghaus, 1999). Moreover, these methods often oversimplify the gastrointestinal conditions, such as pH, temperature and interaction with biological fluids (Lemke et al., 2001; Avantaggio et al., 2003; Moschini et al., 2008).

The objective of this work was to evaluate the efficacy of different typologies of SAs (mineral clays, activated carbon and yeast cell wall-based product) to adsorb AFB₁ and AFG₁, either in aqueous or in simulated monogastric/ruminant gastrointestinal fluids.
Materials and methods

Eight potential-aflatoxin SAs were evaluated for their capability to sequester aflatoxins in vitro. The SAs were: magnesium bentonite (Grupo Tolsa, Madrid, Spain); calcium bentonite (Tecnocozzo, Padova, Italy); sodium bentonite (Amcol International Corp., Arlington Heights, IL, USA); kaolinite (Fluka 03584, Sigma-Aldrich Chemie GmbH, Switzerland); zeolite (Fluka 96096, Sigma-Aldrich Chemie GmbH, Switzerland); clinoptilolite (Tecnocozzo, Padova, Italy); yeast cell wall-based product (Mycosorb®, Alltech Italy, Bologna, Italy), and activated carbon (Acque Nymco, Milan, Italy). Only some of these products (i.e., magnesium bentonite, calcium bentonite, clinoptilolite and yeast cell wall-based product) are specifically marketed as mycotoxin SA by the producers.

Chemicals

Solvents used were of grade ACS-ISO. Acetonitrile, acetone, chloroform and methanol were HPLC grade (purity 99.5%, 99.0%, 99.0% and 99.9%; respectively) from Merck (Darmstadt, Germany). Purified water was obtained from a Milli-Q Gradient A10 water purification device (Millipore, Bedford, MA, USA). Phosphate-buffered saline (PBS) was prepared by dissolving 0.20 g KCl, 0.20 g KH₂PO₄, 1.16 g anhydrous Na₂HPO₄ and 8.00 g NaCl in 1 L of water. The pH was adjusted to 7.4 with NaOH (0.1 mol/L).

Preparation of aflatoxin test solution (solution A)

Aflatoxins (AfB₁, and AfG₁) were extracted from a contaminated corn meal inoculated with the Aspergillus flavus strain MPV92 (Institute of Entomology and Plant Pathology, Università Cattolica del Sacro Cuore, Piacenza, Italy). The corn meal was incubated at 25°C and 0.99 water activity for 7 days, respecting the optimum mycotoxin production conditions (Giorni et al., 2007). The final aflatoxin concentrations of the corn meal were: 82.21±0.01 mg/kg for AfB₁ and 97.20±0.01 mg/kg for AfG₁.

A stock solution (solution A) of AfB₁ and AfG₁ was prepared by extracting aflatoxins from 1 g of the contaminated corn meal using a water/methanol solution (80:20, v/v) in ratio of 1:100 (g/mL), at room temperature and in agitation (150 shake/min) for 120 min. The obtained solution A concentrations were 0.821 µg/mL for AfB₁ and 0.974 µg/mL for AfG₁, respectively.

Single concentration adsorption tests

A simply-water (W), a gastro-intestinal simulating monogastric model (MM) and a ruminal model (RM) were used to test the sequestering capacity of the different SAs. The W and MM were performed in agreement with Lemke et al. (2001), while the RM consisted of a pre-incubation with rumen fluid (Moschini et al., 2008), followed by incubation steps applied for MM model.

The three methods differed for the dilution media, incubation steps and the pH conditions in which the adsorption tests were conducted:

**W method:** one step in deionized water at 39°C for 2h;

**MM method:** two steps at 39°C for 4h in two buffers (pH 2 and 7) to simulate gastrointestinal conditions of monogastric;

**RM method:** one step in rumen fluid (pH 7 for 2h at 39°C) followed by the two MM steps (pH 2 and 7 for 4h at 39°C), to simulate the ruminant digestible tract.

The pH 2 buffer used in the first step of MM method was prepared in agreement with Lemke et al. (2001) as follows: 1.250 g/L of peptin enzyme, 0.500 g/L of citric acid monohydrate, 0.500 g/L of malic acid disodium salt, 0.050 mL/L of acetic acid and 0.042 mL/L of lactic acid. In the second step, the pH was increased up to 7 with sodium bicarbonate, followed by addition of pancreatin (20 mg/sample) and bile salts (70 mg/sample).

The RM method consisted of a pre-incubation in rumen fluid. The rumen fluid was collected from two fistulated cows housed at the CERZOO research and experimental centre (San Bonico, Piacenza, Italy) before the morning meal (08.00), filtered with a two layers cheesecloth, stored at 39°C under CO₂ and used within 1 h. The measured pH of the rumen fluid was 7.0±0.3. After this step, HCI 1M was used to reach pH 2 in test tubes and 50 mg/sample of pepsin was added to each sample. The third step of RM method was carried out by increasing pH up to 7 with sodium bicarbonate followed by the addition of pancreatin (20 mg/sample) and bile salts (70 mg/sample).

The AF-B₅:SA ratio used in the current study were designed to be about 1:500,000 (w/w), in agreement with AF-B₅:SA ratio used in our previous work (Moschini et al., 2008). This ratio was chosen to reflect possible field conditions. In particular, dairy cows fed with an AFB₅ contaminated diet near to the EU limit of 5 µg/kg (EU, 2003) consumed, on average, 100-120 µg/head/day AfB₁ in presence of a SA dose of about 50-60 g/head/day, as suggested by the producers for on farm utilization. A similar approach could be used for swine fed with a diet containing 1% of a SA with an AfB₅ contamination of 20 µg/kg, representing the swine EU feedstuff limit (EU, 2003). The dilution factor, considered as the ratio between the amount of incubated AfB₅ (ng) and the total volume (mL) in which the absorption test was conducted, was similar for the three methods and equal to 4.1 (ng/mL), in agreement with Moschini et al. (2008).

Incubation procedure

Single SAs were weighted (82.0±0.1 mg) in 100-mL test tubes (Pyrex®, Barloworld Scientific Ltd., Staffordshire, UK) for each method (W, MM and RM). Then, 0.200 mL of solution A and 40 mL of the three media, precisely deionized water (W), pH 2 buffer (MM) or rumen fluid (RM), were added to each test tube and gently shaken for 5 minutes every 15 minutes. Each single-concentration adsorption test was repeated twice.

Control samples (n=3) for each method (W, MM and RM, respectively) were prepared by adding 0.200 mL of solution A and 40 mL of specific media into 100-mL test tubes without the addition of the SA.

At the end of incubations for all samples, the supernatant was removed after centrifugation (3500 x g for 15 min at 4°C) and then the precipitate washed twice with 10 mL of water. The recovered supernatant (about 30 mL) as a whole was analyzed by HPLC. In particular for RM samples, to measure AfB₁ and AfG₁, sequestered by rumen fluid, the precipitate of the controls were treated in agreement with Moschini et al. (2008) and analyzed for aflatoxin contents: the supernatant was removed and the precipitate was re-suspended in 10 mL chloroform before centrifugation. This step was repeated twice, and the recovered solution was analyzed by HPLC to measure the AfB₁ and AfG₁ contained in the rumen pellet of controls.

The sequestered aflatoxins (AfB₁ and AfG₁) by the SAs in each sample were calculated as percentage ratio between the aflatoxin quantity of supernatant and its amount in the control sample.

Animals

The ruminal fluid cow donors were fed on a diet formulated in agreement with dairy cow requirements (NRC, 2001) and based on grass hay (700 g/kg), corn silage (200 g/kg) and concentrate (100 g/kg) on a dry matter basis. The research protocols and animal care were performed in accordance with the EC council.
Aflatoxin and SAs analysis and apparatus

Aflatoxins (AfB₁ and AfG₁) were analyzed in agreement with Moschini et al. (2008). Briefly, 10 grams of dried contaminated corn meal were extracted with 100 mL of an acetonewater solution (85:15, v/v), shaken at 150 rpm for 45 min (Universal table Shaker 709) and filtered with Schleicher&Schuell 595.5 filter paper (Dassel, Germany). Elutions of 5 mL with 45 mL of bi-distilled water were performed and passed through an immuno-affinity column (Aflatoxin Easi-extract, Rhône diagnostics technologies, Glasgow, UK). The supernatant solution was passed through an immunoafinity column previously washed with 20 mL of PBS. The column was then washed with 5 mL water and slowly eluted with 2.5 mL of methanol. The extract was dried under nitrogen, re-dissolved in 1 mL of an acetonitrile:water (25:75, v/v) solution and filtered (Millipore Corporation, Bedford, MA, USA, HV 0.45 μm) prior to HPLC analysis for the AfB₁- and AfG₁-contents.

Analysis was performed using an HPLC instrument consisting of a LC-200 pump (Perkin Elmer, Norwalk, CT, USA) an AS-2055 sampling system, a FP-1520 fluorescence detector (Jasco Corporation, Tokyo, Japan), and a UV derivatizer (UVE TM derivatizer, LC tech, Dorfen, Germany); the instrument was controlled by Borwin 1.5 software (Jasco). A Superspher RP-18 column (4 μm particle size, 125x4 mm i.d., Merck) was used at room temperature with a mobile phase of water: methanol:acetonitrile (64:23:13, v/v/v) at 1 mL/min. The AfB₁- and AfG₁- were detected after post-column photochemical derivatization to AfB₁-N and AfG₁-N, respectively. The detector was set at 365 nm excitation and 440 nm emission wavelengths.

The elemental components of the SAs were determined by a semi-quantitative X-ray fluorescence analysis using the scanning electron microscope Phillips XL 30 E-SEM (Phillips electron Optics B.V., Eindhoven, Netherlands) equipped with an energy-dispersive X-ray detector model Genesis (Edax Inc., Mc Kee Drive, Mahwah NY, USA) operating in low vacuum. The SA samples were assayed in duplicate according to the AOAC (1990) to determine total ash content (procedure 942.05).

Statistical analysis

The in vitro data were analyzed using a complete randomized design and the general linear model of the SAS® (SAS Institute Inc., Cary, NC; version 9.1.3). A factorial arrangement was used and fixed effects of model included SA (n=8), method (n=3) and associated first order interaction. When data suggested the first order interaction, the SLICE option of LSMEANS statement was used to compare SAs within each tested method. The correlation between AfB₁ and AfG₁- sequestering efficiency was calculated with the Spearman rank correlation coefficient using PROC CORR of SAS (SAS Institute Inc., 2001) within each method. Significance was declared at P<0.05.

Results and discussion

The most used SAs belong to the silicate mineral, the activated carbon and the yeast cell-wall product (Diaz and Smith, 2005; Jouany, 2007) classes. The widest SA class is the silicate minerals, which could be grouped in phyllosilicate and tectosilicate sub-classes. The SAs belonging to phyllosilicate sub-class are montmorillonite/smectite, such as bentonite and kaolinite clays, while the tectosilicate sub-class includes zeolite and clinoptilolite clays (Diaz and Smith, 2005). In the present work, the tested clays had an ash content higher than 90% (Table 1). In particular for bentonite clays, the dominant cation characterized the type of bentonite, as confirmed by elemental chemical composition of magnesium bentonite (Mg 14.8%), calcium bentonite (Ca 14.8%) and sodium bentonite (Na 3.4%). Zeolite and kaolinite clays appeared different with respect to other clays having a similar Al and Si content (14.1% and 14.6% for zeolite and 18.6% and 21.0% for kaolinite, respectively). The high carbon content of activated carbon (89.6%) and yeast cell wall (61.4%) suggested an organic origin, even if the available analytical data can not exclude a possible clay presence, particularly for the yeast cell wall product.
Table 2. Aflatoxin B$_1$ (AfB$_1$) and aflatoxin G$_1$ (AfG$_1$) recovered in supernatants, in rumen pellet and as total in water, simulating gastro-intestinal monogastric model and ruminant total tract model methods in control samples.

| Method          | Supernatant | Rumen pellet | Total   |
|-----------------|-------------|--------------|---------|
| **AfB$_1$**     | 36.6 (0.18) | 28.6 (0.46)  | 65.2 (0.40) |
| **AfG$_1$**     | 55.6 (0.37) | 26.3 (0.18)  | 81.9 (0.54) |

In the present work, the average AfB$_1$ and AfG$_1$ recovered in control samples in absence of any type of sequestering agents were 92.3% and 104.9% in W method (Table 2), in accordance to our previous results (Moschini et al., 2008). Similar results were obtained in MM method (89.5% and 101.5%, respectively for AfB$_1$ and AfG$_1$), in agreement with Lemke et al. (2001). The acid condition (pH 2) in which the first step of MM method was conducted did not influence the amount of AfB$_1$ recovered, showing the absence of the reaction of AfB$_1$ to AfB$_2a$ (Vekiru et al., 2007). This could be related to the limited incubation time. The AfB$_1$ presence observed in control samples of W and MM methods determinate an AfB$_1$:SA ratio of about 1:5000, as designed before experiments.

In RM method, the aflatoxin determine in the supernatant of the control after the centrifugation of the rumen fluid media were 56.2% and 81.9%, respectively. Therefore, the AfB$_1$:SA ratio for RM method was different with respect to other methods, being about 1:750,000. This supports the idea that some rumen fluid components such as chlorophyllin, bacteria and yeast cell wall could be effective in sequestering aflatoxins (Breinholt et al., 1999; Peltonen et al., 2000; Diaz and Smith, 2005; Gratz et al., 2005; Oatley et al., 2005). In particular, Arimoto-Kohayashi et al. (1997) suggested that chlorophyllin structure could form a complex between porphyrin-like structure of chlorophyllin and the polycyclic structures of carcinogenic molecules, like AfB$_1$. Also specific cell wall components in yeast (Dawson et al., 2001) and bacteria, i.e. lactobacilli, interacted with mycotoxins forming a stable complex (Gratz et al., 2005; Oatley et al., 2005; Niderkom et al., 2009).

Upadhyay et al. (2009) recently reported a loss of AfB$_1$, ranging from 8 to 20% when in vitro incubated at different times (0, 3, 6, 9 and 12 h) with rumen fluids collected by goats and steers. These authors supposed that AfB$_1$ was degraded by rumen microflora. Niderkom et al. (2007), testing more than 200 bacterial strains to verify their ability to bind and/or biotransform mycotoxins (i.e., deoxynivalenol, zearalenon and fumonisin B$_1$ and B$_2$), reported that most strains were capable to bind Fusarium toxins. In particular, these authors indicated Streptococcus spp. and Enterococcus spp. as the most effective genera, while only few strains (i.e., 8 Lactobacilli spp. and 3 Leucinostoc spp.) could be involved in the biotrasformation of zearalenone in α-zearalenol. However, these bacteria did not biotransform other studied mycotoxins as deoxynivalenol and fumonisins B$_1$ and B$_2$.

**SA sequestering activity**

Our results showed that three potential SAs (i.e., activated carbon, Mg bentonite and Na bentonite) exhibited strong AfB$_1$-sequestering capacity, with values higher than 99.0% (Figure 1), in all experimental condition and with no differences among methods (Table 3).
The high affinity of the activated carbon for AFB₁ molecule confirmed the results obtained by other authors (Galvano et al., 1996; Lemke et al., 2001; Rao and Chopra, 2001; Vekiru et al., 2007). The mechanism involved in the sequestering of aflatoxin could be the physical capture by the pore of activated carbon structure, even if it could depend by other factors, such as surface area, structure and dose of mycotoxins (Galvano et al., 2001). However, the use of activated carbons as chemoprotective against aflatoxins produced variable results in vivo. Galvano et al. (1996) reported that only one of the two tested activated carbons at 2.0% inclusion level in the animal diet resulted effective to reduce AFB₁ excretion in rats, while Diaz et al. (2004) did not observe a significant reduction of the AFB₁, milk concentration when an activated carbon was added to the diet at 0.25% inclusion level.

Two bentonites (Mg and Na bentonites) were highly efficient to adsorb the available AFB₁ in vitro. These results were in agreement with the ones by different authors (Dvorak, 1989; Ramos and Hernandez, 1996; Rao and Chopra, 2001; Diaz et al., 2004; Moschini et al., 2008), suggesting bentonite’s ability to adsorb AFB₁ in several media, such as water, buffer solution, saline solution, swine stomach and bovine rumen fluids. More recently, Veriku et al. (2007), testing 61 different bentonite materials, concluded that AFB₁ was generally strongly bound by these clays, both in a pH 5 acetate buffer or in a pH 7 phosphate buffer. These results are consistent with data reported by Diaz et al. (2003), comparing different SAs (i.e., Na and Ca bentonite, esterified glucomannan and activated carbons) at pH 3, 7 and 10. Moreover, Moschini et al. (2008) reported that the AFB₁-bentonite complex was very stable in the gastro-intestinal tract of lactating dairy cows, with an estimated release of the sequestered AFB₁ lower than 5%.

Masoreo et al. (2009) testing the Mg bentonite in vivo on lactating dairy cows, measured a significant reduction in the AFB₁ milk concentration (44%) and in the AFB₁ carry over into milk (47%), when the SA was added at a level of 100 g/cow/day to an AFB₁-contaminated diet causing an ingestion of 174 µg of AFB₁/cow/diet.

In the present study, more than 97% of the available AFB₁ was adsorbed by the Ca bentonite using MM and RM methods, while an inefficient sequestering activity was observed using the W method (i.e., 48%). Similar differences among methods were observed with clinoptilolite clay, that could be considered effective to bind AFB₁ if tested with MM and RM methods (96% and 100% sequestering activities, respectively), while it appeared inefficient in W media. In agreement with our observation, Lemke et al. (2001) reported that under MM conditions, a clinoptilolite showed a sequestering activity not observed in other methods. These authors concluded that the MM model was the most physiologically relevant method to pre-screen potential SAs.

Ineffective or limited sequestering activity was observed for kaolinite and yeast cell wall products in all methods. In particular, these products were not able to sequester AFB₁, when tested in W media, while they showed poor sequestering activities both in MM and in RM (37% and 62% for kaolinite and 32% and 54% for yeast cell wall, respectively in MM and RM method) (Figure 1).

The results obtained for yeast cell wall-derived products confirmed our previous data, in which a low in vitro efficiency was measured in all experimental conditions (Moschini et al., 2008). In particular, the pH and the solvent type appeared to be critical in the reduction of the ability of β-d-glucans, a major component of the inner layer of the yeast cell wall, to complex mycotoxins (Yiannikouris et al., 2005). A previous report by Dawson et al. (2001) suggested that a pH of 4.0 was optimal for yeast cell wall product activity.

Differences among methods showed that the two gastro-intestinal model tests (MM and RM methods) gave higher (P<0.05) sequestering affinity than W method when Ca bentonite, clinoptilolite, kaolinite and yeast cell wall products were tested. This was not true for zeolite, which resulted efficient at binding AFB₁ in W, sequestering about the 80% of the available AFB₁, while it appeared inefficient using the MM method. Also Thieu et al. (2008), found that a zeolite product had a different capacity to adsorb AFB₁, using a single concentration gastro intestinal method at pH 3 or pH 7 (80% and 20% sequestering activities, respectively).

Contrasting in vivo data were obtained adding zeolite to animal diet (Shariatmadari, 2008). Modirzadeh et al. (2004) reported that zeolinite at 0.50% and 0.75% inclusion in the broiler chick diet did not reduce any adverse effects due to AFB₁, ingestion (1.0 mg/kg of diet). On the contrary, Mazzio et al. (2005) suggested that zeolinite is able to counteract same AFB₁-dependent toxic effects in growing broiler chicks ingesting 2.5 mg of AFB₁/kg of diet. Similarly, Abdel-Wahhab et al. (2002) reported improved haematological and bio-

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**Table 3. Analysis of variance (ANOVA) of sequestering agents, method and first order interaction effects on the AFB₁ and AFG₁ sequestering efficiency data.**

| Source                          | Degrees of freedom | AFB₁ (P value) | AFG₁ (P value) |
|---------------------------------|--------------------|----------------|----------------|
| Sequestering agents (SA)        | 7                  | <0.001         | <0.001         |
| Method (M)                      | 2                  | <0.001         | <0.001         |
| SA x M interaction              | 14                 | <0.001         | <0.001         |
| Mean square error               | 24                 | 63.98          | 56.60          |
| SA x M interaction sliced by SA |                    |                |                |
| Activated carbon                | 2                  | 0.996          | 0.996          |
| Mg bentonite                    | 2                  | 0.515          | 0.703          |
| Na bentonite                    | 2                  | 0.683          | 0.657          |
| Ca bentonite                    | 2                  | <0.001         | <0.001         |
| Clinoptilolite                  | 2                  | 0.004          | 0.004          |
| Kaolinite                       | 2                  | <0.001         | <0.001         |
| Yeast cell wall-derived         | 2                  | <0.001         | <0.001         |
| Zeolite                         | 2                  | <0.001         | <0.001         |
chemical parameters in rats fed with a contaminated AFB1 (2.5 mg/kg) diet containing sor- bent compound derived from a natural zeolite.

The AFB1 and AFG sequestering efficiencies observed in this experiment with the three methods resulted very similar (Figure 2). This was confirmed by statistical analysis of the data: strong and positive correlations (P<0.001) were measured between AFB1 and AFG sequestering activity in MM and RM methods (r=0.96 and r=0.99, respectively). Less strong correlation was found for the W method (r=0.79; P<0.001).

Despite some small differences between the chemical structure of the aflatoxin molecules (AB1 and AFG belong to cyclopentenone and lactone series, respectively), these data seem to confirm suggestions of Phillips et al. (2008) concerning the mechanism of aflatoxin absorption by clays: the process should be not site-specific because it involves the β-dicarbonyl system of AFB1 and AFG molecules, thought the chelation of metal ions at the surface and within the interlayer of silicate clays. The carbons of the β-dicarbonyl system have a partial positive charge and could be attracted through an electron donor acceptor mechanism by the negatively charges of platelets of silicates. Phillips et al. (2006) reported a good correlation between the magn- itude of partial positive charges on carbons C1 and C2 of the β-dicarbonyl system and the strength of AF-SA complex.

Figure 2. Data represent the sequestered AFG, from each adsorbent (SA) in the three methods: water (W), gastro-intestinal (MM) and ruminant model (RM), respectively (P of the model <0.001; SEM= 5.320).

Conclusions

Based on the results of the current paper, obtained with the in vitro pre-screening tests, three SA products (activated carbon, Mg ben- tonite and Na bentonite) could be considered efficient at sequestering the available AFB1, resulting as promising agents for in vitro trials. The Ca bentonite and clinoptinolite products were able to adsorb available AFB1 in MM and RM model methods, while they appeared inefficient when the adsorbent test was performed in water media. The adsorption ability of zeo- lite was confirmed only by the W method.

The simulated gastrointestinal methods presented in the current paper (MM and RM, respectively) gave similar results and could be considered useful for in vitro pre-screening of potential sequestering agents. However, the major analytical complexity of the rumen fluid method supports the idea that the 2-steps MM method proposed by Lemke et al. (2001) should be used.

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