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**ISOTHERM MODELING OF ORGANIC ACTIVATED BENTONITE AND HUMIC ACID POLYMER USED AS MYCOTOXIN ADSORBENTS**

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Abstract:	The aim of the current study was to evaluate and to compare two representatives of different classes of adsorbents, intended for use as a feed additive to prevent or reduce adverse effects exerted by mycotoxins, specifically Ochratoxin A and Zearalenone. The adsorbents, an organically activated bentonite (OAB) and a humic acid polymer (HAP) were tested in a common in vitro model with a pH course comparing the maximum pH changes that can be expected in the digestive system of a monogastric animal, i.e. pH 7.4 for the oral cavity, pH 3.0 for the stomach, and pH 8.4 for the intestines. In the 1st experiment, the concentration-dependent adsorbent capacity of OAB and HAB were tested using a fixed concentration of either mycotoxin. Thereafter, adsorption was evaluated applying different isotherms models, such as Freundlich, Langmuir, Brunauer-Emmett-Teller (BET) and Redlich-Peterson, to characterize the adsorption process as being either homo- or heterogeneous, and represents either mono- or multilayer binding.

At the recommended statutory level for the mycotoxins of 0.1 mg Kg<sup>-1</sup> OTA and 0.5 mg Kg<sup>-1</sup> ZEN, OAB showed an adsorbed capacity of >96% towards both mycotoxins, regardless of the pH. The HAP product was also able to absorb >96% of both mycotoxins at pH 3.0, but extensive desorption occurred at pH 8.4. Based on chi-square (Chi<sup>2</sup>) values, Langmuir and Redlich-Peterson equations proved to be the best models to predict monolayer equilibrium sorption of OTA and ZEN, onto the organically activated bentonite and the humic acid polymer. The applied methodology has a sufficient robustness to facilitate further comparative studies with different mycotoxins adsorbing agents.

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5 **HUMIC ACID POLYMER USED AS MYCOTOXIN ADSORBENTS**  
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12 R.R. Santos<sup>a\*</sup>, S. Vermeulen<sup>a</sup>, A. Haritova<sup>b</sup>, J. Fink-Gremmels<sup>a</sup>  
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18  
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21  
22 <sup>a</sup> Department of Equine Sciences, Veterinary Pharmaceuticals, Pharmacology and  
23  
24 Toxicology Division, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The  
25  
26 Netherlands  
27

28  
29 <sup>b</sup> Department of Pharmacology, Physiology of Animals and Physiological Chemistry,  
30  
31 Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria  
32  
33

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41  
42  
43  
44  
45  
46 \* Corresponding author: Regiane Rodrigues dos Santos, DMV, PhD, Utrecht  
47  
48 University, Faculty of Veterinary Medicine, Department of Equine Sciences, Veterinary  
49  
50 Pharmaceuticals, Pharmacology and Toxicology Division, Yalelaan 114, 3584 CM,  
51  
52 Utrecht, The Netherlands  
53  
54 tel. +31 30 253 1078  
55  
56  
57 R.Rodriguesdossantos@pq.cnpq.br  
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## Abstract

The aim of the current study was to evaluate and to compare two representatives of different classes of adsorbents, intended for use as a feed additive to prevent or reduce adverse effects exerted by mycotoxins, specifically ochratoxin A (OTA) and zearalenone (ZEN). The adsorbents, an organically activated bentonite (OAB) and a humic acid polymer (HAP) were tested in a common *in vitro* model with a pH course comparing the maximum pH changes that can be expected in the digestive system of a monogastric animal, i.e. pH 7.4 for the oral cavity, pH 3.0 for the stomach, and pH 8.4 for the intestines. In the 1<sup>st</sup> experiment, the concentration-dependent adsorbent capacity of OAB and HAP were tested using a fixed concentration of either mycotoxin. Thereafter, adsorption was evaluated applying different isotherms models, such as Freundlich, Langmuir, Brunauer-Emmett-Teller (BET) and Redlich-Peterson, to characterize the adsorption process as being either homo- or heterogeneous, and represents either mono- or multilayer binding. At the recommended statutory level for the mycotoxins of 0.1 mg kg<sup>-1</sup> OTA and 0.5 mg kg<sup>-1</sup> ZEN, OAB showed an adsorbed capacity of >96% towards both mycotoxins, regardless of the pH. The HAP product was also able to absorb >96% of both mycotoxins at pH 3.0, but extensive desorption occurred at pH 8.4. Based on chi-square (Chi<sup>2</sup>) values, Langmuir and Redlich-Peterson equations proved to be the best models to predict monolayer equilibrium sorption of OTA and ZEN, onto the organically activated bentonite and the humic acid polymer. The applied methodology has a sufficient robustness to facilitate further comparative studies with different mycotoxins adsorbing agents.

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6 **Keywords:** mycotoxin binders, activated bentonite; humic acid polymers, adsorbent  
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8 isotherms, ochratoxin A, zearalenone.  
9

## 10 11 12 **Introduction**

13  
14  
15 Several strategies have been applied to reduce adverse health effects of  
16  
17 mycotoxins in farm animals and in special cases also in humans (for review see EFSA,  
18  
19 2009). The most widely used approach is the administration of the so-called mycotoxin  
20  
21 adsorbents to animal feeds, aiming at the prevention of mycotoxin absorption in the  
22  
23 gastrointestinal tract (Kabak et al., 2006). The efficacy of such an approach depends on  
24  
25 physical and chemical properties of both, the adsorbent and the mycotoxin. Total charge  
26  
27 and charge distribution, the size of the pores and the accessible surface area of the  
28  
29 adsorbent, and the polarity, solubility, shape and charge distribution of the mycotoxins  
30  
31 targeted are major determinants of adsorption characteristics (Huwig et al., 2001).  
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36 Classes of substances that have been suggested as mycotoxin adsorbents include  
37  
38 inorganic mineral clays and zeolites, as well as organic products such as yeast cell wall  
39  
40 constituents, activated charcoal, humic acid polymers and micronized plant fibres (for  
41  
42 review see Ramos et al., 1996; Galvano et al., 2001; Jouany et al., 2007; EFSA, 2009).  
43  
44

45  
46 Bentonite is widely present in animal feed production as an anti-caking agent. It  
47  
48 is a clay mineral composed mainly of montmorillonite, belonging to the smectite group  
49  
50 of silica clays (WHO, 2005). The crystal structure of a montmorillonite is an octahedral  
51  
52 alumina sheet layered between two tetrahedral silica sheets (Bailey, 1980). Natural  
53  
54 bentonites are amphoteric with a pH-dependent surface charge and the capacity to react  
55  
56 specifically with cations, and to a lesser extent with anions and molecules forming  
57  
58 chemical bonds (WHO, 2005). The cation exchange capacity of montmorillonite is  
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3 rather high ( $\sim 100 \text{ meq } 100\text{g}^{-1}$ ), and is not limited to inorganic ions but also to organic  
4  
5 substances such as aflatoxin B1 (Thieu et al., 2008); organic compounds like alkyl  
6  
7 ammonium can act as balancing cations modifying the overall charge of the sorbent  
8  
9 particles (Majdan et al., 2009). Subsequently, high molecular weight quaternary amines  
10  
11 are often used to improve hydrophobicity of the montmorillonite, resulting in so-called  
12  
13 organically activated bentonites, as used in the current experiment (Balek et al., 2002).  
14  
15 The ability of such activated bentonites to bind diverse structures has been demonstrated  
16  
17 *in vitro* with zearalenone (Bueno et al., 2005), aflatoxin (Thieu et al., 2008) and  
18  
19 ochratoxin A from wine (Kurtbay et al., 2008).  
20  
21  
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24  
25 Humic acid polymers (HAP) originate from natural decaying of organic plant  
26  
27 materials. They are present in soils, sediments, peat, and brown coals. The natural  
28  
29 occurring humic acid polymers contain different reactive moieties, such as hydroxyl,  
30  
31 phenol, carboxyl and methoxyl groups, and have a large specific surface area (Sutton  
32  
33 and Sposito, 2005). Although humic acid polymers are chemically diverse and differ in  
34  
35 molecular size, they are all hydrophilic, reversible colloids composed of 300 to 10,000  
36  
37 units. They can form hydrophobic (van der Waals,  $\pi$ - $\pi$ , CH- $\pi$ ) and hydrogen bonds, the  
38  
39 former becoming more relevant with increasing pH (Piccolo, 2002). The capacity of  
40  
41 humic substances to sequester zearalenone *in vitro* (Sabater-Vilar et al., 2007) and  
42  
43 aflatoxins *in vivo* has been demonstrated (Jansen van Rensburg et al., 2006).  
44  
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48  
49 Commonly, new adsorbents or mixtures of are tested at variable concentrations,  
50  
51 varying between  $1 \text{ g kg}^{-1}$  (Sabater-Vilar et al., 2007),  $2.5 \text{ g kg}^{-1}$  (Kocabagli et al., 2002),  
52  
53 and  $4 \text{ g kg}^{-1}$  feed (Thieu et al., 2008). A regularly used tool to define the optimal  
54  
55 concentration and adsorbent capacity of different substances are *in vitro* assays  
56  
57 conducted in buffer solutions with or without feed materials and enzymes (Avantaggiato  
58  
59 et al., 2007). Originally, *in vitro* tests have been performed at a defined pH, sometimes  
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1  
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3 acidic (Kurtbay et al., 2008), but more often at a neutral pH (Cavret et al., 2010).

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5  
6 However, the binding affinity of a polar substance is influenced by pH, and therefore it  
7  
8 is recommended to measure adsorbent characteristics under the conditions of the  
9  
10 gastrointestinal tract at neutral, acid and basic pH (Sabater-Vilar et al., 2007).

11  
12 Previously, it has been shown that adsorption of zearalenone and aflatoxin B<sub>1</sub> by  
13  
14 mineral clays and humic acid polymers, respectively, is pH dependent (Dakovic et al.,  
15  
16 2007; Ye et al., 2009), and that the transition to alkaline conditions may lead to  
17  
18 desorption.  
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21  
22 Next to single concentration experiments, the evaluation of adsorption isotherms  
23  
24 have been recommended to characterize mycotoxin adsorption (Ringot et al., 2007;  
25  
26 EFSA, 2009), considering that under defined condition an adsorption equilibrium is  
27  
28 reached at which the quantity of the toxin being adsorbed is equal to the quantity being  
29  
30 desorbed. Several theoretical adsorption models have been developed to describe such  
31  
32 isotherms, among others by Freundlich (Freundlich, 1906), Langmuir (Langmuir,  
33  
34 1916), Brunauer-Emmett-Teller (BET) (Brunauer et al., 1938), and Redlich-Peterson  
35  
36 (Redlich and Peterson, 1958), considering homogeneity, as well as monolayer and  
37  
38 multilayer adsorption. Such isotherms analyses allow to (i) identify the best fitted  
39  
40 isotherm model, (ii) predict adsorption capacity of a mycotoxin onto an specific  
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42 adsorbent, and (iii) recognize each adsorbent capacity as homo- or heterogeneous, or as  
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44 mono- or multilayer binding.  
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50  
51 Ochratoxin A (OTA) and zearalenone (ZEN) are two mycotoxins that are  
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53 frequently occurring in animal feeds in Northern Europe, being of concern in the diets  
54  
55 for pigs (EFSA, 2004a,b). OTA, a nephrotoxic and immunosuppressive agent in  
56  
57 animals, consists of a dihydroisocoumarin moiety linked to l-phenylalanine through an  
58  
59 amide bond (Fig. 1A). It has a carboxyl group at the phenylalanine moiety and a phenol  
60



1  
2  
3 group at the dihydroisocoumarin part with a pKa 4.4 and 7.3 respectively (Ringot et al.,  
4  
5 2005). Being a polyaromatic molecule, OTA is hydrophobic ( $\log P_{OW}=4.4$ ) when  
6  
7 unionized (Dakovic et al., 2005). ZEN is a resorcylic acid lactone, and is a  
8  
9 hydrophobic compound ( $\log P_{OW} = 3.66$ ) (Lemke et al., 1998). It is a weak acid due to  
10  
11 the presence of the diphenolic moiety and has a pKa of 7.62 (Fig. 1B). The structure of  
12  
13 ZEN is flexible enough to bind to oestrogen receptors in animals, leading to  
14  
15 hyperoestrogenicity and adverse effects on fertility and reproduction particularly in pigs  
16  
17 (Zinedine et al., 2007). OTA and ZEN were selected as model mycotoxins for the  
18  
19 current study due to their very different structure and physic-chemical properties and in  
20  
21 consideration of their relevance in pig husbandry as for both mycotoxins, pigs are  
22  
23 considered to be the most sensitive animal species (EFSA, 2004a,b).  
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29 Hence, the aim of the present study was to evaluate and compare two chemically  
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31 different sorbents, an organically activated bentonite and a humic acid polymer,  
32  
33 regarding their capacity to bind mycotoxins under different conditions. To this end,  
34  
35 adsorbent concentration dependence, adsorption pH dependence, and mycotoxin  
36  
37 desorption were analysed. Modelling of mycotoxin adsorption was performed to  
38  
39 characterize OTA and ZEN adsorption at neutral, acidic and basic pH and to evaluate  
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41 the robustness of this model for forthcoming assessments of new mycotoxin adsorbents.  
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## 48 **Materials and Methods**

### 49 *Mycotoxins and adsorbents*

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OTA and ZEN were obtained from Sigma (Saint Louis, MI, USA). Solutions with OTA and ZEN were prepared by dissolving each mycotoxin separately in a buffer solution ( $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ , 1.2mM; KCl, 2.7 mM;  $\text{KH}_2\text{PO}_4$ , 1.5 mM;  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 1.1 mM; NaCl, 138 mM;  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 8.1 mM; pH=7.4). The tested sorbents consisted of

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3 commercially available organic activated bentonite (OAB) and humic acid polymer  
4 (HAP) (Poortershaven Industrial Minerals BV, Rotterdam, The Netherlands).  
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10 *Single concentration experiments: effects of adsorbent concentration and pH on*  
11 *adsorption capacity*  
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15 Either OTA or ZEN were added to the buffer solution at a fixed concentration of  
16 0.1 or 0.5  $\mu\text{g mL}^{-1}$ , respectively following the guidance levels as defined in  
17 Commission Recommendation 2006/576/EC. For each mycotoxin, the test protocol  
18 comprised i) negative controls (buffer alone or with each of the five adsorbent  
19 concentrations without mycotoxins, ii) positive controls, i.e. buffer with mycotoxin  
20 without adsorbent, to validate pH dependent recovery of the mycotoxin by the applied  
21 extraction methods and iii) test solutions, containing buffer with the dissolved  
22 mycotoxin (OTA or ZEN) to which the adsorbent (OAB or HAP) at five different  
23 concentrations (0.5, 1.0, 2.0, 4.0 or 5.0  $\text{mg mL}^{-1}$ ) was added. Incubations were at three  
24 different pHs (7.4, 3.0 and 8.4) in a continuous system. The percentage of adsorption by  
25 the adsorbents was computed using the equation:  
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$$41 \quad \% \text{ adsorption} = \frac{(C_0 - C_{\text{eq}}) * 100}{C_0} \quad (1)$$

42  
43 where  $C_0$  and  $C_{\text{eq}}$  are the initial concentration of the mycotoxin in solution ( $\mu\text{g mL}^{-1}$ )  
44 and the residual mycotoxin concentration at equilibrium ( $\mu\text{g mL}^{-1}$ ), respectively.  
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51 *Equilibrium adsorption isotherms*  
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53 Based on the results of the experiment 1, a second series of experiments was  
54 performed to describe the interaction between OTA and ZEN and the two test products  
55 OAB and HAP. These equilibrium experiments were carried out by incubating using the  
56 same buffer solution, with or without addition of one of the adsorbents in the most  
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3 efficient concentration obtained in the experiment 1. In these experiments mycotoxin  
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5 concentrations varied for OTA between 0.025, 0.05, 0.1, 0.15 and 0.2  $\mu\text{g mL}^{-1}$  and for  
6  
7 ZEN between 0.125, 0.250, 0.5, 0.75 and 1.0  $\mu\text{g mL}^{-1}$ . The test protocol was essentially  
8  
9 the same as for the single concentration studies.  
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### 12 13 14 15 *Incubation protocol*

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17 For both experiments, incubations were performed in glass flasks containing 30  
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19 mL of the test solution with a pH gradient including pH 7.4, 3.0 and 8.4, following a  
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21 procedure previously described by Sabater-Vilar et al. (2007). In brief, a first incubation  
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23 was performed at pH 7.4, at 39 °C for 30 min, in a water bath under constant stirring to  
24  
25 allow solution equilibrium. After this first incubation, an aliquot was taken for analysis.  
26  
27 The pH of the remaining solution was decreased to 3.0 with HCl (1 M) and re-incubated  
28  
29 for 1 hour to simulate the pH conditions during gastric passage of a monogastric animal.  
30  
31 Immediately at the end of incubation, a second aliquot was taken for analysis and the  
32  
33 pH of the remaining solution was raised to 8.4 with NaOH (1 M) to simulate pH  
34  
35 conditions during intestinal passage of a monogastric animal. This last incubation was  
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37 performed for 3 hours and, at the end, a third aliquot was taken for analysis. All samples  
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39 were separately filtered by using Minisart-GF filters (Sartorius, Gottingen, Germany),  
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41 and subsequently stored in glass tubes at -20 °C until analysis.  
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### 51 *Determination of binding capacity*

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53 The binding capacity was calculated from the recovery of the unbound mycotoxin  
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55 fraction, measured by standard HPLC methods.  
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58 *Ochratoxin A:* The free (non-bound) concentration of OTA was quantified by a standard  
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60 HPLC method using a Spherisorb ODS-2, stainless steel, 5  $\mu\text{m}$  column (250x4.6mm;

Phenomenex, The Netherlands) and a mobile phase composed of water/acetonitrile/acetic acid; 102/96/2, v:v:v. Fluorescence detection was performed with a FP 920 fluorescence detector (Jasco, Japan) set at 330 nm excitation and 460 nm emission wavelengths. Quantification was conducted by comparing peak areas of samples with the standard curve obtained from the control samples to correct for differences in the percentage of protonated and non-protonated forms of OTA at the different pH conditions.

*Zearalenone*: ZEN was quantified after extraction using as stationary phase a C18 Luna II, stainless steel, 5 µm column (150x4.6mm; Phenomenex, The Netherlands). and as mobile phase: methanol/water; 70:30, v:v as a flow rate of 0.8 mL min<sup>-1</sup>; identification was obtained with a FP 920 fluorescence detector (Jasco, Japan) set at 236 nm excitation and 418 nm emission wavelengths.

### *Isotherm models*

#### *Adsorption equilibrium*

The concentration of adsorbed mycotoxin (OTA or ZEN) was calculated according to equation below (Ringot et al., 2007):

$$Q_{eq} = \frac{(C_0 - C_{eq}) * V}{m} \quad (2)$$

where  $Q_{eq}$  is concentration of adsorbed mycotoxin per gram of adsorbent (mg g<sup>-1</sup>),  $V$  is the volume of aqueous phase (L) and  $m$  is the mass of adsorbent (g).

Four different adsorption equilibrium models (Langmuir, Freundlich, Brunauer-Emmett-Teller and Redlich-Peterson) were used to fit the experimental data, as presented in Table 1. Non-linear regression was used to determine the parameters of each isotherm model (Origin, version 8.1).

### *Statistical analysis*

Each experiment was conducted in quadruple. The data were analyzed with the Statistica 6.1 computer program (Statistica for Windows, StatSoft, Inc., USA, 1984–2002). Statistical analysis was done by using a factorial ANOVA with mycotoxins/adsorbents and concentrations as categorical predictor variables and percentage of adsorption at the tested pH values as dependent variable. Bonferroni test was used as post hoc test. To estimate the parameter values which best describe the data of a nonlinear fitting, chi-square ( $\text{Chi}^2$ ) minimization was used as indicated in the Origin computer program used to evaluate the isotherm models. Threshold for level of significance was set at  $P < 0.05$ .

### **Results and Discussion**

Figure 2 shows the results of the single concentration experiments in three-dimensional diagrams, with pH 7.4 on the x-axis, pH 8.4 on the y-axis, and pH 3.0 on the z-axis, presenting the adsorbent capacity of both products, OAB (Fig. 2A and 2C) and HAP (Fig. 2B and 2D) in different concentrations, to adsorb OTA ( $0.1 \mu\text{g mL}^{-1}$ ) (Fig. 2A and 2B) and ZEN ( $0.5 \mu\text{g mL}^{-1}$ ) (2C and 2D). Concentration-dependent absolute adsorption affinity between the individual adsorbents at different concentrations and at different pH conditions is presented in Table 2.

Both adsorbents, OAB and HAP, presented similar adsorption capacity only at pH 3.0. At pH 7.4 and 8.4, OTA adsorption onto OAB was significantly higher when compared to HAP, with all tested concentrations of the adsorbent. There was no pH effect on the OTA adsorption capacity onto OAB. This can be explained by the fact that

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2  
3 the organic cations that confer hydrophobicity to OAB remain almost unaffected by the  
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5 pH change from 7.4 to 3.0 and back to 8.4. The protonation of OTA on the other hand is  
6  
7 changed in the pH course, as it has a pKa of 4.4 and 7.3. At pH 3.0 almost all OTA will  
8  
9 be in the neutral (non-charged) form, while at pH 7.4 it will be present as a monoanion  
10  
11 or a dianion, and at pH 8.4 most OTA will be in the dianion form. Previous  
12  
13 investigations have demonstrated that minerals through binding of metal cations could  
14  
15 adsorb OTA (Dakovic et al., 2005). However, as in our studies an organically activated  
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17 bentonite was used, interactions with metal cations are unlikely as the OAB surface will  
18  
19 be covered with positively charged groups, presenting a bilayer and probably also  
20  
21 interlayer, of cationic surfactants. It can be assumed that the anionic species of this  
22  
23 mycotoxin will have both partly electrostatic and hydrophobic interaction with OAB,  
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25 while regarding HAP, a coupling between divalent cations and anions on negatively  
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27 charged groups in humic acids can be expected.  
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34 Whereas OAB showed to be an excellent OTA-adsorbent, regardless of pH,  
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36 sequestering >96% of OTA at a minimum concentration of 2.0 mg mL<sup>-1</sup>, OTA  
37  
38 adsorption onto HAP, in all concentrations, was significantly higher at pH 3.0 (>96%)  
39  
40 when compared to pH 7.4 (67%) and 8.4 (58%) (Table 2). As the incubations at pH 3.0  
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42 were followed by incubations at pH 8.4, we suggest that desorption at alkaline pH is  
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44 probably caused by repulsion, as both OTA and humic acid obtain a negative charge at  
45  
46 an alkaline pH through their carboxyl and phenol groups.  
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50  
51 There was no pH effect on ZEN adsorption onto OAB or HAP, although in  
52  
53 presence of OAB it was slightly less efficient at 8.4, compared to pH 7.4 and 3.0. ZEN  
54  
55 has a pKa of 7.62 (Lemke et al., 1998), and will be found almost completely in its  
56  
57 deprotonated form at pH 8.4. HAP only shows a >90% adsorption at pH 3.0. The  
58  
59 negative charge of this compound developed at higher pH through its carboxyl and  
60

phenol groups, is detrimental for the needed hydrophobic character to adsorb OTA or ZEN. When OAB and HAP were compared, at all tested pH conditions, a higher ZEN adsorption capacity of OAB was observed when compared to HAP. However, when both adsorbents were used at concentrations of 4.0 and 5.0 mg mL<sup>-1</sup>, no differences could be noted (Table 2).

Evaluating the concentration-dependency of adsorption, it could be shown that for OAB a minimal concentration of 2.0 mg mL<sup>-1</sup> is requested to adsorb >96% of OTA and ZEN, while HAP adsorbs >90% of ZEN and >60% of OTA at concentrations of 4.0 and 5.0 mg mL<sup>-1</sup>, respectively (Table 2). OTA adsorption onto HAP is increasing with the increase of the amount of the adsorbent added. Therefore, either adsorption affinity or capacity seems to be low. For ZEN adsorption, there was hardly any concentration dependent effect beyond a concentration of 4.0 mg mL<sup>-1</sup> (Table 2), allowing the assumption that binding affinity is relatively higher.

Based on the results of these single concentration experiments, the adsorbent concentration was set at 4.0 mg mL<sup>-1</sup> for OAB and HAP, to perform the equilibrium studies in which increasing mycotoxins concentrations were used in the same model with three different pH conditions. To obtain the parameters and determine an optimal fit, non-linear equations were used instead of linear ones (Kumar, 2007). Figures 3-6 show the isotherm graphs for each mycotoxin following adsorption onto OAB and HAP. Results show that, at pH 3.0, all equilibrium curves were linear, while at pH 7.4 and 8.4 equilibrium curves were non-linear. The correlation coefficient ( $r^2$ ) value is often used to predict the best fit for linear equations (Ringot et al., 2007). However, when applying non-linear models, fit of the isotherm should be evaluated using the Chi<sup>2</sup> parameter (Ho, 2004). From the results for HAP, shown in table 3, the Freundlich model failed to describe adsorption, except when OTA (Chi<sup>2</sup>=7.61E-06) or ZEN (Chi<sup>2</sup>=41.9E-



05) adsorption was performed at pH 3.0 confirming that linearity is only achieved at pH 3 as mentioned above. For ZEN adsorption, most of the values for  $\text{Chi}^2$  ranged between 10.8E-05 to 75.5E-05 for OAB, and between 31.8E-05 to 113E-05 for HAP. Only ZEN binding to OAB at pH 3.0 presented the lowest  $\text{Chi}^2$  values, ranging between 10.8E-05 to 15.1E-05 (Table 3). Chi-square values showed a better fit, indicated by lower  $\text{Chi}^2$  values, for the adsorption of OTA to both adsorbents.

The Langmuir equation (Langmuir, 1916) is valid for monolayer sorption to a surface with a finite number of identical sites (Ringot et al., 2007). For practically all adsorbent-adsorbate pairs the Langmuir model showed a good fit, at all pH conditions, but a large variation was observed in the maximum adsorption capacity. The affinity parameter  $K_L$  showed a complementary variation, with lower values when maximum adsorption capacity  $q_{\text{max}}$  was high, and higher values when  $q_{\text{max}}$  was low. The small value of  $K_L$  indicates that the adsorbate has a high affinity to the adsorbent (Arica et al., 2003), suggesting that OAB exhibits affinity to both, ZEN and OTA.

The BET equation consists in a model for multilayer adsorption. The principal BET isotherm equation (Brunauer et al., 1938) has been developed for gas phase adsorptions. To apply such an equation in mycotoxin adsorption experiments, the BET equation is directly transferred to liquid phase adsorption by replacing saturation partial pressure of adsorbate in the gas phase by liquid phase concentrations (Ringot et al., 2007). However, this approach is not entirely correct as the unlimited adsorption in the classical BET equation for the gas phase cannot be applied to liquid phase adsorptions (Ebadi et al., 2009). Therefore, in the present study, the linear equation was replaced by a non-linear equation as proposed by Ebadi et al. (2009). However, even under these conditions, the BET equation did not adequately describe the observed OTA adsorption, as  $\text{Chi}^2$  was high for all tested conditions, but it was found to be suitable to describe the



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2  
3 interaction between ZEN and HAP, as demonstrated by the low  $\chi^2$  values. This  
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5 confirms that multilayer adsorption will also depend on the chemical structure of the  
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7 mycotoxin.  
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10 In general, the lowest values of  $\chi^2$  were obtained with Langmuir and Redlich-  
11  
12 Peterson models. Redlich-Peterson and Langmuir isotherms overlapped each other,  
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14 when OTA adsorption was evaluated at pH 7.4 (OAB and HAP) and 8.4 (HAP), and  
15  
16 ZEN adsorption onto HAP at all pH conditions. Comparing both models, maximum  
17  
18 adsorption capacity was similar in all analyses, except at pH 3.0. The Redlich-Peterson  
19  
20 isotherm is an empirical equation that combines characteristics of Langmuir and  
21  
22 Freundlich isotherms into a single equation. At low concentrations, it approximates  
23  
24 Henry's law, while at high concentrations it approaches the Freundlich model (Ho,  
25  
26 2006). The Redlich-Peterson isotherm accurately described the adsorption behaviour  
27  
28 observed for all mycotoxin-adsorbent pairs, and gave the best fit in general. The  
29  
30 Redlich-Isotherm parameters did not vary as much as the correspondently parameters of  
31  
32 the Langmuir function. The Redlich-Peterson constant ( $g$ ) was generally 1 (but 0.8 for  
33  
34 OTA at pH 3.0 for both OAB and HAP, and 0.9 for ZEN/OAB, pH 3.0, respectively),  
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36 which indicates that the function is reduced to the Langmuir isotherm equation.  
37  
38 Therefore, the Redlich-Petersen isotherm model can be recommended for further  
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40 comparisons of mycotoxin-binding agents.  
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48 Mycotoxin adsorption by organically modified aluminosilicates has been studied  
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50 previously (Lemke et al., 1998; Tomasevic-Canovic et al., 2003; Dakovic et al., 2005,  
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52 2007; Feng et al., 2008). Feng et al. (2008) measured ZEN binding to montmorillonite  
53  
54 and organically modified montmorillonite, and found a  $\log K_F$  of 2.3 and 3.7  
55  
56 respectively. Lemke et al. (1998) even found an increase of  $\log K_F$  from -0.5 to 4.9. The  
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58 performance at pH 10 was much lower than at pH 2 or 6.5. Dakovic et al. (2003) and  
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3 Tomasevic-Canovic et al. (2003) did not find any pH effect (pH 3.0, 7.0 and 9.0) in  
4  
5 their isotherm studies on organically modified zeolites. Bentonite was initially used to  
6  
7 bind aflatoxins (Thieu et al., 2008), and found less effective as a binding agent for other  
8  
9 mycotoxins. With the intention to broaden the binding spectrum, attempts were made to  
10  
11 apply heat activation (Bueno et al., 2005) or organically activation (Kurtbay et al.,  
12  
13 2008). The last authors showed that OTA adsorption onto non-modified bentonite was  
14  
15 poor, while organically activated bentonite was effective to adsorb OTA, suggesting  
16  
17 that the improved hydrophobicity by organic activation results in a broader application  
18  
19 to mycotoxins that are less polar.  
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24  
25 Aluminosilicates have been studied extensively as mycotoxin adsorbents. In  
26  
27 contrast, the use of humic acid polymers as a mycotoxin sorbent has received much less  
28  
29 attention. Jansen van Rensburg et al. (2006) tested the processed humic acids  
30  
31 (oxihumate) as a binder of aflatoxin B1, both *in vitro* and *in vivo*. Oxihumate proved to  
32  
33 be a good adsorbent *in vitro*, at pH 3.0, 5.0 and 7.0, and *in vivo*, as it was able to  
34  
35 diminish the adverse effects of aflatoxin on body weight, liver, stomach, heart and blood  
36  
37 of male broiler chickens. A further *in vitro* evaluation of sodium humate as an aflatoxin  
38  
39 B1 adsorbent was presented by Ye et al. (2009). Sodium humate was found to be an  
40  
41 efficient adsorbent with increasing adsorption at pH 3.0, 7.0 and 8.0. Sabater-Vilar et al.  
42  
43 (2007) had before investigated some natural humic acid polymers for their capacity of  
44  
45 ZEN adsorption at acidic (pH 2.5) and alkaline (pH 8) conditions. However, in none of  
46  
47 these studies, the nature of the binding applying isotherm studies was included. The  
48  
49 experiments presented here confirm the principal capacity of humic acid polymers, to  
50  
51 adsorb mycotoxins, at least OTA and ZEN.  
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58 The use of OAB and HAP as mycotoxin adsorbents may have some advantages  
59  
60 and disadvantages. Next to mycotoxin binding, bentonites can act as gut protectant

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3 when used in animal feed, and have a capacity to bind heavy metals, polycyclic  
4  
5 aromatic and other non polar substances, such as dioxins (WHO, 2005). In turn, the use  
6  
7 of bentonites in practice requires continuous monitoring of the absence of undesirable  
8  
9 contaminants in these products. Bentonites have a long history of safe use as an  
10  
11 anticaking agent. In addition, the safety and efficacy of bentonite as feed additive has  
12  
13 been evaluated also by the European Food Safety Authority (EFSA, 2007). They  
14  
15 concluded that for ruminants there were no toxicological concerns and approved it also  
16  
17 as aflatoxin binder. Bentonites are not genotoxic and are not absorbed following  
18  
19 application as a feed additive, hence providing no direct toxicological risk for the  
20  
21 animal of consumer. However, the absence of other undesirable effects, such as  
22  
23 significant adsorption of essential elements and vitamins need to be evaluated for each  
24  
25 product and confirmed, like the efficacy, in *in vivo* experiments with target animal  
26  
27 species.  
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34 Humic acids complexes also have been shown to promote health, possibly  
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36 through their anti-inflammatory, antiviral and heavy-metal binding activities (Jansen  
37  
38 van Rensburg, 2005). In contrast to the pro-carcinogenic soluble humic acids, poorly  
39  
40 soluble humic acid complexes occurring in natural peats, and being composed of humic  
41  
42 and fulvic acid complexes have broad applications in soil managements, water cleaning  
43  
44 and industrial decontamination processes (Sutton and Sposito, 2005). The medical  
45  
46 literature of East Europe refers to various beneficial effects of these products (Cozzi et  
47  
48 al., 1993; Lind and Glynn, 1999) and feeding experiments in pigs have confirmed their  
49  
50 value in pig nutrition and as mycotoxin ameliorating agents (Ji et al., 2006, Demeterova  
51  
52 en al., 2009). These broad beneficial effects classify this group of compounds for long  
53  
54 term use in farm animals at risk for exposure to contaminated feed. However, like for  
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3 the bentonides the efficacy and safety of a humates need to be established in target  
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5 animal species prior to large scale use.  
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### 8 **Conclusions**

9  
10 In the present study, we characterized the adsorption of OTA and ZEN by an  
11 organically activated bentonite (OAB) in comparison with a humic acid polymer (HAP).  
12  
13 HAP has an increased OTA adsorption capacity at pH 3.0, but this adsorption is  
14  
15 susceptible to alkaline pH, leading to a partial desorption of this mycotoxin at pH 8.4.  
16  
17 Analysis of the individual results as the efficient isotherm models confirmed that  
18  
19 adsorption capacity and affinity depend on the interactions between mycotoxin,  
20  
21 adsorbent and pH. Therefore, isotherm models to describe adsorption equilibrium  
22  
23 cannot be generalized, but serve as a valuable tool to identify and to compare new  
24  
25 products. The presented *in vitro* approach has shown its robustness in various  
26  
27 experimental protocols.  
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39  
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3 **Figure captions**  
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8 **Fig. 1** Molecular structure of OTA (A) and ZEN (B).  
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12 **Fig. 2** Three-dimensional diagrams, with pH 7.4 on the x-axis, pH 8.4 on the y-axis, and  
13 pH 3.0 on the z-axis, representing the capacity, in percentages, of OAB (A, C) and HAP  
14 (B, D) to adsorb OTA (A, B) and ZEN (C, D).  
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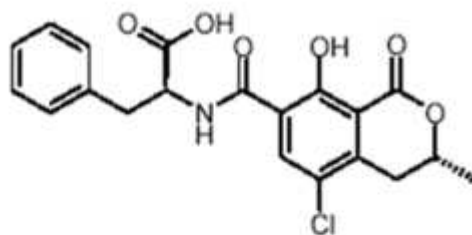
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22 **Fig. 3** Adsorption isotherm modelling of OTA sequestration using OAB at pH 7.4, 3.0  
23 and 8.4 (A); Langmuir, Freundlich, BET and Redlich-Peterson isotherms obtained at pH  
24 7.4 (B), 3.0 (C) and 8.4 (D).  
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32 **Fig. 4** Adsorption isotherm modelling of OTA sequestration using HAP at pH 7.4, 3.0  
33 and 8.4 (A); Langmuir, Freundlich, BET and Redlich-Peterson isotherms obtained at pH  
34 7.4 (B), 3.0 (C) and 8.4 (D).  
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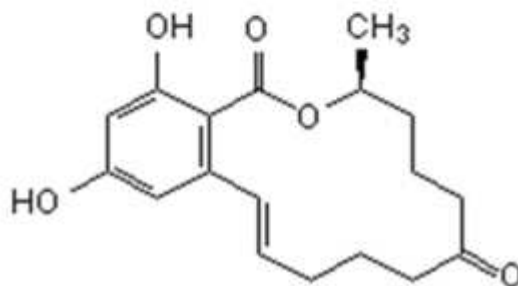
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41 **Fig. 5** Adsorption isotherm modelling of ZEN sequestration using OAB at pH 7.4, 3.0  
42 and 8.4 (A); Langmuir, Freundlich, BET and Redlich-Peterson isotherms obtained at pH  
43 7.4 (B), 3.0 (C) and 8.4 (D).  
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51 **Fig. 6** Adsorption isotherm modelling of ZEN sequestration using HAP 7.4, 3.0 and 8.4  
52 (A); Langmuir, Freundlich, BET and Redlich-Peterson isotherms obtained at pH 7.4  
53 (B), 3.0 (C) and 8.4 (D).  
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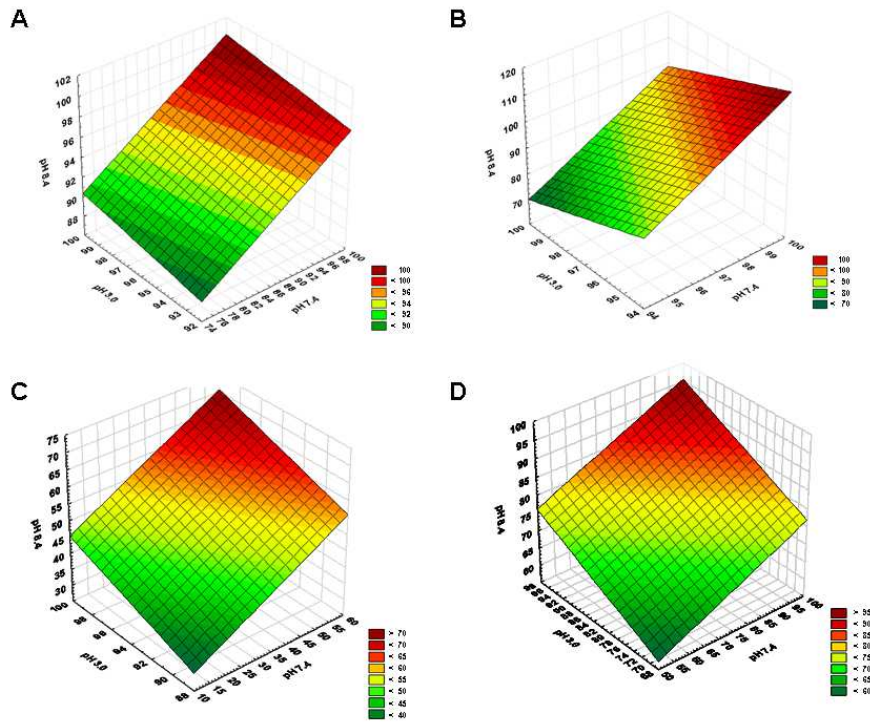
A



B

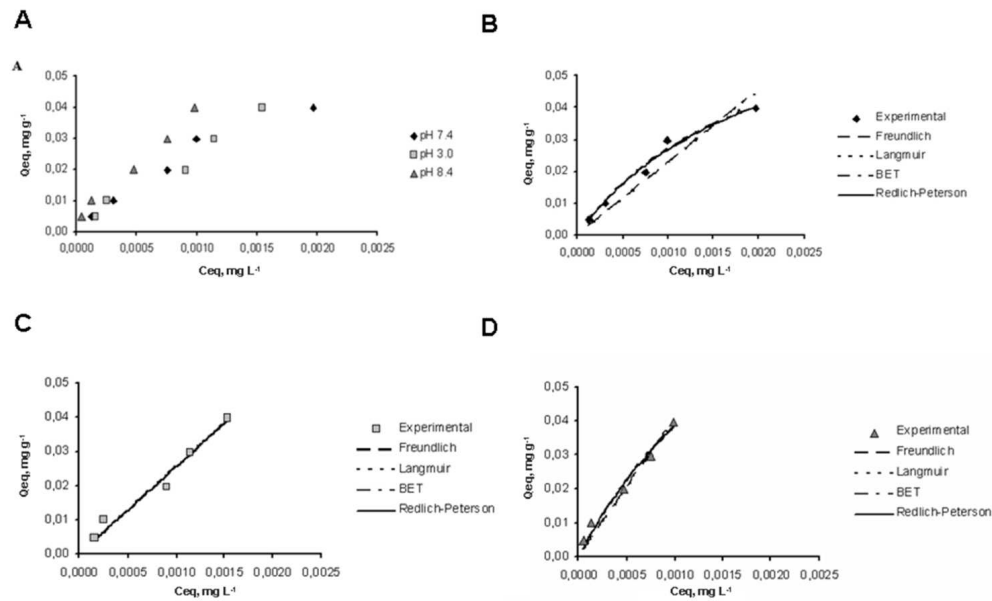


Molecular structure of OTA (A) and ZEA (B).  
12x17mm (600 x 600 DPI)



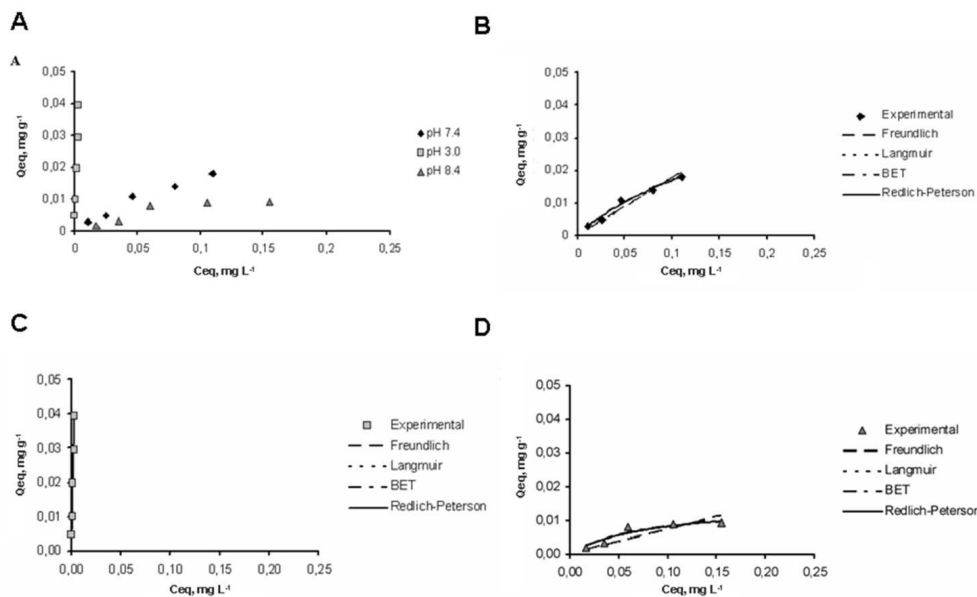
Three-dimensional diagrams, with pH 7.4 on the x-axis, pH 8.4 on the y-axis, and pH 3.0 on the z-axis, representing the capacity, in percentages, of OAB (A, C) and HAP (B, D) to adsorb OTA (A, B) and ZEA (C, D).

80x61mm (300 x 300 DPI)

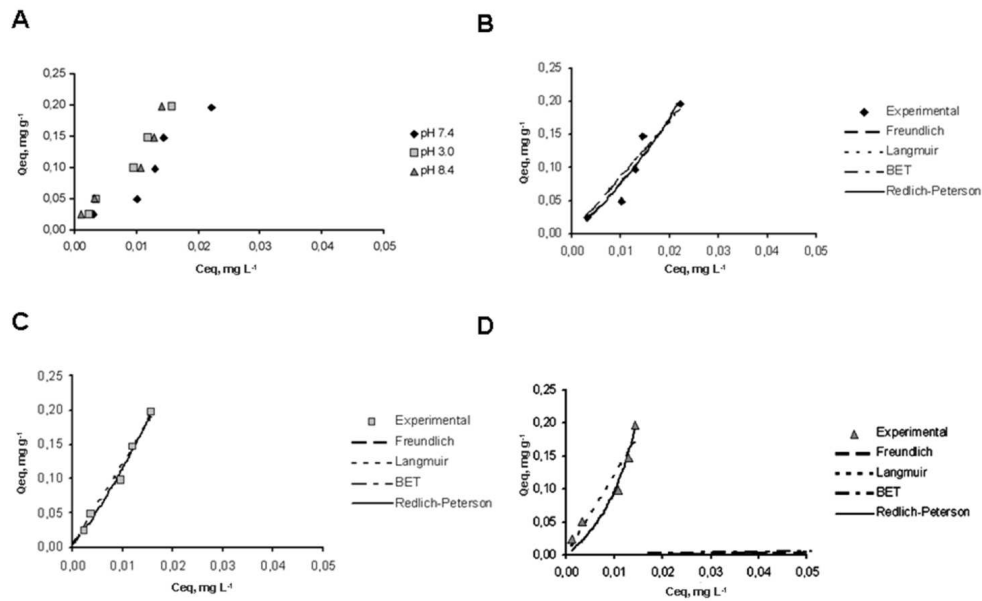


Adsorption isotherm modelling of OTA sequestration using OAB at pH 7.4, 3.0 and 8.4 (A); Langmuir, Freundlich, BET and Redlich-Peterson isotherms obtained at pH 7.4 (B), 3.0 (C) and 8.4 (D).

63x39mm (600 x 600 DPI)



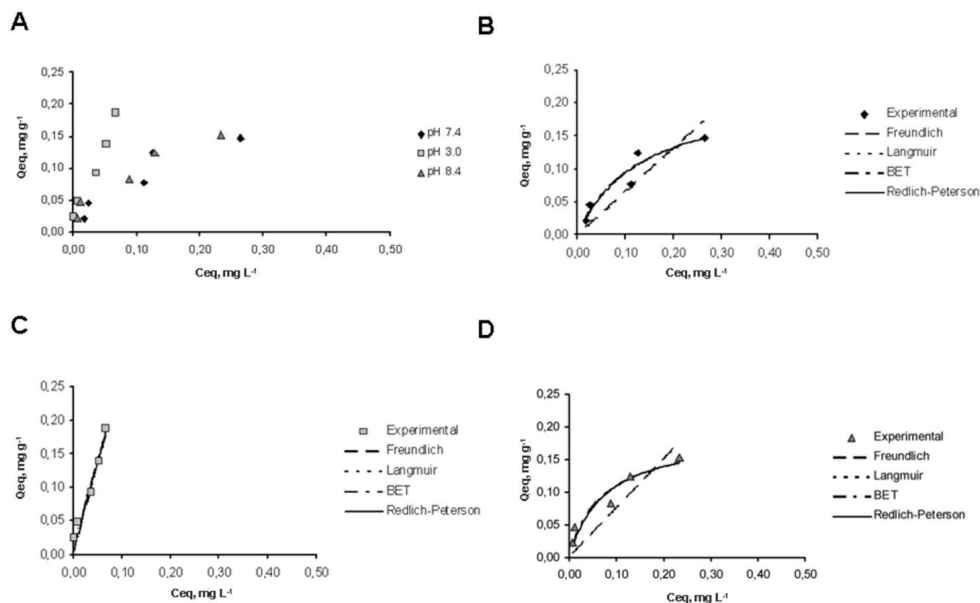
Adsorption isotherm modelling of OTA sequestration using HAP at pH 7.4, 3.0 and 8.4 (A); Langmuir, Freundlich, BET and Redlich-Peterson isotherms obtained at pH 7.4 (B), 3.0 (C) and 8.4 (D).  
62x38mm (600 x 600 DPI)



Adsorption isotherm modelling of ZEA sequestration using OAB at pH 7.4, 3.0 and 8.4 (A); Freundlich, Langmuir, BET and Redlich-Peterson isotherms obtained at pH 7.4 (B), 3.0 (C) and 8.4 (D).

62x38mm (600 x 600 DPI)

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Adsorption isotherm modelling of ZEA sequestration using HAP 7.4, 3.0 and 8.4 (A); Langmuir, Freundlich, BET and Redlich-Peterson isotherms obtained at pH 7.4 (B), 3.0 (C) and 8.4 (D). 63x39mm (600 x 600 DPI)

Review Only



Table 1. Isotherms functions

Isotherms	Expression	Constants	References
Freundlich	$Q_{eq} = K_F \cdot C_{eq}^{1/n_F}$	$K_F, n_F$	(Ringot et al., 2007)
Langmuir	$Q_{eq} = (q_{max} \cdot K_L \cdot C_{eq}) / (1 + K_L \cdot C_{eq})$	$K_L, q_{max}$	(Kumar and Sivanesan, 2005)
BET	$Q_{eq} = \frac{q_{max} \cdot (K_{BET} \cdot C_{eq})}{(1 - K_L \cdot C_{eq}) \cdot (1 - K_L \cdot C_{eq} + K_{BET} \cdot C_{eq})}$	$K_{BET}, q_{max}$	(Ebadi et al., 2009)
Redlich-Peterson	$Q_{eq} = (A \cdot C_{eq}) / (1 + B \cdot C_{eq})^g$	$A, B, g$	(Kumar and Sivanesan, 2005)

$1/n_F$  Freundlich index/exponent

$K_F$  Freundlich isotherm constant ( $\text{mg g}^{-1}$ )

$K_L$  Langmuir isotherm constant ( $\text{L mg}^{-1}$ )

$q_{max}$  maximum adsorption capacity ( $\text{mg g}^{-1}$ )

$K_{BET}$  Bruanauer-Emmett-Teller (BET) isotherm constant ( $\text{L mg}^{-1}$ )

$A$  Redlich-Peterson isotherm constant ( $\text{L mg}^{-1}$ )<sup>g</sup>

$B$  Redlich-Peterson isotherm constant ( $\text{L g}^{-1}$ )

$g$  Redlich-Peterson isotherm constant

Table 2. Mean percentages ( $\pm$ SEM) of adsorbed OTA and ZEA by organic activated bentonite and humic acid polymer.

Mineral concentration	Minerals											
	Organic Activated Bentonite (OAB)						Humic Acid Polymer (HAP)					
	OTA			ZEA			OTA			ZEA		
	pH 7.4	pH 3.0	pH 8.4	pH 7.4	pH 3.0	pH 8.4	pH 7.4	pH 3.0	pH 8.4	pH 7.4	pH 3.0	pH 8.4
0.5 mg mL <sup>-1</sup>	75.9 $\pm$ 4.8 <sup>Ac</sup>	92.8 $\pm$ 1.3 <sup>c</sup>	89.0 $\pm$ 1.9 <sup>c</sup>	94.4 $\pm$ 0.3 <sup>A</sup>	94.7 $\pm$ 0.1 <sup>A</sup>	87.1 $\pm$ 1.4 <sup>Ac</sup>	15.2 $\pm$ 3.6 <sup>BCc</sup>	89.0 $\pm$ 1.5 <sup>D</sup>	35.5 $\pm$ 5.3 <sup>BEc</sup>	56.2 $\pm$ 5.1 <sup>Bc</sup>	69.8 $\pm$ 1.0 <sup>Bc</sup>	60.8 $\pm$ 2.1 <sup>Bc</sup>
1.0 mg mL <sup>-1</sup>	88.1 $\pm$ 2.4 <sup>Ac</sup>	95.7 $\pm$ 0.3 <sup>c</sup>	94.6 $\pm$ 0.6 <sup>Ac</sup>	97.5 $\pm$ 0.1 <sup>A</sup>	98.0 $\pm$ 0.1 <sup>A</sup>	92.2 $\pm$ 0.9 <sup>Ac</sup>	24.9 $\pm$ 2.1 <sup>BCd</sup>	94.1 $\pm$ 0.6 <sup>D</sup>	42.9 $\pm$ 2.0 <sup>BEc</sup>	74.8 $\pm$ 4.1 <sup>Bd</sup>	85.2 $\pm$ 0.2 <sup>Bd</sup>	78.2 $\pm$ 0.8 <sup>Bd</sup>
2.0 mg mL <sup>-1</sup>	96.3 $\pm$ 0.1 <sup>Ad</sup>	97.8 $\pm$ 0.2 <sup>d</sup>	98.2 $\pm$ 0.2 <sup>Ac<sup>d</sup></sup>	98.6 $\pm$ 0.2 <sup>A</sup>	98.5 $\pm$ 0.1 <sup>A</sup>	96.7 $\pm$ 0.2 <sup>Ad</sup>	37.7 $\pm$ 1.6 <sup>BCe</sup>	96.7 $\pm$ 0.2 <sup>D</sup>	55.9 $\pm$ 2.6 <sup>BE<sup>d</sup></sup>	86.6 $\pm$ 2.4 <sup>Bd</sup>	92.0 $\pm$ 0.1 <sup>Bc</sup>	86.1 $\pm$ 1.2 <sup>Bd</sup>
4.0 mg mL <sup>-1</sup>	98.9 $\pm$ 0.2 <sup>Ad</sup>	99.0 $\pm$ 0.1 <sup>d</sup>	99.6 $\pm$ 0.1 <sup>Ad</sup>	99.5 $\pm$ 0.0	99.2 $\pm$ 0.1	98.2 $\pm$ 0.1 <sup>d</sup>	42.7 $\pm$ 1.8 <sup>BCe</sup>	98.3 $\pm$ 0.1 <sup>D</sup>	61.7 $\pm$ 3.2 <sup>BE<sup>d</sup></sup>	95.0 $\pm$ 0.3 <sup>e</sup>	94.7 $\pm$ 0.1 <sup>e</sup>	90.4 $\pm$ 0.9 <sup>e</sup>
5.0 mg mL <sup>-1</sup>	98.9 $\pm$ 0.1 <sup>Ad</sup>	99.4 $\pm$ 0.2 <sup>d</sup>	99.7 $\pm$ 0.1 <sup>Ad</sup>	99.7 $\pm$ 0.0	99.7 $\pm$ 0.1	99.0 $\pm$ 0.1 <sup>d</sup>	58.2 $\pm$ 1.0 <sup>BC<sup>f</sup></sup>	98.7 $\pm$ 0.1 <sup>D</sup>	67.1 $\pm$ 3.1 <sup>BC<sup>d</sup></sup>	96.8 $\pm$ 0.2 <sup>e</sup>	95.9 $\pm$ 0.3 <sup>e</sup>	94.3 $\pm$ 0.5 <sup>e</sup>

A,B Values within rows are significantly different at the same pH, concentration and mycotoxin when minerals (OAB and HAP) are compared.

C-E Values within rows are significantly different at the same mineral and mycotoxin when different pH (7.4, 3.0 and 8.4) are compared.

c-f Values within columns are significantly different at the same mineral, pH and mycotoxin when different concentrations (0.5, 1.0, 2.0, 4.0 and 5.0 mg mL<sup>-1</sup>) are compared.

Table 3. Isotherms data related to the adsorption of OTA and ZEA onto organic activated bentonite and humic acid polymer.

Parameter	Minerals											
	Organic Activated Bentonite (OAB)						Humic Acid Polymer (HAP)					
	OTA			ZEA			OTA			ZEA		
	pH 7.4	pH 3.0	pH 8.4	pH 7.4	pH 3.0	pH 8.4	pH 7.4	pH 3.0	pH 8.4	pH 7.4	pH 3.0	pH 8.4
Freundlich												
$1/n$	16.5896	22.7567	25.3232	20.2633	15.2121	13.6468	5.6042	20.4273	0.7011	2.2934	10.7148	4.9581
$K_F$	1.3724	1.1162	1.6108	2.4021	0.7928	0.8790	0.0315	0.6108	0.1048	0.2854	0.2522	6.5911
$\chi^2$	32.8E-06	8.43E-06	11.5E-06	75.5E-05	15.0E-05	63.2E-05	3.44E-06	7.61E-06	6.42E-06	113E-05	41.9E-05	111E-05
Langmuir												
$q_{max}$	483.4152	6.3864	385.6266	869.2168	2222.6820	406.2835	5.8490	132.1720	10.7499	7.4447	1.7901	13.0581
$K_L$	0.08250	0.4087	0.1394	0.0097	0.0054	0.0295	0.0459	0.1274	0.0158	0.2211	1.6653	0.1927
$\chi^2$	4.92E-06	8.43E-06	8.84E-06	75.5E-05	15.1E-05	63.2E-05	0.86E-06	10.2E-06	2.09E-06	32.1E-05	41.4E-05	36.7E-05
BET												
$q_{max}$	483.8100	116.2170	386.0334	6777.2410	2853.9420	2882.7440	5.9843	132.6000	10.8150	8.8095	6.8011	16.1901
$K_{BET}$	0.8244	0.2163	0.1393	0.0012	0.004230	0.0042	0.0449	0.1270	0.0157	0.1943	0.4649	0.1726
$\chi^2$	6.80E-06	12.6E-06	13.6E-06	75.4E-05	15.0E-05	63.1E-05	1.28E-06	10.7E-06	2.73E-06	31.8E-05	40.6E-05	34.8E-05
Redlich-Peterson												
A	483.4348	25.5306	385.8587	11.8061	9.9959	39.8218	5.8491	54.3784	10.7498	7.4438	2.9810	13.0551
B	39.8810	1.1206	53.7612	6.6551	8.4543	5.7928	0.2683	18.3187	0.1695	1.6458	1.7901	2.5154
g	1	0.8	1	1	0.9	1	1	0.8	1	1	1	1
$\chi^2$	4.92E-06	8.43E-06	8.84E-06	61.9E-05	10.8E-05	57.9E-05	0.86E-06	6.92E-06	2.09E-06	32.1E-05	41.4E-05	36.7E-05