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Abstract:	<p>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.</p>

	<p>At the recommended statutory level for the mycotoxins of 0.1 mg Kg-1 OTA and 0.5 mg Kg-1 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 (Chi2) 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.</p>

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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 (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 1st 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⁻¹ OTA and 0.5 mg kg⁻¹ 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 (Chi²) 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.

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

Introduction

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

Bentonite is widely present in animal feed production as an anti-caking agent. It is a clay mineral composed mainly of montmorillonite, belonging to the smectite group of silica clays (WHO, 2005). The crystal structure of a montmorillonite is an octahedral alumina sheet layered between two tetrahedral silica sheets (Bailey, 1980). Natural bentonites are amphoteric with a pH-dependent surface charge and the capacity to react specifically with cations, and to a lesser extent with anions and molecules forming chemical bonds (WHO, 2005). The cation exchange capacity of montmorillonite is

rather high ($\sim 100 \text{ meq } 100\text{g}^{-1}$), and is not limited to inorganic ions but also to organic substances such as aflatoxin B1 (Thieu et al., 2008); organic compounds like alkyl ammonium can act as balancing cations modifying the overall charge of the sorbent particles (Majdan et al., 2009). Subsequently, high molecular weight quaternary amines are often used to improve hydrophobicity of the montmorillonite, resulting in so-called organically activated bentonites, as used in the current experiment (Balek et al., 2002). The ability of such activated bentonites to bind diverse structures has been demonstrated *in vitro* with zearalenone (Bueno et al., 2005), aflatoxin (Thieu et al., 2008) and ochratoxin A from wine (Kurtbay et al., 2008).

Humic acid polymers (HAP) originate from natural decaying of organic plant materials. They are present in soils, sediments, peat, and brown coals. The natural occurring humic acid polymers contain different reactive moieties, such as hydroxyl, phenol, carboxyl and methoxyl groups, and have a large specific surface area (Sutton and Sposito, 2005). Although humic acid polymers are chemically diverse and differ in molecular size, they are all hydrophilic, reversible colloids composed of 300 to 10,000 units. They can form hydrophobic (van der Waals, π - π , CH- π) and hydrogen bonds, the former becoming more relevant with increasing pH (Piccolo, 2002). The capacity of humic substances to sequester zearalenone *in vitro* (Sabater-Vilar et al., 2007) and aflatoxins *in vivo* has been demonstrated (Jansen van Rensburg et al., 2006).

Commonly, new adsorbents or mixtures of are tested at variable concentrations, varying between 1 g kg^{-1} (Sabater-Vilar et al., 2007), 2.5 g kg^{-1} (Kocabagli et al., 2002), and 4 g kg^{-1} feed (Thieu et al., 2008). A regularly used tool to define the optimal concentration and absorbent capacity of different substances are *in vitro* assays conducted in buffer solutions with or without feed materials and enzymes (Avantaggiato et al., 2007). Originally, *in vitro* tests have been performed at a defined pH, sometimes

acidic (Kurtbay et al., 2008), but more often at a neutral pH (Cavret et al., 2010). However, the binding affinity of a polar substance is influenced by pH, and therefore it is recommended to measure adsorbent characteristics under the conditions of the gastrointestinal tract at neutral, acid and basic pH (Sabater-Vilar et al., 2007). Previously, it has been shown that adsorption of zearalenone and aflatoxin B₁ by mineral clays and humic acid polymers, respectively, is pH dependent (Dakovic et al., 2007; Ye et al., 2009), and that the transition to alkaline conditions may lead to desorption.

Next to single concentration experiments, the evaluation of adsorption isotherms have been recommended to characterize mycotoxin adsorption (Ringot et al., 2007; EFSA, 2009), considering that under defined condition an adsorption equilibrium is reached at which the quantity of the toxin being adsorbed is equal to the quantity being desorbed. Several theoretical adsorption models have been developed to describe such isotherms, among others by Freundlich (Freundlich, 1906), Langmuir (Langmuir, 1916), Brunauer-Emmett-Teller (BET) (Brunauer et al., 1938), and Redlich-Peterson (Redlich and Peterson, 1958), considering homogeneity, as well as monolayer and multilayer adsorption. Such isotherms analyses allow to (i) identify the best fitted isotherm model, (ii) predict adsorption capacity of a mycotoxin onto a specific adsorbent, and (iii) recognize each adsorbent capacity as homo- or heterogeneous, or as mono- or multilayer binding.

Ochratoxin A (OTA) and zearalenone (ZEN) are two mycotoxins that are frequently occurring in animal feeds in Northern Europe, being of concern in the diets for pigs (EFSA, 2004a,b). OTA, a nephrotoxic and immunosuppressive agent in animals, consists of a dihydroisocoumarin moiety linked to l-phenylalanine through an amide bond (Fig. 1A). It has a carboxyl group at the phenylalanine moiety and a phenol

group at the dihydroisocoumarin part with a pKa 4.4 and 7.3 respectively (Ringot et al., 2005). Being a polyaromatic molecule, OTA is hydrophobic ($\log P_{OW}=4.4$) when unionized (Dakovic et al., 2005). ZEN is a resorcylic acid lactone, and is a hydrophobic compound ($\log P_{OW} = 3.66$) (Lemke et al., 1998). It is a weak acid due to the presence of the diphenolic moiety and has a pKa of 7.62 (Fig. 1B). The structure of ZEN is flexible enough to bind to oestrogen receptors in animals, leading to hyperoestrogenicity and adverse effects on fertility and reproduction particularly in pigs (Zinedine et al., 2007). OTA and ZEN were selected as model mycotoxins for the current study due to their very different structure and physic-chemical properties and in consideration of their relevance in pig husbandry as for both mycotoxins, pigs are considered to be the most sensitive animal species (EFSA, 2004a,b).

Hence, the aim of the present study was to evaluate and compare two chemically different sorbents, an organically activated bentonite and a humic acid polymer, regarding their capacity to bind mycotoxins under different conditions. To this end, adsorbent concentration dependence, adsorption pH dependence, and mycotoxin desorption were analysed. Modelling of mycotoxin adsorption was performed to characterize OTA and ZEN adsorption at neutral, acidic and basic pH and to evaluate the robustness of this model for forthcoming assessments of new mycotoxin adsorbents.

Materials and Methods

Mycotoxins and adsorbents

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; KH_2PO_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

commercially available organic activated bentonite (OAB) and humic acid polymer (HAP) (Poortershaven Industrial Minerals BV, Rotterdam, The Netherlands).

Single concentration experiments: effects of adsorbent concentration and pH on adsorption capacity

Either OTA or ZEN were added to the buffer solution at a fixed concentration of 0.1 or 0.5 $\mu\text{g mL}^{-1}$, respectively following the guidance levels as defined in Commission Recommendation 2006/576/EC. For each mycotoxin, the test protocol comprised i) negative controls (buffer alone or with each of the five adsorbent concentrations without mycotoxins, ii) positive controls, i.e. buffer with mycotoxin without adsorbent, to validate pH dependent recovery of the mycotoxin by the applied extraction methods and iii) test solutions, containing buffer with the dissolved mycotoxin (OTA or ZEN) to which the adsorbent (OAB or HAP) at five different concentrations (0.5, 1.0, 2.0, 4.0 or 5.0 mg mL^{-1}) was added. Incubations were at three different pHs (7.4, 3.0 and 8.4) in a continuous system. The percentage of adsorption by the adsorbents was computed using the equation:

$$\% \text{ adsorption} = \frac{(C_0 - C_{\text{eq}}) * 100}{C_0} \quad (1)$$

where C_0 and C_{eq} are the initial concentration of the mycotoxin in solution ($\mu\text{g mL}^{-1}$) and the residual mycotoxin concentration at equilibrium ($\mu\text{g mL}^{-1}$), respectively.

Equilibrium adsorption isotherms

Based on the results of the experiment 1, a second series of experiments was performed to describe the interaction between OTA and ZEN and the two test products OAB and HAP. These equilibrium experiments were carried out by incubating using the same buffer solution, with or without addition of one of the adsorbents in the most

efficient concentration obtained in the experiment 1. In these experiments mycotoxin concentrations varied for OTA between 0.025, 0.05, 0.1, 0.15 and 0.2 $\mu\text{g mL}^{-1}$ and for ZEN between 0.125, 0.250, 0.5, 0.75 and 1.0 $\mu\text{g mL}^{-1}$. The test protocol was essentially the same as for the single concentration studies.

Incubation protocol

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

Determination of binding capacity

The binding capacity was calculated from the recovery of the unbound mycotoxin fraction, measured by standard HPLC methods.

Ochratoxin A: The free (non-bound) concentration of OTA was quantified by a standard HPLC method using a Spherisorb ODS-2, stainless steel, 5 μm 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⁻¹; 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⁻¹), 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 (χ^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

the organic cations that confer hydrophobicity to OAB remain almost unaffected by the pH change from 7.4 to 3.0 and back to 8.4. The protonation of OTA on the other hand is changed in the pH course, as it has a pKa of 4.4 and 7.3. At pH 3.0 almost all OTA will be in the neutral (non-charged) form, while at pH 7.4 it will be present as a monoanion or a dianion, and at pH 8.4 most OTA will be in the dianion form. Previous investigations have demonstrated that minerals through binding of metal cations could adsorb OTA (Dakovic et al., 2005). However, as in our studies an organically activated bentonite was used, interactions with metal cations are unlikely as the OAB surface will be covered with positively charged groups, presenting a bilayer and probably also interlayer, of cationic surfactants. It can be assumed that the anionic species of this mycotoxin will have both partly electrostatic and hydrophobic interaction with OAB, while regarding HAP, a coupling between divalent cations and anions on negatively charged groups in humic acids can be expected.

Whereas OAB showed to be an excellent OTA-adsorbent, regardless of pH, sequestering >96% of OTA at a minimum concentration of 2.0 mg mL⁻¹, OTA adsorption onto HAP, in all concentrations, was significantly higher at pH 3.0 (>96%) when compared to pH 7.4 (67%) and 8.4 (58%) (Table 2). As the incubations at pH 3.0 were followed by incubations at pH 8.4, we suggest that desorption at alkaline pH is probably caused by repulsion, as both OTA and humic acid obtain a negative charge at an alkaline pH through their carboxyl and phenol groups.

There was no pH effect on ZEN adsorption onto OAB or HAP, although in presence of OAB it was slightly less efficient at 8.4, compared to pH 7.4 and 3.0. ZEN has a pKa of 7.62 (Lemke et al., 1998), and will be found almost completely in its deprotonated form at pH 8.4. HAP only shows a >90% adsorption at pH 3.0. The negative charge of this compound developed at higher pH through its carboxyl and

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⁻¹, 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⁻¹ 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⁻¹, 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⁻¹ (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⁻¹ 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 Chi² parameter (Ho, 2004). From the results for HAP, shown in table 3, the Freundlich model failed to describe adsorption, except when OTA (Chi²=7.61E-06) or ZEN (Chi²=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 χ^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 χ^2 values, ranging between 10.8E-05 to 15.1E-05 (Table 3). Chi-square values showed a better fit, indicated by lower χ^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_{\max} was high, and higher values when q_{\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 χ^2 was high for all tested conditions, but it was found to be suitable to describe the

interaction between ZEN and HAP, as demonstrated by the low χ^2 values. This confirms that multilayer adsorption will also depend on the chemical structure of the mycotoxin.

In general, the lowest values of χ^2 were obtained with Langmuir and Redlich-Peterson models. Redlich-Peterson and Langmuir isotherms overlapped each other, when OTA adsorption was evaluated at pH 7.4 (OAB and HAP) and 8.4 (HAP), and ZEN adsorption onto HAP at all pH conditions. Comparing both models, maximum adsorption capacity was similar in all analyses, except at pH 3.0. The Redlich-Peterson isotherm is an empirical equation that combines characteristics of Langmuir and Freundlich isotherms into a single equation. At low concentrations, it approximates Henry's law, while at high concentrations it approaches the Freundlich model (Ho, 2006). The Redlich-Peterson isotherm accurately described the adsorption behaviour observed for all mycotoxin-adsorbent pairs, and gave the best fit in general. The Redlich-Isotherm parameters did not vary as much as the correspondently parameters of the Langmuir function. The Redlich-Peterson constant (g) was generally 1 (but 0.8 for OTA at pH 3.0 for both OAB and HAP, and 0.9 for ZEN/OAB, pH 3.0, respectively), which indicates that the function is reduced to the Langmuir isotherm equation. Therefore, the Redlich-Petersen isotherm model can be recommended for further comparisons of mycotoxin-binding agents.

Mycotoxin adsorption by organically modified aluminosilicates has been studied previously (Lemke et al., 1998; Tomasevic-Canovic et al., 2003; Dakovic et al., 2005, 2007; Feng et al., 2008). Feng et al. (2008) measured ZEN binding to montmorillonite and organically modified montmorillonite, and found a $\log K_F$ of 2.3 and 3.7 respectively. Lemke et al. (1998) even found an increase of $\log K_F$ from -0.5 to 4.9. The performance at pH 10 was much lower than at pH 2 or 6.5. Dakovic et al. (2003) and

Tomasevic-Canovic et al. (2003) did not find any pH effect (pH 3.0, 7.0 and 9.0) in their isotherm studies on organically modified zeolites. Bentonite was initially used to bind aflatoxins (Thieu et al., 2008), and found less effective as a binding agent for other mycotoxins. With the intention to broaden the binding spectrum, attempts were made to apply heat activation (Bueno et al., 2005) or organically activation (Kurtbay et al., 2008). The last authors showed that OTA adsorption onto non-modified bentonite was poor, while organically activated bentonite was effective to adsorb OTA, suggesting that the improved hydrophobicity by organic activation results in a broader application to mycotoxins that are less polar.

Aluminosilicates have been studied extensively as mycotoxin adsorbents. In contrast, the use of humic acid polymers as a mycotoxin sorbent has received much less attention. Jansen van Rensburg et al. (2006) tested the processed humic acids (oxihumate) as a binder of aflatoxin B1, both *in vitro* and *in vivo*. Oxihumate proved to be a good adsorbent *in vitro*, at pH 3.0, 5.0 and 7.0, and *in vivo*, as it was able to diminish the adverse effects of aflatoxin on body weight, liver, stomach, heart and blood of male broiler chickens. A further *in vitro* evaluation of sodium humate as an aflatoxin B1 adsorbent was presented by Ye et al. (2009). Sodium humate was found to be an efficient adsorbent with increasing adsorption at pH 3.0, 7.0 and 8.0. Sabater-Vilar et al. (2007) had before investigated some natural humic acid polymers for their capacity of ZEN adsorption at acidic (pH 2.5) and alkaline (pH 8) conditions. However, in none of these studies, the nature of the binding applying isotherm studies was included. The experiments presented here confirm the principal capacity of humic acid polymers, to adsorb mycotoxins, at least OTA and ZEN.

The use of OAB and HAP as mycotoxin adsorbents may have some advantages and disadvantages. Next to mycotoxin binding, bentonites can act as gut protectant

when used in animal feed, and have a capacity to bind heavy metals, polycyclic aromatic and other non polar substances, such as dioxins (WHO, 2005). In turn, the use of bentonites in practice requires continuous monitoring of the absence of undesirable contaminants in these products. Bentonites have a long history of safe use as an anticaking agent. In addition, the safety and efficacy of bentonite as feed additive has been evaluated also by the European Food Safety Authority (EFSA, 2007). They concluded that for ruminants there were no toxicological concerns and approved it also as aflatoxin binder. Bentonites are not genotoxic and are not absorbed following application as a feed additive, hence providing no direct toxicological risk for the animal of consumer. However, the absence of other undesirable effects, such as significant adsorption of essential elements and vitamins need to be evaluated for each product and confirmed, like the efficacy, in *in vivo* experiments with target animal species.

Humic acids complexes also have been shown to promote health, possibly through their anti-inflammatory, antiviral and heavy-metal binding activities (Jansen van Rensburg, 2005). In contrast to the pro-carcinogenic soluble humic acids, poorly soluble humic acid complexes occurring in natural peats, and being composed of humic and fulvic acid complexes have broad applications in soil managements, water cleaning and industrial decontamination processes (Sutton and Sposito, 2005). The medical literature of East Europe refers to various beneficial effects of these products (Cozzi et al., 1993; Lind and Glynn, 1999) and feeding experiments in pigs have confirmed their value in pig nutrition and as mycotoxin ameliorating agents (Ji et al., 2006, Demeterova et al., 2009). These broad beneficial effects classify this group of compounds for long term use in farm animals at risk for exposure to contaminated feed. However, like for

the bentonides the efficacy and safety of a humates need to be established in target animal species prior to large scale use.

Conclusions

In the present study, we characterized the adsorption of OTA and ZEN by an organically activated bentonite (OAB) in comparison with a humic acid polymer (HAP). HAP has an increased OTA adsorption capacity at pH 3.0, but this adsorption is susceptible to alkaline pH, leading to a partial desorption of this mycotoxin at pH 8.4. Analysis of the individual results as the efficient isotherm models confirmed that adsorption capacity and affinity depend on the interactions between mycotoxin, adsorbent and pH. Therefore, isotherm models to describe adsorption equilibrium cannot be generalized, but serve as a valuable tool to identify and to compare new products. The presented *in vitro* approach has shown its robustness in various experimental protocols.

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Figure captions

Fig. 1 Molecular structure of OTA (A) and ZEN (B).

Fig. 2 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 ZEN (C, D).

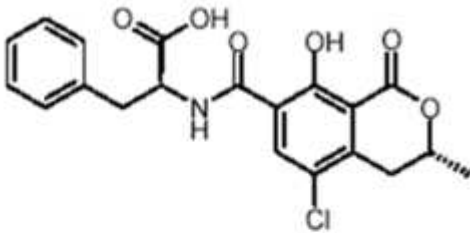
Fig. 3 Adsorption isotherm modelling of OTA sequestration using OAB at pH 7.4, 3.0 and 8.4 (A); Langmuir, Freundlich, BET and Redlich-Peterson isotherms obtained at pH 7.4 (B), 3.0 (C) and 8.4 (D).

Fig. 4 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).

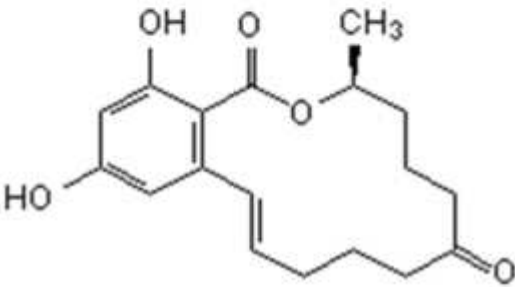
Fig. 5 Adsorption isotherm modelling of ZEN sequestration using OAB at pH 7.4, 3.0 and 8.4 (A); Langmuir, Freundlich, BET and Redlich-Peterson isotherms obtained at pH 7.4 (B), 3.0 (C) and 8.4 (D).

Fig. 6 Adsorption isotherm modelling of ZEN 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).

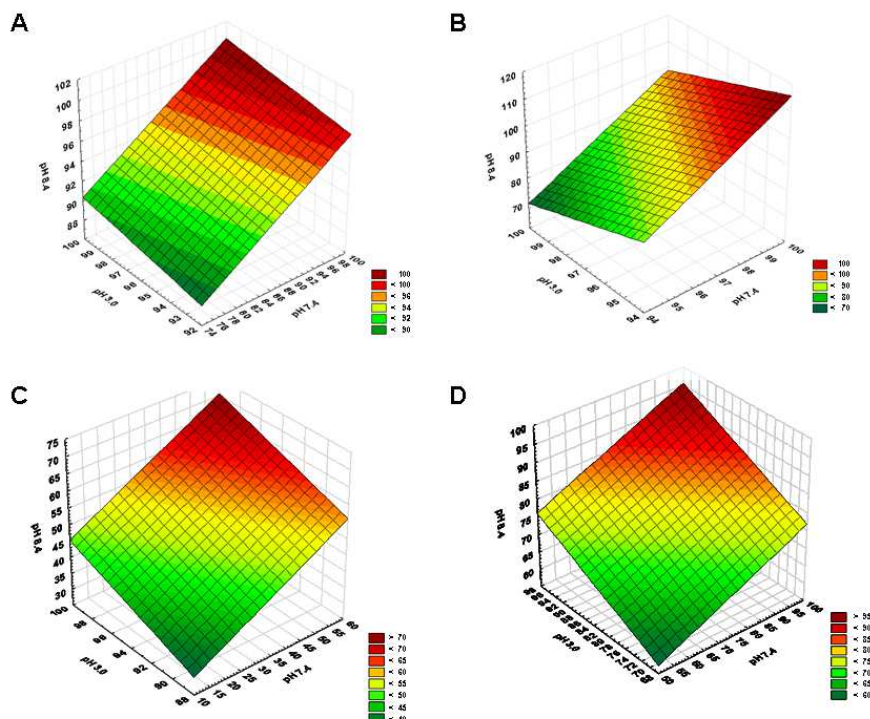
A



B

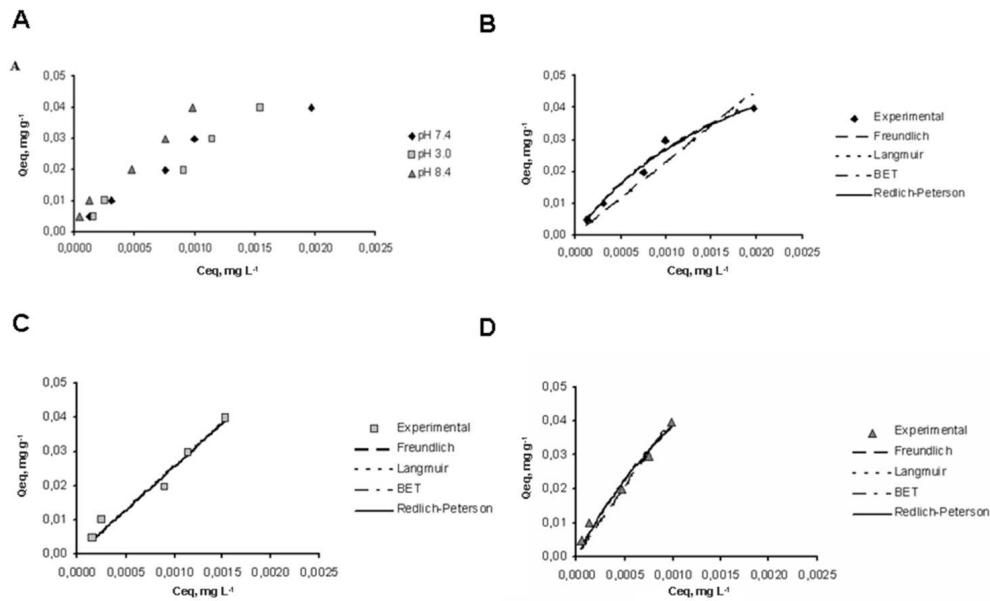


Molecular structure of OTA (A) and ZEA (B).
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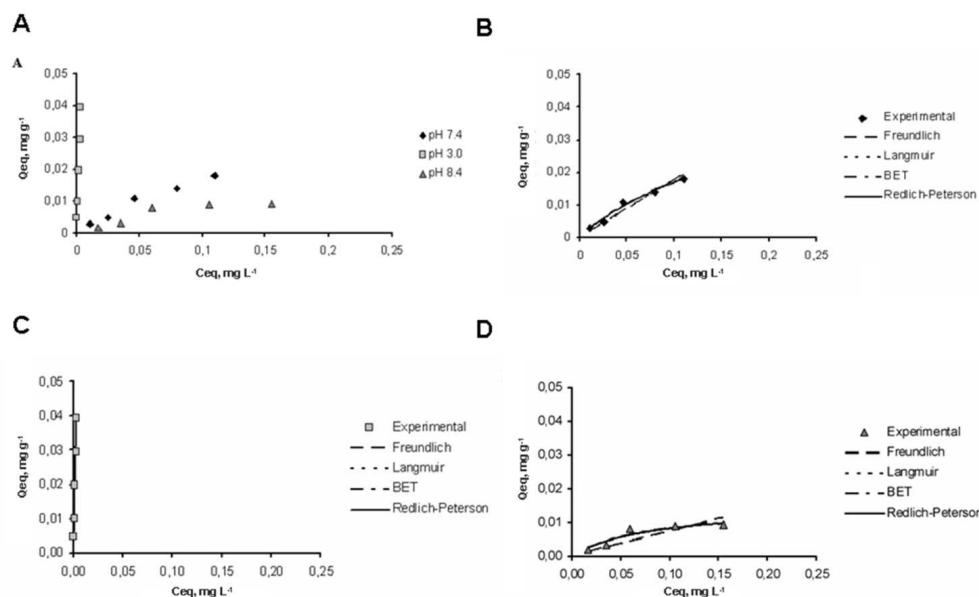


Three-dimensional diagrams, with pH 7.4 on the x-axis, pH 8.4 on the y-axis, and pH 3.0 on the z-axis, representing the capacity, in percentages, of OAB (A, C) and HAP (B, D) to adsorb OTA (A, B) and ZEA (C, D).

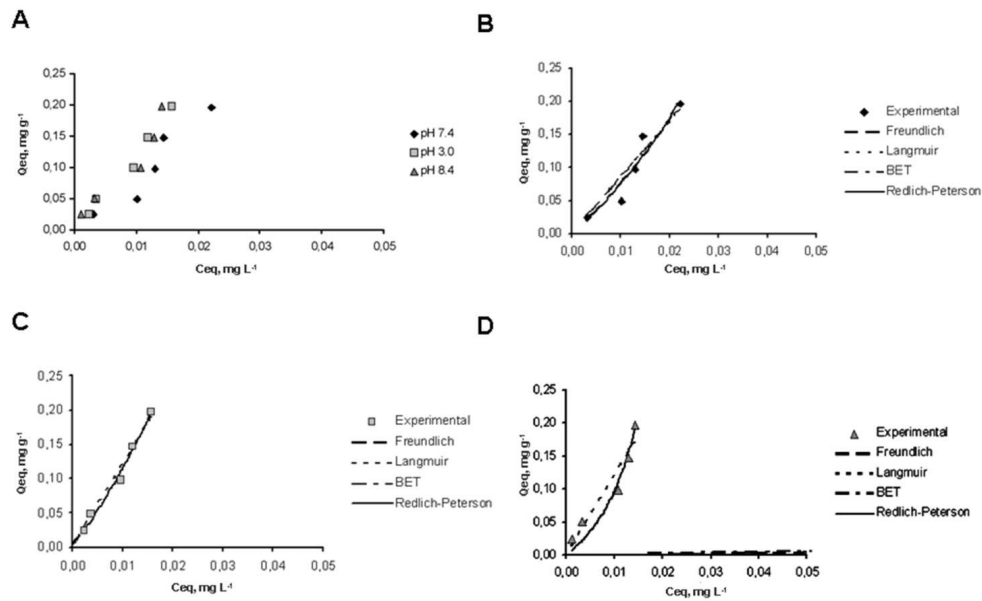
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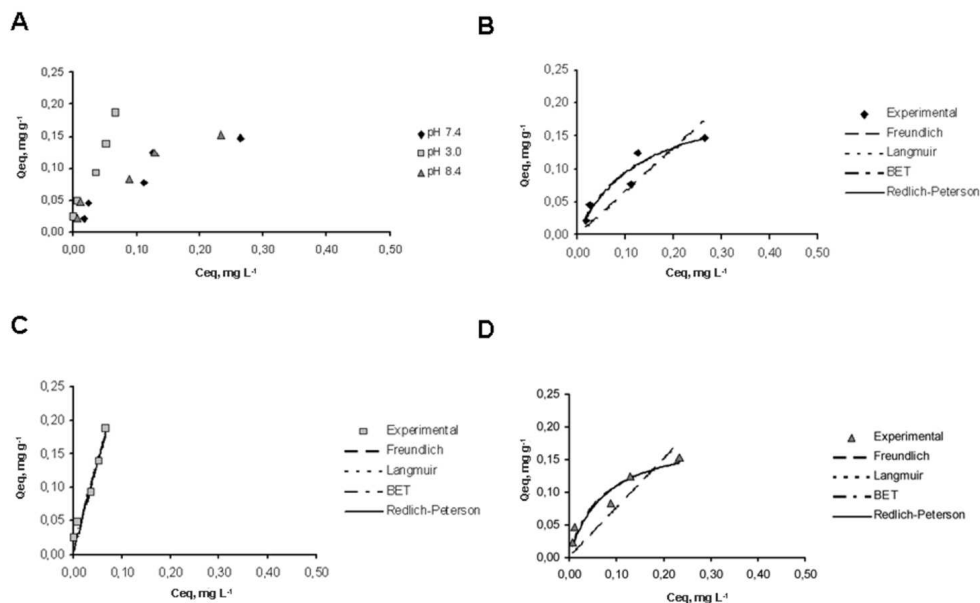
Adsorption isotherm modelling of OTA sequestration using OAB at pH 7.4, 3.0 and 8.4 (A); Langmuir, Freundlich, BET and Redlich-Peterson isotherms obtained at pH 7.4 (B), 3.0 (C) and 8.4 (D).
63x39mm (600 x 600 DPI)



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);
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 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)

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 (mg g ⁻¹)
K_L	Langmuir isotherm constant (L mg ⁻¹)
q_{max}	maximum adsorption capacity (mg g ⁻¹)
K_{BET}	Bruanauer-Emmett-Teller (BET) isotherm constant (L mg ⁻¹)
A	Redlich-Peterson isotherm constant (L mg ⁻¹) ^g
B	Redlich-Peterson isotherm constant (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 ⁻¹	75.9 \pm 4.8 ^{Ac}	92.8 \pm 1.3 ^c	89.0 \pm 1.9 ^c	94.4 \pm 0.3 ^A	94.7 \pm 0.1 ^A	87.1 \pm 1.4 ^{Ac}	15.2 \pm 3.6 ^{BCc}	89.0 \pm 1.5 ^D	35.5 \pm 5.3 ^{BEc}	56.2 \pm 5.1 ^{Bc}	69.8 \pm 1.0 ^{Bc}	60.8 \pm 2.1 ^{Bc}
1.0 mg mL ⁻¹	88.1 \pm 2.4 ^{Ac}	95.7 \pm 0.3 ^c	94.6 \pm 0.6 ^{Ac}	97.5 \pm 0.1 ^A	98.0 \pm 0.1 ^A	92.2 \pm 0.9 ^{Ac}	24.9 \pm 2.1 ^{BCd}	94.1 \pm 0.6 ^D	42.9 \pm 2.0 ^{BEc}	74.8 \pm 4.1 ^{Bd}	85.2 \pm 0.2 ^{Bd}	78.2 \pm 0.8 ^{Bd}
2.0 mg mL ⁻¹	96.3 \pm 0.1 ^{Ad}	97.8 \pm 0.2 ^d	98.2 \pm 0.2 ^{Ac^d}	98.6 \pm 0.2 ^A	98.5 \pm 0.1 ^A	96.7 \pm 0.2 ^{Ad}	37.7 \pm 1.6 ^{BCe}	96.7 \pm 0.2 ^D	55.9 \pm 2.6 ^{BEcd}	86.6 \pm 2.4 ^{Bd}	92.0 \pm 0.1 ^{Be}	86.1 \pm 1.2 ^{Bd}
4.0 mg mL ⁻¹	98.9 \pm 0.2 ^{Ad}	99.0 \pm 0.1 ^d	99.6 \pm 0.1 ^{Ad}	99.5 \pm 0.0	99.2 \pm 0.1	98.2 \pm 0.1 ^d	42.7 \pm 1.8 ^{BCe}	98.3 \pm 0.1 ^D	61.7 \pm 3.2 ^{BE^d}	95.0 \pm 0.3 ^e	94.7 \pm 0.1 ^e	90.4 \pm 0.9 ^e
5.0 mg mL ⁻¹	98.9 \pm 0.1 ^{Ad}	99.4 \pm 0.2 ^d	99.7 \pm 0.1 ^{Ad}	99.7 \pm 0.0	99.7 \pm 0.1	99.0 \pm 0.1 ^d	58.2 \pm 1.0 ^{BCf}	98.7 \pm 0.1 ^D	67.1 \pm 3.1 ^{BCd}	96.8 \pm 0.2 ^e	95.9 \pm 0.3 ^e	94.3 \pm 0.5 ^e

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⁻¹) 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
χ^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
χ^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
χ^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
χ^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