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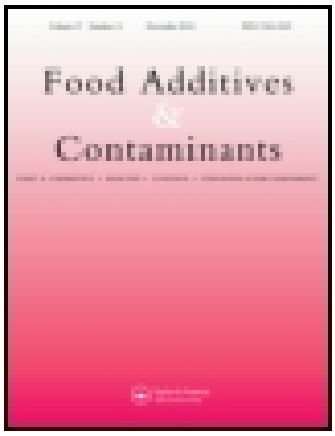

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Negligible effects of tryptophan on the aflatoxin adsorption of sodium bentonite

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Negligible effects of tryptophan on the aflatoxin adsorption of sodium bentonite

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Abstract

The main objective of this study was to determine if the competitive adsorption of tryptophan (Trp) and aflatoxin B_1 (AFB_1) could potentially affect the ability of a sodium bentonite (NaB) to prevent aflatoxicosis in monogastric animals. The adsorption of Trp and AFB_1 on this adsorbent is fast and could be operating on the same time-scale making competition feasible. *In vitro* competitive adsorption experiments under simulated gastrointestinal conditions were performed. A high affinity of the clay for Trp and NaB was observed. The effect of an excess of KCl to mimic the ionic strength of the physiological conditions were also investigated. A six times decrease in the Trp surface excess at saturation was observed. A similar behavior was previously found for AFB_1 adsorption. Taking into account the amount of Trp adsorbed

by the clay and the usual adsorbent supplementation level in diets, a decrease in Trp bioavailability is not expected to occur. Tryptophan adsorption isotherms on NaB were S-shaped and were adjusted by the Frumkin-Fowler-Guggenheim model. The reversibility of the adsorption processes was investigated in order to check a potential decrease in the ability of NaB to protect birds against chronic aflatoxicoses. Adsorption processes were completely reversible for Trp, while almost irreversible for AFB_1. In spite of the high affinity of the NaB for Trp, probably due to the reversible character of Trp adsorption, no changes in the AFB_1 adsorption isotherm were observed when an excess of the amino acid was added to the adsorption medium. As a consequence of the preferential and irreversible AFB_1 adsorption and the reversible weak binding of Trp to the NaB, no changes in the aflatoxin sorption ability of the clay are expected to occur in the gastrointestinal tract of birds.

Keywords: Aflatoxin B_1; sodium bentonite; tryptophan, competitive adsorption.

Introduction

Aflatoxins are secondary metabolites produced in several substrates by some species of *Aspergillus* section *Flavi* (Zain 2011). Numerous aflatoxins (AFs) have been already identified. Figure 1 shows the chemical structure of aflatoxin B_1 (AFB_1), one of the most common and toxic compounds present in avian feeds (Hussein and Brasel 2001). The toxicity of AFB_1 in broilers has been widely investigated due to their carcinogenic, mutagenic, teratogenic, and growth inhibitory effects (Oğuz et al. 2000; Magnoli et al. 2011a, b; Sur and Celik 2003). Animals that consume AF-contaminated feed develop various health problems, including growth retardation, increased feed:gain ratio, and both liver and kidney damage (Bintvihok 2002).

A variety of physical, chemical and biological approaches, employed to counteract the mycotoxin problems, have been reported in the literature (Zaki et al. 2012). However, large scale, practical and cost-effective methods for a complete detoxification of mycotoxin-containing feedstuffs are currently not available. One of the most common strategies used in the feed industry is the dietary inclusion of sorbent materials in order to remove toxins by

adsorption during passage through the gastrointestinal tract. The incorporation of non-nutritive substances such as the sodium bentonite (NaB), hydrated sodium calcium aluminosilicates (HSCAS); among others similar dietary feed additives have been reported in the literature (Oğuz 2011). Some of these substances have shown ability to bind AFB_1 in laboratory *in vitro* assays (Phillips et al. 1988; Jaynes et al. 2007) and have decreased the severity of aflatoxicosis in chickens (Kubena et al. 1990a,b; Huff et al. 1992; Scheideler 1993; Magnoli et al. 2011a, b) and swine (Smith 1984; Schell et al. 1993; Papaioannou et al. 2005). *In vivo* effectiveness of these additives depends on their ability to bind AFs in the gastrointestinal tract, resulting in a decrease in the toxin bioavailability for absorption. An inert, stable and insoluble complex between NaB and AFB_1 was assumed to be the responsible for preventing toxin absorption in poultry (Rosa et al. 2001). Bentonites are smectite clays with a 2:1 layered structure with an inner aluminium octahedral layer that shares oxygen atoms with two outer silicon tetrahedral sheets. The substitution of both Si^{4+} by Al^{3+} in the tetrahedral sheets and of trivalent cations by divalent ones in the octahedral sheet give rise to a negative charge in the clay framework. This charge is counterbalanced by monovalent and divalent cations, such as Na^+, K^+, Ca^{2+} and Mg^{2+}, located in the interlamellar space. These ions are easily exchangeable and are responsible for the cation exchange ability of the clays. Organic substances can be adsorbed in the clay, not only in the external basal surfaces and edges but also in the interlayer spaces (Dakovic et al. 2008). Sodium bentonite is commonly added to feed diets either as pelletization agent and/or as aflatoxin binder. Different factors, such as pH, feed composition and additives among others, can affect the mycotoxin binding ability during digestion. These aluminosilicates are non-selective adsorbents and have some disadvantages when are included in high rates in the diets (Moshtaghian et al., 1991; Phillips 1999). In fact, these adsorbents may interact with critical nutrients in the diets either decreasing the nutrients bioavailability or changing the adsorbent capacity to bind mycotoxins (Chestnut et al., 1992; Abbès et al. 2008).

Recent studies have shown the influence of monensin in the AFs adsorption capacity of a sodium bentonite from Argentina. This coccidiostat agent, commonly added to poultry feed

as a prophylactic tool, has reduced the detoxification potential of the assayed bentonite. (Magnoli et al. 2011a,b). The knowledge of potential interfering agents in the detoxification procedure allows decisions to be taken about prevention protocols, diet formulations and could even highlight the need to seek for new adsorbents. The poultry industry uses different diets along the production cycle based in the use of concentrated feed; mainly composed of cereals, soybean meal and additives (NRC 1994). Tryptophan (Trp), Figure 1, is an essential aromatic amino acid, component of many vegetable and animal proteins. Raw materials of feed usually give the nutritional required levels of Trp for birds and do not need to be supplemented. This amino acid increases enzyme activity favoring the digestion of different nutrients. Recommended Trp levels vary between 0.18 - 0.24% for different diets during the lifetime of birds (Ross Broiler Management Manual, 2009). Lambert (2008) has deeply reviewed the physical-chemical aspects about the adsorption mechanism of different amino-acids on mineral surfaces. On the other hand, several *in vitro* studies have been carried down to test the mycotoxin adsorption ability of clay minerals mainly montmorillonite (Deng et al. 2010 and references therein). However, to the best of our knowledge there are not studies in the literature about competitive adsorption between tryptophan and aflatoxin B_1 onto sodium bentonite. Therefore, the objective of this study was to *in vitro* evaluate the competitive adsorption of tryptophan and aflatoxin B_1 on a sodium bentonite from Argentina.

Materials and Methods

Reagents

Aflatoxin B_1 and tryptophan (Sigma Chemical Co; ST. Louis, MO) purity ≥98% were used without further purification. Demineralized water (HPLC grade) was obtained with Labconco (Labconco Corp., Kansas City, MO) equipment (model 90901-01).

A commercial “Del Lago” bentonite, provided by Castiglioni, Pes y Cía, was used for the assays. The location of source mine is Cinco Saltos, province of Río Negro-Argentina. The clay, previously characterized in Magnoli et al. 2008, is mainly composed by sodium

montmorillonite. The adsorbent was activated for 24 h at 110°C in vacuum oven (Vacuum over Yamato ADP-31).

Analytical methods for adsorbates quantification

Tryptophan was determined by UV-visible spectrometry following the adsorption at 280 nm (diode array spectrophotometer Hewlett Packard Model 8453). Aflatoxin B_1 was either by UV spectroscopy following the AFB_1 absorption band at 362nm or by HPLC of previously derivatized samples according to Trucksess et al. (1994). The mobile phase was methanol:acetonitrile:water (1:1:4, v/v), flow-rate of 1.5 mL/min. For derivatization, aliquots (200 µl) were mixed with 700 µl of acetic acid–trifluoroacetic acid–water (20:10:70) solution, allowed to stand for 9 min at 65ºC in the dark (AOAC, 1994). An HPLC Gilson 302 pump with a fluorescence detector (fluorometer model 121), excitation wavelength of 305-395 nm and emission wavelength of 430-470 nm, and a C_{18} Luna Phenomenex column (150 x 4. 6 mm, 5 µm) was used (AOAC, 1994). The derivatized AFB_1 retention time was 4.7min and the limit of detection was 0.001 µg/ml. Standards for the calibration curve were prepared by dilution of a stock solution of AFB_1 2.06 µg/ml. The concentrations of chromatographic standards were 0.005, 0.010 and 0.015 µg /ml of AFB_1. Standard solutions for the calibration curves were prepared daily.

Adsorption experiments

Tryptophan adsorption isotherms

Different Trp working solutions with concentrations of 53, 88, 197, 264, 285, 292, 360 and 449 µM were prepared at the corresponding experimental conditions. Aliquots of each working solution (4 mL) were allowed to be in contact with 100 mg of NaB. The solutions were agitated in an orbital shaker for 1h at 39.5 ± 0.5°C in order to simulate the gastrointestinal tract temperature. After incubation, the solutions were centrifuged 15 min at 16,000 × g, and the supernatants were carefully decanted into clean tubes. Controls of Trp and

NaB were also included in each assay. The concentration of the supernatant was analyzed by UV-visible spectroscopy. The adsorbed Trp was calculated from the Trp depletion in the supernatant after incubation. Each point in the isotherm is the mean of three assays.

Buffer solution at pH 2 was prepared by mixing 62.5 mL of 0.2 M sodium chloride with 16.25 mL of 0.2 M of hydrochloric acid. Prior to make up the volume to 250 mL, the final pH value was adjusted. The solvents for the adsorption experiment were either buffer pH 2, for a typical experiment, or a KCl (1.5 M) solution made in buffer at pH 2 for the experiment under controlled ionic strength.

Aflatoxin B_1 adsorption isotherms

Different AFB_1 working solutions with concentrations of 1.9, 3.2, 4.4, 12.6, 21.5 μM were prepared in buffer pH 2 at the corresponding experimental condition. Aliquots of 1 mL of a NaB suspension in buffer pH 2 (1 mg/mL) were added to 2 mL of each AFB_1 working solution. The same procedure described for Trp isotherms was followed. Controls of AFB_1, and NaB were also included in the assay. The concentration of the supernatant was determined by UV-visible spectroscopy at the maximum absorption wavelength of the toxin in the adsorption medium (362 nm). The absence of interference due to eventual matrix components upon the spectrometric determination was further confirmed by FD-HPLC of previously derivatized samples. The HPLC procedure was described above.

In order to test the Trp effect on the AFB_1 adsorption, a 300 μM Trp solution was prepared in buffer at pH 2 and further used as solvent for the AFB_1 working solutions and NaB suspension. The isotherm was performed by UV-visible spectroscopy at the AFB_1 362 nm. No interference due to the Trp was observed at this wavelength as shown in Figure 2.

Aflatoxin B_1 and Tryptophan adsorption kinetics

In order to perform the kinetic of the AFB_1 adsorption, aliquots of 2 mL of an AFB_1 solution (31.7 μM) in buffer at pH 2 were placed in capped vials. Aliquots of 1 mL of a NaB suspension (1mg/mL) in buffer at pH 2 were added to each vial and were kept at 39.5 ± 0.5°C in an orbital shaker. Two vials were withdrawn at each sampling time and the AFB_1 concentration in the supernatants were analyzed by UV-visible spectroscopy after

centrifugation at 16,000 × g over a period of 10 min. Sampling times vary from 0.5 to 60 min. Separated controls of both AFB_1 and NaB were also performed. For the Trp adsorption kinetic, aliquots of 4 mL of a Trp solution (300 μM) in buffer at pH 2 were allowed to be in contact with 100 mg of NaB at 39.5 ± 0.5°C, following the same methodology described for AFB_1. Differences in the amounts of NaB employed in both kinetic assays, as well as in the isotherm experiments, were mainly due to both the lower solubility and the higher affinity of the toxin for the adsorbent.

Tryptophan and AFB_1 desorption assays

Pellets (adsorbate containing bentonite) that remained after centrifugation in the adsorption assays were used for further desorption studies. The pellets left over after supernatant withdrawal were re-suspended in 4mL of the extraction solvents, and thoroughly mixed during one hour. Afterward they were centrifuged for adsorbate determination in the supernatant. The procedure was performed with each of the following extraction solvents: buffers at pH 2, 4 and 6 that were prepared as described below. The percentage of adsorbate released was determined comparing the amount of the adsorbate in the supernatant with the initial adsorbed amount for each assayed. Two replicates for each assay.

Buffer at pH 2 was prepared as explained in the Tryptophan adsorption isotherms. Buffer at pH 4 was prepared following the same general procedure but mixing 125 mL of a potassium hydrogen phthalate (0.1 M) with 0.1 mL solution of hydrochloric acid (0.1 M), Buffer at pH 6 was prepared mixing 125 mL of a potassium dibasic phosphate (0.1 M) with 14 mL sodium hydroxide (0.1 M) the final pH value was adjusted either with HCl or sodium hydroxide as required.

Curve fitting and data processing

Curves representing the amount of bound adsorbate as a function of the concentration of the free equilibrium concentration after adsorption were plotted. Two theoretical models Langmuir (L) and Frumkin-Fowler-Guggenheim (FFG) were selected to fit the isotherms

(Giles et al. 1974a; Giles et al.1974b; Hans Jürgen et al. 2003). The selection was made following the criteria suggested by Hinz (2001). Mathematical expressions and parameters of each model are shown in Table 1. The adsorbed amounts were determined by the following equation:

$$\Gamma_{Ads} = \frac{\left[\left(\left[Ads\right]_0 - \left[Ads\right]_{eq}\right) x V\right]}{S}$$

where *Ads* means adsorbate, *i.e.* AFB_1 or Trp whichever correspond, Γ is adsorbate surface excess (mol/m^2), $[Ads]_0$ and $[Ads]_{eq}$ are the initial and equilibrium adsorbate concentrations (mol L^{-1}) and V (L) and S (m^2) are the final volume and the surface of adsorbent in the adsorption experiment, respectively. A nonlinear least squares method, with a tolerance limit of 0.05, was used for curve fitting (Origin® version 7.0, Microcal Software Inc.).

Results and discussion

Previous adsorption studies carried out with NaB from different geological sources demonstrated that the AFB_1 adsorption capacity seems to be related to both the isomorphic substitution and the surface charges of the montmorillonite component in the bentonite mineral (Magnoli et al. 2008). The NaB used for the present study showed the best comparative *in vitro* performance as binder in the above mentioned study.

In order to check if Trp adsorption on NaB occurs in a competitive time scale with respect to AFB_1 adsorption a kinetic study was performed. In both cases the adsorption was fast and during the first three minutes approximately 80% of the total adsorption capacity of NaB has been used (data not shown).

The tryptophan adsorption isotherm at pH 2 at 39.5 ± 0.5°C is shown as solid squares in Figure 3. As can be observed a slightly sigmoid isotherm was obtained. Data were adjusted by the Frumkin-Fowler-Guggenheim (FFG) model (Masel, 1996). The fitting curve is shown as a solid line above experimental data. The mathematical expression and the fitting parameters

for FFG model are shown in Tables 1 and 2, respectively. This type of isotherm is characteristic of systems in which the activation energy for substrates desorption is surface coverage dependent (Giles et al. 1974a,b). Frumkin-Fowler-Guggenheim model assumes the non-equivalence of the adsorption sites on the surface, caused by lateral interactions between adsorbate molecules on the adsorbent surface. A positive value of the "a" parameter indicates a cooperative adsorption mechanism. The high values of the adsorption constant ($\beta = (2.19 \pm 0.01) \times 10^3\ M^{-1}$) showed an elevated *in vitro* affinity between the adsorbent and Trp under our experimental conditions.

Different authors evaluated the adsorption isotherms of Trp on zeolites (Palit and Moulik 2001; Titus et al. 2003; Lambert 2008), but paucity of data is available for smectites and montmorillonites. For instance, Titus et al. (2003) reported that Trp was not adsorbed by a modified zeolite (NaZSM-5) because its kinetic diameter is higher than the zeolite channel diameter. In the present study a highly expansible NaB with a swelling capacity of 31.4 cm^3 was used as adsorbent (Magnoli et. al, 2008). The swelling capacity correspond to the volume (cm^3) of the swollen bentonite measured 24 h after the progressively addition of 2 g of bentonite into a graduated cylinder filled with 100 ml distilled water (Besq et al. 2003).

Therefore Trp molecules that have a kinetic diameter of 0.6nm can easily enter in the interlayer space of montmorillonite clay (Titus et al. 2003). Besides, a mean pore diameter of 3,780 nm for this clay is big enough to allow the mobility of the Trp molecules (Magnoli et al. 2008). In order to explain the adsorption, the effect of the pH should be taken into account because it affects not only the specific amino acid charge but also the pH dependent surface charges of the montmorillonite edges (Magnoli et al., 2008). Carnerio et al. (2011) have stressed the importance of electrostatic interactions on amino acid absorption of zeolites. The effect of pH on the adsorption of amino acids on zeolites was studied by several authors (Munsch et al., 2001; Krohn and Tsapatsis, 2005, 2006). In general, they observed that amino acids with uncharged R-groups or negatively charged R-groups were more adsorbed on zeolites under acidic pH rather than at basic pH. An opposite trend was observed for amino

acids with positively charged R-groups. Also, Hossein et al. (2011) showed that the pH of the medium is the most important parameters influencing the amino acid adsorption.

Figure 3 also shows the effect of ionic strength (1.5 M of KCl) onto the Trp adsorption isotherm in buffer at pH 2 and at 39.5 ± 0.5°C. The isotherm in the presence of KCl was L-shaped and could be fitted according to Langmuir model, indicating a finite number of equivalent adsorption sites on a monolayer arrangement on the absorbent surface (Giles et al. 1974a,b). The Langmuir mathematical expressions and the fitting parameters are shown in Tables 1 and 2, respectively. It can be suggested that under ionic strength control a salt saturated homogeneous surface can be available for Trp adsorption. Therefore, a change in the adsorption mechanism, from a cooperative to a Langmuir type one, is probable mediated by interface salt saturation.

Although the adsorption constants showed the highest affinity [$\beta = (4.1 \pm 0.1) \times 10^3$ M^{-1}] between Trp and the NaB surface under controlled ionic strength. Also, almost a six time decrease in the surface excess at saturation (Γ_{max}) was observed in presence of KCl. The observed reduction in Γ_{max} probably shows the competition between K^+ ions and protonated Trp for the adsorption sites on the NaB surface. This displacement is probably the main process responsible for the adsorption in the salt saturated interface. A decrease in Γ_{max} under ionic strength control was previously also observed for AFB_1 adsorption (Magnoli et al. 2013). Tessis et al., (1999); Franchi et al., (2003); Gao et al., (2008) have shown that Na^+, Ca^{2+} , and Mg^{2+} ions affect the adsorption of biomolecules (ATP, nucleic acids, amino acids) on montmorillonite among other minerals.

Desorption assays for Trp saturated NaB showed that 93% of adsorbed amino acid was recovered after the first wash with pH 2 buffer solution, achieving full recovery after a second wash. On the contrary AFB_1 was kept irreversibly bounded to NaB.

Taking into account the reversible character of Trp adsorption as well as the Trp surface excess at monolayer coverage (7.4 ± 0.1 μmoles of Trp g^{-1} of NaB), the recommended amount of Trp in bird diets (0.18-0.24% w/w) and the usual NaB supplementation levels (0.3

and 0.5% w/w), a reduction effect of the adsorbent on the Trp availability is unexpected. These studies are further confirmed with *in vivo* experiments carried down with poultry (Magnoli et al. 2011a,b). In fact, sodium bentonite supplementation (0.3%) did not affected the productive parameters compared to pair-fed controls. The corresponding grower and finisher basal diets contained 2.3 and 1.8 mg of Trp/Kg of feed, respectively. None Trp deficiency mediated by NaB has to be occurring because it is known that low levels of Trp not only reduces bone growth but also increases feed conversion ratio (Carew et al. 1983).

However, since Trp adsorption, even reversible, could still be affecting the ability of NaB to protect birds against chronic aflatoxicosis. Therefore competitive adsorption studies were performed.

Figure 4 shows the influence of tryptophan on the AFB_1 adsorption isotherm at pH 2 and 39.5 ± 0.5°C. S-shaped isotherms were adjusted using the FFG mathematical expression and the fitting curves are shown as solid lines on the top of the experimental points. The experimental data show that for Trp/AFB_1 ratios equal or higher than 12 were obtained and that the isotherms shapes were exactly the same. The same agreement can be observed in the fitting parameters (Table 2). The lack of competition effect from Trp on AFB_1 was observed even at low AFB_1 concentration. A completely different behavior has been previously observed in the AFB_1 adsorption isotherm when a coccidiostatic drug (i.e. monensin) was added to the adsorption medium. Liver histopathology showed that Monensin co-adsorption occurs at low toxin concentration, affecting the sequestering ability of NaB by AFB_1 (Magnoli et al. 2011 a,b).

As can be observed in Table 2 the positive "a" values suggest a cooperative mechanism for AFB_1 adsorption both in absence or presence of Trp. The amino acid does not affect the great affinity of the adsorbent by AFB_1. On the other hand, the AFB_1 adsorption constant is around to 100 times higher than those obtained for Trp indicating the highest affinity of the NaB for AFB_1.

From our results, it can be interpreted that the number of surface sites occupied by tryptophan was lower than for AFB_1. Different binding mechanisms have been proposed for

the AFB_1 adsorption on smectites (Deng et al. 2010). Grant and Phillips (1999) suggested that electron donor acceptor (EDA) complexation is the main binding mechanism responsible for AFB_1 adsorption on a related hydrated sodium calcium aluminosilicate (HSCA). Therefore, taking into account the moderated electron donor capacity of Trp (Pullman 1958, Kaletas et al. 2005), a fairly weak EDA complex between Trp molecules and the positive charged surface edges could be the responsible for the amino acid adsorption on NaB (Wang et al. 2014). Differences in the action sites of Trp and AFB_1 adsorption could explain the great difference between the adsorbates Γ_{max}. Assuming the main adsorption sites of Trp and AFB_1 are the montmorillonite edges and the interlaminar spaces respectively, the great Γ_{max} ratio of AFB_1 to Trp could be explained. In fact, for the assayed clay, the ratio between internal to external areas (587 and 34 m^2/g, respectively) is approx. 17 (Magnoli et al. 2008). The high swelling capacity (31.4 cm^3) of the montmorillonite portion of the clay, which corresponds to 84% of the mineral composition, is likely to be involved in the observed Γ_{max} differences (Magnoli et al. 2008).

In conclusions, from the practical point of view, this study demonstrates that although Trp is adsorbed by sodium bentonite, it may not appreciably affect the amino acid bioavailability. Due to the reversible nature of Trp adsorption onto the clay, the potential NaB ability to prevent chronic aflatoxicosis is anticipated to remain unaffected. However further *in vivo* studies are guaranteed in order to evaluate if Trp and others essential diet components could affect NaB performance on chronic aflatoxicosis.

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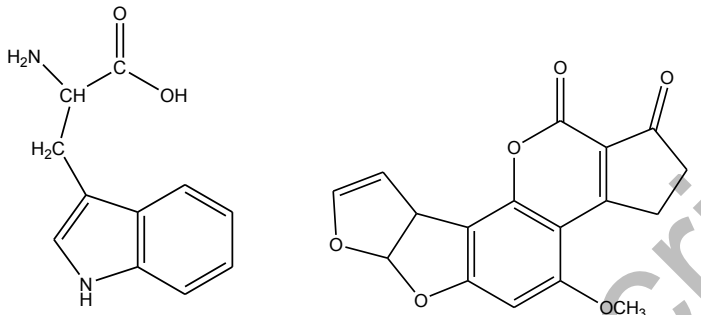
Figure 1 Molecular structures of tryptophan (a) and aflatoxin B_1 (b)



(a) (b)

Figure 2: Spectra of adsorption by UV-visible spectroscopy.
___ AFB1 2.2 e^{-5}M,--- Trp 2.01e^{-4} M.

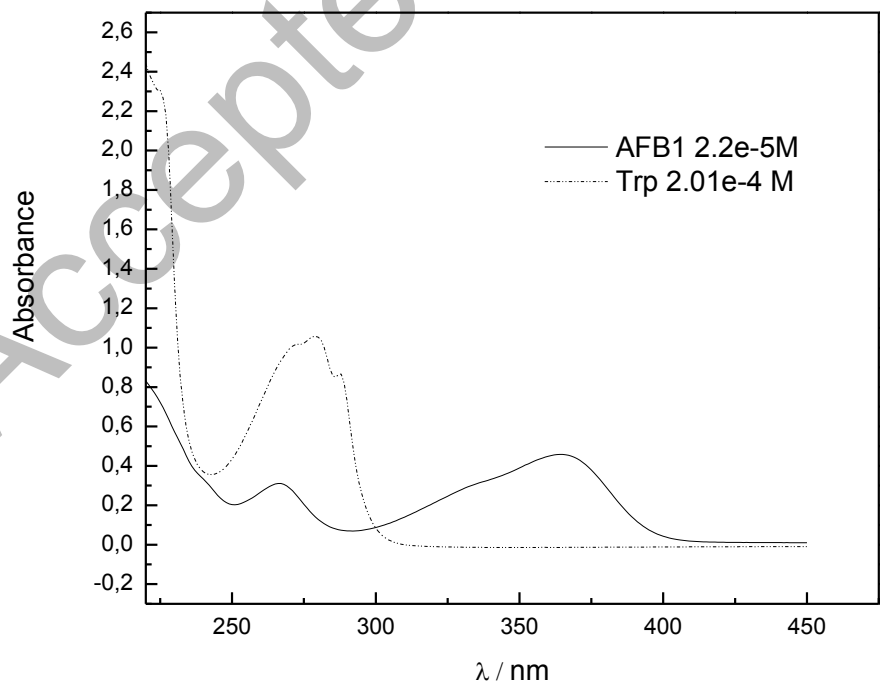


Figure 3: Tryptophan adsorption isotherm at pH 2 and 39.5 ± 0.5°C
■ without KCl ○ with KCl 1.5 M

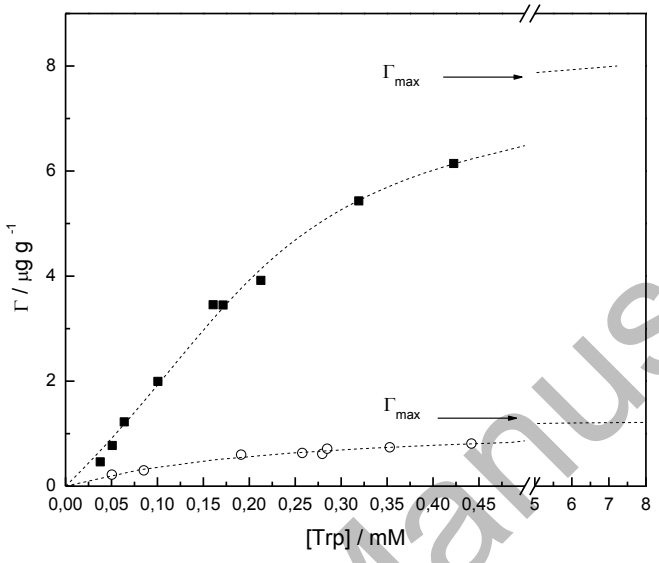


Figure 4: adsorption isotherm of AFB_1 on NaB at pH 2 and 39.5 ± 0.5 °C.
○ with 3 x 10^{-4} M of Trp. ■ without Trp.

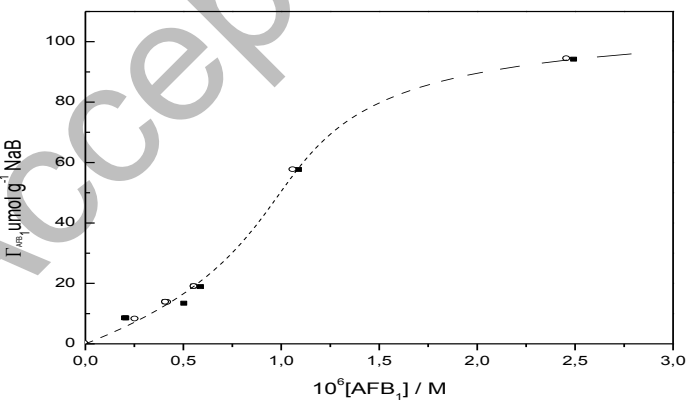


Table 1. Theoretical adsorption models, mathematical equations and adjusting parameters

Models	Mathematical Expression	Parameters
Langmuir	$\beta_{Ads} = \frac{\Gamma_{Ads}}{(\Gamma_{Ads,\max} - \Gamma_{Ads})[Ads]}$	$\Gamma_{Ads,\max}$, β_{Ads}
FFG	$\beta_{Ads} = \left[\frac{\Gamma_{Ads}}{(\Gamma_{Ads,\max} - \Gamma_{Ads})[Ads]}\right] \exp(-2a\Gamma / \Gamma_{\max})$	$\Gamma_{Ads,max}$, β_{Ads}, a

Γ_{Ads} and Γ_{max} are the adsorbate surface excesses (mol/m^2) measured before and after saturation respectively, [*Ads*] is either the Trp or AFB_1 equilibrium concentration, β_{Ads} is the adsorption constant and "a" is the FFG parameter that measures the interaction between adsorbed molecules.

Table 2. Adjustment parameters

Adsorbate / Experimental Conditions	Γ_{max} µmoles/g	$10^{-3}\beta$ / M^{-1}	**a**	R^2
Trp Buffer pH 2 KCl (0 M)	7.4 ± 0.1	2.19 ± 0.01	0.79 ± 0.04	0.9999
Trp Buffer pH 2 KCl (1.5 M)	1.3 ± 0.3	4.1 ± 0.1	0	0.9899
AFB_1 Buffer pH 2 Trp (0 mM)	108 ± 3	232 ± 2	1.4 ± 0.1	0.9945
AFB_1 Buffer pH 2 Trp (0.3 mM)	108.0 ± 0.5	239 ± 4	1.41 ± 0.05	0.9998