ORIGINAL ARTICLE

In vitro adsorption of aflatoxin B₁, ochratoxin A, and zearalenone by micronized grape stems and olive pomace in buffer solutions

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Abstract

This work characterizes the adsorption of aflatoxin B₁ (AFB₁), ochratoxin A (OTA), and zearalenone (ZEN) by dry micronized olive pomace (OliPom) and grape stems (GrapStem). Their performance was compared with that of three other materials, activated carbon (ActCarb), bentonite (Bent), and a commercial product (ComProd). Experiments were conducted in vitro at several pH values using buffer solutions. For OTA and ZEA, the strongest adsorbent was ActCarb, with 5 mg/mL being sufficient to bind > 99% of all the mycotoxins. For AFB₁, ComProd and Bent were the most effective adsorbents, as 0.5 mg/mL bound > 95% of this mycotoxin. Among the two agro by-products, GrapStem was the strongest binder, with 10 mg/mL being sufficient to bind at least 90% of all the mycotoxins (except OTA at pH 7). OliPom was the least efficient material, but at a concentration of 30 mg/mL, its performance was similar to GrapStem. Adsorption isotherms were calculated, and ActCarb showed the maximum adsorption capacity (Q_{max}), with values that ranged from 19 to 24 µg/mg for pH 2 and from 17 to 20 µg/mg for pH 7. ComProd, Bent, and GrapStem showed more similar Q_{max} between them (1.4–4.4 µg/mg for pH 2 and 0.5–4.8 µg/mg for pH 7).

Keywords Aflatoxin B₁ · Binders · Feed additives · Isotherms · Ochratoxin A · Zearalenone

Introduction

Many fungi can produce toxins as secondary metabolites with a variety of different structures and toxic effects. These toxins, called mycotoxins, are produced by filamentous fungi that grow in food crops at pre- or post-harvesting stages. The main mycotoxigenic fungi belong to the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, and *Claviceps* (Frisvad et al. 2006). Mycotoxins are characterized by low molecular weight, high diversity of molecular structures, and triggering

several adverse toxic effects in humans and animals, even when consumed in low concentrations (Bennett and Klich 2003). Their toxicity includes, among others, immunosuppressive, teratogenic, mutagenic, carcinogenic, cytotoxic, hepatotoxic, nephrotoxic, and neurotoxic effects (Hussein and Brasel 2001). It has been estimated that approximately 600 fungal metabolites can be classified as mycotoxins, but only a few constitute a real threat to human and animal health (Sakuda and Kimura 2010). Those of most concern are aflatoxins (B₁, B₂, G₁, G₂, and M₁), ochratoxin A, zearalenone, fumonisins (B₁ and B₂), deoxynivalenol, T-2 and HT-2 toxin, patulin, citrinin, and ergot alkaloids. Presently, they are all included in European legislation (EC 2016), but regulations for many of them have also been established in almost 100 countries worldwide (van Egmond et al. 2007). Additionally, it was estimated that approximately 25% of the total global output of food and feed is affected by mycotoxin contamination, representing significant economic losses worldwide (CAST 1989).

Because of the impact of mycotoxins on the economy and health, it is essential that there is proper management of their occurrence in food and feed, in order to maintain the contamination levels as low as possible. In the particular case of the

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animal feed sector, dietary additives that possess mycotoxin-adsorbing properties are widely used to reduce the bioavailability of mycotoxins in the gastrointestinal tract of animals (Jard et al. 2011). These additives are usually composed of inorganic and organic binders, complemented, in some cases, with biotransforming agents (microorganisms or enzymes that modify the chemical structure of some mycotoxins, which eliminate their toxicity) and with health enhancers that protect organisms from toxic effects of mycotoxins. These additives are the last solution to combat mycotoxins, which otherwise would affect the health of farm animals and, inevitably, humans as their primary consumers (Boudergue et al. 2009). Their use is rather simple, as they need only to be added to the animal feed. These binders are able to adsorb and retain several mycotoxins until they are expelled through feces. The efficiency of the procedure depends on the properties of the mycotoxins (such as polarity, solubility, molecular size, shape, and charge distribution) and of the adsorbent in use (the physical structure, total charge distribution, pore size, surface accessibility, etc.). There are already a large variety of adsorbents patented and in the market, including natural clay products as well as synthetic polymers (Kolossova and Stroka 2011).

Silicate clays, which include aluminosilicates (HSCAS), bentonites, montmorillonites, and zeolites, are the most significant class of mycotoxin binders and are an excellent example of efficient adsorbents for specific mycotoxins (Jard et al. 2011). For example, HSCAS could adsorb *in vitro* up to 80% of AFB₁ and prevent aflatoxicosis in chickens (Galvano et al. 2001). Another well-known mycotoxin binder is activated carbon, which has multiple applications in the industry (Ramos et al. 1996). Many tests *in vitro* and *in vivo* were performed using activated carbon as a mycotoxin binder, with positive results for principal mycotoxins (Galvano et al. 1998; Ramos et al. 1996). More recently, low-cost agro by-products, such as grape pomace, were studied as biosorbents and were shown to bind different mycotoxins simultaneously *in vitro* (Avantaggiato et al. 2014). Grape pomace efficiency was later evaluated in pigs using urinary biomarkers, which showed a reduction in the gastrointestinal absorption of several mycotoxins (Gambacorta et al. 2016).

Regardless of the advantages of present mycotoxin adsorbents, a considerable lack of efficiency in the removal of some mycotoxins still exists, and there is the concern that some of these adsorbents can remove essential nutrients too (as it happens with activated carbon), justifying further studies and the characterization of new materials. Considering the efficiency obtained with some biosorbents and the benefits of its use *in vivo*, the objective of the presented work was to evaluate, *in vitro*, the binding efficiency of micronized dry grape stems and olive pomace and to compare them with the efficiencies of a commercial product, bentonite, and activated carbon.

Materials and methods

Adsorbents and mycotoxins solutions

Adsorbents from organic and mineral origin were used to test the adsorption of mycotoxins in buffer solutions. The adsorbents tested as reference materials were a commercial product (ComProd), bentonite (Bent) from Sigma (CAS no. 1302-78-9, ref. 285234), and activated carbon (ActCarb) from Panreac AppliChem (CAS no. 7440-44-0, ref. 211240). The organic materials tested were obtained from agro-industrial by-products and consisted of micronized dry olive pomace obtained from the traditional extraction process (OliPom) and micronized dry grape stems obtained from a winery (GrapStem). The mycotoxin standards used were obtained from Sigma-Aldrich. Mycotoxin adsorption was studied at different pH values using the following buffers: 0.1 M KCl/HCl at pH 2, 0.1 M citrate/phosphate at pH 5, and 0.1 M phosphate at pH 7 and 8. Buffer solutions containing a mixture of the three mycotoxins were prepared using stock solutions in acetonitrile of aflatoxin B₁ (AFB₁—2 mg/mL, Sigma A6636), ochratoxin A (OTA—1 mg/mL, Sigma O1877), and zearalenone (ZEN—2 mg/mL, Sigma Z2125) to obtain final concentrations of 0.05, 0.5, 1, 2, 4, 6, and 10 µg/mL.

Characterization of adsorbing materials

The particle size, moisture, and organic matter content of materials were determined in triplicate as follows. Moisture was determined using a MAC 50/1/NH moisture analyzer (Radwag, Poland) using 0.5 g of sample and following equipment instructions. The determination of organic matter was performed by incineration and ash weighing. Two grams of sample was dried overnight at 105 °C and placed in a furnace at 550 °C until a constant weight was attained. Organic matter content was calculated in percentage by subtracting ash and moisture content. The particle size of materials was determined with a Mastersizer 3000 (Malvern Instruments, UK) by Paralab, SA (Portugal).

Mycotoxin adsorption experiments

Experiments were conducted in 2-mL microcentrifuge tubes. The adsorbent was weighed in the tubes, and 1 mL of buffer containing the mycotoxins mixture at each particular concentration was added. The tubes were incubated at 37 °C for 1 h with rotary agitation (40 rpm). After the incubation, tubes were centrifuged (10 min at 10621×g) and 0.8 mL of supernatant was collected to clean 2-mL microcentrifuge tubes. Then, 0.8 mL of a solution of acetonitrile/methanol/acetic acid

(78:20:2, v/v/v) was added, and after a vigorous vortex agitation, the tubes were centrifuged again. Finally, clear supernatants were transferred to clean 2-mL amber vials. All experiments were performed in triplicate and analyzed by high-performance liquid chromatography with fluorescence detection (HPLC-FL). Samples were preserved at $-20\text{ }^{\circ}\text{C}$ until their analysis.

First, to evaluate the influence of pH, experiments were conducted with 20.0 mg of adsorbent and a mycotoxin concentration of $1\text{ }\mu\text{g/mL}$ in buffers at pH values 2, 5, 7, and 8. Next, to evaluate the influence of adsorbent concentration, 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, and 30.0 mg of the adsorbent were tested with a mycotoxin concentration of $1\text{ }\mu\text{g/mL}$ at pH 2 and 7. Finally, to calculate the sorption isotherms, experiments with a fixed concentration of adsorbent and different amounts of mycotoxin were conducted. The amounts of adsorbent used were 2.5 mg for ComProd and Bent, 0.5 mg for ActCarb, and 10 mg for OliPom and GrapStem. Those experiments were performed only at pH 2 and 7 using the same buffers described above. The concentrations of mycotoxins used were 0.05, 0.5, 1, 2, 4, 6, and $10\text{ }\mu\text{g/mL}$ for each mycotoxin studied.

Mycotoxin desorption

Following the adsorption tests, the desorption of the mycotoxins from the adsorbents was investigated to evaluate the bond stability between mycotoxin/adsorbent and to confirm that mycotoxins were bonded efficiently. The centrifuged sediments containing the adsorbed mycotoxins from experiments conducted with 10 and 30 mg of adsorbent at pH 2 and 7 and $1\text{ }\mu\text{g/mL}$ of mycotoxin was used for that purpose.

To test the bond strength and understand if the mycotoxin remained bonded to the adsorbent after a change of pH, the centrifuged sediments of 10 mg samples were washed with 1 mL of buffer at pH 7. For that purpose, the buffer was added to tubes, vigorously vortexed and incubated at $37\text{ }^{\circ}\text{C}$ for 1 h with rotary agitation (40 rpm). Then, the tubes were centrifuged (10 min at $10621\times g$), and the supernatants were collected and prepared for HPLC-FL as described before.

To confirm that mycotoxins were efficiently bonded to the adsorbent, the centrifuged sediments of 30 mg samples were extracted as follows. First, 500 μL of a solution of acetonitrile/methanol/acetic acid (78:20:2, v/v/v) was added to tubes. Then, after vigorous vortex agitation, 500 μL of distilled H_2O was added, and the tubes were incubated at $37\text{ }^{\circ}\text{C}$ for 1 h with rotary agitation (40 rpm). Finally, the tubes were centrifuged twice (10 min at $10621\times g$), and the supernatant was collected and analyzed by HPLC-FL. All samples were preserved at $-20\text{ }^{\circ}\text{C}$ until analyzed by HPLC-FL. The amounts of mycotoxin desorbed were expressed as the percentage of the adsorbed mycotoxin.

Mycotoxin analysis

The mycotoxins were analyzed by HPLC-FL. The mobile phase used for AFB₁ determination was H_2O_d /methanol/acetonitrile (3:1:1, v/v/v), and for OTA and ZEN, it was methanol/ H_2O_d /acetic acid (65:35:1, v/v/v). Both solutions were filtered with $0.2\text{ }\mu\text{m}$ membrane filters (GHP, Gelman) and degassed with ultrasounds. The HPLC system used was composed of a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, and a Jasco FP-920 fluorescence detector that were connected through the Varian 850-MIB data system interface and operated in the computer through the Galaxie chromatography software. The column was an analytical C18 reversed-phase YMC-Pack ODS-AQ ($250\times 4.6\text{ mm I.D.}$, $5\text{ }\mu\text{m}$) protected by a pre-column. The parameters used for AFB₁ detection were as follows: excitation = 365 nm, emission = 435 nm, and gain = 10 or 100, depending if samples contained more or less than $6\text{ }\mu\text{g/mL}$ of mycotoxin, respectively (Soares et al. 2010). The parameters of detection for OTA and ZEN were excitation = 280 nm, emission = 460 nm, and gain = 100 or 1000, also depending on mycotoxin concentration in samples (Niderkorn et al. 2007).

Calibration curves for AFB₁ were obtained using standards in the concentration range of 1.0 to $10.0\text{ }\mu\text{g/mL}$ for a gain of 10 and 0.05 to $1.0\text{ }\mu\text{g/mL}$ for a gain of 100. OTA and ZEN calibration curves were obtained using standards in the concentration range of 1.0 to $10.0\text{ }\mu\text{g/mL}$ for a gain of 100 and 0.5 to $1.0\text{ }\mu\text{g/mL}$ for a gain of 1000. The injection volume was of 50 μL . The retention times of AFB₁, OTA, and ZEN were approximately 22.1 min, 17.8 min, and 16.7 min, respectively. The analyses were performed at $30\text{ }^{\circ}\text{C}$. Mycotoxins were identified by comparing the retention times of the peak samples with the standards.

Data calculation and curve fitting for isotherms

The results obtained from the adsorption experiment with different mycotoxin concentration were used to calculate the quantity of mycotoxin adsorbed per mass of adsorbent (Q_{eq} , $\mu\text{g/mg}$), as given by Eq. (1):

$$Q_{eq} = [(C_0 - C_{eq}) \cdot V] / m \quad (1)$$

Q_{eq} was calculated as the difference between the mycotoxin concentration in control tubes (C_0 , $\mu\text{g/mL}$) and the residual mycotoxin concentration determined in the supernatant of the experimental tubes (C_{eq} , $\mu\text{g/mL}$), in the solution volume (V , mL), and per quantity of adsorbent (m , mg). Adsorption isotherms were calculated using GraphPad Prism version 7.02 software for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com) by plotting the quantity of mycotoxin adsorbed per mass of adsorbent (Q_{eq} , $\mu\text{g/mg}$) against the residual mycotoxin concentration

remaining in solution (C_{eq} , $\mu\text{g/mL}$) (Kinniburgh 1986). Then, these data were nonlinearly fitted to the Freundlich, Langmuir, and Hill isotherm models according to Eqs. (2), (3), and (4), respectively (Saadi et al. 2015). The models were compared to each other to obtain the best fit and associated parameters.

$$Q_{eq} = K_f \cdot C_{eq}^{1/n} \quad (2)$$

where K_f is the Freundlich isotherm constant (which is an indicator of the adsorption capacity of the adsorbent for the mycotoxin) and $1/n$ gives information on the intensity of adsorption and heterogeneity of the adsorbent surface. Values of $1/n$ between 0 and 1 indicates a favorable adsorption and a homogeneous surface. As $1/n$ gets closer to zero, the surface becomes more heterogeneous. Also, a value below 1 implies a chemisorption process, whereas a value above 1 is indicative of a cooperative adsorption.

$$Q_{eq} = (Q_{max} \cdot K_L \cdot C_{eq}) / (1 + K_L \cdot C_{eq}) \quad (3)$$

where Q_{max} is the maximum adsorption capacity (which represents the maximum of mycotoxin adsorbed by adsorbent unit) and K_L is the Langmuir affinity coefficient (which refers to the energy of adsorption and the extent of the interaction between adsorbent and mycotoxin)

$$Q_{eq} = (Q_{max} \cdot C_{eq}^{nH}) / (K_D + C_{eq}^{nH}) \quad (4)$$

where, Q_{max} is the maximum adsorption capacity as in the Langmuir model, K_D is the Hill equilibrium dissociation constant (which represent the concentration of mycotoxin necessary to achieve half-maximum binding at equilibrium), and nH is the Hill cooperativity coefficient of the binding interaction (when $nH > 1$, there is a positive cooperativity; $nH = 1$, non-cooperative or hyperbolic binding; and $nH < 1$, negative cooperativity).

Results and discussion

Evaluation of adsorption efficiency at different pH

In these experiments, the materials (20 mg/mL) were tested in buffer solutions containing a mixture of three mycotoxins (AFB₁, OTA, and ZEN). Two of the substances used (bentonite and activated carbon) are well known for having excellent adsorbing properties for certain mycotoxins (Ramos et al. 1996); the other one (ComProd) is a commercial feed additive available in the market. These three were used as positive controls and for comparison purposes to estimate the adsorbing capacity of micronized olive pomace and grape stems. Table 1 shows some physicochemical characteristics of these materials.

The results obtained are presented in Fig. 1. Generally, it was observed that OTA was the most difficult mycotoxin to be adsorbed. Although it was well adsorbed by all the materials at pH 2 (> 89%) and by ActCarb at all pH (100%), its adsorption by ComProd, Bent, OliPom, and GrapStem was poor at pH 5, 7 and 8 (11% to 77%). This weak performance can be the result of conformational changes of the OTA molecule induced by pH since OTA takes a monoanionic form (OTA¹⁻) at pH > 4.0, a dianionic form (OTA²⁻) at pH > 7.0, and a lactone-opened form (OP-OTA) under alkaline conditions (Bazin et al. 2013). Eventually, it can also be the result of changes in charge of the adsorbents themselves, also induced by the pH of the solutions (Huwig et al. 2001). At pH 5, 7, and 8, GrapStem was the substance that best adsorbed OTA (35% to 77%).

Apart from this mycotoxin, good adsorption efficiencies were achieved for AFB₁ and ZEN in all pH values. ActCarb produced reductions of almost 100% in all pH conditions for all mycotoxins, which is in agreement with other studies (Avantaggiato et al. 2004; Galvano et al. 1998; Jard et al. 2011). ComProd and Bent were more efficient in adsorbing AFB₁, with nearly 100% of this mycotoxin being adsorbed at all pH values. The agro-residue OliPom removed 70 to 77% of this mycotoxin, and GrapStem removed approx. 96% at all pH values. In turn, the removal of ZEN was stronger with GrapStem (avg. 99%) and OliPom (avg. 93%) than with ComProd (avg. 85%) and Bent (avg. 73%). Therefore, these results suggest that the agro by-products have, at these particular conditions, similar performances to products available commercially.

Evaluation of adsorption efficiency with different concentration of adsorbents

At this point, instead of using only 20 mg/mL of adsorbent, concentrations between 0.5 and 30.0 mg/mL were tested to determine the influence of adsorbent concentration in the adsorption of the mycotoxins. This time, only two buffers (0.1 M KCl/HCl at pH 2 and 0.1 M phosphate buffer at pH 7) were used to evaluate the adsorption performances. One of the buffers provided an acidic environment, reproducing the acidic conditions of the stomach, while the other provided a neutral environment, reproducing the conditions found in the intestines.

The results are presented in Fig. 2. If we compare the graphics of each mycotoxin for both pH values, it is visible that the adsorptions are more effective at pH 2 than at pH 7. This is more evident for OTA and less evident for the adsorption of AFB₁. Still, this is indicative that the adsorption may benefit from the acidic stomach conditions. In general, ActCarb was the most effective adsorbent, reaching removals superior to 99% for all the mycotoxins, with a concentration as low as 5 mg/mL and for both pH values. These results are in

Table 1 Properties of materials studied

	Moisture (%)	Organic matter (%)	Ash (%)	Particle size median (μm)
OliPom	5.3 ± 0.5	93.5 ± 0.1	1.3 ± 0.1	336
GrapStem	6.1 ± 0.5	90.1 ± 0.1	4.0 ± 0.1	318
ComProd	6.6 ± 0.5	48.7 ± 0.6	47.9 ± 0.6	68
Bent	6.7 ± 0.2	1.6 ± 0.2	98.2 ± 0.1	37
ActCarb	9.9 ± 0.4	79.3 ± 0.3	11.9 ± 0.3	16

agreement with those of other studies that also tested activated carbon for the adsorption of mycotoxins (Avantaggiato et al. 2004; Bueno et al. 2005; Galvano et al. 1997, 1998, 1996). More specifically, it was reported that 2 mg of some

commercial ActCarb (0.04%, w/v) could adsorb more than 95% of AFB₁ and over 99% of OTA present in aqueous solutions (Galvano et al. 1998, 1996), and that doses between 0.1 and 1% of ActCarb could all bind 100% of ZEN in solutions

Fig. 1 Percentage of mycotoxin adsorbed by 20 mg of adsorbent at different pH values. **a** AFB₁. **b** OTA. **c** ZEN. Results are expressed as mean ± SD (*n* = 3)

Fig. 2 Percentage of mycotoxin adsorbed by different concentrations of adsorbent at pH 2 **a** AFB₁, **b** OTA, **c** ZEN, and pH 7 **d** AFB₁, **e** OTA, **f** ZEN. Results are expressed as mean $\pm$ SD ($n = 3$)

with pH 3 and 7.3 (Bueno et al. 2005). Interestingly, in these studies, some of the activated carbons tested also showed low binding performances, demonstrating that not all products have the same efficacy. According to Galvano et al. (1996), their performance depends on the type of carbonaceous substances and activation process used to produce the activated carbon, and on the number and distribution of the medium-size pores in the carbon.

High percentages of mycotoxin adsorption with low amounts of substance were also obtained with ComProd and Bent for AFB₁ since 0.5 mg/mL removed approx. 99% and 96% of this mycotoxin at pH 2 and 7, respectively. Nonetheless, for ZEN, it was necessary 20 mg/mL of those products to remove approx. 85% of this mycotoxin at both pH values. For OTA, 10 mg/mL was sufficient to remove approx. 95% at pH 2; but at pH 7, the maximum adsorption reached only 51% with 30 mg/mL. Concerning the performance of agro by-products, OliPom was the least efficient adsorbing material since it was necessary at least 30 mg/mL of this substance to achieve a substantial removal of the mycotoxins. With this amount of OliPom, the adsorption of AFB₁ reached 76% at pH 2 and 90% at pH 7, the adsorption of ZEN reached approx. 92% for both pH values, and the adsorption of OTA reached 93% at pH 2 and 37% at pH 7. With less than 10 mg/mL of OliPom, the percentages of adsorption dropped to less

than 60% in the case of AFB₁. On the other hand, GrapStem performance was more similar to ComProd and Bent, but more quantity of material was necessary to achieve similar percentages of adsorption. In general, 10 mg/mL of GrapStem was sufficient to adsorb around 90% of all mycotoxins at all pH values, except OTA at pH 7. For this particular case, 30 mg/mL of GrapStem removed only 54% of OTA, which still is the best performance among tested products, with the exception of ActCarb.

Additionally, the adsorbents tested showed good reproducibility, as they obtained similar results in triplicate, which can be perceived by the error bars depicted in Fig. 2. Generally, the efficiency of the adsorption increased with the increase in the adsorbent concentration. Nonetheless, for some adsorbents, the full removal of the mycotoxins from the solution was not achieved with the highest concentration used.

Mycotoxin desorption

The desorption results are presented in Fig. 3. When centrifuged sediments of experiments done with 10 mg/mL of adsorbent were extracted with buffer at pH 7, some amounts of the mycotoxins were released in most of the cases (dark gray bars in Fig. 3). The observed desorptions were probably caused by pH-induced conformational changes of both

Fig. 3 Percentage of mycotoxin desorbed from materials using the buffer solution (dark gray) and the solvent solution (light gray). Centrifuged sediments used were from assays done with 10 and 30 mg/mL of adsorbent conducted at pH 2 and 7. Results are expressed as mean $\pm$ SD ($n = 3$)

mycotoxins and adsorbents, which disturbed the binding interactions existing between them, as observed by other researchers (Bazin et al. 2013). The release of OTA was the most pronounced, confirming that the adsorption of this mycotoxin is pH dependent and unstable. ActCarb was the adsorbent that retained better the three mycotoxins, with only minor quantities of OTA and ZEA being released from sediments (< 1%). The highly porous structure (in particular, the medium-size

pores) and the low surface acidity of this material have been considered the main responsible for its superior ability to adsorb organic molecules such as mycotoxins (Galvano et al. 1996).

Additionally, a strong interaction was observed between AFB₁ and ComProd and Bent (< 1%). In the case of Bent, the interlayers that are typical of these clays are considered the determinant attribute involved in the retention of AFB₁ (Grant

and Phillips 1998; Vekiru et al. 2007). ZEN was also well retained by these two materials, with desorptions < 22%. However, they did not retain OTA very well, since desorption percentages were between 20% and 57%. Among the agro by-products, GrapStem was the material that retained the mycotoxins better, with ZEN being fully retained, AFB₁ released in small amounts (< 5%), and OTA in quantities inferior to 24%. On the other hand, OliPom capacity to retain the mycotoxins was substantially inferior, since desorptions for AFB₁ and OTA were as high as 40% and 61%, respectively. OliPom capacity to retain ZEN was almost as good as GrapStem with only 5% being released.

When samples with 30 mg/mL were extracted with organic solvents, the totality of the mycotoxins was desorbed in almost all cases (light gray bars in Fig. 3), proving that the mycotoxins were retained by the adsorbents during the experimental procedure and not chemically converted. The exceptions were the experiments conducted with ComProd and Bent at pH 2 with AFB₁ since this mycotoxin was not recovered entirely from centrifuged sediments. Eventually, its chemical conversion into AFB_{2a} may have occurred due to the acidic conditions used (pH 2). This conversion has been described previously by Vekiru et al. (2007) when using several types of bentonites under strongly acidic conditions. Furthermore, none of the adsorbed mycotoxins into the ActCarb were recuperated, suggesting that a very stable bond was formed between them.

Adsorption isotherms

The calculated parameters concerning the adsorption of AFB₁, OTA, and ZEN by the different materials are presented in Tables S1, S2, and S3, supplied in Online Resource 1. In the majority of the cases, the adsorption isotherms provided good fits, with $R^2 > 0.98$, and displayed, graphically, an asymptotic curve that tended to reach a plateau (Fig. 4), revealing that the sorption capacity of these materials is limited. The adsorption isotherms of ActCarb were the exception to this since most of their R^2 values were inferior to 0.90. Generally, the Hill model

showed better correlations than the Freundlich and Langmuir model for most of the studied conditions. Among the adsorbents tested, ActCarb showed the highest maximum adsorption capacity (Q_{max}) for the three mycotoxins tested, with values that ranged from 19 to 24 µg/mg for pH 2 and from 17 to 20 µg/mg for pH 7. The adsorbents ComProd and Bent had the second most elevated Q_{max} , ranging from 2.4 to 4.4 µg/mg for pH 2 and from 0.8 to 4.8 µg/mg for pH 7. Finally, the micronized agro by-products showed the lower Q_{max} , ranging from 1.4 to 3.8 µg/mg at pH 2 and from 0.5 to 1.9 µg/mg at pH 7. These results agree with previous reports, where other agro by-products were successfully tested for the adsorption of mycotoxins (Aoudia et al. 2009; Avantaggiato et al. 2014; Bočarov-Stančić et al. 2012; Gambacorta et al. 2016).

In the particular case of AFB₁ (Table S1), the Q_{max} of ActCarb was 19 and 17 µg/mg at pH 2 and 7, respectively. The Q_{max} of ComProd and Bent, which were very similar between them, was 4 and 3.8 µg/mg for pH 2, and 3.7 and 4.8 µg/mg for pH 7. The Q_{max} of agro by-products was lower and in the range of 1.4 to 1.9 µg/mg for both pH values, except for OliPom at pH 2, which was 3 µg/mg but calculated by the Langmuir model. For this mycotoxin, the adsorbents that fitted the Hill model presented a cooperativity coefficient of the binding interaction (nH) superior to 1, which implies the existence of positive cooperative binding (Saadi et al. 2015). On the other hand, the adsorbents that fitted the Langmuir model (OliPom and GrapStem) had a K_L constant inferior to 1 but superior to 0. This constant is related to the free energy of adsorption with larger values implying greater interaction between the adsorbent and the mycotoxin. With K_L , the Langmuir separation factor (R_L) can also be calculated. This parameter characterizes the adsorption nature according to the following criteria: $R_L > 1$, unfavorable; $R_L = 1$, linear; $R_L = 0$, irreversible; $0 < R_L < 1$, favorable (Avantaggiato et al. 2014). The R_L obtained in these cases ranged from 0 to 1, suggesting favorable adsorption of AFB₁ by the two agro by-products. Additionally, for OliPom and GrapStem, the exponent from the Freundlich model ($1/n$), which indicates the heterogeneity

Fig. 4 Examples of adsorption isotherms. Error bars indicate the standard deviation of replicates. Dotted lines indicate 95% confidence intervals. **a** AFB₁ isotherm for GrapStem at pH 2. **b** OTA isotherm for ComProd at

pH 7. **c** ZEN isotherm for Bent at pH 2. These isotherms were all fitted with the Hill model using GraphPad Prism version 7.02

of the adsorption sites, was close to 1, suggesting the presence of homogeneous surfaces (Saadi et al. 2015).

In the case of OTA (Table S2), the maximum adsorption capacities were found to be substantially higher at pH 2 than at pH 7. This behavior was also observed for AFB₁ and ZEN, but less markedly. Again, ActCarb was shown to be the adsorbent with the higher Q_{max} , 22.9 µg/mg at pH 2 and 20.2 µg/mg at pH 7. On the other hand, the remaining adsorbents had a Q_{max} very similar between them, ranging from 2.6 to 4.2 µg/mg at pH 2 and 0.5 to 1.5 µg/mg at pH 7. The adsorbents that were fitted by the Hills model had all $nH > 1$, which implies positive cooperativity in the binding of OTA. The values of R_L for the materials that fitted the Langmuir model are again indicative of favorable adsorption of OTA, as they were between 0 and 1. Also, $1/n$ values of OliPom and GrapStem were close to 1, suggesting homogeneous surfaces.

The calculation of isotherms for adsorption of ZEN (Table S3) showed better adjustments to the Freundlich and Langmuir models than for AFB₁ and OTA. The maximum adsorption capacity of the adsorbents followed the tendencies observed for OTA. That is, ActCarb had the best adsorption capacity (24.4 µg/mg at pH 2 and 18.6 µg/mg at pH 7), while the remaining adsorbents presented Q_{max} values without any clear distinction between them (1.9 to 4.4 µg/mg at pH 2 and 0.9 to 2.4 µg/mg at pH 7). The Hill cooperativity coefficient (nH) was also positive for ZEN, the Langmuir separation factors (R_L) indicate favorable adsorption to the different materials, and the exponent from the Freundlich model ($1/n$) was also between 0 and 1, suggesting homogeneous surfaces.

In summary, all the adsorbents tested showed favorable adsorption for the three mycotoxins tested (AFB₁, OTA, and ZEN), but their maximum adsorption capacities varied considerably according to pH and type of mycotoxin. ActCarb proved to be the best adsorbent, with a Q_{max} often 10 times higher than the others. Its efficiency was consistently superior in all experiments performed, as it required smaller quantities to adsorb the totality of the mycotoxins. It also formed very strong interactions with the mycotoxins, since no desorption was observed under the tested conditions. The adsorbents ComProd and Bent also showed good performances for AFB₁ (at both pH values), OTA (at pH 2), and ZEN (at both pH values). In the case of ZEN, satisfactory percentages of adsorption with ComProd and Bent were only achieved at the maximum concentration of adsorbent tested (30 mg/mL). Concerning the agro by-products, the micronized grape stems demonstrated the strongest mycotoxin-binding capacities, which are comparable to ComProd and Bent, or even superior in the case of ZEN. On the other hand, the micronized olive pomace showed lower binding performances, but the use of higher quantities of this material could compensate for this. It should, however, be highlighted that some practical limitations exist to the use of high quantities of adsorbents in animal

feed. For example, the bioavailability of micro-nutrients such as minerals or vitamins can be impaired. These two agro by-products are inexpensive and abundantly available in countries with tradition in wine and olive oil production. They are usually used for energy generation, composting, animal feed or to obtain natural bioactive compounds (Berbel and Posadillo 2018; Ruiz-Moreno et al. 2015). In the feed industry, they can be used directly in low doses to complement ruminant diet without any relevant adverse effect (Christman 2018; Moate et al. 2014; Molina-Alcaide and Yáñez-Ruiz 2008). Considering the mycotoxin-binding characteristics of these agro by-products, their use as a mycotoxin binding agent to complement the performance of other materials already existing in the market would be an additional form to value these by-products.

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Compliance with ethical standards

Conflicts of interest The authors declare that there are no conflicts of interest.

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