Universidade do Minho
Escola de Engenharia

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In vitro evaluation and characterization of mineral, organic and polymers mycotoxin binders

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Trabalho efetuado sob a orientação de

Professor Doutor Luís Abrunhosa

Doutora Carla Oliveira

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DECLARAÇÃO

Nome: Jean-Michel Fernandes

Endereço eletrónico: michel.tkdt.93@gmail.com Telefone: 913168272

Bilhete de Identidade/Cartão do Cidadão: 14420263

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Orientadores:

Professor Doutor Luís Abrunhosa

Doutora Carla Oliveira

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RESUMO

Micotoxinas são metabolitos secundários sintetizados por fungos filamentosos que têm uma grande diversidade de estruturas químicas e baixo peso molecular. Estes compostos apresentam variadas propriedades tóxicas mesmo em baixas quantidades e provocam doenças ou mesmo morte em humanos e animais. A presença de fungos no meio ambiente e sua capacidade de crescimento em diversas culturas agrícolas leva à ocorrência de contaminações por micotoxinas nos mais variados produtos alimentares, causando grandes perdas económicas. De entre todas as micotoxinas, as mais importantes são as aflatoxinas, ocratoxina A, tricotecenos, fumonisinas, zearalenona e patulina.

Neste trabalho, adsorventes de origem mineral, orgânica e polímeros foram avaliados e caracterizados *in vitro* na adsorção de uma mistura de três micotoxinas (AFB1, OTA, ZEA). Os ensaios preliminares demonstraram que adsorventes de origem orgânica e mineral são mais eficazes na remoção das micotoxinas sendo capazes de remover nalgumas das condições testadas 100% das micotoxinas. A variação da concentração dos adsorventes demonstrou que o uso de apenas 5 mg/mL de carvão ativado (ActCarb) possibilitou a remoção de 100% das micotoxinas e o uso de 10 mg/mL do produto comercial 1 (ComProd1), bentonite (Bent) e engaço de uva (GrapStem) removeram mais de 80% das micotoxinas, excetuando OTA e ZEA a pH 7.0. No geral, o aumento da concentração dos adsorventes aumentou a eficácia da adsorção. Estes adsorventes demonstraram elevada força de ligação às micotoxinas testadas, uma vez que as percentagens de dessorção com tampão não superaram os 50%. Noutro ensaio com solventes orgânicos, a extração das micotoxinas retidas nos adsorventes foi quase total, deduzindo-se assim que a adsorção não causou alterações conformacionais nas micotoxinas. As isotérmicas de adsorção calculadas apresentaram bons ajustes. O ActCarb obteve valores de capacidade máxima de adsorção (Qmax) 3 a 4 vezes superiores aos restantes, seguindo-se ComProd1, Bent e GrapStem, que obtiveram valores de Qmax indicativos de uma adsorção também ela favorável para as três micotoxinas.

Por outro lado, a proteína BSA foi testada como adsorvente de OTA. Esta foi ligada às resinas de níquel His-Pur e Sepharose activada formando uma matriz estável, mas não com celulose Avicel. A presença da BSA melhorou substancialmente a adsorção da OTA (> 50%), sugerindo a possibilidade do seu uso como adsorvente.

Palavras-Chave: Micotoxinas, descontaminação, adsorção, adsorventes, isotérmicas.

ABSTRACT

Mycotoxins are secondary metabolites synthetized by filamentous fungi. They have a great diversity of chemical structures, low molecular weight and present great variety of toxic properties at very low levels, making them responsible for diseases or even dead in humans and animals. The worldwide propagation of fungi and the diversity of crops and foodstuffs they grow on increases the chance of mycotoxins contamination in several agricultural commodities, causing great economical losses. The mycotoxins that are better known are aflatoxins, ochratoxin A, trichothecenes, fumonisins, zearalenone and patulin.

Decontamination methods for mycotoxin removal were studied. Mineral, organic and polymers mycotoxin binders were characterized and evaluated *in vitro* as adsorbent of a mixture of three mycotoxins (AFB1, OTA and ZEA). A preliminary screening showed that adsorbents from organic and mineral origin, such as commercialized product 1 (ComProd1), activated carbon (ActCarb) and grape stems (GrapStem) have better adsorption efficiencies reaching nearly 100% of mycotoxin removal in certain conditions. When experimenting with different concentrations of adsorbents, just 5 mg/mL of ActCarb removed 100% of the mycotoxins and 10 mg/mL of ComProd1, GrapStem and Bent reached mycotoxin removals >80% (except for OTA and ZEA at pH 7.0). The increase in the concentration of adsorbent resulted in the increase of the adsorption efficiency. The adsorbents tested also showed a strong bond with the adsorbed mycotoxins, since the mycotoxin desorption with buffer never reached values superior to 50%. In another experiment with organic solvents, mycotoxin extraction from adsorbents was almost total, suggesting that the experimental procedure did not change mycotoxin conformation. Adsorption isotherms delivered good fits. From the adsorbents tested, ActCarb showed the maximum adsorption capacity (Qmax) 3 to 4 times higher than other adsorbent, followed by ComProd1, Bent and GrapStem, which obtained good Qmax values, indicating favorable adsorption for all mycotoxin and conditions tested.

Additionally, the use of BSA protein in different resins was studied as an OTA sorbent. BSA was bond to His-Pur nickel resin and activated-Sepharose resin forming a stable matrix, but not with cellulose Avicel. The presence of BSA in the matrix improved substantially the retention of OTA (over 50%), suggesting the possibility of the use of BSA as an OTA adsorbent.

KEYWORDS: MYCOTOXINS, DECONTAMINATION, ADSORPTION, ADSORBENT, ISOTHERMS

INDEX

LIST OF FIGURES

LIST OF TABLES

List of Abbreviations

ActCarb	Activated carbon
ADON	3- and 15-acetyldeoynivalenol
AFB1	Aflatoxin B1
AFM1	Aflatoxin M1
ALARA	As low as reasonably achievable
Bent	Bentonite
BSA	Bovine Serum Albumin
ComProd1	Commercial product 1
ComProd2	Commercial product 2
DAS	Diacetoxyscipenol
DiatEarth	Diatomaceous earth
DON	Deoxynivalenol
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
FB2	Fumonisin B2
FB4	Fumonisin B4
FB6	Fumonisin B6
GrapStem	Micronized dry grape stems
GRAS	Generally Regarded as Safe
HPLC-FL	High Performance Liquid Chromatography with fluorescence detection
HSCAS	Hydrated Sodium Calcium Aluminosilicates
IARC	International Agency for Research on Cancer
NIV	Nivalenol
OliPom1	Micronized dry olive pomace extracted
OliPom2	Micronized dry olive pomace centrifuged
OliPom3	Micronized dry olive pomace pressed
OTA	Ochratoxin A
PE	Polyethylene

P(E-MA)	Poly(ethylene-all-maleic-anhydre)
P(MVE-MA)	Poly(methy vinyl ether-all-maleic anhydre)
PP1	Aquatex
PP2	Propyltex
PSS	Poly(sodium 4-styrenesulfonate)
PVOH	Mowiol
PVPP	Poly(vynilvinylpolypyrrolidone)
S.bayanus	*Saccharomyces bayanus*
TRIS	Tris(hydroxymethyl)aminomethane
ZEA	Zearalenone

1. INTRODUCTION

1.1 Fungi

Fungi is one kingdom, on the biological system, that belongs to the Eukaryota domain. It is composed by eukaryotic organisms such as yeast (unicellular fungi), molds (filamentous fungi) or mushrooms (multicellular fungi). Fungi is among the biggest kingdom as there is estimated to exists around 1.5 million different species on earth of which only 100 thousand are presently documented and described (Celio, 2006). Fungi have some specific characteristic which differentiates them from other domains, such as the presence of chitin in theirs cells walls (plants and protists contain cellulose) and the presence of ergosterol on their cytoplasmic membranes (which is a target of some antifungal agents). Nutritionally fungi are classified as chemoorganoheterotrophs, and most of them have saprotrophic nutrition with is a process of extracellular digestion that involves the process of decayed or dead of organic matter, for these reason they play a very important role on nutrient recycling and in decomposition of organic matter (Hawksworth, 1991).

Fungi are microorganisms with reduce mobility and its propagation results mostly from their radial growth and the dissemination of their spores through the elements. Nevertheless, they can be found abundantly worldwide and fully adapted to the environment in site. That is also a consequence of fungal reproduction since they develop structures for sexual and asexual reproduction. Asexual reproduction can occur through mycelial fragmentation or through the release of spores called conidia. This method allows a faster dispersion than sexual reproduction and creates clonal population that are adapted to a specific environment. Sexual reproduction, on the other hand, is different from that found in animals and plants, as the fungus can produce sexual spores (ascospores in ascomycete fungi and basidiospores in basidiomycete fungi), mating with individuals of the opposite mating type or mate with another individual or with itself. This type of reproduction allows actively dispersion of spores or sporangioespores that can travel through the air for great distances (Deacon, 2013).

Fungi, although we might not notice, are involved in many daily processes and offer many utilities. For centuries they have been consumed as food, in the form of mushrooms, or involved in many food process such as fermentations. Fermentation leads to various forms of foods, that otherwise would not exist, some examples are wine, beer, bread, yogurt, soy sauce, etc. As science start evolving, they start

to be used as antibiotics producers, and recently alcohols, steroids, enzymes, etc. Their presence in crops fields also can be valuable, as they act in some cases as biological pesticides that control weeds, prevents plants diseases and insect pests.

Fungi can also be harmful and cause damage in many ways, and when it happens they can deteriorate food, cause allergies, mycosis, break down textiles, etc., provoking great impact in economics, food industries and in human and animal health. There also are many species that are pathogenic and/or producers of toxic compounds known as mycotoxins that affects the health of animals and humans (Magan, 2006).

1.1.1 Fungal diseases and Human Health

Fungi is a large kingdom in which good or harmful species exist. Fungi are major insects and plant pathogens, but from a medical point of view there are few species that matters. Diseases caused by fungi can be called mycosis, when fungi grow on animal hosts, or mycotoxicosis, when the dietary, respiratory, dermal, and other animal systems are exposed to toxic fungal metabolites known as mycotoxins. In this sense, mycotoxicosis are consequences of "poisoning by natural means", and the symptoms that it causes differs from type of mycotoxin, amount ingested and length of exposure, as well as the physical characteristics of the human/animal in cause.

Around the world people is affected by mycosis and mycotoxicosis, but it is difficult to define exactly how many are affected. While in developed world mycosis is caused more by opportunistic fungi and are acquired via inhalation of spores or by unusual growth of a commensal species that are normally resident on human skin and became pathogenic in the presence of certain substances. On the other end, mycotoxicosis are more common in underdeveloped nations, where they are practiced poor methods of food handling and storage, which leads to the consumption of contaminated food.

Like all toxicological syndromes, mycotoxicosis can be categorized as acute or chronic. Acute toxicity generally has a rapid onset and an obvious toxic response, while chronic toxicity is characterized by low-dose exposure over a long time period, resulting in cancers and other generally irreversible effects (Williams, 1985).

1.1.2 Mycotoxigenic fungi

As we know, fungi are involved in many areas and processes that affect directly or indirectly humans. They can be used as food or producers of metabolites. Among all the metabolites they produce some are toxigenic compounds denominated mycotoxins. The fungi that produce mycotoxins are called mycotoxigenic fungi, and they occur mainly in crops. Mycotoxin production occurs naturally, affecting approximately 25% of the total food and feed global output, which leads to great economical losses (Summerell, 2010). Although there are many species of fungi producing mycotoxins, they belong mainly to the genera *Aspergillus, Penicillum* and *Fusarium*. They are able to grow and contaminate crops with mycotoxins before or immediately after harvesting, in the case of *Fusarium* and *Alternaria*; or after harvesting when crops are drying or stored in the case of *Aspergillus* and *Penicillum* (Sweeney, 1999).

Although mycotoxin production may be abundant, in order to do so, filamentous fungi have need of more restrict conditions that those required for their reproduction. Generally, field fungi (before harvest) require temperatures between 20-25 °C, moisture contents around 70 to 90% and a water activity superior to 0.85; while storage fungi (post-harvest or when the food/feed is drying) needs higher temperatures and lower aw (minimum of 0.75-0.85 and optimum of 0.93-0.98) (Magan, 2006).

1.1.3 Genus *Aspergillus*

Aspergillus is a genus of Ascomycetous fungi that as more than 250 species described, including some that present a great economic importance due to the production of numerous useful extracellular products (enzymes, organic acids, various drugs and many secondary metabolites of importance in biotechnology) and some that function as pathogens infecting plants, humans and animals. *Aspergillus* is one of the oldest characterized genera of fungi and was first discovered in 1729 by the botanist Pier Antonio Micheli, which named it that way because, when viewed at the microscope, it resembles a spore-bearing instrument called *asperge*, used to sprinkle holy water. Currently aspergillum is also the name given to the asexual structure (conidiophore with a different morphology) found common to all *Aspergillus* species. *Aspergillus* are widespread molds found commonly in the air in the form of spores that plays an important role in natural ecosystems as well to human bioprocess mechanisms. Some *Aspergilli* have been used for more than 1,500 years in food and beverage production (Bennett, 2010).

Concerning the various species in this genera, *Aspergillus niger* and *Aspergillus flavus* are among the better understood, as many studies were performed regarding this species.

Aspergillus niger belongs to the called Back aspergilli, and it represents one of the most important species used for biotechnological applications. The exceptional protein excretion capability that it possesses makes it one of the most important organism used for industrial fermentations, as it can yield protein at concentrations higher than 20 g/L (high-yield cultivations). Also the existence of industrial facilities, availability of easy biomass separation procedures are all advantages of its use as cell factory in biotechnology (Yoder, 2004). In fact, its advantages are exploited commercially for production of innumerous industrial enzymes (like alpha-amylases, cellulases, pectinases, chymosin, glucose oxidases, catalases, lipases, proteases, phytases and xylanases), for production of citric acid used in foods and for production of cosmetics and pharmaceutical preparations. *A. niger* is capable of correctly process proteins that are difficult to express in other host organisms because of the possession of posttranslational mechanisms, such glycosylation. Also, under industrial conditions *A. niger* is nontoxic and generally regarded as safe (GRAS). For all these reasons summed up, it become extensively used as a cell factory for heterologous expression of proteins (Schuster, 2002). On the other end and more recently, some wild strains were found to produce ochratoxin A (OTA), fumonisin B2 (FB2), fumonisin B6 (FB6) and fumonisin B4 (FB4) (Frisvad, 2007). Since *A. niger* damages a large number of crops and foods worldwide because it has a wide distribution, in fact, currently it is among the most important mycotoxigenic contaminants of food and feed, due to their fast growth, pH tolerance and highly abundant existence. Also because *A. niger* is used widely in industry, it is a requirement of insurance the use of strains that do not produce mycotoxins (Pitt, 2009).

Aspergillus flavus is a species extensively studied because it is one of the main producers of aflatoxins, which is a powerful carcinogenic mycotoxin, and for that reason is described as pathogenic fungus that affect humans, various animal species and also plants. *A. flavus* phytopathogenic ability is responsible for the infection of crops (such as corn, peanuts, cotton and nut trees) before harvest, because, as for most fungi, *A. flavus* is predominately a saprophyte and grows on dead plant and animal tissue in the soil (Scheidegger, 2003). *A. flavus* unlike the majority of fungi is favored by hot dry conditions. It has an optimal growth temperature around 37 °C, making it predominant in field areas with high temperatures and drought (CAST, 2003). Other important aflatoxin producers are *Aspergillus parasiticus* and *Aspergillus nominus*.

1.1.4 Genus *Penicillium*

Penicillium is a genus of Acomycetous fungus, like *Aspergillus*, first described by Johann Heinrich Friedrich Link in 1809. The name *Penicillium* was given from the resemblance of the asexual structure (conidiophore) to *penicillatis* (a hair pencil brush), that means "pencil-like". As is for *Aspergillus* it has a range of habitats that goes from soil, vegetation, air, indoor environments to food products in a worldwide distribution. *Penicillium* is a well-known genus of fungus because it was the source of a revolution on medical care due to the discovery, in 1928, of the production of penicillin by some species with great application on the treatment of bacterial diseases, in fact this fungal metabolite is used as antibiotic by humans since then (Fleming, 1929). Nowadays, some species of this genus are used in the food industry (productions of specialty cheeses like Camembert or Roquefort, and fermented sausages), and also in the production of enzymes (Frisvad, 2004; Thom, 1906). There are already 354 accepted species in this genus being frequently described new species (Samson, 2010). Because of its nutrition, its main function in nature is the decomposition of organic materials, causing impact in material recycling (Frisvad, 2004).

Several metabolites are produced by the species of genus *Penicillium* and most of them produce mycotoxins, but even though they produce toxic compounds the same isn't observed under industrial conditions, which makes them safe for industrial use. Plus, they do not produce some of the most important mycotoxins such as fumonisins, trichothecenes, zearalenone and aflatoxins (Visagie, 2014; Weidenbörner, 2001). On the other hand, an important nephrotoxic and possibly carcinogenic mycotoxin, the ochratoxin A is produced by *P. verrucosum* (common contaminant of cereals) and *P. nordicum* (found on cheese and meat products) within the genus *Penicillium* (Hymery, 2014). Citrinin is another nephrotoxic mycotoxin that can be produced by *P. expansum, P. radicicola* and *P. verrucosum*. Another common *Penicillium* mycotoxin is patulin mainly produced by *P. carneum* (found on rye-bread and dry meat products), *P. expansum* (found on pomaceous fruits and nuts), *P. griseofulvum* (found on cereals), *P. paneum* (found on rye-bread) and *P. sclerotigenum* (found on yams). Along these main mycotoxins there is still the production of penicillic acid, verrucosidin, penitrem A, fumitremorgin A and B, cyclopiazonic acid, secalonic acid D and F, and as there are new species being described there is yet more mycotoxins to be found (Frisvad, 2004).

1.1.5 Genus *Fusarium*

Another important and well-studied fungal genera is *Fusarium*. It is distributed worldwide and most species are soil fungi and associated with plants. The taxonomy of the genus is complex as identification of species has variability between isolates (size and shape of conidia and colony colour). The number of recognized species had included more than 1000 species, but nowadays the genus *Fusarium* contains at least 300 phylogenetically distinct species (Espinel-Ingroff, 2015). *Fusarium* species exist as plant pathogens, causing root and stem rot, vascular wilt or fruit rot, or saprophytes on plant debris and in soil. It mainly affects crops of tomatoes, bananas, sweet potatoes, pigeon peas, pears, and some are commonly isolated from seeds (particularly those of cereals), occurring around the world on tropical and temperate regions. This genus causes some significant diseases in plants, like head blight of wheat and *Fusarium* wilt of bananas (also known as Panama disease of banana) (Summerell, 2010).

Some *Fusarium* species are important mycotoxin producers, as they can produce trichothecenes (T-2 toxin, HT-2 toxin, diacetoxyscirpenol (DAS), deoxynivalenol (DON), 3- and 15-acetyldeoxynivalenol (ADON) and nivalenol (NIV)), zearalenone and fumonisins, which makes *Fusarium* species one of the most prevalent toxin-producing fungi and commonly found on cereals grown in the temperate regions of America, Europe and Asia (Kelly, 2015). Several species have emerged as important opportunistic pathogens in humans causing hyalohyphomycosis (especially in burn victims and bone marrow transplant patients), mycotic keratitis and onychomycosis (Guarro, 2013).

1.2 Mycotoxins

Mycotoxins are secondary metabolites synthetized by filamentous fungi (frequently called molds). These naturally occurring chemicals present a great diversity of chemical structures and are characterized by a low molecular weight. Due to the worldwide propagation of fungi, mycotoxins can contaminate almost all natural materials since filamentous fungi grows on a diversity of crops and foodstuffs such as cereals, nuts, spices, dried fruits, apple juice and coffee, etc. and can even contaminate many materials made by man. However filamentous fungi do not always produce mycotoxins, they do it when they are "stressed" (stress can be caused by drought, excess of moisture, plant disease, etc.), or as a form of defense mechanism, or they need to be in favorable environmental conditions that are warms temperatures and higher humidity (Bennett, 2003).

Mycotoxins are toxic in low concentrations to humans, animals and to plants, and its ingestion can cause a variety of adverse toxic effects, diseases (known as Mycotoxicosis) or even dead (Bennett, 2003). There are more than 500 mycotoxins described and the number is steadily increasing, but only a few constitute a real threat to human and animal health. Those of most concern are aflatoxins (B1, B2, G1, G2 and M1), ochratoxin A, zearalenone, fumonisins (B1, B2 and B3), trichothecenes (principally nivalenol, deoxynivalenol, T-2 and HT-2 toxin), patulin, and citrinin, which are included in current European legislation (Commision, 2006).

The characterization of mycotoxins isn't easy, because they assume many forms and they are typically found at low concentrations, but its toxic properties are well recognized and vast. They can be immunosuppressive, teratogenic, mutagenic, carcinogenic, allergenic, cytotoxic, hepatotoxic, nephrotoxic, neurotoxic, and estrogenic at very low levels (Hussein, 2001). Due to their varied pharmacological activity, mycotoxins and its derivatives were also experimentally employed in many kinds of drugs such antibiotics and growth promotants, and others have been implicated as chemical warfare agents. Mycotoxins, for all the reasons showed before, poses globally a great economical and health threat. Among the principal problems of agriculture is the contamination of food and feed with mycotoxins, which involves the losses of crops and consequently animal productivity. To these is added the cost of the mycotoxin management causing a great reduction in the profitability in whole food production chain.

Managing mycotoxins is also a hard task, because it is not possible to eliminate entirely mycotoxins from the food chain since they are natural contaminants. The solution found to minimize associated health risks is the reduction of their levels in food and feed that can be achieved using preventive methods to avoid fungal contamination and subsequent production of mycotoxins (e.g. by applying good agricultural practices, transportation and storage conditions), and by implementing methods of decontamination and detoxification when necessary (Lisker, 1991). Regarding most mycotoxins, there is also rules and strict legislative limits established by the European Commission. They exist also several international organizations that established tolerable daily intakes (TDI) that estimates the quantity of mycotoxin to which someone can be exposed daily over a lifetime without posing a significant risk to health.

1.2.1 Aflatoxins

Aflatoxins are described as one of the major groups of mycotoxins. They were first isolated and characterized back in the 1960s after the death of more than 100,000 turkeys by a disease called turkey

X disease that was found to be caused by the consumption of peanut meal contaminated with moulds (Goldblatt, 1969). Among all different forms of aflatoxins, the most important one are named aflatoxin B1, B2, G1 and G2 due their blue or green fluorescence under UV light and relative chromatographic mobility during thin-layer chromatography. Aflatoxin B1 (AFB1) is the most potent natural carcinogenic known, and for that reason the International Agency for Research on Cancer (IARC) has classified AFB1 as a group I carcinogen, more specifically a liver carcinogen (Squire, 1981; Wogan, 1999). For that reason, plus for being the major aflatoxin produced by toxigenic strains, AFB1 is the subject of a lot of studies and published papers. Aflatoxins are mainly produced by *Aspergillus flavus*, *A. parasiticus* and *A. nominus*, that are common contaminants in agriculture (especially *A. flavus*), although there are other producing species encountered less frequently such as *A. bombycis, A. ochraceoroseus*, *A. nomius*, and *A. pseudotamari* (Jelinek, 1988; Klich, 2000).

Aflatoxins are associated with toxicity and carcinogenicity either in humans as in others animals, and the disease caused by aflatoxins consumption are called aflatoxicoses. The severity of this disease depends mainly on the quantities ingested, for instance acute aflatoxicoses result in death and chronic aflatoxicoses result in hepatitis, immunosuppression and cancer, being the liver the primary target organ (Eaton, 1994). Aflatoxins are natural contaminants of cereals, figs, oilseeds, nuts, tobacco, among others commodities. Dairy products can be an indirect source of aflatoxins, as cows can metabolize the ingested AFB1 into a hydroxylated form called aflatoxin M1 (AFM1) that is passed to its milk (JØrgensen, 2005).

1.2.2 Ochratoxin A

Along with aflatoxin, ochratoxin A (OTA) is also one of the most important mycotoxins produced by *Aspergillus*. It was first discovered and isolated as a metabolite of *A. ochraceus* back in 1965 (Van der Merwe, 1965). This mycotoxin is mainly produced by *A. ochraceus*, *A. carbonarius*, *Penicillium verrucosum* and *Penicillium nordicum* (Bayman, 2002). It was also isolated in low amounts from *A. niger,* and because it is widely used in the production of enzymes, it's important to ensure that the strains used are non-producers of OTA (Abarca, 1994).

Ochratoxin A has been found in barley, oats, rye, coffee beans, wheat and other plant products, equally it may be present in certain wines (due to contamination of grapes, especially with *A. carbonarius*) (Van Egmond, 1994). In addition to aflatoxins, only ochratoxin A, is equally important among the *Aspergillus* toxins. The primary target of this metabolite is the kidney, and it can be classified as a nephrotoxin to all animal species studied to date (it is frequently found in pork). In addition, it is a liver

toxin, immunosuppressant, potent teratogen and it is rated as a possible human carcinogen by IARC. Even though the effects of ochratoxin A in humans is still unconfirmed, human exposure has been detected in a lot of countries, like Canada, Sweden, West Germany, Yugoslavia and Sierra Leone (Kuiper-Goodman, 1989). Due to its toxicity many risk assessments have been conducted and with the data available from animal studies, the Scientific Committee of European Union recommended a level of OTA consumption below 5 ng/kg of body weight per day. This level was later revised by the European Food Safety Authority (EFSA) to 17 ng/kg of body weight per day because of OTA indirect effect on genotoxicity (Sweeney, 2000).

1.2.3 Zearalenone

Zearalenone (ZEA), also known as F-2 toxins, is one of the most common mycotoxin produced by *Fusarium*, specifically by *F. graminearum* (where it was first described), *F. equiseti*, *F. crookwellense* and *F. culmorum*. All the species producers of ZEA were found to be regular contaminants of cereal crops worldwide, with contamination occurring primarily on hot climates before harvesting in crops of corn, oatmeal, rye, wheat and barley (Logrieco, 2003). ZEA (and its reduced form zearalenol) resembles the principal hormone produced by the human ovary 17β-estradiol. Also a synthetic formulation (zeranol (Ralgro)) was patented as an anabolic agent for sheep and cattle, but it was banned in some parts of the world and in the European Union in 1989 (Hagler Jr, 2001). Although the biological potency of these compound is high, its toxicity is considered low compared with other mycotoxins. Nevertheless, it is estimated that the safe human intake of ZEA is 0.05 μg/kg of body weight per day (Kuiper-Goodman, 1987). Despite some reviews of epidemiological data, which concluded that the risk to human populations is minimal, it's recommended to stay alert for adverse health effects.

1.2.4 Fumonisins

Fumonisins were first isolated and characterized in *Fusarium* species, in 1988, and their main producers are *F. verticillioides* and *F. proliferatum* (among a large number of *Fusarium* species) that are found in corn all around the world, making this species economical important (Marasas, 2001). They contaminate mainly corn but they can sometimes be found in wheat, tea, rice, wine, raisins and they are mostly associated with pre-harvest contaminations. Chemically, they are a class of mycotoxins that is divided into several structural groups, being the most relevant those who belong to the series B.

Fumonisins B1 is the most important one because it represents about 70% of all fumonisins produced by the toxigenic strains. It is classified as phytotoxic and, by the IARC, as probably carcinogenic (group 2B), since it has been correlated with esophageal cancer in humans due to the consumption of contaminated corn (Rheeder, 2002). Also, it was shown that fumonisin can cause hepatotoxic and carcinogenic effects, leukoencephalomalacia in equines, pulmonary edemas in swines, apoptosis in the liver of rats (Dutton, 1996).

1.2.5 Trichothecenes

Trichothecenes are a class of mycotoxins consisting of about 200 metabolites produced by several fungal genera, like *Fusarium*, *Myrothecium*, *Phomopsis*, *Stachybotrys*, *Trichoderma*, *Trichothecium*, etc. (Scott, 1989). All trichothecenes have in common a 12,13-epoxytrichothecene skeleton and various side chain substitutions by an olefinic bond. They are found as food and feed contaminants and its consumption results in alimentary hemorrhage and vomiting, while the direct contact causes dermatitis (Ueno, 1983). Since they are favored by the presence of high humidity a way to prevent its occurrence is to dry the grain at humidity lower than 14% (Bennett, 2003). This is also valid for other mycotoxigenic fungi too. Trichothecenes, as other *Fusarium* mycotoxins, are mostly found in barley, corn, oat, rye safflower seed, wheat, and mixed feed but their occurrence vary a lot from year to year because the development of their producers depend a lot on the weather conditions (Joffe, 1986).

Among all trichothecenes the best understood are nivalenol (NIV), deoxynivalenol (DON), T-2 and HT-2 toxin, which are produced by *Fusarium* species. DON is one of the most commons mycotoxins present in grains. It is also named vomitoxin because its ingestion causes nausea, vomiting, diarrhea or food refusal at lower doses. T-2 toxins are cytotoxic and have an immunosuppressive effect leaving a lower resistance to infectious microbes (Rotter, 1996).

1.2.6 Patulin

Patulin is a mycotoxin produced by many different molds but predominantly by *Penicillium expansum*, which is found mostly in apples and its food derivatives, due to the association of these fungus with the fruit. Despite that, it does not survive the process of fermentation used to produce cider products. It was first isolated from *P. patulum* (now called *P. griseofulvum*) as an antimicrobial active principle around 1940s. Its antibiotic activity was studied and it became apparent, during the 1950s and 1960s,

that in addition to its antibacterial activity is was also toxic to both animals and plants, causing it to be reclassified as a mycotoxin (Ciegler, 1971). Nowadays, patulin is also found contaminating peach, pears, grapes, apricot, quince, fruit marmalades and some fresh vegetables. Patulin is also produced by some species in the genus *Aspergillus*, *Penicillium*, *Byssochlamys* and *Paecilomyces*. This mycotoxin was proved to be toxic in high concentration in laboratories but their impact on food is not too much relevant. Regardless the Joint Food and Agriculture Organization-World Health Organization Expert Committee on Food Additives set a maximum tolerable daily intake of 0.4 mg/kg of body weight per day as a provisional measure and estimated an action level of 50 µg/kg for patulin in apple products (apple juice, apple juice concentrate, etc.) (Trucksess, 2001).

1.2.7 Citrinin

Citrinin was first isolated prior to World War II from *Penicillium citrinum* (Hetherington, 1931). Afterwards it was also isolated from several species of *Aspergillus* (e.g. *A. carneus*, *A. terreus*, *A. niveus, etc.*), species of *Penicillium* (e.g. *P. expansum* and some strains of *P. camemberti*) and from species of *Monascus* (e.g. *M. ruber* and more recently from *M. purpureus* that is used to produce red pigments and a food supplement called red rice) (Blanc, 1995). Citrinin is considered a nephrotoxin that have been implicated as a contributor to porcine nephropathy and it's associated with yellow rice disease in Japan (Saito, 1971). Although it has been reported the presence of citrinin in wheat, oats, rye, corn, barley, rice, as well in naturally fermented sausages from Italy and certain vegetarian food colored with *Monascus* pigments (Abramson, 2001). Its significance for human health is unknown.

1.2.8 Mycotoxin management

The uneven propagation of fungi and production of mycotoxins makes the search for those contaminants difficult, time consuming and expensive. Mycotoxins are natural contaminants, being impossible to predict where its presence is more likely to occur. Removing mycotoxins, or avoid entirely their presence in food and feed it's impossible due to their characteristics, and their formation is often inevitable when climatic conditions are favorable to the development of mycotoxigenic fungi. There are however some preventive methods and practices for controlling mycotoxins in order to reduce their levels "as low as reasonably achievable" (ALARA). The ALARA principle proposes keeping the exposure to

contaminants at the lowest achievable level without compromising the availability of major food supplied or without increasing grossly their costs.

In order to do so, many methods have been studied to minimize the presence of mycotoxins in food and feed. Since it is very difficult to remove mycotoxins, most of these methods are based on prevention in order to avoid the development of fungi and of their metabolic activity, thus preventing mycotoxin accumulation. They include good agricultural practices at pre-harvest, harvesting and post-harvest stages and may consist in using crop varieties resistant to fungi, in crops rotation, proper application of fungicides/insecticides, management of grain moisture before storage (drying below 15%), and management of moisture, temperature and insect infestations in storage facilities (Bennett, 2003).

Despite these efforts, fungus find a way to growth, making the mycotoxin contamination inevitable. As a result, new methods were developed focusing on decontamination strategies that can be used to improve the safety of products. Decontamination methods can use physical methods (such as segregation, sorting or cleaning of contaminated grains or kernels after harvest, shelling, roasting, dry and wet milling and extrusion), or use chemical methods (with the use of ammonia hydroxide, calcium hydroxide, acid or bases, reducing or oxidizing agents and chlorinated agents). There are also others methods that involve the additions of dietetic additives (such adsorbent and health enhancers). These in specific are being widely used on animal nutrition to reduce the bioavailability of mycotoxins in the gastrointestinal tract, as also health protecting agents to counteract their toxic effects (Venâncio, 2007).

Recently, new strategies involving the use of microorganisms and enzymes that can biotransform or adsorb mycotoxins have also been proposed and developed.

1.3 Adsorbents

Mycotoxin detoxifying agents are a group of feed additives used to reduce the contamination of feed by mycotoxins. They can be defined in two categories, adsorbing agents (binders) that are supposed to decrease the bioavailability of the mycotoxin in the feed, and biotransforming agents (modifiers) that are supposed to degrade or biotransform mycotoxins in non-toxic compounds, leading ultimately to a reduction on the intake of mycotoxin.

The use of mycotoxin-adsorbing agents is a very reliable method to reduce the consumption of mycotoxins. Adsorbents are dietetic additives with large molecular weight that are able to bind mycotoxins in contaminated feed. They are frequently used as a decontamination method to avoid mycotoxin toxic

effects in animals, and they act by binding to mycotoxins in the gastrointestinal tract reducing their bioavailability and consequently their absorption by the animal. The method of mycotoxins adsorption has a great economic significance since it is the last measurement to reduce the contamination of feed by mycotoxins, that otherwise would affect the health of animals and inevitably humans as primary consumers (Boudergue, 2009). The process involved in this decontamination method is rather simple. It consists in the addition of the adsorbent (organic or mineral) to the animal feed. These mycotoxin-adsorbing agents should be able to bind the mycotoxin, which will remain bonded until it is expelled through faeces. Due to the properties of the mycotoxins (such as polarity, solubility, molecular size, shape, and charge distribution, etc.) and those of the adsorbent in use (the physical structure, total charge and distribution, pore size, surface accessibility, etc.), the efficiency of the adsorption varies. Also one important criterion of evaluation of this adsorbents of mycotoxins is their effectiveness at different pH levels because they need to be effective through all the gastro-intestinal tract.

These adsorbing agents can be mineral, organic as well as biological (such as yeast, bacteria, etc.). There are already a large variety of adsorbents patented and in the market including natural clay products as well as synthetic polymers. HSCAS (hydrated sodium calcium aluminosilicate) is one of the most studied mycotoxin-binders and one of the most effective (Kolosova, 2011).

Regardless of the advance in the utilization of dietetic additives, there still a considerably lack of efficiency in the removal of some mycotoxins, and also the concern that these adsorbents can remove essentials nutrients too. Thus new additives are being characterized and studied, in order to find more effective mycotoxin-adsorbing agents.

1.3.1 Silicate binders

Silicates are minerals that constitute the majority of Earth's crust (approximately 90 percent). They can be formed by a wide range of methods such as melting, crystallization, fractionation, metamorphism, weathering and diagenesis. These minerals are the largest class of mycotoxins binders and the most studied. Silicates are divided into subclasses accordingly to their structure, specifically the phyllosilicates that includes aluminosilicates (HSCAS), bentonites, montmorillonites and the tectosilicates that includes zeolites (Jard, 2011).

Aluminosilicates (hydrated sodium calcium aluminosilicates, or HSCAS) is the most extensively studied of these materials. It was showed *in vitro* that HSCAS could adsorb up to 80% of AFB1, being able to prevent aflatoxicosis, but others tests showed that it has low affinity for OTA (Galvano, 2001).

Others than HSCAS more minerals adsorbents have been studied, like bentonites, which are crafted from volcanic ash and constituted mainly by montmorillonites (montmorillonites are a layered silicate that is able to adsorb either in its external surface as well in its inter-laminar spaces), and zeolites, that are crystalline hydrated aluminosilicates formed with large pores that gives them the ability of adsorb and lose water, and exchange constituent cations (Ramos, 1996).

Generally, silicate binder, and more concretely aluminosilicates, are good examples of efficient adsorbent. They are stable and have elevated porosity, being able to adsorb high concentrations of mycotoxins. Because of all these reasons, these adsorbents have been commercialized for use in animal feed.

1.3.2 Activated carbon

Activated carbon, also known as activated charcoal, is a non-soluble powder also used as adsorbent, and applied to a wide variety of drugs and toxic agents. It is manufactured using several organic compounds by activation process that allows to form a highly porous structure and as a consequence achieve an excellent adsorptive capacity. It has multiple applications in industry, analytical chemistry, environment, agriculture, purifications of fuel, gas, alcoholic beverages, and in medicine, and it has been used as a treatment for severe intoxications since the 19^{th} century (Ramos, 1996). Many tests were done using activated carbon as a mycotoxin binder *in vitro* and *in vivo*, and the results were positive, with better efficiency regarding some mycotoxins (such as AFB1, ZEA, etc.) (Galvano, 2001). The properties of activated carbon influence the efficiency of its adsorption, as the pore size, surface area and structure differs due to its manufacturing process. The manufacturing process can lead to a form that is called superactivated carbon, which has a surface area elevated from 500 m^2/g to 3500 m^2/g.

This adsorbent, likewise those obtain from silicate based clays, are very effective adsorbents, but, as they are non-specific, they also adsorb others compounds, changing the final composition of the product (e.g. the removal of some nutrients from food and feed).

1.3.3 Biological adsorbents

The limitations of using minerals as adsorbent lead to the search of a more specific adsorbent that would obtain a greater efficiency, and at the same time reduce the nutritional impact in food products. The answer to this search could be biological adsorbents. Biological adsorbents have been targets of

studies during the last decades in order to see its use as adsorbent. Several biological organisms and compounds were tested, but those better known are yeast, yeast extracts and bacteria. They all have a cell and membrane walls constituted by proteins, carbohydrates (from different sources, e.g. glucose, glucosamine, mannose) and lipids that can act as adsorption centers.

One example is *Saccharomyces cerevisiae*, that when used as adsorbent was proved to bind to AFB1 reducing its effects on rats (Madrigal-Santillán, 2006). More recently, some studies show that yeast and yeast cell walls has shown a good capacity of removing ochratoxin A (Evans, 2000). Bacteria, in specific some strains of lactic acid bacteria (LAB), were tested and are used to remove mycotoxins (El-Nezami, 1998). Usually they are found decomposing plants and lactic products.

Although these microorganisms can act as adsorbents, its use as mycotoxin-biotransforming agents is also abundant. Some studies have shown that some have the ability to degrade or biotransform mycotoxins. These include bacteria (e.g. *Nocardia asteroids and Corynebcterium* spp (gram-positive), *Flavobacterium aurantiacum* (gram-negative), *Eubacterium* spp (anaerobic), *Mycobacteria fluoranthenivorans* and *Pseudomonas fluorescens* (aerobic bacteria), etc.), yeasts (e.g. *Trichosporon mycotoxinivorans*, *Phaffia rhodozyma*, etc.), fungi (e.g. *Aspergillus* spp., *Eurotium herbariorum*, *Penicillium raistricki*, etc.) and enzymes (e.g. proteases A, pancreatin, carboxypeptidade A, epoxidase, lactonohydrolase, etc.) (Wu, 2009; Péteri, 2007; Abrunhosa, 2006). The idea is to use a specific enzyme that will specifically degrade each mycotoxin, or class of mycotoxins, and such enzymes can be prevenient from bacteria, yeast, fungi or even purified enzymes.

1.3.4 Adsorption models

In order to better understand and explain the mechanism of adsorption some calculation can be done to obtain sorption isotherms. They quantify the amount of adsorbate on the adsorbent as a function of its pressure/concentration at constant temperature (Foo, 2010). The first adsorption model was defined by Freundlich and Kuster in 1895 and consisted in an empirical formula used for gaseous adsorbates ($\frac{x}{m} = kP^{\frac{1}{n}}$), where x is the quantity of adsorbate absorbed (mol), m is the mass of the adsorbent, P is the pressure/concentration of the adsorbate and k and n are empirical constants. A few years later, in 1918, was described the Langmuir model ($Qeq = Qmax\left(\frac{KL \cdot Ceq}{1+KL \cdot Ceq}\right)$), that is appropriate to explain the adsorption of a single ligand to a single type of site on a particular sorbent. This equation relates the quantity of mycotoxin adsorbed per mass of adsorbent (Qeq) with the residual

mycotoxin concentration in the supernatant of the experimental tubes (Ceq), describing parameters related with the maximum mycotoxin capacity (Qmax) and affinity of the adsorbent (KL) (Langmuir, 1916). From these models and with the objective of applying these equations to heterogeneous solid surfaces, others models were created, such as the Freundlich ($Qeq = Qmax\left(\left(\frac{Kf \cdot Ceq}{1+(Kf \cdot Ceq)}\right)^{n}\right)$) that describes the additional parameter, n, which represent the heterogeneity of the adsorption. Another important sorption model is the Hill model ($Qeq = \left(\frac{Qmax \cdot Ceq^{nH}}{KD+Ceq^{nH}}\right)$) that assumes the adsorption as a cooperative phenomenon in which the binding affinity of one ligand in one site may influence other binding sites on the molecule and describes the Hill constant (KD) and Hill cooperativity coefficient of the binding interaction (nH) (Hill, 1910). Nowadays, many others equations were derivate from these mathematical equations to estimate Qmax and KD of adsorption, like, the Eadie-Hofstee, Lineweaver-Burk, Scatchard and reciprocal Langmuir models (Kinniburgh, 1986). These mathematical equations give us a way to measure and compare different adsorbent, facilitating the study of adsorbents and their efficiency.

1.4 BSA Protein

Bovine serum albumin (BSA) is a serum albumin protein found in the plasma of bovines. It is the most abundant blood protein in mammals and function as a carrier protein. BSA is easy to obtain (is purified from bovine blood) and has various biochemical applications such as in immunoblots, in ELISAs (Enzyme-Linked Immunosorbent Assays), as nutrient for microbial and cell cultures, as enzyme stabilizer, as standard for protein quantification (Bradford protein assay), etc. (Putnam, 1975).

Regarding mycotoxins, studies shown that when OTA is adsorbed by the gastrointestinal tract, it binds primarily to albumin, resulting in extended half-life that could go from a few days to one month and that one molecule of BSA is able to adsorb more than 2 molecules OTA (Peraica, 1999; Chu, 1971). These properties suggest that BSA could be used as mycotoxin binder. Also, considering that BSA is used in the blockage of unspecific antibody-binding sites of cellulosic matrixes, new detoxification methods (e.g. using a matrix formed with BSA adsorbed into cellulose) can be hypothesized to remove mycotoxins.

2. OBJECTIVES

The worldwide mycotoxin contamination of food and feed causes great economical and health related losses. Many methods have been described in order to attempt to reduce these contaminants, thus improving agriculture production and decreasing health issues caused by these toxins.

During the course of this master thesis we studied adsorption methods by evaluating and characterizing several mineral and organic mycotoxin binders *in vitro*. The experiments performed consisted in testing the efficiency of adsorbent agents with mineral origin (e.g. zeolite, bentonite, activated carbon, aluminosilicate and diatomaceous earth), organic origin (e.g. micronized olive pomace and grape pomace), and polymers (e.g. aquatex, propyltex, poly(vinylpolypyrrolidone), polyethylene, poly(methyl vinyl ether-all-maleic anhydre), Mowiol, poly(sodium 4-styrenesulfonate) and poly(ethylene-alt-maleic-anhydre)); and to compare their efficiency with two different commercial products available in the market. Mycotoxins were aflatoxin B1, ochratoxin A and zearalenone. Furthermore, adsorption isotherms were calculated for the adsorbents that showed better adsorption results.

In addition to the characterization of mycotoxin-binders, experimental methods, relaying in the use of proteins as adsorbents were conducted. The protein bovine serum albumin (BSA) was tested and characterized as an ochratoxin A specific binder.

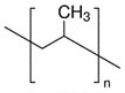
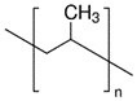
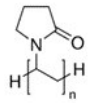
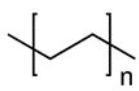
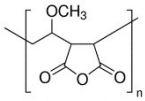
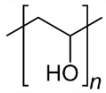
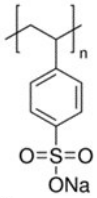
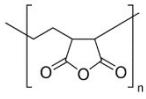
3. MATERIALS AND METHODS

3.1 Adsorbents

3.1.1 Proprieties of adsorbents tested

In these experiments several adsorbents with different properties (organic, mineral, animal, etc.) were used to test the adsorption of mycotoxins in buffer solutions. The adsorbents tested were commercial product 1, commercial product 2, bentonite, zeolite, activated carbon, diatomaceous earth, micronized dry olive pomace obtained from three different extraction processes (obtained after solvent extraction, two-phases with centrifugation and traditional pressing), micronized dry grape stems, *Saccharomyces bayanus*, aquatex, propyltex, poly(vinylpolypyrrolidone), polyethylene, poly(methy vinyl ether-all-maleic anhydre), Mowiol, poly(sodium 4-styrenesulfonate) and poly(ethylene-all-maleic-anhydre). The properties of the different products tested are described in Table 1.

Table 1. Properties of the products tested.

Adsorbent	Abbreviation	Origin	Particle size	MW	Structure
Commercial product 1	ComProd1	-	-	-	-
Commercial product 2	ComProd2	-	-	-	-
Bentonite	Bent	Sigma 285234-500G	-	-	-
Zeolite	Zeolite	-	-	-	-
Activated carbon	ActCarb	Panreac	-	12.01	-
Diatomaceous earth	DiatEarth	Panreac	-	-	-
Micronized dry olive pomace extracted	OliPom1	-	-	-	-
Micronized dry olive pomace centrifuged	OliPom2	-	-	-	-
Micronized dry olive pomace pressed	OliPom3	-	-	-	-
Micronized dry grape stems	GrapStem	-	-	-	-
S. bayanus	S.bayanus	-	-	-	-
Aquatex	PP1	Micro Powder, Inc.	149 µm	-	
Propyltex	PP2	Micro Powder, Inc.	45 – 100 µm	-	
Poly(vinylpolypyrrolidone)	PVPP	Sigma 77627-100G	110 µm	-	
Polyethylene	PE	Sigma 434272-100G	53 - 75 µm	Ultra-high molecular weight	
Poly(methy vinyl ether-all-maleic anhydre)	P(MVE-MA)	Sigma 416339-100G	-	216,000	
P	PVOH	Sigma 81365-250G	-	130,000	
Poly(sodium 4-styrenesulfonate)	PSS	Sigma 243051-100G	-	70,000	
Poly(ethylene-all-maleic-anhydre)	P(E-MA)	Sigma 188050-100G	-	100,000-500,000	

3.1.2 Buffer preparation

The buffers used to obtain the different pH were 0.1 M KCl/HCl at pH 2; 0.1 M citrate/phosphate buffer at pH 5; 0.1 M phosphate buffer at pH 7 and 0.1 M phosphate buffer at pH 8. They were prepared as shown in Table 2. For each buffer it was prepared a solution containing a mixture of the three mycotoxins in test (AFB1, OTA, ZEA). In a 50 mL glass flask, it was added to 10 mL of buffer, 10 µL of AFB1 (Sigma A6636, 2000 µg/mL), 10 µL of ZEA (Sigma Z2125, 2000 µg/mL,) and 20 µL of OTA (Sigma O1877, 1000 µg/mL) and then added more 9.96 mL of the same buffer to obtain a final volume of 20 mL with mycotoxin concentration of 1 µg/mL.

Table 2. Preparation of buffers used in mycotoxins adsorption experiments.

Buffer	Reagents	Molarity (M)	Quantity In 500 mL stock	Volume (mL) for 200 mL of buffer
0.1 M KCl/HCl pH 2	KCl (MW: 74.551 g/mol)	0.2	7.46 g	50
	HCl 37% (MW: 36.46 g/mol, ρ : 1.190 g/l)	0.2	8.21 mL	13
	H_2O_d			Add until 200
0.1 M citrate/ phosphate pH 5	Citric acid (MW: 192,124 g/mol)	0.1	9.6 g	48.6
	$Na_2HPO_4.2H_2O$ (MW: 177.99 g/mol)	0.2	17.8 g	51.4
	H_2O_d			Add until 200
0.1 M phosphate pH 7	$NaH_2PO_4.H_2O$ (MW: 137.99 g/mol)	0.2	13.8 g	39
	$Na_2HPO_4.2H_2O$ (MW: 177.99 g/mol)	0.2	17.8 g	61
	H_2O_d			Add until 200
0.1 M phosphate pH 8	$NaH_2PO_4.H_2O$ (MW: 137.99 g/mol)	0.2	13.8 g	5.3
	$Na_2HPO_4.2H_2O$ (MW: 177.99 g/mol)	0.2	17.8 g	94.7
	H_2O_d			Add until 200

3.1.3 Preliminary evaluation of mycotoxins adsorption

The procedure used to test adsorbents for mycotoxins is well-established and delivers good results, even if it can be found in the literature with many modifications. Basically, the procedure consist in adding a certain quantity of the adsorbent to a tube containing a buffer solution with the mycotoxin to be studied. After a certain period of incubation under agitation the adsorbent is removed from the liquid fraction and the concentration of mycotoxin is determined in this one.

In this case, the mycotoxins tested were aflatoxin B1 (AFB1), ochratoxin A (OTA) and zearalenone (ZEA), all at a final concentration of 1 µg/mL in the buffer solutions at pH 2, pH 5, pH 7 and pH 8. First,

20 mg of the adsorbent were added to 2 mL Eppendorf tubes and then, 1 mL of the buffers containing the mixture of the 3 mycotoxins was added. Then the mixture was vortexed and incubated at 37 °C for 1 hour with rotary agitation. After the incubation, samples were centrifuged at 10621 g for 10 minutes and 0.8 mL of the supernatants were collected to clean 4 mL amber vials. Then, it was added to the supernatants, 0.8 mL of acetonitrile/methanol/acetic acid (78/20/2, v/v/v), and after a strong vortex agitation, samples were filtered using a syringe filter (PP, 0.45 µm) to clean 2 mL amber vials. All experiments were performed in triplicates and analyzed by High Performance Liquid Chromatography with Fluorescence Detection (HPLC-FL), described in 3.1.7. Samples were preserved at -20 °C until their analysis.

3.1.4 Evaluation of adsorption efficiency with different concentration of adsorbents

Gathering the results from first tests, we selected the adsorbents that showed the best results and decided to test if their concentration would impact significantly the mycotoxin adsorption, or if with less concentration of adsorbent we could get similar results. The adsorbents selected were ComProd1, Bent, ActCarb, OliPom3, GrapStem, S.bayanus and PVPP, P(MVE-MA),.

The procedure applied was the same as previously described with the exception of the adsorbent concentration, instead of using 20 mg/mL we tested with 0.5, 1.0, 2.5, 5.0, 10.0, 20.0 and 30.0 mg/mL (respectively, 0.05%, 0.1%, 0.25%, 0.5%, 1%, 2% and 3%). The concentration of mycotoxin and the buffers used were the same. All samples were treated in triplicates and analyzed by HPLC-FL.

3.1.5 Mycotoxin desorption

Following the adsorption tests, we intended to check the stability of the mycotoxin/adsorbent bond and to confirm that the mycotoxins effectively bonded to the adsorbent. To do so, we took the remaining pellets of assays performed with 10 and 30 mg/mL of adsorbent from experiments of previous point. The samples with 10 mg/mL were used to test the bond strength and the samples with 30 mg/mL were used to check if the mycotoxin was really bonded to the adsorbent.

The pellets of 10 mg/mL samples were extracted through the addition of 1 mL of buffer at pH 7 and the tubes were strongly vortexed and incubated at 37 °C for 1 hour with rotary agitation. Then, the tubes were centrifuged (10 min at 10621 g) and the supernatant was collected and prepared for HPLC-

FL analysis as described before. In this case, the main goal was to understand if the mycotoxin will remain bonded to the adsorbent after this process.

The pellets of 30 mg/mL samples were extracted by adding 500 µL of acetonitrile/methanol/acetic acid (78/20/2, v/v/v). The tubes were vortexed for a few seconds and 500 µL of distillated H_2O was added to each one. The tubes were incubated at 37 °C for 1 hour with rotary agitation. Then, the tubes were centrifuged (10 min at 10621 g) and the supernatant was collected and prepared for HPLC-FL analysis by only filtering the solutions to clean 2 mL amber vials as described before. Here it was intended to remove all the mycotoxin that remained in the adsorbent using organic solvents. All samples were preserved at -20 °C until be analyzed by HPLC-FL.

3.1.6 Evaluation of adsorption efficiency with different concentration of Mycotoxin

In previous experiments it was proved the adsorption efficiency of some adsorbent and the stability of the bond mycotoxin/adsorbent. With that, we defined the minimum concentration of adsorbent that demonstrate sufficient capacity to adsorb the mycotoxins and performed experiments with varied concentrations of the mycotoxins with the objective to calculate adsorption isotherms.

The adsorbents tested were those selected and used in the previous evaluation of adsorption efficiency. It was used ComProd1 and Bent at a concentration of 2.5 mg/mL (0.25%), ActCarb at a concentration of 0.5 mg/mL (0.05%), OliPom3 and GrapStem at a concentration of 10 mg/mL (1.0%) and S.bayanus, PVPP and P(MVE-MA) at a concentration of 20 mg/mL (2.0%). The buffers used were 0.1 M KCl/HCl at pH 2 and 0.1 M phosphate buffer at pH 7 and the concentration of mycotoxins were 0.05 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL, 6 µg/mL and 10 µg/mL, all prepared as described before.

The procedure involved the incubation of adsorbents in 1 mL of mycotoxins solutions as described previously. Experiments were conducted in triplicate and all samples were preserved at -20 °C until being analyzed by HPLC-FL.

3.1.7 Determination of mycotoxins

The mycotoxins were quantified by high performance liquid chromatography with fluorescence detection (HPLC-FL). For AFB1 determination the mobile phase used was H_2O_d/methanol/acetonitrile (3:1:1, v/v/v) and for OTA and ZEA determination it was H_2O_d/methanol/acetic acid (65:35:1, v/v/v). Both solutions were filtered and degassed in ultrasounds. The system used was composed by a Varian Prostar 210 pump, a Varian Prostar 410 autosampler, a Jasco FP-920 fluorescence detector, which were connected through the Varian 850-MIB data system interface and operated in the computer through the Galaxie chromatography software. The parameters used for AFB1 detection were: excitation = 365 nm, emission = 435 nm and Gain = 10 for samples containing mycotoxin concentration $\geq$ 6 µg/mL or Gain = 100 for samples containing mycotoxin concentration < 6 µg/mL. For OTA and ZEA these parameters were: excitation = 280 nm, emission = 460 nm and Gain = 100 for samples containing mycotoxin concentration $\geq$ 6 µg/mL or Gain = 1000 for samples containing mycotoxin concentration < 6 µg/mL.

Calibration curves for AFB1 were obtained using standards in a concentration with a 1.0 – 10.0 µg/mL range at Gain = 10 and in a concertation with a 0.05 – 0.5 µg/mL range for Gain = 100. For OTA and ZEA calibration curves were obtained using standards in a concentration with a 1.0 – 10.0 µg/mL range at Gain = 100 and in a concertation with a 0.5 – 1.0 µg/mL range for Gain = 1000.

The setup of the system involved turning on all modules followed by their detection in the software. Then, the samples were placed in the injector tray and was started the stabilization (by running mobile phase solution to achieve a stable pressure in the system). With a good pressure achieved, the protocol is selected, samples identified and the mycotoxin analysis started. The results were exported as Excel files and the graphs were exported as OneNote files.

3.1.8 Data calculation and curve fitting for isotherms

With the results of the experiment performed as described in 5.1.6, the quantity of mycotoxin adsorbed per mass of adsorbent (Qeq (µg/mg)) was calculated as the difference between the mycotoxin concentration in control tubes (C0 (µg/mL)) and residual mycotoxin concentration in the supernatant of the experimental tubes (Ceq (µg/mL)) in the solution volume (V (mL)), per quantity of adsorbent (m (mg)), as the equation:

$$Qeq = \frac{[(C0 - Ceq)V]}{m}$$

The adsorption isotherms were calculated in the software Graphpad Prism version 7.02 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com), by plotting the quantity of mycotoxin adsorbed per mass of adsorbent (Qeq) against the mycotoxin concentration in the tube with adsorbents (Ceq). Then, this data was fitted to the Freundlich, Langmuir and Hill isotherm models. The models were compared to each other in order to obtain the best fit and associated parameters.

3.2 BSA protein

3.2.1 Binding of ochratoxin A to BSA adsorbed in Avicel cellulose columns

As mentioned before, bovine serum albumin (BSA) is a protein largely used in protein assays due to its binding capabilities and is also known to bind ochratoxin A. This experiment was planned with the objective of immobilizing BSA into a cellulose matrix in a gravity-flow column and study the mycotoxin retention when passing through it.

It was prepared a solution by diluting BSA in 50 mM acetate buffer at pH 5.0 in a final concentration of 1 mg/mL and weighed 100 mg of Avicel cellulose in 2 eppendorf tubes of 2 mL. To one of the tubes is was added 1 mL of the BSA solution and, in the other one, it was added 1 mL of acetate buffer to be our negative control. The tubes were incubated for 2 hours with gentle agitation using a platform rocker PMR-30 at room temperature, then, they were centrifuged at 2795 g for 10 min and the supernatants collected to quantify protein and calculate the immobilization efficiency. Next, the pellet was re-suspended in 1 mL of acetate buffer and centrifuged at same conditions to guarantee good protein adsorption. After centrifuge the supernatant is again collected and the cellulose was re-suspended and transferred to the respective 6 mL SPE column. Once on the column, the Avicel was washed with 3 mL of 50 mM acetate buffer at pH 5.0 and the flow-through collected in fraction of 1 mL to eppendorf tubes. One mL of OTA in 50 mM acetate buffer at pH 5.0 with a concentration of 90 ng/mL was then added and the flow-through collected. After the passage of the mycotoxin, the column was washed with 3 to 9 mL of 50 mM acetate buffer at pH 5.0 and fractions of 1 mL collected. Different quantities of Avicel were tested (100, 300 and 600 mg) and different buffers (pH 2.0, 5.0, 7.0, 8.0). It was also performed the same experiment with 100 mg of Avicel and different buffers (pH 2.0, 5.0, 8.0) in 2 mL eppendorfs. Buffers used were prepared as shown in Table 3. All samples were analyzed for OTA and protein concentration as described in section 3.2.5.

Table 3. Preparation of buffers used in BSA protein experiments.

Buffer	Reagents	Molarity (M)	Quantity In 500 mL stock	Volume (mL) for 200 mL of buffer
0.1 M KCl/HCl pH 2.0	KCl (MW: 74.551 g/mol)	0.2	7.46 g	50
	HCl 37% (MW: 36.46 g/mol, ρ : 1.190 g/l)	0.2	8.21 mL	13
	H_2O_d			Add until 200
0.05 M acetate pH 5.0	Acetic Acid, $C_2H_4O_2$ (MW: 60.05 g/mol; d = 1.049 g/L)	0.2	5.78 mL	6.8
	$C_2H_3O_2Na$ (MW: 82.03 g/mol)	0.2	8.2 g	102.8
	H_2O_d			Add until 200
0.1 M phosphate pH 7.0	$NaH_2PO_4.H_2O$ (MW: 137.99 g/mol)	0.2	13.8 g	39
	$Na_2HPO_4.2H_2O$ (MW: 177.99 g/mol)	0.2	17.80 g	61
	H_2O_d			Add until 200
0.1 M TRIS pH 8.0	$C_4H_{11}NO_3$ (MW: 121.14 g/mol)	0.1	1.21 g/200 mL	
	H_2O_d			Add until 200
0.1 M NaHCO3 pH 8.3	$NaHCO_3$ (MW: 84.01 g/mol)	0,1	1.68 g/200 mL	
	NaCl (MW: 58.44 g/mol)	0.5	5.84 g/200 mL	
	H_2O_d			Add until 200
0.2 M glycine pH 8.0	$C_2H_5NO_2$ (MW: 75.07 g/mol)	0.2	3.0 g/200 mL	
	H2Odd			Add until 200
0.1 M acetate/0.5 M NaCl pH 4.0	Acetic Acid, $C_2H_4O_2$ (MW: 60.05 g/mol; d = 1.049 g/L)	0.2	5.78 mL	82
	$C_2H_3O_2Na$ (MW: 82.03 g/mol)	0.2	8.2 g	18
	NaCl (MW: 58.44 g/mol)	0.5	5.84 g/200 mL	
	H_2O_d			Add until 200

3.2.2 Quantification of protein BSA and Avicel cellulose interactions

This experiment aimed at quantifying the interaction between BSA and the Avicel cellulose using a fix concentration of protein and different quantities of cellulose. BSA solutions in 50 mM acetate buffer at pH 5.0 were prepared at a final concentration of 1 mg/mL. In 2 mL Eppendorf tubes, it was weighed 50, 100, 200, 300, 400 and 500 mg of Avicel and 1 mL of BSA solution was added to each one. The tubes were incubated with gentle agitation using a platform rocker PMR-30 at ambient temperature for 2 hours, centrifuged at 2795 g for 10 minutes and supernatants collected for future analysis. The pellets were then re-suspended and washed twice with 1 mL of 50 mM acetate buffer at pH 5.0, centrifuge and supernatants also collected for future analysis. Buffers used were prepared as shown in Table 3. Protein content of supernatants was determined by the Bradford method described in section 3.2.5.

3.2.3 Binding of ochratoxin A to BSA immobilized in Nickel resin columns

In this experiment it was used a nickel resin to bond the protein BSA and to construct a gravity-flow column as an alternative to Avicel cellulose and study the mycotoxin retention when passing through it. The resin used was HisPur™ Ni-NTA Resin (Cat N #88222), from Thermo Fisher Scientific (nickel resin) in a gravity-flow 3 mL SPE column, which has a claim binding capacity up to about 60 mg of 28 kDa 6xHis-tagged protein per mL of resin and enables effective immobilized metal affinity chromatography (IMAC) purification of polyhistidine-tagged proteins.

Was prepared a solution by diluting BSA in 100 mM TRIS buffer at pH 8.0 in a final concentration of 10 mg/mL and pipetted 500 μL of nickel resin in 2 gravity-flow 3 mL SPE column. The columns were washed with 1.5 mL of ultrapure water and stabilized with 3 mL of 100 mM TRIS buffer pH 8.0 and the flow-through collected in fraction of 1 mL to eppendorf tubes. To one of the columns was added 1 mL of the BSA solution and in the other one was added 1 mL of TRIS buffer to be our negative control. One mL of OTA in 50 mM acetate buffer at pH 5.0 with a concentration of 90 ng/mL was then added and the flow-through collected. After the passage of the mycotoxin, the column was washed with 3 mL of 100 mM TRIS buffer at pH 8.0 and fractions of 1 mL collected. The experiment was replicated in the same conditions and using 50 mM acetate buffer pH 5.0. Buffers used were prepared as shown in Table 3. All samples were analyzed for OTA and protein concentration as described in section 3.2.5.

3.2.4 Binding of ochratoxin A to BSA immobilized in activated-Sepharose resin columns

This experiment was planned and performed with the objective of study the immobilization of BSA in Cyanogen Bromide-activated-Sepharose (C9210), from Sigma-Aldrich resin and the mycotoxin retention provided by this matrix. The Cyanogen Bromide-activated-Sepharose resin has shown a coupling capacity of 5 mg of BSA per mL of hydrated resin.

It was weighed to a 15 mL falcon tube 5 mg of BSA that was dissolved in 2 mL of 0.1 M $NaHCO_3$ coupling buffer containing 0.5 M NaCl at pH 8.3 to obtain a final concentration of 2.5 mg/mL. To 250 mg of dry resin it was added 50 mL of cold 1 mM HCl and incubated 2 times for 1 hour at 4 °C, allowing it to swell. The resin was centrifuged at 1000 g for 5 minutes, the supernatant discarded and washed with 10 mL of distilled water, followed by the repetition of the centrifugation step. Next it was added 1.25 mL of coupling buffer $NaHCO_3$/NaCl to the resin and immediately transferred to the BSA solution in coupling buffer. This solution was incubated overnight at 4 °C. The solution was centrifuged at 1000 g

for 5 minutes and the supernatant collected to quantify unbound protein. Then the resin was incubated with 5 mL of coupling buffer $NaHCO_3$/NaCl for 30 minutes at room temperature being centrifuged and the supernatant collected as previously. In order to block unreacted groups, the resin was incubated with 5 mL of 0.2 M glycine at pH 8.0 for 2 hours at room temperature. The resin was centrifuged, the supernatant discarded and washed with 10 mL of coupling buffer $NaHCO_3$/NaCl at pH 8.3. This step was repeated three times using 10 mL of 0.1 M acetate buffer containing 0.5 M NaCl at pH 4.

The resin was equilibrated with 3 mL of 50 mM acetate buffer at pH 5 and two columns were prepared as previous. To one of the columns it was added 1 mL of the BSA solution and in the other it was added 1 mL of TRIS buffer to be our negative control. Then it was added 1 mL of OTA in 50 mM acetate buffer at pH 5.0 with a concentration of 90 ng/mL and the flow-through collected. The column was washed with 3 mL of 50 mM acetate buffer at pH 5.0 and fractions of 1 mL collected. Buffers used were prepared as shown in Table 3. All samples were analyzed for OTA and protein concentration as described in section 3.2.5.

3.2.5 Analytical determinations

The protein content of samples was determined by the Bradford method following the microtiter plate protocol from Bio-Rad Protein Assay. Briefly, 10 µL of each sample were pipetted to separate wells into a 96-well microplate, 200 µL of diluted dye reagent (1 part Dye Reagent Concentrate with 4 parts H_2O_{dd}) was add to each well and mixed. After an incubation period of 5 min in de dark at room temperature without agitation, plates were read in a microplate reader (Citation™ 3 from Biotek) at 595 nm. A calibration curve was prepared with BSA in 0.05-0.5 mg/mL range.

Ochratoxin A was determined by HPLC-FL as described in section 3.1.7 and by spectrofluorimetry using a microplate reader (Citation™ 3 from Biotek) in a dark 96 wells plate. The parameters of detection were: excitation = 333 nm, emission = 460 nm and Gain = 140 by adding 100 µL of sample to wells. A calibration curve was prepared with standards of OTA (Sigma-Aldrich) prepared in 50 mM acetate buffer at pH 5.0 in 1-90 ng/mL range. All samples were analyzed in triplicates.

4. RESULTS AND DISCUSSION

4.1 Adsorbents

4.1.1 Preliminary evaluation of mycotoxins adsorption

The use of adsorbents to sequester mycotoxins in the digestive tract is common in animal nutrition being available for commercialization several adsorbents that have been tested and studied (Huwig, 2001). In this experiments several organic and inorganic substances were tested with buffer solutions containing a mixture of three mycotoxins (AFB1, OTA and ZEA). Some of the substances used are well known for having good adsorbing efficiencies for certain mycotoxins and are easy to obtain since they are commercialized (Ramos, 1996). The list of substances tested is presented in Table 1 in the section 3.1.1. All experiments were performed with 20 mg of adsorbent in buffers solutions with different pH values (2.0, 5.0, 7.0 and 8.0) using a mycotoxin concentration of 1 µg/mL. This experiment was a screening to select the adsorbents with better efficiency for further analysis.

Observing the histograms presented in Figure 1, it can be verified that OTA is the most difficult mycotoxin to be adsorbed, especially at higher pH (pH 7.0 and 8.0). This effect maybe due to the conformational change of OTA in alkaline solutions, which switches to the open ring form of OTA (OP-OTA) and changes its interaction with the adsorbent (Bazin, 2013). Generally, the adsorbents from organic and mineral origin such as ComProd1, Bent, ActCarb, OliPom3, GrapStem, etc. showed better adsorption efficiencies than polymer based adsorbents, which proved to be inconsistent, delivering good efficiencies at a certain pH and weak performances at others. For example, PSS reduced 72% of ZEA and 62% of OTA at pH 7.0, but at pH 2.0 or 5.0 it was unable to adsorb any of the mycotoxins.

In resume, the adsorbents that had better efficiencies in reducing the concentration of mycotoxins and chosen to additional analysis were ComProd1, Bent, ActCarb, OliPom3, GrapStem, S.bayanus, PVPP and P(MVE-MA). ComProd1 reached nearly 100% of reduction for OTA at pH 2.0, nearly 100% of reduction for AFB1 and around 85% for ZEA in all pH tested. Bent performed similarly to ComProd1, reaching nearly 100% reduction for OTA at pH 2.0, nearly 100% reduction for AFB1 in all pH tested and 66% to 85% reductions for ZEA. ActCarb produced reductions of almost 100% in all conditions, which demonstrates why it is one of the most used adsorbents (Galvano, 1998). OliPom3 removed 70 to 75% of AFB1 at all pH, around 93% of ZEA in all tested pH and 89% for OTA at pH 2.0. GrapStem reduced the content of

AFB1 and ZEA in approx. 95 to 100% independently of the pH tested and 95% for OTA at pH 2.0. S.bayanus reduced AFB1 by approx. 50%, OTA by 21 to 85% and ZEA by 84 to 89%. PVPP was essentially able to reduce ZEA (83 to 89%) independently of pH. P(MVE-MA) showed good efficiencies at acidic pH (50 to 80%) but poor performances at basic pH (20 to 50%).

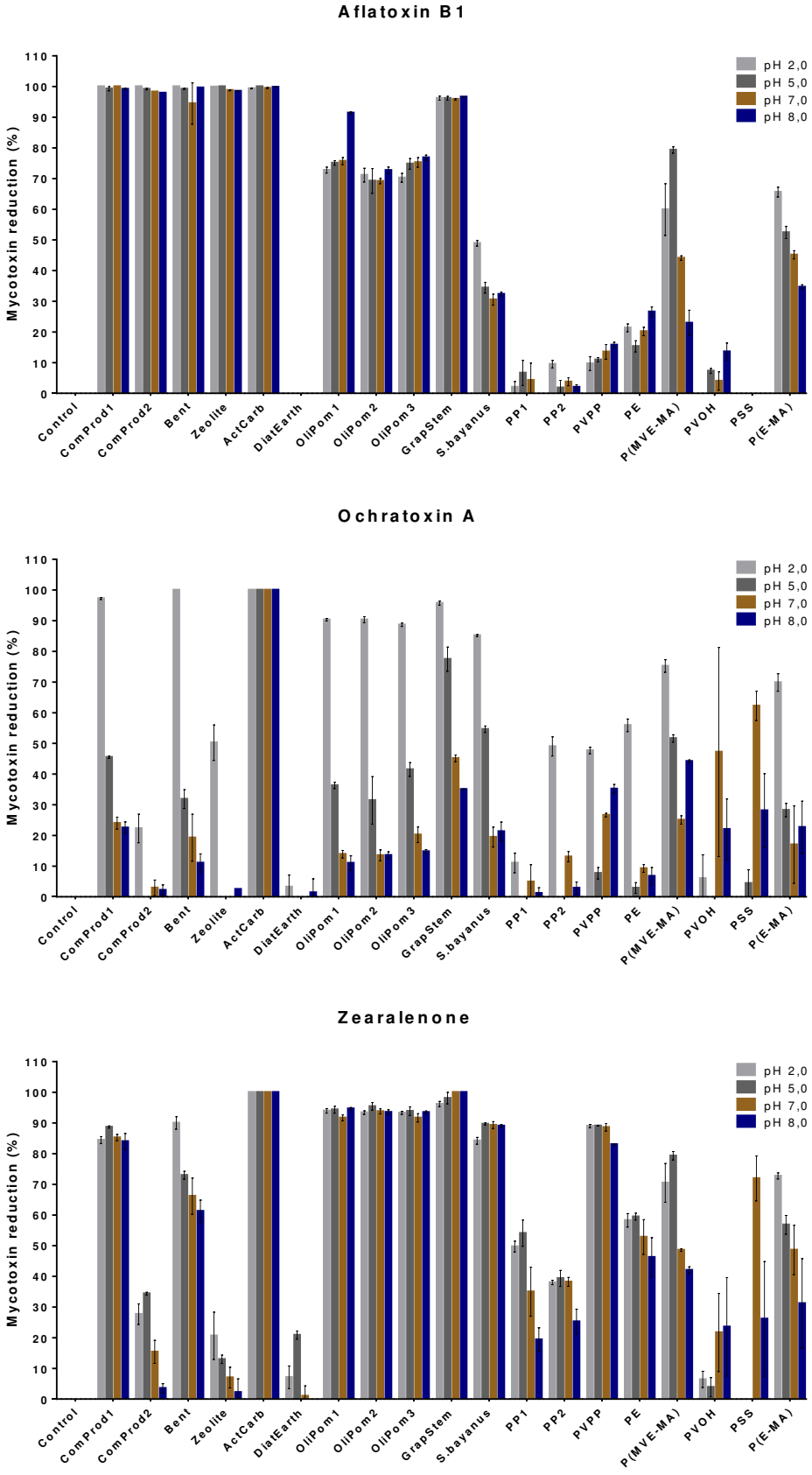


Figure 1. Percentage of mycotoxin removal (AFB1, OTA and ZEA) by different adsorbents at different pH. Results are expressed as the average of three replicates and the error bars indicate standard deviation.

4.1.2 Evaluation of adsorption efficiency with different concentration of adsorbents

The previous experiments, provided a good description of the interaction and efficiency of these adsorbents with the mycotoxins used. We were able to understand that some adsorbents were not efficient in removing the mycotoxins (e.g. diatomaceous earth), or in some cases they were able to perform well in limited conditions (e.g. only at pH 2.0). These results can be explained by the type of structure of molecules and the interactions they form between each other, which is the most important feature of the adsorption process (Huwig, 2001).

The adsorbents that had best results in mycotoxins removal (ComProd1, Bent, ActCarb, OliPom3, GrapStem, S.bayanus, PVPP and P(MVE-MA)) were selected to additional experiments. In this experiment, instead of using 20 mg/mL of adsorbent, it was tested concentrations of 0.5, 1.0, 2.5, 5.0, 10.0, 20.0 and 30.0 mg/mL (0.05%, 0.1%, 0.25%, 0.5%, 1%, 2% and 3% respectively) to determine the influence of adsorbent concentration in the adsorption of mycotoxins. Two different buffer (0.1 M KCl/HCl at pH 2.0 and 0.1 M phosphate buffer at pH 7.0) were used in order to have an acidic medium reproducing the conditions found in the stomach and a neutral medium reproducing the condition found in the intestines. All other conditions and procedures of the experiment were maintained the same.

The results are presented in Figure 2. If we compare the graphs of both pH, it is visible that the adsorption is more effective when performed at pH 2.0 than at pH 7.0. This is more evident for OTA and less evident in the adsorption of ZEA. As previously observed, ActCarb was able to reach mycotoxin removals of 100% even when used at low concentrations (less than 5 mg/mL) in all conditions tested, being the most effective adsorbent, compliant with others studies (Galvano, 1998). High percentage of mycotoxin removal were also obtained with ComProd1, Bent and GrapStem with less than 10 mg/mL of adsorbent (except for OTA at pH 7.0) (Ramos, 1996; Avantaggiato, 2014). On the other hand, the yeast and the polymer based adsorbents (S.bayanus, PVPP and P(MVE-MA)) were the less efficient in adsorbing the mycotoxins, showing the lowest percentages of mycotoxin removal. Also mineral and organic adsorbents shown better replicability, obtaining similar results in the triplicates, while the polymer based adsorbents shown more discrepancy between them, which is shown in the errors bars depicted in the Figure 2. Generally, the efficiency of the adsorption increased with the increase of the adsorbent concentration. Nonetheless, for some adsorbents the full removal of the mycotoxins from the solution was not achieved with concentration used. In some cases, it was even observed a tendency for the stabilization of removal percentages, which implies that a substantially high concentration of adsorbent will be needed to adsorb the totality of the mycotoxins from solutions.

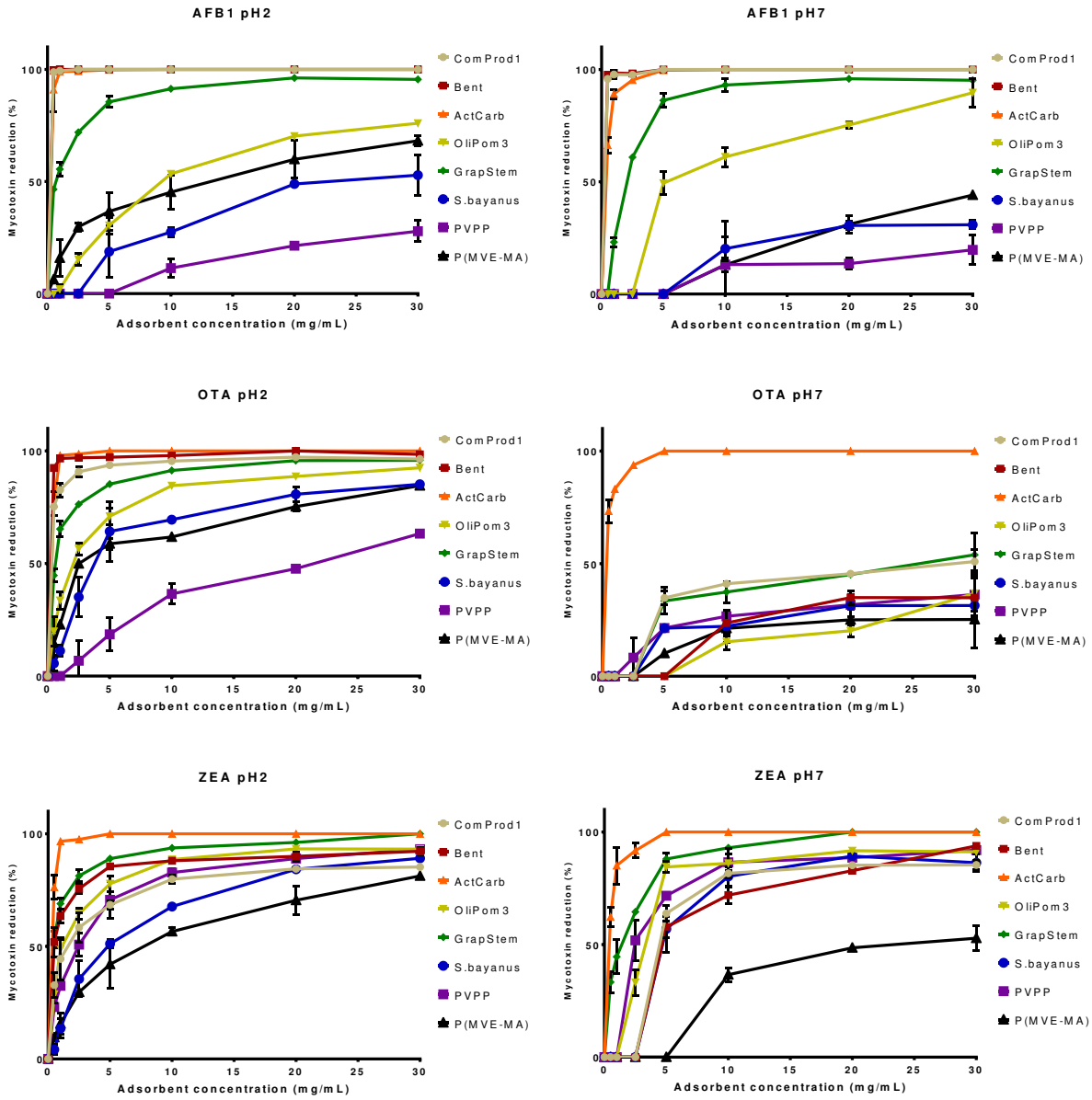


Figure 2. Percentages of mycotoxin reduction at different adsorbent concentration. Each graph represents the adsorption of one mycotoxin at a certain pH with different amounts of adsorbent. Results are expressed as the average of three replicates and the error bars indicate standard deviation.

4.1.3 Mycotoxin desorption

In the previous experiments it was obtained high percentages of mycotoxin removal when the highest concentrations of adsorbent (10 to 30 mg/mL) were used. In order to achieve such results, there must have been a strong bond between the adsorbent and the mycotoxin that was retain. The quantification method used was only able to quantify the specific structure of the mycotoxin tested and not their conformational changes, being uncertain that the reduction was due to the adsorption or due to conformational changes occurred during the experimental process.

These experiments were designed to desorb the mycotoxins retained by the adsorbents and to test the strength of the interaction formed. The strength of the bonds was tested using the pellets from samples containing 10 mg/mL of adsorbent by adding 1 mL of buffer at pH 7.0. The quantification of the adsorbed mycotoxin was performed with the pellets from samples containing 30 mg/mL by adding 0.5 mL of extraction solution following the protocol presented in section 3.1.5.

The results are presented as histograms in the Figure 3 and 4. When testing the bond strength in the experiments with 10 mg/mL, in many cases a small amount of mycotoxin was released. The release of mycotoxin was more visible in the case of OTA, which could be caused by the increase of pH, triggering conformational changes and altering its interactions with the adsorbent (Bazin, 2013). Even though, the concentration released never reached half of the adsorbed quantities of mycotoxin, implying that the bond was strong. For the experiments with 30 mg/mL almost the totality of the mycotoxins were desorbed, meaning that the mycotoxins were retained by the adsorbent and didn't change conformation during the experimental procedure, with the exception of the removal of AFB1 at pH 2.0 from ComProd1 and Bent. It was described that some phyllosilicates clays (such bentonite) can chemisorb aflatoxins (Phillips, 1995; Jaynes, 2011). Also, none of the adsorbed mycotoxins by ActCarb were recuperated, suggesting that a very strong bond was formed between them. When extracting the mycotoxins from samples with 30 mg/mL at pH 7.0, it was obtained percentages of desorption superiors to those of mycotoxins adsorption. These values can be the result of experimental and systematic errors, such as quantifications errors of the adsorbed mycotoxin in the HPLC-FL (presence of air in the system), conformational changes of mycotoxins, detection of other compounds present in the sample, etc.

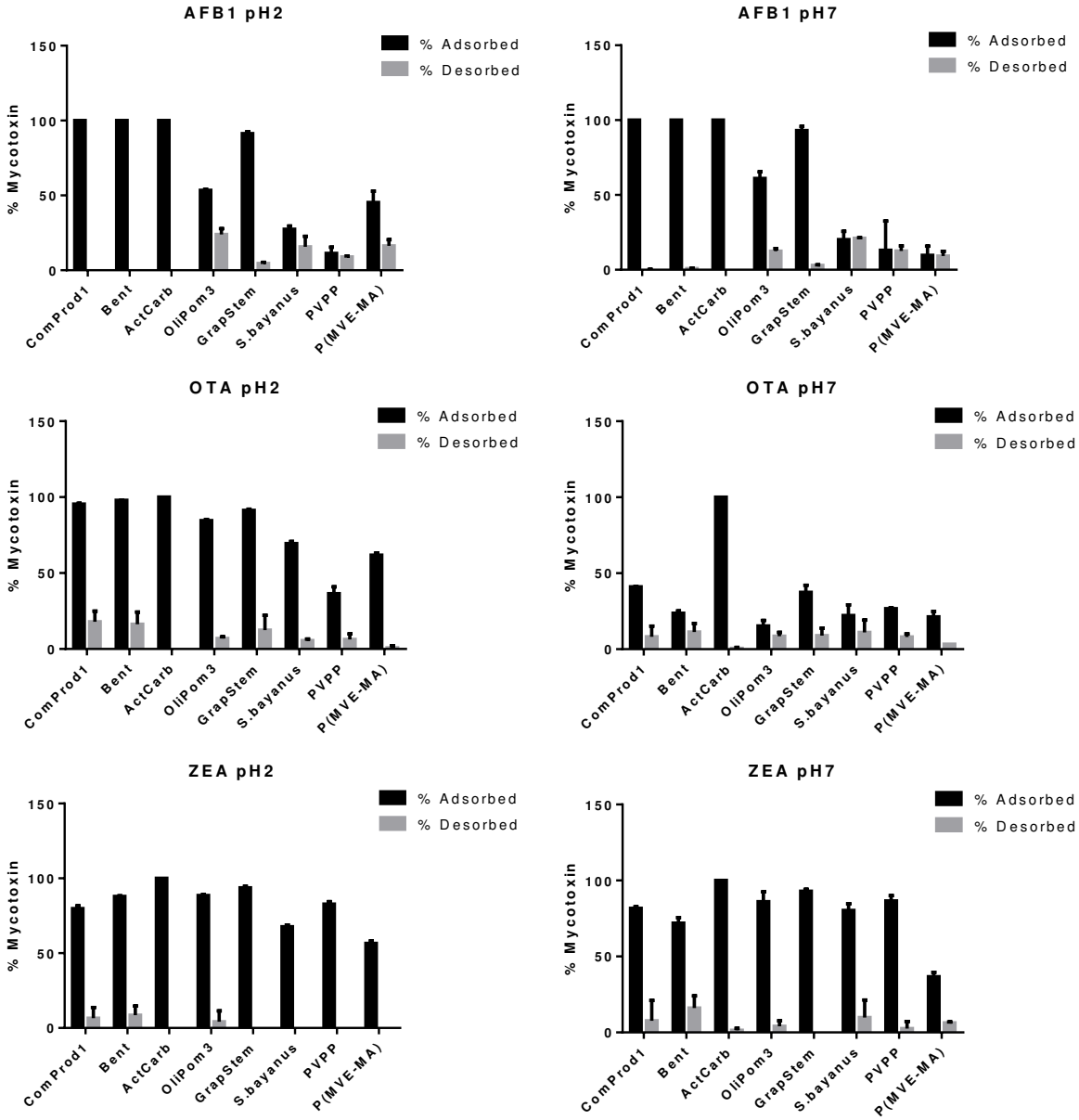


Figure 3. Desorption study of samples with 10 mg/mL of adsorbent. Mycotoxins were desorbed using buffer pH 7.0. Results are expressed as the average of three replicates and the error bars indicate standard deviation.

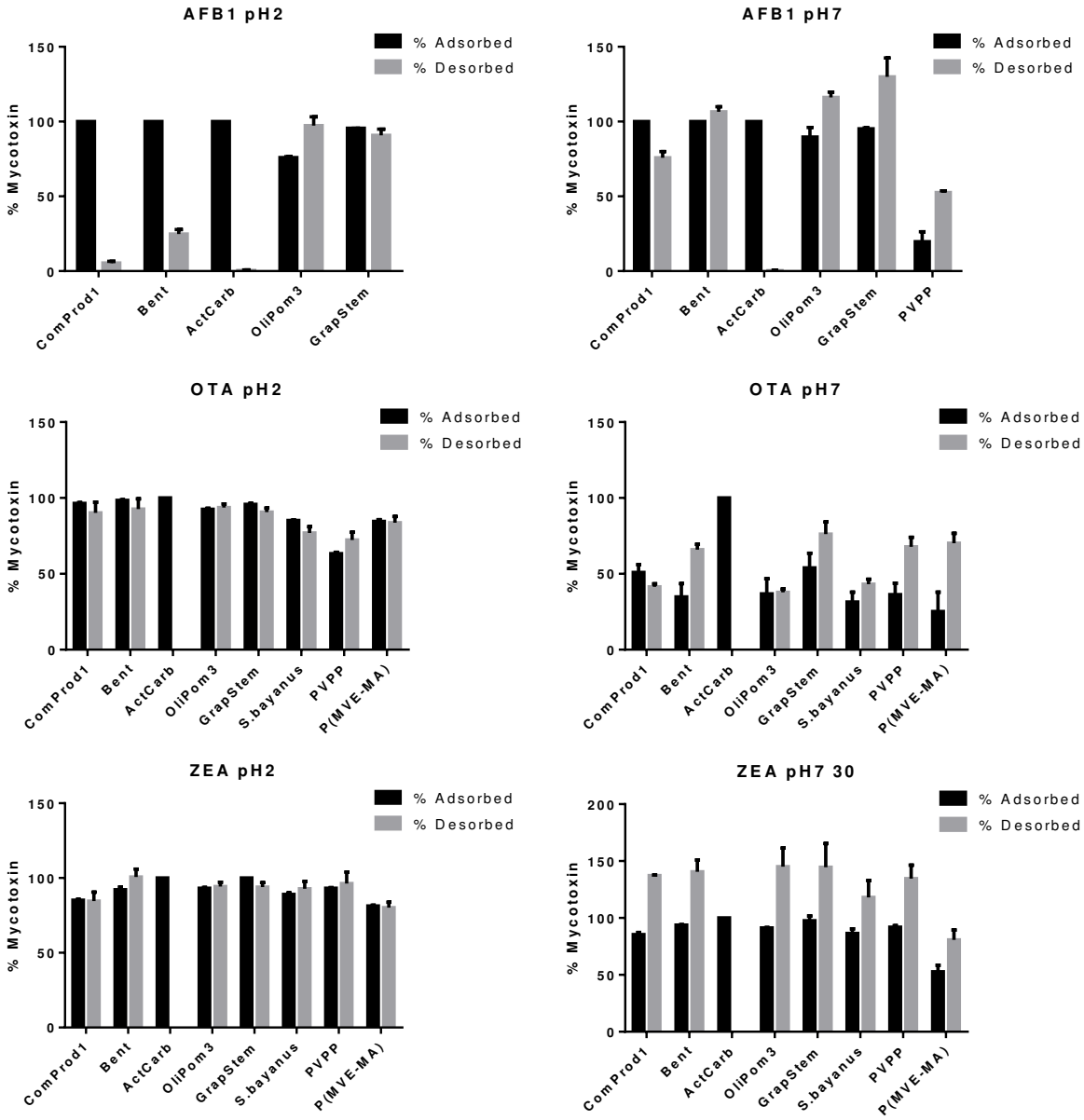


Figure 4. Desorption study of samples with 30 mg/mL of adsorbent. Mycotoxins were desorbed using extraction solution. Results are expressed as the average of three replicates and the error bars indicate standard deviation.

4.1.4 Evaluation of adsorption efficiency with different concentration of mycotoxin

In order to calculate sorption isotherms, a fix concentration of adsorbent was used in different amounts of mycotoxin. It was used adsorbents in the concentrations of 2.5 mg/mL (ComProd1 and Bent), 0.5 mg/mL (ActCarb), 10 mg/mL (OliPom3 and GrapStem) and 20 mg/mL (S.bayanus, PVPP and P(MVE-MA)) with buffers 0.1 M KCl/HCl at pH 2.0 and 0.1 M phosphate at pH 7.0. The concentration of mycotoxins was 0.05 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL, 6 µg/mL and 10 µg/mL. All other conditions and procedures of the experiment were maintained the same. After the performance of the experiment, the quantity of mycotoxin adsorbed per milligram of adsorbent (Qeq) was calculated and plotted against the residual mycotoxin concentration in the supernatant of the experimental tubes (Ceq), as shown in Figure 5. This data was then fitted using 3 different adsorption models (Freundlich, Langmuir and Hill) to obtain the parameters involved in the adsorption of the mycotoxins.

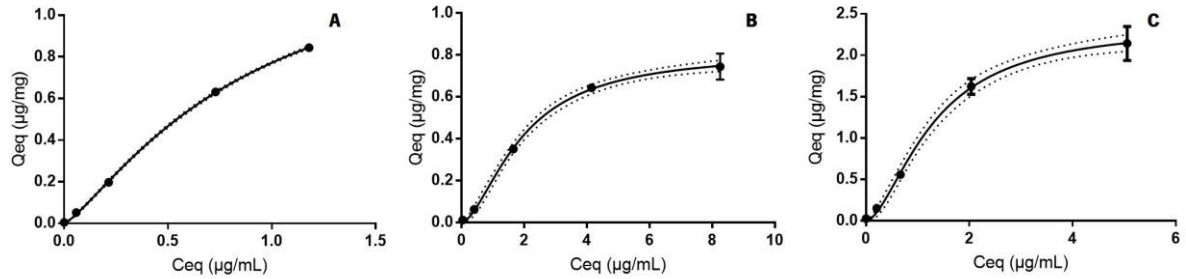


Figure 5. Examples of adsorption isotherms. Graph were obtained in GraphPad Prism version 7.02. Errors bars and points indicate 95% confidence intervals. A: AFB1 isotherm with GrapStem at pH 2; B: OTA isotherm with ComProd1 at pH 7; C: ZEA isotherm with Bent at pH 7.

The parameter calculated related with adsorption of AFB1, OTA and ZEA are presented in Tables 4, 5 and 6, respectively. With the exception of ActCarb and PVPP (at pH 7), the isotherms models delivered a good fit ($R^2 > 0.96$) and displayed exponential growth, since the amount of mycotoxin adsorbed per quantity of adsorbent (Qeq) increased with higher concentrations of mycotoxin. The Hill model had better correlations than the Freundlich and Langmuir model because it was developed for adsorption on heterogeneous solids (Hill, 1910). This model was applied to ComProd1, Bent, GrapStem, S.bayanus and P(MVE-MA). For the adsorbents ActCarb, OliPom3 and PVPP, the Freundlich and Langmuir models fitted better and produced very similar results.

From the adsorbents tested (Table 4), ActCarb showed the maximum AFB1 adsorption capacity with 19.40 ± 0.95 µg/mg at pH 2 and 16.47 ± 0.73 µg/mg at pH 7. ComProd1, Bent, OliPom3 and GrapStem had a lower Qmax, of 1.43 ± 0.69 µg/mg to 4.03 ± 0.08 µg/mg at pH 2 and 1.23 ± 0.09

μg/mg to 4.82 ± 0.30 μg/mg at pH 7. These results are in agreement with earlier reports and other studies (Grant, 1998; Avantaggiato, 2014). S.bayanus and P(MVE-MA) had the lowest values of Qmax (0.30 ± 0.05 μg/mg to 0.63 ± 0.07 μg/mg at pH 2 and 0.19 ± 0.01 μg/mg to 0.32 ± 0.11 μg/mg at pH 7). PVPP didn't show a good fit to models tested ($R^2 < 0.9538$) and displayed high values of associated errors in the calculated parameters. The adsorbents adjusted to Hills model (ComProd1, Bent, ActCarb, GrapStem, S.baynus and P(MVE-MA)) obtained the cooperativity coefficient of the binding interaction (nH) superior to 1, implying a positive cooperativity of the binding interaction (Hill, 1910). The constant KL from the Langmuir model for OliPom3 was 0.04 ± 0.02 at pH 2 and 0.05 ± 0.04 at pH 7. This constant is related to the free energy of adsorption and can be used to calculate the Langmuir separation factor (RL) which represent the adsorption nature (RL > 1, unfavorable; RL = 1, linear; RL = 0, irreversible; 0 < RL < 1, favorable). For OliPom3, RL is between 0 and 1, suggesting a favorable adsorption of AFB1 (data not shown). Also the exponent from the Freundlich model (1/n), that indicates the heterogeneity of the adsorption sites (0 < 1/n < 1, heterogeneous systems; 1/n = 1 relatively homogenous system), was between 0 and 1, suggesting a heterogeneous system (Avantaggiato, 2014).

The adsorption of OTA obtained better maximum adsorption capacity at pH 2, with significantly lower results at pH 7 (Table 5). This effect was already explained by the formation of an open ring form of OTA (OP-OA) in basic solutions (Bazin, 2013). The parameters obtained were similar with those obtained in adsorption of AFB1. Again, ActCarb showed to be the adsorbent with maximum adsorption capacity, specifically 22.90 ± 2.81 μg/mg at pH 2 and 20.16 ± 2.95 μg/mg at pH 7. Adsorbents from organic origin (OliPom3, GrapStem, ComProd1 and Bent) obtained a Qmax from 2.58 ± 0.47 μg/mg to 4.20 ± 0.39 μg/mg at pH 2 and 0.45 ± 0.02 μg/mg to 1.47 ± 0.07 μg/mg at pH 7. Polymers (P(MVE-MA) and PVPP) showed lower maximum adsorption capacity than organic adsorbents (0.46 ± 0.01 μg/mg to 0.46 ± 0.02 μg/mg at pH 2 and 0.12 ± 0.01 μg/mg to 0.18 ± 0.01 μg/mg at pH 7) and S.bayanus had the lowest value of Qmax (0.12 ± 0.01 μg/mg at pH 7). The adsorbents fitted to Hills model (ComProd1, Bent, GrapStem, S.baynus and P(MVE-MA)) obtained nH > 1, implying a positive cooperativity of the binding interaction to OTA (Hill, 1910). The values of the RL for ActCarb and OliPom3 are again indicatives of favorable OTA adsorption (data not shown). ActCarb and OliPom3 also obtained 1/n values between 0 and 1, suggesting a heterogeneous system (data not shown) (Avantaggiato, 2014).

The calculation of isotherms for adsorption of ZEA (Table 6) showed more adjustments to the Freundlich and Langmuir models than AFB1 and OTA. The maximum adsorption capacity of the adsorbents was distributed as for the adsorption of AFB1 and OTA. That is, ActCarb had the best result

of Qmax (24.44 ± 3.19 µg/mg at pH 2 and 18.56 ± 2.26 µg/mg at pH 7), followed by the organic adsorbents such as OliPom3, GrapStem, ComProd1 and Bent (with Qmax from 1.86 ± 0.15 µg/mg to 4.37 ± 1.02 µg/mg at pH 2 and 0.99 ± 0.01 µg/mg to 2.37 ± 0.05 µg/mg at pH 7). The lower values of maximum adsorption capacity were obtained with S.bayanus and the polymers (P(MVE-MA) and PVPP) (0.45 ± 0.02 µg/mg to 0.78 ± 0.11 µg/mg at pH 2 and 0.24 ± 0.01 µg/mg to 0.96 ± 0.06 µg/mg at pH 7). The cooperativity of the binding interaction to ZEA for the adsorbents fitted to Hills model (GrapStem, Bent, S.bayanus and P(MVE-MA)) was positive as the parameter nH was superior to 1. For ComProd1, ActCarb and OliPom3 RL values indicate favorable ZEA adsorption (data not shown). The adsorption intensity (n) obtained for ComProd1, ActCarb and OliPom3 were higher than 1, therefore the value 1/n was between 0 and 1 suggesting a heterogeneous system (data not shown).

In summary, the adsorbents tested showed favorable adsorption of the three tested mycotoxins (AFB1, OTA and ZEA). ActCarb prove to be the best adsorbent tested with a mycotoxin uptake capacity, often, 3 to 4 times higher than the remaining adsorbents. Mineral adsorbents, such as ComProd1 and Bent, also showed good performance for all mycotoxins, which is expectable since booth of these are well known as mycotoxin adsorbents and have been target of multiple studies that shows their efficiency. Another adsorbent that obtained good performance reducing mycotoxins is GrapStem. This adsorbent may be interesting for commercial uses, because it is cheap to produce (obtained from remaining of grape stems) and delivers high efficiency, comparable to bentonite. Some adsorbents that fitted Langmuir and Freundlich models (ComProd1, ActCarb and OliPom3) suggested a heterogeneous system, which is able to establish different interactions mycotoxins. As for polymer based adsorbents, such as PVPP, isotherms fitting provided weak correlations and high values of associated errors.

Table 4. AFB1 isotherms parameters from different adsorbents.

Model		pH 2								pH 7							
		ComProd1	Bent	ActCarb	OliPom3	GrapStem	S.bayanus	PVPP	P(MVE-MA)	ComProd1	Bent	ActCarb	OliPom3	GrapStem	S.bayanus	PVPP	P(MVE-MA)
Freundlich	Kf (±SD)	-	-	-	0.12 ± 0.01	-	-	9.8 e-4 ± 8.8 e-4	-	-	-	-	0.16 ± 0.01	-	-	0.01 ± 0.002	-
	n (±SD)	-	-	-	1.12 ± 0.04	-	-	0.53 ± 0.12	-	-	-	-	1.10 ± 0.09	-	-	0.77 ± 0.12	-
	R^2	-	-	-	0.9963	-	-	0.9156	-	-	-	-	0.984	-	-	0.9538	-
Langmuir	Qmax (±SD)	5.20 ± 0.42	-	-	3.03 ± 1.29	2.45 ± 0.24	1.72 ± 1.85	175.6 ± 253840	2.40 ± 1.07	-	-	-	3.27 ± 2.24	2.79 ± 0.53	-	210.1 ± 145140	-
	KL (±SD)	55.34 ± 11.47	-	-	0.04 ± 0.02	0.45 ± 0.06	0.03 ± 0.04	3.5e-5 ± 0.05	0.14 ± 0.08	-	-	-	0.05 ± 0.04	0.56 ± 0.14	-	5.24e-5 ± 0.04	-
	R^2	0.9628	-	-	0.9953	0.9978	0.9721	0.8749	0.9904	-	-	-	0.9845	0.9924	-	0.9437	-
Hill	Qmax (±SD)	4.03 ± 0.08	3.82 ± 0.04	19.40 ± 0.95	-	1.43 ± 0.69	0.30 ± 0.05	-	0.63 ± 0.07	3.70 ± 0.08	4.82 ± 0.30	16.47 ± 0.73	-	1.23 ± 0.09	0.19 ± 0.01	-	0.32 ± 0.11
	KD (±SD)	8.1e-5 ± 5.1e-5	6.9e-8 ± 3.8e-8	1.6e-6 ± 3.9e-6	-	0.85 ± 0.09	7.89 ± 1.78	-	0.96 ± 0.23	4.5e-8 ± 5.6e-8	1.8e-4 ± 1.5e-4	2.8e-11 ±1.2e-10	-	0.31 ± 0.08	20.85 ± 7.38	-	10.34 ± 3.24
	nH (±SD)	2.06 ± 0.13	2.86 ±0.09	3.13 ± 0.57	-	1.28 ± 0.04	2.01 ± 0.39	-	1.63 ± 0.14	4.56 ± 0.33	2.52 ± 0.21	5.08 ± 0.88	-	1.57 ± 0.11	3.72 ± 0.63	-	1.57 ± 0.35
	R^2	0.9957	0.9984	0.9638	-	0.9996	0.9825	-	0.9967	0.9945	0.9963	0.9712	-	0.9977	0.9887	-	0.9856

Note: -, does not converge.

Table 5. OTA isotherms parameters from different adsorbents.

Model		pH 2								pH 7							
		ComProd1	Bent	ActCarb	OliPom3	GrapStem	S.bayanus	PVPP	P(MVE-MA)	ComProd1	Bent	ActCarb	OliPom3	GrapStem	S.bayanus	PVPP	P(MVE-MA)
Freundlich	Kf (±SD)	-	-	31.04 ± 5.89	0.38 ± 0.01	-	-	0.04 ± 0.004	-	-	-	27.04 ± 6.04	-	-	-	0.03 ± 0.003	-
	n (±SD)	-	-	3.50 ± 1.07	1.16 ± 0.02	-	-	0.83 ± 0.05	-	-	-	3.47 ± 1.25	-	-	-	1.78 ± 0.20	-
	R^2	-	-	0.8696	0.9984	-	-	0.9901	-	-	-	0.8404	-	-	-	0.9485	-
Langmuir	Qmax (±SD)	-	-	22.90 ± 2.81	3.78 ± 0.71	-	-	4055 ± 8.2e+6	-	-	-	20.16 ± 2.95	-	-	-	0.12 ± 0.01	-
	KL (±SD)	-	-	32.42 ± 14.71	0.11 ± 0.03	-	-	1.2e-5 ± 0.03	-	-	-	28.54 ± 15.16	-	-	-	0.28 ± 0.07	-
	R^2	-	-	0.8693	0.9974	-	-	0.9822	-	-	-	0.8408	-	-	-	0.9617	-
Hill	Qmax (±SD)	4.20 ± 0.39	3.89 ± 0.06	-	-	2.58 ± 0.47	0.46 ± 0.02	-	0.46 ± 0.01	0.81 ± 0.04	1.47 ± 0.07	-	0.45 ± 0.02	0.45 ± 0.02	0.12 ± 0.01	-	0.18 ± 0.01
	KD (±SD)	1.09 ± 0.24	1.2e-3 ± 0.5e-3	-	-	2.24 ± 0.60	0.48 ± 0.09	-	7.6e-3 ± 4.6e-3	2.98 ± 0.30	2329 ± 2869	-	40.49 ± 17.13	43.45 ± 22.58	38.49 ± 26.53	-	61.39 ± 45.98
	nH (±SD)	1.10 ± 0.11	3.22 ± 0.20	-	-	1.20 ± 0.06	2.58 ± 0.25	-	4.51 ± 0.52	1.66 ± 0.18	8.19 ± 1.28	-	3.66 ± 0.49	4.28 ± 0.63	4.15 ± 0.92	-	5.83 ± 1.16
	R^2	0.9941	0.9966	-	-	0.9992	0.9943	-	0.9884	0.9933	0.9738	-	0.9865	0.9804	0.9687	-	0.9636

Note: -, does not converge.

Table 6. ZEA isotherms parameters from different adsorbents.

Model		pH 2								pH 7							
		ComProd1	Bent	ActCarb	OliPom3	GrapStem	S.bayanus	PVPP	P(MVE-MA)	ComProd1	Bent	ActCarb	OliPom3	GrapStem	S.bayanus	PVPP	P(MVE-MA)
Freundlich	Kf (±SD)	0.36 ± 0.03	-	28.58 ± 4.28	0.46 ± 0.01	-	-	0.35 ± 0.01	-	0.26 ± 0.03	-	19.59 ± 2.55	-	-	-	0.40 ± 0.01	-
	n (±SD)	1.29 ± 0.08	-	3.06 ± 0.86	1.18 ± 0.02	-	-	0.91 ± 0.02	-	1.54 ± 0.14	-	4.54 ± 1.76	-	-	-	1.36 ± 0.06	-
	R^2	0.9853	-	0.8932	0.9989	-	-	0.9983	-	0.9696	-	0.8655	-	-	-	0.9919	-
Langmuir	Qmax (±SD)	4.37 ± 1.02	-	24.44 ± 3.19	3.30 ± 0.32	15.68 ± 13.78	-	-	-	1.70 ± 0.22	-	18.56 ± 2.26	1.25 ± 0.08	19.17 ± 70.64	-	0.96 ± 0.06	-
	KL (±SD)	0.08 ± 0.03	-	14.05 ± 6.16	0.17 ± 0.02	0.07 ± 0.07	-	-	-	0.17 ± 0.04	-	19.33 ± 10.08	1.16 ± 0.15	0.10 ± 0.40	-	0.73 ± 0.07	-
	R^2	0.9841	-	0.8932	0.9989	0.9962	-	-	-	0.9777	-	0.8655	0.9896	0.9309	-	0.9978	-
Hill	Qmax (±SD)	-	2.37 ± 0.13	-	-	1.86 ± 0.15	0.45 ± 0.02	-	0.78 ± 0.11	-	2.37 ± 0.05	-	-	0.99 ± 0.01	0.46 ± 0.01	-	0.24 ± 0.01
	KD (±SD)	-	1.56 ± 0.23	-	-	0.84 ± 0.14	0.66 ± 0.09	-	1.14 ± 0.33	-	32.84 ± 8.29	-	-	2.6e-3 ± 3.9e-4	0.16 ± 0.02	-	3.72 ± 0.85
	nH (±SD)	-	1.68 ± 0.19	-	-	1.44 ± 0.06	2.24 ± 0.17	-	1.48 ± 0.14	-	10.80 ± 0.74	-	-	3.73 ± 0.08	1.95 ± 0.08	-	2.97 ± 0.49
	R^2	-	0.9891	-	-	0.9994	0.9958	-	0.9962	-	0.9944	-	-	0.9995	0.9985	-	0.9765

Note: -, does not converge.

4.2 BSA protein

4.2.1 Binding of ochratoxin A to BSA adsorbed in Avicel cellulose columns

BSA, or bovine serum albumin, is a serum protein from cows. It is easy to obtain and frequently used as a standard in protein quantification, in ELISAs (Enzyme-Linked Immunosorbent Assays), in immunoblots, as nutrient for cellular and microbial cultures, to stabilize enzymes in restriction digestions, etc. Some studies have pointed out that BSA adsorbs OTA, being able to bind 2 molecules of OTA by each molecule of BSA (Chu, 1971).

The adsorption of OTA by BSA was studied. Several protocols were tested (these can be found in material and methods, sections 3.2.1 to 3.2.5). In these protocols, different matrixes were used in an attempt to retain or immobilize BSA and further test the binding of OTA to this protein. The results of the experiments are presented in Tables 7, 8 and 9. The experiments were initially performed in gravity-flow columns packaged with different amounts of Avicel - microcrystalline cellulose (100, 300 and 600 mg). Cellulose was chosen as support because BSA is known to adsorb to cellulosic matrixes (Wang, 2015). BSA solutions with different pH values and a fixed OTA concentration of 90 ng/mL were applied into the columns. After the addition of BSA and after the addition of OTA, the columns were washed with three column volumes, according to resin bed weight. The use of column is simple and can serve multiple purposes. If a stable matrix is formed with BSA adsorbed in Avicel, this system could function as a filter for mycotoxins in which contaminated solutions (e.g. wine) are passed through and decontaminated.

BSA (at the concentration of 1.1 mg/mL) dissolved in two different buffers (acetate pH 5.0 and TRIS pH 8.2), when added to 100 mg of Avicel, was able to adsorb OTA. In the column using acetate buffer pH 5.0, 90.6% of OTA was adsorbed, while in the column using TRIS buffer pH 8.2, only 58.8% was adsorbed. Although OTA was retained in the column, it was completely removed after washing the column with 3 volumes of the respective buffer. BSA was also removed after washing the column. In the negative control (column with 100 mg of Avicel without BSA) with acetate buffer pH 5.0, OTA was also adsorbed (63.3%) and completely removed after washing (Table 7). This result indicates that cellulose can also adsorb temporarily OTA. However, the amounts of OTA adsorbed was much higher when BSA was in the column, thus indicating adsorption of OTA by BSA. An identical experiment was performed using buffer phosphate pH 7.0, but the results obtained in the column with BSA and in the respective

control were similar (60.7% and 61.5% of OTA adsorption, respectively). After washing both columns, and despite the removal of the protein and all OTA in the negative control, 13.8% of OTA remained in the column with BSA. Even though BSA was washed from the column, it is possible that a small amount of the protein remained there and retained OTA.

The adsorption of OTA by BSA was also tested in eppendorfs containing 100 mg of Avicel (Table 7). These experiments were performed in an attempt to minimize the removal of the protein when washing the resin. In the three buffers tested (KCL/HCl pH 2.0, acetate pH 5.0 and TRIS pH 8.2), OTA was always adsorbed, but the best efficiency was obtained when using acetate buffer pH 5.0 (48.7%). When comparing the results obtained for BSA with the results of the negative controls (eppendorfs without BSA) at pH 5.0 and 8.2, cellulose with BSA showed better OTA adsorption than the cellulose alone (48.7% versus 36.2% and 44.8% versus 24%, in pH 5.0 and 8.2, respectively). The opposite effect was obtained at pH 2.0 (33.7% with BSA versus 40.5% in negative control). In all cases, BSA was removed together with OTA washing. Interestingly, the best results for OTA adsorption were obtained in the gravity-flow columns.

Therefore, experiments in gravity-flow columns were replicated using higher quantities of Avicel (300 and 600 mg). The best conditions for OTA adsorption found in previous column assays were used (acetate buffer pH 5.0 with approximately 1.0 mg/mL of BSA). In the experiment using 300 mg of cellulose the adsorption of OTA was better in the column with BSA, reaching 96.5% in comparison to 53.8% in the column without protein. Also the increment of cellulose was able to preserve 0.4 mg of BSA after washing the columns, but it was unable to retain any of the adsorbed OTA. This may indicate that for these conditions the interaction OTA-BSA is weak. In the experiment using 600 mg of cellulose similar results were obtained (Table 7). After washing the column, BSA was completely removed, but 60.4% of the initial OTA remained in the column (in the negative control OTA was completely removed). The replication of this experiment did not reproduce the same results, since the OTA adsorption was lower (83.9% in the test with BSA and 86.1% in the negative control) and more OTA was removed after washing the columns (only 9.1% of the adsorbed OTA remained in the column where BSA was added). In summary, these sets of results show better OTA adsorption in the columns where BSA was added and when higher quantities of Avicel were used. As stated before, BSA was washed from the columns but it is possible that a small quantity of protein might have been retained, explaining the increase of retention of the mycotoxin with the increase of cellulose quantity.

Table 7. Results of OTA adsorption by BSA in experiments performed in gravity-flow columns with Avicel and different buffers used. In experiments performed in eppendorfs Avicel with different buffers was used.

	Resin	Buffer	Components present	Protein added (mg)	Protein retained [a] (mg)	% OTA adsorbed [b]	% Final OTA [c]
Experiments in gravity-flow columns	Avicel 100 mg	Acetate pH 5.0	BSA + OTA	1.1	0	90.6	0
		TRIS pH 8.2	BSA + OTA	1.1	0	58.8	0
		Acetate pH 5.0	BSA + OTA	0.9	0	96,5	0
			NC	-	-	63.3	0.5
		Phosphate pH 7.0	BSA + OTA	0.9	0	60.7	13.8
			NC	-	-	61.5	1.3
	Avicel 300 mg	Acetate pH 5.0	BSA + OTA	0.9	0.5	96.5	0
			NC	-	-	53.8	0
	Avicel 600 mg	Acetate pH 5.0	BSA + OTA	1.0	0	94.7	60.4
			NC	-	-	63.8	0
			BSA + OTA	1.3	0	83.9	9.1
			NC	-	-	86.1	0
Experiments in Eppendorfs	Avicel 100 mg	KCL/HCl pH 2.0	BSA + OTA	1.0	-	33.7	0
			NC	-	-	40.5	0
		Acetate pH 5.0	BSA + OTA	1.0	-	48.7	0
			NC	-	-	36.2	0
		TRIS pH 8.2	BSA + OTA	1.0	-	44.8	0
			NC	-	-	24.0	0

OTA concentration used was 90 ng/mL; NC: Negative control without BSA; [a] Protein retained is the quantity of protein adsorbed after washing the column; [b] % OTA adsorbed is the percentage of OTA retained after the passage through the column; [c] % Final OTA is the OTA retained in the column in the end of the experiment; -, protein quantification was not performed.

4.2.2 Quantification of protein BSA and Avicel cellulose interactions

The use of a higher quantity of Avicel lead to an increase of the OTA retention, also suggesting an increase of the BSA adsorption, therefore, the interaction between BSA and cellulose Avicel was studied (by incubating 1.0 mg of protein with 50, 100, 200, 300, 400 and 500 mg of cellulose). The results presented in Table 8 show that BSA is removed gradually as a result of the washes performed. When 50 mg of cellulose was used, BSA was removed just after two washes (2 mL of buffer). With 100 and 200 mg of cellulose, the protein was removed after three washes (3 mL of buffer). Only more than 300 mg of Avicel were able to maintain a small amount of BSA adsorbed. Thus, the higher the quantity of cellulose, the harder is the removal of the protein through the washes. These results are in accordance with previous ones, since more OTA was retained in experiments with higher amounts of Avicel and, possibly, BSA (Table 8).

Table 8. Results of BSA and Avicel interaction. Experiments were performed in 2 mL eppendorfs. The same BSA solution was used for all experiments.

	Buffer	Avicel (mg)	BSA added (mg)	BSA retained (mg)	BSA retained after 1st wash (mg)	BSA retained after 2nd wash (mg)	BSA retained after 3rd wash (mg)
Experiments in Eppendorfs	Acetate pH 5.0	50	1.026	1.044	0.062	0.0	0.0
		100	1.026	0.996	0.132	0.011	0.0
		200	1.026	1.063	0.253	0.055	0.0
		300	1.026	0.946	0.306	0.107	0.018
		400	1.026	0.944	0.394	0.145	0.048
		500	1.026	1.030	0.443	0.155	0.070

4.2.3 Binding of ochratoxin A to BSA immobilized in HisPur™ Ni-NTA and Cyanogen Bromide-activated-Sepharose resin columns

Because it was observed an unstable interaction between BSA and cellulose, experiments using HisPur™ Ni-NTA (nickel resin) and Cyanogen Bromide-activated-Sepharose resin were performed to effectively immobilize BSA and construct gravity-flow columns with a stable ligand. The nickel resin was produced by immobilizing the desired metal ion in an immobilized metal affinity chromatography (IMAC) basis for purification of His-tagged proteins. Since BSA have multiples histidines, it might be able to bind to this resin and form a stable matrix (Besselink, 2013). The activated-Sepharose resin can immobilize ligands containing primary amines, and thus it is suitable for immobilizing the BSA protein. The results obtained with these resins are presented in Table 9.

When BSA was immobilized in the nickel resin at pH 8.0, 97.8% of the OTA was adsorbed and 82.4% remained there after the washing steps. When replicating the experiment with a negative control, 81.9% of the OTA was adsorbed into the column containing BSA and 85.6% in the negative control column. After washing of the columns with buffer, 55.1% of OTA remained in the column with BSA (together with 4.0 mg of protein), and only 1.5% of the mycotoxin remained in the control column. When using a different buffer (acetate pH 5.0), the protein added was washed and a lower percentage (23%) of OTA remained into the column after the washing step. Indeed, we confirmed by SDS-PAGE electrophoresis that BSA binds to the nickel resin at pH 8.0 but not at pH 5.0 (data not shown).

In the experiment with 250 mg of the activated-Sepharose resin, the OTA adsorption reached 85.7% when using BSA and 32.3% in the negative control. However, after washing the column, the OTA adsorbed decreased to 28.3%, suggesting, once again, a weak OTA-BSA interaction at pH 5.0 and corroborating the previous results of the cellulose resin. Also, the presence of 1.3 mg of BSA in the activated-Sepharose

resin after multiple washing steps indicates that the interaction between BSA and Sepharose was stable. As in previous results for the nickel resin, the presence of BSA in the resin delivered better results for OTA adsorption, but the removal of large percentage of OTA suggests that the OTA-BSA interaction is not very stable.

Table 9. Results of OTA adsorption by BSA in experiments performed in gravity-flow columns with HisPur™ Ni-NTA resin and Cyanogen Bromide-activated-Sepharose resin and different buffers used.

	Resin	Buffer	Components present	Protein added (mg)	Protein retained [a] (mg)	% OTA adsorbed [b]	% Final OTA [c]
Experiments in gravity-flow columns	Nickel 500 µL	TRIS pH 8.0	BSA + OTA	-	-	97.8	84.2
			BSA + OTA	11.3	4.0	81.9	55.1
			NC	-	-	85.6	1.5
		Acetate pH 5.0	BSA + OTA	8.2	0	74.4	22.5
			NC	-	-	63.8	23.2
	Activated Sepharose 250 mg	Acetate pH 5.0	BSA + OTA	2.9	1.3	85.7	28.3
			NC	-	-	32.3	4.7

OTA concentration used was 90 ng/mL; NC: Negative control without BSA; [a] Protein retained is the quantity of protein adsorbed after washing the column; [b] % OTA adsorbed is the percentage of OTA retained after the passage through the column; [c] % Final OTA is the OTA retained in the column in the end of the experiment; -, protein quantification was not performed.

In summary, both nickel and Sepharose resins were able to stably retain BSA, which did not happen with the cellulose Avicel. The results obtained also indicate that the presence of BSA increases significantly the retention of OTA and proves BSA-OTA interaction. Thus, the adsorption of OTA is possible using the protein BSA as adsorbent.

5. CONCLUSIONS

Mycotoxin adsorbents were evaluated and characterized *in vitro* for the adsorption of AFB1, OTA and ZEA. Adsorbents are used in decontaminations methods, being responsible for binding to mycotoxins and reduce their bioactivity in humans and animals.

In a preliminary evaluation, 19 adsorbents were tested, including adsorbent agents with mineral origin, organic origin, polymers and two commercialized products. From these it was perceptible that adsorbents from organic and mineral origin have better efficiency removing mycotoxins, even when present in different pH solutions. The adsorption of ochratoxin A proved to be less effective in higher pH (pH 7.0 and 8.0) due, probably, to its conformational change in alkaline solutions to OP-OTA. ComProd1, Bent, ActCarb, OliPom3, GrapStem, S.bayanus, PVPP and P(MVE-MA) were the adsorbent that obtained better efficiencies in the preliminary evaluation. The earlier conclusion was supported in other experiment using different concentrations of adsorbents, in which lowers amounts of adsorbents from organic and mineral origin (< 5 mg/mL for ActCarb and < 10 mg/mL for ComProd1, Bent, OliPom3, GrapStem) were sufficient to adsorbed more than 80% of the mycotoxins. In addition to demonstrate high efficiency, the adsorption was also characterized by a strong interaction, since the recuperation of the adsorbed mycotoxins, using buffer at pH 7.0, was not very effective (< 50%), suggesting the ability to withstand conditions found in the intestines. In other hand, adsorbed mycotoxins were extracted almost entirely, implying that the procedure did not modify the mycotoxin characteristic or its toxicity. The adsorption isotherms calculated for these adsorbents had better correlations for the Hill model, that was develop for adsorption on heterogeneous solids. Generally, isotherms models delivered good fits (R2 > 0.96) and the adsorbents tested showed favorable adsorption of the three tested mycotoxins (AFB1, OTA and ZEA). ActCarb obtained values of mycotoxin uptake capacity from 16.47 ± 0.73 µg/mg (AFB1 at pH 7) to 24.44 ± 3.19 µg/mg (ZEA at pH 2), proving to be substantially superior (3 to 4 times higher) in comparison with other adsorbents tested. As in earlier experiments, mineral adsorbents (ComProd1 and Bent) obtained Qmax values between 0.81 ± 0.04 µg/mg (OTA at pH 7) and 4.82 ± 0.30 µg/mg (AFB1 at pH 7). Organic adsorbents (OliPom3 and GrapStem) obtained similar result to mineral adsorbents, with Qmax ranging from 0.45 ± 0.02 µg/mg (OTA at pH 7) to 3.78 ± 0.71 µg/mg (OTA at pH 7). Isotherms fitting for polymer based adsorbents (PVPP and P(MVE-MA)) delivered weak correlations and high values of associated errors. ActCarb efficiency was consistently superior in all experiments performed, as it required smaller quantities to adsorb the totality of the mycotoxins and formed very strong interactions never

releasing adsorbed mycotoxins. Also the results obtained for mineral adsorbents are accordingly to the literature, describing these as good adsorbents with high adsorption efficiency. Organic adsorbents demonstrated to be paired with mineral adsorbent with the advantage of being cheaper to obtain and able to be decomposed naturally. Adsorbents based on polymers did not produce consistent results during the experiments, obtaining high levels of discrepancy between experiments.

In addition to the characterization of these mycotoxin-binders, BSA as mycotoxin sorbent was tested. This protein was intended to be immobilized in resin to adsorb OTA, but the formation of a stable matrix was only achieved in nickel and sepharose resins. OTA retention was substantially increased and sustained when BSA was adsorbed in the resin even in small amounts. This BSA effect on OTA suggests a BSA-OTA interaction.

In conclusion, some of the adsorbents tested proved to be very capable at binding to mycotoxins AFB1, ZEA and OTA and thus have potential to reduce its bioactivity in the gastro intestinal tract. Mycotoxin adsorption was more effective at acid mediums (pH 2.0) and remained stable at neutral medium (pH 7.0). Although these experiments were performed *in vitro*, they are indicatives of the capacity to adsorb mycotoxins in the gut and expel them, making them prone to be used as food and feed additives. The downside of using this mycotoxin-binders is that they are not specific to mycotoxins, being able to bind to other compounds such as nutrients. The results of BSA experiments are indicatives of its capacity to function as OTA adsorbent. The challenge posed was the formation of a stable matrix with a high concentration of BSA.

To further understand the adsorbents tested, *in vitro* experiments using different compounds, or feed and food stocks contaminated with mycotoxins could be performed, in order to understand adsorption in a competitive medium. Ultimately, *in vivo* experiments would be able to demonstrate with high detail their effect on the field.

As for BSA, the experiments performed could be replicated using higher concentrations of resins, in order to immobilize a higher quantity of protein. From there, the system formed could be applied as a filter to reduce mycotoxins concentration in liquid solutions such as wine.

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ATTACHMENT I – TABLES AND GRAPHS OF ADSORPTION ISOTHERMS

Table 10. Ceq and Qeq values for AFB1 adsorption isotherms at pH 2.

ComProd1				Bent				ActCarb				OliPom3			
Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)		
0,00045	0,01820	0,01810	0,01779	0,00054	0,01753	0,01917	0,01981	0,00073	0,07298	0,08967	0,07673	0,01933	0,00260	0,00265	0,00275
0,00177	0,22916	0,25176	0,23257	0,00131	0,23064	0,24007	0,24240	0,00225	0,98675	1,25333	1,01997	0,25377	0,03512	0,03573	0,03219
0,00571	0,88974	0,85900	0,87910	0,00201	0,89477	0,86051	0,89099	0,00991	5,19026	4,54697	4,73858	0,98628	0,12125	0,11091	0,13186
0,01597	2,93962	2,72794	2,98620	0,00463	2,89569	2,72298	2,99159	0,01907	14,57379	12,14912	15,47765	3,64731	0,36978	0,37368	0,33313
0,06333	4,09739	3,91963	3,76299	0,03000	3,78961	3,86305	3,79262	0,17833	22,81783	16,06011	19,24837	4,94333	0,50212	0,47784	0,48836
GrapStem				S.bayanus				PVPP				P(MVE-MA)			
Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)		
0,00000	0,00462	0,00465	0,00461	0,02347	0,00116	0,00122	0,00119	0,03587	0,00068	0,00044	0,00054	0,00783	0,00204	0,00192	0,00195
0,05880	0,05286	0,05076	0,05275	0,31253	0,01542	0,01543	0,01407	0,52925	0,00178	0,00460	0,00456	0,09661	0,02663	0,02462	0,02566
0,21445	0,19339	0,19969	0,19837	1,26507	0,04845	0,04474	0,04991	2,10202	0,00599	0,00565	0,00829	0,34536	0,09409	0,09280	0,09041
0,72981	0,64239	0,62747	0,62390	3,52179	0,19229	0,17856	0,18378	6,60995	0,03837	0,04570	0,01577	0,96603	0,31539	0,31622	0,31139
1,18000	0,85060	0,83907	0,84189	5,21333	0,20279	0,26267	0,22769	8,74333	0,05883	0,07004	0,05249	1,51000	0,43895	0,42233	0,39794

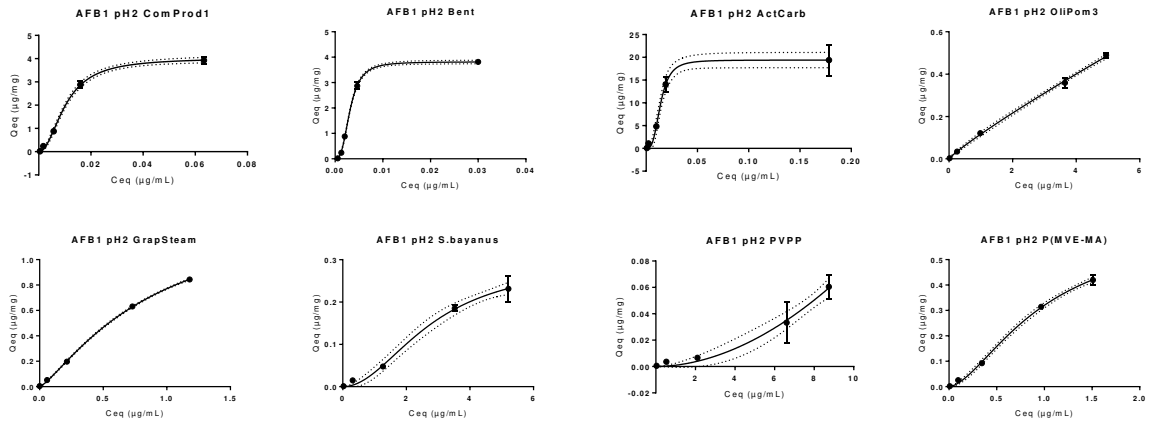

Figure 6. Graphs from AFB1 adsorption isotherms at pH 2.

Table 11. Ceq and Qeq values for AFB1 adsorption isotherms at pH 7.

ComProd1				Bent				ActCarb				OliPom3			
Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)		
0,00000	0,02092	0,02287	0,02131	0,00120	0,02102	0,02106	0,02155	0,00031	0,12366	0,10208	0,08629	0,02027	0,00349	0,00336	0,00373
0,00395	0,20977	0,21032	0,21241	0,00621	0,21651	0,21809	0,22351	0,00200	0,95606	1,00717	0,95543	0,20716	0,03140	0,03382	0,03211
0,01924	0,94980	0,93017	0,94314	0,01903	0,94188	0,94217	0,95004	0,00714	5,23765	5,47079	4,47595	0,95188	0,13243	0,15337	0,16453
0,03093	2,79101	2,74643	2,76964	0,03761	2,84353	3,02368	2,74428	0,01099	11,23610	14,97890	13,32091	2,85621	0,45860	0,34659	0,47059
0,07367	3,62333	3,56969	3,83456	0,05633	3,87682	3,74868	3,89287	0,15730	14,60401	16,07218	18,71133	3,88469	0,52243	0,56801	0,54337
GrapStem				S.bayanus				PVPP				P(MVE-MA)			
Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)		
0,00000	0,00557	0,00541	0,00536	0,03857	0,00103	0,00069	0,00085	0,04674	0,00064	0,00055	0,00024	0,03758	0,00110	0,00100	0,00069
0,04191	0,04823	0,05046	0,04960	0,41769	0,00456	0,00887	0,00582	0,47440	0,00390	0,00316	0,00378	0,35189	0,01036	0,00895	0,00914
0,18473	0,22671	0,21380	0,22147	1,61701	0,04597	0,04748	0,03217	2,10838	0,01748	0,02220	0,01372	1,49761	0,04729	0,04606	0,05037
0,50049	0,62843	0,64879	0,67110	3,90244	0,15832	0,17224	0,17100	5,97673	0,06578	0,04251	0,07605	4,13472	0,12952	0,16709	0,16357
0,83633	0,84528	0,89035	0,87269	5,85017	0,20487	0,17331	0,17215	7,66307	0,10166	0,08305	0,08317	5,59867	0,17388	0,19959	0,19451

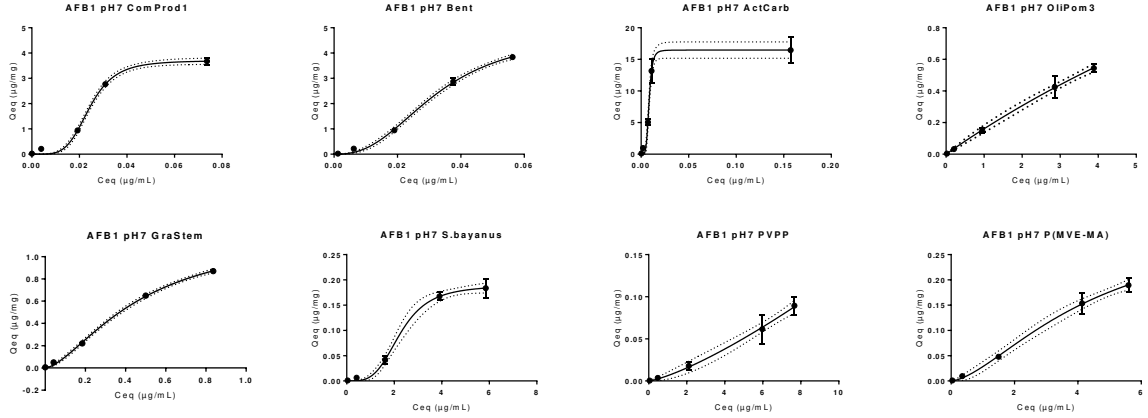

Figure 7. Graphs from AFB1 adsorption isotherms at pH 7.

Table 12. Ceq and Qeq values for OTA adsorption isotherms at pH 2.

ComProd1				Bent				ActCarb				OliPom3			
Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)		
0,01365	0,02137	0,02141	0,02041	0,00000	0,02557	0,02821	0,02880	0,00000	0,10709	0,13181	0,11236	0,00536	0,00644	0,00659	0,00496
0,07939	0,22886	0,24246	0,22876	0,04134	0,24303	0,24957	0,25193	0,00000	1,09948	1,39725	1,13675	0,11817	0,05546	0,05630	0,05079
0,29553	0,83742	0,77769	0,80259	0,08665	0,90589	0,86781	0,90361	0,00000	5,46440	4,79529	4,99935	0,41826	0,18616	0,17815	0,19690
0,97846	2,06277	1,84402	2,05408	0,13700	2,32463	2,18520	2,40211	0,03123	11,94193	9,92664	12,69345	1,29774	0,46682	0,46804	0,44895
2,64115	3,29114	2,94991	2,92964	0,41607	3,78647	3,88295	3,76508	0,21664	23,56973	16,56373	20,00071	2,32342	0,80474	0,76971	0,76677
GrapStem				S.bayanus				PVPP				P(MVE-MA)			
Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)		
0,00000	0,00668	0,00672	0,00665	0,00000	0,00337	0,00348	0,00336	0,00000	0,00323	0,00333	0,00326	0,01676	X	X	0,00258
0,07092	0,05760	0,05712	0,05729	0,12703	0,02845	0,02872	0,02661	0,31257	0,01890	0,01598	0,01730	0,07871	0,03135	0,02872	0,02966
0,26249	0,20055	0,20663	0,20256	0,45089	0,09453	0,09024	0,09424	1,25415	0,05247	0,05478	0,05239	0,25695	0,10436	0,10215	0,09996
0,61336	0,52969	0,51597	0,50505	0,80261	0,25897	0,24580	0,25699	3,12346	0,14205	0,15506	0,11783	0,37145	0,29725	0,27255	0,26715
1,13778	0,89815	0,87411	0,87973	1,76362	0,40509	0,42142	0,42231	5,00057	0,26257	0,26745	0,25198	1,07609	0,48467	0,46058	0,43460

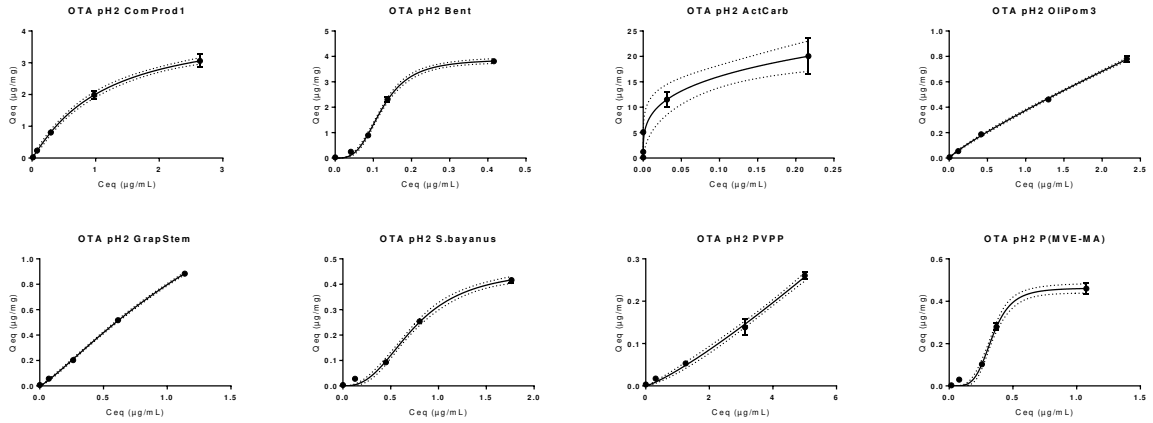

Figure 8. Graphs from OTA adsorption isotherms at pH 2.

Table 13. Ceq and Qeq values for OTA adsorption isotherms at pH 7.

ComProd1				Bent				ActCarb				OliPom3			
Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)		
0,03491	0,01083	0,01213	0,01115	0,04947	0,00667	0,00362	0,00702	0,00000	0,14307	0,11706	0,09905	0,04337	0,00124	0,00173	0,00323
0,41018	0,06406	0,05972	0,06188	0,54154	0,01178	0,01142	0,01076	0,00000	0,99902	1,05452	0,99902	0,45109	0,00453	0,01630	0,01318
1,65265	0,34844	0,36078	0,34193	2,05258	0,20305	0,21959	0,16983	0,00000	5,45729	5,69984	4,66351	1,78889	0,07789	0,06350	0,08896
4,14916	0,65618	0,63268	0,63888	2,99283	1,20168	1,35752	0,84898	0,03871	9,02307	12,00724	10,71391	3,06882	0,29388	0,27006	0,24409
8,13201	0,73155	0,81152	0,68897	6,43452	1,40258	1,43487	1,56743	0,21339	15,53290	16,83938	19,57693	5,71352	0,38822	0,40567	0,46740
GrapStem				S.bayanus				PVPP				P(MVE-MA)			
Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)		
0,04195	0,00268	0,00171	0,00215	0,05546	0,00048	0,00039	X	0,05439	0,00037	0,00054	0,00058	0,03851	0,00141	0,00138	0,00108
0,45904	0,00975	0,02023	0,00252	0,45948	0,00677	0,00709	0,00262	0,37167	0,01074	0,00937	0,00967	0,36265	0,00992	0,01022	0,01008
1,70437	0,09326	0,07913	0,07723	1,91013	0,03288	0,03287	0,03064	1,71687	0,04231	0,04035	0,04322	1,66690	0,04264	0,04379	0,04648
2,78213	0,26259	0,33596	0,28264	3,80635	0,09364	0,10232	0,10914	4,51905	0,06678	0,05205	0,07592	2,71648	0,12962	0,18893	0,14547
5,64185	0,50184	0,42017	0,40441	7,74324	0,13542	0,09489	0,11734	8,27413	0,08562	0,07493	0,09785	6,28792	0,18850	0,20124	0,15755

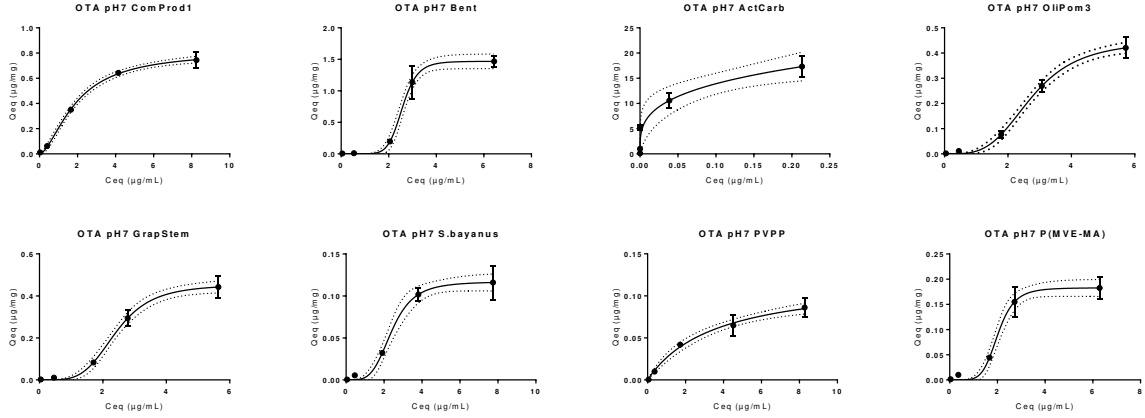

Figure 9. Graphs from OTA adsorption isotherms at pH 7.

Table 14. Ceq and Qeq values for ZEA adsorption isotherms at pH 2.

ComProd1				Bent				ActCarb				OliPom3			
Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)		
0,04475	0,00920	0,00661	0,01199	0,00000	0,02574	0,02839	0,02898	0,00000	0,10778	0,13265	0,11308	0,00000	0,00648	0,00663	0,00653
0,27277	0,13659	0,12642	0,12065	0,20885	0,15577	0,15239	0,15122	0,00000	0,97932	1,24456	1,01252	0,09429	0,04974	0,05229	0,04602
1,10003	0,46358	0,32803	0,36971	0,66933	0,56246	0,52983	0,58323	0,00000	4,84932	4,25552	4,43661	0,30348	0,17131	0,16488	0,18153
3,68258	1,02551	0,81224	1,12098	2,03916	1,66322	1,51431	1,69169	0,06618	12,21355	10,15718	12,96866	1,07953	0,50134	0,50675	0,49003
6,69990	1,65079	1,48689	1,54358	5,05729	2,06958	2,37354	1,98243	0,35272	23,97620	16,70741	20,33949	2,01577	0,85604	0,83177	0,82488
GrapStem				S.bayanus				PVPP				P(MVE-MA)			
Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)		
0,00000	0,00672	0,00676	0,00670	0,00000	0,00339	0,00350	0,00338	0,00000	0,00325	0,00335	0,00328	0,02974	X	X	0,00195
0,08566	X	0,04897	X	0,12965	0,02420	0,02484	0,02305	0,08217	0,02531	0,02562	0,02430	0,09707	0,02657	0,02411	0,02517
0,18837	0,18344	0,18719	0,18347	0,43497	0,08151	0,07806	0,08290	0,26610	0,08803	0,09021	0,08681	0,27755	0,09042	0,08824	0,08608
0,48112	0,55797	0,54398	0,53567	0,92956	0,26111	0,24765	0,25925	0,79543	0,25896	0,25894	0,25879	0,70912	0,27218	0,27218	0,26755
0,88951	0,94877	0,92708	0,92833	2,24169	0,39900	0,39873	0,42283	1,27329	0,44642	0,46254	0,45802	1,38644	0,48294	0,46153	0,43109

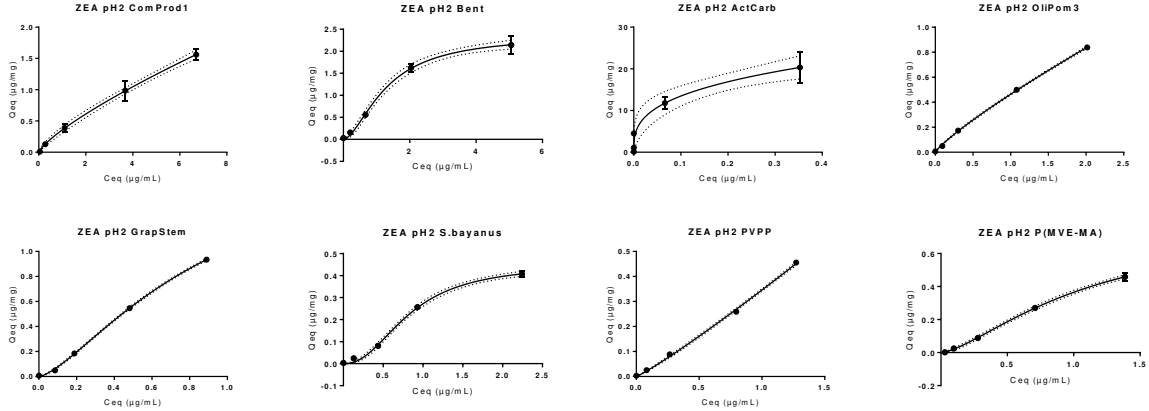

Figure 10. Graphs from ZEA adsorption isotherms at pH 2.

Table 15. Ceq and Qeq values for ZEA adsorption isotherms at pH 7.

ComProd1				Bent				ActCarb				OliPom3			
Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)		
0,00000	0,02237	0,02446	0,02279	0,04158	0,00792	0,00594	0,00766	0,00000	0,13372	0,10941	0,09258	0,00000	0,00578	0,00598	0,00594
0,32061	0,14519	0,05199	0,06145	0,40915	0,05588	0,05292	0,05408	0,00000	0,95247	1,00538	0,95247	0,05123	0,04674	0,04768	0,04661
1,39292	0,24485	0,26244	0,42902	1,18959	0,25276	0,46879	0,45402	0,00000	4,69231	4,90086	4,00979	0,20335	0,19480	0,19494	0,20395
4,27934	0,75166	0,71750	0,73011	1,55617	1,80417	1,99376	1,75986	0,07840	9,45509	12,70998	11,38187	0,57799	0,57119	0,53186	0,54335
7,59489	1,02769	0,96670	0,85536	4,22139	2,40806	2,27417	2,41456	0,50054	15,59765	14,96236	19,90355	1,65510	0,79397	0,83112	0,81371
GrapStem				S.bayanus				PVPP				P(MVE-MA)			
Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)			Ceq (µg/mL)	Qeq (µg/mg)		
0,00000	0,00596	0,00579	0,00574	0,00000	0,00292	0,00294	0,00290	0,00000	0,00301	0,00303	0,00294	0,05226	0,00039	0,00037	0,00042
0,09092	0,04444	0,04410	0,04426	0,07757	0,02330	0,02436	0,02173	0,05215	0,02474	0,02509	0,02413	0,21743	0,01623	0,01568	0,01566
0,14029	0,20691	0,19146	0,20021	0,19936	0,09932	0,09934	0,09659	0,16373	0,09854	0,10074	0,10374	1,05931	0,04290	0,06316	0,06381
0,22309	0,55987	0,57822	0,59285	0,49486	0,27616	0,29610	0,29144	0,56297	0,28379	0,27932	0,28215	2,42545	0,15558	0,21430	0,19286
0,51612	0,95757	0,96522	0,94451	1,46789	0,44098	0,41746	0,43371	1,14327	0,44913	0,41831	0,43019	5,28643	0,23607	0,24123	0,21472

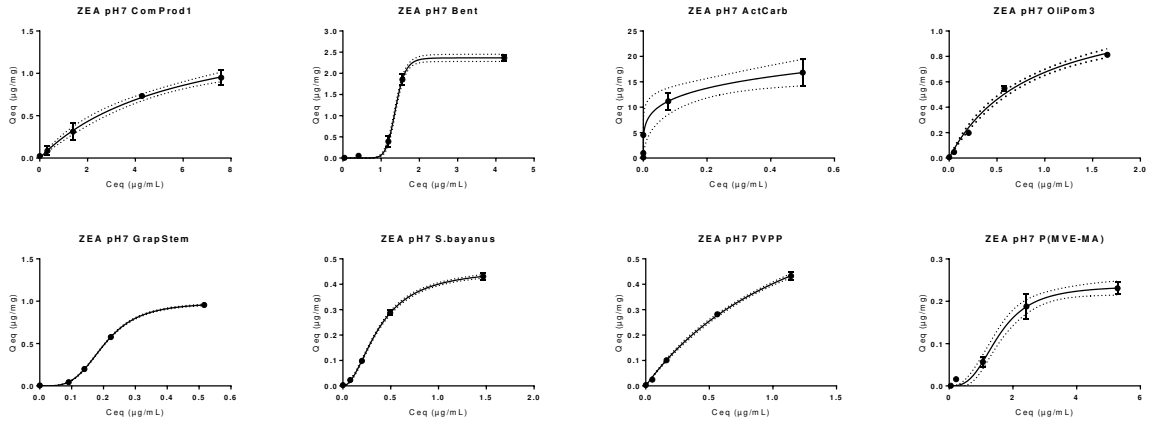

Figure 11. Graphs from ZEA adsorption isotherms at pH 7

ATTACHMENT II – CALIBRATIONS CURVES USED IN EXPERIMENTS WITH BSA PROTEIN

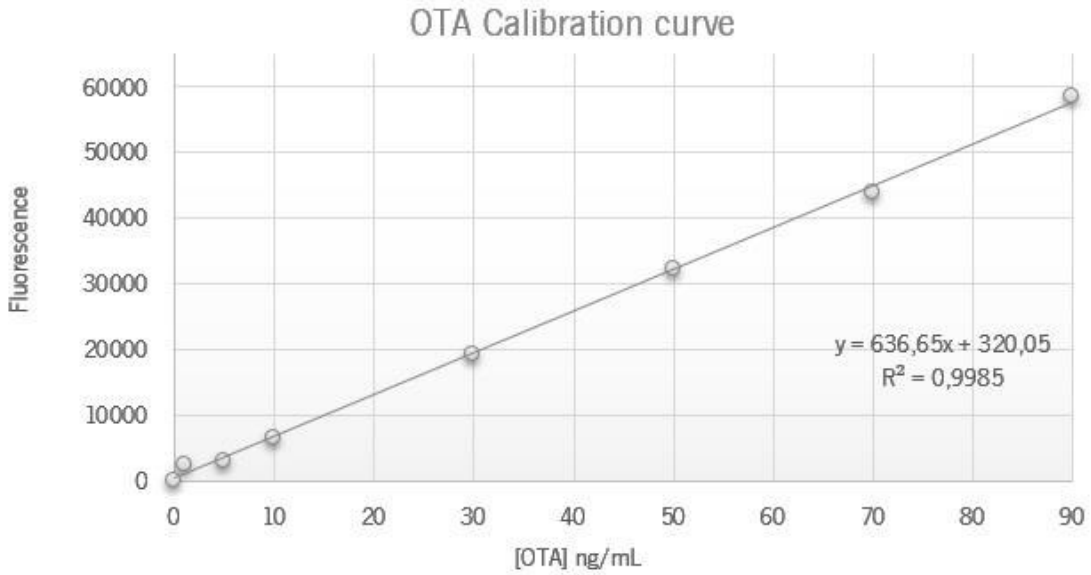

Figure 12. OTA calibration curve. OTA standards with concentrations between 1 and 90 ng/mL were analyzed in a microplate reader (Citation™ 3 from Biotek) in dark 96 wells plate, with excitation = 333 nm, emission = 460 and Gain = 140. The results were fitted to a linear regression (R^2 = 0.9985) to obtain the equation (Y = 636.65×X + 320.05).

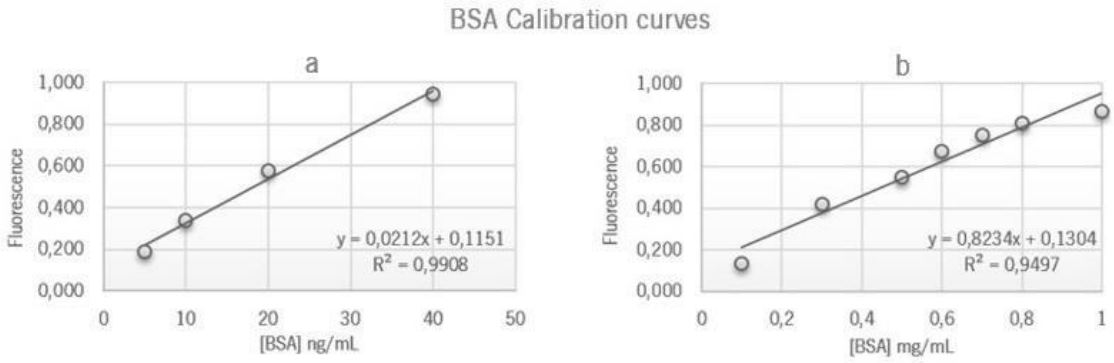

Figure 13. BSA calibration curves. BSA standards with concentration between 5 to 40 ng/mL (a) and 0.1 to 1.0 mg/mL were analyzed in microplate reader (Citation™ 3 from Biotek) in 96 wells plate in a wavelength of 595 nm. The results were fitted to a linear regression to obtain the equations ((a) Y = 0.0212×X + 0.1151; (b) Y = 0.8234×X + 0.1304).