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DEVELOPMENT OF AN IN VITRO METHOD TO DETERMINE THE BINDING CAPACITY OF BENTONITE AND DIATOMITE MYCOTOXIN BINDERS WITH DEOXYNIVALENOL

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DEVELOPMENT OF AN IN VITRO METHOD TO DETERMINE THE BINDING CAPACITY OF BENTONITE AND DIATOMITE MYCOTOXIN BINDERS WITH DEOXYNIVALENOL

The aim of this thesis was to examine mycotoxin binders´ adsorption capacity of deoxynivalenol and aflatoxin B1 mycotoxins in a single-concentration experiment. This study is part of the Bioproton Europe Oy project, which aims to explore the efficacy of some mineral binders.

The experiment was conducted with two different mycotoxin binders suitable for mycotoxins adsorption in gastrointestinal simulated conditions. Unbound deoxynivalenol was analysed with high-performance liquid chromatography using a variable wavelength detector (VWD). The choice of these methods for analyzing unbound deoxynivalenol contents was based on previous literature.

In this experiment unbound deoxynivalenol content was analyzed. The aflatoxin samples were not analyzed due to insufficient equipment; they were sent to an external laboratory for further analysis and thus, are missing in this thesis. Only the results of deoxynivalenol are discussed and they show that bentonite-based and the two diatomite-based mycotoxin binders did not adsorb the deoxynivalenol. In this study the limit of detection (LOD) and the limit of quantification (LOQ) were 2.22 µg/mL and 7.39 µg/mL, respectively.

KEYWORDS:

HPLC-UV, deoxynivalenol, aflatoxin B1, mycotoxin binder

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ABBREVIATIONS AND TERMS

Abbreviations

AFB1	Aflatoxin B1
BEN	Bentonite
DIA	Diatomaceous earth, Diatomite
DON	Deoxynivalenol
LOD	Limit of detection
LOQ	Limit of quantification
PBS	Phosphate-buffered saline
RP-HPLC	Reverse-phase high-performance chromatography

1 INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by a number of fungi. The latter grow in human and animal foods. A large number of animal feeds can be contaminated with mycotoxins which might cause disease and even death in animals (Robinson & Batt, 2014). Regarding this, mycotoxin binders which act as “chemical sponges” are used to bind mycotoxins in the gastrointestinal tract to reduce the negative health risks in animals (Bočarov-Stančić et al., 2018). A significant number of experiments have been conducted to find approaches to prevent the harmful effects of mycotoxins in animals by detoxification and inactivation of these fungal metabolites using mycotoxin binders (Bočarov-Stančić et al., 2018). In fact, studies on mycotoxins and mycotoxins binders could contribute to preventing various health problems in both animals and humans.

The detection of mycotoxin contamination by organoleptic methods, such as the sense of smell, taste, and sight, is quite challenging. In fact, mycotoxins are characterized as being invisible and odourless. A number of studies have examined mycotoxins adsorption capacity with mycotoxin binders to observe the possible binding efficacy (Binder et al., 2007; Bočarov-Stančić et al., 2018; Avantaggiato et al., 2005). In this regard, different criteria should be considered in the evaluation of potential mycotoxin binders, including price, availability, and possible nutrient adsorption. In particular, Binder et al. (2007) specify two conditions to be considered: firstly, the stability of the sorbent-toxin bond to prevent desorption of the toxin, and secondly, the effectiveness of mycotoxin binders within a broad pH level to ensure their performance throughout the gastrointestinal tract.

Having said this, the aim of the present study was to learn more about mycotoxin, and in particular, to investigate if mycotoxin binders adsorb the mycotoxin in a single-concentration study. In other words, the goal was to observe bentonite- and diatomite-based mycotoxin binders´ adsorption capacity with the mycotoxins deoxynivalenol and aflatoxin B1. This study was conducted as a single-concentration study in simulated gastrointestinal conditions with phosphate-buffered saline (PBS) at two different pH values, 6.5 and 3.0. The objective of this thesis was twofold. Firstly, to develop reliable methodology to quantify and compare the binding performance of bentonite- and diatomite-based mycotoxin binders for deoxynivalenol. Secondly, to prepare aflatoxin B1 samples for further analysis in an external laboratory.

2 MYCOTOXINS

Mycotoxins are toxic secondary metabolites produced by several fungi such as *Aspergillus*, *Fusarium*, *Penicillium*, *Claviceps*, and *Alternaria*. Aflatoxin B1 (AFB1), ochratoxin A zearalenone, deoxynivalenol (DON, vomitoxin), T-2 and HT-2 toxins, and fumonisins are among the most investigated mycotoxins in the literature (Kolossova & Stroka, 2012). It is considered that an important number of these fungal secondary metabolites are harmful to humans and animals. In fact, animals can be exposed to mycotoxins through the consumption of contaminated feed. Later, when animals enter the food chain, they constitute a source of mycotoxin exposure to humans too. In addition, a work environment that includes the growth, handling, and storage of agricultural commodities is considered a high-risk place for mycotoxin exposure and contamination. In this regard, it is estimated that 25% of crops are affected by mycotoxins (Kolossova & Stroka, 2012).

The presence of toxins produced by fungi in food and feed poses a health risk to both human and animal. The health risks can be manifested in different biological effects which may be carcinogenic, mutagenic, teratogenic, oestrogenic, neurotoxic, immunotoxic, etc. (Kolossova & Stroka, 2012). In addition, mycotoxins can have harmful effect on the weight gain, immunity, and reproductivity of animals (Kolossova & Stroka, 2012).

2.1 Deoxynivalenol

Deoxynivalenol (DON), also referred to as the trichothecene mycotoxin vomitoxin, is a type B trichothecene that is a naturally occurring mycotoxin generally produced by the fungus *Fusarium* genus (Holanda et al., 2021). The genus includes *F. graminearum* and *F. culmorum* which are the main species that produce DON globally and are considered important plant pathogens, causing *Fusarium* head blight in wheat and Gibberealla ear rot in maize (Holanda et al., 2021, FAO/WHO, 2001).

The trichothecenes is composed of over 200 structurally related secondary metabolites produced by the fungi *Fusarium*, *Stachybotrys*, and *Myrothecium* during growth in food and the environment (Grove 1988, 1993). Among these fungi, *Fusarium* species produce most of the mycotoxins such as DON, nivalenol and T-2 toxin (Sobrova et al., 2010).

Structurally, DON is a polar organic compound, whose chemical name is 12,13-epoxy-3α,7α,15- trihydroxytrichothec-9-en-8-on. In its molecule it contains 3 free hydroxy groups (-OH) as demonstrated in Figure 1, which are associated with its toxicity (Nagy et al., 2005). One of DON's important physicochemical properties is its ability to resist high temperatures. This property increases the risk of its presence in food (Hughes et al., 1999). A number of studies document that DON is heat stable and is very stable under temperatures within the range of 170°C to 350°C, with no reduction of DON concentration after 30 min at 170°C (Sobrova et al., 2010).

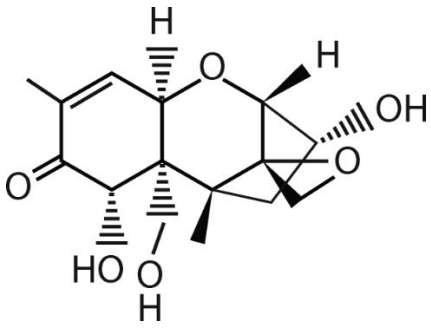

Figure 1. The chemical structure of deoxynivalenol (Sobrova et al., 2010)

DON is the most frequently occurring trichothecene that can be found in wheat, barley, corn, rye, oats, and rice. DON is produced by *F. graminearum*, *F. culmorum*, and *F. crookwellense*. Derivatives of DON also occur, such as nivalenol, 3-acetyldeoxynivalenol (3-ADON), and 15-acetyldeoxynivalenol (15-ADON). Nivalenol, although considered structurally similar to DON, is regarded as more toxic. These derivatives occur at different frequencies in different countries. For instance, the derivative 3-ADON is more commonly found in Europe, Asia, Australia, and New Zealand. The derivative 15-ADON, however, is more common in North America (Robinson & Batt, 2014).

DON is considered a highly common grain contaminator with more than 90% of its occurrence in food and feed. It has various toxic effects on animals causing gastroenteritis, feed refusal, and haemorrhage in the digestive tract. It might also destroy the bone marrow and the immune system. These problems can be manifested in animals as follows: gastrointestinal problems, vomiting, loss of appetite, bloody diarrhoea, reproductive problems, abortions, and death, mouth lesions and extensive haemorrhaging in the intestines. In humans it can lead to gastroenteritis with vomiting. It is suggested that DON and its derivatives might be the origin of a variety of gastrointestinal syndromes (Robinson & Batt, 2014).

In addition, it is suggested that DON can cause acute human illness. Between 1946 and 1963 in Japan and Korea, Fusarium-infested foods were frequently associated with outbreaks of human gastroenteritis that had nausea, diarrhea and vomiting as their primary symptoms (Yoshizawa 1983). Furthermore, during the years (1984–1991) Chinese people witnessed a gastroenteritis outbreak which was subsequently associated with scabby cereals containing DON and/or other trichothecenes, with the largest event affecting 130,000 people. In this latter outbreak, DON was found in 10 wheat samples ranging from 2 to 93 ppm (Pestka, 2010).

2.2 Aflatoxin B1

Aflatoxins are secondary metabolites produced by fungi among which *Aspergillus flavus*, *A. parasiticus*, *A. nomius* and *A. pseudotamarii* (Cary et al., 2005; Jamgampalli & Matcha, 2018). *A. nomius*, has only been found in the soils of Western United states (Batt, 2014, p.856). As seen in Figure 2, the following aflatoxins are: B1, B2, G1, G2, M1, and M2 (Batt, 2014, p.856).

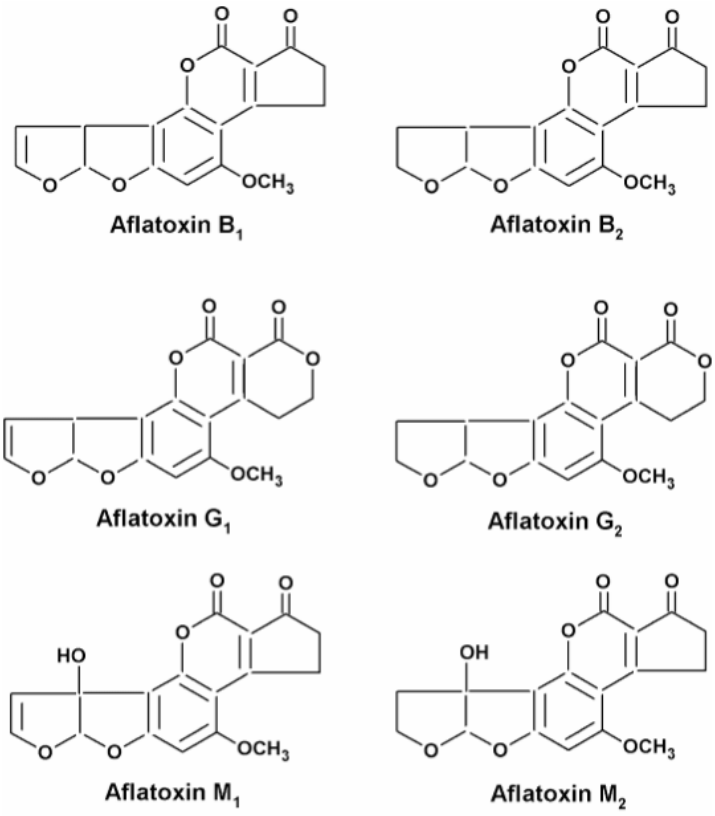

Figure 2. The chemical structures of aflatoxins (Batt, 2014, p.856)

Aflatoxins are difuranocoumarin derivatives formed from the polyketide pathway. The main four aflatoxins produced naturally are B1, B2, G1 and G2. Aflatoxin B1 and B2 have monohydroxylated derivatives M1 and M2 which are composed in the milk of lactating animals that have consumed B1 and B2 contaminated feed. The highest concentration of the aflatoxins in the contaminated feed and food is usually B1 (Abrar et al. 2013; Streit et al., 2012). Aflatoxins are aromatic planar, relatively hydrophilic, molecules that have very strong tendency to adsorb onto planar surfaces (Boudergue et al., 2009).

Aflatoxins may be found in cereals (such as corn), oil seeds (such as cottonseed, peanuts, and sun flower seeds), tree nuts (such as cashew, pistachios, and pecans), and dried fruits (such as dried figs) (Batt, 2014 p.856). In fact, fish and poultry feed are suspected to have a high percentage of aflatoxin contamination (Jamgampalli & Matcha, 2018). It has been documented that aflatoxin presents a major threat to agriculture and to human and animal health. *A. flavus* occurs mainly in soils and plant products, particularly oil-rich seeds, and in living plants (Geiser et al., 2000). Furthermore, tropical regions that are characterised by warm temperature and high humidity are considered the best weather conditions for aflatoxin production.

The way aflatoxins affect animals varies according to the dose, the exposure length, species, and the diet. If consumed in large doses, exposure could lead to cancer, particularly liver cancer. Most of the time, young animals are more susceptible to the acute toxic effects. Evidence from previous studies shows that susceptibility also differs from one breed to another. For instance, mature ruminants and chickens are more resistant compared to swine, young calves (Batt, 2014 p. 856). In fact, the main target organ of aflatoxin toxicity in animals is the liver (Streit et al., 2012).

It has been reported that aflatoxins are the most potent toxic, carcinogenic and mutagenic compound of all fungal toxins (Keyl and Booth 1971; Jamgampalli & Matcha, 2018). Out of all these aflatoxins, B1 is considered the most toxic and carcinogenic of the group (Batt, 2014 p. 856). In fact, The International Agency for Research on Cancer (IARC) considers aflatoxin B1 (AFB1) as a human carcinogen (Batt, 2014 p. 856). The evidence shows that aflatoxins (B1, B2, G1 and G2) are the group of mycotoxins that cause the greatest concern globally (Sweeney & Dobson, 1998).

2.3 Mycotoxin binders and modifiers

Mycotoxins can be the cause of feed contamination which causes serious economic losses in animal production (Wu, 2007). To prevent these losses, different methods have been developed such as the addition of mycotoxin detoxifiers to the feed which is the most used for the decontamination of feed (Kolossova & Stroka 2011; De Mil et al., 2015). The additives used for this purpose can be divided into two groups: binders and modifiers. Mycotoxin binders can usually be clay- (inorganic) or yeast-derived (organic) products (Kolossova & Stroka 2011). These binders are a substance added to animal feed in small quantities to prevent the absorption of the mycotoxins from the intestinal tract of the animal by adsorbing the toxins to their surface. Mycotoxin modifiers, however, are generally of microbiological origin comprised of whole cultures of bacteria, yeasts, or enzymes. The role of the mycotoxin modifiers is to alter the chemical structure of the mycotoxins and, consequently, reduce their toxicity (De Mil et al., 2015). Two mycotoxin adsorbents are explained in this chapter, which are bentonite, and diatomaceous earth.

2.3.1 Mineral adsorbents

There are several kinds of adsorbing agent which can be (Boudergue et al., 2009):

- inorganic (silica-based): aluminosilicates, bentonites, montmorillonites, zeolite, HSCAS (Hydrated sodium calcium aluminosilicate)
- organic such as: yeast cell walls, micronized fibres, activated carbons, enzymes, and bacteria
- polymers such as: cholestyramine and polyvinylpyrrolidone.

Today, in the market, the inorganic adsorbing agents compromise not only natural clay products but also synthetic polymers. Mycotoxin-adsorbing agents are high molecular weight compounds that could bind mycotoxins found in contaminated feed without dissociation in the digestive tract of the animal. In this manner, the toxin-adsorbing agent complex passes through the animal and is excreted through faeces. As a result, the animal will be less exposed to mycotoxins (Boudergue et al., 2009).

2.3.2 Bentonites

Bentonites (BEN) are hydrated alumina silicates. The impure clay is composed of minerals of the smectite group, mainly of montmorillonite (50-90%) (Bočarov-Stančić et al., 2018). BENs are generated from volcanic ash alteration. In particular, they are created by the weathering of volcanic ash in situ (Ramos et al., 1996). Structurally, these mineral absorbents are composed of SiO_2 tetrahedrons and Al_2O_3 octahedrons. The SiO_2 and Al_20_3 are associated to construct three-layer plates with a negative charge, whereas the edges of the lamellae have positive charges. When water is involved, the lamellae are separated, and their volume is increased (Bočarov-Stančić et al., 2018).

The European Bentonite Association (EUBA) has classified bentonites into the following types: sodium bentonite, calcium bentonite, acid activated bentonite and organophilic bentonite. Bentonite clays are characterized by a high adsorption and absorption property. Among all the naturally occurring minerals, bentonites have the largest specific surface area characterized by a vast external and internal channel network (Mishra et al., 2015).

BENs are used for different purposes. In the feed industry, for instance, they are used to increase the hardness and toughness of pellets. In addition, BENs have the ability to adsorb not only some mycotoxins (Huwig et al., 2001) but also radionuclides, toxic metals and ammonia (Adamović et al., 2009).

2.3.3 Diatomaceous earth

Diatomites (DIA) occur in a natural environment mainly in the sea and in lakes. DIA is siliceous sedimentary rock that is considered a mineral of organic origin. DIA consists of fossilized skeletal remains of the diatom which is a unicellular photosynthetic plant related to algae (Closceri et al., 1989). It has large porosity and therefore a high adsorption capacity due to a high content of silicon dioxide. DIA is characterized by a small mass that ranges from 0.5 to 0.8 g/cm^3(Bočarov-Stančić et al., 2018).

DIA is not a pure hydrous silica but also contains inorganic matter, mainly aluminium, and to a lesser extent, alkaline earth metals, alkali metals and other minor constituents such as boron, copper, and manganese (Closceri et al., 1989; Al-Ghouti et al. 2003). Furthermore, DIA is also used as a remedy of diarrhoea in animals (Adamović et al.,

2011). DIA is usually used as an anti-caking agent during feed processing (Weaver et al., 2013).

3 DETECTION OF MYCOTOXINS

3.1 In vitro method

There are many methods used to detect mycotoxin adsorption such as single-concentration, multi-mycotoxin, static and dynamic gastrointestinal models (Boudergue et al., 2009). The single-concentration method is defined as an efficient tool for analyzing mycotoxin binders' efficacy. It is simple to perform and produce less toxic waste. This measurement takes place in aqueous medium, where a known amount of mycotoxin is reacted with a known amount of test product in solutions. The results of *in vitro* studies sometimes differ from the ones in *in vivo* studies. In other words, adsorption *in vivo* is complicated due to the physiological variables and the composition of the feed; factors which are rarely accounted for *in vitro.* The single-concentration studies are effective methods to identify and rank potential mycotoxin binders. Through these studies, it would be possible to determine the mechanisms and suitable conditions in adsorption tests (Diaz and Smith, 2005).

Regarding in vitro studies, it is possible to evaluate the efficiency of mycotoxin-detoxifying agents in binding mycotoxins through gastrointestinal model stimulation. As a result, it would be possible to identify the physiological conditions that are crucial for the binding. A number of *in vitro* methods in static and dynamic gastrointestinal models have been created to evaluate the binding performance of mycotoxin-detoxifying agents (Boudergue et al., 2009). The complex and single-concentration studies mentioned above are designed to simulate gastrointestinal conditions. These studies are regarded as crucial for a most favourable pre-screen strategy to select and rank the adsorbing materials (Boudergue et al., 2009).

DON and other trichothecenes are non-ionisable molecules with a bulky epoxy group and do not adsorb well to plane surfaces (Boudergue et al., 2009). In fact, they have the ability to adsorb onto very few mycotoxin binders such as commercial activated carbons. These mycotoxins range from hydrophilic (NIV and DON) to moderately hydrophobic (HT-2 andT-2) (Boudergue et al., 2009).

On the other hand, unlike DON and other trichothecenes, aflatoxins which are relatively hydrophilic aromatic planar molecules, have a very high tendency to adsorb onto planar surfaces. Moreover, their beta-dicarbonyl system allows for the formation of coordination

bonds with metallic cations (Al^{3+} or others) present in clays (Phillips et al.,1995). Thus, they have the ability to exhibit very high affinities for planar clays (Boudergue et al., 2009).

The important parameters for the single-concentration method were the duration of the incubation time, which was 1 hour, two different (3.0 and 6.5) pH values, and the temperature of the test solutions. The incubation varies between studies from one to several hours depending on the method. However, the same incubation time (1 hour) as was used in this thesis, commonly appears in many studies (Diaz D.E et al. 2002; Bočarov-Stančić et al., 2018; Avantaggiato, et al., 2004). The pH and the temperature of the test solutions vary from highly acidic to slightly alkaline (pH 2 to 8) and the temperature from ambient to high within the normal range of mammalian temperature.

3.2 Chromatography

Chromatography is the most widely used and accepted analysis technique in modern analytical chemistry (Vitha, 2016). It is an effective separation method for the identification and purification of the components of a mixture for qualitative and quantitative analysis (Coskun, 2016). Chromatographic analysis separates different mixtures of components based on the distribution or partition of a sample between two phases: mobile and stationary phase (ThermoFisher Scientific, 2019). It introduces a small volume of the sample mixture which travels through the stationary phase at different speeds causing them to be separated from each other. Unlike the sample, the mobile phase flows continuously through the column which pushes the molecules in the sample through the column to elute from the other end. The nature of the specific stationary and mobile phase determines which substances travel faster and which more slowly through the column (Vitha, 2016).

A wide variety of chromatographic methods have been created and they serve different purposes and are optimal for different mixtures. Some of the most common forms are gas, liquid, thin-layer, and ion-exchange chromatography (ThermoFisher Scientific, 2019). The chromatography method used to detect DON in this thesis appears in many studies (Sabater-Vilar et al., 2004, Kotal et al., 2002, Antonios et al. 2010)

3.2.1 High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is an analytical technique used to separate, identify, and quantify components in a mixture. It is one of the most used chromatographic techniques in most laboratories worldwide (Moldoveanu & Victor David 2012, 2).

There are several types of HPLC methods that have been mentioned in the literature which have differences and similarities. Various criteria are at play when comparing these types, for instance, the nature of the stationary and mobile phases and the range of concentration of solvents. These types include normal-phase HPLC, ion-pair chromatography (IPC), hydrophobic interaction chromatography (HIC), nonaqueous reverse-phase chromatography (NARP), hydrophilic interaction liquid chromatography (HILIC), and the reversed-phase HPLC (RP-HPLC) which is the most common technique used worldwide (Moldoveanu & Victor David 2012, 9). In this thesis, RP-HPLC was used and thus, it will be further discussed.

RP-HPLC is a type of chromatography that is carried out on a nonpolar stationary phase with a polar mobile phase. The RP-HPLC stationary phase is acquired by chemically bonding long hydrocarbon chains to a solid surface such as silica (Moldoveanu & Victor David, 2012, 9). C18 column contains 18 carbon atoms and is regarded as the most common chain bound to silica. Furthermore, C 18 has very high hydrophobic character. In the bonded phase, for instance, C18 has a higher hydrophobicity than C8 (Moldoveanu & Victor David, 2012, 9). This is the reason behind selecting C18 in this thesis.

The separation in RP-HPLC is based on the distribution of the analyte between the stationary phase (immobilized liquid) and the mobile phase, although some experiments can be explained by adsorption equilibrium. In RP-HPLC, the mobile phase is a combination of water and an organic solvent (methanol, acetonitrile, etc), with a range of content in the organic solvent. The interactions in RP-HPLC are regarded as the hydrophobic forces (Moldoveanu & Victor David, 2012, 10).

3.2.2 Detectors

In the past, several existing methods for detecting mycotoxins were chromatographic. The chromatographic assays have continued to evolve with improvements of instrumentation. In fact, not only has the instrumentation cost decreased but also the detector sensitivity and chromatographic techniques affordability have improved dramatically (Maragos et al., 2010).

Among the available detectors, HPLC coupled with ultraviolet (UV), a diode array detector (DAD) and a fluorescence detector (FLD) are the most frequently used techniques for identification of the main mycotoxins in feed and food. For example, Aflatoxin M1, ochratoxin A, zearalenone, patulin and DON are analysed by using FLD or UV detectors with good accuracy and precision (France, J. et al., 2008). The HPLC-UV technique was an early method for the determination of mycotoxins in grains and was based on an acidic mobile phase with phosphoric acid. HPLC-FLD is selective, repeatable, and highly sensitive. FLD detectors have a disadvantage concerning some of the mycotoxins that are not fluorescent, for example, fumonisins. However, specific labelling reagents have been developed for derivatization for nonfluorescent mycotoxins to produce fluorescent derivatives (France, J. et al., 2008; Rahmani, A. et al., 2009).

The High-pressure liquid chromatography with tandem mass spectrometry (HPLC/MS-MS) has very selective and sensitive detection. This technique contains all the advantages for the identification and quantification for mycotoxins. Methods have been developed for simultaneous determination of various mycotoxins with great diversity in molecular level. Today, the great potential with liquid chromatography with tandem mass spectrometry (LC-MS/MS) is its ability to screen several mycotoxins in samples. Studies have demonstrated that as many as 35 different mycotoxins can be detected within one run by LC-MS/MS in herbal medicine matrices. In fact, this multiscreen ability is not possible with the use of HPLC, UV, and FL detectors (Zhang et al., 2018; Rahmani, A. et al., 2009).

4 MATERIALS AND METHODS

4.1 Materials

To conduct this research, DON (1 mg serum bottle, ≥ 98.0 % purity) was purchased from Sigma (St. Louis, MO) and AFB1 (1 mg serum bottle, > 95.0 % purity) was purchased from Fisher Scientific. The mycotoxin binders, bentonite and two different diatomite samples, were provided by Bioproton Europe Oy. Stock solutions for DON were prepared at 1 mg/mL and 0.5 mg/mL in acetonitrile and AFB1 was prepared at 1 mg/mL in methanol, respectively. All the solvents used in chromatography were HPLC grade and all other reagents were of analytical grade. In addition, all the measurements were performed with analytical scale, volumetric pipettes, volumetric flasks, graduated cylinder, and mechanical pipettes.

In this study, the Agilent HP1100 series HPLC was equipped with autosampler injector, thermostated column compartment, variable wavelength detector (VWD), degasser, pump, and solvent tray. The reverse-phased column used in the analysis was an Agilent Eclipse XDB-C18, 4.6 x 150 mm, 5 µm.

The mobile phase was prepared by measuring 50 mL 100% acetonitrile into a 500 mL volumetric flask. In addition, ultrapure water (Milli-Q) was added to the mark of the volumetric flask and then the solution was transferred to a storage bottle.

4.1.1 Phosphate-buffer solution preparation

The 10x PBS was prepared as follows:

The 10x PBS was composed of 4.008 g of sodium chloride (NaCl), 0.12115 g of potassium dihydrogen phosphate (KH_2PO_4), 0.8921 g of disodium hydrogen phosphate dihydrate (Na_2HPO_4) and 0.10933 g of potassium chloride (KCL) dissolved in 50 ml of ultrapure water. A Sigma-Aldrich PBS calculator was used to calculate the components of the PBS solution. These calculations were performed with the Sigma-Aldrich website.

The 1x PBS was prepared as follows:

The 1x PBS at pH 3.0 and 6.5 was prepared by adding 5 mL of 10x PBS solution into a 50 mL volumetric glass and the pH was adjusted to 3.0 and 6.5 by adding 0.1 M Hydrochloric acid (HCL), and then the volume was increased to 50 mL.

4.1.2 Deoxynivalenol stock solution preparation

The 1000 µg/mL stock solution was prepared by pipetting 1 mL of 100 % acetonitrile with a volumetric pipette into a DON serum bottle (1 mg).

The 500 µg/mL stock solution was prepared by pipetting 2 mL of 100 % acetonitrile with the volumetric pipette into the DON serum bottle (1 mg).

The 250 µg/mL working solution was prepared from 1 mL of 500 µg/mL stock solution, mentioned above, by adding 1 mL of 100 % acetonitrile into the DON serum bottle.

4.1.3 Aflatoxin B1 stock solution preparation

The 1000 µg/mL stock solution was prepared by pipetting 1 mL of 100 % methanol with a volumetric pipette into an aflatoxin B1 plastic bottle (1 mg)

The 100 µg/mL working solution was prepared by pipetting 0.2 mL of 1000 µg/mL stock solution with a mechanical pipette into a 2 mL volumetric flask. In addition, methanol was added to the mark of the volumetric flask.

The 50 µg/mL working solution was prepared by pipetting 0.1 mL of 1000 µg/mL stock solution with a mechanical pipette into the 2 mL volumetric flask. In addition, methanol was added to the mark of the volumetric flask.

4.2 Standard preparation

An external standard calibration procedure was used for the quantification process. The external standard was prepared from stock solution of 1000 µg/mL by diluting to 100 µg/mL with ultrapure water (Milli-Q water). The standard solutions of 70 µg/mL, 50 µg/mL and 5 µg/mL were prepared by diluting from 100 µg/mL standard sample concentration with mobile phase. In addition, a 25 µg/mL was diluted with mobile phase from the 50 µg/mL standard sample due to low volume of 100 µg/mL for the calibration curve, as

demonstrated in Table 1. All standard samples were filtered with a 2 mL syringe and a Whatman PVDF 0.45 µm syringe filter to an HPLC glass vial.

Table 1. Standard preparation of the DON samples from the 100 and 50 µg/mL DON working solutions. Concentrations 70, 50 and 5 µg/mL were prepared from 100 µg/mL working solution and concentration 25 µg/mL from 50 µg/mL working solution

Sample	Concentration (µg/mL)	Amount of working solution (mL)	Total Volume (mL)
STD100	100	0.2	2
STD70	70	0.7	1
STD50	50	0.5	1
STD25	25	0.5	1
STD5	5	0.1	2

4.3 Sample preparation

The three tested mineral clay products in this study were one bentonite- and two diatomite-based mycotoxin adsorbents. Each adsorbent was labelled with a letter code (BEN bentonites, 1./2. DIA diatomite and toxin concentration). The amount of mycotoxin adsorbent in the single-concentration experiment was 10.0 mg of bentonite-based product and 100.0 mg of diatomite-based product. These values refer to the dosage used in the feed.

For the DON, the sample solutions were prepared from two different stock solutions, 500 and 1000 µg/mL, as mentioned in Section 4.1. First, 100 µg/mL solution was prepared by adding 0.5 mL of 1000 µg/mL stock solution into the 5 mL volumetric flask. In addition, PBS was added to the mark of the volumetric flask depending on the needed pH of the solution. Second, 50 µg/mL solution was prepared by adding 1 mL of 500 µg/mL stock solution to the 10 mL volumetric flask. In addition, PBS was added to the mark of the volumetric flask depending on the pH of the solution. Lastly, 25 µg/mL solution was prepared by adding 1 mL of 250 µg/mL into the 10 mL volumetric flask. In addition, PBS was added to the mark of the volumetric flask depending on the pH of the solution. Table 2 below presents the sample preparation of the DON samples.

Table 2. Preparation of the 25, 50 and 100 µg/mL DON sample solutions from stock solutions of 1000, 500 and 250 µg/mL. Buffer solution was added to the mark of the volumetric flask

Sample	Stock solution	Total volume (mL)
100 µg/mL	0.5 mL of the 1000 µg/mL stock solution	5
50 µg/mL	1 mL of the 500 µg/mL stock solution	10
25 µg/mL	1 mL of the 250 µg/mL stock solution	10

For the AFB1, the sample solutions were prepared from two different solutions 50 and 100 µg/mL, as mentioned in Section 4.1. First, 10 µg/mL solution was prepared by adding 1 mL of 100 µg/mL stock solution to the 10 mL volumetric flask. In addition, PBS was added to the mark of the volumetric flask depending on the pH of the solution. Second, 1 µg/mL solution was prepared by adding 0.1 mL of 100 µg/mL stock solution to the 10 mL volumetric flask. In addition, PBS was added to the mark of the volumetric flask depending on the pH of the solution. Lastly, 5 µg/mL solution was prepared by adding 1 mL of 50 µg/mL stock solution to the 10 mL volumetric flask. In addition, PBS was added to the mark of the volumetric flask depending on the pH of the solution. Table 3 below presents the sample preparation of the AFB1 samples with stock solutions and buffer solution.

Table 3. Preparation of the 1, 5 and 10 µg/mL AFB1 sample solutions from working solutions of 100 and 50 µg/mL. Buffer solution was added to the mark of the volumetric flask

Sample	Stock solution	Total volume (mL)
10 µg/mL	1 mL of the 100 µg/mL stock solution	10

5 µg/mL	1 mL of the 50 µg/mL stock solution	10
1 µg/mL	0.1 mL of the 100 µg/mL stock solution	10

4.3.1 Deoxynivalenol experiment

All DON samples were incubated in 1 mL of mycotoxin-buffer solution in three different concentration 25, 50 and 100 µg/mL at two different pHs 3 and 6.5, as mentioned in Section 4.3.

All the sample suspensions were shaken in a thermostatically controlled shaker at 37ºC for 1 hour at 175 rpm and then centrifuged at 6,000 *x g* for 10 min. The supernatants were collected and used in HPLC separation.

For each experiment, a control treatment without adsorbent (blank control) was included. The experiments were performed in duplicate except sample concentration 100 µg/mL due to low quantity of DON. All DON samples were filtered with 2 mL syringe and Whatman PVDF 0.45 µm syringe filter to HPLC glass vial.

4.3.2 Aflatoxin B1 experiment

All AFB1 samples were incubated in 1 mL of mycotoxin-buffer solution in three different concentration 1, 5 and 10 µg/mL at two different pHs 3 and 6.5, as mentioned in Section 4.3.

The suspensions were shaken in a thermostatically controlled shaker at 37ºC for 1 hour at 175 rpm and then centrifuged at 6,000 *x g* for 10 min. The supernatants were collected and send to external laboratory for further analysis.

For each experiment, a positive control treatment without adsorbent (blank control) and a negative control treatment with only adsorbent were included. The experiments were performed in duplicate. All AFB1 samples were filtered with 2 mL syringe and Whatman PVDF 0.45 µm syringe filter to HPLC glass vial.

4.4 High-performance liquid chromatography analysis

The unbound residual of DON was detected with high-performance liquid chromatography. Mobile phase which consists of acetonitrile-water (10:90, v/v) was the optimum mobile phase to determine DON. The often-used wavelength in the DON determination studies is within a range of 214-229 nm, and it is found that the maximum is around 218 nm (Kotal et al., 2002). The usage of HPLC with UV detector has good precision and accuracy to detect trichothecenes. In addition, HPLC-UV has the advantage of not requiring a derivatization step to detect DON. Also, the increased temperature of the analytical column moderately speeds the elution of the analyte from the column, which is highly desirable (Feltrin et al., 2018).

For this study, all the standard solutions were injected once except for the system test which included six injections. As seen in Table 4, the column temperature was increased from standard temperature to +25ºC to speed up the elution of the analyte from the column. DON was determined at a wavelength of 218 nm by using a VWD detector. The 8-minute runtime was determined with the standard test-run which showed no peaks after the ~5.5 min DON peak. The method was performed by isocratic elution (flow rate 1.0 mL/min) with acetonitrile-water (10:90, v/v) mobile phase. In the end the column was washed with a higher percentage of acetonitrile.

Table 4. HPLC method information for the determination of DON

Column	**Agilent Eclipse XDB-C18, 4.6 x 150 mm, 5 µm, reverse-phased**
Eluent	Acetonitrile–water (10:90, v/v)
Wavelength	218 nm
Injection volume	50 µL
Column temperature	+25ºC
Runtime	8 min
Deoxynivalenol retention time	~5.5 min
Pressure	~70 bar
Flow rate	1.0 mL/min

4.5 Performance of HPLC method

Linearity was determined by the injection of DON standard from 5 to 100 µg/mL. First, the correlation coefficient (R^2) was expected to be more than 0.99. Second, the accepted standard linearity for DON plotted using calibration solutions and the correlation coefficient was found to be 0.9989.

The limit of detection (LOD) is calculated through this formula: LOD = 3 × S with S referring to standard deviation. The samples used should either be blank samples (no detectable analyte) or test samples with concentration levels close to or below the expected LOD. The limit of quantification (LOQ) is calculated through this formula: LOQ = 10 × S with S referring to standard deviation. However, it is worth mentioning that in chromatography technique, which rely on detecting a peak above the noise, sample concentrations close to or above are required (Eurachem, 2014). In this study LOD and LOQ were calculated using 5 ppm sample concentration. The results of LOD and LOQ were 2.22 µg/mL and 7.39 µg/mL respectively.

5 RESULTS

The objective of this study was to determine DON's ability to be absorbed by one bentonite-based and the two diatomite-based mycotoxin binders. The chapter is divided into two sections. Section 5.1 discusses the DON standard curve and its linearity. Section 5.2 focuses on DON sample results that were carried out in three different concentrations 25, 50 and 100 µg/mL at two different pHs 3 and 6.5.

5.1 Standard linearity of standard curve

The standard curve was formed from the absorbances of the following standard solution concentrations: 5, 25, 50, 70 and 100 µg/mL. The standard curve, as observed in Figure 3, is linear with the correlation coefficient square at R^2=0.9989.

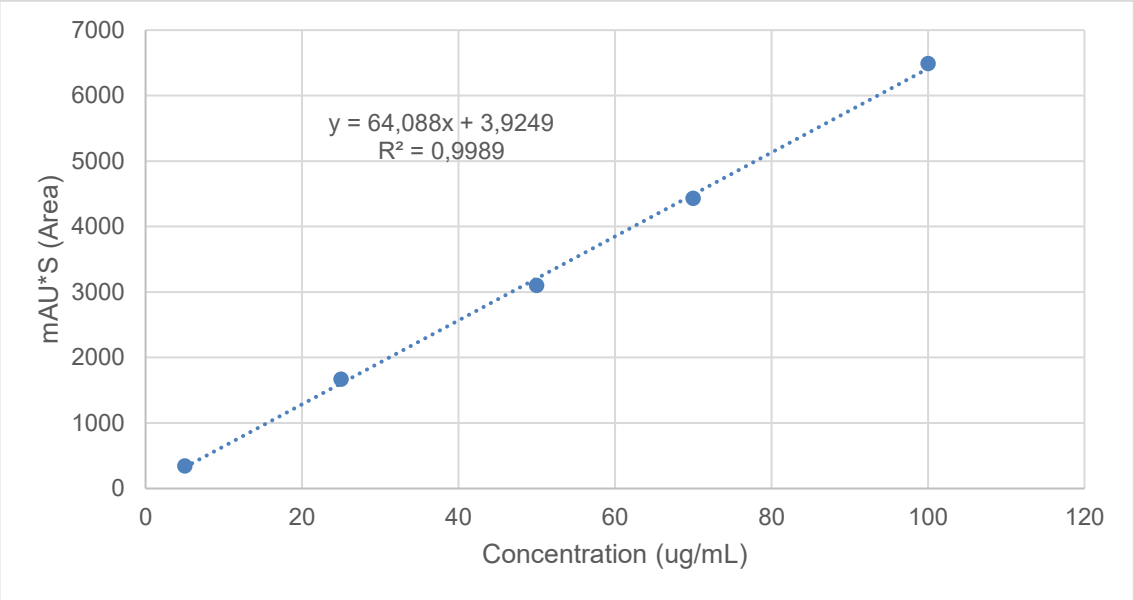

Figure 3. DON standard curve formed with concentrations of 5, 25, 50, 70 and 100 µg/mL

As demonstrated in Figure 4, the points in the DON residual plot are on both sides of the line. This indicates that the first two residuals are positive, the next two are negative and the last one is positive. The points in Figure 4 show a random dispersed pattern and therefore the linear regression model is appropriate.

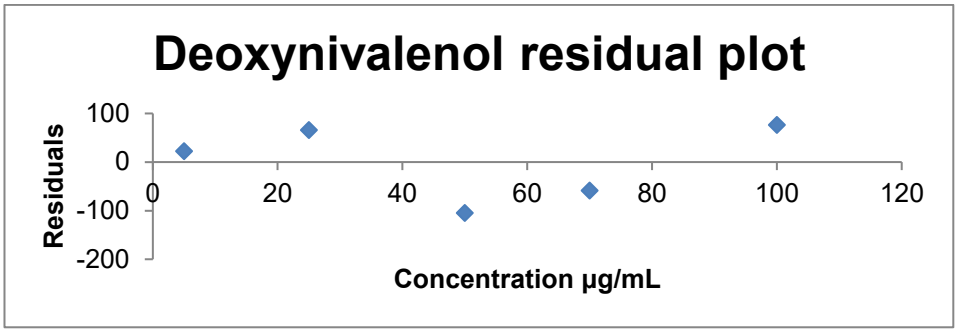

Figure 4. Variable residual plot of DON samples

Table 5 indicates that the standard curve goes through the origin because the upper 95% level is above zero and the lower 95% is under zero. This shows that the intercepts are not statistically different from zero therefore errors are low at lower concentrations.

Table 5. The DON standard intersection points of the y-axis

	Lower 95%	*Upper 95%*
Intercept	-230.7048204	238.554625
X Variable 1	60.18303809	67.99318894

5.2 Deoxynivalenol samples

In all of the DON chromograms, it has been observed that there were multiple peaks before the DON peak as seen in Figure 5. The unknown peaks are most likely due to impurities found in the samples. The variation between the duplicates of the chromatogram samples was not noticeable in this study.

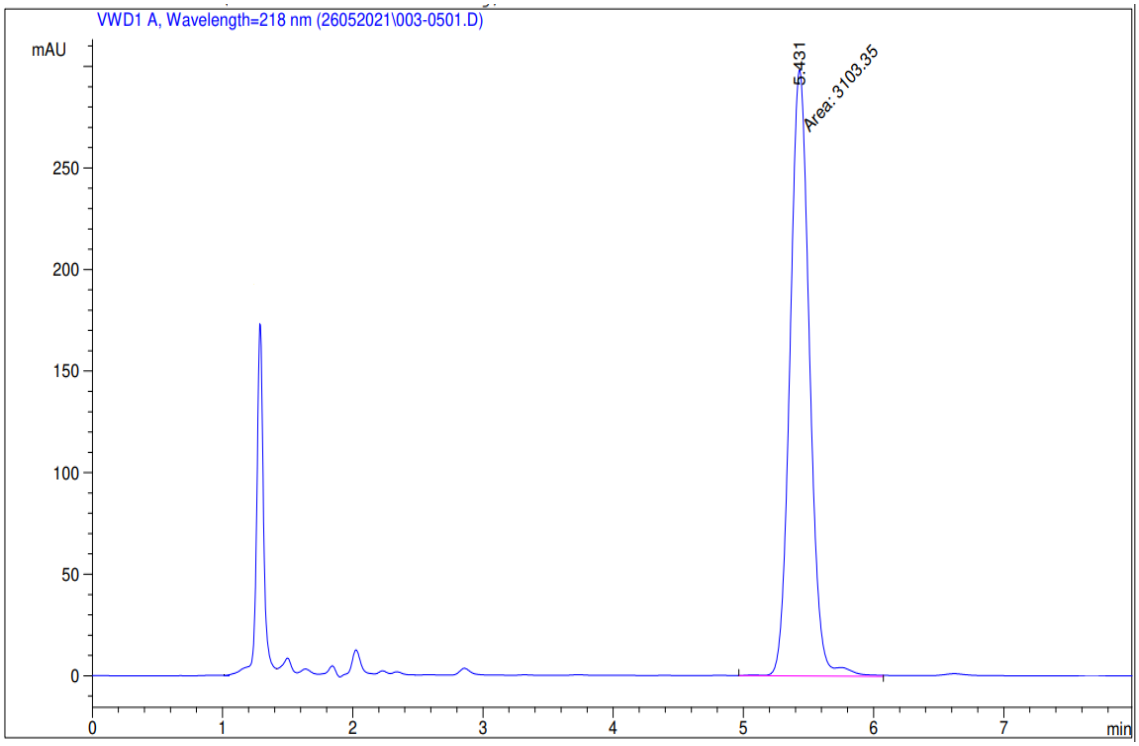

Figure 5. DON 50 µg/mL standard sample chromatogram (50 µL injection volume)

All the adsorption experiment test samples show that no adsorption between DON and mycotoxin binder has occurred. All adsorption experiment samples exhibit more than 100 % recovery, most likely due to measurement uncertainty, except for the 2. Diatomite at pH 3 sample, as seen in Table 9. Therefore, it is possible to conclude that the mycotoxin binders used in this study present no efficient binding towards DON mycotoxin.

All DON samples with different concentrations had higher or equal measured concentrations of DON left in the sample after interaction with mycotoxin binder compared to control samples without mycotoxin binder as seen in Figures 6-11.

As seen in Figures 6 and 7 below, the 25 µg/mL of DON concentration results at pH 6.5 and 3 in PBS solutions show that the first duplicates (in blue colour) were much closer to the control sample (in green colour) concentrations compared to the second duplicates (in red colour).

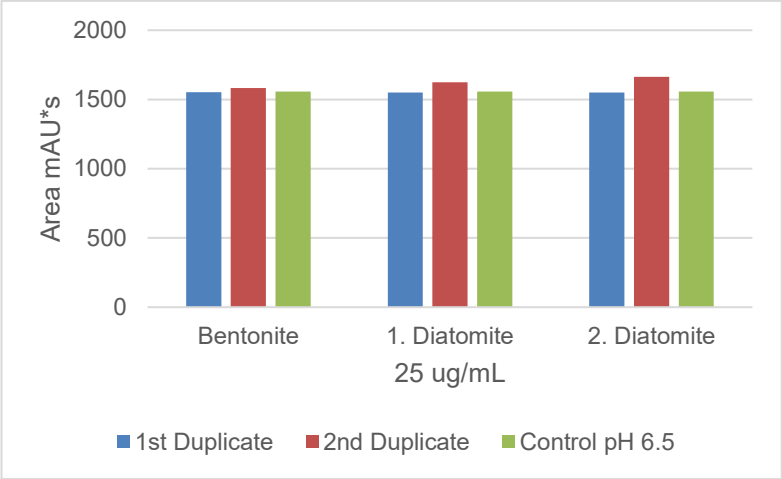

Figure 6. The 25 µg/mL of DON concentration results in duplicates with control (without mycotoxin binder) in PBS pH 6.5

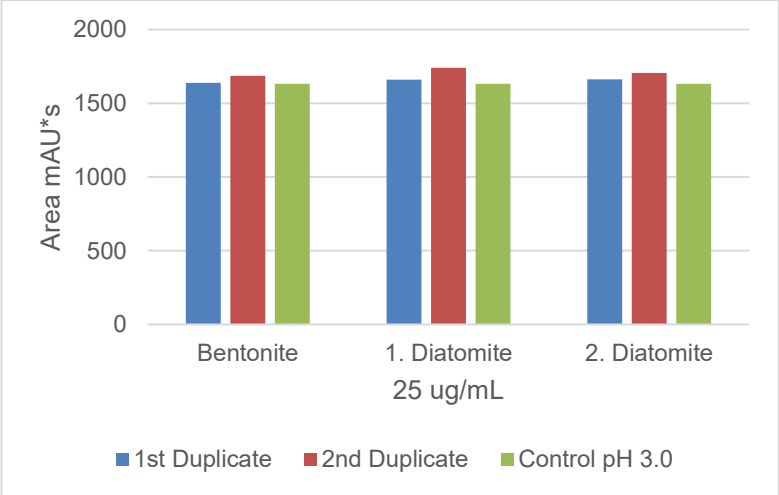

Figure 7. The 25 µg/mL of DON concentration results in duplicates with control (without mycotoxin binder) in PBS pH 3.0

As seen in Figures 8 and 9 below, the 50 µg/mL of DON concentration results at pH 6.5 and 3 in PBS solutions show that the first duplicates (in blue colour) were much closer to the control sample (in green colour) concentrations compared to the second duplicates (in red colour). The only exception was with Bentonite and 2. Diatomite samples' values which were below the control sample concentrations as seen in Figure 8.

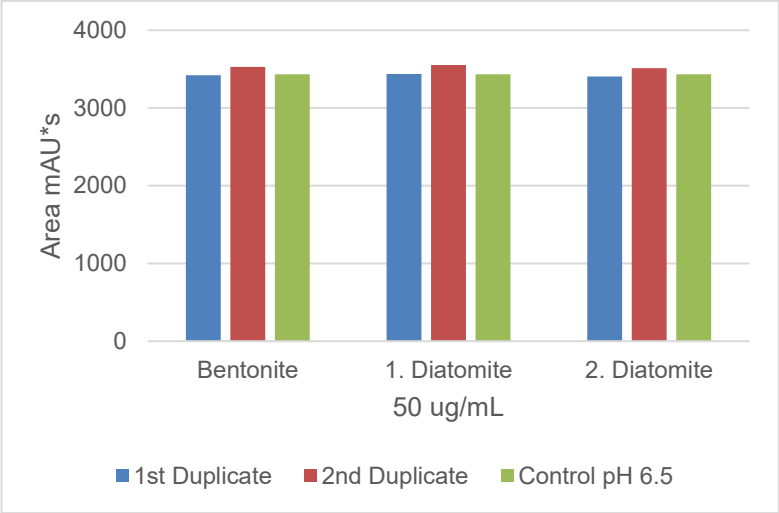

Figure 8. The 50 µg/mL of DON concentration results in duplicates with control (without mycotoxin binder) in PBS pH 6.5

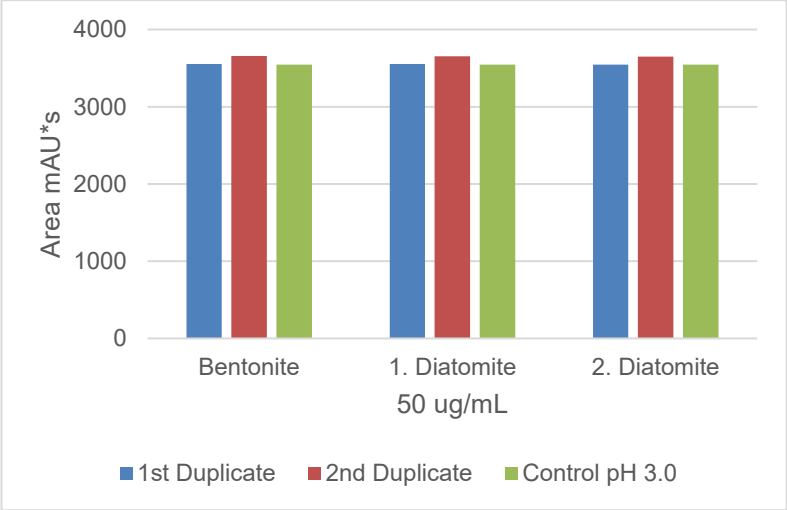

Figure 9. The 50 µg/mL of DON concentration results in duplicates with control (without mycotoxin binder) in PBS pH 3.0

In Figures 10 and 11 below, 100 µg/mL of DON concentrations were used at two different pHs 6.5 and 3 in PBS solutions. Figure 10 illustrates that DON concentration samples (in blue) were lower than the control concentration samples which can be seen in green. However, the opposite observation is seen in Figure 11 which illustrates that DON concentration samples (in blue) were higher than the control concentration samples (in green). There are many possible reasons for this: sample preparation, human error, or analytical equipment problems.

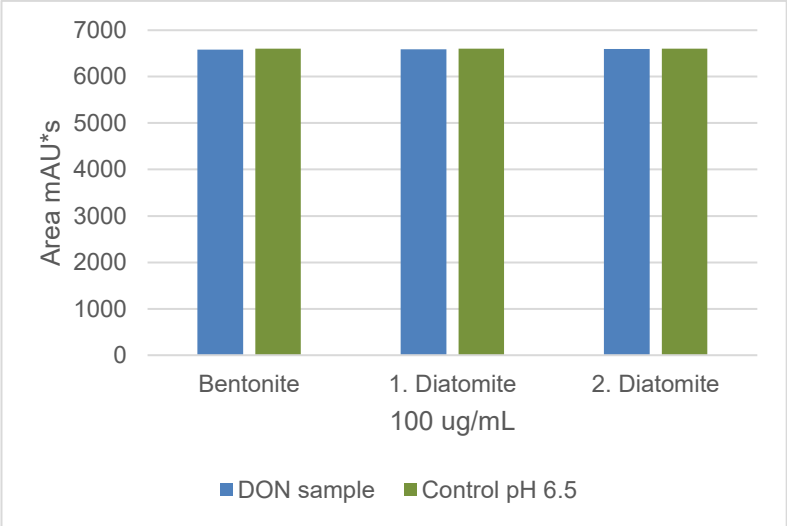

Figure 10. The 100 µg/mL of DON concentration results with control (without mycotoxin binder) in PBS pH 6.5

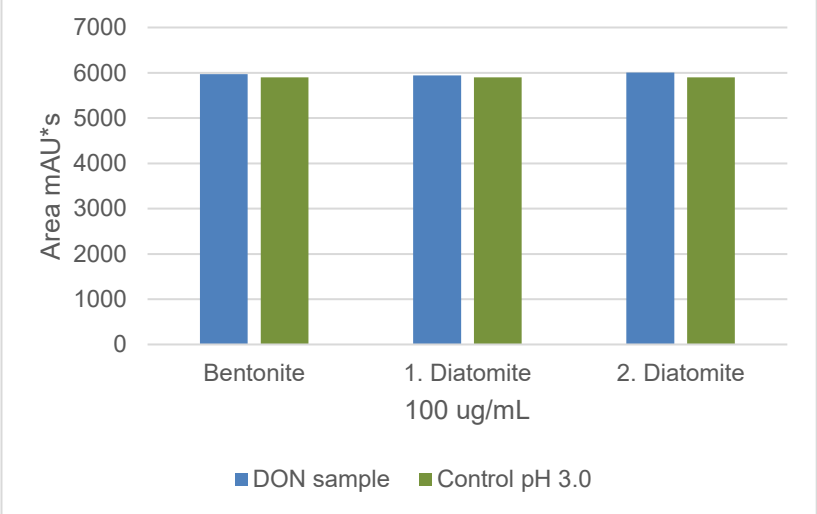

Figure 11. The 100 µg/mL of DON concentration results with control (without mycotoxin binder) in PBS pH 3.0

The recovery percentages of sample concentrations (25 µg/mL and 50 µg/mL) of all three mycotoxin binders at pH 6.5 and 3.0 are shown in Figure 12. The relation between the recovery percentage of DON and the pH indicates that the recovery percentage was lower at pH 6.5 compared to pH 3.0 in Bentonite mycotoxin binder. The opposite observation was detected with 2. Diatomite mycotoxin binder. Another interesting observation concerns the relation between recovery percentage of DON and pH with 1. Diatomite mycotoxin binder. In 25 µg/mL concentration samples, the recovery

percentage of 1. Diatomite mycotoxin binder was lower at pH 6.5 compared to pH 3.0; and in 50 µg/mL concentration samples, the opposite was observed.

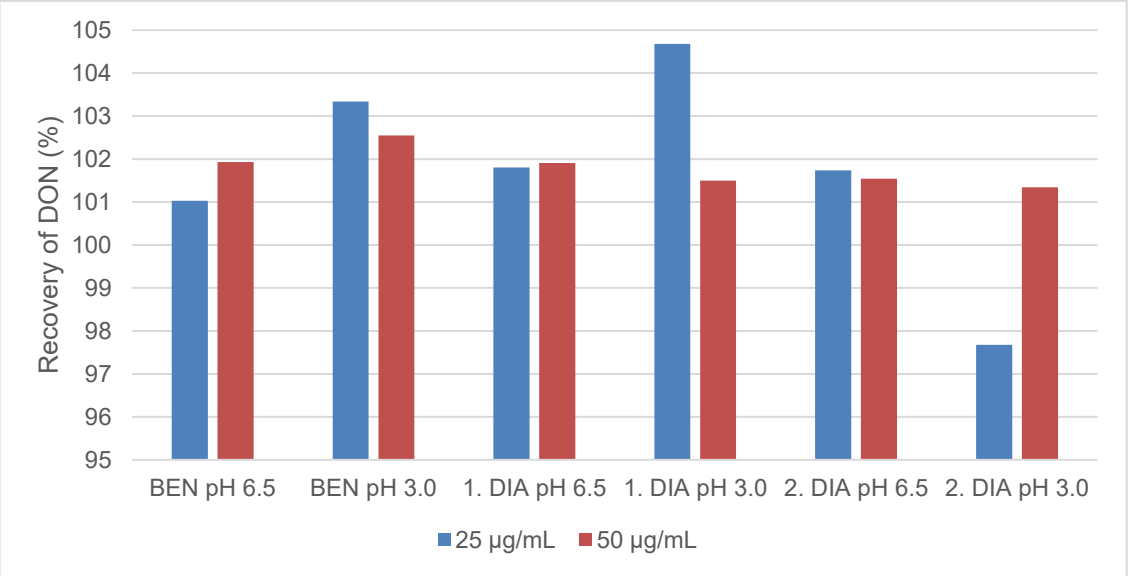

Figure 12. Demonstration of DON recovery percentage of all the mycotoxin binders with sample concentrations 25 µg/mL and 50 µg/mL at pH 6.5 and 3.0.

5.2.1 Bentonite

In BEN mycotoxin binder samples, the DON concentrations 25 and 50 µg/mL in both pH environments (pH 3.0 and 6.5) show that adsorption did not occur. The recovery of DON mycotoxin is higher than 100 % in every sample, most likely due to measurement uncertainty. In addition, the measured concentrations (µg/mL) of DON prove that it was below the estimated concentration in only one sample, as seen in Tables 6 and 7.

Table 6. Results of DON concentrations 25 and 50 µg/mL in BEN mycotoxin binder samples at pH 6.5

Sample	Concentration (µg/mL)	Measured concentration (µg/mL)	Recovery (Mean)
BEN	25	24.49	101.03
BEN	50	54.55	101.93

Table 7. Results of DON concentrations 25 and 50 µg/mL in BEN mycotoxin binder samples at pH 3.0

Sample	Concentration (µg/mL)	Measured concentration (µg/mL)	Recovery (Mean)
BEN	25	26.24	103.34
BEN	50	56.65	102.55

5.2.2 Diatomite

As for DIA mycotoxin binder samples, DON concentrations 25 and 50 µg/mL in both pH environments (pH 3.0 and 6.5) show that adsorption did not occur. The recovery of DON mycotoxin is higher than 100 % in every sample except in sample 2, most likely due to measurement uncertainty, as demonstrated in Table 9. The measured concentrations (µg/mL) of DON demonstrate the value is below the estimated concentration only in DON concentration 25 (µg/mL) at pH 6.5, as demonstrated in Tables 8 and 9.

Table 8. Results of DON concentrations 25 and 50 µg/mL in DIA mycotoxin binder samples at pH 6.5

Sample	Concentration (µg/mL)	Measured concentration (µg/mL)	Recovery (Mean)
1.DIA	25	24.68	101.80
1.DIA	50	54.53	101.91
2.DIA	25	24.66	101.74
2.DIA	50	54.34	101.54

Table 9. Results of DON concentrations 25 and 50 µg/mL in DIA mycotoxin binder samples at pH 3.0

Sample	Concentration (µg/mL)	Measured concentration (µg/mL)	Recovery (Mean)
1.DIA	25	25.58	104.68
1.DIA	50	56.07	101.50
2.DIA	25	25.86	97.68
2.DIA	50	55.99	101.34

6 CONCLUSIONS & DISCUSSION

Many mycotoxin binder adsorption studies are conducted *in vitro* as they are considered to be a fast and accurate method for showing the adsorption–desorption potential ability of mycotoxins under various simulated conditions (Prapapanpong et al. 2019).

This study investigates the possible effect of mycotoxin binders in an adapted single-concentration adsorption experiment. In particular, the sample preparation procedure was inspired by the work of Avantaggiato, et al. (2004) and Sabater-Vilar et al., (2004). The interaction of the binders was performed with mycotoxins, in this case DON. The analysis of AFB1 was conducted in an external laboratory due to the lack of the necessary equipment for performing the analysis. Therefore, the results are missing from this study due to time reasons.

The findings show that all DON samples had more or the same amount of DON in the sample compared to the control samples. Therefore, it could be concluded that in this thesis, the bentonite-based and the two diatomite-based mycotoxin binders did not adsorb the DON. In fact, several studies showed that *in vitro* experiments with natural and modified clay minerals revealed extensive binding of aflatoxins as opposed to little or no binding of DON (FAO/WHO, 2001; Avantaggiato, et al.; 2004, Thimm et al., 2000). Therefore, results of the AFB1 analysis would have brought more value to this study by giving the opportunity to compare the results with DON adsorption. It is worth mentioning that most mineral mycotoxin binders are not very effective *in vitro* studies, which is the case in this thesis. All the mycotoxin binders showed poor affinity towards DON with maximum adsorption level of 18% (Avantaggiato, et al., 2004). Thus, further research needs to be performed *in vivo* to prove the binding capacity for DON (Döll et al., 2004; Avantaggiato, et al., 2004).

In addition, there could be a connection between the recovery percentage of DON and the pH with bentonite and diatomite mycotoxin binders in sample concentrations 25 µg/mL and 50 µg/mL. The recovery percentage was lower in pH 6.5 compared to pH 3.0 in Bentonite mycotoxin binder and the opposite in 2. Diatomite mycotoxin binder. The only exception is with 1. Diatomite mycotoxin binder, which showed that in 25 µg/mL sample concentration, the recovery percentage was lower at pH 6.5 compared to pH 3.0 and the opposite occurred in 50 µg/mL sample concentration. Based on these

observations, it is difficult to make any definite conclusion on whether pH has any influence on mycotoxin adsorption.

As regards to the methodology, the used methods were suitable for the determination of the mycotoxin binder's adsorption capacity with DON. This method was a good starting point for this type of research as it was simple enough and despite the time limit it was successful. On this basis it would be possible to conduct similar experiments. However, some problems were encountered working with low volumes. The low amount of DON (1 mg serum bottle) has proved to be a limiting reagent in this thesis; therefore, 5 mg serum bottle of DON should preferably be considered in future analyses to minimize measurement uncertainty.

In future analyses, method validation should be considered. For example, it would be interesting to modify the experiment parameters such as solution pH, buffer solution composition, incubation time, and temperature. It is possible that pH level of the sample had an impact on DON binding efficacy. Thus, it is worth investigating how different pH variations might contribute to DON binding efficacy. Another parameter to consider is the composition of the buffer solution. The PBS does not reflect to the digestive tract condition since it is missing for example feed, digestive enzymes, bile salts, nutrients etc. which may interfere with the binding of DON. It is also important to consider the incubation duration. The incubation duration could be longer than one hour such as 2 to 3 hours to see if the duration affects the binding capacity. A final thought regarding the parameters that might affect the results is the incubation temperature (37ºC). As mammalians' body temperature varies from around 34 to 40ºC, it would be interesting to conduct this study using all the possible temperatures.

In addition, there is one detail that should be taken into consideration: following the analysis of the unbound mycotoxin from the supernatant, the bound mycotoxins in the clay could be washed and collected for further analyses. The analysis should be carried out by coupling a multi-mycotoxin experiment with detectors. In this way, the competition of mycotoxin adsorption capacity could be seen with the mycotoxin binders. As mentioned above, in vivo studies should be considered for a better demonstration of the adsorption-desorption ability of the mycotoxin binders. In fact, the method used in this thesis cannot follow the variations in pH as in the animal intestinal environment.

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