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POTENTIAL OF DIFFERENT MYCOTOXIN ADSORBENTS UNDER *IN VITRO* CONDITIONS

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Abstract

Mycotoxins are a large and chemically diverse group of toxic secondary metabolites. Regarding their prevalence in animal feed and the effect on animal health, the biggest problems in terms of safety and economic losses are caused by aflatoxins, fumonisins, ochratoxins, trichothecenes and zearalenone. Adsorbents are substances that are added to food contaminated with mycotoxins, in order to bind them in the gastrointestinal tract and thereby prevent or reduce their effect. The aim of this study was to examine the possibility of using pyrophyllite as a mineral adsorbent, as well as preparations made of ground peach pits of different particle sizes as organic adsorbents, for adsorption of deoxynivalenol and ochratoxin A. Mycotoxin adsorption experiments were performed *in vitro* in electrolyte solutions at pH 3 and 7. The adsorption efficiency of the adsorbent was expressed as adsorption index. Pyrophyllite had adsorption index of 13.47% for ochratoxin A at pH 3, while at pH 7, as well as for deoxynivalenol, the same mycotoxin produced a negligible degree of adsorption. Ground peach stones (of larger diameter, d = 0.1 mm) had considerable adsorption rates for ochratoxin A at pH 3 (34.41%) and deoxynivalenol at pH 7 (18.57%). The values were similar for smaller diameter (d < 0.1 mm) for ochratoxin A at pH 3 (42.71%) and deoxynivalenol at pH 7 (20.11%). The obtained results suggest that the potential of the preparation of ground peach stones for the adsorption of tested mycotoxins is higher compared to the potential of pyrophyllite, but there are differences in their efficiency depending on the pH value of the adsorption environment.

Key words: ochratoxin A, deoxynivalenol, adsorbents, pyrophyllite, peach stones

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POTENCIJAL RAZLIČITIH ADSORBENATA MIKOTOKSINA U *IN VITRO* USLOVIMA

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Kratak sadržaj

Mikotoksini su relativno velika i hemijski raznolika grupa toksičnih sekundarnih metabolita. Uzimajući u obzir prevalencu u hrani za životinje i efekat na zdravlje životinja, najveće bezbednosne i ekonomske probleme predstavljaju aflatoksini, fumonizini, ohratoksini, trihoteceni i zearalenon. Adsorbenti predstavljaju materije koje se dodaju hrani kontaminiranoj mikotoksinima, kako bi ih u gastrointestinalnim traktu vezivali i time sprečili ili umanjili njihovo dejstvo. Cilj ovog rada je da se ispita mogućnost primene pirofilita kao predstavnika mineralnih, kao i preparata mlevenih koštica breskve različitih veličina čestica kao predstavnika organskih adsorbenata za adsorpciju deoksinivalenola i ohratoksina A. Eksperimenti adsorpcije mikotoksina su izvođeni *in vitro* u rastvorima elektrolita na pH 3 i 7. Efikasnost adsorpcije adsorbenta je izražena kao indeks adsorpcije. Rezultati su ukazali da pirofilit ima vrednost indeksa adsorpcije od 13,47% za ohratoksin A pri pH 3, dok je za isti mikotoksin pri pH 7, kao i za deoksinivalenol, utvrđen zanemarljiv nivo adsorpcije. Mlevene koštice breskve većeg dijametra (d = 0,1 mm) su pokazale odgovarajuće indekse adsorpcije za ohratoksin A pri pH 3 (34,41%) i deoksinivalenol pri pH 7 (18,57%), dok su mlevene koštice breskve manjeg dijametra (d < 0,1 mm) pokazale slične vrednosti indeksa adsorpcije za ohratoksin A pri pH 3 (42,71%) i za deoksinivalenol pri pH 7 (20,11%). Iz dobijenih rezultata se može zaključiti da je potencijal preparata mlevenih koštica breskve za adsorpciju ispitanih mikotoksina veći u odnosu na potencijal pirofilita, ali da postoje razlike u njihovoj efikasnosti u zavisnosti od pH vrednosti sredine u kojoj se adsorpcija odvija.

Ključne reči: ohratoksin A, deoksinivalenol, adsorbenti, pirofilit, koštice breskve

INTRODUCTION

Agriculture is becoming increasingly important due to the rise in world population and urbanization. This implies a rapid growth and boost of livestock production, resulting in the safety of animal feed becoming even more significant considering the potential threat of hazards reaching the human food chain. Among the undesirable substances, according to Directive 2002/32/EC (European Commission, 2002), mycotoxins are considered one of the greatest hazards in feed raw materials (Santos Pereira et al., 2019). Among other mycotoxins, deoxynivalenol (DON) and ochratoxin A (OTA) certainly represent the most important causes of diseases in animals. Namely, global economic losses caused by DON are in the range of about one billion dollars per year. The Food and Agriculture Organization and the World Health Organization identified it as one of the most dangerous food contaminants as early as 1973 (Neme and Mohammed, 2017). This mycotoxin mainly contaminates agricultural crops such as corn, wheat, and barley, and has immunogenic, carcinogenic, and teratogenic effects in animals. It is also detected in animal products such as meat, eggs, and milk, and poses a danger to human health (Yao and Long, 2020). OTA causes great economic losses to animal husbandry, as the intake of contaminated feed can significantly impair animal health and safety of animal products (Battacone et al., 2010). It can be found in a wide range of agricultural and livestock products, and in processed foods as well, while human exposure to this mycotoxin is mainly attributed to contaminated grains (Abrunhosa et al., 2010). Juodeikiene et al. (2012) stated three possible solutions for avoiding the harmful effect of mycotoxin contamination of both food and feed. They include the following: prevention of contamination, decontamination of mycotoxin-containing food and feed, and inhibition of mycotoxin absorption into the digestive tract. Nevertheless, if mycotoxin contamination already exists, application of decontamination methods is advised (Jouany, 2007). Various methods for mycotoxin reduction were studied, including the application of irradiation, thermal or microwave heating, ozone, chemical compounds, and microbials (Binder and Binder, 2004; Bullerman and Bianchini, 2007; Herzallah et al., 2008; Garg et al., 2013; Krstović et al., 2021). Moreover, the use of existing feed additives, such as enzymes showed a potential for degradation of some mycotoxins (Jakšić et al., 2022). Finally, measures can be applied for inhibition of mycotoxin absorption in the gastrointestinal tract, e.g., the use of adsorbents to reduce the bioavailability of mycotoxins in the digestive tract of animals (Abdel-Wahhab and Kholif, 2010). The use of adsorbents is one of the most suitable options for treatment of mycotoxins in practice (Li et al., 2018). In general, the adsorption method includes both physical and chemical force, which can not only reduce the toxic impact of mycotoxins, but also enable the avoidance of toxic residues, thus becoming the most commonly applied method of protecting animals from mycotoxins (Li et al., 2018). Adsorbents can be used as single formulations, but are recently combined for better efficiency, usually as mineral-organic adsorbent mixture (Nešić et al., 2020).

The aim of this study was to examine the possibility of using two types of mycotoxin adsorbents. The first is pyrophyllite, a non-metallic aluminosilicate mineral that has adsorptive properties, primarily for ions of heavy metals and colors from aqueous solutions. The second adsorbent included two preparations of ground peach stones of different particle sizes. This type of adsorbent is organic, its waste biomass of agro-industrial source, and as an economically and ecologically profitable material, it is generally considered cheaper than mineral (inorganic) adsorbents. The adsorptive capacity of these preparations was tested for the adsorption of DON and OTA under *in vitro* conditions.

MATERIAL AND METHODS

Adsorbents

Adsorption potential test was performed for two preparations. Pyrophyllite, which belongs to the group of non-metallic aluminosilicate minerals, was selected as a potential inorganic adsorbent. Its use is already permitted in animal feed at up to 2% to prevent ruminal acidosis in ruminants (US Food and Drug Administration, 2019; Adamović et al., 2020). Another potential adsorbent is a preparation of ground peach stones (Lopičić et al., 2013). It was prepared in two fractions: larger (particle size d = 0.1 mm) and smaller (particle size d < 0.1 mm) fraction. The preparations were not commercially available. All preparations were prepared by and obtained from the Institute for Technology of Nuclear and Other Mineral Raw Materials, Belgrade, Serbia.

Mycotoxin removal procedure in vitro

To simulate *in vivo* conditions, where mycotoxin adsorption is performed on adsorbents, mycotoxin adsorption experiments were performed *in vitro* in solutions at pH 3 and 7. These pH values are expected in the digestive tract of monogastric animals and humans. The crude extract of DON, produced in house on wet maize kernels after artificial inoculation with toxigenic *Fusarium graminearum* (Krstović et al., 2018), as well as the standard solution of OTA (Sigma, St. Louis, MO, USA) were used for the adsorption experiments. 90 mg of adsorbent was weighed into glass tubes using Teflon stoppers (volume of 20 cm^3), and then 10 mL of 0.1 M KH2PO4 (13.609 g KH2PO4 in 1000 mL of water, pH set to pH = 3 and pH = 7) was added. After that, a volume of 100 μL of crude toxin solution (c = 300 μg/mL) was added in order to obtain the final concentration of 3 μg/ml (crude toxin to adsorbent ratio was 1:3000). Mixing magnets were added to the tubes and then placed in an incubation system at 37 °C for 120 minutes with continuous mixing. The control was set up in the same way, without adsorbent. After the contact of the adsorbent and the mycotoxin was completed, the solution was filtered through quantitative filter paper for slow filtration and then additionally through nylon micro syringe filters with a porosity of 0.2 μm. The solutions prepared in this way were analyzed for HPLC for the mycotoxins' contents. All experiments were performed in duplicate. The adsorption efficiency of the natural adsorbent is expressed as an adsorption index, where:

Ci – mycotoxin initial concentration,

Ceq – mycotoxin concentration at equilibrium.

Adsorption index, % = [((Ci – Ceq)/Ci)] × 100

HPLC analysis

DON and OTA determinations were carried out on a 1260 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) using photodiode (DAD) and fluorescence (FLD) detectors (Agilent Technologies, USA) and a Hypersil ODS (150 x 4.6 mm i.e., particle size 5 μm) column (Agilent Technologies, USA). Stock calibration solutions at a concentration of 0.1 mg/mL were prepared in a mixture of ethyl acetate and methanol (19:1, v/v) for DON (Sigma, St. Louis, MO, USA), and in acetonitrile for OTA (Sigma, St. Louis, MO, USA). Working calibrant solutions were prepared by measuring the appropriate volume of the stock solution, evaporating to dryness under a stream of nitrogen at 50 °C and dissolving in the appropriate volume of the mobile phase. The stock solutions were stored at a temperature of -18 °C, while the working solutions were stored in a refrigerator at a temperature of 4 – 8 °C. HPLC conditions for DON determination were set as proposed by Abramović et al. (2005) and for OTA a method by Sugita-Konishi et al. (2006) was used. All analyses were done in duplicate. A volume of 20 μL of solution obtained after adsorption was injected into a HPLC system. The mobile phase consisted of an isocratic mixture of acetonitrile and water (HPLC grade, Sigma, St. Louis, MO, USA). For the determination of DON, the ratio of the solvents was the following: acetonitrile: water (16:84, *v/v*), and in the case of OTA, this ratio acetonitrile: water was (50:50, *v/v*) and 1% of acetic acid was added (HPLC purity, Fisher Scientific, USA). In both cases, a mobile phase flow rate of 0.8 mL/min was applied. Before use, the mobile phase was filtered through regenerated cellulose membrane filters (0.45 μm) (Agilent Technologies, USA). DON was detected using a DAD detector at a wavelength of λ = 220 nm, for the detection of OTA, FLD detector was used at wavelengths of λ_{ex} = 333 nm and λ_{em} = 470 nm. The identification of DON and OTA was performed by comparing the retention times and spectra of the standards with the retention times and spectra of the samples.

RESULTS

The results of mycotoxin adsorption using the *in vitro* method are shown in tables 1-3.

Table 1. Results of mycotoxin adsorption using pyrophyllite, n = 2

		DON	**OTA**
Control	Ci, ng/mL	3012 ± 15	2986 ± 10
	Ceq, ng/mL	2996 ± 11	2981 ± 13
	A, %	0.53	0.17
pH 3	Ci, ng/mL	2946 ± 53	2969 ± 18
	Ceq, ng/mL	2945 ± 23	2569 ± 21
	A, %	0.03	13.47
pH 7	Ci, ng/mL	2978 ± 31	2980 ± 11
	Ceq, ng/mL	2974 ± 13	2948 ± 32
	A, %	0.15	1.1

Ci - mycotoxin initial concentration, Ceq - mycotoxin concentration at equilibrium, A - adsorption index.

Table 2. Results of mycotoxin adsorption using the preparation of ground peach stones (GPS), particle size *d* = 0.1 mm, n = 2

		DON	OTA
Control	Ci, ng/mL	3012 ± 15	2986 ± 10
	Ceq, ng/mL	2996 ± 11	2981 ± 13
	A, %	0.5	0.2
pH 3	Ci, ng/mL	2946 ± 53	2969 ± 18
	Ceq, ng/mL	2940 ± 56	1948 ± 89
	A, %	0.20	34.41
pH 7	Ci, ng/ml	2978 ± 31	2980 ± 11
	Ceq, ng/ml	2425 ± 38	2972 ± 12
	A, %	18.57	0.29

Ci - mycotoxin initial concentration, Ceq - mycotoxin concentration at equilibrium, A - adsorption index.

Table 3. Results of mycotoxin adsorption using the preparation of ground peach stones (GPS), particle size *d* < 0.1 mm, n = 2

		DON	OTA
Control	Ci, ng/mL	3012 ± 15	2986 ± 10
	Ceq, ng/mL	2996 ± 11	2981 ± 13
	A, %	0.5	0.2
pH 3	Ci, ng/mL	2946 ± 53	2969 ± 18
	Ceq, ng/mL	2941 ± 28	1701 ± 98
	A, %	0.19	42.71
pH 7	Ci, ng/mL	2978 ± 31	2980 ± 11
	Ceq, ng/mL	2379 ± 89	2897 ± 56
	A, %	20.11	2.80

Ci - mycotoxin initial concentration, Ceq - mycotoxin concentration at equilibrium, A - adsorption index.

The adsorption index of DON was below 1% using pyrophyllite for both pH values, as well as in the case of using the preparation of ground peach stones at pH = 3 (Figure 1). As for the preparation of ground peach stones at pH = 7, adsorption index values of 18.57% (larger fraction) and 20.11% (smaller fraction) were obtained. This indicates that there is a certain potential of the ground peach stone preparation for DON adsorption at pH = 7, while the adsorption potential of pyrophyllite has not been proven.

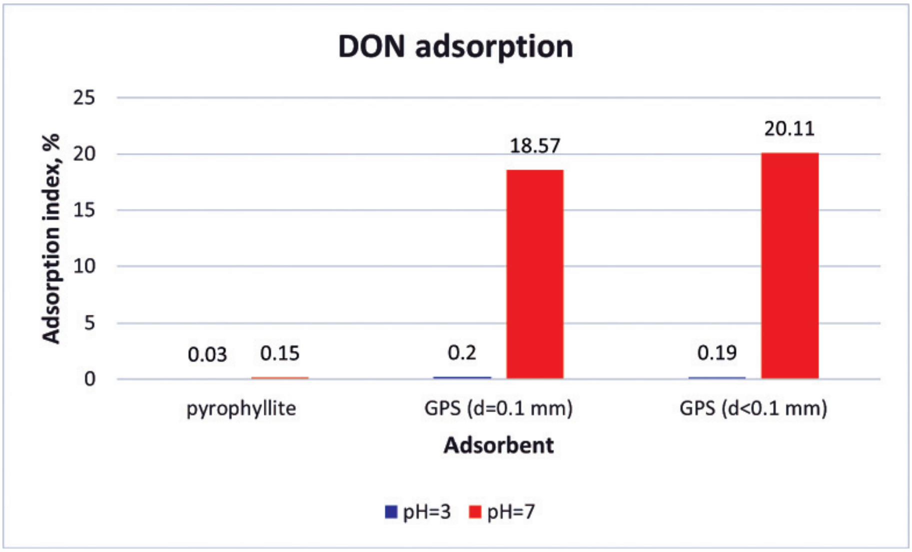

Figure 1. Comparative overview of the adsorption index of ground peach stones (GPS) and pyrophyllite for DON adsorption

When it comes to the adsorption index of pyrophyllite for OTA, it was affected by the pH value (Figure 2). Namely, at pH=3, the adsorption index was found to be 13.47%, while at pH = 7, it was as low as 1.1%. However, using the preparation of ground peach stones, these differences in the adsorption index were opposing compared to the application of pyrophyllite, where high values of the adsorption index of 34.41% (larger fraction) and 42.71% (smaller fraction) were found at pH = 3, and low values (0.29% and 2.80%) at pH = 7. These results indicate that the potential of the preparation of ground peach stones for OTA adsorption is higher compared to the potential of pyrophyllite, but there are still differences in their efficiency depending on the pH value of the environment in which the adsorption takes place.

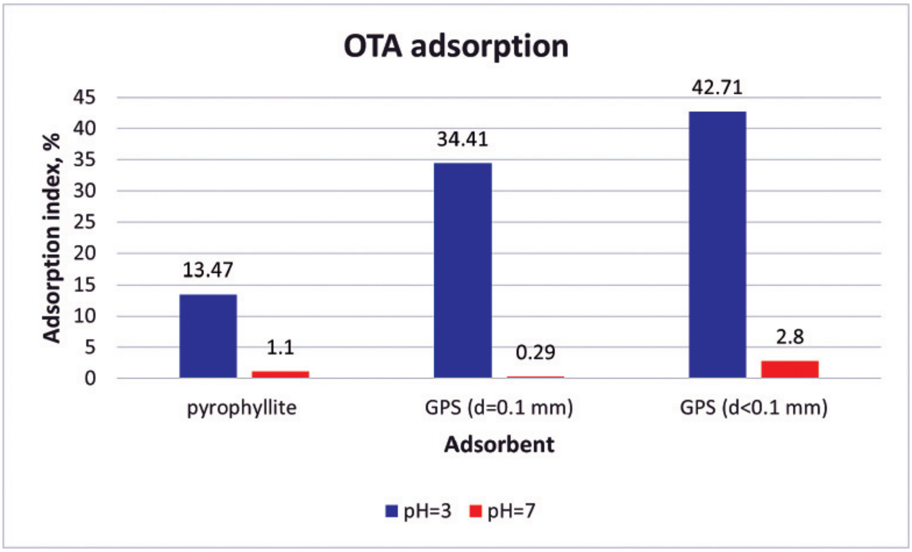

Figure 2. Comparative overview of the adsorption index of ground peach stones (GPS) and pyrophyllite for OTA adsorption

DISCUSSION

Pyrophyllite is already a recognized adsorbent of various substances such as cationic (due to negative hydrophilic surface sites) and anionic (after the addition of trivalent aluminium cation) dyes (Gücek et al., 2005). It is also known for its ability to bind ions of heavy metals such as cobalt (98.97%), lead (97.53%), nickel (98.87%) and cadmium (98.65%) from aqueous solution (maximum adsorption in optimized conditions) (Panda et al., 2018). Furthermore, in the research conducted by Murtić et al. (2020), it was demonstrated that the application of pyrophyllite, due to its adsorbent and ion exchange capabilities can reduce the use of mineral fertilizers in the production of lettuce without adverse effects on its yield and quality, in the amount of 25% or 50% of the recommended amount of fertilizer. However, despite the results of the aforementioned research, as well as the proven adsorption capacity of numerous other aluminosilicates against mycotoxins, literature data on the adsorption of mycotoxins, specifically by pyrophyllite, is very scarce. In contrast, the potential of peach stones to adsorb mycotoxins has been studied more extensively. Hence, in the research by Adamović et al. (2013) the adsorption of the following six mycotoxins was tested: AFB1, zearalenone, diacetoxyscirpenol (DAS), T-2 toxin, OTA and DON using the *in vitro* method. Similar to our study, the adsorption index was tested at two pH values, pH = 3.0 and 6.9.

Peach stones showed the highest affinity to aflatoxin (80.00% and 73.30%) and the lowest to DAS (0% at both pH values). When it comes to OTA and DON, a higher adsorption capacity was demonstrated in comparison with our research, given that the adsorption index at pH=3.0 and 6.9 were 66.67% and 50% for OTA, and 25% and 50% for DON, respectively. However, there were certain differences in the experimental design that could produce a different result (the ratio of individual mycotoxins and adsorbents in this research was 1:5000 and the particle size of the investigated biomass < 100 μm). Comparable research was conducted by Lopičić et al. (2013), at pH values of 3.0 and 7.0. Similar to the previous study, the best adsorption index of peach stones was obtained for aflatoxin (58.82% at both pH values), the poorest for DAS (0% and 16.67%), while for OTA it was 66.67% and 19.98% and 23.08% and 39.97% for DON, at pH 3.0 and 7.0, respectively. It is interesting to point out that in both studies, in addition to the unmodified, modified peach stones (activated with 1 M HCl) were used, and they achieved a somewhat different, weaker result compared to the unmodified. Furthermore, in the research by Bočarov-Stančić et al. (2011), the adsorption capacities of mineral adsorbents bentonite, diatomite and zeolite against aflatoxin, DAS, OTA, and DON were investigated. Like in the previous research, the highest adsorption index was obtained for aflatoxins (at pH 3.0 and 6.9: bentonite 96.90%; zeolite 95.50% and diatomite 95%) which is slightly higher compared to organic adsorbents, while the weakest was obtained for DAS (0% in all adsorbents). However, it is noted that organic adsorbents were more effective for OTA and DON. With OTA, only diatomite had adsorption properties (66.67%) at pH = 3. On the other hand, at pH = 6.9, as well as for bentonite and zeolite at both pH values, the adsorption index was 0%. Considering DON, mineral adsorbents showed affinity at pH = 3, bentonite and zeolite 50% and diatomite 25%, whereas at pH = 7 they did not display any affinity (0%). These results may lead to a conclusion that mineral adsorbents are more effective when it comes to aflatoxins, while organic adsorbents are more successful for OTA and DON adsorption. This was confirmed by the results of our research, where pyrophyllite was used as a mineral adsorbent. This adsorbent had an effect (13.47%) only for OTA at pH = 3, while it was ineffective in other cases. Ground peach stones were more efficient, as a larger diameter showed the degree of adsorption for OTA at pH = 3 (34.41%) and DON at pH 7 (18.57%), and the smaller diameter similarly, for OTA at pH = 3 (42.71%) and for DON at pH = 7 (20.11%). However, it is certainly necessary to prove this result under *in vivo* conditions.

The obtained results in our and similar research indicate that the degree of binding depends primarily on the type of adsorbent (mineral or organic), and on individual types within these groups, as well as on the mycotoxin. These characteristics should be considered when creating formulations for use in practice, and certain adsorbents should be used depending on the conditions on the farm and occurrence of certain types of mycotoxins. In the future, formulations containing several types of adsorbents need to be tested, in order to enable action against several types of mycotoxins or use one of them that has the widest spectrum of adsorption capacity when different types of mycotoxins are involved.

CONCLUSION

The results of this study point to several conclusions. Namely, the potential of the preparation of ground peach stones for the adsorption of DON at pH = 7 was found, while its application at pH=3, the adsorption potential was not confirmed. The adsorption potential of pyrophyllite was not established at any of the pH values to which it was applied. The potential of the preparation of ground peach stones for OTA adsorption is higher compared to the potential of pyrophyllite, but there are differences in their efficiency depending on the pH value of the environment in which the adsorption takes place. Like with other *in vitro* research, this research cannot fully simulate conditions in the gastrointestinal tract of animals, so further *in vivo* experiments are necessary in order to evaluate the effectiveness of these materials as mycotoxin adsorbents.

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Author’s Contribution:

S.K. basic idea, conception and design; D.G. acquisition of data; J.K. interpretation of results, manuscript revision; M.D. acquisition of data, drafting the manuscript; I.J. conception and design, final approval.

Competing interest

The author(s) declare that they have no competing interests.

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