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IN VITRO EVALUATION OF THE EFFICACY OF PEACH STONES AS MYCOTOXIN BINDERS

ABSTRACT: The paper describes *in vitro* model for the evaluation of ability of peach shell (unmodified and modified), prepared at the Institute for Technology of Nuclear and Other Mineral Raw Materials, Belgrade, to adsorb different mycotoxins.

Peach stones were obtained from "Vino Župa" Company from Aleksandrovac, where they have been disposed of as by-products from their Juice Factory. After proper preparation, two sorts of peach shell particles were used: one as unmodified peach shell particles (PS) and another one obtained by acid modification, denoted as MPS.

Adsorption of six mycotoxins: aflatoxin B1 (AFL), ochratoxin A (OTA), deoxynivalenol (DON), zearalenone (ZON), diacetoxyscirpenol (DAS) and T-2 toxin by PS and MPS was tested *in vitro*. Crude extracts of mycotoxins, produced at the Department of Microbiology of Bio-ecological Center, Zrenjanin, were used for adsorption experiments. The ability of binding mycotoxins was evaluated in the electrolyte 0.1 M K_2HPO_4, which pH value was adjusted to 3.0 and 7.0, respectively. Mass ratio of individual mycotoxin and peach shell samples was 1:5000. The experimental mixtures were incubated for 1 hour on a rotary shaker (185 rpm) at room temperature (22-25°C). After incubation, the extractions of non-adsorbed mycotoxins from the filtrates were performed with organic solvents, and their quantification was done by thin-layer chromatography (TLC).

KEY WORDS: peach stones, acid modification, adsorption, **in vitro,** mycotoxins

INTRODUCTION

Biosorption has proved to be efficient, low cost and sustainable technique that uses cheap and abundant biomaterials, usually declared as waste, for removing heavy metals and organic waste mostly dyes (N a j a et al., 2010; S u d et al., 2008; D a s et al., 2008). The authors have used one type of agricultural waste material-peach stone for *in vitro* removing of the new type of pollutants in biosorption – mycotoxins.

Among all substances that can contaminate feedstuffs, the outstanding place belongs to mycotoxins. These naturally occurring components, secondary

fungal metabolites, can have deleterious health effects on animals and humans due to the consumption of animal products. One approach in finding method(s) for prevention of toxic effects of mycotoxins is detoxification and inactivation of these fungal metabolites by using mycotoxin binders. These additives inhibit the uptake of mycotoxins by animals *in vivo.*

Agricultural waste materials mostly consist of cellulose, lignin, hemicellulose, pectin, extracts, lipids and other organic compounds that are rich in different functional groups responsible for pollutants binding. The presence of these three biological polymers (cellulose, lignin, and hemicellulose) causes richness of peach shell in hydroxyl and phenol groups which can be further chemically modified to produce adsorbent materials with improved adsorbing properties. They also have multilayer porous structure filled with openings and channels that provide large volume per sorbent surface unit, which is favorable for biosorption process (H u b b e et al., 2011). Chemical modification of cellulosic materials is often used to improve certain properties of the material or some of its components, such as material hydrophilic or hydrophobic characteristics, its elasticity, adsorptive or ion-exchange capability, thermal properties of the material or its resistance to microbiological attack, but in most cases the chemical modification serves to improve absorbing capacity of materials (S u n, 2010). Many researchers have conducted their investigations using different chemical agents (acids, bases, etc.) in order to improve absorbing properties of biomaterials toward heavy elements (W a n N g a h and H a n a f i a h, 2008). Acid pretreatment serves for removal of some soluble organic impurities and it can be used to change the structure of functional cell compounds and expose binding sites to pollutants in order to improve biosorption capacity. In the case of heavy elements biosorption, acid pretreatment increased the overall negative charge of the adsorbents, which improved biosorption capacities for negative cations (E l a n g o v a n et al., 2008).

So, the aim of the presented investigation was to evaluate and compare *in vitro* the binding capacity of unmodified peach shell particles and peach shell particles treated with hydrochloric acid to six different mycotoxins: aflatoxin B1, ochratoxin A, deoxynivalenol, zearalenone, diacetoxyscirpenol and T-2 toxin.

MATERIALS AND METHODS

Biosorbent preparation

Lignocellulosis material – peach stones were obtained from "Vino Župa" Company from Aleksandrovac, where they were disposed of as by-products from their Juice Factory. The samples were manually crushed and separated from kernels, and in that way only hard stone parts were taken for further analysis. The crushed peach stones were further milled to different fractions and washed several times in tap and distilled water. The sample was sent to

chemical analysis. The content of micro and macroelements in unmodified peach stones was analyzed using standard chemical methods (Službeni Glasnik SFRJ, broj. 15/87) and the morphology of the untreated material was obtained with dried sample coated with gold and observed using JEOL JSM-6610LV SEM model.

For mycotoxins adsorption, only the fraction with diameter less than 100 μm was used. Prior to experiments, samples were dried at 60°C for 24 h, washed three times in 0.01 M HCl, and then in distilled water until negative reaction with Cl^- ions was reached. After drying, one part of these particles was directly used for mycotoxin adsorption as unmodified material (PS). Another sample marked as modified peach shell particles (MPS) was activated by 1M hydrochloric acid on thermostatic orbital shaker (25°C and 200 rpm). After 1 hour, flask content was filtered, particles were washed with distilled water several times, and the procedure with 1M HCl was repeated two more times. At the end, MPS was washed with distilled water until negative reaction with Cl^- ions was reached. All the samples were marked and stored in polypropylene bags until the experiment started.

Production, quantification and isolation of mycotoxins

Aflatoxin B1 (AFL), ochratoxin A (OTA), deoxynivalenol (DON) and zearalenone (ZON) were produced by solid substrate fermentations based on the methods of Bočarov-Stančić et al. (2009a and 2009b) and Bočarov-Stančić et al. (2010), respectively. Type A trichothecenes (diacetoxyscirpenol – DAS and T-2 toxin) were biosynthesized by submerged fermentation in liquid medium (Bočarov-Stančić et al., 2007). For the toxin production, the following fungal cultures were used: *Aspergillus flavus* GD-2 (leg. prof. dr G. Dimić, Technological Faculty, Novi Sad, Serbia), *A. ochraceus* CBS 108.08, *Fusarium graminearum* GZ-LES (leg. dr J. Lević, Maize Research Institute, Belgrade-Zemun, Serbia), *F. graminearum* D2 (leg. dr A. Bočarov-Stančić, Bio-Ecological Centre, Zrenjanin, Serbia), *F. semitectum* SL-B (leg. dr A. Bočarov-Stančić, Bio-Ecological Centre, Zrenjanin, Serbia), and *F. sporotrichioides* ITM-391 (leg. dr A. Bottalico, Consiglio Nazionalle delle Richerce, Istituto Tossine a Micotossine da Parassiti Vegetali, Bari, Italy).

Isolations of mycotoxins and determinations of single mycotoxin content in solid substrates were done according to standard thin-layer chromatographic method for fodder analysis (Službeni Glasnik SFRJ, br.15/87). Isolations of type A trichothecenes were done by ethyl acetate and their quantities were determined by thin-layer chromatographic (TLC) method according to Rukmini and Bhat (1978). Isolated crude toxins were evaporated to dryness and dissolved in following solvents: ethanol (AFL, OTA, ZON), ethyl acetate (DAS, T-2) and methanol (DON). The final concentrations of stock mycotoxin solutions were 0.1 μg/μl (AFL) and 1 μg/μl (OTA, DON, ZON, DAS and T-2), respectively.

Experimental procedure

In order to perform adsorption experiments, stock solutions of mycotoxins were diluted as follows: AFL to 0.2 μg/ml, ZON to 0.8 μg/ml, and all other mycotoxins to 2.0 μg/ml with electrolyte (0.1M K_2HPO_4). pH value of electrolyte was adjusted with 0.1M HCl or 0.1 NaOH to 3.0 and 6.9, respectively.

The binding ability of peach stones was tested *in vitro* as follows: aliquots (50 ml) of test solutions were added to Erlenmayer flasks (250 ml) containing 500 mg of single adsorbent in the case of OTA, DON, DAS and T-2 toxin, 200 mg in the case of ZON, and 50 mg in the case of AFL. Controls were prepared by adding 50 ml of the test solutions without mineral adsorbent. The flasks were stoppered, incubated for 1 hour on rotary shaker (185 rpm) at room temperature (22-25^{o}C) and then filtered. In 25 ml aliquots of electrolyte with adsorbent (C) and without it (C_o), concentrations of mycotoxins were determined, after extraction with 2 x 15 ml of organic solvents: benzene (ZON), benzene-acetonitrile (AFL), and ethyl acetate (OTA, DON, DAS and T-2) respectively, by TLC methods (Službeni Glasnik SFRJ, br. 15/87; R u k m i n i and B h a t, 1978). All analyses were performed in three replications.

The adsorption index of individual mycotoxin, in percentages, was calculated with the following formula:

$$Adsorption\,index = [\frac{C_0 - C}{C_0}]\,x\,100$$

RESULTS AND DISCUSSION

The chemical analyses of unmodified peach stone particles were performed in order to elucidate the composition and content of micro and macroelements present in the materials. Peach shell consisted mostly of cellulose (58.02%), hemicellulose (16.54%), and lignin (5.02%) (B o č a r o v-S t a n č i ć et al., 2012a) (Table 1).

On the other hand, results shown in Table 2 indicate that peach stone particles contained several important minerals such as calcium (0.14%) and potassium (0.089%); toxic elements were not present in significant amounts.

Such chemical compositions of the examined peach stone samples implied that these materials could be used in animal feed as energetic materials or even as carriers of certain active substances used in agriculture and industry.

The morphology and the surface nature of the grounded PS were presented on the SEM micrograph (Figure 1) at 3000 x magnification. As it can be seen from Figure 1, the PS particles have multilayer porous surface with irregular laminated structure. The average pore diameter was less than 1 μm, which might be beneficial for mycotoxin diffusion and adsorption.

Tab. 1 – Chemical composition of unmodified peach shell (PS) in %

Parameter (%)	PS	dPS*	JUS/ISO/ Documented methods
Dry matter	92.23	100.00	Službeni Glasnik SFRJ, br. 15/87, Method 6
Moisture	7.77	-	Službeni Glasnik SFRJ, br. 15/87, Method 6
Crude protein	1.26	1.37	Službeni Glasnik SFRJ, br. 15/87, Method 7
Crude fat	0.05	0.05	Službeni Glasnik SFRJ, br. 15/87, Method 12
Crude cellulose	58.05	62.94	Method VDM-111
Ash	0.42	0.46	Službeni Glasnik SFRJ, br. 15/87, Method 18
Nitrogen free extracts (NFE)	32.45	35.18	Službeni Glasnik SFRJ, br. 15/87, Method 20
Neutral detergent fiber (NDF)	71.12	77.11	Method VDM-118
Acid detergent fiber (ADF)	66.12	71.69	Method VDM-119
Lignin	16.54	17.93	Method VDM-119

Legende: *dPS-dry basis

Tab. 2 – Micro and macroeleements present in unmodified peach shell

Parameter	K	Na	Ca	Mg	Fe	Mn	Al
Amount (%)	0.089	0.042	0.14	0.031	0.016	<0.01	0.005
Parameter	P_2O_5	Pb	Ni	Cd	Zn	Cu	SO_3
Amount (%)	0.54	0.008	0.001	<0.01	0.0015	0.003	<0.02

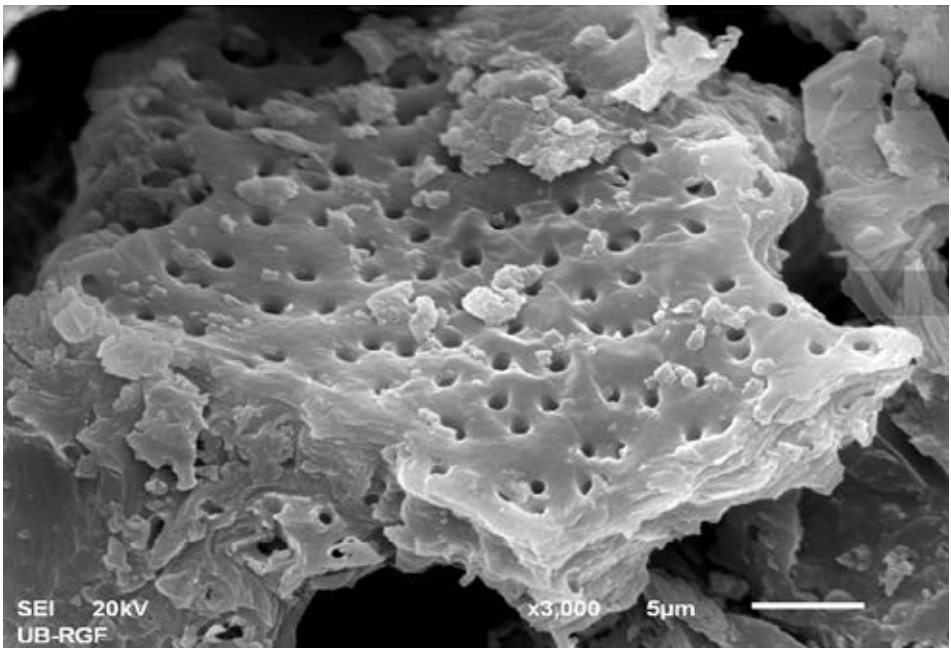

Fig. 1 – SEM micrograph of unmodified peach shell particles

Table 3 shows the adsorption indices of six mycotoxins by unmodified biosorbent – peach shell particles (PS) and peach shell particles modified by acid (MPS), calculated at two pH levels.

Tab. 3 – Adsorption indices of six mycotoxins in modified and unmodified peach shell particles at different pH values

Adsorbent	pH	Adsorption Index (%)					
		AFL	OTA	DON	ZON	DAS	T-2
Peach shell (PS)	*3.0*	58.82	42.86	23.08	50.00	0	25.00
	7.0	58.82	33.32	40.00	33.33	16.67	40.00
Modified peach shell (MPS)	*3.0*	41.18	42.86	40.00	33.33	16.67	50.00
	7.0	41.18	33.32	50.00	58.33	33.33	40.00

Data presented in Table 3 can provide better explanation by looking the graphs given in Figure 2 and Figure 3.

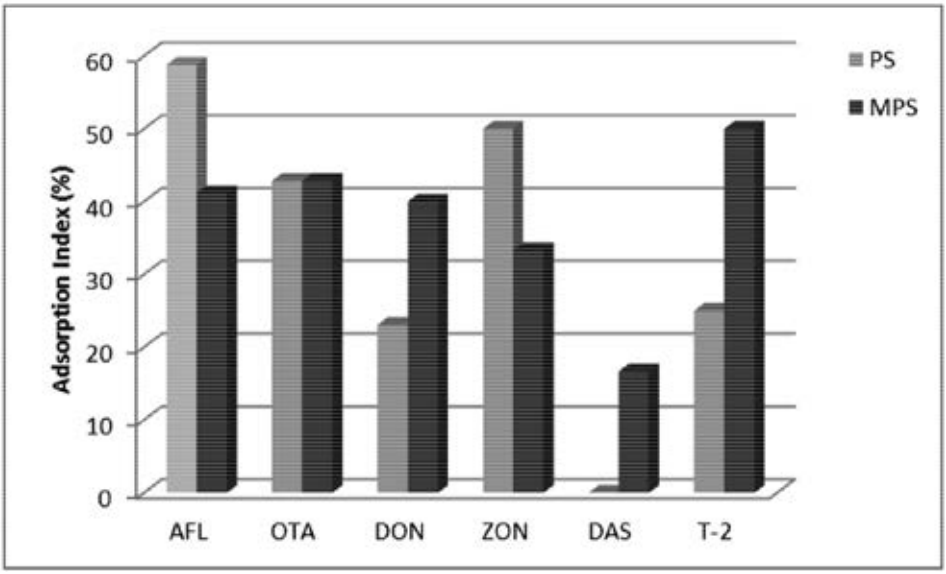

Fig. 2 – Comparison of adsorption indices (%) for modified and unmodified peach shell particles at pH 3.0

By using the TLC method, it was noted that unmodified peach shell bound more (58.82%) of applied AFL than the modified peach stones (41.18%); quantity of adsorbed AFL B1 was the same at pH 3.0 and pH 7.0 (Tab. 3). The effect of pH level on binding capacity of particular mycotoxin was quite different for other tested mycotoxins. In case of OTA, adsorption indices were the same for PS and MPS (42.86% at pH 3.0 and 33.32% at pH 7.0, respectively). Although binding of DON was observed in both analyzed samples, its adsorp-

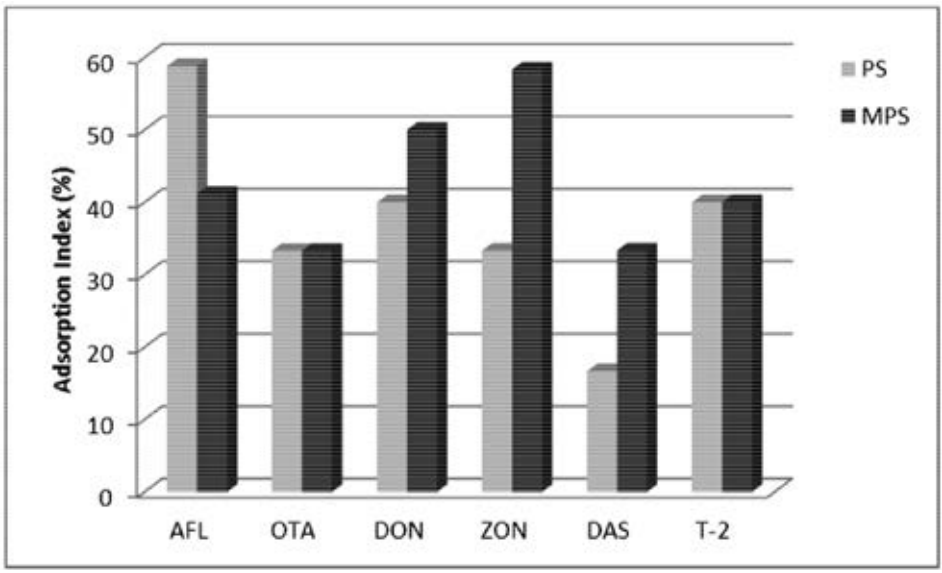

Fig. 3 – Comparison of adsorption indices (%) for modified and unmodified peach shell particles at pH 7.0

tion indices were higher in modified peach shell (40.0% at pH 3.0 and 50.0% at pH 7.0, respectively). Effect of electrolyte pH value on the binding of ZON was different for different samples; PS adsorbed more ZON at pH 3.0 (50.0%) while MPS adsorbed more ZON at pH 7.0 (58.33). In case of type A trichothecenes (DAS and T-2 toxin), PS bound DAS only at pH 7.0 (16.67%). Higher adsorption indices for the same fusariotoxin were obtained in modified PS (16.67% at pH 3.0 and 33.33% at pH 7.0, respectively). The amount of the bound T-2 toxin was the same at pH 7.0 (40.0%) but higher for MPS at pH 3.0 (50.0% compared with 25.0% in unmodified PS).

Peach stones produced from food industries as solid waste are often known as binding material in the form of ash or activated carbon, for example lead (R a s h e d, 2006) or aqueous ammonia (S o t t o-G a r r i d o et al., 2003), some textile dyes (G e r c e l et al., 2009), but not often as unmodified material that can adsorb heavy element ions (Cu) and radionuclide (uranium) (S t o j a n o v i ć et al., 2012). Numerous data about the adsorption capacity of agricultural waste materials for toxic fungal metabolites are not available in Serbia (B o č a r o v-S t a n č i ć et al., 2012a; B o č a r o v-S t a n č i ć et al., 2012b, S t o j a n o v i ć et al., 2012). In case of fusariotoxins, the presented results (Tab. 3) were similar to the results obtained from our previous investigation (B o č a r o v-S t a n č i ć et al., 2012b).

The application of different lignocellulose materials such as apricot stones as one of the ingredients of mycotoxin binding additive for food and animal feed, and which has at the same time fungistatic and bacteriostatic effect, is described in patent No. 20120070516 (www.faqs.org/pattents/app/20120070516). Beside

apricot stones, this additive also contains plant material from other prunus (e.g. prune, cherry, plum, almonds etc.) as well as plant extracts (e.g. herbal remedy, herbal extracts, powder, oil etc.). The resulting adsorbent can bind different mycotoxins, including OTA, DON, nivalenol and T-2, which are the mycotoxins difficult to be bound. According to the authors of the patent No. 20120070516, the ability of porous lignocellulosic materials to thermally collapse during melting can be used to adsorb mycotoxins irreversibly in wet system and then to entrap them after closing lignin pore structures under high temperature treatment.

As it can be seen from the presented results (Tab. 3), acid modification by 1M HCl changed the adsorption index for five out of six mycotoxins, leading in most cases to the improvement of removal, except in the case of aflatoxin B1, where decrease from 58.82 % to 41.18 % at both pH values occurred. In case of OTA, acid modification gave no changes in adsorption index at both pH values. In case of other four mycotoxins: DON, ZON, DAS and T-2, acid modification mostly improved the adsorption indices, except in the case of ZON for pH value of 7.0 where the decrease from 50.00% to 33.33% occurred.

It is not surprising that peach stones particles demonstrated ability for biosorption of mycotoxins **in vitro** conditions because of their rather high cellulose content (58.5%).

CONCLUSION

The results presented here indicate that the peach shell particles can be used as effective biosorbents of mycotoxins. Acid modification leads to the improvement of biosorption capacity in most cases, but further investigations should be performed in order to elucidate the nature of interaction between the biosorbent and specific mycotoxin.

Similar to other *in vitro* assays, the presented assay cannot completely simulate the conditions in gastro-intestinal tract of animals, so further *in vivo* experiments are necessary to assess the efficacy of peach stones and other waste materials as mycotoxin binders.

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IN VITRO ОЦЕНА КОШТИЦЕ БРЕСКВЕ КАО АДСОРБЕНСА МИКОТОКСИНА

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Резиме

Рад описује *in vitro* модел за процену способности коштице бресквe (немодификоване и модификоване), припремљене у Институту за технологију нуклеарних и других минералних сировина у Београду, да адсорбује различите микотоксине.

Коштице бресквe су набављене од „Вино Жупе" из Александровца, компаније где су одложене као отпадни материјал њихове Фабрике сокова. Након одговарајуће припреме, у испитивању су коришћене две врсте честица коштице бресквe: једна немодификована (PS) и друга добијена киселинском модификацијом (MPS).

In vitro методом је тестирана адсорпција шест микотоксина: афлатоксина Б1 (AFL), охратоксина А (OTA), деоксиниваленола (DON), зеараленона (ZON), диацетокисцирпенола (DAS) и Т-2 токсина. За експерименте адсорпције коришћени су сирови екстракти микотоксина произведени у Одељењу за микробиологију Био-еколошког центра у Зрењанину. Способност честица немодификоване и модификоване коштице бресквe за везивање микотоксина је оцењивана у електролиту 0,1 M K_2HPO_4 чија је pH вредност подешена на 3,0 односно 7,0. Масени однос појединачних микотоксина и узорака коштице бресквe је био 1:5000. Експерименталне смеше су инкубиране 1 сат на ротационој тресилици (185 о/мин) на собној температури (22-25 ^oC). После инкубације, екстракција неадсорбованих микотоксина из филтрата експерименталних смеша су извршене органским растварачима, а њихова квантификација методом танкослојне хроматографије.

КЉУЧНЕ РЕЧИ: коштице бресквe, киселинска модификација, адсорпција, *in vitro*, микотоксини

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