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Intestinal absorption of zearalenone and in vitro study of non-nutritive sorbent materials

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Abstract

Zearalenone is a mycotoxin produced by several members of the genus *Fusarium* that elicits oestrogenic effects on mammalian reproductive systems. Methods for an effective detoxification of contaminated feedstuffs are not currently available, but one of the new approaches to the problem is the addition of non-nutritive sorptive materials to the diets of animals in order to reduce gastrointestinal absorption of mycotoxins. The objectives of this study were to examine the intestinal absorption of zearalenone, to evaluate several sorbent materials for zearalenone affinity in vitro, and to select a potentially efficacious candidate for protection against zearalenone intoxication. In situ results obtained showed that the absorption of zearalenone in the rat small intestine follows first-order kinetics, with an absorption rate constant k_a, of 9.27 $\pm$ (0.69)/h. In vitro adsorption tests of zearalenone by selected materials showed that cholestyramine was the best adsorbent, followed in decreasing order by crospovidone, montmorillonite, bentonite, sepiolite and magnesium trisilicate. The Freundlich isotherm showed a better fit than the Langmuir isotherm. This could suggest the existence of a heterogeneous sorbent surface, the existence of different adsorption mechanisms or both. Results demonstrated that crospovidone was able to adsorb 313.7 μg zearalenone/g adsorbent, whereas montmorillonite, bentonite, sepiolite and magnesium trisilicate were able to adsorb 192.2, 112.4, 74.37 and 22.61 μg zearalenone/g adsorbent, respectively. Cholestyramine adsorption parameters were above these levels.

Keywords: Mycotoxin; Zearalenone; *Fusarium graminearum*; Intestinal absorption; Adsorbents; Adsorption isotherms

1. Introduction

Zearalenone is a phenolic ß-resorcylic acid lactone naturally produced by several members of the genus *Fusarium*, including *Fusarium graminearum* and *Fusarium roseum*. Of all the *Fusarium* species, *F. graminearum* is the major causative agent of zearalenone contamination of grains because it frequently infects grains used for feed and food, mainly corn and other cereals (Viñas et al., 1985; Trenholm et al., 1991), and is

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capable of producing large quantities of this mycotoxin (Eugenio et al., 1970; Merino et al., 1993).

This fusariotoxin elicits oestrogenic effects on mammalian reproductive systems and has specifically been associated with field cases of swine hyperoestrogenism (Rainey et al., 1991), including tumescence and oedema of the vagina, mammary gland enlargement, and swelling and reddening of the vulva. The role of zearalenone in vulvo-vaginitis and vaginal and rectal prolapses in prepubertal gilts is widely acknowledged (Kurtz and Mirocha, 1978). In addition, zearalenone causes anestrus, nymphomania, and reduced litter size in sexually mature swine (Dieckman and Long, 1987).

As an oestrogen, zearalenone is a luteotropic agent that has also been considered to play a role in infertility and abortions in animals in the first trimester (Miller et al., 1973), but does not cause abortion in the second or third trimester of gestation (Osweiler, 1990). Although zearalenone intoxication is not usually fatal, secondary bacterial infections with clinical signs such as vaginal and rectal prolapses can result in death (Kurtz and Mirocha, 1978).

Evidence of the carcinogenicity of zearalenone is, at present, not clear, and for this reason this mycotoxin has been placed for the moment in the category of "limited evidence" of carcinogenicity by the International Agency for Research in Cancer (International Agency for Research on Cancer, 1983). The possible role of zearalenone in carcinogenesis is uncertain since there is a great deal of evidence that it does not induce point mutation in somatic cells.

Methods to detoxify mycotoxin-containing feedstuffs on a large scale and in a cost-effective manner are not currently available. A large number of physical, chemical and biological methods for detoxifying mycotoxin have been reported (Ramos and Hernández, 1991), but these methods have been employed with limited success. One of the more encouraging approaches to solve the problem is the use of non-nutritive sorptive materials in the diet to reduce gastrointestinal absorption of mycotoxins. In vitro studies have shown that several sorptive materials can form highly stable complexes with some mycotoxins, like aflatoxins (Masimango et al., 1978; Phillips et al., 1988; Ramos and Hernández, 1990a,b; Maryamma et al., 1991) and ochratoxin A (Rotter et al., 1989), while dietary additions of zeolite, bentonite, kaolin, bleaching powders and a specific hydrated sodium calcium aluminosilicate (HSCAS) have been shown to reduce the in vivo toxic effects in farm animals of several mycotoxins like zearalenone (Smith, 1980), T-2 toxin (Carson, 1982; Smith, 1984) and aflatoxins (Phillips et al., 1988; Maryamma et al., 1991; Huff et al., 1992).

Cholestyramine is an anionic-exchange resin that is used by the pharmaceutical industry to bind bile acids in the gastrointestinal tract and to reduce plasma low density lipoprotein (LDL) and cholesterol (Neuvonen et al., 1988). This quaternary ammonium resin can also interfere with the gastrointestinal absorption of several molecules (Kivistö and Neuvonen, 1990), but no data are currently available on the ability of this resin to prevent the gastrointestinal absorption of mycotoxins.

Polyvinylpyrrolidone (PVP) is a highly polar, amphoteric soluble polymer. It is used in the cosmetic, food and textile industries, and in the medical and pharmaceutical field as an excipient. This compound forms complexes with a wide variety of substances. These complexes are insoluble in water or alcohol, but dissolve readily in caustic solutions, which means that the binding mechanism is of the hydrogen bound type (Adeyeye and Barabas, 1993).

The objectives of this study were: (i) to examine the intestinal absorption of zearalenone, (ii) to evaluate several sorbent materials, including the pharmaceutical drugs cholestyramine and insoluble polyvinylpyrrolidone, for zearalenone affinity in vitro, and (iii) to select a potentially efficacious candidate for protection against zearalenone intoxication.

2. Materials and methods

2.1. Organism and culture conditions

Fusarium graminearum ATCC 26557, a zearalenone producer strain kindly supplied by the Spanish Type Culture Collection (CECT, strain

no. 2150), was used in this study. Culture conditions and toxin production were carried out as previously described (Merino et al., 1993).

2.2. Zearalenone standards

A stock solution of pure crystalline zearalenone (Sigma Chemical Co., St. Louis, MO) in acetonitrile (200 μg/ml) was prepared. Then a solution in methanol was prepared from the acetonitrile solution, and the actual concentration of the toxin was determined by the AOAC procedure (Association of Official Analytical Chemists, 1990).

Standard solutions of the toxin at diverse concentrations were prepared by removing the adequate volume from the stock solution, thus evaporating the solvent to dryness under a gentle nitrogen stream and dissolving the residue with the appropriate volume of solvent.

2.3. Absorption experiments

Male Wistar rats weighing from 200 to 300 g were used. Twenty hours before the beginning of the absorption experiment rats were maintained without food, but water was available ad libitum. The in situ perfusion technique was employed (Doluisio et al., 1969), using the whole small intestine, adapted as previously described (Martin-Villodre et al., 1986; Merino et al., 1989a,b).

After rinsing with a physiological saline solution to eliminate faecal residues and debris, 10 ml of an isotonic solution of toxin at 4 μg/ml (pH of 6.5) were perfused into the small intestine at 37°C. Aliquots of 250 μl of sample were taken at 5, 8, 11, 14, 17 and 20 min, and the concentration of the remaining zearalenone was measured. Mean values from six animals were used to determine the absorption rate constant.

Water reabsorption was evaluated separately for each animal. This process follows apparent zero-order kinetics (Martin-Villodre et al., 1986; Gabus and Buri, 1987). To characterize the zero-order equation in each experiment, the volume remaining at 20 min was measured. After taking the last sample, this volume was assessed by separating the intestinal fraction and pulling out the residual liquid. The remaining volume at 0 min was evaluated, using five animals, as previously reported (Martin-Villodre et al., 1986).

With the mean remaining value at 0 min ($n = 5$) and the individual one at 20 min, a straight line was obtained. Thus, theoretical volumes at each sampling time were obtained and the remaining toxin concentration was properly corrected.

2.4. Absorption rate measurements

The zearalenone absorption rate constant, k_a, was estimated by fitting the remaining luminal toxin concentrations at the time of sample collection to first-order kinetics. For this purpose, we used the classical first-order equation:

$$A = A_o e^{-k_a t} \quad (1)$$

where A is the toxin concentration remaining in the intestinal lumen, A_0 is the theoretical concentration of solute at zero time, t is the time of sample collection, and k_a is the absorption rate constant. Both parameters (A_0 and k_a) were calculated according to a non-linear least squares regression.

A values were calculated by HPLC. Intestinal samples were centrifuged at 3000 rev./min for 10 min, and 10 μl of supernatant were injected into the chromatograph. Samples were analyzed using the HPLC conditions described in a previous paper (Merino et al., 1993).

A_0 was not exactly the initial concentration perfused because a very quick process of toxin adsorption to the intestinal mucosa occurred when the solution was introduced into the intestinal lumen (Doluisio et al., 1969). Therefore, only the A values obtained from 5 to 20 min were used for calculations, i.e. the zero time sample was not used for regression.

2.5. Adsorbents studied

Six adsorbents were evaluated in this study: bentonite (No. B-3378, Sigma Chemical Co., St. Louis, MO), cholestyramine resin (No. C-4650, Sigma Chemical Co., St. Louis, MO), insoluble polyvinylpyrrolidone or crospovidone (No. P-6755, Sigma Chemical Co., St. Louis, MO), magnesium trisilicate (No. 151796, Panreac,

Barcelona, Spain), montmorillonite (No. 28152-2, Aldrich-Chemie, Steinheim, Germany), and sepiolite (No. T 1-400, Tolsa S.A., Madrid, Spain).

2.6. Source of zearalenone for adsorption experiments

For the in vitro studies of adsorption, zearalenone was extracted from a sterile corn kernel medium inoculated with the *F. graminearum* strain, using a procedure previously described (Merino et al., 1993). The final chloroformic extract was completely evaporated under a gentle stream of nitrogen and redissolved in 1 ml of methanol. This extract was diluted to 100 ml with distilled water.

Using different amounts of this zearalenone-containing water, simulated intestinal fluids were made according to the United States Pharmacopeia (The United States Pharmacopeia, 1990). Final zearalenone concentration ranged from 10 to 1 μg/ml.

2.7. Adsorption experiments

An aliquot of 30 ml of the simulated intestinal fluid at a known concentration of zearalenone was added to a 40 ml centrifuge tube containing 1.5 g of the tested adsorbent. The tubes were shaken in a thermostatically controlled water bath at the 37 ($\pm$0.5)°C at a speed of 70 rev./min for the required equilibration time (60 min). A control without adsorbent was also carried out for each experiment to check for any change in zearalenone stability.

The suspensions were centrifuged for 10 min at 3000 rev./min, and a 20 ml subsample of the supernatant was extracted with 30 ml of chloroform; 5 ml of this extract was cleaned up and analyzed using the high pressure liquid chromatography method previously described (Merino et al., 1993). In brief, samples were cleaned up by applying the extract to a disposable silica cartridge and by eluting the toxin with a mixture of hexane/dry ethyl ether (5:5). Separation was achieved by a reverse phase μBondapak C_{18} column followed by fluorescence detection using an excitation wavelength at 274 nm and an emission wavelength at 440 nm. The detection limit was about 5 ng.

For each assay, a calibration curve using zearalenone standards in acetonitrile was made. These standard samples were applied to a Sep-Pak cartridge as they were problem samples, too. Thus, any loss originated by the cartridge was quantified. Recoveries ranging from 85.37 to 100.97% were obtained.

Peak areas from problem samples were interpolated in the calibration curve and zearalenone concentrations were obtained from supernatants. The amount of zearalenone joined to the sorbent used was obtained by the difference between the initial amount and remaining amount of zearalenone in the supernatant.

2.8. Fitting of models to data

Adsorption data were assayed to fit Langmuir and Freundlich isotherms. The Langmuir isotherm is expressed by the equation:

$$C_a = \frac{K_1 K_2 C_r}{1 + K_1 C_r} \tag{2}$$

where C_a is the amount of zearalenone sorbed per unit of weight of sorbent (μg/g), C_r is the concentration of unadsorbed zearalenone at equilibrium (μg/ml), k_1 is the affinity constant, and k_2 is the capacity of the adsorbent for the mycotoxin studied.

The Freundlich isotherm is expressed by the equation:

$$C_a = KC_r^a \tag{3}$$

where k is a constant related to the capacity of the adsorbent for zearalenone, and a is a constant related to the affinity of the adsorbent for zearalenone. Fittings were performed by means of the PCNONLIN 3.0 program (simplex algorithm).

The Akaike Information Criterion (AIC) (Akaike, 1986), sum of squares of residuals, *SS*, and correlation coefficients between theoretical and experimental values, r, were used to assess the goodness of the fits.

Table 1
Luminal disappearane of zearalenone and absorption constants. Percent zearalenone concentrations, relative to initial (4 μg/ml), remaining in the intestinal fluid at each sampling time are given. Absorption rate constants (k_a), intercept values at zero time (A_0) and correlation coefficients (r) are also shown

Sampling time (min)	Percentage remaining in intestinal lumen						Mean value (± S.D.)
	Experimentation animal number						
	1	2	3	4	5	6	
5	41.08	43.19	44.97	42.61	39.15	41.20	42.03 ± 2.01
8	28.33	27.03	27.21	26.27	24.04	25.62	26.42 ± 1.48
11	17.67	18.17	17.69	15.97	14.82	13.85	16.36 ± 1.77
14	11.6	12.51	9.77	9.95	9.18	9.12	10.36 ± 1.39
17	7.41	8.09	6.07	6.39	6.21	6.98	6.86 ± 0.79
20	4.86	6.04	4.27	4.26	3.76	3.95	4.52 ± 0.83
k_a (/h)	8.41	8.38	9.79	9.62	9.55	9.89	9.24
(± S.D.)	0.22	0.25	0.22	0.11	0.12	0.44	0.22
A_0 (%)	83.76	85.72	101.5	94.82	86.52	93.79	90.61
(± S.D.)	2.36	2.79	2.69	1.26	1.27	4.95	1.18
r (>)	0.999	0.998	0.999	0.999	0.999	0.997	0.999
Mean $k_a = (9.273 \pm 0.689)/h$							

3. Results

The average concentrations of zearalenone remaining in the intestinal lumen samples at each sampling time (A values, mean from six animals), expressed as the percentages of the initial perfused concentration, are given in Table 1. Absorption rate constants, k_a, and concentrations of zearalenone at the intercept, A_0, calculated according Eq. (1), are also given.

The Langmuir and Freundlich adsorption isotherms obtained for zearalenone (5.0%) at pH 7.5 are given in Fig. 1. In Table 2 the remaining zearalenone concentration and the amount of zearalenone sorbed per unit of weight of sorbent at each starting toxin concentration are shown. In Table 3, parameter values found for each model equation are given; statistical figures are also indicated.

4. Discussion

The main way to incorporate mycotoxins into the organism is orally by means of their joint incorporation into contaminated foods. Studies on the gastrointestinal absorption process of mycotoxins are of great interest because they give an idea of the bioavailability of these toxic metabolites.

For the first studies on absorption through cellular membranes, isolation of purified cellular

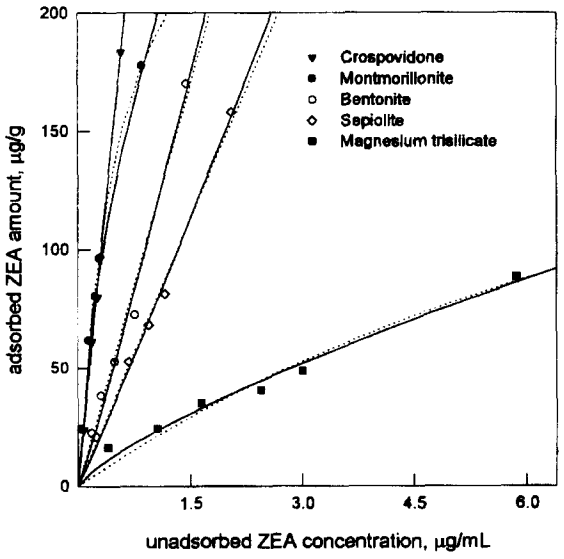

Fig. 1. Non-linear regression plots of the Langmuir (· · ·) and Freundlich (——) adsorption isotherms for zearalenone. The symbols represent the different adsorbents assayed.

Table 2
Unadsorbed zearalenone concentration at equilibrium (C_r, μg/ml) and amount of zearalenone sorbed per unit of weight of sorbent (C_a, μg/g) at each starting toxin concentration found for each adsorbent assayed at 5%

Initial concentration (μg/ml)	Adsorbents assayed											
	Cholestyramine		Crospovidone		Montmorillonite		Bentonite		Sepiolite		Magnesium trisilicate	
	C_a	C_r	C_a	C_r	C_a	C_r	C_a	C_r	C_a	C_r	C_a	C_r
1.293	25.86	N.D.	23.88	0.073	24.41	0.040	22.41	0.176	21.04	0.236	16.33	0.401
2.155	43.10	N.D.	–	–	–	–	38.30	0.310	–	–	24.25	1.047
3.341	66.82	N.D.	61.35	0.180	61.89	0.135	52.82	0.486	52.79	0.673	34.89	1.638
4.642	92.84	N.D.	79.98	0.261	80.45	0.225	72.54	0.756	68.14	0.943	40.41	2.445
5.237	104.7	N.D.	96.19	0.292	96.28	0.281			81.44	1.156	48.54	3.006
10.131	202.6	N.D.	183.4	0.577	177.9	0.849	170.4	1.445	158.1	2.046	88.43	5.862

N.D. denotes non-detected concentrations.

membrane pieces like vesicles, or the everted sac technique were used. With these methods, the integrity of the plasmatic membrane was broken and blood circulation was not taken into account.

We used an in situ technique which does not involve recirculation and prevents total or partial destruction of the aqueous diffusion layer adjacent to the luminal membrane. This layer is characteristic of gastrointestinal tract physiology, and therefore the methods used must preserve its physiological conditions. Moreover, this technique maintains the blood circulation in the gastrointestinal tract and the vital constants of the animal.

In our study we used a modification of the method developed by Doluisio et al. (Doluisio et al., 1969) which allows an accurate evaluation of the water reabsorption process and of the small intestine mycotoxin absorption through the luminal membrane.

The results obtained (Table 1) showed that intestinal absorption of zearalenone perfectly fits a first-order kinetics, with a mean correlation coefficient greater than 0.999. This indicates the passive nature of the process, as other authors using the everted sac gut technique have reported for aflatoxins (Thompson et al., 1992).

The absorption rate constants obtained showed very high k_a values, which suggest that this mycotoxin can be absorbed by passive diffusion through the small intestine at a very high rate. These elevated k_a, together with the toxic properties of this mycotoxin, made this fungal metabolite even more dangerous.

The mean k_a obtained is 9.27 ($\pm$0.69)/h (Table 1), that means that 1 h after the perfusion of the solution with the mycotoxin only 0.0095% of the zearalenone remained unabsorbed. The half-life absorption rate of zearalenone — that is, the time needed to reduce the zearalenone concentration in the intestinal lumen to a half — is only about 4.48 min. Given the intestinal passage time, these data show that complete zearalenone absorption takes place in the gastrointestinal tract.

The mean absorption rate constant obtained corresponds to the mean value of the absorption constants along the perfused area. Perfusion of different parts of the small intestine could give the k_a for each intestinal area assayed — that is, a different absorption rate for the duodenum, jejunum and ileum, as other authors have determined for aflatoxins using an in situ technique with recirculation (Kumagai, 1989).

Since zearalenone is able to cross the intestinal lumen at such a high rate, if we want to use sorbent compounds to avoid the gastrointestinal absorption of this mycotoxin, we need a compound able to form a stable mycotoxin-sorbent complex at a high rate, too. At the same time this compound must have a high affinity for this toxin

Table 3
Parameter values found according to the Freundlich and Langmuir isotherms for zearalenone at pH 7.5

Adsorbent assayed	Parameter value (S.D.)	
	Freundlich equation	Langmuir equation
Cholestyramine	N.D.	N.D.
Crospovidone	$a = (0.977 \pm 0.031$	$k_1 = (6.245 \pm 9.285)10^{-2}$ ml/μg
	$k = (313.7 \pm 9.545)$ μg/g	$k_2 = (5266 \pm 7551)$ μg/g
	$SS = 2.3552$	$SS = 2.3693$
	AIC = 12.283	AIC = 12.3130
Montmorillonite	$a = (0.593 \pm 0.029)$	$k_1 = (1.802 \pm 0.234)$ ml/μg
	$k = (192.2 \pm 4.367)$ μg/g	$k_2 = (292.6 \pm 20.62)$ μg/g
	$SS = 2.5137$	$SS = 2.8371$
	AIC = 12.609	AIC = 13.2140
Bentonite	$a = (1.077 \pm 0.092)$	$k_1 = (1.913 \pm 115)10^{-3}$ ml/μg
	$k = (112.4 \pm 4.707)$ μg/g	$k_2 = (59340 \pm 3510000)$ μg/g
	$SS = 3.7112$	$SS = 3.9183$
	AIC = 14.5569	AIC = 14.8283
Sepiolite	$a = (1.035 \pm 0.057)$	$k_1 = (1.020 \pm 5.077)10^{-2}$ ml/μg
	$k = (74.37 \pm 2.50)$ μg/g	$k_2 = (7488 \pm 320400)$ μg/g
	$SS = 2.8185$	$SS = 3.0076$
	AIC = 13.1811	AIC = 13.5058
Magnesium trisilicate	$a = (0.755 \pm 0.061)$	$k_1 = (8.264 \pm 4.584)10^{-2}$ ml/μg
	$k = (22.61 \pm 1.979)$ μg/g	$k_2 = (265 \pm 111)$ μg/g
	$SS = 2.8213$	$SS = 3.3134$
	AIC = 14.2233	AIC = 15.1878

to avoid a rupture of the complex allowing the absorption of this fungal metabolite.

To describe the different kinds of adsorption processes several mathematical approaches have been developed. The most useful one involves the study of the isotherm, in which the amount of compound sorbed per unit of weight of sorbent is plotted against the concentration of the compound in the external phase, at a fixed temperature and under equilibrium conditions. Langmuir and Freundlich isotherms are the most helpful and we therefore chose them to fit our results.

The results show that cholestyramine is the best of the assayed adsorbents for sequestering zearalenone from the medium. At a concentration of 5% (1 h shaking, pH 7.5, 37°C) this sorbent is able to adsorb zearalenone to levels that make it impossible to detect in the external phase with our analytical method, regardless of the initial concentration of the toxin (Table 2). Within our experimental design, calculation of adsorption isotherms for this compound is not possible, so that we can conclude that cholestyramine showed the highest affinity and capacity for zearalenone adsorption of the assayed compounds.

With regard to the other compounds, the best sorbent material was the insoluble polyvinylpyrrolidone, or crospovidone, followed in decreasing order by montmorillonite, bentonite, sepiolite and magnesium trisilicate, as can be seen in Fig. 1.

The Freundlich isotherm fits the data better than the Langmuir isotherm, as is demonstrated by the more homogeneous standard deviation of each parameter observed and by the lower sum of squares as well as the AIC values obtained (Table 3).

The better results obtained with the Freundlich isotherm suggest the presence of a heterogeneous sorbent surface (because of the existence of adsorption centres with different affinity for the adsorbate), the coincidence of different adsorption mechanisms or both things at the same time. In the assayed concentration range, a better fitting of

data to the Freundlich isotherm shows that most of the adsorbed molecules are filling the more active centres of the sorbent, far from saturation of the adsorbent material.

The results shown in Table 3 demonstrate that, according to the Freundlich isotherm, polyvinylpyrrolidone is able to adsorb 313.7 μg zearalenone/g adsorbent, whereas montmorillonite, bentonite, sepiolite and magnesium trisilicate are able to adsorb 192.2, 112.4, 74.37 and 22.61 μg zearalenone/g adsorbent, respectively. Cholestyramine adsorption parameters have not been evaluated but the levels are similar.

Addition of cholestyramine or crospovidone to the initial composition of feedstuffs would efficiently prevent the mycotoxicosis originated by zearalenone by inducing the formation of a zearalenone-cholestyramine complex that impedes the gastrointestinal absorption of this toxic metabolite. Complete in vitro and in vivo studies are needed to determine the stability of the complex formed in the gastrointestinal tract. Similar in vitro studies have been done by other authors on the complexes formed between adsorbents and aflatoxins (Ramos, 1994). The in vivo studies needed to establish the innocuousness of these complexes, with long-term experiments, will be similar to those performed by other authors working on aflatoxin adsorption (Sova et al., 1991; Scheideler, 1993). The possible interferences with mineral and essential nutrient absorption must be kept in mind. In acute intoxications, cholestyramine at adequate doses could be used as an antidote against zearalenone poisoning.

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