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Development of an in vitro method for the prediction of mycotoxin binding on yeast-based products: case of aflatoxin B_1, zearalenone and ochratoxin A

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Abstract To date, no official method is available to accurately define the binding capacity of binders. The goal is to define general in vitro parameters (equilibrium time, pH, mycotoxin/binder ratio) for the determination of binding efficacy, which can be used to calculate the relevant equilibrium adsorption constants. For this purpose, aflatoxin B_1 (AFB_1), zearalenone (ZEA) or ochratoxin A (OTA) were incubated with one yeast cell wall in pH 3, pH 5 or pH 7 buffers. The percentage of adsorption was recorded by quantitation of remaining mycotoxins in the supernatant and amount of mycotoxin adsorbed on the residue. The incubation of yeast cell wall in the presence of mycotoxins solved in buffer, lead to unexpected high adsorption percentage when the analysis was based only on remaining mycotoxins in the supernatant. The decrease of mycotoxins in the supernatant was not correlated to the amount of mycotoxins found in the residue. For this reason we modified the conditions of incubation. Yeast cell wall (5 mg) was pre-incubated in buffer (990 μl) at 37 °C during 5 min and then 10 μl of an alcoholic solution of mycotoxin (concentration 100 times higher than the final concentration required in the test tube) were added. After incubation, the solution was centrifuged, and the amount of mycotoxins were analysed both in the supernatant and in the residue. A plateau of binding was reached after 15 min of incubation whatever the mycotoxins and the concentrations tested. The adsorption of ZEA was better at pH 5 (75 %), versus 60 % at pH 3 and 7. OTA was only significantly adsorbed at pH 3 (50 %). Depending on the pH, the adsorptions of OTA or ZEA were increased or decreased when they were together, indicative of a cooperative effect.

Keywords Binder · Aflatoxin · Ochratoxin · Zearalenone · Yeast · In vitro screening method

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Introduction

Mycotoxins are fungal secondary metabolic products growing on a variety of crops. These compounds pose a potential threat to human and animal health through the ingestion of food or feed Yannikouris and Jouany (2002). Aflatoxin B_1 (AFB_1) is a potent human carcinogen (group 1) (International Agency for Research on Cancer [IARC] 1993) mainly produced by *Aspergillus flavus* and *A. parasiticus*. Aflatoxin contamination occurs frequently in agricultural products, particularly in maize and groundnuts. This mycotoxin is a potent liver toxin that can be lethal when consumed in large amount, and it induces cancer by chronic exposure. Ochratoxin A (OTA) produced by several *Aspergillus* and some *Penicillium* species (Varga et al. 2003; Ostry et al. 2013), is a main contaminant of

cereals (corn, barley, wheat) and to some extent beans (coffee, soy, cocoa). The IARC rated OTA as a possible carcinogen (group 2B) (IARC 1993). The presence of OTA in several commodities (feed, food and beverages) is considered as a serious health hazard in view of its nephrotoxic, teratogenic, hepatotoxic and carcinogenic properties (Varga et al. 2001; Pfohl-Leszkowicz and Manderville 2007, 2012). Zearalenone (ZEA), produced by numerous *Fusarium* species, exhibits an estrogenic activity (Mirocha et al. 1971). It contaminates grains (wheat, barley, sorghum and corn) and fruits. ZEA is frequently implicated in reproductive disorders or physical changes in genital organs of farm animals (Zinedine et al. 2007; Fink-Gremmels and Malekinejad 2007).

Primary strategies to reduce the risk of mycotoxin contamination include good agricultural practices in the field (crop rotation, soil cultivation, weed and insect control, careful use of fungicides) and upon harvest, as well as transportation and storage under dry and cool conditions (Jouany 2007). However, complete avoidance of mycotoxins is not possible.

Several approaches have been investigated to reduce the risk of mycotoxicosis in livestock (Jouany 2007; Kolosova and Stroka 2011). Biological, chemical, or physical treatments can minimize toxin production and eliminate contaminants in food and feed. The most applied method to prevent mycotoxicosis in animals consists in the addition of adsorbents to animal feed, in order to bind the mycotoxins in the gastro-intestinal tract (Regulation EC No. 386/2009). The efficacy of binders appears to depend on the chemical structure of both the adsorbent and the mycotoxin. The most important feature is the physical structure of the adsorbent, i.e., the total charge and charge distribution, the size of the pores and the accessible surface area (Di Natale et al. 2009). On the other hand, the properties of the adsorbed mycotoxins, like polarity, solubility, shape and charge distribution, also play a significant role (Huwig et al. 2001; Avantaggiato et al. 2005).

Silicates are the most widespread commercially available feed additives for mycotoxins binding. Mineral adsorbents such as clays or activated carbons have been extensively tested for their potential to bind aflatoxin and impair their gastrointestinal absorption (Kabak et al. 2006). However, these compounds are relatively inefficient toward others mycotoxins and can also impair the absorption of some micronutrients (Huwig et al. 2001).

Organic adsorbents such as yeast cell wall (YCW) both in vitro and in vivo have shown much larger sorption capabilities across a wider spectrum of mycotoxins (Binder 2007; Huwig et al. 2001; Jouany 2007; Yiannikouris et al. 2004, 2006, 2013; Karaman et al. 2005; Kabak et al. 2006; Kabak and Dobson 2009; Shetty and Jespersen 2006; Shetty et al. 2007; Ringot et al. 2007; Sabater-Vilar et al. 2007; Korosteleva et al. 2007; Matur et al. 2010; Firmin et al. 2010, 2011; Joannis-Cassan et al. 2011). *Saccharomyces cerevisiae* occurs as part of natural microbial population in food fermentation and as starter cultures in the food and beverage industries. Yeast glucans are major cell wall components often present as the inner wall layer and associated with other cell wall components such as chitin (together, they represent 50–60 % of the wall dry weight) which insured the mechanical strength (physical protection and osmotic support). The outer cell wall layer is made of heavily glycosylated mannoproteins, which are involved in cell–cell recognition events and limitation of wall porosity (Kollar et al. 1997; Manners et al. 1973; Zekovic et al. 2005).

Adsorption on the cell wall surface is an interaction between the toxins and functional groups of the cell surface, based on physical adsorption, ion exchange and complexation. The cell walls harbouring polysaccharides (glucan, mannan), proteins and lipids exhibit numerous different and easily accessible adsorption centers as well as different binding mechanisms (i.e., hydrogen bonds, ionic or hydrophobic interactions; Huwig et al. 2001; Ringot et al. 2007). The mannoproteins contain hydrophobic domain enabling hydrophobic interactions to occur with OTA especially at the pH of the YCW adsorption system (pH 3) where OTA is mainly in its non-ionized form (Ringot et al. 2005). The measurement of enthalpy/entropy of OTA adsorption on YCW suggested a possible role of the phenylalanine moiety of OTA (Ringot et al. 2005). OTA biosorption onto YCW involves both polar and non-polar non-covalent interactions and the concomitant reorganization of the water molecules of the solvent. The non-polar interactions involve the aromatic rings of OTA and hydrophobic amino acids of yeast. The polar interactions can be explained in different complementary ways: (1) electrostatic ionic interactions involving carboxyl group of OTA and basic amino acids of YCW, (2) electrostatic π–π interactions involving aromatic ring of OTA and aromatic amino acids of YCW, (3) hydrogen bounds of OTA phenol and amide group as donor and acceptor group of YCW, (4) hydrogen bounds of OTA involving aromatic rings as acceptors interacting with donor groups of YCW (Ringot et al. 2005).

The beta-glucan fraction of YCW is directly involved in the binding strength (Jouany 2007; Shetty and Jespersen 2006; Yiannikouris et al. 2004). Carvet et al. (2010) tested different β-glycans and showed that the bound type (1–3 or 1–6) or ramification might not be sufficient to characterize their adsorption properties. The binding efficiency is a more quantitative phenomenon (large surface area) than a qualitative phenomenon (chemical structure involved in the interaction), and in this sense the analysis of the glucomannan and peptidoglycan chemical structures did not indicate major differences in the capacity to produce hydrophobic interactions or hydrogen bonds, which are presumably involved in the binding (Pizzolitto et al. 2011). The interaction between β-D-glucans and the mycotoxins is driven by steric complementarities enabling a marked involvement of van der Waals interaction causing some stacking effects as well as stable

intermolecular hydrogen bonding involving the hydroxyl, lactone, and ketone groups commonly found on mycotoxins. (1–3)- β-D-Glucans were involved in both mechanisms, whereas (1–6)-β-D-glucans seemed to strengthen the van der Waals bonds and consequently to strongly stabilize the toxin–glucan interaction. However, the mycotoxins were not all equivalent in their ability to bind with β-D-glucans. Also, the environmental conditions such as pH were determining for the stability of the toxin–glucan complexes generated. The stereo-chemistry and hydrophobic properties of mycotoxins are of prime importance and account for the differences in their affinity for β-D-glucans (Yiannikouris et al. 2006). The cell diameter/cell wall thickness relation showed a correlation between cell wall amount and mycotoxin removal ability (Armando et al. 2012).

The obvious advantage of in vitro model is the possibility to rapidly screen the efficacy of high numbers of different substances enabling a pre-selection of products.

The experimental conditions for in vitro experiments reported in literature are often not sufficiently described to be reproduced or the designs are very different (e.g., pH, filtration versus centrifugation for binder/toxin separation, one single concentration or equilibrium point, percentage of binder, concentration of the toxin, incubation parameters)impeding comparison of the results. Until now, no official method is available to accurately define the adsorption capacity of yeast products. Only some guidelines have been recently published (EFSA 2009). The aim of this work was to test different conditions to define a protocol which is reproducible. In the end, as a validation of the protocol several yeast-based products were tested for their ability to bind together AFB_1, OTA and ZEA.

Material and methods

Binders

The binding material used to develop the in vitro procedure was YCW from baker industry called Y0. This YCW contains 27.1 g/100 g of proteins, 14.5 g/100 g of lipids, 27.1 g/100 g of mannans, 20.1 g/100 g of glucans, with a ration Mannans/Glucans of 1.08. Eight other binders including inactivated *yeasts* and *yeast* cell walls from baker and brewer yeast industries have been used to validate the new protocol. The products were labelled with a letter code (Y1 to Y8), and some of their characteristics including proteins contents and lipids are summarized in Table S1.

Chemicals

AFB_1, OTA, ZEA were supplied by Sigma-Aldrich (France). All chemicals used were of analytical grade unless otherwise stated. All solvents (HPLC grade) were purchased from ICS (France).

The binding of mycotoxins was studied under three different pHs: pH 3 (citrate buffer), pH 5 (acetate buffer) and pH 7 (phosphate buffer). These pHs were chosen to determine the effects of pH on mycotoxin binding within the range found in the gastrointestinal tract.

Preparations of the buffers

Citrate buffer (pH 3), 0.1 mol/l: 4.27 g of tri-sodium citrate 2-hydrate ($C_6H_5Na_3O_7$–$2H_2O$, MW=294.1) were dissolved in approximately 900 ml of distilled water. Then, the solution was adjusted to pH 3 with 17.96 g of citric acid ($C_6H_8O_7$–H_2O, MW=210.13, d=1.5) and filled up to 1,000 ml with distilled water.

Acetate buffer (pH 5), 0.1 mol/l: 13.608 g of sodium acetate 3-hydrate ($C_2H_3NaO_2$–$3H_2O$, MW=136.08) were dissolved in approximately 900 ml of distilled water. Then, the solution was adjusted to pH 3 with acetic acid ($C_2H_4O_2$, MW=60.05, d=1.05) and filled up to 1000 ml with distilled water.

Phosphate buffer (pH 7), 0.1 mol/l: a 0.1 mol/l solution of sodium di-hydrogen phosphate 2-hydrate was prepared by dissolving 15.601 g of NaH_2PO_4–$2H_2O$ (MW=156.01) in 1,000 ml of distilled water. A 0.1 mol/l solution of di-sodium hydrogen phosphate 12-hydrate was prepared by dissolving 35.814 g of Na_2HPO_4–$12H_2O$ (MW=358.14) in 1,000 ml of distilled water. Then, the Na_2HPO_4 solution was adjusted to pH 7 by adding the NaH_2PO_4 solution.

Incubation conditions for optimisation of the method

Preliminary tests/evaluation of the relative concentrations mycotoxin/yeast both solved in buffers

The YCW numbered Y0 (1, 5, 50 mg/ml) was incubated during 90 min at 37 °C in the presence of AFB_1 (2 μg/ml) or ZEA (20 μg/ml) dissolved in the buffers. The adsorption was compared to control tube containing buffer (pH 3, 5, 7) and either 2 μg of AFB_1 or 20 μg of ZEA solved in the buffer (final volume=1 ml). After 90 min of incubation at 37 °C under shaking, the mixture was centrifuged 10 min at 9,200×g. The amount of unbound mycotoxins was determined without any extraction directly in the supernatant and the adsorbed mycotoxin was determined after extraction in the residue. Data are expressed as the mean value of three incubations per conditions.

Kinetic of adsorption

YCW (Y0, 5 mg) was pre-incubated during 5 min in the presence of 990 μl of buffer (pH=3, 5, 7). Then 10 μl

mycotoxins solved in methanol (100 times higher than final concentration required in the test tube) were added. The amounts of mycotoxins in 1 ml (final volume) were 0.5 or 10 μg for either AFB_1 or OTA; 1 or 50 μg for ZEA. The mixture was shaken from 0 to 90 min at 37 °C, and centrifuged as described above. The amount of unbound mycotoxins was determined without any extraction directly in the supernatant and the adsorbed mycotoxin was determined after extraction in the residue. Data are expressed as the mean value of three incubations per conditions.

Equilibrium point

YCW (Y0, 5 mg) was pre-incubated for 5 min in the presence of 990 μl of buffer (pH=3, 5, 7). Then increasing amounts of mycotoxins solved in methanol (10 μl) were added. The amounts of OTA tested in 1 ml were 0.05, 0.5, 2, 5, 10 μg; and for ZEA 1, 5, 20, 50, 80 μg. The mixture was shaken 15 min at 37 °C and centrifuged 10 min at 9,200×*g*. The amount of unbound mycotoxins was determined without any extraction directly in the supernatant and the adsorbed mycotoxin was determined after extraction in the residue.

Simultaneous binding of the three mycotoxins

YCW (Y0, 5 mg) was pre-incubated during 5 min in the presence of 990 μl of buffer (pH 3, 5). Then, 10 μl of methanol mycotoxin solution (concentration 100 times higher than final concentration requited in the test tube) was added. Two different mixtures of mycotoxins were tested: (1) ZEA (1 μg/ml)+OTA (0.5 μg/ml)+AFB_1 (0.5 μg/ml); (2) ZEA (20 μg/ml)+OTA (2 μg/ml)+AFB_1 (2 mg/ml). The mixture was shaken15 min at 37 °C and centrifuged 10 min at 9,200×*g*. The amount of unbound mycotoxins was determined without any extraction directly in the supernatant and the adsorbed mycotoxin was determined after extraction in the residue. Data are expressed as the mean value of three incubations per conditions.

Evaluation of binding capacities of eight yeast-based products compared to Y0

To validate the new method, comparison of binding capacities of eight yeast-based products (Y1-Y8) against AFB_1, OTA, ZEA — alone or in combination — was done and compared to Y0 (called Y9) as described in the following paragraphs.

Five milligrams of each yeast-based product was pre-incubated during 5 min in the presence of 990 μl of buffer pH 3 (citrate buffer). Then, 10 μl of methanol mycotoxin solutions (at concentration 100 times higher than the final concentration required in the test tube) was added.

The amount of ZEA was 20 μg/ml; the amount of AFB_1 or OTA was 0.5 μg/ml. Two different mixtures of mycotoxins were tested: (1) ZEA (20 μg/ml)+AFB1 (0.5 μg/ml); (2) ZEA (20 μg/ml)+AFB1 (0.5 μg/ml)+OTA (0.5 μg/ml)

The mixtures were shaken 15 min at 37 °C and centrifuged 10 min at 9,200×*g*. The amount of mycotoxins was determined in the supernatant without any extraction and on the residue after extraction. The % of binding were calculated using the both results, taking as reference the amount of mycotoxin engaged and measured in the control tube. Activated carbon was tested as reference binding substance for each mycotoxin. Data are expressed as the mean value of three incubations per conditions.

Extraction of mycotoxin from residue

Extraction of OTA

OTA was extracted from residue by 900 μl of the following mixture $MgCl_2$ (0.2 M)/HCL (1 M) vol/vol. Chloroform (900 μl) was added to the mixture and shaken for 10 min. After 10 min of centrifugation at 4 °C, and 10,000×*g*, the aqueous phase was isolated. The chloroform phase (at the bottom of the tube) containing the mycotoxins was taken off and kept in a new tube for following purification. Again, 900 μl of chloroform was added to the aqueous phase, shaken for 10 min, and centrifuged for the collection of the chloroform phase, which was added to the previous one. The chloroform phase was evaporated to dryness. The dry extract was dissolved in 1 ml methanol and put in ultrasonic bath for 1 min. The solution was filtered on a filter SPARTAN 0.2 μm. Before adding the solution, the filter received 500 μl of methanol. After the solution pass through the filter, 500 μl methanol was added again. The methanol was evaporated to dryness under nitrogen. Finally, the extract was solved in 300 μl of methanol, stored in glass tube at minus −20 °C until HPLC analysis.

Extraction of AFB_1

AFB_1 was extracted by addition of 300 μl of methanol to the yeast residue and shaken10 min. The mixture was centrifuged at 9,200×*g* for 10 min, at 4 °C. The supernatant was taken off for analysis by HPLC.

Extraction of ZEA

ZEA was extracted by 300 μl of mixture water/acetonitrile (AcN) (1/3). The mixture was shaken for 10 min, and then centrifuged at 9,200×*g* during 10 min. The supernatant was recovered and used for HPLC.

Chromatographic conditions

Mycotoxin concentration was analysed by HPLC with fluorescence detection. Unbound mycotoxin was analysed using 20 µl of the supernatant. Bound mycotoxin was analysed using 20 µl of the extract. HPLC system (ICS, France) was equipped with an injector 20 µl loop, a C18 spherisorb column (Prontosil; 25×0.4 cm) with inner porosity of 3 µm, and a fluorescence detector Shimadzu Fluorescence Detector RF-10AXK. The HPLC was run in a thermostatic room (25 °C).

Zearalenone analysis

The mobile phase was acetonitrile/water (70:30, v/v) at a flow rate of 0.5 ml/min. The spectrofluorimetric conditions were of 275 nm for ZEA excitation and 450 nm for emission.

Ochratoxin A analysis

The mobile phase for separation of OTA was methanol/acetonitrile/natrium acetate 0.005 M (0.68 g/l of water) (300:300:400, v/v/v) at a flow rate of 0.5 ml/min. The spectrofluorimetric conditions for OTA were 330 nm for excitation and 465 nm for emission.

Aflatoxin B_1 analysis

AFB_1 was detected after derivatization in Kobra® cells. The mobile phase was methanol/acetonitrile/water (200:200:600, v/v/v) added with 119 mg/l of kalium bromide and 350 µl/l of nitric acid 4 M, at a flow rate of 0.5 ml/min. The spectrofluorimetric conditions for AFB_1 were 362 nm for excitation and 425 nm for emission.

Separation for simultaneous evaluation of the three toxins

To separate in one run AFB_1, OTA and ZEA, HPLC was run using gradient conditions.

> Phase A: methanol/acetonitrile/water (200:200:600) + ammonium formate 0.34 g (6.5 mM). The pH was adjusted by addition to formic acid till pH 3.5.
> Phase B: methanol/acetonitrile/water (350:350:300) + ammonium formate 0.34 g (6.5 mM). The pH was adjusted by addition to formic acid till pH 3.5.

The gradient was run as follows: T0min 100 % A, T15min 100 % A, T25min 65 % A, T40min 65 % A, T60min 100 % B (0 % A), T70min 100 % B, T75min 100 % A.

The elution times were 27.5 min for AFB1, 60.7 min for ZEA, and 61.9 min for OTA (see Fig. S1).

Quantitation of the percentage of mycotoxins bound

Percentage of adsorption was calculated either using amount of remaining mycotoxin in the supernatant or using mycotoxin bound on the residue.

$$\% \text{ binding } = [(C_i - C_s)/C_i] \times 100,$$

where C_i is the initial amount of mycotoxin; Cs the amount of mycotoxin in the supernatant

$$\% \text{ binding } = C_r/C_i \times 100,$$

where Cr is the amount extracted from the residue.

Statistical analysis

For the comparison of binding capacities of several yeast-based products when mycotoxins are in binary or ternary mixture, the results are expressed as means±SD. Data were analysed by one way analysis of variance, and multiple comparisons of each treatment were calculated by applying the Tukey test (Keppel 1973).

Results

Development of an in vitro screening method for mycotoxin binding

Evaluation of the best relative concentrations of binder and mycotoxin

The physical properties of mycotoxins like polarity, solubility, size, specific shape and in the case of ionized compounds, charge distribution and dissociation constants play a significant role in the binding processes. For this reason, the binding tests have been done at different pHs to mimic the gastrointestinal tract.

Binding capacity of YCW against 2 µg of AFB_1 or 20 µg of ZEA dissolved in buffer was tested using increasing amount of YCW in different buffers. The final volume of incubation was 1 ml. Control tube contained only AFB_1 or ZEA in buffers and was incubated in the same conditions to test the stability of the mycotoxin in the buffer. After 90 min of incubation tubes were centrifuged and the amount of AFB_1 or ZEA in the supernatant and in the residue were analysed.

Based on remainingAFB_1 in the supernatant compared to the control tube, no adsorption could be observed with 1 mg of yeast-based product, whereas it seems that 50 mg of YCW was highly efficient to adsorb 2 µg of AFB_1 (Fig. 1). Nevertheless, analysis of AFB_1 in the residues did not show

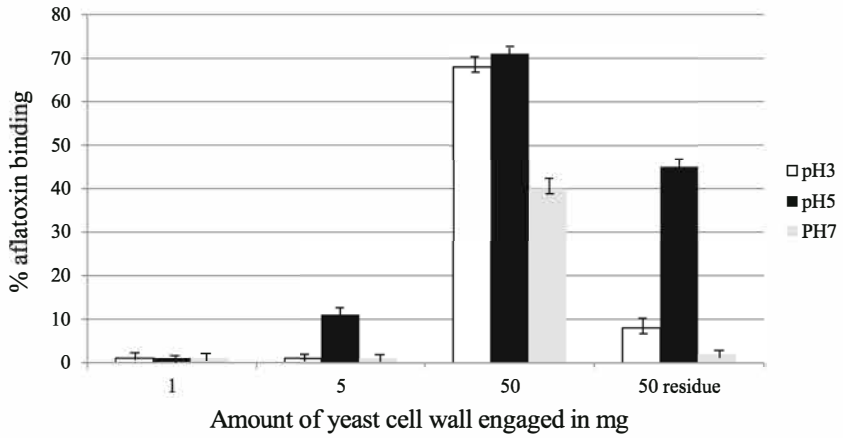

Fig. 1 Binding efficacy of 2 μg/ml AFB_1 by increasing amounts of yeast cell wall (Y0) in different buffers: pH 3 (*white*); pH 5 (*black*); pH 7; (*grey*). The data are expressed as percentage of binding calculated on amount of ABF_1 remained in the supernatant in the presence of 1, 5 or 50 mg/ml yeast; or based on mycotoxin bound on yeast cell walls (50 mg/ml)

relevant AFB_1 adsorption at pH 3 and pH 7 with this high amount of YCW. Indeed, the percentage of adsorption calculated on AFB_1 extracted from the residue was 8 %, 45 % and 2 % at pH 3, pH 5 and pH 7, respectively.

Concerning ZEA, the % of adsorption increased with the amount of YCW. With 50 mg/ml of yeast whatever the pH, again it seems that almost 100 % of ZEA were adsorbed. Nevertheless, based on ZEA in residue the % of adsorption is about 60 % at pH 3; 80 % at pH 5; 55 % at pH 7.

Influence of the pH on fluorimetric response of mycotoxin

The reason of the unexpected high adsorption seen above is due to the fact that large amount of YCW (50 mg/ml) modified the pH of the buffer which was dramatically lowered (Table 1).

The fluorimetric response of AFB_1 but also of ZEA was lower in acidic condition (Fig. 2), and thus virtually less mycotoxin was detected when they are analysed directly on supernatant leading to a misinterpretation on high adsorption. In contrast in the same conditions (2 μg/ml OTA; 50 mg/ml YCW) a lower % of adsorption as expected compared to OTA found in the residue was observed. Acidification of media by YCW in the case of OTA, virtually increases the amount of residual OTA in supernatant because fluorimetric response is higher at pH 3 compared to pH 5 or pH 7 for OTA (see Fig. S2).

Using 5 mg YCW/ml, the pH of the buffer was stable whatever the buffer (Table 1).

Table 1 Stability of pH in the presence of yeast (50 or 5 mg/ml)

Initial pH	Water	pH 3	pH 5	pH 7
Without yeast	6.5	3	5	7
With yeast (Y0) (50 mg/ml)	1.63	2.5	4.08	5.5
With yeast (Y0) (5 mg/ml)	4.5	3.1	4.9	6.99

For all of these reasons, further experiments have been done with 5 mg of YCW pre-incubated 5 min in the buffer followed by the addition of a minimal volume (10 μl) of mycotoxin solved in methanol in a final volume of 1 ml buffer.

Evaluation of the best incubation time and the stability of binding

Figure 3 shows the kinetic of binding of ZEA. Whatever the mycotoxins and the concentrations of mycotoxin, the adsorption equilibrium was reached before 15 min, with almost no change after longer incubation times. The adsorption of ZEA was better at pH 5 than at pH 3 or 7 (Fig. 3). The maximum of adsorption reached 80 % at pH 5 and 60 % at pH 3, whatever the concentration of ZEA. The adsorption of OTA was around 50–60 % for 0.5 μg/ml in buffer pH 3 (Fig. 4). At pH 5, the adsorption of OTA was negligible. No adsorption of OTA could be observed in pH 7.

As a plateau was reached after 15 min of incubation, the following experiments have been done with 15 min incubation time.

Equilibrium points at different pH values/validation step of the in vitro method

To check the best pH and validate concentration ranges, increasing amounts of mycotoxins were incubated with fixed concentration of binder (5 mg) during 15 min at 37 °C. The binding capacities were calculated using both the amount of mycotoxin remaining in the supernatant and the amount of mycotoxins bound on the residue.

As no binding of AFB_1 can be observed with Y0, equilibrium point has been evaluated only with ZEA and OTA. Adsorption equilibrium was established when the quantity of the toxin being adsorbed (Q_{eq}) was equal to the quantity being desorbed. Then, the equilibrium concentration in solution (C_{eq}) remained constant. Whatever the pH, the isotherm curves drawn using mycotoxins in the supernatant, are similar

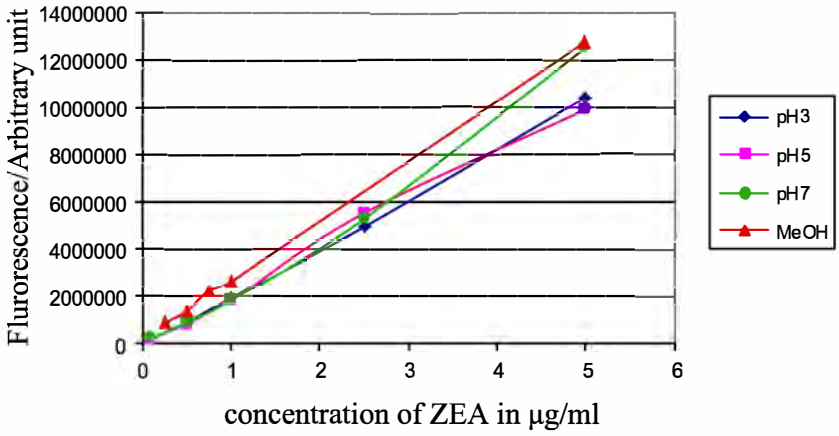

Fig. 2 Calibration curves and fluorescence signal of ZEA solved in different buffers: *red triangle*: methanol, *blue diamond*: buffer pH 3; *pink square*: buffer pH 5; *green circle*: buffer pH 7

to curves drawn using mycotoxins in the residue (see Figs. S3 and S4). This lead to the conclusion that evaluation could be done using only the amount of mycotoxin in the supernatant or both data can be mixed.

For ZEA whatever the concentration, the percentage of binding was around 60 % at pH 3 or pH 7, and 70 % at pH 5. The saturation point was not reached even with 80 µg/ml (Fig. 5). The % of binding of OTA increased with the concentration of ochratoxin from 0.05 to 5 µg/ml. Whatever the concentration, the OTA binding was better at pH 3 than at pH 5, and no adsorption occurred at pH 7. A saturation of binding was reached at 5 µg/ml (Fig. 6).

Simultaneous binding of ZEA and OTA

To check if the presence of one mycotoxin can modify the adsorption of another, ZEA (1 or 20 µg/ml) and OTA (0.5 or 2 µg/ml) were incubated together with 5 mg of Y0 in buffer pH 3 and pH 5. The binding of low concentration of ZEA (1 µg/ml) in the presence of OTA was significantly reduced (50 % vs. 60 %) at pH 3 ($p<0.05$) and slightly decreased (68 % vs. 75 %) at pH 5. In contrast with higher amount of ZEA (20 µg/ml), the binding of ZEA at pH 3 was significantly higher ($p<0.01$) in the mixture (80 % vs. 60 %) whereas at pH 5, the binding was similar when ZEA was alone or in mixture (Fig. 7). Concerning OTA, the presence of low concentration of OTA (0.5 µg/ml) in the mixture favoured the adsorption of this toxin on YCW whatever the pH. For higher concentration (2 µg/ml), at pH 3 the adsorption was similar, and a trend to decrease was observed at pH 5. For AFB, the presence of the other mycotoxins decreased the adsorption of low concentration of AFB_1 at pH 3 (18 % vs. 30 %). The decrease was significant ($p<0.01$) at pH 5 (5 % vs. 22 %). In contrast for high concentration, the adsorption of AFB_1 was similar at pH 3 (about 20 %) and a decrease at pH 5 was observed (5 % vs. 10 %).

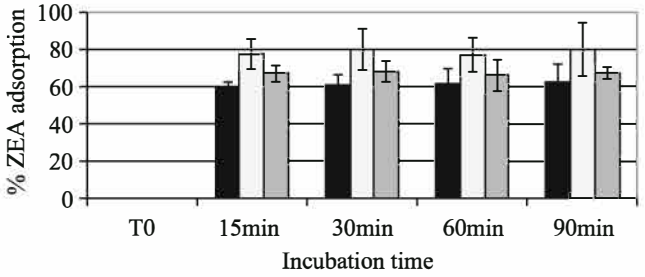

Fig. 3 Binding kinetic of ZEA. Data are expressed as average percentage of binding of ZEA (20 µg/ml), calculated taking into account amount of ZEA in the supernatant and on the residue. Incubation in different buffers: pH 3 (*black*); pH 5 (*white*); pH 7 (*grey*) in the presence of 5 mg/ml of Yeast cell wall (Y0)

Comparisons of several yeast-based products on adsorption of mycotoxins

Comparison of the binding capacity of nine yeast-based products using the new validated in vitro screening method

Nine yeast-based products (Table S1) were used to evaluate the pertinence of the new in vitro procedure to compare efficacy to bind the three mycotoxins (AFB_1, ZEA, OTA). The incubation was done only in buffer pH 3 (citrate) as it was the buffer for which relevant adsorption has been observed for the three mycotoxins in the presence of 20 µg/ml ZEA or 0.5 µg/ml AFB1 or 0.5 µg/ml OTA individually.

The percentage of adsorption of ZEA ranges from 30 % to 60 %. The less effective products are product Y1, Y2, Y7 and Y8 (about 30 %). Three of them are from brewer origin. The efficacy of products Y4, Y6, Y9, Y3 and Y5 is almost similar (about 60 %). The percentage of adsorption of ZEA on charcoal was 99.6±0.4 %.

The % of binding of AFB_1 ranged from 15 % (products Y2, Y9 and Y6) to 45 % (product Y5). The percentage of AFB_1 binding on charcoal was 99.9±0.05 %.

The % of binding of OTA ranged from 40 % (products Y1, Y2) to 70 % (product Y3). There was almost no difference between binding capacity of products Y3, Y4, Y5, Y8, Y9 for

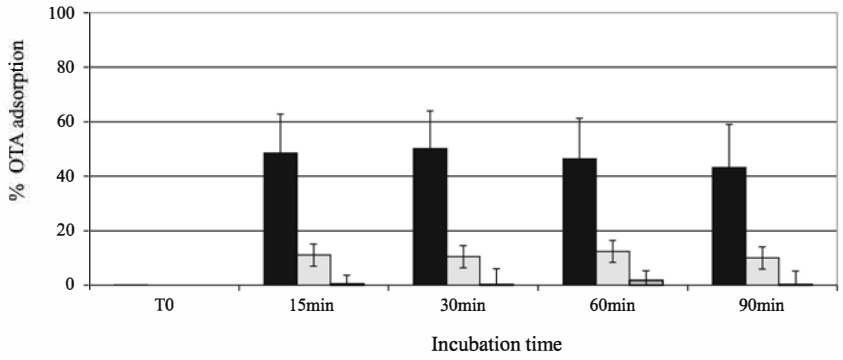

Fig. 4 Binding kinetic of OTA. Data are expressed as average percentage of binding of OTA (0.5 μg/ml), calculated taking into account amount of OTA in the supernatant and on the residue. Incubation in different buffers: pH 3 (*black*); pH 5 (*white*); pH 7 (*grey*) in the presence of 5 mg/ml of yeast cell wall (Y0)

OTA (more than 65 %). The % of binding of OTA on charcoal was 98.7±0.3 %.

The product Y5 (inactivated yeast-based product enriched in glutathione) seemed to be the most interesting products as it bound the three mycotoxins to an extent higher than 50 % for OTA and ZEA and 45 % for AFB_1.

Comparison of the adsorption of two or three mycotoxins simultaneously on several yeast products

To test if the binding of one toxin was modified by the others we tested the binding capacities of the several products in two conditions: (1) ZEA (20 μg/ml)+AFB (0.5 μg/ml); (2) ZEA (20 μg/ml)+AFB (0.5 μg/ml)+OTA (0.5 μg/ml) (Fig. 8).

The simultaneous presence of ZEA with OTA and AFB_1 modified the % of binding on yeast products. Depending on the products this adsorption could be increased or decreased. With yeast products Y1 and Y7 (YCW brewer), Y9; Y6 (YCW baker) and Y8 (alcoholyeast) the simultaneous presence of the three mycotoxins increased the binding of ZEA. This increase was already observed when ZEA was only in the presence of AFB_1(data not shown).

With yeast products Y3 (Se Yeast) and Y5 (GSH Yeast), the binding of ZEA was similar when ZEA was alone or in the presence of the two other toxins but was lower when ZEA was in the presence only of AFB_1. With product Y2 (YCW from brewer) a trend of decreasing ZEA binding was observed when ZEA was in the presence of AFB_1 or both toxins.

OTA binding is increased on Y7, whereas it is decreased on products Y1, Y2, Y3, Y4, Y9 when the two other mycotoxins are simultaneously present (Fig. 8).

Discussion

Adsorption on the cell wall surface is an interaction between the toxins and functional groups of the cell surface, based on physical adsorption, ion exchange and complexation. The cell walls harbouring polysaccharides (glucan, mannan), proteins and lipids exhibit numerous different and easily accessible adsorption centers as well as different binding mechanisms (i.e., hydrogen bonds, ionic or hydrophobic interactions (Huwig et al. 2001; Ringot et al. 2007). On the other hand, the properties of the adsorbed mycotoxins, like polarity, solubility, shape and charge distribution also play a significant role (Huwig et al. 2001; Avantaggiato et al. 2005).

Most studies concerning the effects of mycotoxins binders are focused on AFB_1. OTA and ZEA were selected in addition to AFB_1 as model mycotoxins for the current study due to their very different structure and physico-chemical properties and, due to their toxic properties notably in pig husbandry,

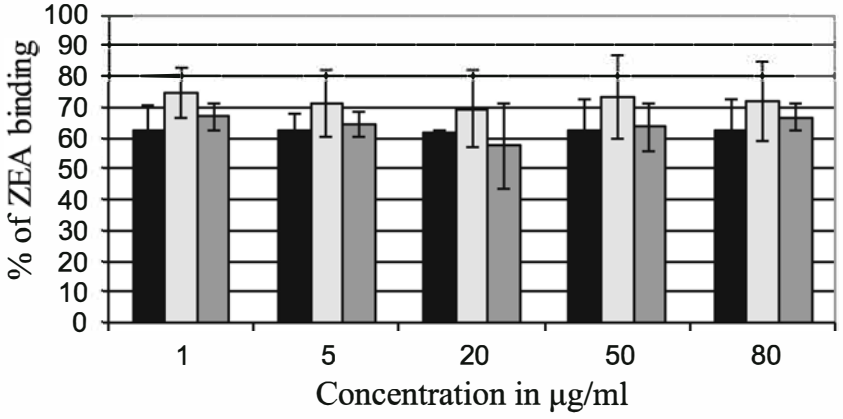

Fig. 5 ZEA binding of increasing amount of ZEA in different buffers in the presence of 5 mg/ml Y0. Percentage of ZEA binding in function of initial concentration of ZEA and according to the pH: pH 3 (*black*); pH 5 (*white*); pH 7 (*grey*)

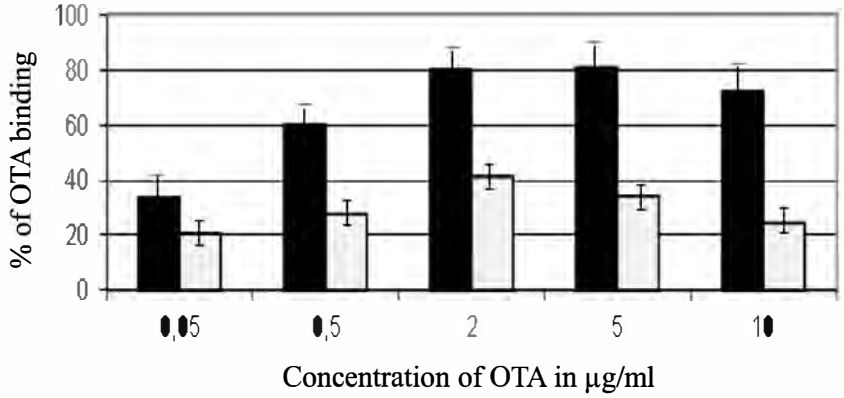

Fig. 6 OTA binding of increasing amount of OTA in different buffers in the presence of 5 mg/ml Y0. Percentage of OTA binding in function of initial concentration of OTA and according to the pH: pH 3 (*black*); pH 5 (*white*); pH 7 (*grey*)

which is considered as the most sensitive animal species (EFSA 2004a, b). OTA has a carboxyl group at the phenylalanine moiety and a phenol group at the dihydroisocoumarin part with a pK_a of 4.4 and 7.3, respectively. Being a polyaromatic molecule, OTA is hydrophobic when unionized. ZEA is a resorcyclic acid lactone. It has a pK_a of 7.62 and is also a weak acid due to the presence of the diphenolic moiety. ZEA will be found almost completely in its deprotonated form at pH 8.4. (Dakovic et al. 2005).

The aim of the present work was to adapt a reliable method to explore, using an in vitro test, the efficacy of mycotoxin adsorption on a natural organic adsorbent made of isolated *S. cerevisiae* cell wall fraction.

Originally, in vitro tests have been performed at a defined pH, sometimes acidic (Kurtbay et al. 2008), but more often at a neutral pH (Carvet et al. 2010; Yiannikouris et al. 2004) and using a single mycotoxin concentration (Shetty and Jespersen 2006). The methods of selection using a unique mycotoxin concentration may lead to erroneous result when the concentrations of mycotoxin changed (Shetty and Jespersen 2006; Bueno et al. 2007; Pizzolitto et al. 2011). Next to single concentration experiments, the evaluation of adsorption isotherms have been recommended to characterize mycotoxin adsorption (Ringot et al. 2007; EFSA 2009; Joannis-Cassan et al. 2011). Under defined conditions, an adsorption equilibrium is reached at which the quantity of the toxin being adsorbed is equal to the quantity being desorbed. Table 2 summarizes the different protocols used for testing yeast binding capacity of mycotoxins.

However, the binding affinity of a polar substance is influenced by pH and, therefore, it is recommended to measure adsorbent characteristics under the conditions of the gastrointestinal tract at neutral, acid and basic pH (Sabater-Vilar et al. 2007). Previously, it has been shown that adsorption of ZEA and AFB_1 by mineral clays and humic acid polymers, respectively, is pH-dependent (Dakovic et al. 2005; Ye et al. 2009), and that the transition to alkaline conditions may lead to desorption.

The first step of this study was to define the best ratio (binder/mycotoxin) using the most common incubation time (90 min) and temperature (37 °C) during the analysis ofresidual mycotoxins in the supernatant. Comparison with real amount of mycotoxin bound in the pellet, pinpointed inaccurate conclusion of high biosorption with huge amount

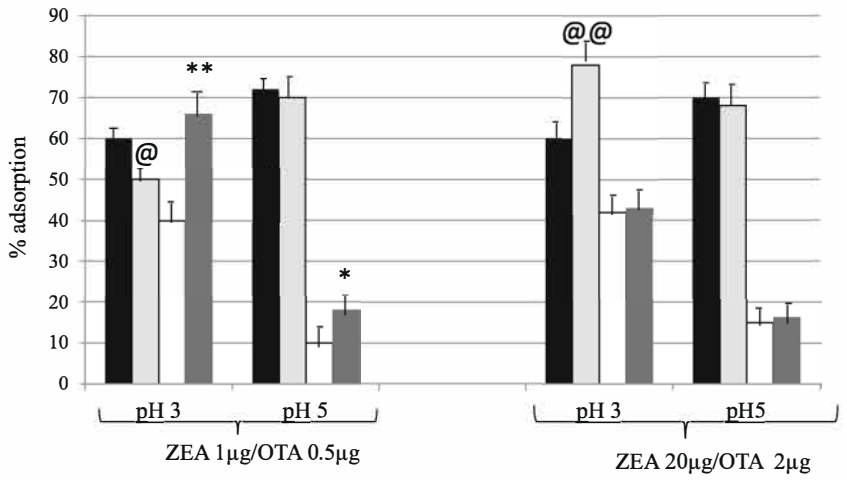

Fig. 7 Comparison ZEA or OTA binding in mixture in the presence of 5 mg/ml Y0 either in buffer pH 3 or pH 5. Percentage of ZEA binding: *black* (alone); *light grey* (in the presence of OTA); percentage of OTA binding: *white* (alone); *dark grey* (in the presence of ZEA). Significant differences of ZEA binding in mixture compare to ZEA alone @@p<0.01, @p<0.05. Significant differences of OTA binding in mixture compare to OTA alone: **p<0.01, *p<0.05

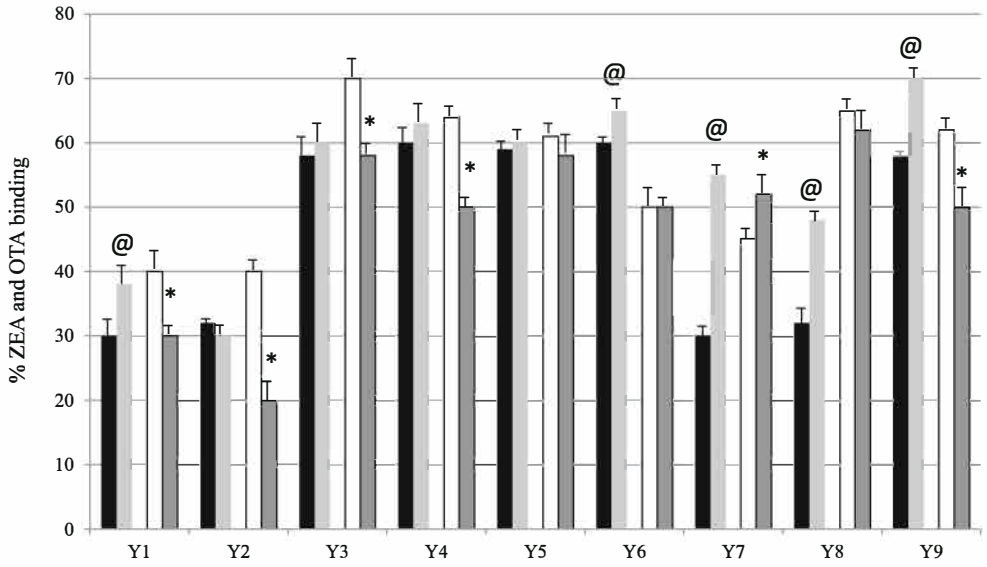

Fig. 8 Percentage of ZEA and OTA binding in mixture by several yeast-based products. *Black column*: ZEA alone; *light grey column*: ZEA+ AFB_1 + OTA; *white column*: OTA alone; *dark grey column*: OTA+ ZEA + AFB_1. The percentages are expressed as means value using ZEA remaining in supernatant and ZEA bound in residue. @Significant difference between ZEA alone and ZEA in mixture. The percentages are expressed as means value using OTA remaining in supernatant and OTA bound in residue. *Significant difference between OTA alone and OTA in mixture

of YCW both with AFB1 and ZEA. High quantity of YCW (50 mg/ml) even in buffer solution lowered significantly the pH, modifying the analytical performance leading to incorrect analytical findings and as consequence to a misclassification of binder ability. Such interference with analytical method of OTA has been previously observed (Bazin et al. 2013). Yiannikouris et al. 2013 excluded data obtained with pH 7 because ZEA eluted in two peaks instead of a single peak with the pH values of 3.0 and 5.0. Another interference with analytical method was due to the precipitation of the mycotoxins over some concentrations (>10 μg/ml for OTA; >60 μg/ml ZEA). This was also observed by Yiannikouris et al. (2013), who reported a large variation between replicate samples from 60 μg/ml of ZEA. Thus, for avoiding any interference for the subsequent experiments, the incubations were done with 5 mg/ml of binder in the presence of the mycotoxins solved in methanol.

Secondly, the best incubation time was checked. By varying the incubation time, no significant difference in the amount of AFB_1, ZEA or OTA removed from YCW were observed. The process was fast, since in less than 5 min (data not shown) the YCW was able to bind the same amount of mycotoxin as in 90 min. This result was consistent with those of Pizzolitto et al. (2012) and Shetty et al. (2007), who have not observed differences between 1 min and 6 h, or 0.5 and 12 h of time contact, respectively. Fifty minutes has been chosen for the following experiments.

Third, the modelling of mycotoxin adsorption was performed to characterize OTA and ZEA adsorption at neutral, acidic and basic pHs and with increasing mycotoxin concentrations to evaluate the robustness of the method for forthcoming assessments of new mycotoxin adsorbents. There was almost no pH effect on ZEA adsorption (eventhough the binding was slightly better at pH 5) and the binding capacity is almost independent of the concentrations of ZEA. In contrast, OTA was only correctly bound at pH 3. Moreover, OTA binding was dependent on its concentration (0.05–10 μg/ml). It was always linear at low values of OTA and showed the transition to a plateau with higher toxin concentrations. Such curve can be modelled using Hill model (data not shown) indicating several binding sites, and a putative cooperative effect. The slight decrease observed with highest OTA dose could be explained by the fact that yeast has several binding sites for OTA, and a cooperative effect play a major role. The first molecule of OTA bound on YCW modifies the affinity for the following one. Even a decreasing capacity with OTA concentration over 5 μg/ml suggests a dynamic equilibrium between the binder concentration and relative binding efficiency. The process reached equilibrium between bound toxins (occupied sites) and unbound toxins (free sites) and therefore a reversible process could be involved as it was demonstrated with AFB_1 by Pizzolitto et al. (2012) and with ZEA by Yiannikouris et al. (2013). These results indicate that the sorption capacity of yeast products depends greatly on the initial concentration of the mycotoxin. Therefore, a comparison from single tests, frequently used in previous studies by assuming the linear sorption of the mycotoxin, is not adequate. When the isotherms are not linear, the comparison of adsorption capacity of yeast products could lead to opposite conclusions, depending on the initial mycotoxin concentration tested. Thus, adsorption of mycotoxin by yeast products was not a linear phenomenon, which means that isotherm studies are necessary to compare the sorption capacity of yeast products. Analysis of individual results of the efficient isotherm models confirmed that adsorption capacity and affinity depend on the interactions between mycotoxin, adsorbent and pH. Therefore, isotherm models to describe adsorption equilibrium cannot be

Table 2 Comparison of the method use for binding screening

Study	Mycotoxins (concentrations)	Binder (concentration)	pH (buffer)	Incubation time (incubation volume)
Shetty and Jespersen 2006	AFB_1 (5 μg/ml)	Yeast/lactic bacteria (Unknown amount)	Phosphate buffer (pH unknown)	30 min 37 °C (1 ml final volume)
Sabater-Vilar et al. 2007	ZEA (1 μg/ml) DON (1 μg/ml)	Yeast (1; 2.5; 5 mg/ml)	pH 2.5 (citrate buffer)	1 h 37 °C (1 ml final volume)
Carvet et al. 2010	DON (1 and 2 mM)	Yeast (1 mg/ml)	pH 3 and 7	90 min 37 °C (1 ml final volume)
Fruhauf et al. 2012	AFB1 0.2 mg/l ZEA 0.5 mg/l	Yeast (10 mg/5 ml)	pH 3 and 6.5	1 h 37 °C (10 ml final volume)
Pereyra et al. 2012	AF (2 μg/ml) ZEA (1 μg/ml)	Yeast (5 concentrations ranging 2–500 μg/ml)	pH 2 and 6	30 min 37 °C (1 ml final volume)
Manafi et al. 2009	AF (500 ppb) OTA (500 ppb) T-2 (2 ppm)	Herbal binder including minerals and antioxidant)	Citrate buffer adjusted pH 4.5 and 6.5	3 h 37 °C (250 ml final volume)
Santos et al. 2011	OTA (0.1 μg/ml) ZEA (0.5 μg/ml)	Bentonite Humic acid (5 concentrations ranging 0.5–5 mg/ml)	Successive pH (pH 7.2; pH 3; pH 8.4)	30 min 39 °C (pH 7.2), then 1 h (pH 3); then 3 h (pH 8.4) (30 ml final volume)
Santos et al. 2011	Isotherm OTA (0.025–0.1 μg/ml) ZEA (0.125–1 μg/ml)	Bentonite Humic acid (5 concentrations ranging 0.5 –5 mg/ml)	Successive pH (pH 7.2; pH 3; pH 8.4)	30 min 39 °C (pH 7.2), then 1 h (pH 3); then 3 h (pH 8.4) (30 ml final volume)
Joannis-Cassan et al. 2011	Isotherm AF (1–10 μg/ml) OTA (1–10 μg/ml) ZEA (1–80 μg/ml)	Yeast (5 mg/ml)	pH 3 (citrate) pH 5 (acetate) pH 7 (phosphate)	15 min 37 °C
Yiannikouris et al. 2003, 2004	Isotherm ZEA (2–20 μg/ml)	Yeast (100 μg/ml)	In water	90 min 37 °C (1 ml final volume)
Yiannikouris et al. 2006	Isotherm ZEA (2–100 μg/ml)	Yeast (1 mg/ml)	pH 3 (citrate) pH 6 (succinate) pH 8 (Tris)	90 min 39 °C (1 ml final volume)
Ringot et al. 2005	Isotherm OTA (0.5–10 mg/l=5–100 μg/10 ml)	Yeast (500 mg/10 ml water)	water	90 min 4°, 25°, 37 °C (10 ml final volume)
Ringot et al. 2007	Isotherm OTA (0.5–10 mg/l=5–100 μg/10 ml)	Yeast (500 mg/10 ml water)	water	90 min 25 °C (10 ml final volume)
Yiannikouris et al. 2013	Isotherm ZEA (10–50 μg/ml)	Yeast (10 mg/ml)	pH 3 (citrate buffer) pH 5 (Acetate buffer)	90 min 37 °C
Pizzolitto et al. 2012	Isotherm AFB_1 (2–20 μg/ml)	Yeast (100 μg/ml)	phosphate buffer (pH unknown)	30 min 37 °C
Pizzolitto et al. 2012	Isotherm AFB_1 (0.3–2.5 μg/ml)	Yeast ($2.8–5\times10^7$ cell/ml)	pH 7.3 (phosphate buffer)	30 min 37 °C
Armando et al. 2011	Isotherm AFB1 (50–500 ng/ml)	Yeast (10^7 cell/ml)	pH 7.3	60 min 37 °C
Armando et al. 2012	Isotherm OTA (1–100 μg/ml) ZEA (1–50 μg/ml)	Yeast (10^7 cell/ml)	pH 7.2	60 min 37 °C

generalized, but serve as a valuable tool to identify and compare new products (Joannis-Cassan et al. 2011).

The stereo chemistry and hydrophobic properties of mycotoxins are of prime importance and account for the differences in their affinity for β-D-glucans. The environmental conditions such as pH were determining for the stability of the toxin-glucan complexes generated. Acid and neutral conditions gave the highest affinity rates for AFB_1>deoxynivalenol >OTA and involved both the (1–3)-β-D-glucans and the (1–6)-β-D-glucans. Alkaline conditions, owing to their destructuring action on glucans, were favorable only for the adsorption of patulin (Guo et al. 2012). Using molecular mechanics, Yiannikouris et al. (2004, 2006) found that hydroxyl, ketone, and lactone groups are involved in the formation of both hydrogen bonds and van der Waals interactions between aflatoxins B_1, deoxynivalenol, ZEA and patulin, and

β-D-glucans (Yiannikouris et al. 2006). Protonation of OTA is changed in the pH course, as it has a pK_a of 4.4 and 7.3. At pH 3.0, almost all OTA will be in the neutral (non-charged) form, while at pH 7.4 it will be present as a monoanion or a dianion, and at pH 8.4 most OTA will be in the dianion form (Santos et al. 2011; Bazin et al. 2013).

As the balance (amount of mycotoxin adsorbed + amount in the supernatant) was correct and the acidic pH was the best one for the three mycotoxins, we compared several yeast derivatives by incubating fixed amount of mycotoxins (0.5 μg/ml of OTA or AFB_1 and 20 μg/ml of ZEA, individually or in binary or ternary mixture) and analysing the binding capacity taking into account the mycotoxins remaining in the supernatant and bound on the residue. This method allows making discrimination between the different products against the three mycotoxins tested. It is also applicable to analyse binding capacity of yeast-based products in multi-contamination conditions and gives a piece of information about interaction (cooperative effect/competition).

The products Y9 and Y4 (YCW from baker) and Y5 (GSH yeast), had a similar efficacy for ZEA and OTA binding. Products Y1, Y2 (YCW from brewer), Y3 (Se yeast), Y7 (YCW from brewer) and Y8 (alcohol yeast) bound more OTA than ZEA. Only Y6 (YCW from baker) bound a little bit more ZEA than OTA. This later product Y6 was the best binder for ZEA, whereas it was the worst for AFB_1. Product Y5 (GSH yeast) seems to be an interesting product as it boundthe three toxins with an efficacy close to 50 % (AFB_1) and over 60 % (ZEA and OTA). In the same way, product Y3 (SE yeast) was a relatively interesting binder against AFB_1, and good binder for the two others mycotoxins. Nevertheless, these two latter products are not YCW but enriched yeasts. Thus the decrease could be due to some biotransformation of the mycotoxins and not exlusively due to binding.

In general, when the binding of ZEA increased in the presence of the two other toxins, the binding of OTA decreased (i.e., Y1 [YCW brewer], Y9 [YCW baker]). In case of an increase of ZEA binding associated with a decrease of OTA binding (or the reverse), this means that both toxins act on the same binding site, but with a different affinity; or two binding sites exit and the binding of one mycotoxin modifies the binding capacities of the other site (cooperative affect).

There was also a competition between OTA and AFB_1. Although AFB_1 binding on Y1 and Y9 was decreased in the presence of ZEA, this decrease was no more pronounced when OTA was also present. In contrast, with product Y7 the binding of both ZEA and OTA increased, indicating a cooperative effect.

Product Y6 (YCW baker) was the sole product for which ZEA's binding increased in the presence of the two other mycotoxins without decreasing the adsorption either of AFB_1 or OTA. Probably the binding sites are multiple and different for each mycotoxin.

These data could be explained by cooperative interaction. The biosorption capacity of yeast β-D-glucans in vitro was shown to be modulated by the amount of ZEA added to the medium according to a cooperative phenomenon. The binding of the first mycotoxin molecules induces conformation changes in β-glucans that facilitates access to new sites of fixation improving binding efficiency until saturation of all sites of adsorption (Yiannikouris et al. 2003, 2004).

In this study, no correlation could be draw between mycotoxins binding capacity and the amount of glucans and/or mannan. This data is in line of that of Carvet et al. (2010), who tested different β-glycans and shown that bound type (1–3 or 1–6) or the ramifications might not be sufficient criteria to characterize their adsorption properties. In contrast to ZEA binding, the AFB_1 binding did not correlate with the mannoligosaccharides (MOS) and glucan content of the investigated products (Fruhauf et al. 2012; Pizzolitto et al. 2012).

Pereyra et al. 2012 tested AFB_1 and ZEA binding capacity of two YCWs differing in their chemical compositions. The first YCW contained5.9 and 17.4 % of mannans and β-glucans while the second YCW contained a higher percentage of mannans (21 %) and β-glucans (23 %), respectively. The both YCWs adsorbed ZEA at the studied pH conditions. Concerning ZEA at pH 2 there was a much more pronounced cooperative effect than at pH 6 with the first YCW1. It was the opposite for second YWC. The authors concluded that ZEA binding can be attributed to the presence of β-glucans in the walls as the effect of pH on binding constants was systematic and similar for both YCW. They bound both a similar amount of AFB_1 at pH 2. The cooperative indexes obtained for both adsorbents were higher at pH 2. In contrast, they bound much less AFB_1 at pH 6. The interaction of AFB1 with glucomannan of YCW was presumably through hydrogen bonds (Pereyra et al. 2012).

Altogether, to be able to obtain a good and reproducible response to screen different adsorbent materials, ZEA, AFB_1 or OTA should be dissolved in methanol, because dissolution directly in buffer induces a precipitation and distorts the results. Mycotoxins should be dissolved in methanol to appropriate concentration (100× the final concentration required in the test tube) and 10 μl of this solution is added to the yeast-based product (5 mg) pre-incubated in the buffer (990 μl). In these conditions, the balance (amount of mycotoxin adsorbed + amount in the supernatant) is correct. With this protocol, it is possible to make a pre-screening of adsorbent capacities against several mycotoxins present together in range of mycotoxin concentrations corresponding to permitted value in food and feed. This method will be applied to other adsorbent materials such as bentonite and humic acid, and to other mycotoxins including deoxynivalenol or fumonisins.

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