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Abstract

The extraction efficiency of aflatoxin B_1 (AFB_1) in cattle feed containing 9 adsorbents (ADSs), was investigated using two organic/aqueous solvents, composed of methanol/water (80/20 v/v; MeOH) and acetone/water (85/15 v/v; AC). Samples were obtained including a highly AFB_1 contaminated (HC) and a low level AFB_1 contaminated (LC) feedstuffs (15.33 and 7.57 μg kg^{-1}, respectively), nine ADSs (4 clay minerals; 1 yeast cell wall-based product; 1 activated carbon and 3 commercial ADS products) at two different levels of inclusion (10 and 20 g kg^{-1}). After solvent extraction and immunoaffinity column clean-up, all samples were analysed for AFB_1 by HPLC with fluorescence detection. For each contamination level (HC and LC), the data obtained were analysed using a factorial arrangement in a completely randomized design. Means were compared to the correspondent controls using the Dunnett's test. No statistical difference was found in AFB_1 levels of feedstuffs not containing ADSs, when extracted with AC or MeOH, even if numerically higher values were obtained with AC. A dose-dependent effect ($P < 0.01$) of ADSs inclusion was observed on AFB_1 recoveries, that were lower when the higher ADS level (20 g kg^{-1}) was included in the HC and LC feedstuffs. Higher AFB_1 recoveries were obtained using AC compared to MeOH, both in HC (75.0 *vs*. 12.0%, respectively) and in LC (84.0 *vs*. 22.8%, respectively) ADSs containing feedstuffs. However, when activated carbon and the sodium bentonite were included in feeds, lower AFB_1 concentrations with respect to control values

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($P < 0.001$ and $P < 0.05$, respectively) were obtained also using AC. The data obtained in this study indicate that routine use of the MeOH solvent for AFB_1 analysis of unknown feedstuffs, can produce misleading results if they contain an ADS.

Keywords: aflatoxin B_1, analysis, feedstuffs, adsorbents, solvents

Introduction

Crops such as corn, cotton and peanuts and their industrial by-products are frequently contaminated by aflatoxins (AFs), hepatocarcinogenic molecules (IARC, 2002) produced primarily by *Aspergillus flavus* and *A. parasiticus*, either in field or during transport or storage (Scheidegger and Payne, 2003).

Aflatoxin B_1 (AFB_1), the most toxic and carcinogenic aflatoxins (AFs) (Roebuck and Maxuitenko, 1994), once ingested by mammals is absorbed in the gastro-intestinal tract and appears rapidly in blood (Gallo et al., 2008) and in milk (Veldman et al., 1992a; Masoero et al., 2007) as aflatoxin M_1 (AFM_1), the principal AFB_1 hydroxylated metabolite. The AFB_1 carry over (CO) rate into milk as AFM_1 has been determined to range from 1% to 3% in lactating dairy cows and to be principally affected by milk yield (Diaz et al., 2004; Van Eijkeren et al., 2006; Masoero et al., 2007), with a reported maximum value of about 6% (Veldman et al., 1992a).

The limits for AFB_1 fixed by the European Commision (EC) in animal feeds and complete feedingstuffs for dairy animals are 20 and 5 μg kg^{-1}, respectively (European Commission, 2003). In milk, the EC set the AFM_1 maximum permitted level at 0.050 μg kg^{-1} (European Commission, 2006), while in the USA the maximum AFM_1 concentration is regulated by the Food and Drug Administration (FDA) at 0.500 μg kg^{-1} (Berg, 2003).

Various equations to predict the AFM_1 level in milk (ng kg^{-1}) from AFB_1 intake (μg *per* cow *per* day) have been proposed (Veldman et al., 1992a; Pettersson et al., 1989; Van Eijkeren et al., 2006). In dairy farms, the precise AFB_1 determination in animal feeds is useful to predict the AFM_1 concentration in milk and to avoid contamination levels exceeding the legal limits.

The analytical methods for AFB_1 detection/quantification in foods and feedstuffs are based on different separation/detection techniques (Trucksess and Wood, 1994; Stroka et al., 1999), such as enzyme linked immunosorbent assay (ELISA) (Aldao et al., 1995), HPLC (Sharma and Marquez, 2001; Stroka et al., 2003; Arranz et al., 2006) or TLC (Bradburn et al., 1995). All these techniques need an efficient sample extraction method. Several studies have been conducted to investigate the extraction capacity of different organic/aqueous solvents, like methanol (Reif and Metzger, 1995; Stroka et al., 1999; Sharma and Marquez, 2001; Senyuva and Gilbert, 2005; Brera et al., 2007), acetone (Brandurn et al., 1995; Stroka et al., 2003; Arranz et al., 2006), acetonitrile (Stroka et al., 1999) and chloroform (Moller and Nyberg, 2004) solvents. Some studies investigated the organic solvent to water ratio (ml solvent/ml water), the ratio of solvent to sample (ml solvent/g sample) and the matrix effects (Brandburn et al., 1995; Stroka et al., 1999; Moller and Nyberg, 2004).

The AFB_1 method based on a methanol:water (80:20 v/v) extraction procedure (MeOH) and on HPLC determination after an immunoaffinity clean-up step, seems to be the most common method in AFs-specialized laboratories (Trucksess and Wong, 1994; Stroka et al., 1999; Sharma and Màrquez, 2001; Moller and Nyberg, 2004). However, the method based on an acetone:water (85:15 v/v) solvent (AC) to extract AFs from feeds is also used in many laboratories, being the AOAC reference method (2003.2) for AFB_1 analysis (Stroka et al., 2003; AOAC, 2006).

One of the most recent approaches to the prevention of mycotoxicoses in livestock is the addition of adsorbents (ADSs) in the diet, that bind mycotoxins in the gastro-intestinal tract and are capable of reducing their bioavailability (Jouany et al., 2007; Masoero et al., 2009). Certain ADSs can decrease the aflatoxin CO in milk of dairy animals (Diaz and Smith, 2005; Jouany; 2007). Mycotoxin binders belong to three different groups: silicate materials or clay minerals (Phillips et al., 1991; Ramos and Hernandez, 1996), yeast cell wall-based products (Karaman et al., 2005; Yiannikouris et al., 2005), and activated charcoals (Galvano et al., 1996). However, these additives are not at present authorized in

Europe for this purpose and they are currently added to industrial feeds as ingredients (yeast cell wall-based products) or anti-caking agents to improve feed pelleting efficiency (clay minerals) (Jaynes et al., 2007).

No specific work has been published regarding the AF extraction efficiency of solvents (acetone and methanol mixed with different percentages of water) in animal feedstuffs containing mycotoxin ADSs, even if some authors (Veldman, 1992b; Galvano et al., 1996) indicated that the presence of clay ADSs in feeds could reduce the analytical recovery of AFB_1.

The aim of this study was to examine if the inclusion of sequestering agents in AFB_1 contaminated feedstuffs can affect the extraction capacity of AC or MeOH extracting solvents.

Materials and methods

Preparation of feeds

Two AFB_1 contaminated complete feedstuffs (1350 kg each) were produced in an industrial feed mill, using AFB_1 naturally contaminated ingredients: two corn meals (10.21 ± 1.27 and 32.87 ± 2.32 µg kg^{-1}), wheat bran (1.40 ± 0.31 µg kg^{-1}), and soybean meal (1.11 ± 0.23 µg kg^{-1}); the other ingredients were uncontaminated barley and sunflower meal, and a mineral/vitamin supplement. The two maize meals were used in order to obtain, respectively, a low contaminated (LC; 7.57 ± 0.65 µg kg^{-1}) feedstuff with a contamination close to the EC limit of 5 µg kg^{-1} (European Commission, 2003) and a highly contaminated (HC; 15.33 ± 1.12 µg kg^{-1}) feedstuff with a contamination three times higher than the EU limit (Table 1). Both feeds, free of clay minerals or other anti-caking agents, were mixed for 4 minutes in a 2000 kg industrial mixer (MO/20, Grespan, Treviso, Italy).

The two contaminated feeds were divided into 108 sub-samples (25 kg each), 54 for LC and 54 for HC, and tested for the AFB_1 contamination homogeneity, randomly selecting and checking 9 samples for LC and 9 samples for HC. The 18 samples were analyzed in duplicate with AC and MeOH

extraction solvents (Table 1).

In this study we tested four different types of silicate minerals (sodium and calcium bentonite clays; zeolite and kaolinite), one yeast cell wall-based product, an activated carbon, and three commercial products commonly used in industrial feed mills and in dairy farms (Table 2) (Masoero et al., 2009).

The ADSs were characterized for elementary composition (Table 2) and used at two inclusion levels (10 and 20 g kg^{-1}) in LC and HC feedstuffs. The nine ADSs were a sodium bentonite (Amcol International Corp., Arlington Heights, IL, USA), a calcium bentonite (Tecnozoo, Padova, Italy), a zeolite (Fluka 96096, Sigma-Aldrich Chemie Gmbh, Switzerland), a kaolinite (Fluka 03584, Sigma-Aldrich Chemie Gmbh, Switzerland); a yeast cell wall-based product (Mycosorb®, Alltech Italy, Bologna, Italy), an activated carbon product (Acque Nymco, Milan, Italy), and three commercial ADSs (Atox®, Grupo Tolsa, Madrid, Spain; Myco AD AZ, Ascor Chimici, Forlì-Cesena, Italy; Novasil™ plus, Trouw Nutrition Int., Verona, Italy). The manufacturer's specifications of the commercial products classify Atox® and Myco AD AZ as clay mixtures where the presence of aluminosilicates of the smectite group exceeds 85% of the product. In particular, the first could be considered a magnesium bentonite, being magnesium the dominant cation, while the second is a mixture of sodium and calcium bentonite. Novasil™ plus is generally described by the producer as a hydrated sodium calcium aluminosilicate. Recently, some authors reported that this product could be classified as a montmorillonite clay (Pimpukdee *et al.*, 2004, Bailey *et al.*, 2006).

The 108 feed sub-samples were randomly assigned to the 9 ADSs (12 sub-samples each) to obtain 3 replicates for the two inclusion levels (10 and 20 g kg^{-1}) and the two contamination levels (LC and HC). Then, all the sub-samples were mixed for 4 minutes in a 50 kg mixer before being analyzed for the AFB_1 content.

The experimental model studied the effects of: contamination levels (LC and HC); ADSs (n=9); doses (10 and 20 g kg^{-1}) and replicates (n = 3) for a total of 108 samples.

Chemicals

Solvents and reagents. Solvents used were of grade ACS-ISO. Acetonitrile, acetone and methanol were HPLC grade (purity 99.5%, 99.0% and 99.9%; respectively) from Merck (Darmstadt, Germany).

Water. Purified water was obtained from a Milli-Q Gradient A10 water purification device (Millipore, Bedford, MA, USA).

Phosphate-buffered saline (PBS). PBS was prepared by dissolving 0.20 g KCl, 0.20 g KH_2PO_4, 1.16 g anhydrous Na_2HPO_4 and 8.00 g NaCl in 1 l of water. The pH was adjusted to 7.4 with NaOH (0.1 mol l^{-1}).

Standards. The AFB_1 standard was obtained from Sigma-Aldrich (St. Louis, Mo, USA). A stock solution (9.517 mg l^{-1}) was prepared and checked according to the AOAC method 970.44 (AOAC, 1995) and stored at -20°C when not in use. The working standard solution was prepared after evaporation at room temperature under nitrogen of an aliquot (100 µl) of the stock solution and re-dissolution in chloroform (10 ml) by ultrasonication, resulting in an AFB_1 concentration of 95.176 µg l^{-1}. An aliquot (100 µl) of this solution was evaporated at room temperature under nitrogen and re-dissolved in the HPLC mobile phase (0.5-20 ml), to obtain 11 calibration solutions at individual concentrations between 0.475 and 19.035 µg l^{-1}.

Extraction procedures

Sample preparation and clean up. To determine AFB_1 in feeds, 25 g of sample were extracted with 250 ml of a methanol/water mixture (MeOH, 80:20, v/v) according to Stroka et al. (1999), or with 250 ml of an acetone/water mixture (AC, 85:15 v/v) according to AOAC method 2003.2 (AOAC, 2006); samples were shaken using a rotary shaker for 45 min and filtered through a Schleicher & Schuell 595

½ filter paper (Dassel, Germany). Five ml of the filtrate were diluted with 45 ml of distilled water and the solution was purified through an immunoaffinity column (R-Biopharm Rhône Ltd, Glasgow, UK), previously conditioned with 20 ml of PBS. After washing, the column with 5 ml distilled water, AFB_1 was slowly eluted with 2.5 ml of methanol.

The eluate was dried under a gentle stream of nitrogen, re-dissolved in 1 ml of acetonitrile:water (25:75, v/v), and vortex-mixed for a few seconds; the extract was then filtered (Millipore Corporation, Bedford, Massachusetts, USA; HV 0.45 µm) and injected (30 µl) into the HPLC.

Apparatus

Analysis was performed using an HPLC instrument consisting of a LC-200 pump (Perkin Elmer, Norwalk, CT, USA) an AS-2055 sampling system, a FP-1520 fluorescence detector (Jasco Corporation, Tokyo, Japan), and a UV derivatizer (UVE™ derivatizer, LC tech, Dorfen, Germany); the instrument was controlled by Borwin 1.5 software (Jasco). A Superspher RP-18 column (4 µm particle size, 125 x 4 mm i.d., Merck) was used at ambient temperature with a mobile phase of water:methanol:acetonitrile (64:23:13, v/v/v) at 1 ml min^{-1}. The AFB_1 was detected after post-column photochemical derivatization to $AFB_{2\alpha}$. The detector was set at 365 nm excitation and 440 nm emission wavelengths.

The elemental components of the ADSs were determined by a semi-quantitative X-ray fluorescence analysis (Sànchez-Ramos *et al.*, 2008*)* using the scanning electron microscope Phillips XL 30 E-SEM (Phillips electron Optics B.V., Eindhoven, Netherlands) equipped with an energy-dispersive X-ray detector model Genesis (Edax Inc., Mc Kee Drive, Mahwah NY, USA) operating in low vacuum.

Recovery experiment

Before analysis of feeds, the AFB_1 recoveries were performed with an AF-free cattle feed (830 g kg^{-1}

of a cereal mix meals; 115 g kg^{-1} of a soybean meal; 50 g kg^{-1} of a sunflower meal; and 5 g kg^{-1} of a mineral/vitamin supplement) without addition of any ADSs. The recovery values were estimated by spiking eight blank samples (25 g each) with 0.5 ml of an AFB_1 standard solution (250 µg l^{-1} dissolved in methanol) in order to obtain a concentration of 5 µg kg^{-1}. After addition of the spiking solution, the organic solvent was evaporated at room temperature (22°C) under a ventilated hood for 2 hours. The spiked samples were then analysed according to the two extraction procedures (MeOH and AC,, four replicates each) as described above. Four replicates of a certified reference material (ground corn, R-Biopharm Rhône LTD) were also analysed.

Statistical analyses

The AFB_1 contents in feedstuffs without ADSs were tested for homogeneity using the one-way analysis of variance procedure of SAS® (version 9.1, SAS Institute Inc., Cary, NC).

The AFB_1 contents of the feedstuffs supplemented with ADSs were evaluated in a completely randomized design using the general linear model procedure of SAS®. A 2 x 2 x 9 factorial arrangement for each AF contamination level (HC and LC) was used and fixed effects in the model included extraction procedure (Method; 2 levels), inclusion doses (Dose; 2 levels), ADSs (ADS; 9 levels) and associated first order and second order interactions. Dunnett's test was used for comparing treatments with the appropriate control (Lowry, 1992). Significance was declared at $P < 0.05$.

Results and discussion

The average recovery values were 96.6±1.8% and 97.8±2.1% for MeOH and AC extraction procedures, respectively. The declared AFB_1 contamination of the certified reference material was 4.1±1.0 µg kg^{-1}; the results obtained were 4.2±0.2 and 4.3±0.1 µg kg^{-1} using the MeOH and AC procedures, respectively. The limit of detection (LOD, signal-to-noise ratio of 3:1) and of

quantification (LOQ, signal-to-noise ratio of 10:1) were respectively 0.02 and 0.05 µg kg^{-1}.

No statistical differences were found in AFB_1 levels of feedstuffs non containing ADSs, when extracted with AC or MeOH, even if numerically higher values were obtained with AC. The *F*-test at the 95% confidence level demonstrated that the sub-sample variance was not different from the analytical variance (F_{calc}=0.479 and F_{calc}=0.358 *versus* F_{crit}=3.500, respectively for HC and LC), thus demonstrating good homogeneity of the prepared feeds (table 1).

Considering the composition of commercial clays (table 2), Novasil™ plus can be classified as a calcium montmorillonite (Pimpukdee et al., 2004; Bailey et al., 2006; Afriyie-Gyawu et al., 2008), a mineral clay that represents the main constituent of bentonite clays (Diaz and Smith, 2005). Atox®, which is characterized by a high Mg level (14.8 ± 0.39%), can be classified as a magnesium bentonite, in agreement with the manufacturer's specifications; Myco AD AZ exhibited the highest carbon and the lowest ash level (41.0 and 75%, respectively) among clays.

The statistical analysis showed a dose-dependent effect ($P < 0.01$) of ADSs inclusion on AFB_1 recoveries, that were lower when the higher ADS dose (20 g kg^{-1}) was included in the HC and LC feedstuffs (tables 3 and 4).

In particular, in HC feedstuff integrated with ADSs and extracted using the AC solvent, the average recovery was 75.0%, while a very low AFB_1 recovery (12.0%) was obtained when the MeOH solvent was used. A similar trend, but higher AFB_1 recoveries, was observed for the LC feedstuffs (84.0 and 22.8% using AC and MeOH, respectively). Therefore, the AFB_1 extraction capacity of MeOH in feedstuffs containing ADSs resulted significantly lower ($P < 0.001$) with respect to AC: the MeOH method in presence of ADSs showed figures that are far from performance requirements for AF methods fixed in the EC Regulation 401/06 (recoveries of 70-110% and 80-110% in the range 1-10 and >10 µg kg^{-1}, respectively). Besides, when the MeOH extraction method was applied to HC feedstuffs integrated with ADSs, the AFB_1 concentrations resulted lower ($P < 0.001$) than the control value (13.8

µg kg^{-1}) for all the samples (table 3) and under the EU limit of 5 µg kg^{-1} (EC, 2003). On the contrary, when AC was used the observed AFB_1 concentrations exceeded this limit, with values ranging from 5.7 µg kg^{-1} (activated carbon) to 13.6 µg kg^{-1} (Novasil^{TM} plus). Similar results were obtained in LC feedstuffs (table 4), with AFB_1 concentrations exceeding the EU limit only when the analyses were performed using the AC solvent (with the exception of activated carbon).

In particular when the activated carbon was added to the feedstuffs the AFB_1 content measured using the AC method differed ($P < 0.001$) from control values (15.3 and 7.6 µg kg^{-1}, respectively for HC and LC feeds) in all the experimental conditions (Tables 3 and 4). In presence of activated carbon, the AFB_1 recoveries measured with AC at 10 and 20 g kg^{-1} were respectively 52 % and 37 % in the HC; 55 % and 39 % in the LC feeds,. This can be due to a high affinity of the activated carbons *versus* the AFB_1 molecule (Lemke et al., 2001; Vekiru et al., 2007) that could cause a reduction of extraction capacity of the AC mixture. In agreement with our data, Galvano et al. (1996) reported a similar reduction (from 41 % to 74 %) of analytical content of AFB_1 in a pelleted feed containing activated carbons.

Also when the sodium bentonite was added to contaminated feeds, lower ($P < 0.05$) AFB_1 concentrations with respect to controls were measured both in HC (20 g kg^{-1} dose) and LC (10 and 20 g kg-1 doses) feedstuffs. However, the AFB_1 recoveries were higher than those obtained for activated carbon, ranging from 63 % to 76 %.

The data obtained in this experiment indicate that a routine use of the MeOH solvent for AFB_1 analysis of unknown feedstuffs, can produce misleading results if they contain an ADS. Consequently, when an ADS is included in a contaminated feed, the analytical result for AFB_1 could comply with or largely exceed the EU limit in feedstuffs for dairy animals (EU, 2003) according to whether it was obtained using MeOH or AC as extracting solvent, respectively. In any case, also AC did not result fully adequate for AFB_1 quantification in experimental feeds containing two types of ADS (i.e., sodium

bentonite and activated carbon). This fact suggests that other solvent mixtures should be evaluated to improve AFB_1 extraction from feeds containing ADSs.

Conclusions

Erroneous data regarding the true AFB_1 contamination level of a feedstuff may lead to incorrect managerial decisions in dairy farms and consequently to higher than expected AFM_1 levels in milk. In fact, the AFM_1 level in bulk milk (µg kg^{-1}) depends on the quantity of AFB_1 ingested (µg *per* cow *per* day). According to Veldman et al. (1992a), this relationship can be described by the following equation:

$$AFM_1 \text{ (µg kg}^{-1} \text{ of milk)} = (1.19 * x + 1.9) / 1000$$

where x is the AFB_1 (µg) ingested daily by each cow in the herd.

According to our study, if 10 kg of the HC feedstuff is daily administered to cows, considering the AFB_1 result obtained by AC extraction (15.33±1.18 µg kg^{-1}), 153.3 µg *per* cow *per* day can be calculated and the predicted AFM_1 bulk milk concentration should be 0.184 µg kg^{-1}. Otherwise, if 10 kg of the HC feedstuff containing 10 g kg^{-1} of an ADS is given to cows, the AFB_1 level in the feed will be 12.4 or 2.3 µg kg^{-1} (mean values excluding the activated carbon thesis) according to whether the AC or MeOH solvent had been adopted, respectively. Consequently, a daily AFB_1 consumption of 123.7 or 23.3 µg *per* cow *per* day can be calculated, and a milk contamination of 0.149 or 0.030 µg kg^{-1} of AFM_1 can be predicted. The latter value, calculated on the basis of the MeOH result, is well below the limit of 0.050 µg kg^{-1} set by the EU (EU, 2006), but the real situation is quite different, as reported in our previous work (Masoero et al., 2009). In conclusion, it is clear that the ascertainment of the real AFB_1 concentration of feedstuffs containing ADS is essential to avoid putting dairy farmers at risk of unknowingly producing contaminated milk, which will have to be discarded.

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Table 1. AFB_1 content (µg kg^{-1}) of ingredients and feedstuffs using acetone:water (AC; 85:15 v/v) and methanol:water (MeOH; 80:20 v/v) extraction solvents and feedstuff composition (g kg^{-1}).

Ingredients	n°	AFB_1 (µg kg^{-1})		Feedstuff composition (g kg^{-1})	
		AC	MeOH	High contamination (HC)	Low contamination (LC)
High contaminated corn meal	3	32.87 ± 2.32	29.46 ± 3.01	400	50
Low contaminated corn meal	3	10.21 ± 1.27	8.78 ± 1.57	160	500
Wheat bran	3	1.40 ± 0.31	1.36 ± 0.22	120	120
Barley meal	3	n.d.	n.d.	150	160
Soybean meal	3	1.11 ± 0.23	1.08 ± 0.27	115	115
Sunflower meal	3	n.d.	n.d.	50	50
Trace-min./vit. Supplement[a]	3	n.d.	n.d.	5	5
HC feedstuff	**9**	**15.33 ± 1.18**	**13.81 ± 1.05**		
LC feedstuff	**9**	**7.57 ± 0.65**	**7.02 ± 0.63**		

n.d.: not detectable (under the LOD).

[a]Content per 100 g of trace min./vit. Supplement: 120000 IU of vit. A; 9000 IU of vit. D_3; 90 mg of vit. E; 3.6 mg of Co; 19.2 mg of I; 1.44 mg of Se; 600 mg of Mn; 62.4 mg of Cu; 2240 mg of Zn; 1.92 mg of Mo; 360 mg of Fe.

Table 2. Elementary composition and total ash content of the tested adsorbents.

Adsorbents	Elements (%)									Total Ash
	C	O	Al	Si	Na	Mg	K	Ca	Fe	(%)
Sodium bentonite	3.3 ± 0.61	54.7 ± 0.21	9.7 ± 0.13	25.1 ± 0.43	1.4 ± 0.03	1.6 ± 0.06	0.2 ± 0.01	0.7 ± 0.22	2.7 ± 0.09	90.7 ± 1.1
Calcium bentonite	9.7 ± 0.17	52.6 ± 0.25	6.0 ± 0.09	15.5 ± 0.47	0.6 ± 0.10	1.4 ± 0.05	1.6 ± 0.19	8.3 ± 0.16	3.8 ± 0.21	93.5 ± 0.7
Zeolite	2.7 ± 0.18	56.0 ± 0.18	14.1 ± 0.63	14.6 ± 1.05	12.2 ± 0.38	0.1 ± 0.02	0.3 ± 0.03	0.1 ± 0.02	0.1 ± 0.06	99.8 ± 0.1
Kaolinite	0.8 ± 0.44	57.0 ± 1.07	18.6 ± 0.17	21.0 ± 0.35	n.d.	0.2 ± 0.04	1.3 ± 0.10	0.1 ± 0.05	0.4 ± 0.05	88.8 ± 1.2
Yeast cell wall-based	61.4 ± 1.2	34.8 ± 0.04	n.d.	n.d.	0.1 ± 0.01	0.2 ± 0.04	0.9 ± 0.11	1.4 ± 0.76	n.d.	8.1 ± 0.3
Activated Carbon	89.6 ± 0.84	10.0 ± 0.85	n.d.	0.1 ± 0.01	n.d.	n.d.	n.d.	0.1 ± 0.07	n.d.	2.7 ± 0.1
Atox®	3.8 ± 0.79	53.7 ± 0.58	1.6 ± 0.04	21.8 ± 0.47	1.5 ± 0.03	14.8 ± 0.39	0.4 ± 0.02	0.6 ± 0.25	0.9 ± 0.12	94.6 ± 0.9
Myco AD AZ	41.0 ± 0.62	34.6 ± 0.38	5.2 ± 0.17	14.4 ± 0.57	1.2 ± 0.03	0.9 ± 0.02	0.5 ± 0.04	0.6 ± 0.07	1.2 ± 0.10	75.1 ± 0.1
Novasil™ plus	4.2 ± 1.02	56.0 ± 0.44	8.5 ± 0.35	22.6 ± 0.50	0.2 ± 0.06	2.0 ± 0.04	0.6 ± 0.30	1.8 ± 0.12	3.5 ± 0.13	92.4 ± 0.7

n.d.: not detectable (under the LOD)

Table 3. AFB_1 concentrations (µg kg^{-1}) detected using acetone:water (AC, 85:15 v/v) and methanol:water (MeOH, 80:20 v/v) extraction solvents in HC feedstuff mixed with different adsorbents at two doses (10 and 20 g kg^{-1}). The AFB_1 concentrations in untreated HC (control) were 15.33 ± 1.18 and 13.81 ± 1.05 µg kg^{-1} using AC and MeOH, respectively.

Adsorbent	Dose			
	10 g kg^{-1}		20 g kg^{-1}	
	AC	MeOH	AC	MeOH
Sodium bentonite	13.01	0.96	9.93	0.45
	9.73	0.75	8.53	0.52
	12.44	0.82	10.76	0.66
mean	**11.72 ± 1.75**	**0.84 ± 0.11*** **	**9.74 ± 1.13***	**0.54 ± 0.11*** **
Calcium bentonite	12.70	1.69	10.30	1.44
	12.48	1.59	11.67	0.40
	12.50	1.67	12.53	1.02
mean	**12.56 ± 0.12**	**1.65 ± 0.05*** **	**11.50 ± 1.13**	**0.95 ± 0.52*** **
Zeolite	10.32	2.98	13.69	3.08
	12.70	2.84	12.52	2.79
	12.87	2.75	11.55	2.50
mean	**11.96 ± 1.43**	**2.86 ± 0.12*** **	**12.59 ± 1.07**	**2.79 ± 0.29*** **
Kaolinite	13.37	3.15	12.96	4.84
	12.83	3.01	12.36	2.49
	12.30	3.26	12.29	3.46
mean	**12.83 ± 0.53**	**3.14 ± 0.13*** **	**12.54 ± 0.37**	**3.59 ± 1.18*** **
Yeast cell wall-based	10.10	2.71	9.57	2.78
	13.79	2.74	11.45	3.10
	12.73	2.70	11.08	2.70
mean	**12.21 ± 1.90**	**2.71 ± 0.02*** **	**10.70 ± 1.00**	**2.86 ± 0.21*** **
Activated Carbon	6.99	1.39	5.55	1.03
	9.01	1.33	5.86	1.04
	7.99	1.36	5.78	1.00
mean	**7.99 ± 1.01*** **	**1.36 ± 0.03*** **	**5.73 ± 0.16*** **	**1.03 ± 0.02*** **
Atox®	14,15	1.42	12,91	0.83
	15,07	1.05	14,02	0.76
	10,25	1.56	10,37	0.33
mean	**13.16 ± 2.56**	**1.34 ± 0.26*** **	**12.43 ± 1.87**	**0.64 ± 0.27*** **
Myco AD AZ	12,97	4.41	11,19	0.50
	14,09	4.22	9,90	0.59
	10,66	4.05	13,81	0.55
mean	**12.58 ± 1.75**	**4.22 ± 0.18*** **	**11.63 ± 1.99**	**0.55 ± 0.04*** **
Novasil™ plus	9.78	0.89	13.18	0.92
	13.07	1.16	15.71	0.91
	11.73	1.09	11.89	0.68
mean	**11.53 ± 1.66**	**1.05 ± 0.14*** **	**13.59 ± 1.94**	**0.84 ± 0.13*** **

P of the model < 0.001 S.E.M.[a] 0.6124

Source of variation (*P* value)

ADS < 0.001 Dose < 0.01 Method < 0.001

ADS x Dose < 0.05 ADS x Method < 0.001 Dose x Method = 0.8503

ADS x Dose x Method = 0.0702

[a]S.E.M.: standard error of the mean.

Superscripts signify means differ from respective controls; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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Table 4. AFB_1 concentrations (µg kg^{-1}) detected using acetone:water (AC, 85:15 v/v) and methanol:water (MeOH, 80:20 v/v) extraction solvents in LC feedstuff mixed with different adsorbents at two doses (10 and 20 g kg^{-1}). The AFB_1 concentrations in untreated LC (control) were 7.57 ± 0.65 and 7.02 ± 0.63 µg kg^{-1} using AC and MeOH, respectively.

Adsorbent	Dose			
	10 g kg^{-1}		20 g kg^{-1}	
	AC	MeOH	AC	MeOH
Sodium bentonite	5.46	0.55	6.80	0.44
	5.39	0.52	4.90	0.41
	5.41	0.51	5.11	0.48
mean	**5.42 ± 0.46***	**0.53 ± 0.02*****	**5.60 ± 1.04***	**0.45 ± 0.03*****
Calcium bentonite	8.31	2.28	6.90	1.35
	7.19	1.66	7.78	1.44
	7.25	1.89	6.78	1.33
mean	**7.58 ± 0.63**	**1.94 ± 0.31*****	**7.15 ± 0.55**	**1.37 ± 0.06*****
Zeolite	8.29	2.36	7.11	2.55
	7.23	2.78	6.49	2.54
	7.15	2.15	6.56	2.36
mean	**7.56 ± 0.64**	**2.43 ± 0.32*****	**6.72 ± 0.34**	**2.48 ± 0.11*****
Kaolinite	5.89	2.27	6.07	2.16
	7.32	2.17	6.61	1.97
	6.29	2.35	6.12	2.09
mean	**6.50 ± 0.73**	**2.27 ± 0.09*****	**6.27 ± 0.30**	**2.07 ± 0.09*****
Yeast cell wall-based	6.96	2.16	6.19	2.36
	6.73	2.50	6.74	2.40
	6.75	2.13	6.53	2.56
mean	**6.81 ± 0.13**	**2.26 ± 0.20*****	**6.49 ± 0.28**	**2.44 ± 0.11*****
Activated Carbon	4.38	1.40	2.88	0.71
	4.03	1.20	3.04	0.83
	4.11	1.30	3.01	1.04
Mean	**4.17 ± 0.18*****	**1.30 ± 0.10*****	**2.98 ± 0.09*****	**0.86 ± 0.17*****
Atox®	7.10	0.86	7.66	0.58
	7.74	0.84	6.70	0.66
	6.92	0.82	7.38	0.55
Mean	**7.25 ± 0.43**	**0.84 ± 0.02*****	**7.25 ± 0.49**	**0.60 ± 0.06*****
Myco AD AZ	6.91	4.19	6.50	2.68
	7.06	4.94	6.26	2.94
	6.07	5.59	6.36	2.92
Mean	**6.68 ± 0.53**	**4.91 ± 0.70*****	**6.37 ± 0.12**	**2.85 ± 0.14*****
Novasil™ plus	8.11	0.80	5.54	0.57
	6.90	0.88	5.47	0.69
	7.21	0.83	5.74	0.54
mean	**7.41 ± 0.63**	**0.83 ± 0.04*****	**5.58 ± 0.14**	**0.60 ± 0.08*****
P of the model < 0.001	S.E.M.[a] 0.2254			

Source of variation (*P* value)

ADS < 0.001 Dose < 0.010 Method < 0.001

ADS x Dose = 0.001 ADS x Method < 0.001 Dose x Method = 0.3331

ADS x Dose x Method = 0.0610

[a]S.E.M.: standard error of the mean.

Superscripts signify means differ from respective controls: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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