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► **To cite this version:**

Antonio Gallo, Francesco Masoero, Terenzio Bertuzzi, Gianfranco Piva, Amedeo Pietri. Effect of the inclusion of adsorbents on aflatoxin B1 quantification in animal feedstuffs. *Food Additives and Contaminants*, 2009, 27 (01), pp.54-63. 10.1080/02652030903207219 . hal-00573908

HAL Id: hal-00573908

<https://hal.science/hal-00573908>

Submitted on 5 Mar 2011

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Effect of the inclusion of adsorbents on aflatoxin B₁ quantification in animal feedstuffs

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2009-070.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	15-Jul-2009
Complete List of Authors:	Gallo, Antonio; Università Cattolica del Sacro Cuore, Istituto di Scienze degli Alimenti e della Nutrizione Masoero, Francesco; Università Cattolica del Sacro Cuore, Istituto di Scienze degli Alimenti e della Nutrizione Bertuzzi, Terenzio; Istituto di Scienze degli Alimenti e della Nutrizione Piva, Gianfranco; Università Cattolica del Sacro Cuore, Istituto di Scienze degli Alimenti e della Nutrizione Pietri, Amedeo; Università Cattolica del Sacro Cuore, Istituto di Scienze degli Alimenti e della Nutrizione
Methods/Techniques:	Chromatography - HPLC, Extraction
Additives/Contaminants:	Aflatoxins
Food Types:	Animal feedingstuffs, Milk

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Manuscripts

Running head A. Gallo et al. AFB₁ extraction in feeds containing adsorbents

Effect of the inclusion of adsorbents on aflatoxin B₁ quantification in animal feedstuffs

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Abstract

The extraction efficiency of aflatoxin B₁ (AFB₁) 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 AFB₁ contaminated (HC) and a low level AFB₁ 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 AFB₁ 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 AFB₁ 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 AFB₁ recoveries, that were lower when the higher ADS level (20 g kg⁻¹) was included in the HC and LC feedstuffs. Higher AFB₁ 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 AFB₁ 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 AFB₁ analysis of unknown feedstuffs, can produce misleading results if they contain an ADS.

Keywords: aflatoxin B₁, 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 B₁ (AFB₁), 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 M₁ (AFM₁), the principal AFB₁ hydroxylated metabolite. The AFB₁ carry over (CO) rate into milk as AFM₁ 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 AFB₁ fixed by the European Commission (EC) in animal feeds and complete feedingstuffs for dairy animals are 20 and 5 $\mu\text{g kg}^{-1}$, respectively (European Commission, 2003). In milk, the EC set the AFM₁ maximum permitted level at 0.050 $\mu\text{g kg}^{-1}$ (European Commission, 2006), while in the USA the maximum AFM₁ concentration is regulated by the Food and Drug Administration (FDA) at 0.500 $\mu\text{g kg}^{-1}$ (Berg, 2003).

Various equations to predict the AFM₁ level in milk (ng kg^{-1}) from AFB₁ intake ($\mu\text{g per cow per day}$) have been proposed (Veldman et al., 1992a; Pettersson et al., 1989; Van Eijkeren et al., 2006). In dairy farms, the precise AFB₁ determination in animal feeds is useful to predict the AFM₁ concentration in milk and to avoid contamination levels exceeding the legal limits.

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3 51 The analytical methods for AFB₁ detection/quantification in foods and feedstuffs are based on
4
5 52 different separation/detection techniques (Trucksess and Wood, 1994; Stroka et al., 1999), such as
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8 53 enzyme linked immunosorbent assay (ELISA) (Aldao et al., 1995), HPLC (Sharma and Marquez,
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10 54 2001; Stroka et al., 2003; Arranz et al., 2006) or TLC (Bradburn et al., 1995). All these techniques need
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12
13 55 an efficient sample extraction method. Several studies have been conducted to investigate the
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15 56 extraction capacity of different organic/aqueous solvents, like methanol (Reif and Metzger, 1995;
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17 57 Stroka et al., 1999; Sharma and Marquez, 2001; Senyuva and Gilbert, 2005; Brera et al., 2007), acetone
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20 58 (Brandurn et al., 1995; Stroka et al., 2003; Arranz et al., 2006), acetonitrile (Stroka et al., 1999) and
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22 59 chloroform (Moller and Nyberg, 2004) solvents. Some studies investigated the organic solvent to water
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24 60 ratio (ml solvent/ml water), the ratio of solvent to sample (ml solvent/g sample) and the matrix effects
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26
27 61 (Brandburn et al., 1995; Stroka et al., 1999; Moller and Nyberg, 2004).

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29 62 The AFB₁ method based on a methanol:water (80:20 v/v) extraction procedure (MeOH) and on
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31 63 HPLC determination after an immunoaffinity clean-up step, seems to be the most common method in
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33
34 64 AFs-specialized laboratories (Trucksess and Wong, 1994; Stroka et al., 1999; Sharma and Marquez,
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36 65 2001; Moller and Nyberg, 2004). However, the method based on an acetone:water (85:15 v/v) solvent
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38
39 66 (AC) to extract AFs from feeds is also used in many laboratories, being the AOAC reference method
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41 67 (2003.2) for AFB₁ analysis (Stroka et al., 2003; AOAC, 2006).

42
43 68 One of the most recent approaches to the prevention of mycotoxicoses in livestock is the addition of
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45 69 adsorbents (ADSs) in the diet, that bind mycotoxins in the gastro-intestinal tract and are capable of
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48 70 reducing their bioavailability (Jouany et al., 2007; Masoero et al., 2009). Certain ADSs can decrease
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51 71 the aflatoxin CO in milk of dairy animals (Diaz and Smith, 2005; Jouany; 2007). Mycotoxin binders
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53 72 belong to three different groups: silicate materials or clay minerals (Phillips et al., 1991; Ramos and
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55 73 Hernandez, 1996), yeast cell wall-based products (Karaman et al., 2005; Yiannikouris et al., 2005), and
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58 74 activated charcoals (Galvano et al., 1996). However, these additives are not at present authorized in
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3 75 Europe for this purpose and they are currently added to industrial feeds as ingredients (yeast cell wall-
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6 76 based products) or anti-caking agents to improve feed pelleting efficiency (clay minerals) (Jaynes et al.,
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8 77 2007).

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10 78 No specific work has been published regarding the AF extraction efficiency of solvents (acetone and
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12
13 79 methanol mixed with different percentages of water) in animal feedstuffs containing mycotoxin ADSs,
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15 80 even if some authors (Veldman, 1992b; Galvano et al., 1996) indicated that the presence of clay ADSs
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17 81 in feeds could reduce the analytical recovery of AFB₁.

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20 82 The aim of this study was to examine if the inclusion of sequestering agents in AFB₁ contaminated
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22 83 feedstuffs can affect the extraction capacity of AC or MeOH extracting solvents.
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26 27 85 **Materials and methods**

28 29 86 *Preparation of feeds*

30
31 87 Two AFB₁ contaminated complete feedstuffs (1350 kg each) were produced in an industrial feed
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34 88 mill, using AFB₁ naturally contaminated ingredients: two corn meals (10.21 ± 1.27 and $32.87 \pm 2.32 \mu\text{g}$
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36 89 kg^{-1}), wheat bran ($1.40 \pm 0.31 \mu\text{g kg}^{-1}$), and soybean meal ($1.11 \pm 0.23 \mu\text{g kg}^{-1}$); the other ingredients
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38
39 90 were uncontaminated barley and sunflower meal, and a mineral/vitamin supplement. The two maize
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41 91 meals were used in order to obtain, respectively, a low contaminated (LC; $7.57 \pm 0.65 \mu\text{g kg}^{-1}$)
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43 92 feedstuff with a contamination close to the EC limit of $5 \mu\text{g kg}^{-1}$ (European Commission, 2003) and a
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45 93 highly contaminated (HC; $15.33 \pm 1.12 \mu\text{g kg}^{-1}$) feedstuff with a contamination three times higher than
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48 94 the EU limit (Table 1). Both feeds, free of clay minerals or other anti-caking agents, were mixed for 4
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50 95 minutes in a 2000 kg industrial mixer (MO/20, Grespan, Treviso, Italy).

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53 96 The two contaminated feeds were divided into 108 sub-samples (25 kg each), 54 for LC and 54 for
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55 97 HC, and tested for the AFB₁ contamination homogeneity, randomly selecting and checking 9 samples
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58 98 for LC and 9 samples for HC. The 18 samples were analyzed in duplicate with AC and MeOH
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3 99 extraction solvents (Table 1).
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6 100 In this study we tested four different types of silicate minerals (sodium and calcium bentonite clays;
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8 101 zeolite and kaolinite), one yeast cell wall-based product, an activated carbon, and three commercial
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10 102 products commonly used in industrial feed mills and in dairy farms (Table 2) (Masoero et al., 2009).
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12
13 103 The ADSs were characterized for elementary composition (Table 2) and used at two inclusion levels
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15 104 (10 and 20 g kg⁻¹) in LC and HC feedstuffs. The nine ADSs were a sodium bentonite (Amcol
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17 105 International Corp., Arlington Heights, IL, USA), a calcium bentonite (Tecnozoo, Padova, Italy), a
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20 106 zeolite (Fluka 96096, Sigma-Aldrich Chemie GmbH, Switzerland), a kaolinite (Fluka 03584, Sigma-
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22 107 Aldrich Chemie GmbH, Switzerland); a yeast cell wall-based product (Mycosorb[®], Alltech Italy,
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24 108 Bologna, Italy), an activated carbon product (Acque Nymco, Milan, Italy), and three commercial ADSs
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27 109 (Atox[®], Grupo Tolsa, Madrid, Spain; Myco AD AZ, Ascor Chimici, Forlì-Cesena, Italy; Novasil[™]
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29 110 plus, Trouw Nutrition Int., Verona, Italy). The manufacturer's specifications of the commercial
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32 111 products classify Atox[®] and Myco AD AZ as clay mixtures where the presence of aluminosilicates of
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34 112 the smectite group exceeds 85% of the product. In particular, the first could be considered a magnesium
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36 113 bentonite, being magnesium the dominant cation, while the second is a mixture of sodium and calcium
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39 114 bentonite. Novasil[™] plus is generally described by the producer as a hydrated sodium calcium
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41 115 aluminosilicate. Recently, some authors reported that this product could be classified as a
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43 116 montmorillonite clay (Pimpukdee *et al.*, 2004, Bailey *et al.*, 2006).
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45
46 117 The 108 feed sub-samples were randomly assigned to the 9 ADSs (12 sub-samples each) to obtain 3
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48 118 replicates for the two inclusion levels (10 and 20 g kg⁻¹) and the two contamination levels (LC and
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50
51 119 HC). Then, all the sub-samples were mixed for 4 minutes in a 50 kg mixer before being analyzed for
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53 120 the AFB₁ content.
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55 121 The experimental model studied the effects of: contamination levels (LC and HC); ADSs (n=9);
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58 122 doses (10 and 20 g kg⁻¹) and replicates (n = 3) for a total of 108 samples.
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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 AFB₁ 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 AFB₁ concentration of $95.176 \mu\text{g l}^{-1}$. 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 $19.035 \mu\text{g l}^{-1}$.

Extraction procedures

Sample preparation and clean up. To determine AFB₁ 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

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3 147 ½ filter paper (Dassel, Germany). Five ml of the filtrate were diluted with 45 ml of distilled water and
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6 148 the solution was purified through an immunoaffinity column (R-Biopharm Rhône Ltd, Glasgow, UK),
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8 149 previously conditioned with 20 ml of PBS. After washing, the column with 5 ml distilled water, AFB₁
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10 150 was slowly eluted with 2.5 ml of methanol.
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13 151 The eluate was dried under a gentle stream of nitrogen, re-dissolved in 1 ml of acetonitrile:water
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15 152 (25:75, v/v), and vortex-mixed for a few seconds; the extract was then filtered (Millipore Corporation,
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17 153 Bedford, Massachusetts, USA; HV 0.45 µm) and injected (30 µl) into the HPLC.
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21 22 155 **Apparatus**

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24 156 Analysis was performed using an HPLC instrument consisting of a LC-200 pump (Perkin Elmer,
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27 157 Norwalk, CT, USA) an AS-2055 sampling system, a FP-1520 fluorescence detector (Jasco
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29 158 Corporation, Tokyo, Japan), and a UV derivatizer (UVETM derivatizer, LC tech, Dorfen, Germany); the
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32 159 instrument was controlled by Borwin 1.5 software (Jasco). A Superspher RP-18 column (4 µm particle
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34 160 size, 125 x 4 mm i.d., Merck) was used at ambient temperature with a mobile phase of
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36 161 water:methanol:acetonitrile (64:23:13, v/v/v) at 1 ml min⁻¹. The AFB₁ was detected after post-column
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38
39 162 photochemical derivatization to AFB_{2a}. The detector was set at 365 nm excitation and 440 nm emission
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41 163 wavelengths.
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43 164 The elemental components of the ADSs were determined by a semi-quantitative X-ray fluorescence
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46 165 analysis (Sánchez-Ramos *et al.*, 2008) using the scanning electron microscope Phillips XL 30 E-SEM
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48 166 (Phillips electron Optics B.V., Eindhoven, Netherlands) equipped with an energy-dispersive X-ray
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51 167 detector model Genesis (Edax Inc., Mc Kee Drive, Mahwah NY, USA) operating in low vacuum.
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54 55 169 **Recovery experiment**

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58 170 Before analysis of feeds, the AFB₁ recoveries were performed with an AF-free cattle feed (830 g kg⁻¹
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3 171 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
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6 172 mineral/vitamin supplement) without addition of any ADSs. The recovery values were estimated by
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8 173 spiking eight blank samples (25 g each) with 0.5 ml of an AFB₁ standard solution (250 µg l⁻¹ dissolved
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10 174 in methanol) in order to obtain a concentration of 5 µg kg⁻¹. After addition of the spiking solution, the
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13 175 organic solvent was evaporated at room temperature (22°C) under a ventilated hood for 2 hours. The
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15 176 spiked samples were then analysed according to the two extraction procedures (MeOH and AC, four
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17 177 replicates each) as described above. Four replicates of a certified reference material (ground corn, R-
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20 178 Biopharm Rhône LTD) were also analysed.
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22 179 23 24 180 *Statistical analyses*

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27 181 The AFB₁ contents in feedstuffs without ADSs were tested for homogeneity using the one-way
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29 182 analysis of variance procedure of SAS[®] (version 9.1, SAS Institute Inc., Cary, NC).
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32 183 The AFB₁ contents of the feedstuffs supplemented with ADSs were evaluated in a completely
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34 184 randomized design using the general linear model procedure of SAS[®]. A 2 x 2 x 9 factorial
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36 185 arrangement for each AF contamination level (HC and LC) was used and fixed effects in the model
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39 186 included extraction procedure (Method; 2 levels), inclusion doses (Dose; 2 levels), ADSs (ADS; 9
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41 187 levels) and associated first order and second order interactions. Dunnett's test was used for comparing
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43 188 treatments with the appropriate control (Lowry, 1992). Significance was declared at $P < 0.05$.
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46 189 47 48 190 **Results and discussion**

49
50 191 The average recovery values were 96.6±1.8% and 97.8±2.1% for MeOH and AC extraction
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52
53 192 procedures, respectively. The declared AFB₁ contamination of the certified reference material was
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55 193 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
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58 194 procedures, respectively. The limit of detection (LOD, signal-to-noise ratio of 3:1) and of
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3 195 quantification (LOQ, signal-to-noise ratio of 10:1) were respectively 0.02 and 0.05 $\mu\text{g kg}^{-1}$.

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5 196 No statistical differences were found in AFB₁ levels of feedstuffs non containing ADSs, when
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8 197 extracted with AC or MeOH, even if numerically higher values were obtained with AC. The *F*-test at
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10 198 the 95% confidence level demonstrated that the sub-sample variance was not different from the
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12 199 analytical variance ($F_{calc}=0.479$ and $F_{calc}=0.358$ versus $F_{crit}=3.500$, respectively for HC and LC), thus
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15 200 demonstrating good homogeneity of the prepared feeds (table 1).

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17 201 Considering the composition of commercial clays (table 2), NovasilTM plus can be classified as a
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19 202 calcium montmorillonite (Pimpukdee et al., 2004; Bailey et al., 2006; Afriyie-Gyawu et al., 2008), a
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21 203 mineral clay that represents the main constituent of bentonite clays (Diaz and Smith, 2005). Atox[®],
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23 204 which is characterized by a high Mg level ($14.8 \pm 0.39\%$), can be classified as a magnesium bentonite,
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25
26 205 in agreement with the manufacturer's specifications; Myco AD AZ exhibited the highest carbon and
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28 206 the lowest ash level (41.0 and 75%, respectively) among clays.

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30 207 The statistical analysis showed a dose-dependent effect ($P < 0.01$) of ADSs inclusion on AFB₁
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32 208 recoveries, that were lower when the higher ADS dose (20 g kg^{-1}) was included in the HC and LC
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34
35 209 feedstuffs (tables 3 and 4).

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37 210 In particular, in HC feedstuff integrated with ADSs and extracted using the AC solvent, the average
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39 211 recovery was 75.0%, while a very low AFB₁ recovery (12.0%) was obtained when the MeOH solvent
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41 212 was used. A similar trend, but higher AFB₁ recoveries, was observed for the LC feedstuffs (84.0 and
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43 213 22.8% using AC and MeOH, respectively). Therefore, the AFB₁ extraction capacity of MeOH in
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46 214 feedstuffs containing ADSs resulted significantly lower ($P < 0.001$) with respect to AC: the MeOH
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48 215 method in presence of ADSs showed figures that are far from performance requirements for AF
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51 216 methods fixed in the EC Regulation 401/06 (recoveries of 70-110% and 80-110% in the range 1-10 and
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53 217 $>10 \mu\text{g kg}^{-1}$, respectively). Besides, when the MeOH extraction method was applied to HC feedstuffs
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56 218 integrated with ADSs, the AFB₁ concentrations resulted lower ($P < 0.001$) than the control value (13.8
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3 219 $\mu\text{g kg}^{-1}$) for all the samples (table 3) and under the EU limit of $5 \mu\text{g kg}^{-1}$ (EC, 2003). On the contrary,
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6 220 when AC was used the observed AFB₁ concentrations exceeded this limit, with values ranging from 5.7
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8 221 $\mu\text{g kg}^{-1}$ (activated carbon) to $13.6 \mu\text{g kg}^{-1}$ (NovasilTM plus). Similar results were obtained in LC
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10 222 feedstuffs (table 4), with AFB₁ concentrations exceeding the EU limit only when the analyses were
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13 223 performed using the AC solvent (with the exception of activated carbon).
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15 224 In particular when the activated carbon was added to the feedstuffs the AFB₁ content measured using
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17 225 the AC method differed ($P < 0.001$) from control values (15.3 and $7.6 \mu\text{g kg}^{-1}$, respectively for HC and
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20 226 LC feeds) in all the experimental conditions (Tables 3 and 4). In presence of activated carbon, the
21
22 227 AFB₁ recoveries measured with AC at 10 and 20 g kg^{-1} were respectively 52% and 37% in the HC; 55
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24 228 $\%$ and 39% in the LC feeds,. This can be due to a high affinity of the activated carbons *versus* the
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26
27 229 AFB₁ molecule (Lemke et al., 2001; Vekiru et al., 2007) that could cause a reduction of extraction
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29 230 capacity of the AC mixture. In agreement with our data, Galvano et al. (1996) reported a similar
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32 231 reduction (from 41% to 74%) of analytical content of AFB₁ in a pelleted feed containing activated
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34 232 carbons.
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36 233 Also when the sodium bentonite was added to contaminated feeds, lower ($P < 0.05$) AFB₁
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39 234 concentrations with respect to controls were measured both in HC (20 g kg^{-1} dose) and LC (10 and 20 g
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41 235 kg^{-1} doses) feedstuffs. However, the AFB₁ recoveries were higher than those obtained for activated
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44 236 carbon, ranging from 63% to 76% .
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46 237 The data obtained in this experiment indicate that a routine use of the MeOH solvent for AFB₁
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48 238 analysis of unknown feedstuffs, can produce misleading results if they contain an ADS. Consequently,
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51 239 when an ADS is included in a contaminated feed, the analytical result for AFB₁ could comply with or
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53 240 largely exceed the EU limit in feedstuffs for dairy animals (EU, 2003) according to whether it was
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55 241 obtained using MeOH or AC as extracting solvent, respectively. In any case, also AC did not result
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58 242 fully adequate for AFB₁ quantification in experimental feeds containing two types of ADS (i.e., sodium
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3 243 bentonite and activated carbon). This fact suggests that other solvent mixtures should be evaluated to
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6 244 improve AFB₁ extraction from feeds containing ADSs.

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9 10 246 **Conclusions**

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13 247 Erroneous data regarding the true AFB₁ contamination level of a feedstuff may lead to incorrect
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15 248 managerial decisions in dairy farms and consequently to higher than expected AFM₁ levels in milk. In
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17 249 fact, the AFM₁ level in bulk milk ($\mu\text{g kg}^{-1}$) depends on the quantity of AFB₁ ingested ($\mu\text{g per cow per}$
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20 250 day). According to Veldman et al. (1992a), this relationship can be described by the following
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22 251 equation:

$$23 \quad \text{AFM}_1 (\mu\text{g kg}^{-1} \text{ of milk}) = (1.19 * x + 1.9) / 1000$$

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27 253 where x is the AFB₁ (μg) ingested daily by each cow in the herd.

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29 254 According to our study, if 10 kg of the HC feedstuff is daily administered to cows, considering the
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32 255 AFB₁ result obtained by AC extraction ($15.33 \pm 1.18 \mu\text{g kg}^{-1}$), $153.3 \mu\text{g per cow per day}$ can be
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34 256 calculated and the predicted AFM₁ bulk milk concentration should be $0.184 \mu\text{g kg}^{-1}$. Otherwise, if 10
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36 257 kg of the HC feedstuff containing 10 g kg^{-1} of an ADS is given to cows, the AFB₁ level in the feed will
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39 258 be 12.4 or $2.3 \mu\text{g kg}^{-1}$ (mean values excluding the activated carbon thesis) according to whether the AC
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41 259 or MeOH solvent had been adopted, respectively. Consequently, a daily AFB₁ consumption of 123.7 or
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44 260 $23.3 \mu\text{g per cow per day}$ can be calculated, and a milk contamination of 0.149 or $0.030 \mu\text{g kg}^{-1}$ of
45
46 261 AFM₁ can be predicted. The latter value, calculated on the basis of the MeOH result, is well below the
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48 262 limit of $0.050 \mu\text{g kg}^{-1}$ set by the EU (EU, 2006), but the real situation is quite different, as reported in
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51 263 our previous work (Masoero et al., 2009). In conclusion, it is clear that the ascertainment of the real
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53 264 AFB₁ concentration of feedstuffs containing ADS is essential to avoid putting dairy farmers at risk of
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55 265 unknowingly producing contaminated milk, which will have to be discarded.

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3 267 **Acknowledgments**
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6 268 This research was supported by the AFLARID project of Ministero delle Politiche Agricole,
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8 269 Alimentari e Forestali (MiPAAF - Italy).
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Table 1. AFB₁ content ($\mu\text{g kg}^{-1}$) 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 ($\mu\text{g kg}^{-1}$)		Feedstuff composition (g kg^{-1})	
		AC	MeOH	High contamination (HC)	Low contamination (LC)
High contaminated corn meal	3	32.87 \pm 2.32	29.46 \pm 3.01	400	50
Low contaminated corn meal	3	10.21 \pm 1.27	8.78 \pm 1.57	160	500
Wheat bran	3	1.40 \pm 0.31	1.36 \pm 0.22	120	120
Barley meal	3	n.d.	n.d.	150	160
Soybean meal	3	1.11 \pm 0.23	1.08 \pm 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 \pm 1.18	13.81 \pm 1.05		
LC feedstuff	9	7.57 \pm 0.65	7.02 \pm 0.63		

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

^aContent per 100 g of trace min./vit. Supplement: 120000 IU of vit. A; 9000 IU of vit. D₃; 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.

1 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

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

Table 3. AFB₁ 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 AFB₁ 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*
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
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
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
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
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***
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
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
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

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

^aS.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. AFB₁ concentrations ($\mu\text{g kg}^{-1}$) 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 AFB₁ concentrations in untreated LC (control) were 7.57 ± 0.65 and $7.02 \pm 0.63 \mu\text{g kg}^{-1}$ 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

^aS.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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