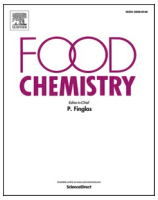
Food Chemistry 432 (2024) 137198

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Optimization and *in-house* validation of the analytical procedure for official control of bentonites as aflatoxin inactivators

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ARTICLE INFO

Keywords:
Aflatoxins
Bentonite
Adsorption
Safety
Animal Feed
Analytical Chemistry Methods

ABSTRACT

In Europe, bentonites are allowed as feed additives for aflatoxin mitigation (1m558) provided they have specific mineralogical characteristics and an aflatoxin-binding capacity (BC_{AfB1}) above 90%. BC_{AfB1} is determined by an official adsorption assay using an aflatoxin solution (4 mg/L) in acetate buffer (pH 5.0) and a bentonite at 0.02% (w/v). To date, the robustness of this method has not been investigated.

In this work, we addressed this challenge and performed a robustness study by analyzing six bentonite samples that met the mineralogical requirements for claim code 1 m558. Leading factors selected for robustness testing were (*1*) preparation mode of bentonite suspension, (*2*) residual amount of acetonitrile in the test trial, (*3*) acetate buffer concentration, (*4*) incubation time, and (*5*) centrifugation. It was statistically evinced that factors *2* and *5* affected the results. Due to its weakness, the method excluded 4 out of six bentonites to be marketed in EU, being $BC_{AfB1} < 90\%$. A new protocol was developed by keeping the main experimental parameters of the official assay and was in-house validated. This protocol yielded $BC_{AfB1} > 90\%$ for all test bentonites and showed satisfactory precisions with a RSD_I of 3.4% and HorRat < 2. Its validity was proven by the isotherm approach, comparing Langmuir adsorption parameters with BC_{AfB1} values. Application of the protocol to bentonites other than montmorillonite was demonstrated.

1. Introduction

The use of feed additives that reduce the exposure of animals to mycotoxins is regarded as a way to improve animal welfare. These additives are defined as substances that are mixed into feed and then adsorb or denature mycotoxins in the digestive tract of animals. There is a vast scientific literature covering mycotoxin detoxification in animal feed and a large number of substances have been proposed as physical or biological adsorbents or microbiological/enzymatic transformation agents (Liu et al., 2022; Avantaggiato, Greco, D'Ascanio, & Logrieco, 2021; Colovic et al., 2019; Vila-Donat, Marín, Sanchis & Ramos, 2018; Zhu, Hassan, Lepp, Shao, & Zhou, 2017; Karlovsky et al., 2016; Avantaggiato, Greco, Damascelli, Solfrizzo & Visconti, 2014; Boudergue et al., 2009).

Efficacy/safety assessment and authorisation of additives for mycotoxin reduction in feeds differs across the world. So far, most countries where these additives are used on regular basis lacks on regulations regarding their use and/or evaluation. In the European Union (EU), mycotoxin-detoxifying agents are regulated by the European Commission (EC) No. 386/2009, which amended the Regulation (EC) No 1831/2003 on additives for use in animal nutrition, opening a new functional group in the category of technological feed additives, i.e., "substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action" (EC, 2003; EC, 2009). In July 2010, the Eupean Food Safety Autorithy (EFSA) issued a statement where it detailed the additional information that are required to perform an assessment of the safety and efficacy of this new group of additives (EFSA 2010). To date, three additives have received authorisation by the EC to be used as substances for reduction of the contamination of feed by mycotoxins. They are the micro-organism strain DSM 11798 for trichothecenes detoxification of feeds for pigs (EC, 2013a) and all avian species (EC, 2017); the fumonisin esterase EC 3.1.1.87 produced by *Komagataella pastoris* DSM 26643 for fumonisin detoxification of feeds for all animal species (EC, 2021a); and a clay mineral (the bentonite in the form of di-octahedral smectite/montmorillonite) as aflatoxin adsorbent for all animal species (EC, 2013b).

Clay minerals, as the materials in "greening 21st century material

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https://doi.org/10.1016/j.foodchem.2023.137198
Received 19 May 2023; Received in revised form 14 July 2023; Accepted 16 August 2023
Available online 24 August 2023

worlds", have attracted attention owing to their adsorption performance, high chemical stability and biocompatibility advantages. More, they are naturally abundant, green, non-toxic and low-cost. Aluminosilicates are the largest and most important class of clay minerals (Kumari & Mohan, 2021; Elliott, Connolly & Kolawole, 2020; D'Ascanio et al., 2019). There are two major sub-classes in this group, phyllosilicate and tectosilicate, with a wide range of applications, including mycotoxin adsorption. The phyllosilicate sub-class mineral clays include significant adsorbents such as the montmorillonite/smectite group, the kaolinite group and the illite (or clay-mica) group. Bentonites are usually impure smectite clays belonging to the class of the phyllosilicates and containing a wide variety of other minerals as impurities, e.g. quartz, mica, feldspar, pyrite or calcium carbonate (Akisanmi, 2022; Deng, Barrientos Velázquez, Billes & Dixon, 2010; Phillips et al., 1995).

As stated by the EC Regulation 1060/2013, bentonites (as smectite clays) are allowed as feed additives (binders, substances for control of radionuclide contamination, and anticaking agents) (1m558*i*) for all animal species, as well as for mitigation of aflatoxin contamination for ruminants, swine, and poultry (1m558). This was based on several, published studies indicating good correlation between the *in vitro* and *in vivo* efficacy of bentonites in adsorbing aflatoxins as reported by EFSA (2011). In this opinion, EFSA concluded that, under the proposed conditions of use, bentonite does not have an adverse effect on animal health, human health or the environment, and that it has the potential to be efficacious as aflatoxin binder for all ruminants. In 2013, the EC considered the aflatoxin binding capacity of bentonite as a characteristic of this clay and extended its application to poultry and pigs (EC, 2013b). According to this regulation, a bentonite used as an aflatoxin adsorbent (1m558) should contain $\geq$ 70% of di-octahedral montmorillonite, <10% opal and feldspar, <4% quartz and calcite; and it has to demonstrate an aflatoxin-binding capacity (BC_{AfB1}) above 90%. The latter is a key point of the Regulation and is determined by a simple adsorption test which is carried out in a buffer solution at pH 5.0 using "intensified" conditions, i.e. a low adsorbent dosage (0.02 %, w/v) and a high toxin concentration (4 mg/L). This adsorption test is applied as a certified method to reliably and reproducibly select efficacious bentonites to be used as aflatoxin-binders, i.e. binders with aflatoxin-adsorption equal or higher than 90%. In the frame of EC regulation of bentonite as aflatoxin-adsorbent, the adsorption test to measure BC_{AfB1} was developed and *in-house* "validated" by an applicant and was evaluated by the EU Reference Laboratory (EURL), which concluded that the method is suitable for the monitoring of aflatoxin-binding capacities above 90% (Von Holst, Robouch, Bellorini, González de la Huebra & Ezerskis, 2016).

Although the validation/verification study of the method for BC_{AfB1} determination showed satisfactory results, several assumptions suggest that it leaves room for analytical interpretation of some experimental parameters. It has been observed that slight variations of the experimental conditions laid down by the method can accumulate significant differences for a single bentonite sample. As a consequence, the method can lead to misleading results, and a bentonite sample complying with the Regulation could be improperly excluded from the EU market.

By addressing this issue, the present study intends to solve the problem of adapting to the needs of other laboratories the method for the determination of BC_{AfB1} of bentonites, and to check for its robustness. The factors selected for the robustness study were related to the requirements and characteristics of the method, and the purpose for which it is used. Five leading factors were analyzed and, for statistical reasons, six different bentonite samples were tested. In accordance with the EC Regulation 1060/2013, these samples were all bentonites containing > 70% of di-octahedral smectite (montmorillonite) as determined by XRD analysis. A new protocol for BC_{AfB1} measurement of bentonites was developed by keeping constant the main experimental parameters of the official adsorption assay. This optimized method was *in-house* validated using internationally recognized guidelines, and its validity was proven by the adsorption isotherms approach. Application of the optimized protocol to smectites other than those belonging to the di-octahedral group is demonstrated.

2. Materials and methods

2.1. Chemicals and AFB1 analysis

Aflatoxin B_1 (AFB_1) standard (purity > 99%) was supplied by Sigma-Aldrich (Milan, Italy). All chemicals used were of analytical grade unless otherwise stated. All solvents (HPLC grade) were purchased from J.T. Baker (Deventer, The Netherlands). Water was of Milli-Q quality (Millipore, Bedford, MA). Stock solutions of AFB_1 (1 mg/mL) were prepared by dissolving the powder of AFB_1 in acetonitrile and stored in the dark at 4 °C. The actual concentration of these solutions was verified by UV–vis spectrophotometric analysis according to the AOAC Official Methods of Analysis (2016).

AFB_1 was analyzed by high performance liquid chromatography with fluorometric detection (HPLC-FLD) as detailed by D'Ascanio et al. (2019). The method of AFB_1 analysis in buffer solutions or in aqueous supernatants of test materials was sensitive, and showed a good selectivity, accuracy, and precision, with relative standard deviations (RSD_s) <6%. Quantitative analysis of AFB_1 in supernatant samples was performed by standards calibration curves and peak-area measurement. The HPLC method was linear ($p < 0.001$) in the AFB_1 concentration range of 0.02–5.0 μg/mL (seven mycotoxin levels, $n = 3$). Calibrants were prepared in buffered solutions at pH 5. The regression coefficient (R^2) was acceptable (≥0.998). The limit of quantitation was 20 ng/mL of AFB_1 (S/N ratio = 10). This limit was almost 2 orders of magnitude below toxin concentration of the working solutions used for adsorption tests and safeguarded the ability to perform accurate liquid chromatography (LC) measurements even when > 90% mycotoxin adsorption occurred. The method was selective and no compound in the aqueous supernatants of test materials (matrix blank controls) interfered with identification and quantification of AFB_1 peaks. Chemical precipitation and losses of AFB_1 due to nonspecific adsorption were not detected. Area values of LC peaks of AFB_1 for blank control samples were comparable to those for standards for aqueous supernatants of test materials spiked with toxins, at the concentrations of the standard calibration curves.

2.2. AFB_1 adsorbing agents

Six test samples of bentonite were used for AFB_1 adsorption studies. Five, out of six samples, were provided by Laviosa Chimica Mineraria S.p.A (Livorno, Italy) and were labeled with a number code from bentonite 1 (B1) to 5 (B5). A bentonite purchased from Sigma (B-3378 Lot 18H0934) was used as a reference material and was named as Ref Bent. All bentonites were examined for mineralogical and physico-chemical properties, namely mineral, and metal contents, carbonate content, pH, total moisture content, particle size distribution, cation exchange capacity (MBI/CEC), swell index, loss of ignition at 960 °C, and viscosity, as described by D'Ascanio et al. (2019).

In addition, the *in-house* validated method developed by the study was applied to determine the BC_{AfB1} of 13 minerals belonging to the group of di-octahedral smectites and 17 samples of the tri-octahedral smectite group, which all were kindly provided by Laviosa Chimica Mineraria S.p.A.

2.3. In vitro assays to determine the BC_{AfB1}

2.3.1. Official adsorption assay

Bentonite test samples were assayed for AFB_1 binding capacity in accordance with the method verified by EURL and described in the EC Regulation 1060/2013. In brief, a working solution of AFB_1 containing 4 μg/mL of toxin, in acetate buffer (pH 5), was mixed with the test substance to have a final concentration of the binder at 0.02% w/v (1 mg binder/5 mL – using the "indirect weighing") and was incubated for 60 min at 37 °C under permanent shaking. To prepare the AFB_1 working

solution, 1.74 mL of an AFB_1 stock solution (115 μg/mL in acetonitrile) was mixed with 50.0 mL of acetate buffer (100 mM) in a volumetric flask. In a 50 mL PP-test tube, 100 mg of each test product were weighed by using an analytical balance. 10 mL-buffer solution was added to each tube (giving a concentration of 1.0% w/v) and vigorously mixed by vortex for few seconds. In a 15 mL PP-test tube, 4.9 mL of AFB_1 working solution in acetate buffer (4 μg/mL) were supplemented with 100 μL of the bentonite suspension, giving a final concentration of 0.02% w/v. The tubes were mixed by vortex for few seconds and shaken in a thermostatically controlled shaker at 37 ± 0.5 °C, at a speed of 250 rpm for 60 min. After the incubation period, experimental tubes and blanks were centrifuged for 15 min at 3660 rpm and at 25 °C. Then, 500 μL of supernatants were transferred into glass amber vials and analyzed for AFB_1 by HPLC-FLD.

Three blank control samples (5 mL of AFB_1 working solution without the test products) were also prepared. These samples were subjected to the same test procedure and served as background control during the analysis to investigate the stability of toxin or any possible nonspecific adsorption. In addition, matrix blanks were prepared for each adsorbing agent. These tubes contained the adsorbents in toxin-free buffer solution and were treated like the experimental tubes containing the toxin. Matrix blanks were prepared to check for any component of the matrix interfering with the LC analysis of AFB_1. All adsorption tests were performed in triplicate.

2.3.2. Optimization of the official adsorption assay (robustness study)

To optimize the adsorption assay for BC_{AfB1} determination, experimental conditions set in the official method (EC, 2013b) were slightly modified (EC, 2021b; Eurachem Guide, 2014). Fixing experimental conditions of the EURL method (§0.2.3.1), main leading parameters of this method were analyzed and varied one-by-one. Leading factors selected for robustness testing were (*1*) preparation mode of bentonite suspension, (*2*) residual amount of acetonitrile in the test trial, (*3*) acetate buffer concentration, (*4*) incubation time, and (*5*) centrifugation. In some cases, different levels of each parameter were evaluated. Thereof, bentonites were first tested according to the experimental condition described in the EURL method (§ 2.3.1). Then, they were tested again by making some changes, each one in independent, triplicate experiments. Each variation of the method was made keeping constant the others, and then statistically analyzing the experimental results obtained for each sample and for each leading factor (as a whole of samples).

1) Bentonite suspension

The official method states that bentonites should be tested in "stringent" conditions, using a low concentration of sample set at 0.02% w/v. In our study, two methods to prepare the bentonite suspension were attempted. The method *1b* refers to the official EURL method (EC, 2013b).

1a) In a 4 mL-clear glass vial, 8 mg of each test product was suspended by 2 mL of buffer (giving a concentration of 0.4% w/v). After that, in a 4 mL-amber glass vial, 1.9 mL of AFB_1 working solution in buffer (4 μg/mL) was mixed with 100 μL of the bentonite suspension, giving a final concentration of 0.02% w/v.
1b) In a 50 mL PP-test tube, 100 mg of test product was suspended by 10 mL of buffer (final concentration at 1.0% w/v). After that, in a 15 mL PP-test tube, 4.9 mL of AFB_1 working solution in buffer (4 μg/mL) was mixed with 100 μL of the bentonite suspension, giving a final concentration of 0.02% w/v.

2) Residual amount of organic solvent in the test sample

This study assessed the effect of organic solvent residue (acetonitrile, ACN) in the test solutions of toxin on BC_{AfB1} values. So that, six working solutions of AFB_1 at 4 μg/mL (in buffer) were prepared by using proper volumes taken from different AFB_1 stock solutions, all prepared in ACN (Table 1). This yielded working solutions with a different residual concentration of ACN (as percentage, v/v). The mode *2c* in Table 1 refers to the EURL protocol (EC, 2013b).

3) Acetate buffer

As requested by the EURL protocol (EC, 2013b), working solutions of AFB_1 are prepared in acetate buffer (100 mM) at pH 5. Since for some LC analysis, a high concentration of buffers of analytical samples can result in salt precipitation into the chromatographic system (injector), four buffers at pH5 and different concentration were assayed, i.e. 1, 10, 50 and 100 mmol/L.

4) Incubation time

According to the EURL method (EC, 2013b), bentonites are incubated with AFB_1 working solutions for 60 min and permanent shaking. To study the effect of incubation time on AFB_1 adsorption, the suspension of bentonites was shaken in a thermostatically controlled shaker at 37.0 ± 0.5 °C, at a speed of 250 rpm, and for 30, 60 and 90 min.

5) Centrifugation

As requested by the adsorption assay, after the incubation period of bentonites with the toxin, all samples are centrifuged, and then the supernatants are analyzed for the residual mycotoxin content. Two ways to centrifuge the samples were assayed, with the *5a* referring to the EURL method (EC, 2013b):

5a) the whole sample in a 15 mL-PP test tube was centrifuged at 3660 rpm, for 15 min.
5b) the sample was allowed to settle, and then 1.0 mL of suspension was transferred into an Eppendorf tube and centrifuged at 14000 rpm, for 20 min.

2.3.3. Optimized adsorption assay

At the end of the optimization study, an adsorption assay was developed to determine the BC_{AfB1} of bentonites. This assay differed from the official method (EC, 2013b) in few items, namely the type of test tubes (4 mL-amber glass vials instead of 15 mL-PP tubes); preparation of the bentonite suspension for adsorption trials; final concentration of ACN in the test samples; concentration of acetate buffer; incubation time; and centrifugation. Main adsorption parameters of the official adsorption assay (EC, 2013b) were maintained.

The AFB_1 working solution (4 μg/mL) was prepared by diluting 0.2 mL of an AFB_1 stock solution (1 mg/mL, in ACN) with buffer (pH5, 1 mM) into a calibrated volumetric flask (50 mL). The bentonite suspension (0.02% w/v) was prepared as described above (§*1a*). This suspension was shaken in a thermostatically controlled shaker at 37 ± 0.5 °C, at a speed of 250 rpm for 90 min. After the incubation period, samples

Table 1
Residual amount of organic solvent (ACN) remaining in the AFB_1 working solutions used for the adsorption assay.

	AFB_1 concentration of the stock solution in ACN (*μg/mL*)	Volume of the stock solution in ACN (*mL*)	Final volume of the working solution in buffer (*mL*)	Residual amount of ACN in the working solutions (% *vol.*)
2a)	1000	0.2	50	0.4
2b)	400	0.5	50	1.0
2c)	115	1.74	50	3.5
2d)	80	1.25	25	5.0
2e)	57.5	3.48	50	7.0
2f)	40	2.5	25	10.0

were allowed to settle and 1.0 mL of the suspension was transferred into an Eppendorf tube and centrifuged for 20 min at 14000 rpm, and at 25 °C. Then, an aliquot of supernatants was analyzed for AFB_1 content.

2.3.4. Data calculations of BC_{AfB1}

AFB_1 adsorption is the percentage of AFB_1 adsorbed by the bentonites and is related to the quantity present at the beginning of the test, under the test conditions. It was calculated as the difference between the amount of AFB_1 in the supernatant of the blank tubes with no bentonite and the amount found in the supernatant of the experimental tubes with the bentonite. This amount was related then to the quantity present in the supernatant of the blank tubes and expressed in percent. Adsorption at 100% is obtained when no AFB_1 is detected in the supernatants of the experimental tubes.

2.4. In-house validation of the optimized adsorption assay

The optimized adsorption assay (§ 2.3.3) was validated by an intra-laboratory study according to the EC Regulation 519/2014 and AOAC Requirements for Single Laboratory Validation of Chemical Methods (AOAC, 2016), and its performances (repeatability and intermediate precision) were determined.

For the repeatability study (intra-assay precision), six bentonites were analyzed by a single operator (operator 1), and in a short period of time, using the same operative conditions. Adsorption experiments were performed in triplicate and by three consecutive days. For each sample, the amount of toxin (expressed as a concentration, μg/mL) sequestered by the bentonite was also calculated considering the initial AFB_1 concentration in the test sample (4 μg/mL) and the residual amount of toxin in the supernatants. Experimental values were expressed as mean of 9 independent replicates. The SD and the relative SD (RSD_r) were measured to assess, respectively, the variability and the precision of each data set. For these data sets, the normality of population composed by nine measurements was verified by Shapiro-Wilk test. In case of normality test failure, the outliers were identified by Grubbs' test and removed from the data set.

To determine the intermediate precision, the AFB_1 adsorption ability of each bentonite was determined by operator 1 and other two operators (operators 2 and 3), in a long period of time (6 months) using different operative conditions (HPLC systems, vessels, pipettes, etc.). Triplicate independent experiments were repeated three times (months 1, 3 and 6) by the operators. As described above, the amount of toxin (μg/mL) adsorbed by each bentonite was calculated and expressed as mean ± SD ($n = 27$). The relative standard deviation of the intermediate precision (RSD_I) was also measured.

To evaluate the acceptability of the precision of the method under intermediate conditions, the Horwitz Ratio (HorRat) was calculated (AOAC, 2016, Appendix F). HorRat represents the ratio of the repeatability relative standard deviation calculated from the data (RSD_I) to the predicted relative standard deviation of reproducibility ($PRSD_R$) calculated from the Horwitz formula (Rivera and Rodríguez, 2015):

$$- \ PRSD_R\% = 2^{[1 \ - \ 0{,}5 \ \log(C)]},$$

where C is the concentration of the analyte expressed as dimensionless mass fraction.

To determine the $PRSD_R$, aflatoxin concentration was set at 3.6 μg/mL corresponding to the amount of toxin adsorbed by a bentonite sample with a $BC_{AfB1} = 90\%$.

Under repeatability conditions, accepted values for HorRat(r) are between 0.3 and 1.3.

2.5. Adsorption isotherms

Equilibrium adsorption isotherms were performed in triplicate at constant temperature (37.0 ± 0.5 °C), pH 5 and 90 min of contact time, testing standard solutions containing an increasing AFB_1 concentration (1–10 μg/mL) with a fixed amount of bentonite (0.005%, w/v). This adsorbent amount was chosen as it yielded for all samples a range of aflatoxin adsorption values suitable for curve fitting and mathematical modeling.

Isotherms were obtained by plotting the amount of mycotoxin adsorbed per unit of mass of adsorbent (Q_{eq}) against the concentration of the toxin in the external phase (C_{eq}), under equilibrium conditions ($Q_{eq} = f(C_{eq})$. These data were transferred to SigmaPlot and fitted by the Langmuir, Freundlich and Sips models using the non-linear regression method and the Marquardt-Levenberg algorithm. Statistical analysis was performed using a one-way ANOVA with concentration (C_{eq}) as predictor variable and quantity of mycotoxin adsorbed (Q_{eq}) as dependent variable, as described by D'Ascanio et al. (2019).

2.6. Statistical analysis

Experimental data of BC_{AfB1} calculated by the robustness study were expressed as mean ± SD. For each parameter studied herein, means of groups (different experimental conditions inside the effect) were compared. The paired *t*-test was used for normally distributed data. ANOVA was applied to compare means between more than two groups (El-Hadary, Sulieman & El-Shorbagy, 2023). Significant level for comparisons was set in all cases at $p < 0.05$. The normality of the measures was tested in each group using Shapiro-Wilk test, whereas the homogeneity of variances across groups was verified using Levene's test. Not normally distributed measures were compared across groups using Kruskal–Wallis test. In the case of significant results, pairwise comparisons using Tukey test or Dunn's method for parametric or non-parametric ANOVA analysis, respectively, was performed using the SigmaPlot® Software, version 12 for Windows.

3. Results and discussion

3.1. Mineralogical and physico-chemical characterization of test bentonites

To study the performances of the analytical method to determine the BC_{AfB1} of bentonites, six minerals were selected considering their mineralogical and physico-chemical properties, as summarized in Table S1. Clay samples containing smectite in the form of montmorillonite as major mineral (>70%) and with traces of other minerals (<10%) were chosen.

XRD analysis of clay samples and determination of the d_{060} and d_{001} values provided definitive information on the mineralogical composition of these minerals, both in terms of clay and non-clay constituents (D'Ascanio et al., 2019). All samples showed presence of di-octahedral smectite as major mineral, which had the d_{060} line located in 1.496–1.499 Å, typical for montmorillonite (Table S1). In addition, trace amounts of some silica impurities, such as quartz, possibly cristobalite, opal, feldspars, were also listed. In all cases, carbonate content was ≤ 3.2%. Referring to the d_{001}-values, B1, B3 and B5 samples were identified as calcium-dominated smectites; B4 and Ref Bent were sodium-smectites; and sample B2 was identified as a sodic/calcic-smectite. The sodium-smectites (B4 and Ref Bent) and the sodic/calcic-smectite (B2) showed the highest swelling properties (Table S1). The calcium-smectites (B1, B3 and B5) exhibited low swelling tendency. All samples had values of loss of ignification at 960 °C ≤ 10% suggesting a low organic matter content. CEC values ranged from 68 to 105 cmol/kg. To avoid the effect of particle size on AFB_1 adsorption, all bentonites (except the Ref Bent) were finely ground and sieved to obtain samples with uniform and fine particle size (<45 μm).

3.2. Determination of BC_{AfB1} using the official method

The experimental values of BC_{AfB1} (mean ± SD, $n = 3$) obtained using

the official method (§2.3.1) ranged from 74.0 ± 1.5 to 96.0 ± 1.5%. Two out of six bentonites showed BC_{AfB1} higher than 90%, and were the calcium-smectite B5 (91.7 ± 3.7%) and the sodium-smectite Ref Bent (96.0 ± 1.5%). The remaining samples adsorbed the toxin with comparable capacity, but lower than 90% (i.e., 88.6 ± 1.7% for B4, 82.0 ± 1.3% for B2, 81.2 ± 2.2% for B3, 74.0 ± 1.5% for B1), and they should not be allowed in the EU market as feed additives for aflatoxin decontamination even though they meet the mineralogical requirements to claim 1m558 code.

3.3. Optimization of the official adsorption assay to determine the BC_{AfB1}

The purpose of this study was to evaluate the robustness of the official adsorption assay and to demonstrate which factors may affect the measurement of BC_{AfB1}. The study intends to assess the "fitness-for-purpose" of the method, and the degree to which the data produced by the method enables a user to make technically and administratively correct decisions for the stated purpose, i.e., selection of bentonites with $BC_{AfB1} \geq 90\%$. To this scope, we kept constant the main experimental parameters of the official assay and introduced slight variations on the protocol. Some parameters were selected as leading factors and were supposed to affect the performance of the procedure. These factors were analyzed singularly and for different levels as detailed in §2.3.2.

3.3.1. Preparation mode of bentonite suspension

According to the official method, efficient aflatoxin-adsorbing bentonites should sequester > 90% of the toxin when tested at a dosage as low as 0.02% w/v. This bentonite suspension was prepared using two different methods as detailed in §2.3.2, and relevant experimental results are shown in Table S2 as data recorded for each bentonite ($n = 3$) and as pool of data ($n = 18$). These values ranged from 74.6 to 96.6% using the first method (*1a*), and from 74.5 to 94.5% using the second one (*1b*). The mean values of the pooled data for the methods *1a* and *1b* were, respectively, 86.5 ± 7.4 and 84.9 ± 6.8%, and were not significantly different ($p = 0.060$). It seems that the method to prepare the bentonite suspension does not affect the determination of the BC_{AfB1} value. However, the first preparation mode (*1a*) could be preferred as it helps to save the toxin by reducing the volume of mycotoxin working solution. In addition, single bentonites behaved differently (Table S2). Most of test bentonites yielded slightly higher adsorption values when the mode *1a* was used, and for few of them (B4 and Ref Bent) this increase was significant ($p < 0.05$). Because of this change, three bentonites met the requirement of the Regulation showing $BC_{AfB1} \geq 90\%$ (Table S2).

3.3.2. Residual amount of ACN in the test sample

ACN is a polar organic solvent widely used to prepare stock solutions of mycotoxins. Some little amount of ACN contained in the working solutions may impact the toxin uptake by the adsorbing materials. The effect of ACN on BC_{AfB1} was studied as detailed in §2.3.2 and the experimental results are shown in Table S3.

The residual amount of ACN in the AFB_1 working solution significantly affected the ability of bentonites in adsorbing the mycotoxin ($p < 0.001$). The mean values of AFB_1 adsorption decreased from 90.2 ± 4.0 to 28.3 ± 13.9% when ACN raised from 0.4 to 10% v/v (Table S3). The pool of samples tested with a working solution containing 3.5% v/v ACN (as per official protocol) had an average value of 85.7 ± 7.9%. Bentonite samples behaved differently in sequestering the toxin depending on ACN content (Table S3). ACN concentration up to 5% v/v had no impact on the capability of the strongest adsorbent sample (Ref. Bent). Samples B5 and B4 adsorbed > 90% of AFB_1 up to 3.5% v/v of ACN, while samples B3, B2 and B1 showed the highest AFB_1 adsorption values (82–90%) at the lowest contents (0.4 and 1% v/v) of ACN. For all test bentonites, BC_{AfB1} values recorded using working solutions with ACN contents at 0.4 and 1% v/v did not significantly differ. At these low ACN contents, four out six bentonites met the requirement of the Regulation, showing $BC_{AfB1} \geq 90\%$.

Organic solvents, including ACN and methanol, are known to affect the swelling behavior of clays in water, since an increasing of their content can lead to a decrease in the values of the basal spacing (d_{001}) of the clay minerals (Kunz et al., 2019). Up to a maximum ACN content of 65% v/v, delamination by osmotic swelling can even occur. The study of Kunz et al. (2019) proved that both ACN and water are intercalated into the interlayer space of the clay, which is an important binding site for AFB_1 (Deng et al., 2012). In the model proposed by Deng et al. (2012) for the aflatoxin–smectite bonding, aflatoxin molecules occupy the interlayer space together with exchange cations and water molecules. It can be suggested that organic solvents that intercalate into the interlayer space of the clays can decrease the stability and selectivity of aflatoxin adsorption by the clays.

3.3.3. Acetate buffer concentration

According to the official method, working solutions of AFB_1 should be prepared in acetate buffer (100 mmol/L) at pH5. This study assessed the effect of acetate buffer concentration on BC_{AfB1} (Table S4). Mean adsorption values recorded for the pool of bentonites slightly increased from 86.8 ± 9.3 to 91.2 ± 3.8 % when concentration of buffer decreased from 100 to 1 mmol/L, but the difference was not statistically significant ($p = 0.667$). However, individual samples behaved differently. AFB_1 adsorption by the Ref Bent and sample B5 was not affected by the buffer concentration ($p > 0.068$), and yielded $BC_{AfB1} \geq 90\%$ in all adsorption tests. AFB_1 adsorption by samples B3 and B4 rose from 83 up to 91% when the concentration of the buffer decreased from 100 to 1 mM ($p < 0.002$). So that, four out of six test bentonites met the requirement of the Regulation with values of $BC_{AfB1} \geq 90\%$. Samples B2 and B1 did not achieve these values but showed significantly higher AFB_1 adsorption values when tested with the 1 mM acetate buffer ($p < 0.001$).

3.3.4. Incubation time

To study the effect of incubation time on BC_{AfB1}, the test materials were assayed as described in the official method, but their suspension was incubated for different times with the AFB_1 working solution (i.e., 30, 60, and 90 min). Experimental values of AFB_1 adsorption are presented in Table S5. The mean adsorption value recorded for the bentonites as a pool was 86%, and it did not differ by changing the time. All samples, except B5 ($p = 0.047$), showed an AFB_1 adsorption capacity consistent over time. This implies that a minimum contact time of 30 min is sufficient for effective AFB_1 adsorption in the tested samples. It can be concluded that time points from 30 to 90 min should not affect the performance of the method.

The effect of incubation time on the mycotoxin adsorption process by different materials has been evaluated by several studies (Hojati, Norouzian, Assadi, Alamouti & Afzalzadeh, 2021; Gonçalves, Gonçalves, Rosim, Oliveira, & Corassin, 2015; Faucet-Marquis, Joannis-Cassan, Hadjeba-Medjdoub, Ballet & Pfohl-Leszkowicz, 2014; Avantaggiato et al., 2014) and as in our study most of them found no difference among the time points. Goncalves et al. (2015) studied the effect of incubation time on the AFB_1 adsorption by different *Saccharomyces cerevisiae*-based products and found no difference ($p > 0.05$) among the time points. Similarly, Faucet-Marquis et al. (2014) showed as adsorption equilibrium of zearalenone and ochratoxin by yeast-based products was reached after a short time of incubation, and an increase in time did not improve mycotoxin uptake. Avantaggiato et al. (2014) also studied the effect of contact time during mycotoxins adsorption process by grape pomace. For all tested mycotoxins, including AFB_1, maximum adsorption was reached in 15 min and no further change was obtained after 15 min up to 2 h.

3.3.5. Centrifugation

During the toxin adsorption assay, the particles of the adsorbing material suspended in the buffered working solution can be separated by centrifugation. This is a critical step of the method, and a high-speed

centrifugation should be used for those finest materials that leave small particles floating in the supernatants. These particles that contain a fraction of the adsorbed toxin are not discarded with the pellet and prior to LC determination of residual amount of toxin in the supernatant, thus producing misleading results on the extent of adsorption, as well as causing technical problems to the chromatography systems. As in the official method for BC_{AfB1} assessment of bentonites, testing solutions are centrifuged in 15 mL-PP test tubes at 3660 rpm for 15 min. Based on our observations, higher centrifugation speed and longer time can be required to centrifuge materials with very small particle size. To study the effect of centrifugation, test samples were centrifuged as in the official method (3660 rpm, 15 min), either in Eppendorf tubes at higher centrifugation speed and time (14000 rpm, 20 min). The experimental results of this study are shown in Table S6. The mean values of aflatoxin adsorption obtained by the two modes of centrifugation and for the pool of bentonites were 90% using high centrifugation speed and 84% using low speed. Significantly higher BC_{AfB1} values ($p < 0.002$) were recorded with most of the test bentonites when centrifuged at a higher speed (Table S6), and three out of six bentonites had BC_{AfB1} values $\geq$ 90%. It can be concluded that centrifugation significantly affects the performance of the method for BC_{AfB1} determination, and that high-speed centrifugation produces better aflatoxin adsorption compared to low speed. To the best of our knowledge, the effect of centrifugation speed on BC_{AfB1} values has not been studied. However, several previous studies dealing with aflatoxin adsorption by bentonites (Vila-Donat et al., 2020; D'Ascanio et al., 2019; Greco et al., 2019; Faucet-Marquis et al., 2014; Grant and Phillips, 1998) centrifuge test samples at high speed (> 10000 rpm).

3.3.6. Determination of BC_{AfB1} using the optimized adsorption assay

The preliminary optimization study showed that some protocol variations can impact the reliability of the EURL method for BC_{AfB1} measurement. A new protocol was set up by choosing the experimental conditions that in the optimization study produced higher BC_{AfB1} values. The main characteristics of the EURL assay were maintained, and the new protocol differed from the official method in few items, namely the type of test tubes, preparation of the bentonite suspension for adsorption trials, final concentration of ACN in the test samples, concentration of acetate buffer, incubation time, and centrifugation.

Table 2 and Fig. 1 report the results obtained by using the official protocol (EURL method), the protocols that varied in one factor (those that achieved higher BC_{AfB1} values), and the new optimized method, where all these variations were introduced at once.

As shown in Fig. 1, the optimized protocol yielded significantly higher mean values of AFB_1 adsorption. These values calculated for the bentonites as a whole (97%) were higher than those determined by using the EURL method (86%). Same effect was observed for the single bentonite samples (Table 2).

Although, some variations in the protocol, i.e., the optimization of ACN concentration in the working solutions, centrifugation conditions, and buffer concentration, produced an effect on the AFB_1 adsorption by materials, only a combination of all variations determined a strong, significant effect on AFB_1 adsorption values, exceeding the 90% threshold for all bentonites. Because of the method optimization, all bentonites showed a $BC_{AfB1} \geq 90\%$, while with the EURL method only 2, out of six bentonites, achieved this value.

3.4. In-house validation of the optimized adsorption assay

The new protocol to measure the BC_{AfB1} of a bentonite was *in-house* validated according to international harmonized guidelines as in §2.4.

The repeatability study (intra-assay precision) was carried out by a single operator (operator 1) in a short period of time, using the same operative conditions. Adsorption experiments were performed in triplicate and by three consecutive days on six bentonites. The SD and the RSD_r were measured to assess, respectively, the variability and the precision of each data set. Except for B5 (RSD_r = 3.6%), all samples showed a low RSD_r ($\leq$ 1.5%) (Table S7). Significant outliers were identified by the Grubbs' test for samples B4 (2) and Ref Bent (1), and then removed from the data set. The overall mean adsorption value (n = 51) was 94.4 ± 2.8%, while the overall RSD_r% was 3.0%. Moreover, considering the initial AFB_1 concentration in the test sample (4 μg/mL) and the residual amount of toxin in the supernatants, data of aflatoxin adsorption were used to calculate the amount of toxin sequestered by each bentonite sample and expressed as a concentration (μg/mL). Also in this case, the outliers were not included in the data set. The overall mean adsorption value (n = 51) was 3.8 ± 0.1 μg/mL, with an overall RSD_r of 3.0% (Table S7).

The intermediate precision of the optimized method was calculated by involving other two operators and for a long period of time. These operators used different operative conditions, and performed triplicate independent experiments which were repeated three times at months 1, 3 and 6. For the operators 2 and 3, the values of RSD_r were in the ranges of 0.1–1.9% and 0.5–1.8% (Tables S8 and S9). The number of outliers was 9 for the operator 2 and 3 for the operator 3. The overall mean adsorption values ± SD and the relevant RSD_r% values (between brackets) were 95.1 ± 3.0% (3.2%) for the operator 2 (n = 45), and 94.2 ± 3.0% (3.2%) for the operator 3 (n = 51).

To calculate the intermediate precision of the method, the amount of toxin (μg/mL) adsorbed by each bentonite was also calculated and expressed as mean ± SD. The RSD_I was measured for each bentonite sample, considering the set of data produced by the operators 1, 2 and 3, and at months 1, 3 and 6. The values of RSD_I were satisfying being in the range of 0.5–2.7% (Table S10).

To assess the acceptability of the precision of the method, the HorRat was calculated. The HorRat is a widely used measure of the precision of analytical methods, and it is often used as a benchmark for method performance. The results of the calculation can provide valuable information about the reliability and accuracy of the method under intermediate conditions, with values < 2 indicating good precision. The *in-*

Table 2
Experimental data of aflatoxin adsorption obtained by testing six bentonites with different protocols, i.e. the EURL method, the methods obtained by varying one factor (buffer, sample preparation mode, incubation time, residual amount of ACN or centrifugation) and the optimized method. EURL method refers to the official protocol, while optimized method refers to the protocol bearing all changes. Values are means ± SD of triplicate independent experiments (n = 3).

	AFB_1 adsorption (%)														
	EURL method		*Buffer*		*Sample preparation*		*Incubation time*		*ACN amount*		*Centrifugation*		*Optimized method*		
Sample	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**p_{value}**
B1	74.0^{a}	1.5	87.8^{b}	0.4	74.6^{a}	0.4	72.6^{a}	1.0	82.6^{c}	0.3	80.2^{c}	1.4	92.9^{d}	0.1	**<0.001**
B2	82.0^{a}	1.3	86.5^{b}	0.6	82.1^{a}	1.4	80.6^{a}	0.8	85.5^{b}	2.1	88.9^{c}	0.5	96.3^{d}	0.4	**<0.001**
B3	81.2^{a}	2.2	90.4^{b}	0.3	83.9^{a}	1.8	82.4^{a}	0.9	88.8^{b}	0.5	83.8^{a}	1.5	95.1^{c}	0.1	**<0.001**
B4	88.6^{a}	1.7	91.3^{b}	0.4	90.4^{ab}	0.9	88.6^{a}	0.4	91.8^{b}	0.7	92.7^{b}	0.1	97.4^{c}	0.1	**<0.001**
B5	91.7^{a}	3.7	94.8^{ab}	0.2	91.2^{a}	0.8	94.4^{ab}	0.5	92.9^{a}	0.2	94.5^{ab}	0.4	97.9^{b}	0.3	**0.001**
Ref Bent	96.0^{ab}	1.5	96.3^{a}	0.1	96.6^{a}	0.8	96.8^{a}	0.4	95.8^{a}	0.7	97.9^{bc}	0.2	99.3^{c}	0.1	**<0.001**

$^{a–d}$ Different superscript letters in the same row indicate significant differences ($p < 0.05$).

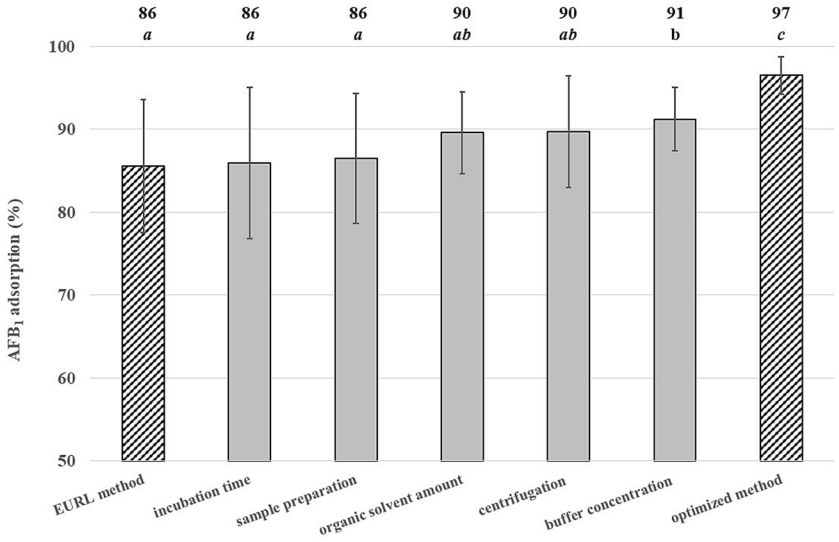

Fig. 1. Experimental data of aflatoxin adsorption obtained by testing six bentonites with different protocols, i.e. the EURL method, the methods obtained by varying one factor (i.e., incubation time, sample preparation mode, residual amount of ACN, centrifugation or buffer concentration) and the optimized method. Values are means ± SD of 6 independent experiments performed in triplicate ($n = 18$).

house validated method to determine BC_{AfB1} was acceptable, and under intermediate conditions it showed a RSD_I of 3.4%. Considering that $PRSD_R$ calculated using Horwitz equation was 13.2%, the HorRat value was 0.3. This value falls within the acceptability criteria stated in the AOAC guidelines for standard method performance requirements (AOAC, 2016).

3.5. Adsorption isotherms

The six test bentonites were analyzed by equilibrium isotherms, which were well fitted by the Freundlich, Langmuir, and Sips models. Fig. 2 represents graphically the experimental and predicted isotherms for AFB_1 adsorption by these materials. Most isotherm graphs show that as the concentration of AFB_1 molecules in the working solution increased, so did the amount of AFB_1 adsorbed per unit mass of bentonite. A typical L-2 or L-1 (Langmuir) shape and an exponential relationship was displayed by the isotherms (Fig. 2). Predicted adsorption isotherms were obtained by fitting experimental adsorption data with the best equation and by using the nonlinear regression analysis method. The Langmuir model provided the best representation for the experimental results of the AFB_1 adsorption isotherms, with $R^2 > 0.912$ and low error values (SSres, sy|x, PRESS), The model allowed the calculation of the adsorption parameters, maximum adsorption capacity

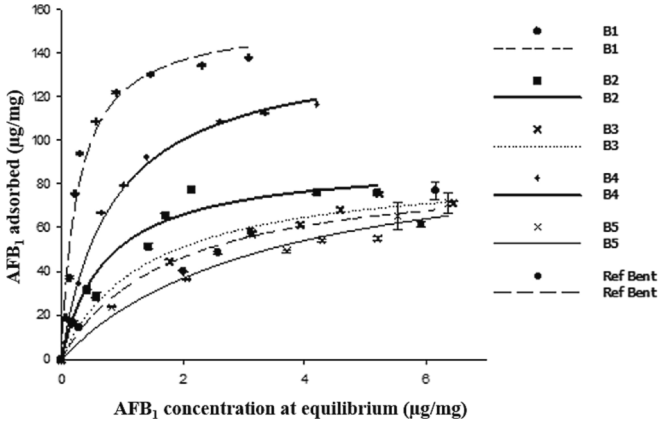

Fig. 2. AFB_1 adsorption isotherms obtained for six bentonites. Experimental adsorption data were fitted using the Langmuir model.

(B_{max}) and affinity (K_d). The Langmuir model assumes a monolayer adsorption, which is consistent with the surface properties of bentonites. It is widely used to study the adsorption of mycotoxins in various matrices (Greco, D'Ascanio, Santovito, Logrieco, & Avantaggiato, 2019; D'Ascanio et al., 2019).

Considering Langmuir parameters, the best AFB_1 adsorbing bentonites were Ref Bent, B4 and B2 samples (Table 3). These samples showed the highest values of B_{max} (≥ 90 µg/mg) and K_d (≥ 1.0 L/mg) and should be ranked as the most promising adsorbents. Accordingly, BC_{AfB1} values determined for these bentonites using the optimized method were all > 96%, while the values calculated using the EURL method were quite different ($p < 0.001$), being 96.0, 88.6, and 82.0%, respectively. So that, by testing these bentonites with the optimized method, all of them should be admitted as aflatoxin binders, while with the EURL protocol, only the Ref Bent sample should be identified as 1 m558.

The bentonites B5, B3 and B1 showed B_{max} values in the range of 75–88 µg/mg and $K_d < 1$ L/mg (0.3–0.7 L/mg). As shown in Table 3, BC_{AfB1} values measured using the optimized method were all higher than 90%, while the values determined with the EURL method were 92, 81 and 74%, respectively ($p < 0.001$). Also in this case, only one bentonite (B5 sample) seems to fulfill the requirements of the regulation if the EURL method is scrupulously applied, whereas all of them complied with the regulation if tested by the optimized protocol. However, it should be noted that B5 sample is a calcium-montmorillonite which showed the lowest adsorption parameters ($p < 0.001$) using the Langmuir model, with B_{max} of 75 µg/mg and K_d of 0.3 L/mg. Due to this, it

Table 3
AFB_1 adsorption values (%) obtained by using both the EURL and the optimized method, and comparison with the Langmuir adsorption parameters (B_{max} and K_d) obtained by equilibrium adsorption isotherms.

	AFB_1 adsorption (%)			
Sample	***EURL***	***Optimized method***	**B_{max} (µg/mg)**	**K_d (L/mg)**
B1	74.0 ± 1.5^{a}	92.9 ± 0.1^{a}	88 ± 7^{a}	0.6 ± 0.1^{a}
B2	82.0 ± 1.3^{b}	96.3 ± 0.4^{b}	91 ± 4^{a}	1.3 ± 0.2^{b}
B3	81.2 ± 2.2^{b}	95.1 ± 0.1^{c}	88 ± 3^{a}	0.7 ± 0.1^{a}
B4	88.6 ± 1.7^{c}	97.4 ± 0.1^{d}	143 ± 4^{b}	1.2 ± 0.1^{b}
B5	91.7 ± 3.7^{cd}	97.9 ± 0.3^{d}	75 ± 1^{c}	0.3 ± 0.1^{a}
Ref Bent	96.0 ± 1.5^{d}	99.3 ± 0.1^{e}	156 ± 5^{d}	3.6 ± 0.4^{c}
p_{value}	<0.001	<0.001	<0.001	<0.001

[a–d] Different superscript letters in the same column indicate significant differences ($p < 0.05$).

may be difficult to understand why, in addition to the Ref Bent sample, only the B5 bentonite should be authorized as an aflatoxin binder for animal feed, whereas it is reasonable that all samples meet the regulation, as demonstrated by using the optimized protocol.

Isotherm adsorption study is internationally recognized as the best approach to explore the adsorption mechanism and rank bentonites on their efficacy in adsorbing AFB_1 (Yeo et al., 2023; D'Ascanio et al., 2019; EFSA 2011; Dixon et al., 2008). The results of the adsorption equilibrium isotherms are used to evaluate the affinity or capacity of a bentonite for AFB_1 and to select a suitable adsorbent and relevant dose. The relationship between isothermal adsorption parameters (B_{max} and K_d) of bentonites and AFB_1 adsorption values expressed as a percentage (Ads %) has been described by D'Ascanio et al. (2019). In this study, adsorption isotherm experiments were also performed using a low adsorbent concentration (0.005% w/v), and at pH 7. Adsorption values expressed either as a percentage (Ads%) as B_{max} and K_d were linearly and positively correlated ($p < 0.001$). B_{max} values of sedimentary bentonites, which were selected as the best AFB_1 adsorbing bentonites, were in the range of 79–165 μg/mg (D'Ascanio et al., 2019). Interestingly, B_{max} values calculated for all the montmorillonites tested herein fall in this range, including those (B1-B4 samples) that, according to the EURL method (Table 3), should not be authorized as an additive for AFB_1 reduction in feed.

This is the first time that adsorption parameter values (B_{max} and K_d) calculated at pH 5 by the equilibrium isotherm approach have been related to BC_{AfB1} values. The study helped us in ranking the bentonites and in confirming the suitability of the optimized protocol for BC_{AfB1} measurement.

3.6. Determination of BC_{AfB1} for di- and tri-octrahedral smectites

The optimized and *in-house* validated method for BC_{AfB1} determination was applied to 13 bentonites belonging to the group of di-octahedral smectites and to 17 samples from the tri-octahedral group (Fig. 3). Most di-octahedral smectites showed $BC_{AfB1} \geq 90\%$, while 2 samples did not fulfill the requirements of the Regulation (Fig. 3). Tri-octahedral smectites behaved differently than montmorillonites (Fig. 3). Nine samples, out of 17, showed BC_{AfB1} values $\leq 90\%$.

According to our findings, Vila-Donat et al. (2019) observed that most tri-octahedral smectites adsorbed >90% of AFB_1 using an experimental protocol that was similar to that optimized herein. So far, tri-octahedral smectites have been poorly evaluated as mycotoxin binders, which can be due to the fact that they are less common. However, recent studies have shown that tri-octahedral smectites have high potential as effective binders for a wide range of mycotoxins, including aflatoxins (Vila-Donat et al., 2019, 2020; Greco D'Ascanio et al., 2022). Further research is needed to fully explore their binding capacity and potential use in the food and feed industry for aflatoxin reduction. *In vivo* studies are required to assess whether tri-octahedral smectites with $BC_{AfB1} \geq 90\%$ are able to prevent aflatoxin exposure in animals.

4. Conclusion

In the EU, feed additives are authorized according to Regulation No. 1831/2003, which also specifies the role played by the EURL in the authorization procedure. Among others, the role of EURL is to issue recommended methods for official control of additives and to evaluate those proposed by the applicants. One of the key issues facing the EURL is determining whether the methods presented by industries are appropriate for official control, given that most of them are single laboratory validated methods. A case study is the method for official control of bentonites as AFB_1 adsorbents. The authorization related to these additives is not "holder-specific", and once a non-holder specific product is authorized, any company can place such products on the market, if they meet the necessary requirements set by EU Regulation No. 1060/2013. About this, the regulation establishes a minimum value of 90% for BC_{AfB1}, which must be determined using a specific procedure. The aim of this method is not to prove the effectiveness of the bentonite but rather to determine whether it is marketed in accordance with the Regulation. The procedure was developed and *in-house* "validated" by an applicant, verified in a second laboratory by conducting independent measurements repeating the protocol of the applicant, and then the EURL evaluated the fitness for purpose on validation data of the two laboratories. EURL concluded that the method is suitable for the monitoring of $BC_{AfB1} \geq 90\%$. However, several laboratories complain about this procedure and believe that it is not robust enough, leaving room for the analytical interpretation of various parameters. Therefore, we performed a robustness study by analyzing five leading factors and six bentonites, which met the mineralogical requirements to claim code 1m558. This study demonstrated that the official method is not robust and does not fit its purpose, as slight variations in some factors can significantly affect reliability during normal use. It implies that some

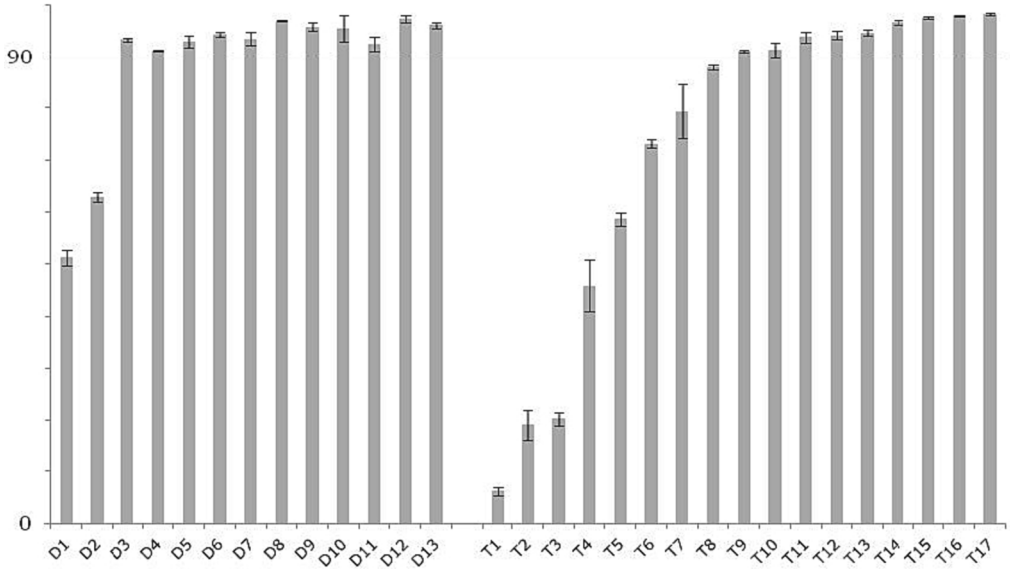

Fig. 3. AFB_1 binding capability (BC_{AfB1}) determined for both di-octahedral ($n = 13$) and tri-octahedral smectites ($n = 17$). Experimental values are means ± SD of triplicate independent experiments.

bentonites can be improperly excluded from the category of 1m558 feed additives, due to the weakness of the analytical procedure. A new protocol was developed by keeping the main experimental parameters of the official adsorption assay and was *in-house* validated. The new protocol yielded significantly higher mean values of AFB_1 adsorption with respect to the EURL method and showed satisfactory precisions with a RSD_I of 3.4% and HorRat < 2. Its validity was proven by the isotherms approach. Of course, an inter-laboratory study for validation of the method is deemed a gold standard and is recommended. The optimized procedure for BC_{AfB1} measurement was successfully applied to tri-octahedral smectites with good results. Tri-octahedral smectites are rising the attention of the scientific community as promising materials to design low-cost adsorbents for mycotoxin inactivation. Further research is required to assess whether tri-octahedral smectites with $BC_{AfB1} \geq 90\%$ can prevent aflatoxin exposure in animals and the transfer of toxic metabolites in animal food products. These studies will help supporting the authorization of these materials for use in animal nutrition as mycotoxin inactivators.

CRediT authorship contribution statement

Vito D'ascanio: Conceptualization, Investigation, Methodology, Validation, Formal analysis, Visualization, Writing – original draft. **Donato Greco:** Investigation, Methodology, Validation, Formal analysis, Visualization, Writing – original draft. **Mariagrazia Abbasciano:** Investigation. **Giuseppina Avantaggiato:** Conceptualization, Methodology, Formal analysis, Visualization, Writing – review & editing, Project administration, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Giuseppina Avantaggiato reports equipment, drugs, or supplies was provided by European Commission. Giuseppina Avantaggiato reports a relationship with Institute of Sciences of Food Production National Research Council that includes: funding grants.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank Laviosa Chimica Mineraria (Livorno, Italy) for its kind support in providing bentonites and physico-chemical properties of minerals. The authors are grateful to Giuseppe Panzarini (CNR-ISPA) for his skilled technical support, and to Marinella Cavallo (CNR-ISPA) and Simonetta Martena (CNR-ISPA) for administrative aid.

Funding

This work was supported by the MycoKey Project (European Union Horizon 2020, Research and Innovation Programme) under Grant Agreement No. 678781.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137198.

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