JRC Scientific and Technical Reports

Evaluation of the Effect of Mycotoxin Binders in Animal Feed on the Analytical Performance of Standardised Methods for the Determination of Mycotoxins in Feed

A. Kolossova, J. Stroka, A. Breidbach,
K. Kroeger, M. Ambrosio, K. Bouten, F. Ulberth

EUR 23997 EN - 2009

JRC
EUROPEAN COMMISSION

irmm
Institute for Reference Materials and Measurements

The mission of the JRC-IRMM is to promote a common and reliable European measurement system in support of EU policies.

European Commission
Joint Research Centre
Institute for Reference Materials and Measurements

Contact information
Address: Joerg Stroka, Retieseweg 111, B-2440 Geel
E-mail: Joerg.stroka@ec.europa.eu
Tel.: +32-14-571229
Fax: +32-14-571783

http://irmm.jrc.ec.europa.eu/
http://www.jrc.ec.europa.eu/

JRC 54375

EUR 23997 EN
ISBN 978-92-79-13106-6
ISSN 1018-5593
DOI 10.2787/15352

Luxembourg: Office for Official Publications of the European Communities

Printed in Belgium

Table of Contents

Summary

The last few years have brought a vast amount of information about mycotoxin inactivation agents (mycotoxin binders) which are applied with the aim to reduce the toxic effects of mycotoxins in animals. The influence of the addition of mycotoxin binders to animal feed on the analytical performance of the methods for the determination of mycotoxins was studied and the results are presented in this report. Standardised methods already available or currently under consideration at the European Standardization Committee (CEN) have been applied for the analysis of mycotoxins in feed materials. Samples of 20 commercial mycotoxin inactivation agents were collected from various companies. The following mycotoxins were included in the study: aflatoxin B1, deoxynivalenol, zearalenone, ochratoxin A, fumonisins B1 + B2, T2 and HT2 toxins. Naturally contaminated or spiked feed materials and the maximum recommended amounts of the mycotoxin detoxifying agents were used in the experiments. A binder (or binders combined in a group) was mixed with feed material containing the corresponding mycotoxin, and the feed material with and without binder was analysed using the appropriate method. For data evaluation, the obtained mean values were compared by Student's *t*-test (independent two-sample *t*-test with unequal sample sizes and equal variance). The repeatability standard deviation of each method based on collaborative trial data was used as an estimate of method variability. No significant differences ($p = 0.05$) in mycotoxin levels between binder free material and the material containing different binders have been found under the applied conditions.

Background Information on Mycotoxin Binders and Their Use

Mycotoxins are toxic secondary metabolites produced by several fungi, particularly by many species of *Aspergillus*, *Fusarium*, *Penicillium*, *Claviceps*, and *Alternaria*. It has been estimated that at least 300 of these fungal metabolites are potentially toxic to animals and humans [1]. The most extensively investigated mycotoxins are aflatoxin B1 (AFB1), ochratoxin A (OTA) zearalenone (ZEA), deoxynivalenol (DON, "vomitoxin"), T-2 and HT-2 toxins, and fumonisins (FUM). Mycotoxins are produced by fungi during growth, handling and storage of agricultural commodities. Their global occurrence is considered to be a major risk factor. Worldwide, approximately 25% of crops are affected by mycotoxins [2]. The economic consequences of mycotoxin contamination are profound, and often crops with large amounts of mycotoxin have to be destroyed.

There is an increasing awareness of the hazards posed to both human and animal health by the presence of toxins produced by fungi in food and feed. Mycotoxins have a diversity of chemical structures which accounts for different biological effects. They can be carcinogenic, mutagenic, teratogenic, oestrogenic, neurotoxic, immunotoxic, etc. In farm animals, mycotoxins can cause, among others, decreased performance, feed refusal, poor feed conversion, diminished body weight gain, immune suppression, reproductive disorders, and residues in animal food products [1, 3]. However, the progression and diversity of symptoms are confusing and diagnosis is difficult [4, 5]. Diagnosis is further complicated by a lack of research, by nonspecific symptoms and by interactions with other stress factors. Mycotoxin effects are also moderated by a number of factors, such as animal species, gender, age, diet, and duration of exposure. Many mycotoxigenic fungi can grow and produce their toxic metabolites under similar conditions. Therefore in animal feed, mycotoxins rarely occur as single contaminants [6]. Apart from that, blends of various raw materials in compound feed can increase the risk of feed contamination with several mycotoxins. Several combinations of mycotoxins have been reported [7-9], such as the co-occurrence of aflatoxin B1 with ochratoxin A or that of deoxynivalenol with zearalenone, nivalenol, or other *Fusarium* toxins. Intake of combinations of mycotoxins may lead to interactive toxic effects. Furthermore, the toxic effect of any single mycotoxin may be amplified due to synergistic interactions with other substances [9]. Regardless of the difficulty of diagnosis, mycotoxins should be considered as a possible cause of production and health problems when such symptoms exist and problems are not attributable to other typical causes [5].

Mycotoxin legislation in the EU

Due to the frequent occurrence of mycotoxins and their severe toxic properties in animals, legislative limits and recommendations for these compounds have been set for feedstuff in Europe. For

animal feed, maximum level set by the European Commission (EC) for aflatoxin B1 is 0.02 mg/kg for all feed materials [10]. For other feeding stuffs, the legal limits vary from 0.005 to 0.02 mg/kg. Recommendations on the presence of deoxynivalenol, zearalenone, ochratoxin A, fumonisins B1+B2, T-2 and HT-2 toxins in products intended for animal feed were given by the EC. The corresponding guidance values for these mycotoxins except for T-2 and HT-2 have been established [11].

Prevention of mycotoxin contamination in the field

Prevention of fungal infections during plant growth, harvest, storage and distribution would seem the most rational and efficient way to avoid mycotoxins in agricultural commodities [1, 12-14]. Common practical measures include planting of more resistant varieties of cereals, selection of high quality seeds, avoiding high plant densities, balanced fertilisation, preventive management towards insect infestations as well as a suitable management of crop residues that are often the primary inoculum of mycotoxigenic fungi. Careful selection of harvest date, equipment and harvesting procedures to minimise crop damage and removal of damaged crops and high moisture plant parts also reduces mould infections. Immediate storage in good storage facilities (moisture, temperature, humidity and insect control) and the addition of antifungal agents may also diminish fungal growth but cannot detoxify contaminated feedstuffs. Furthermore, the growth of fungi and therefore, the production of mycotoxins is limited by the use of propionic acid or ammonium isobutyrate, which can be used for a post-harvest treatment. Calcium propionate has also been suggested as a mould inhibitor [15].

Detoxification of animal feed from mycotoxins

Although the prevention of mycotoxin contamination in the field is the main goal of agricultural and food industries, under certain environmental conditions the contamination of various commodities with mycotoxins is unavoidable. Feed additives like antioxidants, sulphur containing amino acids, vitamins, and trace elements can be useful to reduce the toxic effects observed in animals [16].

The increasing number of reports on the presence of mycotoxins in feeds has given rise to a demand for practical and economical detoxification procedures. A number of approaches have already been used to counteract mycotoxins, though only a few have real practical application.

Physical treatment includes washing, polishing, mechanical sorting and separation, density segregation, flotation, autoclaving, roasting and microwave heating, UV irradiation, ultrasound treatment and solvent extraction [17]. However, the efficiency of these techniques depends on the level of contamination and on the distribution of mycotoxins throughout the grain. Additionally, the results

obtained are uncertain and often connected with high product losses. Moreover, some of these physical treatments are relatively costly and may remove or destroy essential nutrients in feed.

Chemical methods require not only suitable reaction facilities but also additional treatments (drying, cleaning) that can make them time consuming and expensive. Nevertheless, various chemicals including oxidising and reducing agents, acids, bases, salts and chlorinating substances have been tested for their ability to degrade mycotoxins in agricultural commodities. Only a limited number of these are effective without diminishing the feed nutritional value or palatability. Chemically, some mycotoxins can be destroyed with calcium hydroxide, monoethylamine, ozone or ammonia [1, 13, 18, 19]. Particularly, ammoniation is an approved procedure for the detoxication of aflatoxin contaminated feed in several countries [1]. The average ammoniation costs vary between 5 and 20% of the value of the commodity [20]. Main drawbacks of this kind of chemical detoxication are the ineffectiveness against other mycotoxins and the possible deterioration of animal health by excessive residual ammonia in the feed.

With regard to mycotoxin decontamination, the EC is in favour of the use of physical decontamination processes and sorting procedures [21]. However, neither the use of chemical decontamination processes, nor the mixing of batches with the aim of decreasing the level of contamination below the maximum tolerable level are legal within the European Union [21].

Another way of trying to reduce the uptake of mycotoxins from contaminated feed is the use of mycotoxin binders. They are added to the feed with the intention to exhibit a completely different mode of action than the above mentioned physical and chemical treatment. The aim of these additives is to inhibit the uptake of mycotoxins by an animal in vivo. The use of mycotoxin binding agents is occasionally recommended to farmers in order to protect animals against the harmful effects of mycotoxins occurring in contaminated feeds. These adsorbent materials are intended to act like a ‘chemical sponge’ and adsorb mycotoxins in the gastrointestinal tract, thus preventing the uptake and subsequent distribution to target organs. The efficacy of the adsorption appears to depend on the chemical structure of both the adsorbent and the mycotoxin. The most important feature for adsorption is the physical structure of the adsorbent, i.e. the total charge and charge distribution, the size of the pores and the accessible surface area. On the other hand, the properties of the adsorbed mycotoxins, like polarity, solubility, shape and charge distribution, also play a significant role [1, 21].

Several studies have shown that a variety of adsorbent materials have high affinity for mycotoxins by the formation of stable linkages. Examples are activated carbon, hydrated sodium calcium aluminosilicates (HSCAS) and some polymers. These linkages have also been reported to occur in several liquid systems such as water, beer, wine, whole and skimmed milk, and peanut oil [1, 3, 22-25]. Many adsorbents have been extensively studied and are promoted as animal feed additives.

However, most of them appear to bind to only a small group of toxins while showing very little or no binding to others [1]. For example, HSCAS are quite effective with respect to aflatoxins but they fail to prevent toxic effects of Fusarium mycotoxins, such as fumonisins, trichothecenes or zearalenone [21].

Inactivation of mycotoxins by adsorbents has been extensively reviewed elsewhere [1, 17, 21-24]. Most studies related to the alleviation of mycotoxicosis by the use of adsorbents are focused on aluminosilicates, mainly zeolites, HSCAS, and aluminosilicate-containing clays, all consisting of aluminates, silicates and some interchangeable ions, mainly alkali metal and alkaline earth metal ions [1, 26-29]. Mineral clay products such as bentonites, zeolites, and aluminosilicates are the most common feed additives which are effective in binding/adsorbing aflatoxins [21]. When saturated with water, the surfaces of these additives attract polar functional groups of some mycotoxins. This effect isolates the mycotoxin from the digestive process and is thought to inhibit its absorption [30]. The mechanism of mycotoxin binding, however, has not been extensively studied. The high affinity of certain adsorbents for aflatoxin B1 was interpreted as the formation of a complex by the β-carbonyl system of the aflatoxin molecule with aluminium ions [31]. Selection criteria for the evaluation of properties of smectite clays influencing the sequestration of aflatoxin have been suggested. The structural aspects of the interaction between aflatoxin and smectite clay have also been discussed [29].

Hydrated sodium calcium aluminosilicates at 1.0% addition level to the feed was reported to diminish significantly the adverse effects of aflatoxins in young animals [30]. Aluminosilicates are also used at a level up to 2% in complete diets as “anti-caking” agents. Thus, the original intention for the addition is another than mycotoxin binding. These compounds, however, show a number of disadvantages, not least being the impairment of mineral utilization at levels above 2% and a narrow range of binding efficacy [1]. In animals, aluminosilicates appear to be selective in their “chemisorption” of aflatoxins with little or no beneficial effect against zearalenone, fumonisin B1, ochratoxin A, and trichothecenes, including deoxynivalenol, T-2 toxin, or diacetoxyscirpenol [32]. This limitation can be overcome by the use of chemically modified clays. Modifications consist of alterations of surface properties by exchange of structural charge-balance cations with high molecular weight quaternary amines, which results in an increased hydrophobicity [28]. In vitro results have verified the binding efficacy of modified montmorillonite and clinoptilolite against zearalenone and ochratoxin A [28, 33]. However, the potential toxicity of some of these clay types was pointed out [34]. In vitro adsorption of zearalenone by a modified montmorillonite nanocomposite has been reported [35]. This material demonstrated the ability to bind zearalenone in aqueous solutions with little nonspecific adsorption of common nutrients, vitamin E and lysine. Zeolites have also been shown to be efficient against zearalenone toxicosis by some in vivo studies [28].

Regarding the applicability of aluminosilicates for the binding of mycotoxins, it can be concluded that available studies recognise their effect in preventing aflatoxicosis, but their efficacy against zearalenone, ochratoxin, and trichothecenes is limited. In addition to the narrow binding range concerning different mycotoxins, aluminosilicates have the disadvantage of adsorbing micronutrients and showing high inclusion rates for vitamins and minerals. The risk of natural clays to be contaminated with dioxins has also to be considered [1, 17].

Activated carbon can efficiently adsorb most of the mycotoxins in aqueous solution, whereas different activated charcoals have less or even no effects against mycotoxicosis. This might be due to the fact that activated charcoal is a relatively unspecific adsorbent and, hence, essential nutrients are also adsorbed particularly if their concentrations in feed are much higher compared to those of a mycotoxin [1]. However, activated carbons have shown their efficacy in in vivo studies for aflatoxins, ochratoxin A, diacetoxyscirpenol, and T-2 toxin [32] and in experiments in vitro using a gastrointestinal model for deoxynivalenol, nivalenol, and zearalenone [21, 36, 37].

Polymers, such as cholestyramine (an anion exchange resin) and polyvinylpyrrolidone (highly polar amphoteric polymer), have also been demonstrated to bind mycotoxins in vitro and in vivo [1]. Thus, cholestyramine was proven to be an effective binder for fumonisins and zearalenone in vitro. Its efficacy was confirmed by experiments in a dynamic gastrointestinal model for zearalenone and by in vivo experiments for fumonisins [21]. However, the cost of those polymers would be a limiting factor for practical applications.

Facing the relative inefficacy of the clay binders towards mycotoxins other than aflatoxins, natural organic binders have been proposed [17]. A novel strategy to control mycotoxicoses in animals is the application of microorganisms capable of biotransforming certain mycotoxins into less toxic metabolites. The microorganisms act in the intestinal tract of animals prior to the absorption of the mycotoxins. Many species of bacteria and fungi have been shown to enzymatically degrade mycotoxins. However, a question concerning toxicity of products of enzymatic degradation and undesired effects of fermentation with non-native microorganisms on food quality remains open [38]. Yeasts and lactic acid bacteria (LAB) occur as part of natural microbial population in spontaneous food fermentation and as starter cultures in the food and beverage industry. *Saccharomyces cerevisiae* and LAB, the two most important microorganisms in food fermentation, have been shown to bind different mycotoxins strongly to cell wall components [17, 38, 39].

Besides its excellent nutritional value, yeast or yeast cell walls show a potential as mycotoxin binders. Using only yeast cell walls instead of whole cells, the adsorption of mycotoxins can be enhanced. The cell walls harbouring polysaccharides (glucan, mannan), proteins, and lipids exhibit numerous different and easily accessible adsorption centres as well as different binding mechanisms, e.g. hydrogen bonds, ionic, or hydrophobic interactions [1]. It has recently been demonstrated that the

β-d-glucan fraction of yeast cell wall is directly involved in the binding process with zearalenone, and that the structural organization of β-d-glucans modulates the binding strength. Hydrogen and Van Der Waals bonds have been evidenced in the glucans-mycotoxin complexes [17, 38, 40]. Probably, a similar chemical mechanism is involved in the binding process of mycotoxins by LAB. It appears that the carbohydrate components are common sites for binding, with different toxins having different binding sites. It is also known that toxin binding in both *S. cerevisiae* and LAB is strain dependent [38].

A bacterial strain, which belongs to the genus *Eubacteria*, has been originally isolated from bovine rumen fluid. It was found to have trichothecene detoxifying activity and was named BBSH 797. During its metabolism, BBSH 797 produces enzymes (de-epoxidases) that degrade trichothecenes by selective cleavage of their 12,13-epoxy group which is important for the toxicity of these mycotoxins. This detoxification was investigated for several trichothecenes. During its manufacture, BBSH 797 is stabilised by freeze-drying and embedding in protective substances (mainly organic polymers), to guarantee sufficient stability against environmental conditions during storage and its passage through the acidic gastric tract of animals. The mode of action of this strain was demonstrated in vitro and in vivo [7, 41]. Further, a novel yeast strain capable of degrading ochratoxin A and zearalenone was isolated and characterized. Due to its property to degrade mycotoxins this strain was named *Trichosporon mycotoxinivorans* [41, 42].

The recommended dose for the extracted active yeast compounds is in the range of 1-2 kg/ton of feed. Organic binders are efficient against a larger range of mycotoxins than inorganic binders, which makes them more adapted to the most frequent cases of multi-contaminated feeds. Apart from that, they are biodegradable and do not accumulate in the environment after being excreted by animals. On the contrary, clays which are incorporated at a higher rate than organic binders, accumulate in manure and then in the field during spreading and can harm soils and pastures [17].

Various products which combine mycotoxin binding properties of different compounds have recently been developed to counteract the biological effects of co-occurring mycotoxins in animal feed. A study to assess the multimycotoxin binding efficacy of a commercial product has been conducted using a dynamic model simulating the kinetic conditions in the gastrointestinal tract of pigs [6]. The bioavailability of different classes of mycotoxins commonly co-occurring in animal feedstuffs has been simultaneously assessed. On the basis of the results, it has been assumed that this product might significantly decrease in vivo the bioavailable amounts of zearalenone, fumonisins and ochratoxin A, and almost completely prevent intestinal adsorption of aflatoxins in contaminated feed. Due to their composition and the presence of different adsorbent materials, the products with multibinding capacity towards chemically different mycotoxins can be beneficial in reducing both individual and combined adverse effects of mycotoxins in animals.

The extensive use of adsorbent materials in the livestock industry has led to the introduction of a wide range of new products, which still have no legally approved status in the EU market for its intended purpose as a mycotoxin binder. Before any feed additive is applied to prevent mycotoxin intoxication, it is essential to establish its reliability and safety while considering the economic feasibility. Moreover, it should be assured that adsorbent materials do not affect nutritional properties. Many studies have been performed to evaluate different commercial products intended to be used as mycotoxin binding agents and various aspects have been discussed [21, 26, 43-50]. Masoero et al. [49] concluded that physical processing of feed, such as pelleting, had a significant effect on the ability of added sequestering agent to reduce levels of AFM1 in milk when cattle was fed with AFB1 contaminated feed [49]. It was also suggested that even though the ratio of AFB1 to sequestering agent in the contaminated feed could be an important variable to consider when using sequestering agents, it was not as important as the pelleting process. However, the authors also pointed out in their discussion that a partial degradation of AFB1 by heat treatments had been reported by others, but this effect did not occur in their study.

Commonly encountered discrepancies between in vitro and in vivo results for mycotoxin binders outline the need of further research on the fate of the complex of mycotoxin with sequestering agent in the gastrointestinal tract [43, 48]. Not only the effectiveness but also safety aspects of the adsorbent materials for animals have been investigated in some studies [47].

Most of the research, however, deals with the efficacy of sequestering materials to bind mycotoxins in vitro and in vivo. Methods used to study the effects of mycotoxin binders basically include adsorption from aqueous solution, gastro-intestinal models and in vivo studies with different animals. Recently, some extraction studies were performed to investigate aflatoxin B1 adsorption by clays from water and corn meals [26]. It was shown that clays and activated carbon retained less AFB1 from aqueous corn meal dispersions than from water dispersions alone. It should be pointed out that AFB1 was extracted from the aqueous corn meal dispersions with 60% methanol.

In vitro studies do not always predict in vivo results. Adsorption in vivo is complicated by physiological variables and the composition of feed; factors which are rarely accounted for in vitro. Apart from that, animal studies are costly and not easy to perform. However, all potential mycotoxin binding agents must be tested in vivo to confirm their efficacy and safety and lack of interactions with nutrients. An approach for an effective pre-screening mycotoxin/sorbent combinations before testing on animals has been suggested [50]. The in vitro testing system developed so far, involved several investigations:

(1) aqueous binding studies including single concentration sorption studies (isothermal);
(2) studies with the use of a gastrointestinal model;

(3) modified isotherms to compare adsorption in the presence and absence of matrix.

The suggested approach allows the selection of the most promising materials as potential enterosorbents for in vivo testing.

Several requirements have been suggested to assess mycotoxin binder performance in vivo. These requirements include the following criteria: availability of essential nutrients to an animal; a binder should not be a growth promoter (growth promotion may mask mycotoxins); improvement of zootechnical performance; recovery of organ status; excretion of mycotoxins via faeces; broad-spectrum mycotoxin binding performance; recovery of the immune status [51].

Analytical aspects

In response to the risk of great economic losses in the industry and the threat to human and animal health as a result of exposure to mycotoxins, various analytical techniques have been developed for their detection [52-55]. Commonly used methods include thin layer chromatography (TLC), high-performance liquid chromatography (HPLC), gas chromatography (GC), and immunochemical methods such as enzyme-linked immunosorbent assays (ELISA). In addition to the ELISA format, rapid screening tests and a number of new techniques such as biosensors are rapidly emerging. However, chromatographic techniques are usually used as reference methods for mycotoxin analysis. The last few years have brought an incredible amount of information about binders and adsorbents which can be used to decrease the bioavailability of mycotoxins. Various studies concerning the performance of mycotoxin binding agents in vitro and in vivo have been carried out. To our knowledge, no investigation regarding the influence of mycotoxin binders on the analytical characteristics of the methods used for mycotoxin determination was performed. However, this issue is of utmost importance, especially with respect to the authorisation of mycotoxin binding agents. Any interference of mycotoxin binders with the analytical performance of a method can lead to incorrect analytical findings and, as a consequence, to a misclassification with respect to the acceptance or rejection of the material. As a result, the European Commission's Directorate-General for Health and Consumers (DG SANCO) asked the JRC to study the influence of mycotoxin binders on the performance of mycotoxin testing methods. The background for this study is that binders shall not be used to mask any previously non-compliant consignments as compliant by the addition of a binder.

Outline of the Study

The aim of the present research was to investigate the possible effect of mycotoxin binding agents on the analytical performance of standardised methods currently used for mycotoxin determination in feed. The study on the influence of the binders on the analytical results was performed for the following mycotoxins: AFB1, OTA, FUM B1 + B2, ZEA, DON, T2 + HT2 toxins. Naturally contaminated or spiked feed materials were applied for the investigation. Not sufficiently high naturally contaminated feed materials or materials with no corresponding mycotoxin found were spiked to obtain levels approximately 1.5 times higher than the legislative or recommended limits. This was the case for AFB1, DON and ZEA.

Mean values obtained for the untreated samples and the binder treated samples were compared by performing Student's *t*-test. The used variance was derived from the overall method repeatability. These values are based on the method validation data obtained during the collaborative trial studies for each method (Table 1). Differences were assumed to be significant at $p < 0.05$.

Methodology

The test samples of commercial mycotoxin inactivation agents (mycotoxin binders) were collected from various companies. Most of the test samples were obtained via the EU Feed Additives and Premixtures Association (FEFANA). Three companies (Impextraco NV, Belgium; Sud-Chemie AG, Germany; Agromed Austria GmbH, Austria) provided their samples independently. The samples were coded with numbers to preserve the confidentiality of the source (Table 2). In some cases, the identity of the producer/vendor was not disclosed. Type of the commercial products (main components) and the range of their use in feed were communicated by FEFANA, the supplying companies, or were stated in the commercial leaflets.

All samples were tested for pH in aqueous solutions, and infra-red spectra were recorded for identification and characterisation purposes.

For pH measurement, a method for pH measurement of soils [56] was slightly modified to take into account that some binders had a tendency to form stiff suspensions. Briefly, 1.0 g of a test sample was suspended in 12.5 ml Milli-Q water. The pH-value was determined after a period of 60 min directly in the suspension.

Infra-red (IR) spectroscopy studies were performed for the characterisation and identification of the test material. This work has been done in collaboration with the Walloon Agricultural Research Centre (see Annex 2).

Spiking solutions were prepared using mycotoxins from Sigma (Bornem, Belgium). Their concentration was verified by the corresponding chromatographic method. Mycotoxin standard solutions from Biopure (Tulln, Austria) were used for the calibration.

For spiking, 10.0 grams of dry ground feed material was fortified with 0.3 ml of the mycotoxin solution in methanol (for AFB1) or in acetonitrile (for DON and ZEA). The samples were incubated overnight in the dark at room temperature. A binder (or in case of grouped binder experiments, a combination of different binders) was then mixed with the test material prior extraction. Maximum recommended amounts of the mycotoxin binding agents were used in the experiments (Table 2). Feed material without binder was used as a reference. The samples (with and without binders) were prepared as independent triplicates for each experiment and each replicate was injected twice. The samples were analysed according to the method protocol indicated below.

For the experiments with AFB1 and DON, each binder was mixed with a portion of feed test material (in triplicate). The whole set of analyses covering all binders was performed in several batches. Feed material without binder was also analysed in triplicate in parallel with each batch.

For the experiments with OTA, ZEA, T-2 & HT-2 toxins, and fumonisins B1 + B2, the mycotoxin binders were combined in groups according to their type. Thus, group A and group B were aluminosilicate clays. Group A contained samples 2, 3, 4, 12, 13, and group B – samples 15-19. Group C comprised yeast cell walls and included samples 1, 5, 6, 7 and 9. Group D included binders 8, 10, 11, which were mixtures of organic and mineral components. Group E was formed by fibre samples 14 and 20. For the above mentioned mycotoxins, all binders belonging to the same group were mixed with a portion of the test sample (in triplicate). The feed material without binder was analysed in triplicate as a reference.

Table 1. Repeatability data for each method based on collaborative trial data

Mycotoxin	**Average RSD_r (%)**
DON	10.6
AFB1	6.9
ZEA	7.7
T2 & HT2 toxins	9.8
FUM B1 + B2	3.2
OTA	4.1

Table 2. Mycotoxin detoxifying agents and their amounts used in the study

Code	Type of main product(s)	pH value	Amount per 10 g feed material (mg)
1	Yeast cell wall	2.9	20
2	Clay + organic acid	7.9	10
3	Clay	8.4	10
4	Clay	4.6	25
5	Yeast cell wall	6.2	20
6	Yeast cell wall	6.7	20
7	Yeast cell wall	4.7	25
8	Mixture organic + mineral	6.2	25
9	Yeast cell wall	5.0	20
10	Mixture organic + mineral component	8.6	25
11	Mixture organic + mineral component	6.9	25
12	Clay	9.1	50
13	Clay	8.9	100
14	Fibres	6.7	100
15	Montmorillonite	10.4	40
16	Montmorillonite	10.6	40
17	HSCAS	8.8	50
18	HSCAS	9.8	50
19	HSCAS	9.8	50
20	Fibres (lignocellulose)	5.1	250

Methods applied for the determination of mycotoxins

- Aflatoxin B1 has been determined following the standard EN ISO 17375:2006, 'Animal feeding stuffs – determination of aflatoxin B1'.
- Deoxynivalenol has been determined using the method protocol of Stroka et.al. [57], which is currently under consideration as a CEN standard (prEN 15791:2009).
- Zearalenone has been determined using the method protocol of Arranz et.al. [58], which is currently under consideration as a CEN standard (prEN 15792:2009).
- Ochratoxin A has been determined using the method protocol of Stroka et.al. [59], which is currently under consideration as a prospective CEN standard (working document N954 of TC327/WG1).
- Fumonisins B1 and B2 have been determined using the method protocol of Breidbach et.al. [60], which is currently under consideration as a prospective CEN standard (working document N953 of TC327/WG1).
- T2 and HT2 toxins have been determined using the method protocol of Breidbach et.al. [61], which is currently under consideration as a prospective CEN standard.

Data analysis

The data were analysed by performing Student's *t*-test (independent two-sample *t*-test with unequal sample sizes and equal variance). The used variance was derived from the overall method repeatability. These values are based on the method validation data obtained during the collaborative trial studies for each method (Table 1). Differences were assumed to be significant at $p < 0.05$.

Critical *t*-values ($p = 0.05$) were compared with those obtained by the Student's *t*-test according to the following formula:

$$t = \frac{\overline{X}1 - \overline{X}2}{S \times \sqrt{\frac{1}{n1} + \frac{1}{n2}}},$$

where

S – average repeatability relative standard deviation from collaborative trail (%)

$\overline{X}$ 1 - mean value determined for binder free material (set as 100 %)

$\overline{X}$ 2 - mean value determined for the test material with the binder added, relative to $\overline{X}$ 1 (%)

n1 – number of replicates for binder free material

n2 – number of replicates for the material containing mycotoxin binder

Results and Discussion

Aflatoxin B1 is currently the only mycotoxin for which legislative limits in feed materials are set by the EC [10]. Most studies concerning the effects of mycotoxin binders are focused on AFB1. Indeed, the great majority of binding agents, especially mineral adsorbents, are very effective in alleviating aflatoxicosis but their efficacy against other mycotoxins is limited. Most mycotoxins are extracted with an aqueous organic solvent. For example, AFB1 is extracted with a mixture of acetone-water, 85:15 (v/v). This extraction solvent has the highest organic content compared to other solvents used for the extraction of mycotoxins under current investigation. On the other hand, DON is the only mycotoxin which requires the extraction with pure water for the method used in this study [57]. It can be assumed that the mycotoxin binders perform most effectively in neat aqueous solutions. Thus, they should have, if any, the strongest effect in the extraction solvent like it is used for DON. This assumption is also supported by the fact that for the purification of mycotoxins in aqueous organic solvents (e.g. acetonitrile/water), several methods for the determination of mycotoxins make use of substances similar to the mycotoxin binders. These clean-up applications are based on the principle that the purification agents used (e.g. charcoal or Celite®) do not bind to the mycotoxins, but retain interfering co-extracted compounds [62-69]. Thus, they must have no or an insignificant effect on the binding of mycotoxins from extracts with higher organic content. Other authors reported higher retention of AFB1 by some clays from aqueous corn meal dispersions than from water, when 60% methanol was used to extract AFB1 from the corn meal dispersions [26]. Taking into account these aspects, AFB1 and DON were considered as two 'extreme' cases in our study. The effect of mycotoxin binding agents on the analytical determination of these two mycotoxins was investigated extensively for each single mycotoxin binder. For other mycotoxins (OTA, ZEA, FUM B1+B2, T-2 + HT-2), the binders were grouped as described above and added together as a group to the test samples for the extraction. This approach allows minimising the amount of experiments to a reasonable sequence which can be carried out in one run for each mycotoxin. Moreover, it can enhance the effect of similar binders.

For data analysis, critical *t*-values ($p = 0.05$) were compared with those obtained by the Student's independent two-sample *t*-test and applying the repeatability of each method based on collaborative trial data (Table 1). The value corresponding to the method repeatability is a robust estimate of the average performance of the standardised methods when applied in technically competent laboratories. The repeatability of the methods as applied in our laboratory was in all cases lower than the repeatability standard deviation as given in the CEN standards. This fact demonstrates the laboratory's ability of applying the standard methods correctly.

As mentioned in the introduction, the method of addition (here pelleting) of a mycotoxin binder was found to have an effect on the bioavailability of AFB1. It resulted in a reduced excretion of AFM1 to milk when animals were fed with AFB1 contaminated feed [49]. This aspect could not be investigated in this study because of the unavailability of adequate processing machinery. The authors of the above mentioned study claimed that feed processing had an effect only on the functionality of the binder in the animal, and that the reduction of AFM1 in milk was not due to any degradation of AFB1 during the processing. However, such a conclusion can only be made if the analytical method used allows the complete recovery of the mycotoxin from the tested material.

Analysis of aflatoxin B1

No aflatoxin containing feed material was available for this study. Therefore, blank feed material was spiked at 42 µg/kg as described above. Each binder was added in triplicate (Table 2). The whole set of analyses covering all binders was performed in several batches. Spiking was carried out for each analytical batch. The mycotoxin values obtained for the feed material without binder was used as a reference and defined as 100 %. The results are presented in Figure 1. Statistical analysis indicated that there were no significant differences between binder free material and the material containing different binders under the described mixing conditions. Thus, it could be concluded that the addition of the tested binders had no significant effect on the performance of the analytical procedure used for the detection of AFB1.

Analysis of deoxynivalenol

Naturally contaminated feed material was used for these experiments. The amount of DON found in this material was 6000 µg/kg. The material was additionally spiked at 6000 µg/kg to aim at a total amount of DON of approximately 12000 µg/kg. This is approximately 1.5 times higher than the recommended limit for animal feed based on cereals and cereal products with the exception of maize by-products. The experimental design was the same as for AFB1. The results are given in Figure 2. Statistical analysis indicated that there were no significant differences between binder free material and the material containing different binders under the described mixing conditions. Thus, it could be concluded that the addition of the tested binders had no significant effect on the performance of the analytical procedure used for the detection of deoxynivalenol.

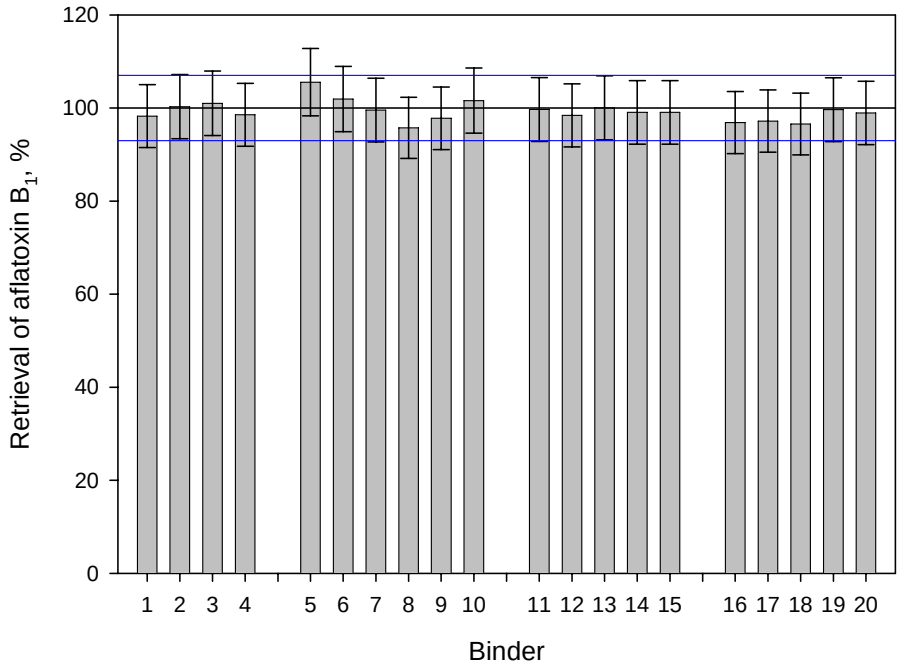

Figure 1. Effect of different binders on the analytical determination of AFB1 by HPLC. The amount of AFB1 determined is related to that in binder free material defined as 100%. Bars are the mean values of triplicates. Error bars correspond to the repeatability (RSDr) of the method. Blue lines represent the RSDr range for binder free material. Binders were analysed in different batches indicated by spaces in the figure.

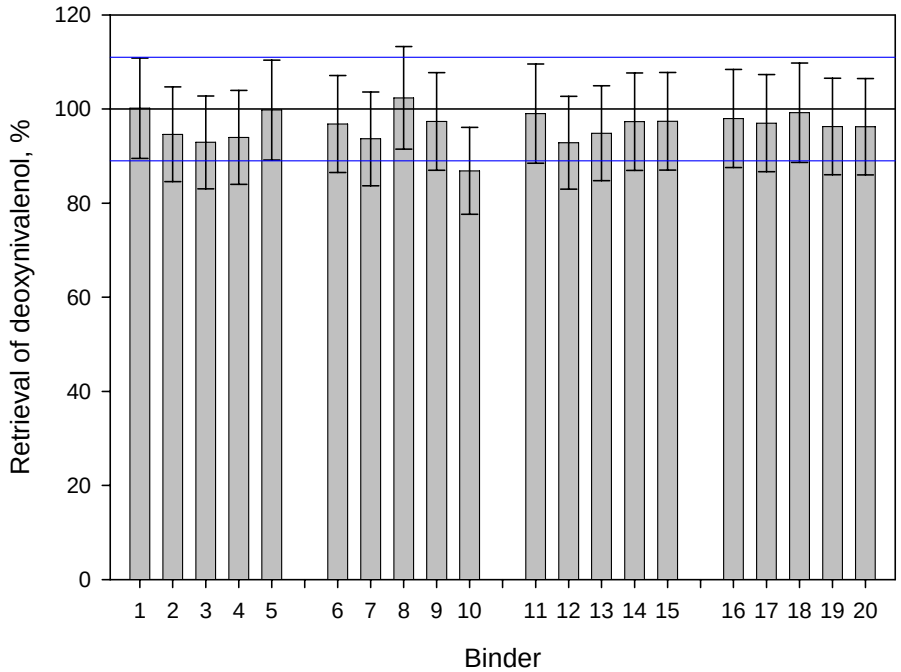

Figure 2. Effect of different binders on the analytical determination of DON by HPLC. The amount of DON determined is related to that in binder free material defined as 100%. Bars are the mean values of triplicates. Error bars correspond to the repeatability (RSDr) of the method. Blue lines represent the RSDr range for binder free material. Binders were analysed in different batches indicated by spaces in the figure.

Analysis of zearalenone

The same material used for the AFB1 experiments was used for the investigation concerning ZEA. The material was found to be free of any naturally occurring ZEA. It was spiked at 2900 µg/kg. For these experiments, binders were combined into groups as described in the section Methodology. The binders, which belonged to the same group, were added together to each portion of feed test material. All the binders were added to the test sample portions at the maximum recommended level (Table 2). The feed material without binder was analysed as a reference and the level found was defined as 100%. The results are presented in Figure 3. Statistical analysis indicated that there were no significant differences between binder free material and the material containing different binders under the described mixing conditions. Thus, it could be concluded that the addition of the tested binders had no significant effect on the performance of the analytical procedure used for the detection of ZEA.

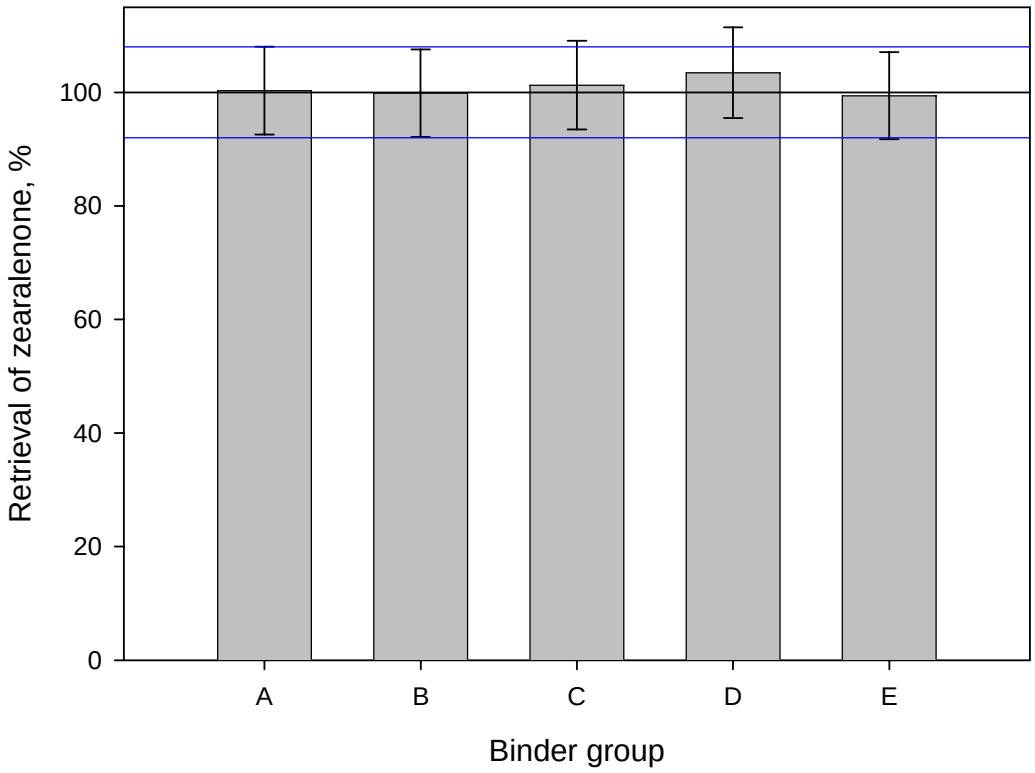

Figure 3. Effect of different binders on the analytical determination of ZEA by HPLC. The amount of ZEA determined is related to that in binder free material defined as 100%. Bars are the mean values of triplicates. Error bars correspond to the repeatability (RSDr) of the method. Blue lines represent the RSDr range for binder free material.

Analysis of ochratoxin A

For the investigation of mycotoxin binder effects for OTA, naturally contaminated feed material was used. The amount of OTA in binder free material has been determined as 367 µg/kg. The experiment was designed in the same way as for ZEA (addition of binders by groups). The results are given in Figure 4. Statistical analysis indicated that there were no significant differences between binder free material and the material containing different binders under the described mixing conditions. Thus, it could be concluded that the addition of the tested binders had no significant effect on the performance of the analytical procedure used for the detection of OTA.

Analysis of fumonisins B1+B2

For the investigation of mycotoxin binder effects concerning fumonisins, naturally contaminated feed material was used. The total amount of FUM B1 and B2 in the binder free material has been determined as 7900 µg/kg. The experiment was designed in the same way as for ZEA and OTA (addition of binders by groups). The results are shown in Figure 5. Statistical analysis indicated that there were no significant differences between binder free material and the material containing different binders under the described mixing conditions. Thus, it could be concluded that the addition of the tested binders had no significant effect on the performance of the analytical procedure used for the detection of fumonisins B1 and B2.

Analysis of T-2 + HT-2 toxins

For the investigation of mycotoxin binder effects concerning T-2 and HT-2 toxins, naturally contaminated feed material (same as for DON experiments) was used. The experiment was designed in the same way as for ZEA, OTA and the fumonisins (addition of binders by groups). The total amount of T2 and HT-2 toxins in the binder free material has been determined as 7850 µg/kg. The results are given in Figure 6. Statistical analysis indicated that there were no significant differences between binder free material and the material containing different binders under the described mixing conditions. Thus, it could be concluded that the addition of the tested binders had no significant effect on the performance of the analytical procedure used for the detection of T-2 and HT-2 toxins.

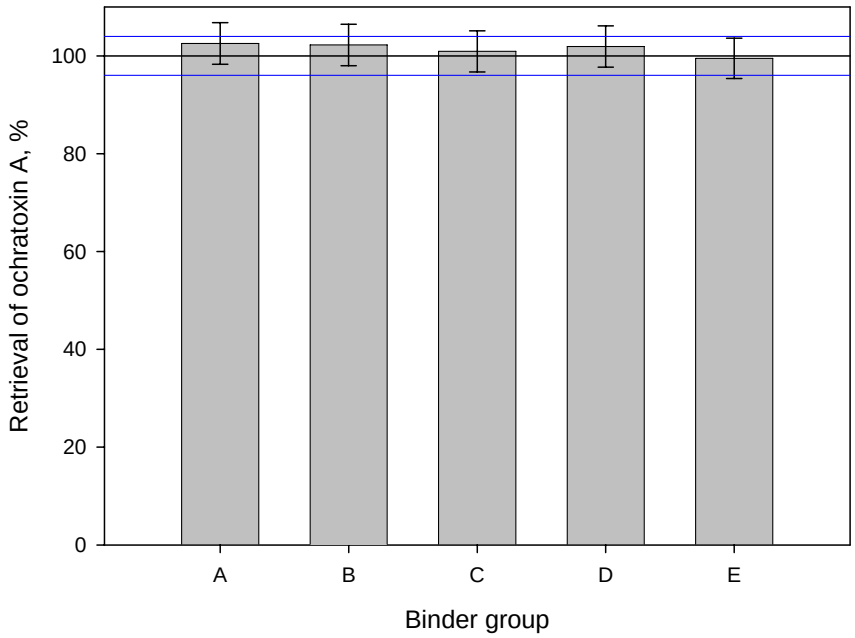

Figure 4. Effect of different binders on the analytical determination of OTA by HPLC. The amount of OTA determined is related to that in binder free material defined as 100%. Bars are the mean values of triplicates. Error bars correspond to the repeatability (RSDr) of the method. Blue lines represent the RSDr range for binder free material.

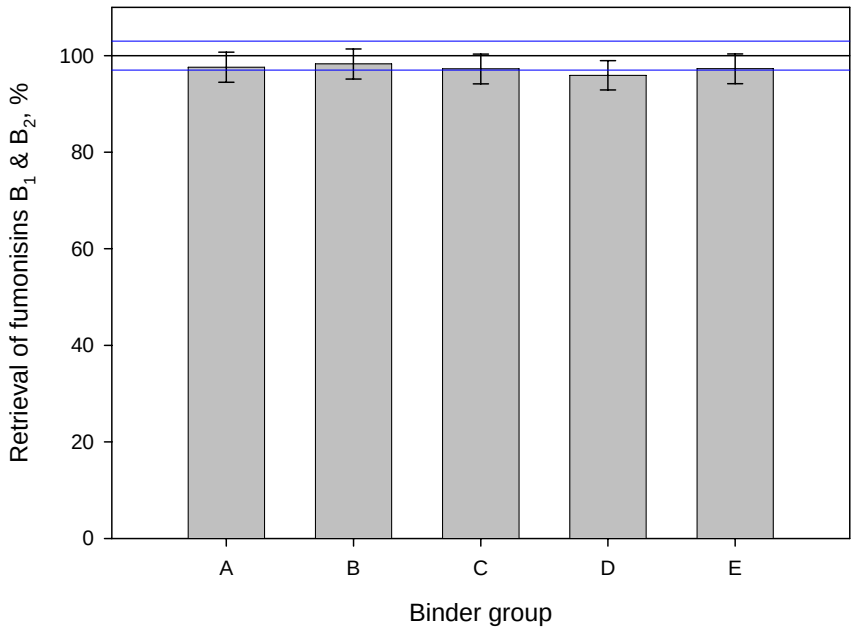

Figure 5. Effect of different binders on the analytical determination of FUM B1+B2 by HPLC. The amount of FUM B1 + B2 determined is related to that in binder free material defined as 100%. Bars are the mean values of triplicates. Error bars correspond to the repeatability (RSDr) of the method. Blue lines represent the RSDr range for binder free material.

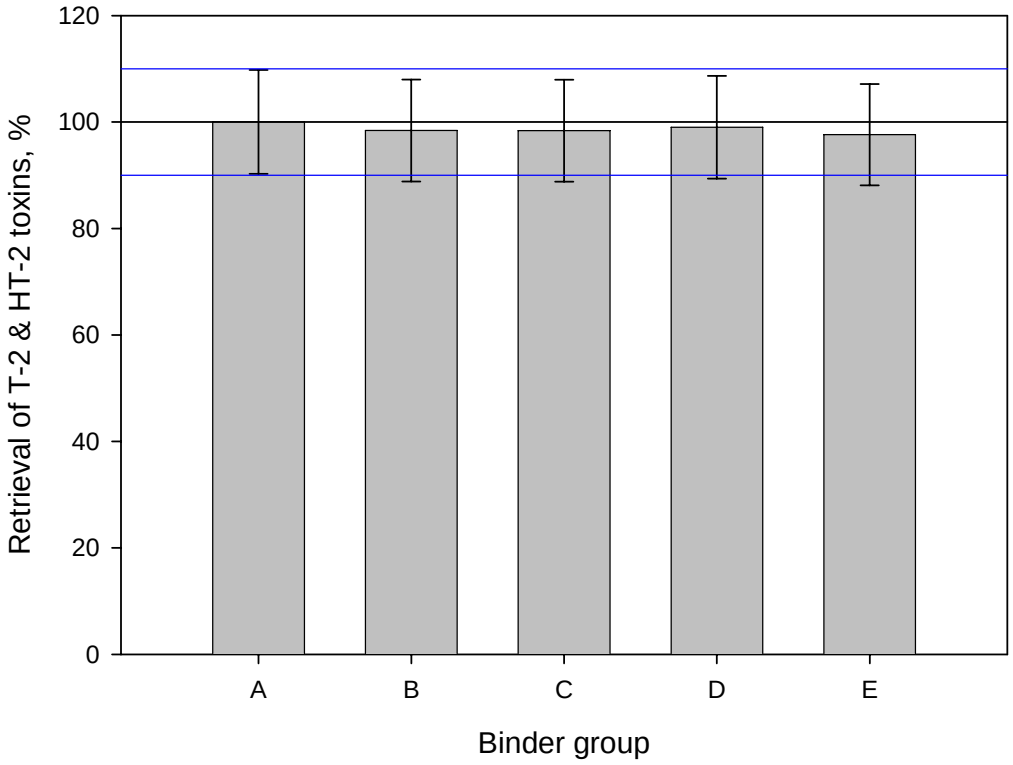

Figure 6. Effect of different binders on the analytical determination of the total amount of T-2 and HT-2 toxins by GC-MS. The amount of T-2 and HT-2 toxins determined is related to that in binder free material defined as 100%. Bars are the mean values of triplicates. Error bars correspond to the repeatability (RSDr) of the method. Blue lines represent the RSDr range for binder free material.

Conclusions

The effect of mycotoxin binders in animal feed on the analytical performance of currently standardised methods for the determination of mycotoxins in feed was investigated. Statistical analysis did not show any significant differences in the selected analytical methods' capacity to determine mycotoxins in binder free material and materials with mycotoxin binders added, respectively. Thus, it could be concluded that the tested binders had no effect on the level of mycotoxins found. It is therefore not possible to use any of the tested binders for masking mycotoxins in contaminated feed under the condition that the binders are used within the recommended range, and that they are mixed into the feed in the same manner as in our study. A combination of binder addition and processing, such as pelleting or extrusion, has not been a subject of this study but could be considered as a next step.

Acknowledgement

We are grateful to Mr. D. Jans (FEFANA) and Mr. G. Bertin (Alltech) for providing us with the test samples of mycotoxin binders collected from various companies.
We would like also to acknowledge Mr. T. Vanderborght (Impextraco NV), Mr. A. Kroismayr (Agromed Austria GmbH) and Sud-Chemie AG for providing the test samples of mycotoxin binders.
We are grateful to Dr. V. Baeten, Dr. O. Abbas and Mr. A. Rodriguez (Walloon Agricultural Research Centre) for the spectroscopy studies on mycotoxin binders.
We also wish to thank Dr. C. Von Holst and Mr. B. Slowikowski (JRC-IRMM) for their support in this research.
The authors wish to thank Anne-Mette Jensen for valuable comments.

References

1. Huwig, A., Freimund, S., Kappeli, O., Dutler, H., 2001. Mycotoxin detoxification of animal feed by different adsorbents. Toxicol. Lett. 122, 179-188.
2. CAST (Council for Agricultural Science and Technology). 1989. "Mycotoxins: Economics and Health Risks". Task Force Report No. 116. Ames, IA.
3. Visconti, A. 1998. Problems associated with Fusarium mycotoxins in cereals. In: Zuxun, J., Quan, L., Yongsheng, L., Zianchang, T., Lianghua, G., (Eds.) Stored product protection. Chengdu: Sichuan, pp. 173–186.
4. Hesseltine, C.W. 1986. Resumé and future needs in the diagnosis of mycotoxins. In: J.L. Richard and J.R. Thurston, (Eds.) Diagnosis of Mycotoxicoses. Martinus Nijhoff Publishers, Dordrecht, the Netherlands, pp. 381-385.
5. Schiefer, H.B., 1990. Mycotoxicosis of domestic animals and their diagnosis. Can. J. Physiol. Pharmacol. 68, 987-990.
6. Avantaggiato, G., Havenaar, R., Visconti, A., 2007. Assessment of the multi-mycotoxin-binding efficacy of a carbon/aluminosilicate-based product in an in vitro gastrointestinal model. J. Agric. Food Chem. 55, 4810-4819.
7. CAST (Council for Agricultural Science and Technology). 2003. "Mycotoxins: Risks in Plant, Animal, and Human Systems". Task Force Report No. 139. Ames, IA.
8. Speijers, G.J., Speijers, M.H., 2004. Combined toxic effects of mycotoxins. Toxicol. Lett. 153, 91-98.
9. Sergent, T., Ribonnet, L., Kolosova, A., Garsou S., Schaut, A., De Saeger, S., Van Peteghem, C., Larondelle, Y., Pussemier, L., Schneider, Y.-J., 2008. Molecular and cellular effects of food contaminants and secondary plant components and their plausible interactions at the intestinal level. Food Chem. Toxicol. 46, 813-841.
10. Commission Directive 2003/100/EC of 31 October 2003 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed. Off. J. Eur. Union L 285/33.
11. Commission recommendation 2006/576/EC of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding L 229/7.
12. Doyle, M.P., Applebaum, R.S., Brackett, R.E., Marth, E.H., 1982. Physical, chemical and biological degradation of mycotoxins in foods and agricultural commodities. J. Food Prot. 45, 946–971.

13. Park, D.L., 1993. Perspectives on mycotoxin decontamination procedures. Food Addit. Contam. 10, 49–60.
14. Ramos, A.J., Hernandez, E., 1997. Prevention of aflatoxicosis in farm animals by means of hydrated sodium calcium aluminosilicate addition to feedstuffs. A review. Anim. Feed Sci. Technol. 65, 197–206.
15. Bintvihok, A., Kositcharoenkul, S., 2006. Effect of dietary calcium propionate on performance, hepatic enzyme activities and aflatoxin residues in broilers fed a diet containing low levels of aflatoxin B1. Toxicon 47, 41-46.
16. Nahm, K.H., 1995. Possibilities for preventing mycotoxicosis in domestic fowl. World Poult. Sci. J. 51, 177–185.
17. Jouany, J.-P., 2007. Methods for preventing, decontaminating and minimizing the toxicity of mycotoxins in feeds. Anim. Feed Sci. Technol. 137, 342-362.
18. McKenzie, K.S., Sarr, A.B., Mayura, K., Bailey, R.H., Miller, D.R., Rogers, T.D., Norred, W.P., Voss, K.A., Plattner, R.D., Kubena, L.F., Phillips, T.D., 1997. Oxidative degradation and detoxification of mycotoxins using a novel source of ozone. Food Chem. Toxicol. 35, 807–820.
19. Lemke, S.L., Mayura, K., Ottinger, S.E., McKenzie, K.S., Wang, N., Fickey, C., Kubena, L.F., Phillips, T.D., 1999. Assessment of the estrogenic effects of zearalenone after treatment with ozone utilizing the mouse uterine weight bioassay. J. Toxicol. Environ. Health A 56, 283–295.
20. Coker, R.D., 1998. The chemical detoxification of aflatoxin contaminated animal feed. Nat. Toxicants Food 284–298.
21. Avantaggiato, A., Solfrizzo, M., Visconti, A., 2005. Recent advances on the use of adsorbent materials for detoxification of Fusarium mycotoxins. Food Addit. Contam. 22, 379-388.
22. Ramos, A.J, Fink-Gremmels, J., Hernandez, E., 1996. Prevention of toxic effects of mycotoxins by means of non-nutritive adsorbent compounds. J. Food Prot. 59, 631–641.
23. Galvano, F., Piva, A., Ritieni, A., Galvano, G., 2001. Dietary strategies to counteract the effects of mycotoxins: a review. J. Food Prot. 64, 120–131.
24. Diaz, D.E., Hagler, W.M., Blackwelder, J.T., Eve, J.A., Hopkins, B.A., Anderson, K.L., Jones, F.T., Whitlow L.W., 2004. Aflatoxin binders II: Reduction of aflatoxin M1 in milk by sequestering agents of cows consuming aflatoxin in feed. Mycopathologia, 157, 233–241.
25. Var, I., Kabak, B., Erginkaya, Z., 2008. Reduction in ochratoxin A levels in white wine, following treatment with activated carbon and sodium bentonite. Food Control 19, 592-598.
26. Jaynes, W.F., Zartman, R.E., Hudnall, W.H., 2007. Aflatoxin B1 adsorption by clays from water and corn meal. Appl. Clay Sci. 36, 197-2005.
27. Barrer, R.M., 1989. Shape-selective sorbents based on clay minerals - a review. Clays Clay Miner. 37, 385–395.

28. Papaioannou, D., Katsoulos, P.D., Panousis, N., Karatzias, H., 2005. The role of natural and synthetic zeolites as feed additives on the prevention and/or the treatment of certain farm animal diseases: A review. Microporous Mesoporous Mat. 84, 161–170.
29. Dixon, J.B., Kannewischer, I., Tenorio Arvide, M.G., Barrientos Velazquez, A.L., 2008. Aflatoxin sequestration in animal feeds by quality-labeled smectite clays: an introductory plan. Appl. Clay Sci. 40, 201–208.
30. Phillips, T.D., 1999. Dietary clay in the chemoprevention of aflatoxin induced disease. Toxicol. Sci. 52, 118-126.
31. Phillips, T.D., Sarr, A.B., Clement, B.A., Kubena, L.F., Harvey, R.B., 1990. Prevention of aflatoxicosis in farm animals via selective chemisorption of aflatoxins. In: Bray, G.A., Ryan, D.H. (Eds.), Pennington Center Nutrition Series. In: Mycotoxins, Cancer, and Health, vol. 1. Louisiana State University Press, Baton Rouge, pp. 223–237.
32. Diaz, D.E., Smith, T.K., 2005. Mycotoxin sequestering agents: practical tools for the neutralisation of mycotoxins. In: Diaz, D.E., (Ed.), The Mycotoxin Blue Book. Nottingham University Press: Nottingham, U.K., pp. 323-338.
33. Lemke, S.L., Grant, P.G., Phillips, T.D., 1998. Adsorption of zearalenone by organophilic montmorillonite clay. J. Agric. Food Chem. 46, 3789-3796.
34. Lemke, S.L., Mayura, K., Reeves, W.R., Wang, N., Fickey, C., Phillips, T.D., 2001. Investigation of organophilic montmorillonite clay inclusion in zearalenone-contaminated diets using the mouse uterine weight bioassay. J. Toxicol. Environ. Health A 62, 243–258.
35. Feng, J., Shan, M., Du, H., Han, X., Xu, Z., 2008. In vitro adsorption of zearalenone by cetyltrimethyl ammonium bromide-modified montmorillonite nanocomposites. Microporous and Mesoporous Mat. 113, 99–105.
36. Avantaggiato, G., Havenaar, R., Visconti, A., 2003. Assessing the zearalenone-binding activity of adsorbent materials during passage through a dynamic *in* V*itro* gastrointestinal model. Food Chem. Toxicol. 41, 1283-1290.
37. Avantaggiato, G., Havenaar, R., Visconti, A., 2004. Evaluation of the intestinal absorption of deoxynivalenol and nivalenol by an *in* V*itro* gastrointestinal model, and the binding efficacy of activated carbon and other adsorbent materials. Food Chem. Toxicol. 42, 817-824.
38. Shetty, P.H., Jespersen, L., 2006. Saccharomyces cerevisiae and lactic acid bacteria as potential mycotoxin decontaminating agents. Trends in Food Sci. Technol. 17, 48-55.
39. Fuchs, S., Sontag, G., Stidl, R., Ehrlich, V., Kundi, M., Knasmuller, S., 2008. Detoxification of patulin and ochratoxin A, two abundant mycotoxins, by lactic acid bacteria. Food Chem. Toxicol. 46, 1398–1407.

40. Yiannikouris, A., Andre, G., Buleon, A., Jeminet, G., Canet, I., Francois, J., Bertin, G., Jouany, J.-P., 2004. Comprehensive conformational study of key interactions involved in zearalenone complexation with β-d-glucans. Biomacromolecules 5 (6), 2176–2185.
41. Schatzmayr, G., Zehner, F., Taubel, M., Schatzmayr, D., Klimitsch, A., Loibner, A.P., Binder, E.M., 2006. Microbiologicals for deactivating mycotoxins. Mol. Nutr. Food Res. 50, 543-551.
42. Molnar, O., Schatzmayr, G., Fuchs, E., Prillinger, H., 2004. Trichosporon mycotoxinivorans sp. nov., a new yeast species useful in biological detoxification of various mycotoxins. Syst. Appl. Microbiol. 27, 661-671.
43. Garcia, A.R., Avila, E., Rosiles, R., Petrone, V.M., 2003. Evaluation of two mycotoxin binders to reduce toxicity of broiler diets containing ochratoxin A and T-2 toxin contaminated grain. Avian Diseases 47, 691–699.
44. Chowdhury, S.R., Smith, T.K., 2004. Effect of feeding blends of grains naturally contaminated with Fusarium mycotoxins on performance and metabolism of laying hens. Poult. Sci. 83, 1849-1856.
45. Sabater-Vilar, M., Malekinejad, H., Selman, M.H.J., Van Der Doelen, M.A.M., Johanna Fink-Gremmels, J., 2007. In vitro assessment of adsorbents aiming to prevent deoxynivalenol and zearalenone mycotoxicoses. Mycopathologia, 163, 81–90.
46. Pasha, T.N., Farooq, M.U., Khattak, F.M., Jabbar, M.A., Khan, A.D., 2007. Effectiveness of sodium bentonite and two commercial products as aflatoxin absorbents in diets for broiler chickens. Anim. Feed Sci. Technol. 132, 103–110.
47. Miles, R.D., Henry, P.R., 2007. Safety of improved Milbond-TX® when fed in broiler diets at greater than recommended levels. Anim. Feed Sci. Technol. 138, 309–317.
48. Moschini, M., Gallo, A., Piva, G., Masoero F., 2008. The effects of rumen fluid on the *in vitro* aflatoxin binding capacity of different sequestering agents and *in vivo* release of the sequestered toxin. Anim. Feed Sci. Technol. 147, 292–309.
49. Masoero, F., Gallo, A., Diaz, D., Piva, G., Moschini, M., 2009. Effects of the procedure of inclusion of a sequestering agent in the total mixed ration on proportional aflatoxin M1 excretion into milk of lactating dairy cows. Anim. Feed Sci. Technol. 150, 34–45.
50. Lemke, S.L., Ottinger, S.E., Mayura, K., Ake, C.L., Pimpukdee, K., Wang, N., Phillips, T.D., 2001. Development of a multi-tiered approach to the in vitro prescreening of clay-based enterosorbents. Anim. Feed Sci. Technol. 93, 17-29.
51. Averkieva, O., Van Dyck, S., 2007. Requirements to assess mycotoxin binder performance. Zootecnica September 2007, 24-28.
52. Krska, R., Welzig, E., Berthiller, F., Molinelli, A., Mizaikoff, B., 2005. Advances in the analysis of mycotoxins and its quality assurance. Food Addit. Contam. 22, 345-353.

53. Zheng, M.Z., Richard, J.L., Binder, J., 2006. A review of rapid methods for the analysis of mycotoxins. Mycopathologia 161, 261–273.
54. Krska, R., Molinelli, A., 2009. Rapid test strips for analysis of mycotoxins in food and feed. Anal. Bioanal. Chem. 393, 67–71.
55. Turner, N.W., Subrahmanyam, S., Piletsky, S.A., 2009. Analytical methods for determination of mycotoxins: A review. Anal. Chim. Acta 632, 168–180.
56. Kogel-Knabner, I., Gottlein, A. Bodenkundliche ubung labormethoden. Skriptum der TU Munchen. http://www.wzw.tum.de/bk/pdfs/uebungen/bk_labor.pdf (accessed on 17.08.2009)
57. Stroka, J., Derbyshire, M., Mischke, C., Ambrosio, M., Kroeger, K., Arranz, I., Sizoo, E., van Egmond, H., 2006. Liquid chromatographic determination of deoxynivalenol in baby food and animal feed: interlaboratory study. J. AOAC Int. 89, 1012-1020.
58. Arranz, I., Mischke, C., Stroka, J., Sizoo, E., Van Egmond H., 2007. Liquid chromatographic method for the quantification of zearalenone in baby food and animal feed: collaborative study. J. AOAC Int. 90, 1598 – 1609.
59. Stroka, J., Ambrosio, M., Doncheva, I., Lerda, D., Mischke, C., Breidbach, A., 2008. JRC Scientific and Technical Report: Determination of Ochratoxin A in Animal Feed by Immunoaffinity Column Clean-up with High Performance Liquid Chromatography using Fluorimetric Detection (EUR 23657 EN – 2008).
60. Breidbach, A., Bouten, K., Kroeger, K., Stroka, J., 2009. JRC Scientific and Technical Report: Determination of the Sum of Fumonisin B1 and B2 in Compound Animal Feed and Maize by Immunoaffinity Column Clean-up and High Performance Liquid Chromatography with Fluorometric Detection (EUR 23941 EN – 2009).
61. Breidbach, A., Povilaityte, V., Mischke, C., Doncheva, I., van Egmond, H., Stroka, J., 2008. JRC Scientific and Technical Report: Validation of an Analytical Method to Determine the Content of T-2 and HT-2 Toxins in Cereals and Compound Animal Feed by Immunoaffinity Column Clean-up and GC-MS (EUR 23560 EN – 2008).
62. Lattanzio, V.M.T., Solfrizzo, M., Visconti, A., 2008. Determination of trichothecenes in cereals and cereal-based products by liquid chromatography–tandem mass spectrometry. Food Addit. Contam. 25, 320–330.
63. Tanaka, H., Takino, M., Sugita-Konishi, Y., Tanaka, T., 2006. Development of a liquid chromatography/time-of-flight mass spectrometric method for the simultaneous determination of trichothecenes, zearalenone and aflatoxins in foodstuffs. Rapid. Commun. Mass Spectrom. 20, 1422-1428.

64. Stecher, G., Jarukamjorn, K., Zaborski, P., Bakry, R., Huck, C.W., Bonn, G.K., 2007. Evaluation of extraction methods for the simultaneous analysis of simple and macrocyclic trichothecenes. Talanta 73, 251–257.
65. Ren, Y., Zhang, Y., Shao, S., Cai, Z., Feng, L., Pan, H., Wang, Z., 2007. Simultaneous determination of multi-component mycotoxin contaminants in foods and feeds by ultra-performance liquid chromatography tandem mass spectrometry. J. Chromatogr. A, 1143, 48–64.
66. Schothorst, R.C., Jekel, A.A., Van Egmond, H.P., De Mul, A., Boon, P.E., Van Klaveren, J.D., 2005. Determination of trichothecenes in duplicate diets of young children by capillary gas chromatography with mass spectrometric detection. Food Addit. Contam. 22, 48–55.
67. Valle-Algarra, F.M., Medina, A., Gimeno-Adelantado, J.V., Llorens, A., Jimenez, M., Mateo, R., 2005. Comparative assessment of solid-phase extraction clean-up procedures, GC columns and perfluoroacylation reagents for determination of type B trichothecenes in wheat by GC–ECD. Talanta, 194–201.
68. Krska, R., 1998. Performance of modern sample preparation techniques in the analysis of Fusarium mycotoxins in cereals. J. Chromatogr. A 815, 49-57.
69. Silva, C.M.G., E. Vargas, E.A., 2001. A survey of zearalenone in corn using Romer Mycosep™ 224 column and high performance liquid chromatography. Food Addit. Contam. 18, 39-45.

Annex 1

Results of the determination of mycotoxins in feed samples by the standardised methods with and without mycotoxin binders

Mycotoxin, batch	Binder(s) added	*t*-value		Amount of mycotoxin, µg/kg		
		critical	calculated	Prep. 1	Prep. 2	Prep. 3
AFB1 Batch 1	Blank	-	-	38.52	39.55	38.71
	Binder 1	2.78	0.31	38.57	38.21	37.95
	Binder 2	2.78	0.05	38.75	38.74	39.62
	Binder 3	2.78	0.18	40.81	37.63	39.52
	Binder 4	2.78	0.26	38.25	38.26	38.58
AFB1 Batch 2	Blank	-	-	37.76	39.84	36.95
	Binder 5	2.78	0.99	37.71	37.61	45.59
	Binder 6	2.78	0.34	39.78	39.37	37.62
	Binder 7	2.78	0.08	39.43	37.44	37.18
	Binder 8	2.78	0.76	37.65	34.46	37.55
	Binder 9	2.78	0.39	38.51	37.05	36.46
	Binder 10	2.78	0.29	37.99	40.12	38.28
AFB1 Batch 3	Blank	-	-	40.35	39.87	40.54
	Binder 11	3.18	0.05	-	39.44	40.81
	Binder 12	2.78	0.28	39.17	39.75	39.92
	Binder 13	2.78	0.00	40.40	41.07	39.32
	Binder 14	3.18	0.15	40.15	-	39.61
	Binder 15	2.78	0.17	39.48	40.82	39.34
AFB1 Batch 4	Blank	-	-	39.23	39.71	39.64
	Binder 16	3.18	0.50	-	38.15	38.44
	Binder 17	2.78	0.50	38.35	38.21	38.69
	Binder 18	2.78	0.61	38.07	38.35	38.09
	Binder 19	2.78	0.06	38.91	39.32	39.94
	Binder 20	2.78	0.19	39.20	38.94	39.18
DON Batch 1	Blank	-	-	10234	10116	10017
	Binder 1	2.78	0.02	10355	10230	9832
	Binder 2	2.78	0.62	9668	9172	9895
	Binder 3	2.78	0.82	10097	8996	9120
	Binder 4	2.78	0.70	9747	9233	9553
	Binder 5	2.78	0.02	10114	10308	9880
DON Batch 2	Blank	-	-	10362	10668	9982
	Binder 6	2.78	0.37	9958	9651	10418
	Binder 7	2.78	0.73	10235	9324	9485
	Binder 8	3.18	0.24	11465	-	9700
	Binder 9	2.78	0.30	10153	10160	9878
	Binder 10	2.78	1.51	8218	8862	9856
DON Batch 3	Blank	-	-	10154	10451	8734
	Binder 11	2.78	0.11	10239	9893	8915
	Binder 12	2.78	0.83	8414	8406	10416
	Binder 13	2.78	0.59	9307	9205	9314
	Binder 14	2.78	0.31	9391	9823	9336
	Binder 15	2.78	0.30	9630	9437	9505

	Binder(s) added	*t*-value		Amount of mycotoxin, µg/kg		
		critical	calucated	Prep. 1	Prep. 2	Prep. 3
DON Batch 4	Blank	-	-	9740	9805	10023
	Binder 16	2.78	0.23	9430	9634	9907
	Binder 17	2.78	0.35	9572	9594	9513
	Binder 18	2.78	0.09	9480	9747	10111
	Binder 19	2.78	0.43	9596	9505	9366
	Binder 20	2.78	0.43	9541	9577	9339
ZEA	Blank	-	-	2716	2777	2778
	Group A	2.78	0.05	2843	2753	2699
	Group B	2.78	0.02	2889	2611	2759
	Group C	3.18	0.18	-	2735	2849
	Group D	2.78	0.55	2811	2928	2818
	Group E	2.78	0.09	2702	2870	2650
OTA	Blank	-	-	370.2	368.6	361.5
	Group A	2.78	0.75	371.9	376.9	379.3
	Group B	2.78	0.66	365.3	384.4	375.3
	Group C	2.78	0.27	369.4	373.4	367.7
	Group D	2.78	0.57	372.5	365.7	383.2
	Group E	2.78	0.15	371.4	353.9	369.3
FUM B1 + B2	Blank	-	-	7955	7822	7923
	Group A	2.78	0.93	7710	7800	7618
	Group B	2.78	0.66	7790	7834	7667
	Group C	2.78	1.06	7736	7730	7581
	Group D	2.78	1.57	7651	7705	7377
	Group E	2.78	1.05	7643	7623	7788
T2 + HT2	Blank	-	-	7705	7690	8165
	Group A	2.78	0.00	7795	8065	7705
	Group B	2.78	0.20	7805	7725	7655
	Group C	3.18	0.18	-	7625	7825
	Group D	2.78	0.13	7830	7790	7705
	Group E	2.78	0.30	7635	7655	7710

Annex 2

(see next page)

Infrared Measurements on Mycotoxin Binders

Report prepared by

Ouissam Abbas and Vincent Baeten

In collaboration with

Alexandro Rodriguez

Walloon Agricultural Research Centre (CRA-W)
Chaussée de Namur 24
B-5030 Gembloux, Belgium

Working document from 17 July 2009
(including the work performed during the
period of April – June 2009)

Walloon Agricultural Research Centre
Quality of Agricultural Products Department
Chaussée de Namur, 24
B – 5030 GEMBLOUX
Tél :++ 32 (0) 81/62.03.50
Fax : ++ 32 (0) 81/62.03.88
baeten@cra.wallonie.be
http://cra.wallonie.be

Walloon Agricultural Research Centre

RÉGION WALLONNE

Contents

1 Summary

The purpose of this work is to check if the different materials from the same group of substances are identical and to characterise their identity. Spectroscopic methods (Raman, mid and near infrared) are used to have additional and complementary information.
Due to the problems encountered with mid infrared and Raman methods, only near infrared results are presented in this report.

1.1 Framework of the study

This work is carried out in the framework of the study asked by the European Commission, DG Joint Research Centre (Institute for Reference Materials and Measurements) in order to use spectroscopic methods to identify mycotoxin binders.

1.2 Objectives

The objectives aimed by this work were as follows:

- The characterization of mycotoxin binders
- The use of spectroscopy to investigate if the different materials from the same group of substances are identical
- To build a small database of different mycotoxin binder classes which allows to check, if needed in future studies, whether there is an evolution of material composition of available mycotoxin binders over time

2 Material and Methods

2.1 Samples

27 samples were measured by Raman and near infrared spectrometry (**Tables 1 and 2**).

Table 1. *Studied mycotoxin binders*

Code	Product
DQ09-0350-001	Yeast cell wall
DQ09-0350-002	Clay + organic acid
DQ09-0350-003	Clay
DQ09-0350-004	Clay
DQ09-0350-005	Yeast cell wall
DQ09-0350-006	Yeast cell wall
DQ09-0350-007	Yeast cell wall
DQ09-0350-008	Mixture organic + mineral
DQ09-0350-009	Yeast cell wall
DQ09-0350-010	Mixture organic + mineral component
DQ09-0350-011	Mixture organic + mineral component
DQ09-0350-012	Clay
DQ09-0350-013	Clay
DQ09-0350-014	Fibres
DQ09-0350-015	Montmorillonite
DQ09-0350-016	Montmorillonite
DQ09-0350-017	HSCAS
DQ09-0350-018	HSCAS
DQ09-0350-019	HSCAS
DQ09-0350-027	Fibres (lignocellulose)

Table 2. *Additional reference substances studied*

Code	Product
DQ09-0350-020	Florisil
DQ09-0350-021	Kieselgur
DQ09-0350-022	Bentonite
DQ09-0350-023	Zeolite
DQ09-0350-024	Activated charcoal
DQ09-0350-025	Sepiolite
DQ09-0350-026	Dowex

2.2 Instruments

Raman analyses were performed using RAMII vertex 70 spectrometer (Bruker).
Near infrared spectroscopic analyses were performed using NirSystem spectrometer (Foss).
Software: OPUS (version 6.5), WinIsi III and The Unscrambler.

2.3 Methodology

The analytical methods followed are presented in the **Table 3**.

Table 3. *Experimental conditions for spectra recording*

	Raman	**Near infrared**	**Mid infrared**
Range	3600 - 0 cm^{-1}	400 – 2498 nm	*Problems encountered with ATR accessory* *No measurements could be done*
Resolution	4 cm^{-1}	2 nm	
Number of scans	128 scans	32 scans	
	Spectra were collected as Raman intensities	*Spectra were collected as log 1/R*	

3 Results and discussion

Three spectroscopic methods were tested on the mycotoxins binders. However when using the MIR instrument, the Attenuated Total Reflectance (ATR) accessory failed. Therefore, no mid-infrared spectra could be generated.
Raman analyses were conducted but spectra obtained were not well resolved. This made it difficult to make interpretations in order to characterize studied samples (data not shown).
As a result only near infrared method was able to give the spectra with good quality.

Because of the use of only one method (NIR), the objective concerning the characterization of mycotoxin binders couldn't be reached. In fact, to have precise and complete information about the composition of the studied samples, two or three methods (NIR/MIR, NIR/Raman or MIR/Raman) should be combined.

Near infrared spectra were used to see if, at a spectroscopic level, the different materials from the same group of substances were identical. CRA-W will proceed by the comparison of spectra of the same family; we will regroup spectra according to their similarity. In fact, the same spectral profile indicates similar composition. Six groups will be studied in this report: yeast cell wall, clay, mixture of organic and mineral components, montmorillonite, hydrated sodium calcium aluminosilicate (HSCAS) and additional reference substances.

3.1 Yeast cell wall

Spectra of yeast cell wall exhibit some differences and they are regrouped by pairs (a, b) (**Figure 1**).
Spectra are regrouped as presented in the table below.

	a	
DQ09-0350-001	Yeast cell wall	500 g-2 kg/T feed
DQ09-0350-007	Yeast cell wall	0,5 - 2,5 kg/T feed
	b	
DQ09-0350-005	Yeast cell wall	1-2 kg/T feed
DQ09-0350-006	Yeast cell wall	1-2 kg/T feed

The spectra have the same bands but differences are present in 492-640 nm and 2008-2230 nm regions. This indicates some difference in the composition of these sub-groups of yeast cell wall.

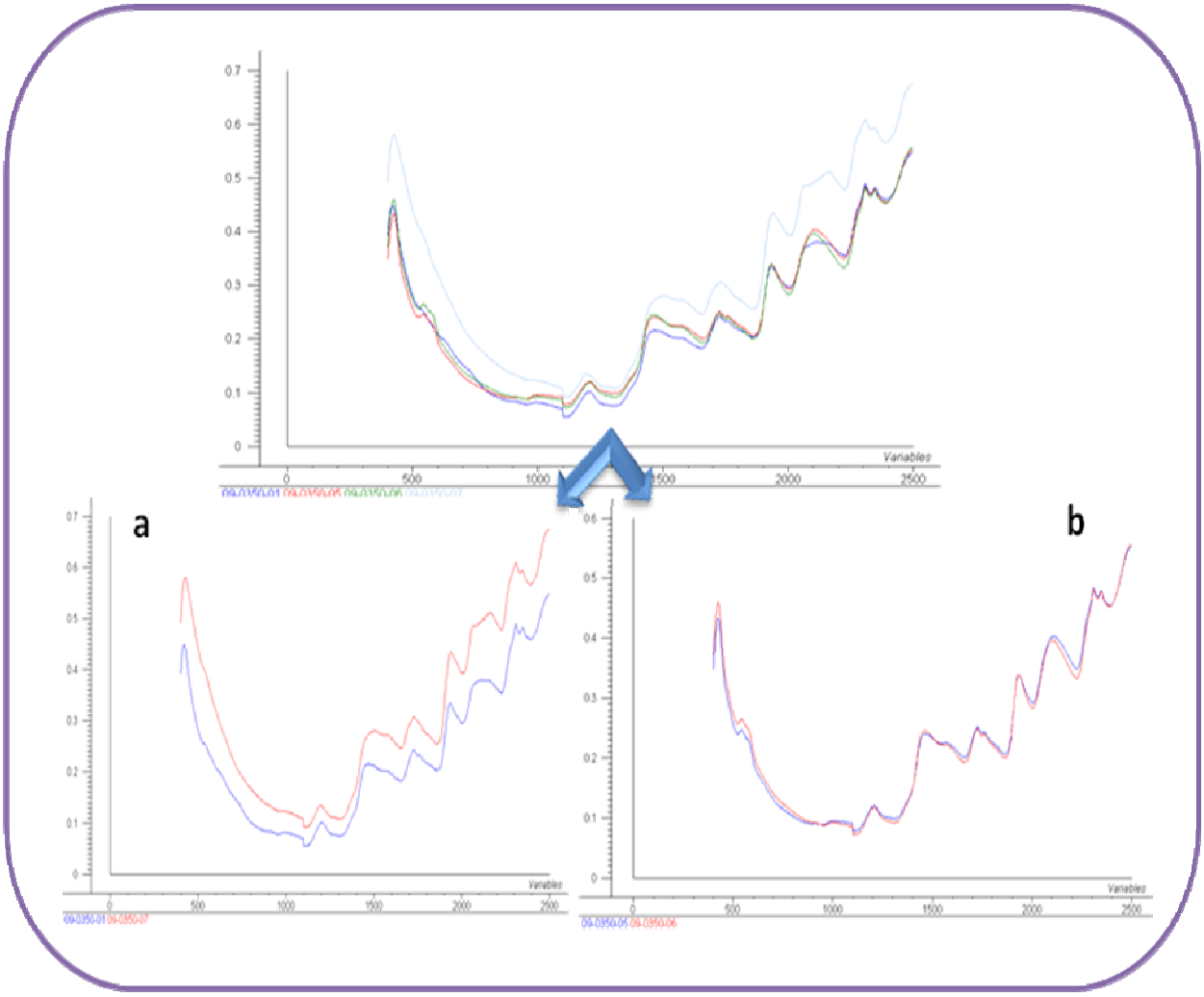

Figure 1: *NIR spectra of yeast cell wall.*

The region below 1100 nm will be excluded because for the rest of the samples, no differences between spectra occur in this region.

3.2 Clay

The spectra generated between 1100 and 2498 nm are compared as shown in **figure 2**. Five clay samples seem to be different. However, there are some similarities between spectra of samples DQ09-0350-02 and DQ09-0350-04 (a) and of samples DQ09-0350-03 and DQ09-0350-12 (b). One band is absent in each case: between 2272 and 2330 nm for sample DQ09-0350-04 and between 2370 and 2436 nm for sample DQ09-0350-12. Sample DQ09-0350-13 has totally different spectrum compared to others indicating another composition. This sample has the highest level of use (2-10 kg/T feed).

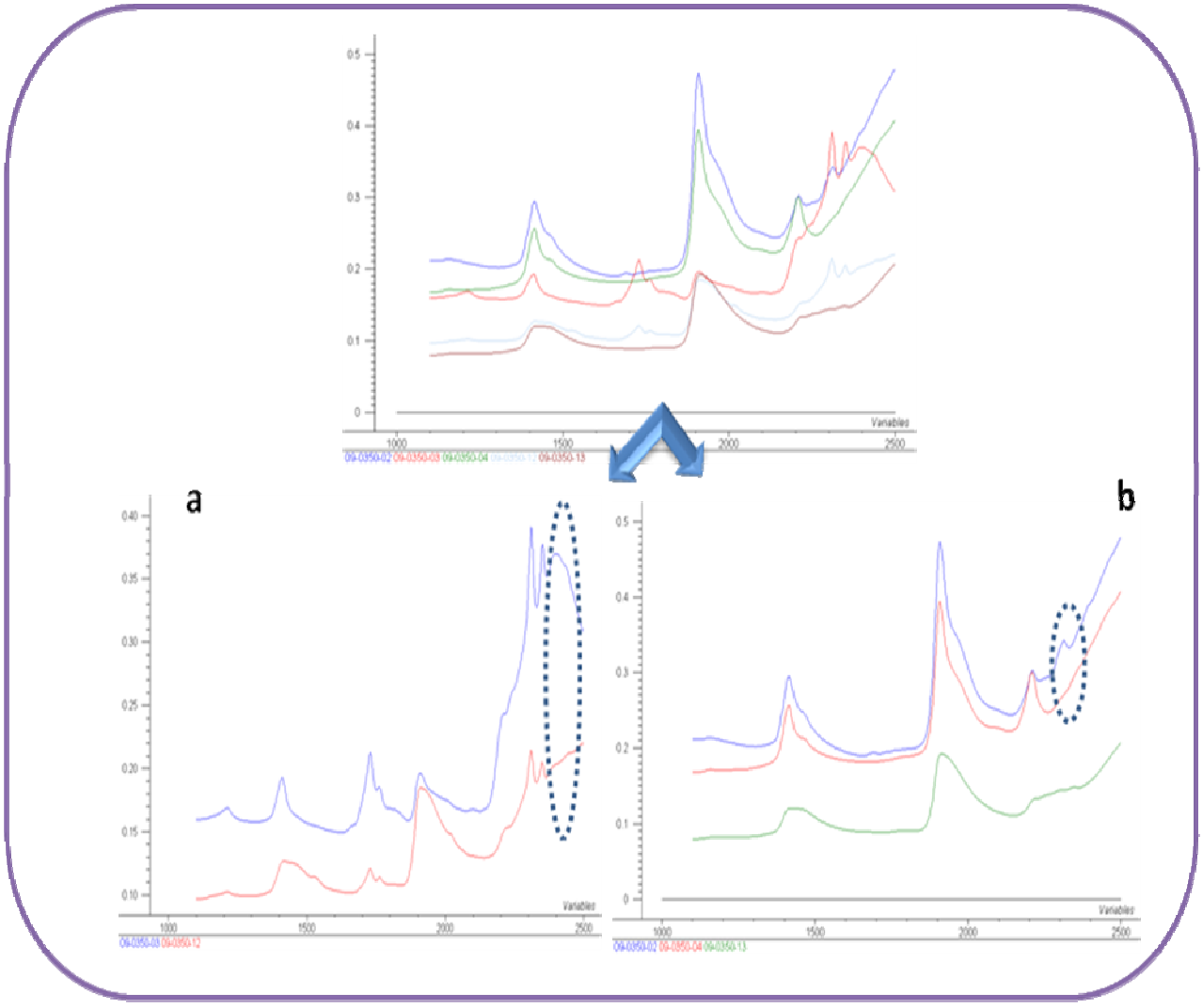

Figure 2: *NIR spectra of clay.*

3.3 Mixture of organic and mineral components

As shown in **figure 3**, spectra of three samples of mixtures of organic and mineral components have the same band between 1860 and 2024 nm but the band of the samples DQ09-0350-11 is slightly shifted and has maximum at 1920 nm while two other spectra have maximum at 1910 nm. The region of the higher wavelengths (> 2024 nm) shows more dissimilarity. Differences in the composition (other proportions or different components) can be assumed.

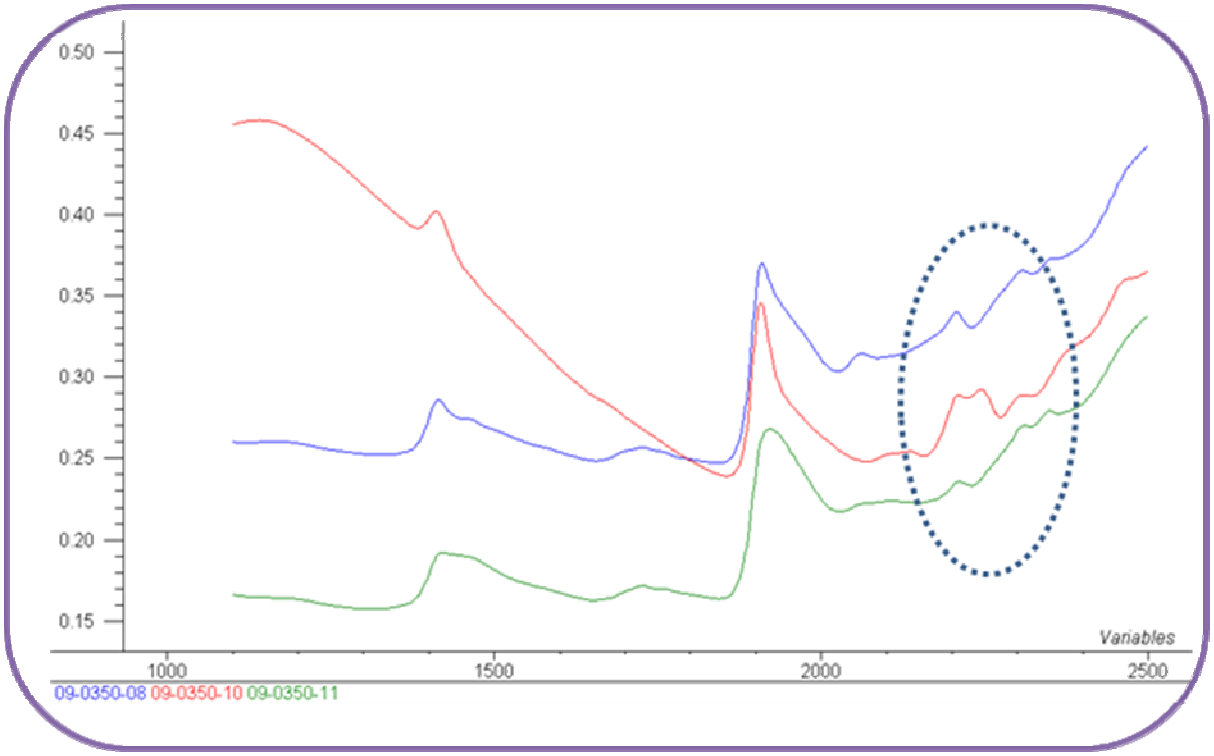

Figure 3: *NIR spectra of mixtures of organic and mineral components.*

3.4 *Montmorillonite*

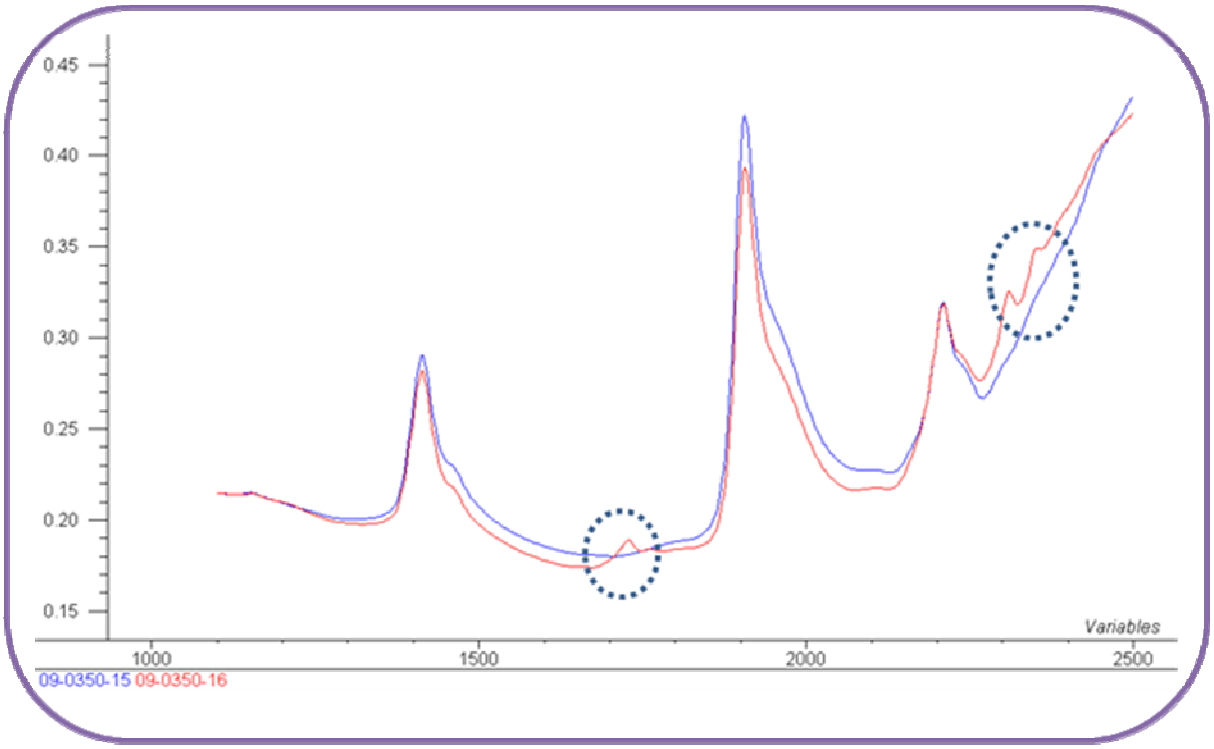

Figure 4: *NIR spectra of montmorillonite.*

Figure 4 indicates that the spectra of two samples of montmorillonite (DQ09-0350-15 and DQ09-0350-16) are different. Differences occur in the regions 1684-1770 nm and 2278-2378 nm.

3.5 Hydrated Sodium Calcium Aluminosilicate (HSCAS)

Spectra of the hydrated sodium calcium aluminosilicate (HSCAS) shown in **figure 5** are very similar. However, for sample DQ09-0350-19, the bands in the region 2164-2230 nm are missing. This compound can be considered as similar to two other samples (DQ09-0350-17 and DQ09-0350-18), with some minor differences.

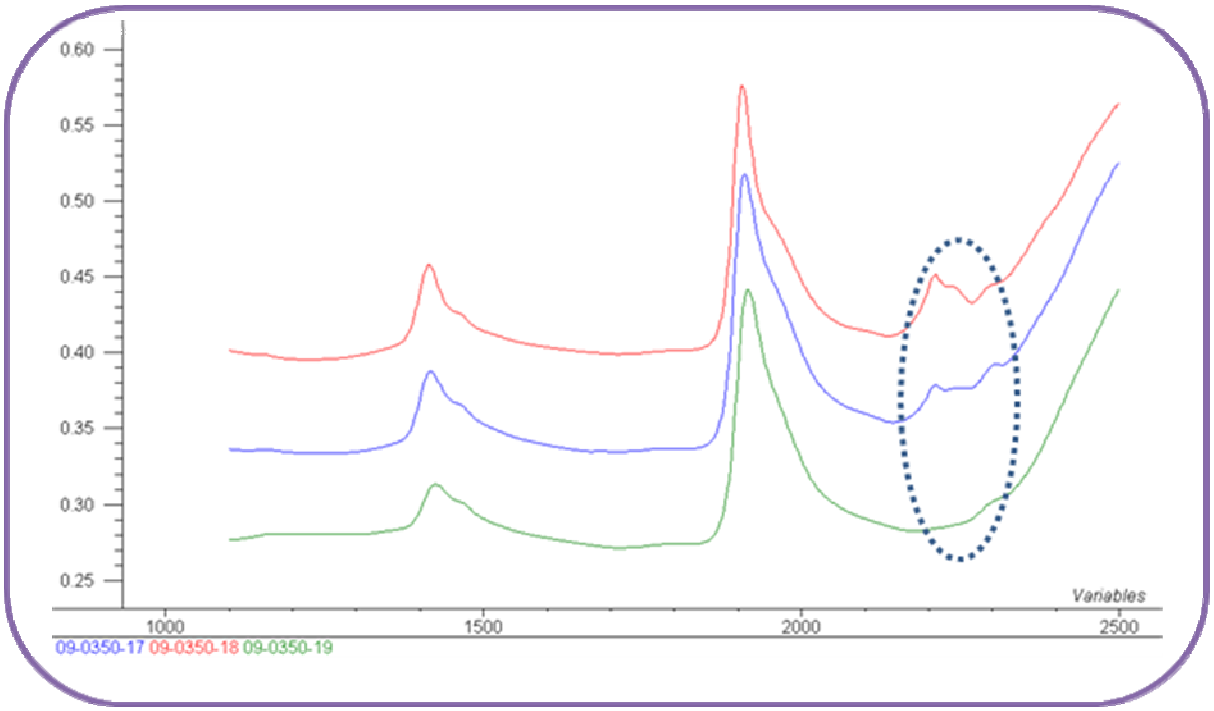

Figure 5: *NIR spectra of hydrated sodium calcium aluminosilicate (HSCAS).*

3.6 Additional reference substances

For those samples, spectra are given without any comparison (**Figure 6**). It can be noted from **figure 6** that each compound has its own spectral profile. Bands present at the appropriate wavelengths inform on the composition of each compound.

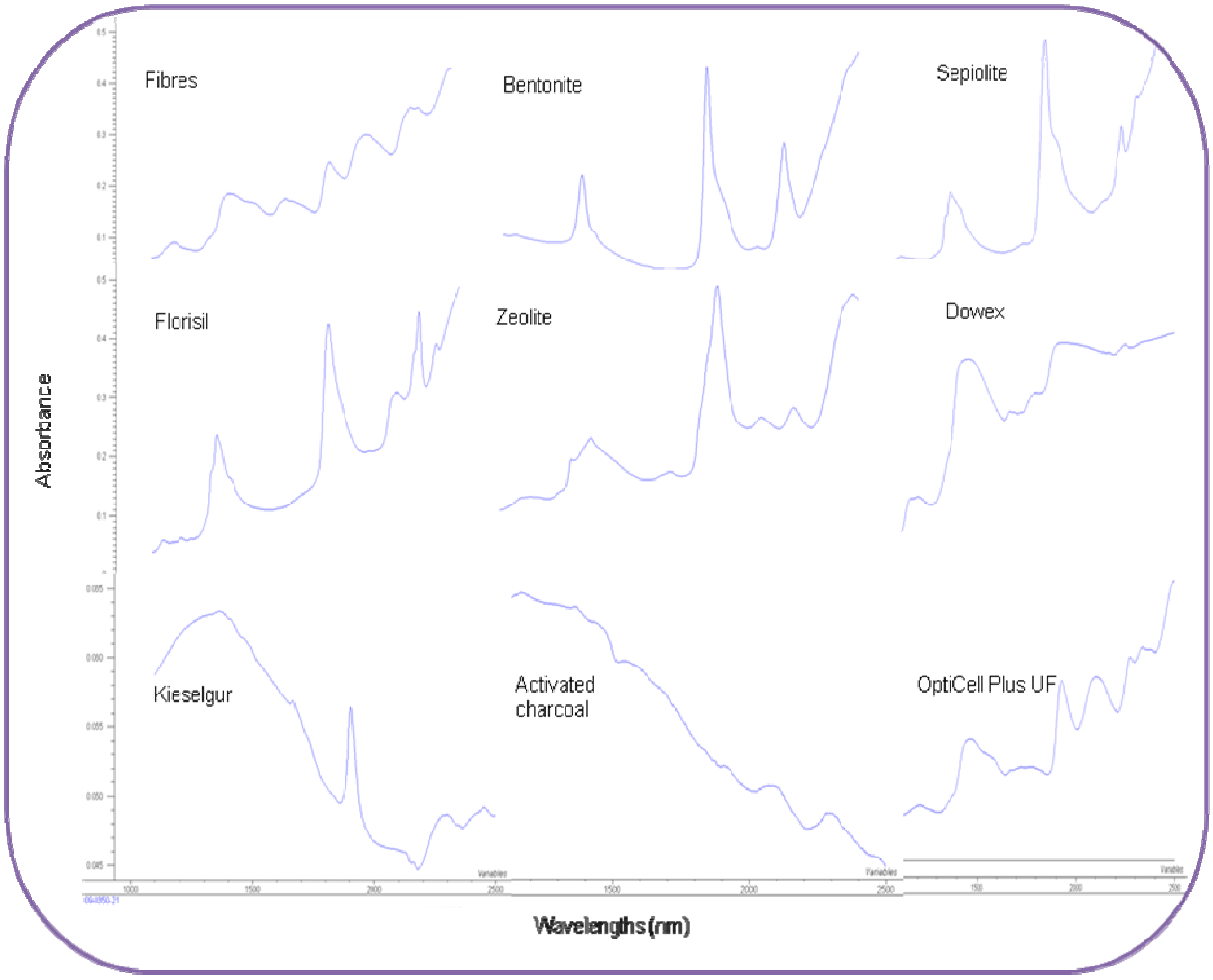

Figure 6: *NIR spectra of the additional reference substances.*

4 Conclusions

Comparison of near infrared spectra of compounds from the same group has shown that some differences can be observed even for the samples from the same family. This indicates some additional components or differences in proportions in the case of mixtures. Near infrared spectroscopy is an effective and rapid tool which can be used for screening purposes and for the evaluation of the composition of a compound or a mixture.

The authors believe that it is interesting to continue the work in the future to estimate the evolution of mycotoxin binding materials in time.

European Commission

EUR 23997 EN – Joint Research Centre – Institute for Reference Materials and Measurements
Title: Evaluation of the Effect of Mycotoxin Binders in Animal Feed on the Analytical Performance of Standardised Methods for the Determination of Mycotoxins in Feed
Author(s): A. Kolossova, J. Stroka, A. Breidbach, K. Kroeger, M. Ambrosio, K. Bouten, F. Ulberth

Luxembourg: Office for Official Publications of the European Communities
2009 – 46 pp. – 21.0 x 29.7 cm
EUR – Scientific and Technical Research series – ISSN 1018-5593
ISBN 978-92-79-13106-6
DOI 10.2787/15352

Abstract
The last few years have brought a vast amount of information about mycotoxin inactivation agents (mycotoxin binders) which are applied with the aim to reduce the toxic effects of mycotoxins in animals. The influence of the addition of mycotoxin binders to animal feed on the analytical performance of the methods for the determination of mycotoxins was studied and the results are presented in this report. Standardised methods already available or currently under consideration at the European Standardization Committee (CEN) have been applied for the analysis of mycotoxins in feed materials. Samples of 20 commercial mycotoxin inactivation agents were collected from various companies. The following mycotoxins were included in the study: aflatoxin B1, deoxynivalenol, zearalenone, ochratoxin A, fumonisins B1 + B2, T2 and HT2 toxins. Naturally contaminated or spiked feed materials and the maximum recommended amounts of the mycotoxin detoxifying agents were used in the experiments. A binder (or binders combined in a group) was mixed with feed material containing the corresponding mycotoxin, and the feed material with and without binder was analysed using the appropriate method. For data evaluation, the obtained mean values were compared by Student's *t*-test (independent two-sample *t*-test with unequal sample sizes and equal variance). The repeatability standard deviation of each method based on collaborative trial data was used as an estimate of method variability. No significant differences ($p = 0.05$) in mycotoxin levels between binder free material and the material containing different binders have been found under the applied conditions.

The mission of the JRC is to provide customer-driven scientific and technical support for the conception, development, implementation and monitoring of EU policies. As a service of the European Commission, the JRC functions as a reference centre of science and technology for the Union. Close to the policy-making process, it serves the common interest of the Member States, while being independent of special interests, whether private or national.

LA-NA-23997-EN-C

JRC
EUROPEAN COMMISSION

ISBN 978-92-79-13106-6

Publications Office
Publications.europa.eu