EUROPEAN COMMISSION
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Standards for Food Bioscience
European Union Reference Laboratory for Feed Additive - Authorisation

JRC.D.5/SFB/CvH/GDA/mds/Ares

EURL Evaluation Report on the Analytical Methods submitted in connection with the Application for Authorisation of a Feed Additive according to Regulation (EC) No 1831/2003

Toxfin® Dry
(FAD-2011-0002; CRL/100361)

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Addendum to the EURL report FAD-2011-0002 JRC.DG.D6/CvH/ZE/mds/ARES(2011)1265818

Background

The Commission forwarded to the EURL supplementary information provided by Kemin Industries related to the characterisation of the Aflatoxin B1 (AfB1) binding capacity of *Toxfin® Dry* to be later specified in the registry entry. The Applicant applied the same experimental method submitted in the frame of FAD 2010-0018 [1], based on the publication by Vekiru *et al.* [2]. The EURL evaluated the suitability of this method.

Evaluation of the analytical method for the determination of the AfB1-binding capacity of Toxfin® Dry

A *Toxfin® Dry* sample (100 mg) was added to 10 mL acetate buffer solution (pH 5); the solution was stirred to homogenise. An aliquot (100 µl) was then added to 5 mL of the acetate buffer containing 4 mg AfB1/L. The mixture was first shaken and incubated for 1 hour at 37 °C, then centrifuged for 5 min. The supernatant (1 mL) was derivatised with 100 µL of trifluoracetic acid (TFA), incubated for 60 minutes at 60 ºC and injected in a high performance liquid chromatograph with fluorescence detection (HPLC-FLD); measurement were performed with an excitation wavelength of 365 nm and an emission wavelength of 440 nm.

According to the Applicant [3], the binding capacity (BC_{AfB1}) is derived from the difference in mycotoxin concentration in the blank solution which contained the initial AfB1 concentration without binder (B) and the supernatant of the incubated binder sample (A). A relative BC_{AfB1} can then be computed as

$$BC_{AfB1}\ \% = (1-A/B)*100$$

The Applicant reported that the limit of detection of the HPLC-FLD method was of the order of 10 µg AfB1/L. The experimental results show satisfactory precisions (a maximum relative standard deviation for intermediate precision of 2.1%) and binding capacities above 83%. The precision was in agreement with the data provided in the frame of FAD 2010-0018 [4].

Based on the experimental evidence presented, the EURL considers the method evaluated in this addendum suitable for determining *AfB1*-binding capacities and confirming the minimum value of 80% proposed by the applicant for *Toxfin® Dry*.

Recommended text for the registry entry (analytical method)

For the determination of the *AfB1-binding capacity*:

> Adsorption test carried out in a buffer solution at pH 5.0 with a concentration of 4 mg/L for AfB1 and 0.02 % (w/v) for the feed additive

Addendum
- Prepared by Gilda D'Arco and Piotr Robouch
- Reviewed and approved by Christoph von Holst (EURL-FA)
Geel, 17/09/2013

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[1] FAD-2010-0018: Supplementary Information – Standard-Operating-Procedure-Binding-Capacity.pdf
[2] E. Vekiru *et al.* Mycotoxin Research 23 (2007) 27-33
[3] FAD 2011-0002: Supplementary Information –Evaluation-of-Aflatoxin-B1-Adsorption
[4] FAD-2010-0018: Supplementary Information – Validation-Report-Binding-Capacity.pdf

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JRC.DG.D.6/CvH/ZE/mds/ARES(2011)1265818

EURL Evaluation Report on the Analytical Methods submitted in connection with the Application for the Authorisation of Feed Additives according to Regulation (EC) No 1831/2003

Dossier related to:	FAD-2011-0002 CRL/100361
Feed additive:	Preparation of Bentonite-Montmorillonite and Sepiolite
Active Substance(s):	- Bentonite-Montmorillonite - Sepiolite
Rapporteur Laboratory:	European Union Reference Laboratory for Feed Additives (EURL-FA) Geel, Belgium
Report prepared by:	Zigmas Ezerskis and Roberto Molteni (EURL-FA)
Report revised by: Date:	Piotr Robouch (EURL-FA) 23/11/2011
Report approved by: Date:	Christoph von Holst 23/11/2011

EXECUTIVE SUMMARY

In the current application authorisation is sought under article 4(1) for *Toxfin®Dry* preparation as *feed additive* under the category "technological feed additives", functional group 1(m) "substances for the reduction of the contamination of feed by mycotoxins" according to the classification system of Annex I of Regulation (EC) No 1831/2003. The authorisation is sought to use the preparation for all animal species and categories.

Toxfin®Dry is a preparation/mixture of two clays consisting of 56 % of *bentonite-montmorillonite* (E558) and 44 % of *sepiolite* (E562). The *feed additive* is intended to be included in *premixtures* or added into *feedingstuffs*. The Applicant suggested an inclusion level of *Toxfin®Dry* preparation in complete *feedingstuffs* ranging from 1 to 20 g/kg.

For the determination of the mineralogical and geological parameters of the individual clays (i.e. *bentonite-montmorillonite* or *sepiolite*) or of the product/preparation (*Toxfin®Dry*) the Applicant submitted X-Ray Diffraction (XRD) and X-Ray Fluorescence (XRF), for elemental analysis. The Applicant analysed five batches of *bentonite*, *sepiolite* and *Toxfin®Dry*. The experimental values obtained for *Toxfin®Dry* samples are in good agreement with the expected/calculated ones, derived from the *bentonite* and *sepiolite* average values.

Based on the experimental evidence presented the EURL recommends for official control X-ray diffraction (XRD) together with X-Ray Fluorescence (XRF) to characterise the preparation *bentonite-montmorillonite*/*sepiolite* (*Toxfin®Dry*).

Furthermore, the Applicant performed a liquid/solid extraction experiment to assess the *Toxfin®Dry* binding capacity to Aflatoxin B1 (AfB1). The experimental results indicate that at least 19 mg AfB1 are adsorbed when 1 g *Toxfin®Dry* is added to a 1 L solution containing 20 mg AfB1. Similar results were obtained by a second independent laboratory in the frame of a verification study. Based on the experimental evidence presented, the EURL considers the method submitted by the Applicant fit for the determination of the *Toxfin®Dry* capacity to bind AfB1.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

KEYWORDS

Toxfin®Dry, bentonite-montmorillonite, sepiolite, technological additives, substances for the reduction of the contamination of feed by mycotoxins, all animal species and categories.

1. BACKGROUND

In the current application authorisation is sought under article 4(1) (new authorisation) for *Toxfin®Dry* preparation as *feed additive* under the category "technological feed additives", functional group 1(m) "substances for the reduction of the contamination of feed by mycotoxins" according to the classification system of Annex I of Regulation (EC) No 1831/2003 [1,2]. The authorisation is sought to use the preparation for all animal species and categories [1].

Toxfin®Dry is a preparation of two clays consisting of 56 % of *bentonite-montmorillonite* (E558) and 44 % of *sepiolite* (E562) [3].

The *feed additive* is intended to be included in *premixtures* or added into *feedingstuffs*. The Applicant suggested an inclusion level of *Toxfin®Dry* preparation in complete *feedingstuffs* ranging from 1 to 20 g/kg [2].

2. TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009, on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the European Union Reference Laboratory concerning applications for authorisations of feed additives, the EURL is requested to submit a full evaluation report to the European Food Safety Authority for each application or group of applications. For this particular dossier, the methods of analysis submitted in connection with *Toxfin®Dry* (Preparation of *Bentonite-Montmorillonite* and *Sepiolite*) and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

3. EVALUATION

Identification/Characterisation of the feed additive

Quantitative and quantitative composition of impurities in the additive

When required by EU legislation, analytical methods for official control of undesirable substances in the additive (such as arsenic, cadmium, lead, mercury, dioxins, microbiological agents and mycotoxins) are available from the respective European Union Reference Laboratories [4].

Description of the analytical methods for the determination of the active agents in the feed additive, premixtures and feedingstuffs

For the determination of the mineralogical and geological parameters of the individual clays (i.e. *bentonite-montmorillonite* or *sepiolite*) or of the product/preparation (*Toxfin® Dry*) the Applicant submitted two analytical methods [5]:

- X-Ray Diffraction (XRD) and

- X-Ray Fluorescence (XRF), using the electron probe micro analysis (EPMA), for elemental analysis.

Bentonite and *Sepiolite* are identified comparing the XRD pattern to the reference patterns published by the International Centre for Diffraction Database. Furthermore, the Applicant provided, upon request of the EURL, the characteristic X-Ray Diffraction pattern of the *Toxfin®Dry* preparation [6].

The electron micro probe analyzer (EMPA) is an analytical tool used for the non-destructive chemical composition analysis. The *bentonite*, *sepiolite* and *Toxfin®Dry* samples are bombarded with a 20keV electron beam, emitting X-rays at wavelengths characteristic to the elements being analyzed. The following mineralogical constituents are identified from the X-ray spectra: Na_2O, MgO, Al_2O_3, SiO_2, K_2O, CaO and FeO [5].

The Applicant analysed five batches of *bentonite*, *sepiolite* and *Toxfin®Dry*. The elemental concentrations obtained are summarised in Table 1. The experimental values obtained for *Toxfin®Dry* samples are in good agreement with the expected/calculated ones, derived from the *bentonite* and *sepiolite* average values.

Based on the experimental evidence presented the EURL recommends for official control X-ray diffraction (XRD) together with X-Ray Fluorescence (XRF) to characterise the preparation *bentonite-montmorillonite*/*sepiolite* (*Toxfin®Dry*).

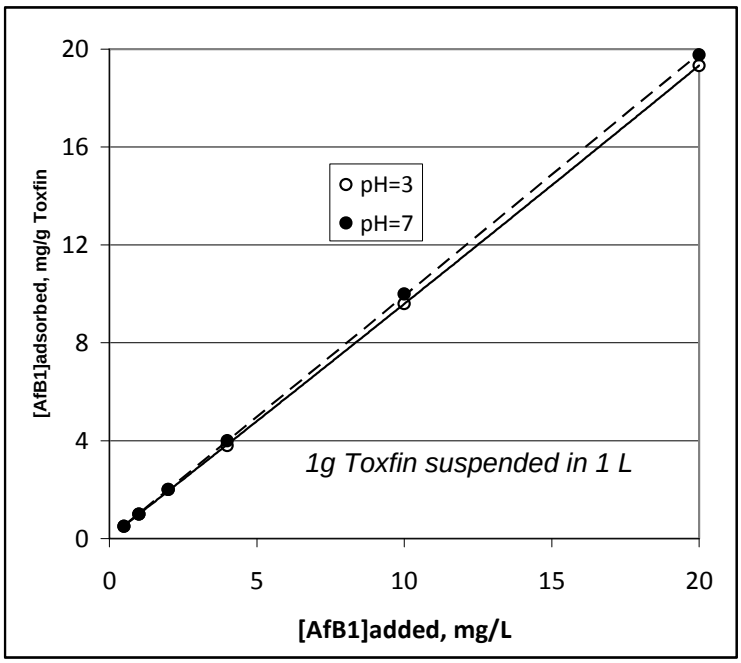
Table 1: Elemental composition of *bentonite, sepiolite* and *Toxfin®Dry* [5], expressed as average ± standard deviation

	Experimental			Calculated
	Bentonite	Sepiolite	Toxfin	Toxfin
Na_2O (%)	3.2 ± 0.4	--	1.9 ± 0.1	1.8
MgO (%)	3.9 ± 0.3	23.1 ± 1.3	13.7 ± 1.0	12.5
Al_2O_3 (%)	19.1 ± 0.3	6.5 ± 1.2	13.4 ± 0.5	13.4
SiO_2 (%)	60.2 ± 0.4	62.3 ± 0.5	60.3 ± 0.6	61.2
K_2O (%)	0.9 ± 0.1	1.6 ± 0.3	1.1 ± 0.1	1.2
CaO (%)	5.2 ± 0.8	3.9 ± 0.7	4.2 ± 0.5	4.6
FeO (%)	5.8 ± 0.3	2.2 ± 0.5	4.5 ± 0.4	4.2

Furthermore, the Applicant performed a liquid/solid extraction experiment [7] to assess the *Toxfin®Dry* binding capacity to AfB1. One gram of *Toxfin®Dry* was added to several 1 L buffered solutions (0.1 M citrate buffer at pH 3 or 0.1 M phosphate buffer at pH 7) containing various amounts of AfB1 in acetonitrile ranging from 0.5 to 20 mg/L. The mixtures were incubated for 90 min at 37°C under continuous shaking. After centrifugation, aliquots of supernatants were submitted to derivatisation with trifluoroacetic acid (TFA), properly diluted and injected in a high performance liquid chromatograph with fluorescence detection (excitation wavelength - 365 nm; emission wavelength - 440 nm). The Applicant reported a limit of detection of 0.05 mg AfB1/L. The same experimental protocol was applied to solutions without *Toxfin®Dry*. The adsorption capacity - expressed as mg AfB1/g *Toxfin®Dry* - is derived from the AfB1 contents in the supernatants in the presence/absence of *Toxfin®Dry*. The experimental results [8] shown in Figure 1 indicate that at least 19 mg AfB1 are adsorbed when 1 g *Toxfin®Dry* is added to a 1 L solution containing 20 mg AfB1. Similar results were obtained by a second independent laboratory in the frame of a verification study [9].

Based on the experimental evidence presented, the EURL considers the method submitted by the Applicant fit for the determination of the *Toxfin®Dry* capacity to bind AfB1.

Figure 1: AfB1 adsorbed by 1 g *Toxfin®Dry* suspended in 1 L solution containing increasing amounts of AfB1 added



The Applicant is aware that the direct determination of *Toxfin®Dry* preparation added to *premixtures* or *feedingstuffs* is not achievable by analysis [3] and provided no experimental data. Therefore the EURL cannot evaluate nor recommend any method for official control to determine *Toxfin®Dry* preparation in *premixtures* and *feedingstuffs*.

Further testing or validation of the methods to be performed through the consortium of National Reference Laboratories as specified by Article 10 (Commission Regulation (EC) No 378/2005) is not considered necessary.

4. CONCLUSIONS AND RECOMMENDATIONS

In the frame of this authorisation the EURL recommends for official control X-ray diffraction (XRD) together with X-Ray Fluorescence (XRF) to characterise the preparation *bentonite-montmorillonite/sepiolite* (*Toxfin®Dry*).

The Applicant did not provide any experimental method or data for the determination of *Toxfin®Dry* preparation in *premixtures* and *feedingstuffs*. Therefore the EURL cannot evaluate nor recommend any method for official control to determine *Toxfin®Dry* preparation in *premixtures* and *feedingstuffs*.

Recommended text for the register entry (analytical method)

For the characterisation of the preparation *bentonite-montmorillonite/sepiolite* in the feed additives:

- X-Ray Fluorescence (XRF) together with
- X-ray Diffraction (XRD)

5. DOCUMENTATION AND SAMPLES PROVIDED TO EURL

In accordance with the requirements of Regulation (EC) No 1831/2003, reference samples of *Toxfin® Dry* have been sent to the European Union Reference Laboratory for Feed Additives. The dossier has been made available to the EURL by EFSA.

6. REFERENCES

[1] *Application, Reference SANCO/D/2 Forw. Appl. 1831/0002 (10529)-2011
[2] *Application, Proposal for Register Entry – Annex A
[3] *Technical dossier, Section II: Identity, characterisation and conditions of use of the additive; Methods of analysis
[4] Commission Regulation (EC) No 776/2006 amending Annex VII to Regulation (EC) No 882/2004 of the European Parliament and of the Council as regards to Community Reference Laboratories
[5] *Supplementary information: XRD and EPMA methods, data
[6] *Supplementary information, Qualitative analysis of Toxfin®Dry
[7] *Technical dossier, Section II - Annex II_14
[8] *Supplementary information, AFB1 binding efficacy study
[9] *Supplementary information, ISPA Final Report

*Refers to Dossier No. FAD-2011-0002

7. RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was European Union Reference Laboratory for Feed Additives, IRMM, Geel, Belgium. This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005, as last amended by Regulation (EC) No 885/2009.

8. ACKNOWLEDGEMENTS

The following National Reference Laboratories contributed to this report:

- Plantedirektoratet, Laboratorium for Foder og Gødning, Lyngby (DK)
- Centro di referenza nazionale per la sorveglianza ed il controllo degli alimenti per gli animali (CReAA), Torino (IT)
- RIKILT - Instituut voor Voedselveiligheid, Wageningen (NL)
- Państwowy Instytut Weterynaryjny, Puławy (PL)
- Ústřední kontrolní a zkušební ústav zemědělský (ÚKZÚZ), Praha (CZ)
- Instytut Zootechniki w Krakowie, Krajowe Laboratorium Pasz, Lublin (PL)
- Thüringer Landesanstalt für Landwirtschaft (TLL), Abteilung Untersuchungswesen, Jena (DE)
- Laboratoire de Rennes, SCL L35, Service Commun des Laboratoires, Rennes (FR)