

*GMP+ Feed Safety Assurance scheme*

## **Guidelines and acceptance criteria for analysis methods Mycotoxins (DON, ZEN and OTA) in raw material(s) for animal feed**

**D**  
**4.11**

### **GMP+ D4.11**

**EN**

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# 1 Introduction

## 1.1 General

The GMP+ Feed Safety Assurance scheme (GMP+ FSA) has been developed since 1992. It was managed from 1992 up until 2009 by the Product Board Animal Feed, The Hague, The Netherlands. Since 2010, this scheme is managed by GMP+ International.

It is a certification scheme for assuring feed safety in all the links in the feed chain. It is also an international scheme, applicable worldwide.

The establishment and development of the scheme was primarily the result of demand from the subsequent links in the animal production chain for better control of feed safety. Another contributory factor was the damage caused by more and less serious contamination incidents.

In the initial phase the demand arose for better differentiation in an increasingly saturated European sales market for animal products. Since 1999, feed & food safety has been a top issue internationally both politically and commercially, because of serious incidents in the feed sector. Because of this, demonstrable assurance of feed safety has become a license to sale.

The basic principle of the GMP+ FSA scheme is that the feed chain is part of the food production chain. Proper assurance of feed safety worldwide is a high priority. Companies must live up to their responsibilities and respond properly and convincingly to the needs of food production chain. The GMP+ Feed Safety Assurance scheme is an aid to realise this.

## 1.2 Structure of the GMP+ Feed Safety Assurance scheme

The documents within the GMP+ FSA scheme are subdivided into a number of series. A description follows of these:

<b>A</b> General (framework) documents	These documents contain the requirements for participation in the certification scheme for companies and certification bodies (framework regulation, the use of logo's, etc.). This series also includes a general list of definitions and abbreviations.
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**B**  
Normative documents.

These documents contain the international standards and additional country notes for use by companies with respect to the various feed products and production phases including cultivation and industrial production, treatment and processing, collection, trade, means of transport, storage and transshipment.

**C**  
Certification requirements

These documents contain the Rules of Certification including those for the approval of certification bodies and auditors, the frequency of audits, minimum audit time, assessment criteria, checklists, etc. There is also an explanation of how the supervision by certification bodies is implemented and of how GMP+ International supervises the certification process.

**D**  
Interpretations and  
accompanying texts

In addition to the above-mentioned normative documents, there are also supporting documents in the D series including a list of frequently-asked questions, manuals and guidances with additional information.

**Document**

**Code**

**Name**

GMP+ D1.1

GMP+ D4.11 Guidelines and acceptance criteria for analyses methods Mycotoxins (DON, ZEN and OTA) in raw material(s) for animal feed

All these documents are available through the website of GMP+ International ([www.gmpplus.org](http://www.gmpplus.org)).

The document in the present case is referred to as standard GMP+ D4.11 *Guidelines and acceptance criteria for analyses methods Mycotoxins (DON, ZEN and OTA) in raw material(s) for animal feed* and is part of the GMP+ FSA scheme. It is not a normative document, but a project in cooperation with the Product Board Animal Feed . In the document you can find the original texts of the report. The information of this project can be used as a guidance for the implementation of the GMP+ FSA norms.

## 2 Preface

This report was prepared by the Group of Experts Analysis Methods Mycotoxins in raw materials for animal feedstuffs. This group of experts was appointed by the Board of Directors of the Product Board Animal Feed in June 2003, after concluding that no uniform guidelines existed for determining the presence of Mycotoxins DON (Deoxynivalenol), OTA (Ochratoxin A) and ZEN (Zearalenon).

In three meetings, the Group of Experts compiled the criteria for confirmation and screening methods for determining the presence of Mycotoxins in raw materials for animal feedstuffs. Additionally, minimum performance criteria were listed and guidelines for determining the presence of Mycotoxins were defined.

Subsequently, the Group of Experts conducted an inventory in order to determine to what extent laboratories that currently conduct Mycotoxins analyses comply with the set criteria.

This report should be regarded as a guideline for the laboratories that intend to determine the presence of Mycotoxins in raw materials for animal feedstuffs. Eventually, a final selection will be conducted after the differences between laboratories have been concluded via ring tests (possibly in a KDLL context).

On behalf of the Group of Experts, I express the hope that this report can contribute to this objective.

Dr. L. Vellenga  
Chairman Group of Experts

### **3 Cause**

After defining the Mycotoxins levels for DON (Deoxynivalenol), OTA (Ochratoxin A) and ZEN (Zearalenon) in various animal feedstuffs on 11 June 2003, the Board of Directors of the Product Board Animal Feed determined that fast, reliable, and if possible, validated analysis methods must become available before the standards are imposed. These standards are part of the GMP scheme. Action and rejection limits for aflatoxin have applied since 1989.

During the same meeting, the Board of Directors of the Product Board Animal Feed agreed to appoint a panel of experts in order to review the analysis methods that are currently available for usability, based on currently available knowledge levels.

## 4 The standards defined by the Board of Directors of the Product Board Animal Feed

The action and rejection limits were determined on a feed intake basis and are applicable for Mycotoxins DON, ZEN and Ochratoxin A. From desktop studies conducted regarding Mycotoxins, it is apparent that the aforementioned Mycotoxins can have a harmful effect on domesticated farm animals. The action and rejection limits for the above-mentioned Mycotoxins will be integrated in the GMP<sup>+</sup> scheme.

Below please find an overview of the standards that apply for the Mycotoxins DON, ZEN and OTA for the various animal feed categories.

The standards for Mycotoxins on feed intake basis are:

		<b>Rejection limit</b>	<b>Action limit</b>
DON			
	Pigs	1.000 µg/kg	800 µg/kg
	Broilers, laying hens and turkeys	4.000 µg/kg	3.200 µg/kg
	Poultry breeders	2.000 µg/kg	1.600 µg/kg
	Calves up to 4 months' old	2.000 µg/kg	1.600 µg/kg
	Dairy cattle	3.000 µg/kg	2.400 µg/kg
	Other cattle	5.000 µg/kg	4.000 µg/kg
Zearalenon			
	Sows / meat-type pig	250 µg/kg	200 µg/kg
	Piglets / Replacement gilt	100 µg/kg	80 µg/kg
	Poultry	no limit	no limit
	Dairy cattle /heifers / calves	500 µg/kg	400 µg/kg
	Beef cattle	no limit	no limit
Ochratoxin A			
	Sows / meat-type pigs / piglets	50 µg/kg	40 µg/kg
	Poultry	200 µg/kg	160 µg/kg
	Ruminants	no limit	no limit

The proposal to the Board of Directors of the Product Board Animal Feed is based on limits at feed intake level. Therefore, it was relevant to determine how to deal with single feedstuffs. For single feedstuffs, a limit of 3 times the limits for end feedstuffs (on feed intake basis) applies, provided that the result is accompanied by an alerting and feeding advice.

## **5 Task of the Group of Experts**

As indicated above, the Board of Directors of the Product Board Animal Feed approved the appointment of a group of experts to conduct an inventory of the availability of methods and their usability and validation. Additionally, this group of experts would conduct an inventory of the need for new methods.

On a more practical level, the group was to make an inventory of the following data regarding confirmation and screening methods:

### **Confirmation methods**

- Reviewing the confirmation methods that are currently available, based on the conditions as described in Decision 2002/657/EG) for Mycotoxins regarding accuracy, reproducibility, repeatability, validation (regarding matrices), recovery, speed, complexity, cost level.
- Determine which methods may be used reliably for which Mycotoxins in which matrices, based on the aforementioned review, and determine if any additional research is required.

### **Rapid testing**

- Reviewing the rapid testing / screening methods that are currently available, determining their practical usability, reliability, speed and cost level, and for which Mycotoxins these are suitable.
- Determine whether rapid testing methods are available for all Mycotoxins and which would still need to be developed; and outlining the technical perspective for the methods to be developed.

## 6 Working method

In order to fulfil its task, the panel of experts used the available literature and the expertise of the panel members in order to review the analysis methods (both confirmation tests and screening methods), and tested these against the following performance criteria:

Regarding confirmation tests:

- Repeatability
- Reproducibility within laboratory
- Reproducibility between laboratories
- Recovery

For screening methods

- Sensitivity
- Specificity

Before conducting such a review, uniform definitions / descriptions of these criteria must be prepared. Where possible, CEN/ISO reports and other sources were consulted. The same applied to preparing the analysis instructions for defining DON, ZEN and OTA.

The results / conclusions / recommendations shall be submitted to the Steering Group Analysis Issues Animal Feed Sector and the Committee Quality Policy Animal Feed Sector with a request for advice before submitting same for approval of the Board of Directors of the Product Board Animal Feed.

## 7 Results

### 7.1 Criteria/ parameters for confirmation methods

The CEN-CR 13505:1999 document (**Appendix 1**) indicates the parameters that are relevant for detecting Mycotoxins. More specifically, this includes parameters for confirmation methods. Below, the definitions and/or specific characteristic of these parameters are listed.

#### 7.1.1 Definitions/description of criteria and parameters

- **Minimum determinability limit**  
This is half the lowest action limit.
- **Repeatability**  
Repeatability,  $RSD_r$  (Relative Standard Deviation within laboratories): this precision indicator relates to incorrect results of a method within a laboratory. This is the standard deviation divided by the average of the test results, obtained using the same methods on identical testing material, under the same conditions (same person, equipment, laboratory and within a short period of time) x 100%. The RSD is expressed as a percentage.
- **Reproducibility (between laboratories);**  
Reproducibility,  $RSD_R$  (Relative Standard Deviation between laboratories): this precision indicator relates to incorrect results of a method between laboratories. This is the standard deviation divided by the average of the test results, obtained using the same methods on identical testing material, but under different conditions (other person, equipment, laboratory and other times) x 100%. The  $RSD_R$  is expressed as a percentage.
- **Reproducibility (within laboratories)**  
Reproducibility  $RSD_{RL}$ : The match of measuring results of the same measuring units, obtained under varying conditions. Varying conditions may include: other observers, measuring instruments, reference standards, places, conditions of use and times.  $RSD_{RL}$  is expressed as a percentage.
- **Extraction method**  
The Mycotoxins DON, ZEN and OTA require (preferably) the use of acetonitril/water. However, water/PEG may be used for DON.  
Pre-treatment with chloroform or dichloromethane is necessary for starchy products or substances with high fat content.
- **Clean up;**  
In order to pre-treat the sample extract in the correct manner, IAC (Immuno-affinity chromatography) must preferably be used for the Mycotoxins DON, OTA and ZEN .

- **Separation / detection**  
The HPLC (High Pressure Liquid Chromatography) method should preferably be used in combination with fluorescence detection or UV for the separation and detection of the Mycotoxins DON, OTA and ZEN. For DON, GC (Gas Chromatography) can also be used in combination with ECD (Electron Capture Detection). Separation and detection is also possible using LC/MS-MS.
- **Detection limit;**  
The detection limit must amount to at least half the minimum determinability limit (see also the definition of minimum determinability limit).
- **Measuring process;**  
This process should at least range from half the action limit to twice the rejection limit.
- **Recovery;**  
Recovery:  $(\text{Measured concentration material with added Mycotoxin} - \text{measured concentration in blank material}) \times 100\% / (\text{pre-determined raise in concentration})$ . Recovery is expressed as a percentage. The added quantity must at least be a substantial fraction of the quantity present in the blank sample. Ideally, the blank sample should contain less of the component to be analysed than its determinability limit.
- **Correctness**  
This is the concentration measured in certified reference material, divided by the true attributed value  $\times 100\%$ . The true attributed value is known only in cases of naturally contaminated materials, certified reference materials or after analysis with another (presumably accurate) method. The concentration of blank material must be obtained by direct analysis or by using the standard addition. In other cases, if the deviation is unknown, the values of the ring test may serve as a reference point.
- **Uncertainty of measurement;**  
This is the same value as the within laboratory reproducibility (RSD<sub>RL</sub>%). For example, if the uncertainty of measurement amounts to 30%, this means that an analysis result of 1,301 shall lead to rejection of the product if the rejection limit is 1,000.

#### 7.1.2 Performance criteria for confirmation methods

Both the analysis methods for determining presence of the Mycotoxins DON, OTA and ZEN in animal feedstuffs and raw materials for animal feedstuffs and the laboratories intending to conduct these Mycotoxins analyses must comply with the following minimum performance criteria. These performance criteria are calculated based on performance characteristics as described in the CEN CR 13505: 1999 document (see Appendix 1).

The minimum performance criteria as applicable for deoxynivalenol (DON) are:

Level ug/kg	Deoxynivalenol (DON)			Recovery %
	RSD <sub>RL</sub> %	RSD <sub>r</sub> %	RSD <sub>R</sub> , %	
> 200	≤ 30	≤ 20	≤ 40	70-110

The minimum performance criteria as applicable for zearalenone are:

Level ug/kg	Zearalenon			Recovery %
	RSD <sub>RL</sub> %	RSD <sub>r</sub> %	RSD <sub>R</sub> , %	
> 20	≤ 32,5	≤ 25	≤ 40	70-110

The minimum performance criteria as applicable for ochratoxin A are:

Level ug/kg	Ochratoxin A			Recovery %
	RSD <sub>RL</sub> %	RSD <sub>r</sub> %	RSD <sub>R</sub> , %	
> 10	≤ 25	≤ 20	≤ 30	70-110

## 7.2 Criteria/parameters for screening methods

The parameters that are relevant for (rapid) screening methods for determining Mycotoxins in raw materials for animal feedstuffs are listed below, as well as definitions and/or specific characteristics of these parameters.

### 7.2.1 Definitions/description of criteria and parameters

- **Sensitivity;**

Sensitivity indicates the possibility of a positive result being classed as positive by the test. This is a measurement of the number of false negative results. High sensitivity (100%) implies that no false negative results are to be expected.

- **Specificity;**

Specificity indicates the possibility of a negative result being classed as negative by the test. This is a measurement of the number of false positive results. High specificity (100%) implies that no false positive results are to be expected.

## 7.2.2 Performance criteria for screening methods

Both the screening methods for determining presence of the Mycotoxins DON, OTA and ZEN in animal feedstuffs and raw materials for animal feedstuffs and the laboratories intending to conduct these Mycotoxins analyses must comply with the following minimum performance criteria.

The minimum performance criteria as applicable for deoxynivalenol (DON), zearalenone (ZEN) and ochratoxin A (OTA) are:

Level ug/kg	Mycotoxin		
	DON	ZEN	OTA
Sensitivity (measurement for false negative results)	100% when exceeding action limit	100% when exceeding action limit	100% when exceeding action limit
Specificity (measurement for false positive results)	Preferably >95%	Preferably >95%	Preferably >95%

The screening methods for determining the above-mentioned Mycotoxins can only serve to determine a positive or negative result. For positive results, a confirmation (using a confirmation test) will always be required.

## 8 6. Guidelines for Analysis of Mycotoxins in raw materials for animal feedstuffs

### 8.1 Confirmation method

Based on NEN procedures for ZEN and OTA, the group of experts developed a guideline for determining the presence of DON, OTA and ZEN in raw materials for animal feedstuffs. These guidelines were subsequently compared to the standard ISO methods and in-house laboratory methods. The guidelines for determining the presence of the Mycotoxins DON, OTA and ZEN are:

#### General:

1. Do not use untreated glass
2. Prevent fungal growth during sample storage and testing process.

#### Specific:

1. Test should be based on at least 500 grams of sample material. Grind the entire quantity to a grain size of  $\leq 1$  mm and homogenise.
2. Transfer 25 gr of the homogenised sample material to a blender or a measuring flask; add 100 ml acetonitril:water (84:16; V:V). (If any problems with phase separation occur, add 2.5 gr of NaCl). For raw materials with a high fat content, extract with chloroform or acid dichloromethane and re-extract with Na bicarbonate.

Please Note: the above is based on sampling material with less than 20% water. For wet-feed, the acetonitril:water proportions must reflect the water content of the wet-feed.

3. Blend the suspension on high speed during 2 minutes, or agitate for 2 to 3 hours.
4. Filter more than 10 ml over a humid filtration paper into a conical flask.
5. Conduct a Solid Phase Extraction (IAC or SPE).
6. If necessary, vaporise (40-60 degrees Celsius) the eluate until dry under mild nitrogen and dissolve the extract in 2 ml mobile phase.
7. Conduct HPLC by injecting 100  $\mu$ l dissolved extract or less, as much as is needed for achieving proper detection and separation.
8. Detection of the Mycotoxins takes place by means of LC with fluorescence detection using the specific excitation and emission wavelengths for the relevant Mycotoxins, or using LC/MS-MS.

## **8.2 Screening methods**

Regarding the screening methods to be accepted for determining the Mycotoxins DON, OTA and ZEN in raw materials for animal feedstuffs, the ELISA methods that are currently on the market as complete kits can be considered satisfactory for determining the presence of the Mycotoxins DON, OTA and ZEN in single feedstuffs.

When using the screening methods for determining the presence of Mycotoxins in raw materials for animal feedstuffs, it is essential to read and follow the user instructions of the manufacturer.

Additionally, it is important to note that pre-treatment with a non-polar organic solvent is required for starchy products and substances with a high fat content.

## 9 Inventory of the performance criteria of the analysis methods and the laboratories already determining Mycotoxins in raw materials for animal feedstuffs (both confirmation and screening methods)

### 9.1 Confirmation methods

The group of experts conducted an inventory in a number of laboratories that are already determining the presence of Mycotoxins in raw materials for animal feedstuffs.

From this inventory, it was safe to conclude that the laboratories can comply with the minimum performance criteria as listed in paragraph 5.1.2. of this report.

This inventory was based on the following performance characteristics

- **Minimum determinability limit**
- **Reproducibility** (within laboratories) RSD<sub>RL</sub> or uncertainty of measurement
- **Repeatability**, RSD<sub>r</sub>

In addition to compliance with the above-mentioned minimum performance criteria, it is important to know for which matrix the test was used and if a full validation (including ring test, for example KDLL) was conducted in order to assess the analysis methods and the laboratories where the guidelines will be implemented.

**The group of experts feels it is important to place special focus on the ring test, since this may expose any differences between laboratories.**

The laboratories that currently already analyse for Mycotoxins and are able to comply with the minimum criteria as described in paragraph 5.1.2. of this report are:

- **Masterlab, Putten**
- **CCL, Veghel**
- **Labco, Rotterdam**
- **TLR, Rotterdam**
- **RIKILT, Wageningen**
- **RIVM, Bilthoven**
- **TNO, Zeist**
- **Faculteit Diergeneeskunde (*Veterinary Faculty*), Utrecht**
- **Gezondheidsdienst voor Dieren (*Animal Health Service*), Deventer**

### 9.2 Screening methods

There are several reasons why rapid (online) screening methods are desirable and are currently applied. The practical usability (online applications), the rapid availability of the analysis results and the costs of the test kit are important aspects.

However, if the speed (direct measuring result) and the practical applicability (user friendliness) of a test kit comply with requirements, people are, in general, inclined to feel satisfied. If the test kit is also on a par with confirmation methods with respect to price, this forms another reason to apply screening methods.

The group of experts is of the opinion that, in addition to the above-mentioned aspects, methods should comply with the requirement that the test kit should give virtually no false negative results. In other words, the test kit must have a high level of sensitivity.

Additionally, from a cost perspective, it is desirable for a screening method to give as few false positive results as possible, since confirmation tests are necessary after each positive result. The group of experts is of the opinion that a test kit with low specificity (with a high number of false positive results) is therefore too expensive.

The (ELISA) test kits that are currently on the market and comply with the above-mentioned criteria are available from:

- Euro-Diagnostica B.V.  
Beijerinckweg 18  
6827 BN Arnhem  
Tel 026 3630364
- R-Biopharm  
Landwehrstrasse 54  
D-64293 Darmstadt  
Germany  
Tel 00 49 6151 80 10 20

## 10 Conclusions, recommendations

Based on the questions that the Product Board Animal Feed handed to the group of experts, and based on the results of the research conducted by the group of experts, the following conclusions were made:

- Guidelines for determining the presence of Mycotoxins (DON, OTA and ZEN) in raw materials for animal feedstuffs were prepared for both confirmation tests and screening methods. This enables laboratories to conduct these analyses.  
For the confirmation methods to be used and for determining the presence of Mycotoxins, the group of experts concludes that HPLC (High Pressure Liquid Chromatography) methods combined with fluorescence detection or UV can be used for the separation and detection of the Mycotoxins DON, OTA and ZEN; or alternatively GC (Gas Chromatography) in combination with ECD (Electron Capture Detection) (for DON). Separation and detection is also possible using LC/MS-MS.
- For guidelines regarding the use of the screening methods, the group of experts refer to the user instructions of the ELISA test kits that are currently on the market.
- From the inventory of laboratories that currently conduct Mycotoxins analyses, it is safe to conclude that all laboratories included in the inventory comply with the minimum performance criteria that apply to confirmation methods.
- Screening methods are available in the Netherlands and Germany that comply with the requirements.
- Based on, and in compliance with, the guidelines as proposed in this report, analysis results regarding the Mycotoxins DON, OTA and ZEN in raw materials for animal feedstuffs can be obtained with satisfactory reliability.
- The reproducibility between laboratories can only be established after conducting a ring test (for example via KDLL). Ring test data are essential for full validation.

The group of experts would like to make the following recommendations:

- In order to obtain reproducibility data between laboratories that are necessary for full validation, a ring test (for example via KDLL) should be launched in the near future (in 2004) among laboratories compliant with the minimum performance criteria.
- The uncertainty of measurement of the analysis results must be calculated in order to enable calculating the level of exceeding the action and/or rejection limits.

## 11 Summary

In June 2003, the Board of Directors of the Product Board Animal Feed defined standards for the Mycotoxins DON (Deoxynivalenol), OTA (Ochratoxin A) and ZEN (Zearalenon) in various animal feedstuffs. The Board indicated that, before implementing these standards, fast, reliable, and (if possible) validated analysis methods had to become available.

At the Board of Directors' proposal, a group of experts was appointed in order to define minimum performance criteria for both confirmation and screening methods (paragraphs 5.1.2 and 5.2.2).

Guidelines for determining the presence of the Mycotoxins DON, OTA and ZEN in raw materials for animal feedstuffs via both confirmation and screening methods were prepared (paragraphs 5.1 and 5.2). For the confirmation methods to be used and for determining the presence of Mycotoxins, the group of experts indicates that HPLC (High Pressure Liquid Chromatography) methods combined with fluorescence detection or UV can be used for the separation and detection of the Mycotoxins DON, OTA and ZEN; or alternatively GC (Gas Chromatography) in combination with ECD (Electron Capture Detection) (for DON). Separation and detection is also possible using LC/MS-MS.

For guidelines regarding the use of the screening methods, the group of experts refer to the user instructions of the ELISA test kits that are currently on the market.

Subsequently, the group of experts conducted an inventory in order to determine to what extent the laboratories that currently conduct Mycotoxins analyses comply with the minimum performance criteria that apply to confirmation methods (paragraph 7). It is apparent that all laboratories included in the inventory comply with the minimum performance criteria that apply to confirmation methods.

Screening methods are available in the Netherlands and Germany that comply with the requirements.

The group of experts concluded that, based on, and in compliance with, the guidelines as proposed in this report, analysis results regarding the Mycotoxins DON, OTA and ZEN in raw materials for animal feedstuffs can be obtained with satisfactory reliability. However, the group wishes to note that reproducibility between laboratories can only be established after conducting a ring test (for example via KDLL). Ring test data are essential for full validation.

Additionally, the group of experts recommends for the uncertainty of measurement of the analysis results to be calculated in order to enable calculating the level of exceeding the action and/or rejection limits.

## **Sources and literature**

- CEN European Committee for Standardization; CR13505:1999; Food Analysis- Biotoxins- Criteria of analytical methods of mycotoxins
- Martin, S.W., Meek, A.H., and Willenberg, P. 1987. Veterinary Epidemiology: principles and methods. Iowa State University Press, Ames
- NEN 7779:2003; Milieu – onzekerheid van meetresultaten (*Environment – uncertainty of measurement*)

## **Appendix 1: CR 13505: 1999 Food analysis – Biotoxins - Criteria of analytical methods of mycotoxins**

This Appendix (5 MB) can be sent per post on request.

## The group of experts consists of:

The following persons were members of the group of experts Analysis Methods Mycotoxins:

- Dr. Cor Arts, Arts Project Support, 's Hertogenbosch
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