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ANNEX

ANNEX

to the

COMMISSION IMPLEMENTING REGULATION

amending Regulation (EC) No 401/2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.

ANNEX I

Annex I to Regulation (EC) No 401/2006 is amended as follows:

(1) The introductory sentence in part 'A. GENERAL PROVISIONS' is replaced by the following:

" Official controls shall be performed in accordance with the provisions of Regulation (EU) 2017/625. The following general provisions shall apply without prejudice to the provisions in Regulation (EU) No 2017/625"

(2) The part "A.1. Purpose and Scope" is replaced by the following

"A.1 Purpose and scope

Samples intended for official control of the levels of mycotoxins content in foodstuffs shall be taken according to the methods set out in this Annex. Aggregate samples thus obtained shall be considered as representative of the lots. Compliance with maximum limits laid down in Regulation (EC) No 1881/2006 shall be established on the basis of the levels determined in the laboratory samples.

To ensure compliance with the provisions in Article 4 of Regulation (EC) No 852/2004, food business operator shall, when samples are taken to control the levels of mycotoxins take the samples according to the methods described in this Annex or apply an equivalent sampling procedure which is demonstrated to have a same level of representativeness as the sampling procedure described in this Annex."

(3) The title of part B is replaced by "B. METHOD OF SAMPLING FOR CEREALS, OILSEEDS OTHER THAN GROUNDNUTS AND CEREAL PRODUCTS"

(4) The introductory sentence in part B is replaced by the following:

"This method of sampling is also of application for the control of the level of mycotoxins in oilseeds other than groundnuts. Whenever reference is made in this part and subparts to "cereals and cereal products", this should be read as "cereals, oilseeds other than groundnuts and cereal products"

(5) The part B.6 Acceptance of a lot or sublot is replaced by the following:

“B.6. Acceptance of a lot or sublot

Control of ergot sclerotia

- acceptance if the first subsample contains less than 50 % of the maximum limit of ergot sclerotia or if the average of two subsamples conforms to the maximum limit;

- rejection if the average of two subsamples exceeds the maximum limit.

Control of mycotoxins other than ergot sclerotia

- acceptance if the laboratory sample conforms to the maximum limit, taking into account the correction for recovery and measurement uncertainty;

- rejection if the laboratory sample exceeds the maximum limit beyond reasonable doubt taking into account the correction for recovery and measurement uncertainty.

(6) The introductory sentence in part “C. METHOD OF SAMPLING FOR DRIED FRUIT, INCLUDING FRUIT AND DERIVED PRODUCTS BUT WITH THE EXCEPTION OF DRIED FIGS” is deleted.

(7) The introductory sentence in part “D. METHOD OF SAMPLING FOR DRIED FIGS, GROUNDNUTS AND NUTS” is deleted.

(8) The introductory sentence in part “D.1. Method of sampling for dried figs” is deleted.

(9) The title of part D2 is replaced by “D2. Method of sampling for groundnuts (peanuts), apricot kernels and tree nuts”

(10) The introductory sentence in part D2 is replaced by the following:

“This method of sampling is also of application for the control of the level of mycotoxins in spices with a relatively large particle size (particle size comparable with peanuts or larger e.g. nutmeg).

Whenever reference is made in this part and subparts to “groundnuts (peanuts), other oilseeds, apricot kernels and tree nuts”, this is replaced by “groundnuts (peanuts), apricot kernels and tree nuts”

(11) In the title of part D.2.5.1. and D.2.5.2., the word “aflatoxin” is replaced by “mycotoxin”.

(12) The introductory sentence in part “E. METHOD OF SAMPLING FOR SPICES” is replaced by the following:

“This method of sampling is of application for the control of the levels of mycotoxins in spices except in cases of spices with a relatively large particle size (heterogeneous distribution of mycotoxin contamination).”

(13) The introductory sentence in part “F. METHOD OF SAMPLING FOR MILK AND MILK PRODUCTS, INFANT FORMULAE AND FOLLOW-ON FORMULAE, INCLUDING INFANT MILK AND FOLLOW-ON MILK is replaced by the following:

“ This method of sampling is of application for the control of the levels of mycotoxins in milk and milk products and infant formulae and follow-on formulae, including infant milk and follow-on milk, young-child formulae and dietary foods (milk and milk products) for special medical purposes intended specifically for infants.”

(14) The title of part G is replaced by “G. METHOD OF SAMPLING FOR COFFEE, COFFEE PRODUCTS, COCOA, COCOA PRODUCTS, LIQUORICE ROOT AND LIQUORICE PRODUCTS”

(15) The introductory sentence in part G is replaced by the following:

“This method of sampling is of application for the control of the levels of mycotoxins in coffee, coffee products, cocoa, cocoa products, liquorice root and liquorice products.

Whenever reference is made in this part and subparts to “roasted coffee, ground roastred coffee, soluble coffee, liquorice root and liquorice extract”, this is replaced by “coffee, coffee products, cocoa, cocoa products, liquorice root and liquorice products”

(16) The title of part H is replaced by “H. METHOD OF SAMPLING FOR FRUIT JUICES INCLUDING GRAPE JUICE, GRAPE MUST, CIDER AND WINE AND OTHER BEVERAGES”

(17) The introductory part in part H is replaced by the following:

“This method of sampling is of application for the control of the levels of mycotoxins in fruit juice is also applicable to beverages other than wine”

(18) The title of part I is replaced by “I. METHOD OF SAMPLING FOR SOLID APPLE PRODUCTS AND PROCESSED PRODUCTS FROM VEGETABLES”

(19) The introductory sentence in part I is deleted.

(20) The introductory part in part “J. METHOD OF SAMPLING FOR BABY FOODS AND PROCESSED CEREAL BASED FOODS FOR INFANTS AND YOUNG CHILDREN” is replaced by the following:

“For the control of the level of mycotoxins in apple juice and solid apple products intended for infants and young children, the method of sampling as described under part H and I of this Annex respectively shall apply”

(21) The introductory sentence in part “K. METHOD OF SAMPLING FOIR VEGETABLE OILS” is deleted.

(22) The part “M. METHOD OF SAMPLING OF FOOD SUPPLEMENTS BASED ON RICE FERMENTED WITH RED YEAST *MONASCUS PURPUREUS”* is replaced by the following:

“M. METHOD OF SAMPLING OF FOOD SUPPLEMENTS

M.1. Weight of incremental sample and method of sampling

The sampling procedure provided for food supplements as capsules/pills is based on retail packages containing usually 30 to 120 capsules/pills per retail package.

Lot size (number of retail packages)	Number of retail packages to be taken for sample	Sample size (minimum amount of the aggregate sample)
1-50	1	Food supplements as capsules/pills: Total content of the retail package
		Other forms of food supplements – incremental samples of approx. 20 g or 20 ml - 100 g for food supplements containing herbal ingredients including extracts (minimum 5 incremental samples) - 50 g or 50 ml for other food supplements (minimum 3 incremental samples)
51-250	2	Food supplements as capsules/pills: total content of the two retail packages
		Other forms of food supplements – incremental samples of approx. 20 g or 20 ml - 200 g for food supplements containing herbal ingredients including extracts (minimum 10 incremental samples) - 100 g or 100 ml for other food supplements (minimum 5 incremental samples)
251-1 000	4	Food supplements as capsules/pills: from each retail package taken for sample, half of the capsules/pills

		Other forms of food supplements – incremental samples of approx. 20 g or 20 ml - 200 g for food supplements containing herbal ingredients including extracts (minimum 10 incremental samples) - 100 g or 100 ml for other food supplements (minimum 5 incremental samples)
> 1 000	4 + 1 retail package per 1 000 retail packages with a maximum of 25 retail packages	Food supplements as capsules and/pills: ≤ 10 retail packages: from each retail package, half of the capsules > 10 retail packages: from each retail package, an equal number of capsules is taken to result in a sample with the equivalent of the content of retail 5 packages
		Other forms of food supplements – incremental samples of approx. 20 g or 20 ml ≤ 10 retail packages : - 200 g for food supplements containing herbal ingredients including extracts (minimum 10 incremental samples) - 100 g or 100 ml for other food supplements (minimum 5 incremental samples) > 10 retail packages – per 5 retail packages: - 100 g for food supplements containing herbal ingredients including extracts (minimum 5 incremental samples) - 50 g or 50 ml for other food supplements (minimum 3 incremental samples)

M.2. Sampling at retail

Sampling of food supplements at the retail stage shall be done where possible in accordance with the sampling provisions set out in this part of Annex I.

Where that is not possible, an alternative method of sampling at retail stage may be used provided that it ensures that the aggregate sample is sufficiently representative of the sampled lot and is fully described and documented. In any case, the aggregate sample shall be at least 0,05 kg.

M.3. Acceptance of a lot

— acceptance if the laboratory sample conforms to the maximum limit, taking into account the correction for recovery and measurement uncertainty;

— rejection if the laboratory sample exceeds the maximum limit beyond reasonable doubt taking into account the correction for recovery and measurement uncertainty.”

(23) The following part N is added:

“N. METHOD OF SAMPLING OF FOR DRIED HERBS, HERBAL INFUSIONS (DRIED PRODUCT) AND TEAS (DRIED PRODUCT)

N.1. Weight of the incremental sample

The weight of the incremental sample shall be about 20 grams, unless otherwise defined in this part N of Annex I.

In the case of lots in retail packings, the weight of the incremental sample depends on the weight of the retail packing.

In the case of retail packs of > 20 grams, this will result in aggregate samples weighing more than 0.5 kg. If the weight of a single retail pack is >> 20 grams, then 20 grams shall be taken from each individual retail pack as an incremental sample. This can be done either when the sample is taken or in the laboratory. However, in cases where such method of sampling would lead to unacceptable commercial consequences resulting from damage to the lot (because of packaging forms, means of transport, etc.), then an alternative method of sampling can be applied. For example, in case where a valuable product is marketed in retail packs of 500 grams or 1 kg, the aggregate sample can be obtained by the aggregation of a number of incremental samples that is smaller than the number indicated in Tables 1 and 2, on the condition that the weight of the aggregate sample corresponds to the required weight of the aggregate sample mentioned in Tables 1 and 2.

Where the retail pack is less than 20 grams and if the difference is not very large, one retail pack shall be considered as one incremental sample, resulting in an aggregate sample of less than 0.5 kg. If the weight of the retail pack is much less than 20 grams, one incremental sample shall consist of two or more retail packs, whereby the 20 grams are approximated as closely as possible.

N.2. General survey of the method of sampling for dried herbs, herbal infusions (dried product) and teas (dried product)

Table 1. Subdivision of lots into sublots depending on product and lot weight

Commodity	**Lot weight (tonnes)**	**Weight or number of sublots**	**Number of incremental samples**	**Aggregate sample Weight (kg)**
dried herbs, herbal infusions (dried product) and teas (dried product)	≥ 15	25 tonnes	25	0.5
	< 15	—	3 – 25 (*)	0,1 - 0.5
(*) Depending on the lot weight — see Table 2 of this part of this Annex.				

N.3. Method of sampling for dried herbs, herbal infusions (dried product) and teas (dried product) (lots ≥ 15 tonnes)

— On condition that the sublot can be separated physically, each lot shall be subdivided into sublots following Table 1. Taking into account that the weight of the lot is not always an exact multiple of the weight of the sublots, the weight of the sublot may exceed the mentioned weight by a maximum of 20 %.

— Each sublot shall be sampled separately.

— Number of incremental samples: 25. Weight of the aggregate sample = 0.5 kg.

— If it is not possible to carry out the method of sampling described above because of the unacceptable commercial consequences resulting from damage to the lot (because of packaging forms, means of transport, etc.) an alternative method of sampling may be applied provided that it is as representative as possible and is fully described and documented.

N.4. Method of sampling for dried herbs, herbal infusions (dried product) and teas (dried product) (lots < 15 tonnes)

For lots of dried herbs, herbal infusions (dried product) and teas (dried product) less than 15 tonnes the sampling plan shall be used with 5 to 25 incremental samples, depending on the lot weight, resulting in an aggregate sample of 0,1 to 0.5 kg.

The figures in the following Table can be used to determine the number of incremental samples to be taken.

Table 2. Minimum number of incremental samples to be taken depending on the weight of the lot of dried herbs, herbal infusions (dried product) and teas (dried product)

Lot weight (tonnes)	**Minimum number of incremental samples**	**Minimum aggregate sample weight (kg)**
≤ 0,1	5	0,1
> 0,1 - ≤ 0,5	10	0.2
> 0.5 - ≤ 5,0	15	0.3
> 5,0 - ≤ 10,0	20	0.4
> 10,0 - ≤ 15,0	25	0.5

N.5. Sampling at retail stage

Sampling of foodstuffs at the retail stage shall be done where possible in accordance with the sampling provisions set out in this part of Annex I.

Where that is not possible, an alternative method of sampling at retail stage may be used provided that it ensures that the aggregate sample is sufficiently representative of the sampled lot and is fully described and documented. In any case, the aggregate sample shall be at least 0,1 kg.

N.6. Acceptance of a lot or sublot

— acceptance if the laboratory sample conforms to the maximum limit, taking into account the correction for recovery and measurement uncertainty;

— rejection if the laboratory sample exceeds the maximum limit beyond reasonable doubt taking into account the correction for recovery and measurement uncertainty.”

ANNEX II

Annex II to Regulation (EC) No 401/2006 is amended as follows:

(1) The following introductory sentence is inserted after the heading“1 INTRODUCTION”

“The requirements set out in this Annex shall be applied where foodstuffs are analysed for the official control of the levels of mycotoxins and for other regulatory purposes, including the controls performed by the food business operator to ensure compliance with provisions in Article 4 of Regulation (EC) No 852/2004.”

(2) Section 4 is replaced by the following

4. METHOD OF ANALYSIS TO BE USED BY THE LABORATORY AND LABORATORY CONTROL REQUIREMENTS

For detailed information regarding interpretation of requirements, a guidance document is available from the European Union Reference Laboratory for mycotoxins and plant toxins in food and feed.

4.1. **Definitions**

For official control the following definitions apply:

Recovery (Rec, %)

Rec = x/xref × 100%

where: x = measured concentration (for spiked samples corrected for background concentration if not blank)

xref = reference concentration (concentration of a Certified Reference Material (CRM), Proficiency Test material, or spiked sample)

Precision

RSDr = repeatability relative standard deviation

Relative standard deviation (%) calculated from results generated under repeatability conditions (repeatability precision): using the same method on the same sample material in one laboratory by the same operator, with the same instrument, within a short interval of time (1 day or 1 sequence).

RSDwR= within-laboratory reproducibility relative standard deviation

Relative standard deviation (%) calculated from results generated under within-laboratory reproducibility conditions (intermediate precision): using the same method on the same sample material in one laboratory but different days (preferably a longer time interval), and may include other conditions, e.g. involving different operators and/or different (equivalent) instruments.

RSDR = reproducibility relative standard deviation

Relative standard deviation (%) calculated from results generated under reproducibility conditions (interlaboratory precision), meaning the same material is analysed by different laboratories. The RSDR can be derived from e.g. collaborative studies and proficiency tests).

Limit of Quantification (LOQ)

LOQ = the lowest content of the analyte which can be measured with reasonable statistical certainty. In the context of this regulation this means the lowest successfully validated level: the lowest tested concentration of analyte in a sample material, for which it has been demonstrated that the criteria for recovery, precision, and identification are met.

4.2. **General requirements**

Confirmatory methods of analysis used for food control purposes shall comply with the provisions of items 1 and 2 of Annex III to Regulation (EU) 2017/625.

Wherever possible, the trueness of the method should be verified by analysis of a certified reference material and/or successful participation in proficiency tests on a regular basis.

4.3. **Specific requirements**

4.3.1. Specific requirements for confirmatory methods

Performance criteria

For confirmatory methods the following performance criteria apply:

Recovery: the average recovery should be between 70 and 120%.

Here the average recovery is the average value from replicates obtained during validation when determining the precision parameters RSDr and RSDwR. The criterion applies to all concentrations and all individual toxins, with the exception of ergot alkaloids.

For ergot alkaloids the criterion applies to the sum of each epimer-pair.

In exceptional cases, average recoveries outside the above range can be acceptable but shall lie within 50-130%, and only when the precision criteria for RSDr and RSDwR are met.

Precision

RSDr shall be ≤ 20%.

RSDwR shall be ≤ 20%.

RSDR shall be ≤ 25%.

These criteria apply to all concentrations.

In case the maximum level applies to a sum of toxins, then the criteria for precision apply to both the sum and the individual toxins. For ergot alkaloids, the criteria for individual toxins applies to the sum of each epimer pair.

Limit of quantification

When a specific requirement for the LOQ of a toxin has been set in the table below, the method shall have an LOQ at or below this value.

Table **LOQ requirements for certain mycotoxins**

Mycotoxin	LOQ requirement (µg/kg)
Aflatoxins	
Cereal based foods for infants and young children B1	≤ 0.1
For all other foods : B1, B2, G1, G2, each of the aflatoxins	≤ 1
Aflatoxin M1	≤ 0.025
Ergot alkaloids (each of 12 epimers included in sum definition of ML)	
Cereals and cereal-based foods	≤ 4
Cereal-based food for infants and young children	≤ 2

In all other cases, the following applies:

LOQ: shall be ≤ 0.5*ML, and should preferably be lower (≤0.2*ML).

In case the maximum level applies to a sum of toxins, then the LOQ of the individual toxins shall be ≤ 0.5*ML/n, with n being the number of toxins included in the ML definition.

Identification

For identification the criteria as laid down in the Guidance document on identification of mycotoxins in food and feed[1].

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[1] Document available at: https://ec.europa.eu/food/sites/food/files/safety/docs/cs_contaminants_sampling_guid-doc-ident-mycotoxins.pdf

4.3.2. Specific requirements for semi-quantitative screening methods

4.3.2.1. Scope

The scope applies to bioanalytical methods based on immuno-recognition or receptor binding (such as ELISA, dip-sticks, lateral flow devices, immuno-sensors) and physicochemical methods based on chromatography or direct detection by mass spectrometry (e.g. ambient MS). Other methods (e.g. thin layer chromatography) are not excluded provided the signals generated relate directly to the mycotoxins of interest and allow that the principle described hereunder is applicable.

The specific requirements apply to methods of which the result of the measurement is a numerical value, for example a (relative) response from a dip-stick reader, a signal from LC-MS, etc., and that normal statistics apply.

The requirements do not apply to methods that do not give numerical values (e.g. only a line that is present or absent), which require different validation approaches. Specific requirements for these methods are provided in point 4.3.3.

This document describes procedures for the validation of screening methods by means of an inter-laboratory validation, the verification of the performance of a method validated by means of an inter-laboratory exercise and the single-laboratory validation of a screening method.

4.3.2.2. Terminology

Screening target concentration (STC): the concentration of interest for detection of the mycotoxin in a sample. When the aim is to test compliance with regulatory limits, the STC is equal to the applicable maximum level. For other purposes or in case no maximum level has been established, the STC is predefined by the laboratory.

Screening method: means method used for selection of those samples with levels of mycotoxins that exceed the screening target concentration (STC), with a given certainty. For the purpose of mycotoxin screening, a certainty of 95% is considered fit-for-purpose. The result of the screening analysis is either “negative” or “suspect”. Screening methods shall allow a cost-effective high sample-throughput, thus increasing the chance to discover new incidents with high exposure and health risks to consumers. These methods shall be based on bio-analytical, LC-MS or HPLC methods. Results from samples exceeding the cut-off value shall be verified by a full re-analysis from the original sample by a confirmatory method.

'*Negative sample'* means the mycotoxin content in the sample is < STC with a certainty of 95% (i.e. there is a 5% chance that samples will be incorrectly reported as negative).

'False negative sample' means the mycotoxin content in the sample is >STC but it has been identified as negative.

'Suspect sample' (screen positive) means the sample exceeds the cut-off level (see below) and may contain the mycotoxin at a level higher than the STC. Any suspect result triggers a confirmatory analysis for unambiguous identification and quantification of the mycotoxin.

'False suspect sample' is a negative sample that has been identified as suspect.

'Confirmatory methods' means methods that provide full or complementary information enabling the mycotoxin to be identified and quantified unequivocally at the level of interest.

Cut-off level: the response, signal, or concentration, obtained with the screening method, above which the sample is classified as ‘suspect’. The cut-off is determined during the validation and takes the variability of the measurement into account.

Negative control (blank matrix) sample: a sample known to be free[2] of the mycotoxin to be screened for, e.g. by previous determination using a confirmatory method of sufficient sensitivity. If no blank samples can be obtained, then material with the lowest obtainable level might be used as long as the level allows the conclusion that the screening method is fit for purpose.

Positive control sample: sample containing the mycotoxin at the screening target concentration, e.g. a certified reference material, a material of known content (e.g. test material of proficiency tests) or otherwise sufficiently characterised by a confirmatory method. In the absence of any of the above, a blend of samples with different levels of contamination or a spiked sample prepared within laboratory and sufficiently characterised can be used, provided it can be proven that the contamination level has been verified.

4.3.2.3. Validation procedure

The aim of the validation is to demonstrate the fitness of purpose of the screening method. This is done by determination of the cut-off value and determination of the false negative and false suspect rate. In these two parameters performance characteristics such as sensitivity, selectivity, and precision are embedded.

Screening methods can be validated by inter-laboratory or by single laboratory validation. If inter-laboratory validation data is already available for a certain mycotoxin/matrix/STC combination, a verification of method performance is sufficient in a laboratory implementing the method.

4.3.2.3.1. Initial validation by single laboratory validation

Mycotoxins:

The validation shall be performed for every individual mycotoxin in the scope. In case of bio-analytical methods that give a combined response for a certain mycotoxin group (e.g. aflatoxins B_1, B_2, G_1 & G_2; fumonisins B_1 & B_2), applicability must be demonstrated and limitations of the test mentioned in the scope of the method. Undesired cross-reactivity (e.g. DON-3-glycoside, 3- or 15-acetyl-DON for immuno-based methods for DON) is not considered to increase the false negative rate of the target mycotoxins, but may increase the false suspect rate. This unwanted increasing will be diminished by confirmatory analysis for unambiguous identification and quantification of the mycotoxins.

Matrices:

An initial validation should be performed for each commodity, or, when the method is known to be applicable to multiple commodities, for each commodity group. In the

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[2] Samples are considered free of analyte if the amount present in the sample does not exceed more than 1/5th of the STC. If the level can be quantified with an confirmatory method, the level must be taken into consideration for the validation assessment.

latter case, one representative and relevant commodity is selected from that group (see table A).

Sample set:

The minimum number of different samples required for validation is 20 homogeneous negative control samples and 20 homogeneous positive control samples that contain the mycotoxin at the STC, analysed under intermediate precision (RSD_{Ri}) conditions spread over 5 different days. Optionally, additional sets of 20 samples containing the mycotoxin at other levels can be added to the validation set to gain insight to what extent the method can distinguish between different mycotoxin concentrations.

Concentration:

For each STC to be used in routine application, a validation has to be performed.

4.3.2.3.2. Initial validation through collaborative trials

Validation through collaborative trials shall be done in accordance with an internationally recognised protocol on collaborative trials (e.g. ISO 5725:1994 or the IUPAC International Harmonised Protocol) which requires inclusion of valid data from at least eight different laboratories. Other than that, the only difference compared to single laboratory validations is that the ≥20 samples per commodity/level can be evenly divided over the participating laboratories, with a minimum of two samples per laboratory.

4.3.2.4. Determination of cut-off level and rate of false suspected results of blank samples

The (relative) responses for the negative control and positive control samples are taken as basis for the calculation of the required parameters.

Screening methods with a response proportional with the mycotoxin concentration

For screening methods with a response proportional with the mycotoxin concentration the following applies:

$$\textit{Cut-off} = R_{STC} - t\text{-}value_{0.05} * SD_{STC}$$

R_{STC} = mean response of the positive control samples (at STC)

t-value: one tailed t-value for a rate of false negative results of 5% (see table B)

SD_{STC} = standard deviation

Screening methods with a response inversely proportional with the mycotoxin concentration

Similarly, for screening methods with a response inversely proportional with the mycotoxin concentration, the cut-off is determined as:

$$\textit{Cut-off} = R_{STC} + t\text{-}value_{0.05} * SD_{STC}$$

By using this specific t-value for establishing the cut-off value, the rate of false negative results is by default set at 5 %.

Fitness for purpose assessment

Results from the negative control samples are used to estimate the corresponding rate of false suspect results. The t-value is calculated corresponding to the event that a result of a negative control sample is above the cut off value, thus erroneously classified as suspect.

$\text{t-value} = (\text{cut off} - \text{mean}_{\text{blank}})/\text{SD}_{\text{blank}}$

for screening methods with a <u>response proportional</u> with the mycotoxin concentration

or

$\text{t-value} = (mean_{blank} - \text{cut off})/\text{SD}_{\text{blank}}$

for screening methods with a <u>response inversely proportional</u> with the mycotoxin concentration

From the obtained t-value, based on the degrees of freedom calculated from the number of experiments, the probability of false suspect samples for a one tailed distribution can either be calculated (e.g. . spread sheet function "TDIST") or taken from a table for t-distribution.

The corresponding value of the one tailed t-distribution specifies the rate of false suspect results.

This concept is described in detail with an example in Analytical and Bioanalytical Chemistry DOI 10.1007/s00216 -013-6922-1.

4.3.2.5. <u>Extension of the scope of the method</u>

4.3.2.5.1. Extension of scope to other mycotoxins:

When new mycotoxins are added to the scope of an existing screening method, a full validation is required to demonstrate the suitability of the method.

4.3.2.5.2. Extension to other commodities:

If the screening method is known or expected to be applicable to other commodities, the validity to these other commodities shall be verified. As long as the new commodity belongs to a commodity group (see Table A) for which an initial validation has already been performed, a limited additional validation is sufficient. For this, a minimum of 10 homogeneous negative control and 10 homogeneous positive control (at STC) samples shall be analysed under intermediate precision conditions. The positive control samples shall all be above the cut-off value. In case this criterion is not met, a full validation is required.

4.3.2.6. <u>Verification of methods already validated through collaborative trials</u>

For screening methods that have already been successfully validated through a collaborative laboratory trial, the method performance shall be verified. For this a minimum of 6 negative control and 6 positive control (at STC) samples shall be analysed. The positive control samples shall all be above the cut-off value. In case this criterion is not met, the laboratory has to perform a root-cause analysis to identify why it cannot meet the specification as obtained in the collaborative trial. Only after taking corrective action it shall re-verify the method performance in its laboratory. In case the laboratory is not capable to verify the results from the collaborative trial, it will need to establish its own cut-off in a complete single laboratory validation.

4.3.2.7. Continuous method verification / on-going method validation

After initial validation, additional validation data are acquired by including at least two positive control samples in each batch of samples screened. One positive control sample is a known sample (e.g. one used during initial validation), the other is a different commodity from the same commodity group (in case only one commodity is analysed, a different sample of that commodity is used instead). Inclusion of a negative control sample is optional. The results obtained for the two positive control samples are added to the existing validation set.

At least once a year the cut-off value is re-established and the validity of the method is re-assessed. The continuous method verification serves several purposes:

quality control for the batch of samples screened

providing information on robustness of the method at conditions in the laboratory that applies the method

justification of applicability of the method to different commodities

allowing to adjust cut-off values in case of gradual drifts over time.

4.3.2.8. Validation report

The validation report shall contain:

- A statement on the STC
- A statement on the obtained cut-off.

Note: The cut-off must have the same number of significant figures as the STC. Numerical values used to calculate the cut-off need at least one more significant figure than the STC.

- A statement on calculated false suspected rate
- A statement on how the false suspected rate was generated.

Note: The statement on the calculated false suspected rate indicates if the method is fit-for-purpose as it indicates the number of blank (or low level contamination) samples that will be subject to verification.

Table A: Commodity groups for the validation of screening methods

Commodity groups	**Commodity categories**	**Typical representative commodities included in the category**
High water content	Fruit Juices Alcoholic beverages Root and tuber vegetables Cereal or fruit based purees	Apple juice, grape juice Wine, beer, cider Fresh ginger Purees intended for infants and small children
High oil content	Tree nuts Oil seeds and products thereof Oily fruits and products thereof	Walnut, hazelnut, chestnut Oilseed rape, sunflower, cotton-seed, soybeans, peanuts, sesame etc. Oils and pastes (e.g. peanut butter, tahina)
High starch and/or protein content and	Cereal grain and products thereof	Wheat, rye, barley, maize, rice, oats Wholemeal bread, white bread,

Commodity groups	**Commodity categories**	**Typical representative commodities included in the category**
low water and fat content	Dietary products	crackers, breakfast cereals, pasta Dried powders for the preparation of food for infants and small children
High acid content and high water content (*)	Citrus products	
"Difficult or unique commodities" (**)		Cocoa beans and products thereof, copra and products thereof, coffee, tea Spices, liquorice
High sugar low water content	Dried fruits	Figs, raisins, currants, sultanas
Milk and milk products	Milk Cheese Dairy products (e.g. milk powder)	Cow, goat and buffalo milk Cow, goat cheese Yogurt, cream

(*) If a buffer is used to stabilise the pH changes in the extraction step, then this commodity group can be merged into one commodity group "High water content".

(**) "Difficult or unique commodities" should only be fully validated if they are frequently analysed. If they are only analysed occasionally, validation may be reduced to just checking the reporting levels using spiked blank extracts.

Table B: One tailed t-value for a false negative rate of 5%

Degrees of Freedom	Number of replicates	t-value (5%)
10	11	1.812
11	12	1.796
12	13	1.782
13	14	1.771
14	15	1.761
15	16	1.753
16	17	1.746
17	18	1.74
18	19	1.734
19	20	1.729
20	21	1.725
21	22	1.721
22	23	1.717
23	24	1.714
24	25	1.711
25	26	1.708
26	27	1.706
27	28	1.703
28	29	1.701

29	30	1.699
30	31	1.697
40	41	1.684
60	61	1.671
120	121	1.658
∞	∞	1.645

4.3.3. Requirements for qualitative screening methods (methods that do not give numerical values)

The development of validation guidelines for binary test methods is currently subject of various standardization bodies (e.g. AOAC, ISO). Very recently AOAC has drafted a guideline on this matter. This document can be regarded as the current state of the art in its field. Therefore methods that give binary results (e.g. visual inspection of dip-stick tests) should be validated according to this guideline

http://www.aoac.org/imis15_prod/AOAC_Docs/ISPAM/Qual_Chem_Guideline_Final_Approved_031412.pdf

4.4. Estimation of measurement uncertainty, recovery calculation and reporting of results[3]

4.4.1. *Confirmatory methods*

The analytical result shall be reported as follows:

(a) Corrected for recovery, the level of recovery being indicated. The correction for recovery is not necessary in case the recovery rate is between 90-110 %.

(b) As x +/– U whereby x is the analytical result and U is the expanded analytical measurement uncertainty, using a coverage factor of 2 which gives a level of confidence of approximately 95 %.

For food of animal origin, the taking into account of the measurement uncertainty can also be done by establishing the decision limit (CCα) in accordance with Commission Implementing Regulation (EU) 2021/XXX[4] (point 2.6 (2) of Annex I) .

In case the maximum level has been set for the sum of toxins (e.g. aflatoxins, T2/HT2-toxin, fumonisins, ergot alkaloids), the analytical results of all individual toxins should be reported. For ergot alkaloids it is also allowed to report the sum of each of the six epimer pairs instead of the 12 individual epimers.

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[3] More details on procedures for the estimation of measurement uncertainty and on procedures for assessing recovery can be found in the report 'Report on the relationship between analytical results, measurement uncertainty, recovery factors and the provisions of EU food and feed legislation' https://ec.europa.eu/food/sites/food/files/safety/docs/cs_contaminants_sampling_analysis-report_2004_en.pdf

[4] Commission Implementing Regulation (EU) 2021/XXX on the performance of analytical methods for residues of pharmacologically active substances used in food-producing animals and on the interpretation of results as well as on the methods to be used for sampling and repealing Decisions 2002/657/EC and 98/179/EC

Recovery correction, if applicable, is done for each of the individual toxins before summation of the concentrations. For ergot alkaloids, the correction can also be done based on the recovery obtained for each of the epimer pairs.

For compliance verification with the sum-ML, a lower-bound approach is applied which means that results for individual toxins that are <LOQ will be replaced by zero for the calculation of the sum.

The present interpretation rules of the analytical result in view of acceptance or rejection of the lot apply to the analytical result obtained on the sample for official control. In case of analysis for defense or referee purposes, the national rules apply.

4.4.2. Screening methods

The result of the screening shall be expressed as compliant or suspected to be non-compliant.

'Suspected to be non-compliant' means the sample exceeds the cut-off level and may contain the mycotoxin at a level higher than the STC. Any suspect result triggers a confirmatory analysis for unambiguous identification and quantification of the mycotoxin.

'Compliant' means that the mycotoxin content in the sample is < STC with a certainty of 95 % (i.e. there is a 5 % chance that samples will be incorrectly reported as negative). The analytical result is reported as '< level of STC' with the level of STC specified.

4.5. Laboratory quality standards

Laboratory must comply with the provisions of Article 37(4) and (5) of Regulation (EU) No 2017/625.

4.6. Method for the determination of ergot sclerotia **(numbering to be updated)**

1. Objective and field of application

The method is used for both qualitative and quantitative determination of ergot in cereals

2. Principle

Ergot in cereals is determined by the macroscopic and microscopic identification of the ergot sclerotia and fragments. Quantification is done by weighing the amount of identified ergot sclerotia and fragments with a particle size > 0,5 mm.

3. Reagents

3.1. Chloralhydrate, ß = 60%
3.2. Sodium hydroxide (pelleted)
3.3. Potassium hydroxide (pelleted)

3.4. Ethanol, o = 50%
3.5. Acetone

The reagents listed can be replaced by others which yield comparable results.

4. Equipment and accessories

4.1. Optical equipment
4.1.1. Stereo microscope (up to 70x magnification)
4.1.2. Magnifier (up to 10x magnification)
4.2. Sieve fitted with square meshes of width of 0.5mm
4.3. Analytical balance (accuracy 0,001 g)
4.4. Reference material

5. Procedure

The examination is performed in cereals.

Qualitative determination of the ergot sclerotia is performed macroscopically and microscopically considering ergot and its fragments in all sieve fractions (sieve fraction > 0.5 mm and < 0.5mm).

However this qualitative determination is not necessary for checking compliance.

Quantification is performed by selecting and weighing of ergot and its fragments with a particle size > 0,5 mm out of the sample.

5.1. Preparation of the test sample

From the 10 kg sample, 4 subsamples of equal weight of approximately 500 grams are taken and used directly for the investigation (5.2 and 5.3).

One subsample is examined. In case the result of the subsamples is equal or below 50 % (analytical threshold) of the maximum level, the sample is compliant with the maximum level. If the result is above 50 % of the maximum level, another subsample need to be examined and the average of the result of the 2 subsamples is used for checking compliance with the maximum level.

The other two subsamples are to be used for defence purposes of for regular quality control.

5.2. Identification of ergot

Ergot sclerotia are identified based on their characteristic features. The identification may be facilitated by comparison to reference material (4.4).

Morphology: *Claviceps purpurea* Tul. sclerotia are elongated with a length up to several centimetres, coloured dark violet to black. The shape is similar to cereal kernels. They only consist of fungal hyphae.

Anatomy: Cross sections through the random parts of ergot sclerotia show very small, narrow interconnected hyphae which yield a dense pseudoparenchymatic tissue. The outer layers of the hyphae are coloured dark violet to black, whereas the inner parts are coloured light pink to violet.

For the identification of ergot fragments the following colour reaction can, if necessary, be used. The staining procedure is only applicable to fresh sclerotia material.
Therefore a filter paper is soaked with a solution of 3 ml ethanol (3.4) and 2 sodium hydroxide pellets (3.2) or 2 potassium hydroxide pellets (3.3).
The sample is distributed on the filter paper.
After approx. 5 min. a red-violet halo around the ergot fragments is observed. The dark violet colouring of the outer hyphae layers is dissolved also in chloralhydrate (3.1) and colours it violet.

5.3. Quantification

The quantification of ergot is performed using the sieve fractions > 0,5 mm.
Material identified as ergot in each fraction is selected and weighed. An aliquot of the sieved fractions may be used if necessary. The ergot content of the fraction > 0.5 mm is summarized and expressed in mg/kg cereals (6.1).

6. Calculation

The amount of ergot fragments in mg/kg cereals (original sample) is calculated using the following formula:

$$C = BC \times 1000/E \text{ [mg/kg]}$$

C = amount of ergot in mg/kg cereals
BC = selected ergot fragments in the test sample (or an aliquot of it) [mg]
E = total weight of the examined sample [g]