EURL-MP-guidance doc_003 (version 1.4)

Guidance document on performance criteria for methods of analysis for mycotoxins and plant toxins in food and feed

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1 Introduction

Performance criteria for methods of analysis used for official control for mycotoxins in food are included in Commission Implementing Regulation (EU) 2023/2782 (Annex II) [1]. This regulation applies from 1 April 2024 and repeals Regulation (EC) No 401/2006. However, given the substantial changes in method performance requirements, a transition period applies until 1 January 2029. This means that the specific requirements regarding recovery, repeatability and reproducibility (point 4.3 in Annex II to (EC) No 401/2006 [2]) remain applicable to methods which have been validated before 1 April 2024.

For plant toxins in food performance criteria for methods of analysis used for official control are included in Commission Implementing Regulation (EU) 2023/2783 (Annex II) [3], which also applies from 1 April 2024. With this regulation, generic method performance criteria are set which are similar to those set for mycotoxins in food. Since such criteria were not existing before, a transition period for complying with the criteria has been set (until 1 July 2028).

This guidance document aims to provide the background and rationale for updated harmonised criteria for mycotoxins and plant toxins. In addition, where considered necessary and in response to questions received from the NRLs, explanations and guidance is given, both on performance criteria that are embedded in legislation and on other performance criteria related to validation and quality control which are not explicitly included in the legislation. NRLs are encouraged to provide feedback that will be taken into account for future updates of this document.

Timeline revision/extension of performance criteria from (EC) 401/2006 to (EU) 2023/2782 and (EU) 2023/2783:

The revised/new performance criteria included in (EU) 2023/2782 [1] and (EU) 2023/2783 [3] were initiated by the EURL-MP in 2018/2019 following a review of the existing regulations at that time. In the existing situation, different performance criteria were specified for the different mycotoxins, and, in addition, for the different levels of the same mycotoxins. Part of the precision criteria were Horwitz-based, others were derived from, in many cases rather old, collaborative studies or expert judgments. With implementation of newer analytical methods, most notably LC-MS/MS, there was no longer any clear scientific rationale for the old mycotoxin/concentration dependent criteria. Furthermore, certain performance parameters like intermediate precision and LOQ requirements were missing in the old legislation. These observations were communicated with the Commission, who then requested the EURL-MP to propose a revision of the method performance criteria as laid down in (EC) No 401/2006 [2].

For plant toxins in food, method performance criteria did not yet exist. However, the type of molecules, matrices, concentrations, and analytical techniques for determination of plant toxins *are mostly similar to those of mycotoxins. Hence, it was the opinion of the EURL-MP that method performance characteristics are, and therefore also the criteria should be, similar to those for mycotoxins. The Commission informed the EURL-MP that for plant toxins establishment of a separate regulation was being prepared, in parallel to a revision of (EC) 401/2006. It was proposed by the EURL-MP to include the same performance criteria as drafted for mycotoxins, given the similarity of mycotoxins and plant toxins analyses, and in order to achieve harmonisation within the EURL-MP/NRL/OL community.*

The revised performance criteria were drafted and discussed within the EURL-MP/NRL/OL community, through iterative review and discussions at the annual EURL-MP workshops in 2018-2023. Additionally, the revised draft regulations were presented, reviewed and discussed during the meetings of the Working Group on Agricultural Contaminants in food in 2020-2023. At the end of 2023 the draft regulations were agreed, finalized and published.

For mycotoxins and plant toxins in feed, some requirements are laid down in Commission Regulation (EC) No 152/2009 [4]. In addition, for mycotoxins in feed, performance criteria have been provided in CEN/TS 17455:2020 [5].

As feed matrices are often similar to food matrices regarding diversity and challenges, it is the opinion of the EURL-MP that for feed the same method performance criteria as for food can and should be applied.

2 Definitions

Below definitions of typical method performance parameters / validation parameters are given. While not all are explicitly included in the legislation, they are addressed here as they are discussed in this guidance document.

2.1 Should and shall

In legislation and this guidance document ‘should’ and ‘shall’ are used to indicate where, with common sense, exceptions/flexibility may be acceptable, and where they may not. Incidental exceptions can be accepted because it is recognised that with the numerous mycotoxins, plant toxins, food, feed, and concentration combinations, especially with multi-toxin methods, it may be very difficult to fully meet all criteria for every individual toxin/matrix/concentration combination.

SHOULD means a recommendation that may be ignored but only in particular circumstances (based on valid reasons) and the full implications of ignoring the recommendation shall be understood and carefully assessed before choosing a different course of action.

SHOULD NOT means not recommended, although it may be acceptable in particular circumstances, but the full implications of ignoring the recommendation shall be understood and carefully assessed.

SHALL means an absolute requirement (the action is mandatory).

SHALL NOT means an absolute no.

2.2 Blanks

Blanks are used to determine method performance characteristics and for quality control. Various types of blanks are defined [18], including:

- Procedural blank: a sample that does not contain the matrix, is brought through the entire measurement procedure and analysed in the same manner as the test sample. The matrix may be replaced by a simulant, e.g. water for aqueous samples.
- Sample blank (also called matrix blank): a sample matrix without the target analyte present.
- Solvent blank: a sample only containing the solvent(s) in the same composition as the final extract injected into the instrument. When injected directly after a calibration standard or a sample containing the analyte, it can be used to assess potential carry-over between injections.

2.3 Matrix effects

Matrix effects are relevant for methods based on LC with MS detection and all GC-based methods.

In LC-MS(/MS): suppression (or enhancement) of analyte response when spiked to a blank sample extract compared to analyte response in solvent at the same concentration. It can occur during ionisation of the analyte in the ion source in the presence of co-extracted matrix and can affect quantification.

In GC-MS(/MS): enhancement (or suppression) of analyte response when spiked to a blank sample extract compared to analyte response in solvent at the same concentration. In GC it usually originates from active sites in the liner of the injector and can affect quantification.

Note: matrix effects should not be confused with matrix interference, which is a detector response from another, (partially) co-eluting, compound that contributes to the analyte response.

The matrix effect (ME) defined as percentage suppression (negative value) or enhancement (positive value) can be calculated as follows:

$$ME = \frac{R_{extract} - R_{solvent}}{R_{solvent}} \times 100 \qquad \textbf{(Eq. 1)}$$

where:

$R_{extract}$ = the response of the toxin spiked to a blank sample extract
$R_{solvent}$ = the response of the toxin in solvent standard at the same concentration

When the ME is -35% this means that the response in spiked final extract is 35% lower than that of the same concentration in a solvent standard, i.e. when the response in the solvent standard is set at 100%, we see 65% of that response in the spiked final extract.

2.4 Linearity

Direct proportionality of response obtained during measurement with the concentration of the analyte.

2.5 Recovery

Several definitions exist for recovery (see Appendix 1), which may also be differently interpreted. Factors that complicate the interpretation are whether or not matrix effects (see 2.2) and/or losses during sample preparation are accounted for, which depends on the approach used for calibration/quantification (see also 3.2, Table 1).

For mycotoxins and plant toxins in food and feed the following definition applies:

$$\text{Recovery (\%)} = x/x_{ref} \times 100 \qquad \textbf{(Eq. 2)}$$

where:

x = measured concentration (for spiked samples corrected for background concentration if applicable)

x_{ref} = reference concentration (the concentration of a Certified Reference Material (CRM), Proficiency Test material, or spiked sample)

Note: it is recognized that, depending on the method of quantification, the recovery as defined above may correspond to different definitions:

a) *Recovery* or *Recovery Factor as defined by IUPAC* [6], also referred to as *'extraction recovery' or 'absolute recovery'*. This is the yield of an analyte from the extraction/cleanup stage. When there are no matrix effects (see 2.2.), it can be determined using quantification method 1 from 3.2 Table 1.
In case matrix-effects are significant, then matrix-effects need to be corrected for in order to obtain the absolute recovery. This can be done by using quantification method 2, 4a, or 5a from 3.2 Table 1.

b) *Apparent recovery* as defined by IUPAC [6]. This is basically any other recovery than described under a). It is the recovery observed with or without correcting for extraction/cleanup losses and/or matrix-effects, using the various options for quantification given in 3.2 Table 1.
Example 1, method using quantification procedure 1 from Table 1 in 3.2: when losing 20% of analyte during sample preparation, and in the LC-MS/MS analysis 20% ion suppression occurs, then the apparent recovery is ~60%.
Example 2, method using quantification procedure 3, 4b or 5b from Table 1 in 3.2: an apparent recovery of around 100% may be obtained even when the extraction yield would be 30% and matrix-effects are significant.

c) *Trueness,* defined as the closeness of agreement between the average value obtained from a series of test results and an accepted reference value (ISO 5725-1), is normally expressed in terms of bias. Bias is the (relative) difference between the measured and the true concentration. The true concentration ideally is a CRM value, if not available the assigned value from a proficiency test or collaborative study, and if also not available, a spiked concentration. Trueness and recovery are sometimes used interchangeably, but are not the same because trueness is expressed as bias. Relative bias and recovery are correlated: bias (%) = recovery (%) – 100%.

2.6 Precision

2.6.1 Reproducibility relative standard deviation, RSD_R

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

Note: Deriving RSD_R from proficiency tests enables to assess the reproducibility within the official control system as a whole (with all the different methods applied) and is therefore an appropriate tool to assess requirements on RSD_R set in this regulation. It is recognized that by expanding the definition of RSD_R to proficiency tests, we deviate from ISO 5725-1 ('conditions where test results are obtained with the same method…'), however, this is considered appropriate because suitability of methods for official control is based on performance criteria rather than the use of standardized methods.

2.6.2 Repeatability relative standard deviation, RSD_r

The 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).

2.6.3 Within-laboratory reproducibility relative standard deviation, RSD_{wR}

The relative standard deviation (%) calculated from results generated under within-laboratory reproducibility conditions (intermediate precision, elsewhere also abbreviated as RSD_{Ri}): using the same method on the same sample material in one laboratory but on different days (preferably a longer time interval), and may include other conditions involving different operators and/or different (equivalent) instruments.

Note*: this parameter was not included in Commission Regulation (EC) No 401/2006, but is a more relevant and realistic in-house precision parameter compared to* RSD_r.

2.7 Limit of detection (LOD) and limit of quantification (LOQ)

2.7.1 Introductory remarks

Various definitions and methods for determination of LOD and LOQ exist, which may result in different outcomes. Example definitions for LOD include ‘analyte content which can be distinguished from the blank with an error probability of (1-ß)’ [7], and ‘minimum amount or concentration of the analyte in a sample which can be detected reliably, but not necessarily quantified’ [5]. Examples for LOQ include ‘lowest level at which the performance is acceptable for a typical application’ [8], ‘analyte content which can be determined with a certain level of precision’ [7], and ‘lowest concentration or amount of the analyte in a test sample which can be quantitatively determined with an acceptable level of precision and accuracy’ [5].

The determination of LOD can be based on statistical approaches or signal-to-noise (S/N) approaches. Statistical approaches involve replicate analyses of pseudo-blanks and procedural calibration at equidistant concentrations in the range close to the (anticipated) LOD [7,8]. Especially in LC-MS/MS-based methods the LOD may be matrix dependent as a result of ion suppression. As a consequence, estimation of the LOD for a wide variety of matrices can be a heavy burden for the laboratory. Estimation of the LOD based on S/N (LOD = concentration corresponding to S/N=3) is relatively straightforward. Visual inspection of chromatograms of a single measurement at low level can give an indication of the lower limit at which the analyte can be detected. However, determination of LOD through S/N is not unambiguous, especially in the case of MS detection, because noise depends on acquisition rate (duty cycle), smoothing, method of S/N calculation (manual or software-based, and in the latter case, the software algorithm).

For the determination of the LOQ, also different options have been described:

- based on the LOD obtained using statistical approaches: LOQ = 3*LOD or 3.3*LOD

- based on S/N approaches: LOQ = concentration corresponding to S/N ≈ 5-10

- based on determination of recovery and precision, iteratively at decreasing levels until the requirements are no longer met or a fit-for-purpose LOQ has been reached. In the latter case, the LOQ may not be the lowest possible level, but the lowest tested level.

Note*: in MS-based methods, for enforcement purposes, normally two (product)ions are needed for identification (see [9]) and the one with the lowest sensitivity/selectivity will determine the LOQ.*

2.7.2 Official control

For official control purposes (checking compliance against maximum levels/guidance values/indicative levels) we define the LOQ as 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 [1,3,10], and identification [9] are met. This definition and the way of determination of the LOQ is less ambiguous than the various options mentioned in 2.7.1. It should be noted that with this definition, there is no relationship between LOQ and LOD. Based on the LOQ requirements (see 3.5), the lowest validation level is chosen. This level may be well above the LOD and the technically feasible LOQ of the method.

According to regulation (EU) 2017/625, methods of analysis should be characterised by the LOD. However, for official control purposes determination of the LOD may be omitted. The rationale for this is that the maximum levels/guidance values/indicative levels for mycotoxins and plant toxins are often well above the method LOD, so its determination has no added value for this purpose, while unnecessarily adding to the validation workload.

2.7.3 Generation of monitoring data for risk assessment

For risk assessment, often the data generated during official control are submitted and used, supplemented with data from dedicated monitoring surveys (other methods/samples). For this reason, for LOQ the same definition as described under 2.7.2 applies [10].

For monitoring purposes, the required LOQs are often (much) lower than strictly necessary for official control. When the method is to be used both for official control and monitoring, then the lowest level included in the validation needs to be adjusted accordingly.

For risk assessment purposes, estimation of a method LOD is often desirable. The exceptions are: a) when the LOQ is low enough to provide quantifiable results in >95% of all samples, b) when the LOQ is below the level in the food/feed matrix considered to be relevant for risk assessment (to be provided by the risk assessor, e.g. EFSA or competent authority).

The estimation of the LOD can be done either following the statistical approach according to [7,9], or by a less laborious and more pragmatic S/N approach.

In case of the statistical approach, for MS-based methods, the calculations are done on the quantifier ion, but the additional requirement is that a signal shall be obtained for the qualifier ion, with $S/N \geq 3$.

In case the LOD is estimated based on the S/N approach, the following applies:

- the LOD is the concentration corresponding to S/N = 3. For MS-based methods, where two ions need to be measured, this requirement applies to both the quantifier and the qualifier ion.
- the LOD shall be estimated using (spiked blank) sample(s) undergoing the entire analytical procedure. The concentration in the sample used for LOD estimation should not be higher than 10 times the resulting LOD. In case no blank matrix exists, and an LOD needs to be provided, then it can be estimated based on a similar matrix that does not contain the toxin, or a spiked procedural blank.

***Note**: S/N is affected by multiple factors, including acquisition rate (duty cycle), smoothing of the data, the way S/N is determined ('visual inspection of chromatograms', software algorithms and their settings), matrix, and the concentration and sensitivity of the analyte. The following should be taken into account:*

a. Noise: peak-to-peak noise is used, and the chromatographic time window used for measurement of the noise should be retention time of the analyte ± at least three times the peak width of the analyte (peak width at base or 10% of the peak height in case of tailing peaks).
b. Signal: at LOD the chromatographic peak should consist of at least 5 data points.
c. Care should be taken with smoothing of the data, over-smoothing may lead to too optimistic LODs and loss of chromatographic resolution (risk of inclusion of interfering signals in analyte signal).
d. LOD should be estimated manually from the signal and noise as observed in the extracted ion chromatogram under conditions and data processing settings as used for sample analysis.
e. While the actual LOD at an individual sample level will vary (e.g. matrix, condition of instrument), risk assessors typically work with a fixed LOD value for their statistics. The LOD reported should be a representative estimate for the samples analysed, taking into account variations in instrument(s) performance over the time period the samples are analysed.

Note: for MS-based methods, the requirement in the determination of the value of the LOD is that for both quantifier and qualifier ion a signal shall be present with S/N ≥ 3. The detection of both ions suffices. Between LOD and LOQ, there is no requirement for the ion ratio. The rational is that at levels below LOQ the response of especially the qualifier ion is typically (very) low and more variable. This less strict criterion is a compromise between achieving an as low as possible LOD (beneficial for risk assessment) and potentially reporting false positives.

In case of reporting toxins below LOQ in monitoring programs: while there is no requirement to comply with the ion ratio criterion, it is recommended to check the relative abundance of the responses of the two ions is in line with expectations, i.e. close to the LOQ you'll expect a ratio not much deviating from the 30% criterion stated in [9].

2.7.4 Dealing with background in LOQ and LOD determinations

Mycotoxins and plant toxins, while not ubiquitous contaminants, can still be present in procedural or solvent blanks through, for example, cross-contamination and carry-over. These should be minimised as much as possible. In case a background signal for procedural or solvent blanks persists (either from carry-over artifacts or interferences), then the responses in the procedural blanks and blank samples shall not be higher than 30% of the LOQ. If it is, effort should be made to reduce it, or the LOQ should be increased accordingly. The LOD shall not be lower than the average background concentration plus 10 times the standard deviation of the background [10].

When toxins are inherently present (e.g. gossypol in cotton seeds, hydrocyanic acid in apricot kernels, quinolizidine alkaloids in lupin seeds) it is by definition impossible to determine an LOQ at levels below that in the available sample(s) for validation. In these cases the LOQ and LOD can be estimated using a highly similar matrix that is free of the toxin, or contains the toxin at a much lower level than the actual matrix (≤30% of the desirable LOQ). For example sweet almonds may be used as proxy for bitter apricot kernels for hydrocyanic acid, and soybeans as proxy for lupin seeds for quinolizidine alkaloids. The LOQ determined this way provides an indication of the LOQ the method can achieve, thereby fulfilling the requirements of ISO 17025.

3 Performance criteria

Below the performance criteria are provided, with the background, explanations, and clarifications to aid in the uniform interpretation of the criteria in the legislation. Not all performance criteria are embedded in legislation, but they are included here as additional information and guidance to the NRLs.

3.1 Matrix effects

Matrix effects in LC-MS(/MS) or GC(-MS/MS) (see 2.2) should be investigated during validation to assess to what extent they occur. In case of strong matrix effects (>50% suppression/enhancement), it is recommended to try to reduce them (e.g. by dilution or applying a cleanup step). However, no criterion applies since matrix-effects are covered by the recovery criteria. When the recovery requirements (see 3.3) are not met due to matrix-effects, a calibration/ quantification procedure shall be used that corrects for matrix-effects, i.e. options 2-5 in Table 1, 3.2.

3.2 Calibration/quantification procedures

For quantification, multi-level calibration (at least 5 concentrations, covering the lowest and highest level to be quantified) is preferred. An appropriate calibration function shall be used (e.g. linear, with or without weighing). The deviation of the back-calculated concentrations of the calibration standards from the true concentrations using the calibration equation used should not be more than ±20%. This criterion is preferable to relying on correlation coefficients.

There are different options for quantification/calibration. Calibration standards can be prepared in solvent/eluent, in sample extracts, in sample material, and with or without the use of isotopically labelled internal standards. Depending on the procedure used, the result obtained is corrected for matrix-effects, and in some cases also for losses during sample preparation. An overview is given in Table 1.

Table 1. Overview calibration/quantification procedures

Quantification method	Calibration procedure	corrects for	
		sample prep. losses	matrix effects
1. solvent standard	calibration standards prepared in solvent	no	no
2. matrix-matched standards	calibration standards prepared in extract of blank sample of the same matrix[a)]	no	yes
3. procedural calibration	calibration standards prepared in sub-portions of blank sample of the same matrix, *added before extraction*	yes	yes
4. isotope labelled internal standard (ILIS) (all responses are normalised to that of the corresponding isotope labelled internal standard)	**4a.** isotope analogue added to the calibration standards, and to the final extract of each sample[b)]	no	yes
	4b. isotope analogue added to the calibration standards, and *to each sample before extraction*	yes	yes
5. standard addition method	**5a**. standards added to aliquots of the extract of each sample	no	yes
	5b. standards added to sub-portions of each sample *before extraction*	yes	yes

[a)] same matrix: same at commodity level as far as it can be expected that analyte response is not affected, e.g. varieties within a commodity such as summer wheat and winter wheat are considered the same. For less well defined matrices, e.g. mixed teas, mixed spices, compound feed, and also in case there is no blank matrix, quantification methods 4 or 5 are more appropriate.

[b)] another option is to add the isotope analogue after extraction, to an aliquot of the extract, before clean-up. In this case, the method corrects for part of the sample preparation losses: it does not correct for low extraction yields, but it does correct for losses during the clean-up step.

3.3 Recovery requirements

3.3.1 Initial and on-going validation

For mycotoxins and plant toxins in food and feed the average recovery should be between 70 and 120%.

Here the average recovery is the average value from replicates (at least 6) obtained during initial or on-going validation (see 5.1 and 5.3) when determining the precision parameters RSD_r and RSD_{wR}. 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 this range can be acceptable but shall lie within 50-130%, and only when the precision criteria for RSD_r and, if available, RSD_{wR} are met. It should be documented why an average recovery of 70-120% could not be achieved, why the deviation is considered acceptable, and what the implications for quantitative measurement are.

3.3.2 Acceptance criteria for individual recovery (batch control)

Recoveries for individual QC samples concurrently analysed with samples should normally be in the range of the average recovery established from on-going validation ± 2x RSD. In case of lack of routine recovery data, as a minimum criterion the recovery of the QC sample should be in the range [average from initial validation ±40% relative of the average recovery value], so e.g. when the average recovery from validation is 100%, the batch recovery should be in the range 60-140%, and when the average recovery from validation is 80%, the batch recovery should be in the range 48-112%. For methods that are frequently used, Shewhart charts can be a tool to verify whether recoveries are within the expected range. In this case, when methods contain a high number of similar toxins, such as pyrrolizidine alkaloids, it is acceptable to use Shewhart charts for $\sqrt{N}$ representative toxins (with N being the number of toxins).

3.3.3 Requirements for extraction/cleanup yields

The yield of the sample preparation steps (extraction, cleanup) should be investigated during method development or validation to gain insight in extraction efficiency and losses during clean-up. Although the aim should always be to use methods with high yields, no criteria for the yields apply since losses, and consistency thereof, during sample preparation are covered by the recovery and precision criteria.

As described in 2.4 and 3.2, there are certain calibration/quantification procedures that correct for incomplete extraction and losses during clean-up (see Table 1, procedure 3, 4b and 5b). This way it is possible to obtain good recoveries (Eq. 2) while in fact e.g. only 30% of the toxin is extracted. This is considered acceptable as long as the recovery and precision criteria are met, the required LOQ can be achieved, and good accuracy can be demonstrated through proficiency test data or CRMs.

3.4 Precision

The criteria for precision apply to all concentrations.

In case a laboratory provides the evidence that the RSD_{wR} criterion is complied with, there is no need to provide that evidence for the RSD_r criterion as compliance with the RSD_{wR} implicates compliance with the RSD_r criterion.

In case the maximum level applies to a sum of toxins, then the criteria for precision apply to the individual toxins and to the sum of the toxins. For calculation of the sum precision, first the concentrations of each individual toxin are calculated. These concentrations are summed to a total. Next the precision is calculated using the total concentrations obtained for each replicate. For ergot alkaloids, the criteria for individual toxins applies to the sum of each epimer pair.

3.4.1 Reproducibility relative standard deviation, RSD_R

As indicated in the definition, this is not a parameter for individual laboratories / single lab validations, but a parameter calculated from the results of multiple laboratories. The criterion for RSD_R is a target value based on what is currently desirable and should be feasible.

Insight in the current situation regarding variability of analysis between laboratories, can be obtained from validation data from collaborative trials (same method), and the (robust) relative standards deviation as observed in proficiency testing (various fit-for-purpose methods). In CEN/Technical Specification for mycotoxins in feed (CEN/TS 17455:2020, Stroka/JRC [7]), it was concluded that a RSD_R of 22% is generally feasible for collaborative studies, irrespective of the concentration, matrix and toxin. An inventory by the EURL-MP of robust RSD_R data from a large number of proficiency tests (>750 mycotoxin/matrix/ concentration combinations, 2013-2018) showed that the median and 75th percentile of the collected RSD_R's were 22% and 26%, respectively. The similarity of the RSD_R's observed in collaborative trials and PTs (although not always calculated using the same statistics) was also noticed by Thompson et al [11]. Based on these available data, it is concluded that a RSD_R of 25% is an appropriate benchmark both as criterion for acceptability of methods validated through collaborative trials, and for use as target relative standard deviation in PTs.

The criterion of 25% for the RSD_R also sets the target for the criteria for RSD_{wR} and RSD_r, as these are expected to be lower than the RSD_R.

***Note**-1: the value of 25% was chosen over the 22% from CEN/TS 17455:2020: i) in order to take into account that different laboratories will use different methods, ii) because of the trend towards increased use of LC-MS-based multi-toxin methods. The 25% criterion is identical to the value used in the field of pesticides in food and feed [12], often analysed by LC-MS/MS, which also covers a wide range of organic molecules in a wide variety of matrices and concentrations.*

***Note**-2: it is recognised that the 25% criterion may be challenging for certain 'new' toxins for which there currently might be a lack of experience. However, for NRLs and OLs there is usually sufficient time between first announcement of upcoming legislation and actual enforcement of maximum levels, to implement, validate and familiarise themselves with fit-for-purpose methods. Another reason to set the* RSD_R *to a value not higher than 25% is that higher values may restrict the possibilities to take legislative action. This is because for enforcement of maximum levels the expanded measurement uncertainty (U) needs to be subtracted from the analysis result to ascertain an exceedance of the maximum level beyond reasonable doubt* [1]*. Taking into account that a food or feed product may be tested at different laboratories in the EU member states, the estimated expanded U (using a coverage factor of k=2, which indicates approximate 95% confidence) in case of a* RSD_R *of 25% is at least* $2*RSD_R = 50\%$*. This means that in this case enforcement action can only be taken (i.e. the analysis result exceeds the ML beyond reasonable doubt) when the analysis result is twice the ML. Example: ML = 100 µg/kg,* $RSD_R = 25\%$ *=> expanded MU = 50%, the toxin concentration exceeds the ML when >200 µg/kg (200 - 50% = 100 µg/kg).*

3.4.2 Repeatability relative standard deviation, RSD_r

Repeatability assessment is typically carried out during initial validation by at least six replicate analyses of the matrix for each concentration tested. The RSD_r shall be ≤20%. See also 5.1.

3.4.3 Within-laboratory reproducibility relative standard deviation, RSD_{wR}

The within-laboratory reproducibility is determined by analysing samples in different batches, on different days.

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[1] Guidance document for competent authorities for the control of compliance with EU legislation on aflatoxins.
https://food.ec.europa.eu/system/files/2021-05/cs_contaminants_catalogue_alfatoxins_guidance-2010_en.pdf

The RSD_{wR} can be determined by analysing (C)RMs or spiked samples (e.g. at LOQ and/or 2-10 times LOQ, or ML) either as part of the initial validation or during routine analysis together with the samples (details see 5.2).

For laboratories that (foresee to) apply the method not more than five times per year, the RSD_{wR} can be assessed during initial validation. The validation design should be such that data are generated on at least three different days/batches. It should be noted that the RSD_{wR} obtained this way may give an underestimation compared to an RSD_{wR} derived from routine analysis (see below).

For laboratories that (foresee to) apply the method on a more routine basis and therefore generate sufficient data through concurrent analysis of quality control samples, the RSD_{wR} is preferably determined based on at least six batches.

The RSD_{wR} shall be ≤20%.

Note: *in theory, the RSD_{wR} is expected to lie between the RSD_r and RSD_R. In (EC) No 401/2006 legislation, for certain mycotoxins a relationship $RSD_r \leq 0.66\ RSD_R$ was suggested, corresponding to 16.5% based on the 25% criterion for RSD_R indicated above. The RSD_{wR} then would lie in between, e.g. 21%. Currently, for mycotoxins and plant toxin in food/feed there is insufficient experimental evidence to support such relationships. Starting from an RSD_R of 25%, an RSD_{wR} of 20% was considered appropriate and set as criterion. Since the RSD_r requirements in (EC) No 401/2006 were in the 15%-40% range, it was decided to set the new requirement not below 20%, despite the resulting equivalence to the RSD_{wR} criterion.*

3.5 Limit of Quantification, LOQ for official analysis

The criteria in (EU) 2023/2782 and (EU) 2023/2783 apply to official control, i.e. analysis for enforcement purposes.

When a specific requirement for the LOQ of a toxin has been included in these regulations, the method shall have an LOQ at or below this value. In all other cases, the following applies:

LOQ shall be ≤ 0.5*ML, and should preferably be ≤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.

From a scientific perspective it does not make sense to sum individual LOQs into a sum-LOQ as parameter to compare against a sum-ML. Therefore, LOQs are determined and reported for individual toxins only.

3.6 Measurement uncertainty

3.6.1 Estimation of measurement uncertainty

For estimation of measurement uncertainty there are different approaches. Detailed background and descriptions can be found in documents from Eurachem [14], Nordtest [15], Eurolab [16] and Codex [17]. Examples have been worked out in these documents and also in SANTE/11312/2021 [13].

A laboratory may report a default expanded measurement uncertainty of 50% as indicated in CR 2023/2782 and 2023/2783, provided that the laboratory meets the precision requirements specified in 3.4, and achieves absolute mean z-scores below 2 (|z| ≤ 2) in proficiency tests for the applicable toxin/commodity group combinations.

3.6.2 Combined measurement uncertainty for sum toxin results

The combined measurement uncertainty for sum toxins is calculated by summation of the individual absolute uncertainties. An example is given in Table 3.

Table 3. Combined measurement uncertainty for summed toxins, example: sum of aflatoxins:

Toxin	Relative u[1]	Sample result (µg/kg)	Absolute u (µg/kg)
AFB1	14.7%	11.6	1.705
AFB2	12.3%	2.11	0.260
AFG1	19.2%	1.02	0.196
AFG2	18.4%	0.91	0.167
sum aflatoxins		15.64	
sum u	14.9%2)		2.328
Sum U (k=2)			4.656

with u = measurement uncertainty. U = expanded measurement uncertainty. k = coverage factor for expanded measurement uncertainty. [1] as established from on-going QC measurements. 2) from 2.328/15.64*100%

Note: the combined measurement uncertainty is often calculated using the law of error propagation, as the square root of the sum of squared absolute uncertainties. In the above example this would result in combined uncertainty (u) of 1.744 (11.2%). For the situation where the concentrations of individual analytes are summed, the method of error propagation is not considered applicable. The errors of the individual toxins are not (fully) independent and random, and that approach leads to an unrealistic low combined uncertainty that decreases with the number of analytes. Especially in case of pyrrolizidine alkaloids (sum of 35) the error propagation approach does not yield meaningful results.

Note: summation of the individual absolute uncertainties is the preferred harmonised approach. Where CRMs are available with a specified sum-toxin concentration, estimation based analysis of the CRM (e.g. Nordtest, using bias and variability for the sum) is also allowed.

4 Reporting

4.1 Recovery correction

Recovery correction (correction for method bias), if applicable (see EU 2023/2782 4.3.1), 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.

4.2 Unit and significant figures

The analytical result and the expanded measurement uncertainty are expressed in the same units as the MLs are stated in legislation. The number of significant figures (SF) of the MLs in legislation (CR (EU) 915/2023) appears not to be coherent (e.g. 0.025 (two SF), 5.0 (two SF), 10.0 (three SF), 15 (two SF), 15.0 (three SF), 1750 µg/kg (three SF), while in other cases the number of SF is not clear (e.g. 200, 1000 µg/kg). For this reason, the SF of MLs in legislation will be ignored. Instead, the number of SF will always be two for the measurement uncertainty and two or three for the analytical result according to the rounding procedure outlined in paragraph 4.4.

4.3 Reporting of sum-toxin results

In case the maximum level has been set for the sum of toxins (e.g. aflatoxins, T-2/HT-2-toxin, fumonisins, ergot alkaloids, pyrrolizidine alkaloids), the analytical results of all individual toxins shall be reported.

Exceptions:

Ergot alkaloids: it is also acceptable to report the sum of each of the six epimer pairs instead of the 12 individual epimers.

Pyrrolizidine alkaloids (PAs): in case of PAs that cannot be sufficiently separated and can only be integrated as one peak, then these PAs are reported together, either named individually or as isomer group, e.g.

PA1: x µg/kg
PA2 and PA3: x µg/kg
PA4-group as sum of x, y, z: x µg/kg
PA5: x µg/kg

Cannabinoids: if the analytical method inherently converts THCA into THC, then THCA cannot be reported and only THC (total) is reported.

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. In case all individual toxins are <LOQ, then either no sum result will be reported, or the sum will be expressed as zero.

As indicated in 3.5, it is not required to report a sum-LOQ since this is meaningless from an analytical perspective.

5 Method validation

Different designs for validation schemes exist. A well-known approach that is already used by many mycotoxin/plant toxin laboratories is the one used in pesticides residue analysis (SANTE/11312/2021 [13]). This approach consists of an initial validation that can be done on one day, followed by on-going validation which is done through QCs samples concurrently analysed with batches of routine samples. The approach is also considered suited for mycotoxins and plant toxins in food and feed. For sake of harmonisation and taking note of existing experiences, reference is made to [13] and a brief outline is included below. However, equivalent alternative approaches may also be used to demonstrate that the method is fit for purpose.

5.1 Initial method validation

Initial method validation is required for at least one matrix from each commodity group (see Appendix 3 and 4) for which the method will be applied. Validation needs to be performed for all toxins in the scope of the method, at least at two levels: the anticipated LOQ and a higher level (2-10x LOQ or ML). For each level typically six replicates are analysed. In addition, one procedural blank and at least one matrix blank should be included in the validation set.

Table 2. *Validation parameters and criteria for official analysis (compliance testing)**

Validation parameter	**What/how**	**Criterion**
Calibration/ quantification	≥ 5 levels, range ≤ LOQ to at least ≥1.5 highest spike level	deviation back calculated concentration from true concentration ≤ ±20% (see 3.2)
Matrix effect	Difference of response from standard in blank matrix extract and standard in solvent (either single point or slope of calibration line)	none (see 3.1)
LOQ	LOQ is defined as lowest level in sample tested that complies with criteria for recovery, precision and identification. Spiked samples at anticipated LOQ and assess recovery (trueness) and precision.	Analytical requirement: at LOQ level in sample, criteria for recovery, precision and identification should be met. Fit-for-purpose requirement for compliance testing: see 3.5

Selectivity (specifity)	Response in procedural blank and blank samples	≤ 30% of LOQ
Recovery	Replicate analysis of spiked samples, average recovery for each level tested	70-120% for each level tested (3.3.1)
RSD_r	Measurement of ≥ 6 replicates on same day/same batch	≤ 20% for each level tested (see 3.4.2)
RSD_{wR}	Single analysis of representative sample(s) on different days Methods not routinely applied (≤5x/year): ≥ 3 days Methods routinely applied (≥ 6x/year): use QCs concurrently analysed with samples, ≥ 6 days	≤ 20% for each level tested (see 3.4.3)
Identification	retention time, for MS: ion ratios and/or mass accuracy	See identification mycotoxins and plant toxins 01/01/2023

* Based on SANTE/11312/2021 [13].

5.2 Assessment of homogeneity of the comminuted laboratory sample

Homogenisation of the laboratory sample is part of the analytical procedure and needs to be assessed as far as possible. Certain mycotoxins (especially aflatoxins in cereals, nuts) and plant toxins (e.g. tropane alkaloids, pyrrolizidine alkaloids in herbs) are highly heterogeneously distributed in the laboratory sample. Appropriate homogenisation by dry-milling or, often better [22], slurry mixing, is essential before taking an analytical portion for extraction. The requirement for homogeneity is embedded in the requirement for repeatability (RSD_r ≤ 20%, see 3.4.2). Homogeneity can be experimentally assessed by analysing multiple (typically ≥6) analytical portions taken from a homogenised laboratory sample that contains the target toxin(s). It is recognized that real samples will not contain all target toxins from the scope of the method, and that it is impossible to assess homogeneity for all individual toxin/matrix combinations. However, for commonly occurring toxin/matrix combinations, this exercise can and should be done as part of the initial validation, using the homogenisation procedure as used in routine sample analysis.

5.3 On-going validation

The purpose of on-going validation is to assess consistency of average recovery to the value obtained during initial validation, and to obtain data on longer term within-laboratory reproducibility (RSD_{wR}). For this purpose, with each batch of routine samples, at least one QC sample (spiked sample, but also CRMs can be included) at LOQ or ≤10x LOQ, ML or a level of particular relevance for the samples being analysed should be included. In case the QC sample is not at LOQ, the sensitivity of the method needs to be verified by inclusion of a blank extract spiked at a level equivalent to the method LOQ, taking into account low absolute recoveries assessed during method development or initial validation.

On-going validation can also be used to demonstrate applicability of the method for other commodities from the same commodity group. For this, a blank sample of another commodity, and a spiked sample of that commodity (LOQ and optionally a higher level) should be included in addition or instead of the regular QC sample.

The batch QCs can be used to calculate the average recovery and the RSD_{wR} at each level (if applicable). Data from different matrices from the same commodity group may be pooled. Compliance of average recovery and RSD_{wR} demonstrates that the method is fit for purpose for the commodity/commodity group.

5.4 Extension of scope to other toxins

When additional analytes are added to the scope of an existing confirmatory method, a full validation is required to demonstrate the suitability of the method.

5.5 Extension of scope to other commodities

If the confirmatory 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 Annex 3 and 4) for which an initial validation has already been performed, a limited additional validation is sufficient. This can be done as described under on-going validation (see 3.7.2) or by analysis of a blank and duplicate spiked sample at LOQ level for the new commodity. In the latter case, the recovery should fall within the range as described under 3.3.2.

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[3] Commission implementing regulation (EU) 2023/2783 of 14 December 2023 laying down the methods of sampling and analysis for the control of the levels of plant toxins in food and repealing Regulation (EU) 2015/705.

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Appendix 1. Definitions for recovery.

1. IUPAC definition

Source: IUPAC Recommendations 2002, Pure Appl. Chem., 2002, 74(11), 2201-2205

RECOVERY or RECOVERY FACTOR: yield of a preconcentration or extraction stage of an analytical process for an analyte divided by amount of analyte in the original sample.

APPARENT RECOVERY: observed value, x(obs), derived from an analytical procedure by means of a calibration graph, divided by reference value, x(ref).

2. EU report 2004

Source: Report on the relationship between analytical results, measurement uncertainty, recovery factors and the provisions of EU food and feed legislation, with particular reference to community legislation concerning contaminants in food and undesirable substances in feed.

https://food.ec.europa.eu/system/files/2016-10/cs_contaminants_sampling_analysis-report_2004_en.pdf

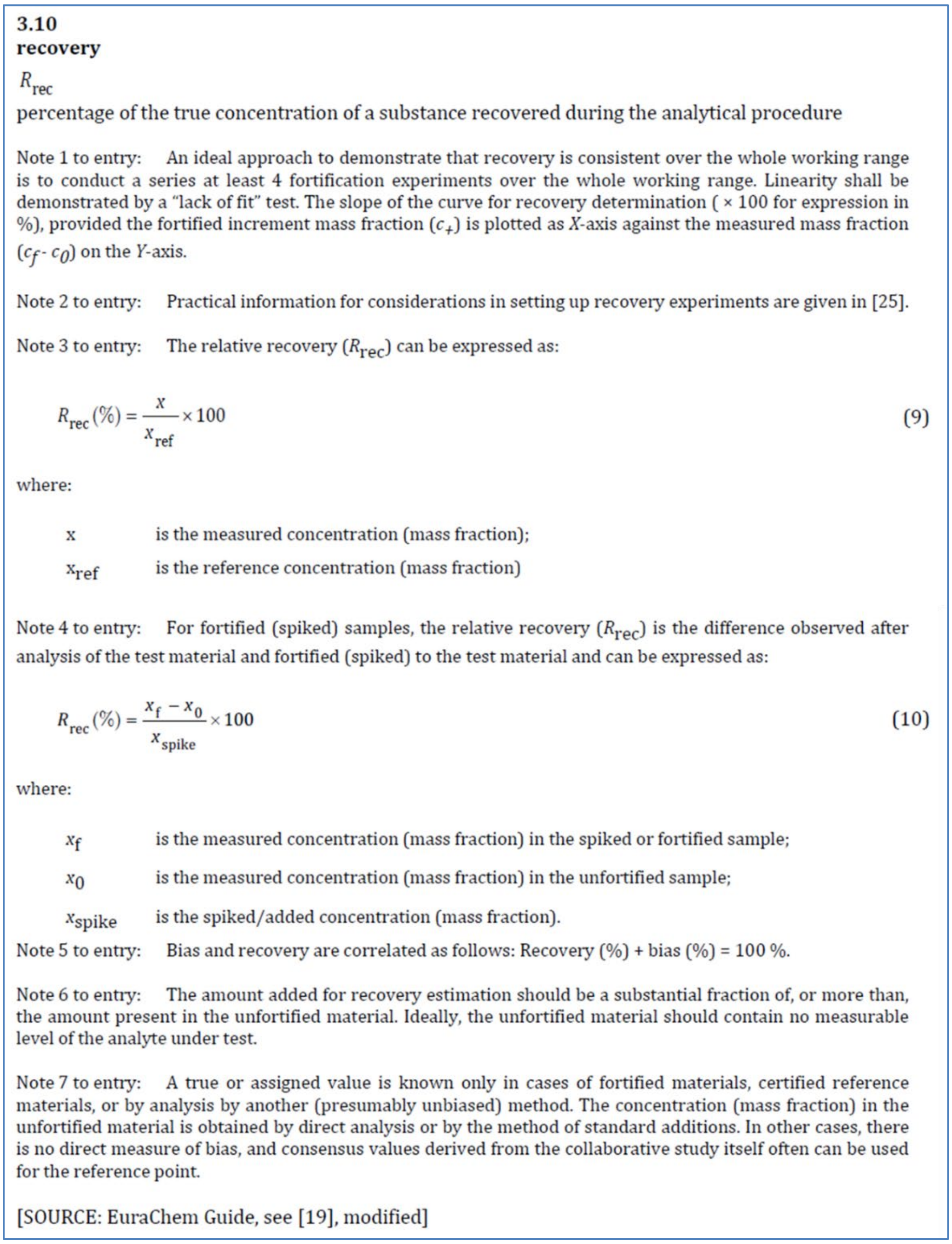
“Recovery” is the amount of material extracted for analysis as a fraction of the amount present. In most analytical work, not all the material is recovered (i.e. the “recovery” is less than 100%).

3. CEN TC327/WG5 (natural toxins in feed)

Source: CEN/TS 17455:2020, Animal feeding stuffs - Methods of sampling and analysis - Performance criteria for single laboratory validated and ring-trial validated methods of analysis for the determination of mycotoxins.

Recovery: percentage of the true concentration of a substance recovered during the analytical procedure (details see next page)

CEN/TS 17455:2020



3.10
recovery

R_{rec}

percentage of the true concentration of a substance recovered during the analytical procedure

Note 1 to entry: An ideal approach to demonstrate that recovery is consistent over the whole working range is to conduct a series at least 4 fortification experiments over the whole working range. Linearity shall be demonstrated by a "lack of fit" test. The slope of the curve for recovery determination (× 100 for expression in %), provided the fortified increment mass fraction (c_+) is plotted as *X*-axis against the measured mass fraction (c_f - c_0) on the *Y*-axis.

Note 2 to entry: Practical information for considerations in setting up recovery experiments are given in [25].

Note 3 to entry: The relative recovery (R_{rec}) can be expressed as:

$$R_{\text{rec}}(\%) = \frac{x}{x_{\text{ref}}} \times 100 \qquad (9)$$

where:

x is the measured concentration (mass fraction);

x_{ref} is the reference concentration (mass fraction)

Note 4 to entry: For fortified (spiked) samples, the relative recovery (R_{rec}) is the difference observed after analysis of the test material and fortified (spiked) to the test material and can be expressed as:

$$R_{\text{rec}}(\%) = \frac{x_{\text{f}} - x_0}{x_{\text{spike}}} \times 100 \qquad (10)$$

where:

x_{f} is the measured concentration (mass fraction) in the spiked or fortified sample;

x_0 is the measured concentration (mass fraction) in the unfortified sample;

x_{spike} is the spiked/added concentration (mass fraction).

Note 5 to entry: Bias and recovery are correlated as follows: Recovery (%) + bias (%) = 100 %.

Note 6 to entry: The amount added for recovery estimation should be a substantial fraction of, or more than, the amount present in the unfortified material. Ideally, the unfortified material should contain no measurable level of the analyte under test.

Note 7 to entry: A true or assigned value is known only in cases of fortified materials, certified reference materials, or by analysis by another (presumably unbiased) method. The concentration (mass fraction) in the unfortified material is obtained by direct analysis or by the method of standard additions. In other cases, there is no direct measure of bias, and consensus values derived from the collaborative study itself often can be used for the reference point.

[SOURCE: EuraChem Guide, see [19], modified]

Appendix 2. Commodity groups for the validation of analysis methods for food.

Commodity groups	Commodity categories	Typical representative commodities included in the category
1. 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, herbal infusions and tea (liquid) Purees intended for infants and small children
2. High oil content	Tree nuts Oil seeds and products thereof Oily fruits and products thereof	Walnuts, hazelnuts, chestnuts, almonds, pistachios Rapeseed, sunflower, cottonseeds, soybeans, peanuts, sesame seeds etc. Oils and pastes (e.g. peanut butter, tahina)
3. High starch and/or protein content and low water and fat content	Cereal grain and products thereof Dietary products	Wheat, rye, barley, maize, rice, oats Wholemeal bread, white bread, crackers, breakfast cereals, pasta Dried powders for the preparation of food for infants and small children
4. High acid content and high water content (*)	Citrus products	
5. Difficult or unique commodities (**)		Cocoa beans and products thereof, copra and products thereof, coffee, tea (dried product) Spices, liquorice root, herbal infusions (dried product), food supplements, pollen, and pollen products
6. High sugar low water content	Dried fruits	Figs, raisins, currants, sultanas
7. Milk and milk products	Milk Cheese Dairy products (e.g. milk powder)	Cow, goat and buffalo milk Cow, goat cheese Yogurt, cream
8. Meat (tissue)	Edible offals Muscle, processed meat products	Kidney, liver ham

(*) 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’ needs only to 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.

Appendix 3. Commodity groups for the validation of analysis methods for feed[1)].

Commodity groups	Commodity categories	Typical representative commodities included in the category
1. High water content	Forage crops	Grasses, clover, alfalfa
	Silage	Maize, clover, grasses
	By-products	Apple pomace, potato peels, molasses
2. High oil content and very low water content	Oil seeds, soil fruits, their products or by-products	Cotton seed, linseed, rapeseed, soybeans
3. Intermediate oil and low water content	Oil seed cake and meal	Cake/meal of soyabeans, sunflower, rapeseed
4. High starch and/or protein content and low water and fat content	Cereal grains, their products, by-products and food waste	Barley, oat, maize, rice, rye, spelt, triticale and wheat kernels, flakes, middlings, hulls and bran. Bread, brewers' and distillers' grains. Cereal based composite feed
	Legume seeds	Dried beans, peas, lentils Seed hulls
5. Difficult or unique commodities (*)	Straw	Barley, oat, maize, rice, rye and wheat straw
	Hay	Grasses
	Premixtures	By-products and food waste such as potato protein and fatty acid distillate
6. Milk and milk products	Milk	Milk replacer By-products and food waste such as whey.

[1)] for a comprehensive list of feed materials, see CR (EU) No 68/2013.

(*) 'Difficult or unique commodities' needs only to 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.