Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food

2016

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Table of Contents

1. Scope and Field of Application

The European Union Reference Laboratory (EURL) for Polycyclic Aromatic Hydrocarbons (EURL PAH), the EURL for Heavy Metals in Feed and Food (EURL HM), the EURL for Mycotoxins (EURL Mycotoxins), and EURL for Dioxins and PCBs in Feed and Food aim to provide with this document guidance to official food control in the EU on the estimation of the limit of quantification of analytical methods for the determination of individual substances in the field of contaminants in feed and food. The document focusses on estimation of the limit of detection (LOD) and/or limit of quantification (LOQ) of polycyclic aromatic hydrocarbons (PAHs), heavy metals (HM), mycotoxins, and polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs).

The concept presented here consists of two major routes: The first route responds to the requirements for determination of PAHs, HMs and Mycotoxins. The second route corresponds to PCDD/F and PCB analysis, where results are calculated as sum-parameters and expressed in toxic equivalents (TEQs), converting thereby congener concentrations together with estimated LOQs using toxic equivalency factor (TEFs) into TEQs. Due to the nature of this more complex procedure, emphasis is put in the area of PCDD/F and PCB analysis on the LOQs, while LODs are of minor consequence.

Terminology in this guidance document was adapted to the chemical analysis of contaminants in feed and food; hence, some of the general terms defined and used in international standards were replaced by more specific terms applicable to analytical chemistry. The presented statistical-mathematical approach is based on elements taken mainly from DIN 32645:2008-11 (DIN 2008) and ISO 11843-2:2000 (ISO 2000).

This document covers only quantitative methods of analysis.

The authors believe that LOD and LOQ values derived from the application of the presented experimental methodologies converge to a certain degree. Estimation of LOD/LOQ values based on blank measurements, and from calibration data is described. Signal-to-noise ratios are applied in the area of PCDD/Fs and PCBs. Mathematical terms and statistical background are presented as well.

This guide document shall be applied systematically if measurement results are used for monitoring purposes and exposure modelling. However, authors are aware that precise knowledge of LOD/LOQ may not be required when assessing compliance with maximum levels exceeding LOD/LOQ. This guidance document shall be applied if maximum levels and analysis results are close to the expected LOQ.

The authors acknowledge that the presented approach has its limitations and may not be generally applicable to all cases.

2. Acronyms and Definitions

a: Intercept of the calibration curve

α: Probability of detecting (erroneously) that a substance is present while truly, it is not (false positive results; type I error)

ß: Probability of (erroneously) not detecting that a substance is present while truly, it is (false negative results; type II error)

b: Slope of the calibration curve (determined by linear regression over a concentration range with a maximum calibration level equal to 10 times the level of LOD)

Background subtraction: Correction of the signal of a test sample by the signal of the background. Blanks, procedural blanks, or only pure solvents may be appropriate for determining the background, depending on the analysis task. The background signal may be representative for a single test sample only, or for a series of test samples.

Blank: Test sample in which the analyte is absent. It is characterized by a mean response value equal to zero ($\overline{y}_b = 0$) and standard deviation σ_b, which is approximated by $s_{y,b}$

Test method: Process covering both sample preparation and instrumental measurement

Confirmatory method: a method that provides full or complementary information enabling the PCDD/Fs and dioxin-like PCBs to be identified and quantified unequivocally at the maximum or in the case of need at the action level. Such a method utilises gas chromatography/high-resolution mass spectrometry (GC-HRMS) or gas chromatography/tandem mass spectrometry (GC-MS/MS)

HM: Heavy metals

iLOD: Instrumental limit of detection

iLOQ: Instrumental limit of quantification

K: Number of replicate analyses of the background/blank sample from which the mean signal forms a single data point for background correction

LOD: Limit of detection: Analyte content which can be distinguished from the blank with an error probability of (1-ß)

LOQ: Limit of quantification: Analyte content which can be determined with a certain level of precision

M: Number of replicate analyses of the test sample from which the mean signal forms a single data point for background correction

m: Number of replicate analyses on the test sample

N: Noise height

n: Number of replicate analyses on the blank sample

p: Number of calibration levels

PAH: Polycyclic aromatic hydrocarbons

PCB: Polychlorinated biphenyls

PCDD/F: Polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans

Prediction interval: Interval derived from a series of measurements, within which the next future measurement is expected with a certain level of probability.

Procedural blank: Sample for which the analytical procedure is executed in all aspects apart from adding the test portion. It consists of all chemicals and reagents. This analysis serves to estimate the background/interferences stemming from the test method, including solvents adsorbents, glassware, etc.

Pseudo-blank: (naturally occurring or spiked) Test sample in which the analyte is present at a concentration level close to (but not exceeding five times) the expected LOD.

q: Number of replicate analyses per calibration level

Q_x: Sum of squared differences of the individual calibration levels from the mean calibration level

$s_{y,b}$: Standard deviation of the blank (pseudo-blank) signals

$s_{y,net}$: Standard deviation of the net signal of paired observations

$s_{y,x}$: Standard deviation of residuals of calibration samples

$s_{y,x}/b$: Standard deviation of the procedure

S/N: Signal-to-noise ratio

TEF: Toxic Equivalency Factor

TEQ: Toxic Equivalents

Upper bound concept: The upper bound concept is applied in PCDD/F and PCB analysis for the calculation of sum TEQ values. Results for measurements that lead to the conclusion that the analyte content is below LOQ are replaced in the calculation of TEQ values by the corresponding LOQ.

WHO-PCDD/F-TEQ: Sum of polychlorinated dibenzo-para-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), expressed as World Health Organisation (WHO) toxic equivalent using the WHO-toxic equivalency factors (WHO-TEFs)

WHO-PCB-TEQ: Sum of polychlorinated biphenyls (PCBs), expressed as WHO toxic equivalent using the WHO-toxic equivalency factors (WHO-TEFs)

WHO-PCDD/F-PCB-TEQ: Sum of polychlorinated dibenzo-para-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and sum of polychlorinated biphenyls (PCBs), expressed as WHO toxic equivalent using the WHO-toxic equivalency factors (WHO-TEFs)

$\bar{x}$: Content value corresponding to mean calibration level

x_c: Critical value of the analyte content; analyte content corresponding to the critical value of the response variable y_c

x_i: Content value of the analyte at calibration level *i*

x_{LOD}: Content value at the limit of detection (LOD)

x_{LOQ}: Content value at the limit of quantification (LOQ)

y_i: Signal value of the measurement system (e.g. peak area) for calibration level *i*

y_j: Signal of the measurement system (e.g. peak area) for the analyte in test sample *j*

y_c: Critical signal value of the measurement system; exceeding this value of the signal (y) will lead with an error probability of α to the conclusion that the analyte is present in the test sample

$\hat{y}_i$: Value derived via the calibration function for level x_i ($\hat{y}_i=a+b*x_i$)

y_{net}: Net signal in paired observations

$\bar{y}_{net}$: Mean net signal of all pairs in paired observations

z_i: Value of noise signal *i*

$\bar{z}$: Average noise value

3. Principles

3.1. General

The limit of detection (LOD) and the limit of quantification (LOQ) are key parameters characterizing the performance of the *whole* test method at low concentrations.

Estimates of LOD and LOQ may be different among sample matrices covered by the analytical method. Hence, they need to be determined for each matrix. However, in multi-analyte / multi-matrix methods, determination of LODs and LOQs for each analyte / matrix combination may require tremendous efforts. Grouping/pooling of matrices based on expert knowledge could then be possible.

Where evidence can be provided that the analytical procedure does not contribute significantly to variability and bias of analytical results, LOD/LOQ may be derived from the instrumental limit of detection (iLOD), or instrumental limit of quantification (iLOQ) obtained from calibration standards.

Actually, in some cases, LOD and LOQ may be far below the working range of an analytical method used for the control of compliance of samples with maximum levels given in legislation. Data generated during validation of the performance of the method in the concentration (working) range relevant for control purposes may not be suitable for estimation of LOD/LOQ as the difference in concentrations between the working range of the method and the LOD/LOQ could lead to unrealistic LOD/LOQ estimates. Therefore, experiments for estimating LOD and LOQ have to be conducted at concentration levels close to the expected LOD and LOQ (this applies especially to estimates derived from calibration measurements). The "expected LOD and LOQ" are in this respect the analyte content levels, which the analysts expects, based on e.g. expert knowledge/experiences obtained in preceding experiments, to be equal to LOD/LOQ.

Instrument signals may be used for the estimation of LOD and LOQ only if the *identification criteria* for the corresponding analyte, e.g. retention time or relative ion intensities in mass spectrometry, are entirely met.

Evidence exists that observed discrepancies in LOD and LOQ values between laboratories may be considerable, and caused by both individual practical performance and the chosen experimental design. This guide aims to harmonise approaches for estimating LOD and LOQ.

Selection of the procedure for estimation of LOD and LOQ primarily depends on legal requirements, as well as availability of blank samples, the appearance of “noise” in chromatograms and its applicability for calculations, or the practicability of calibration experiments.

In the fields of chemical elements, PAHs and mycotoxins preference shall be given to the estimation procedure based on blank (matrix) samples, if available. Alternatively, LOD/LOQ may be estimated based on a calibration model using spiked blank or pseudo-blank (matrix) samples ,or by the paired observations approach. The

presented approaches account for degrees of freedom, probabilities for false positive and false negative decisions, as well as of analyte content/matrix related influences, in accordance to requirements set by the Codex Alimentarius Commission (Codex 2009).

In PCDD/F and PCB analysis using isotope-dilution mass spectrometry, LOQs are assessed either by signal-to-noise ratios or calibration experiments, taking into account procedural blank samples. Also, the approach as described for chemical elements, PAHs and mycotoxins may be applied.

Table 1: Approaches supported in the different areas for the estimation of LOD/LOQ

	S/N	Blank/pseudo-blank, paired observations	Procedural Blank	Calibration (spiked blank/pseudo-blank samples)
Chemical elements		x		x
Mycotoxins		x		x
PAHs		x		x
PCDD/Fs and PCBs	x		x	x

3.2. Mathematical Assumptions

Concepts and statistical-mathematical approaches presented in this guidance document are based on the following assumptions/conditions:

- normal distribution of the analytical response (signal) of blank and calibration samples,
- homoscedasticity (homogeneity of variances) over the calibrated range,
- replicate analyses of blank and calibration samples are independent,
- linearity between the analytical response and the analyte concentration close to the LOQ.
- probabilities for both false positive and false negative decisions of 0.05 are appropriate in the areas covered by the scope of this guidance document

In practice, however, these conditions are frequently not fully met. Hence, the derived estimates might be biased to a certain extent.

4. Samples for LOD/LOQ Estimation Studies

In the field of ubiquitous contaminants, blank samples may not always be available. As an alternative, low contaminated samples (pseudo-blanks) may be used. Homogeneity of variances of both the blank signals and the signals of the pseudo blanks is assumed. According to US EPA (US EPA 2014), the analyte concentrations in such low contaminated samples shall be equal or in the same concentration range as the estimated LODs, but should not exceed 5 times the level of their respective estimated LOD.

Blank samples or pseudo-blank samples must represent the physicochemical properties of the different matrices to be analysed. LOD/LOQ could be determined in each matrix individually. However, when similar matrix effects are expected, pooling of matrices (e.g. based on water, proteins, carbohydrates or fat contents, or food commodity) may be envisaged to reduce the analytical burden.

Depending on the scope of the analytical method, it may be advisable to include in the experiments for the estimation of LOD/LOQ blanks or pseudo-blanks for each matrix (or group of matrices) within the scope of the analytical procedure. This may lead to more conservative LOD/LOQ estimates than if only one particular blank/pseudo-blank sample was repeatedly analysed. Chapter 5.2 offers an experimental design for that purpose.

In both cases the same set of matrices used in the frame of the validation study should be used for the estimation of LOD/LOQ.

Pooling of extracts/digests of blanks or pseudo-blanks, followed by subsampling of the pooled extract/digest is not recommended for the estimation of LOD/LOQ because the influence of individual extractions on the variability of results is lost, which might lead to under-estimated LOD/LOQ estimates.

5. Limit of Detection

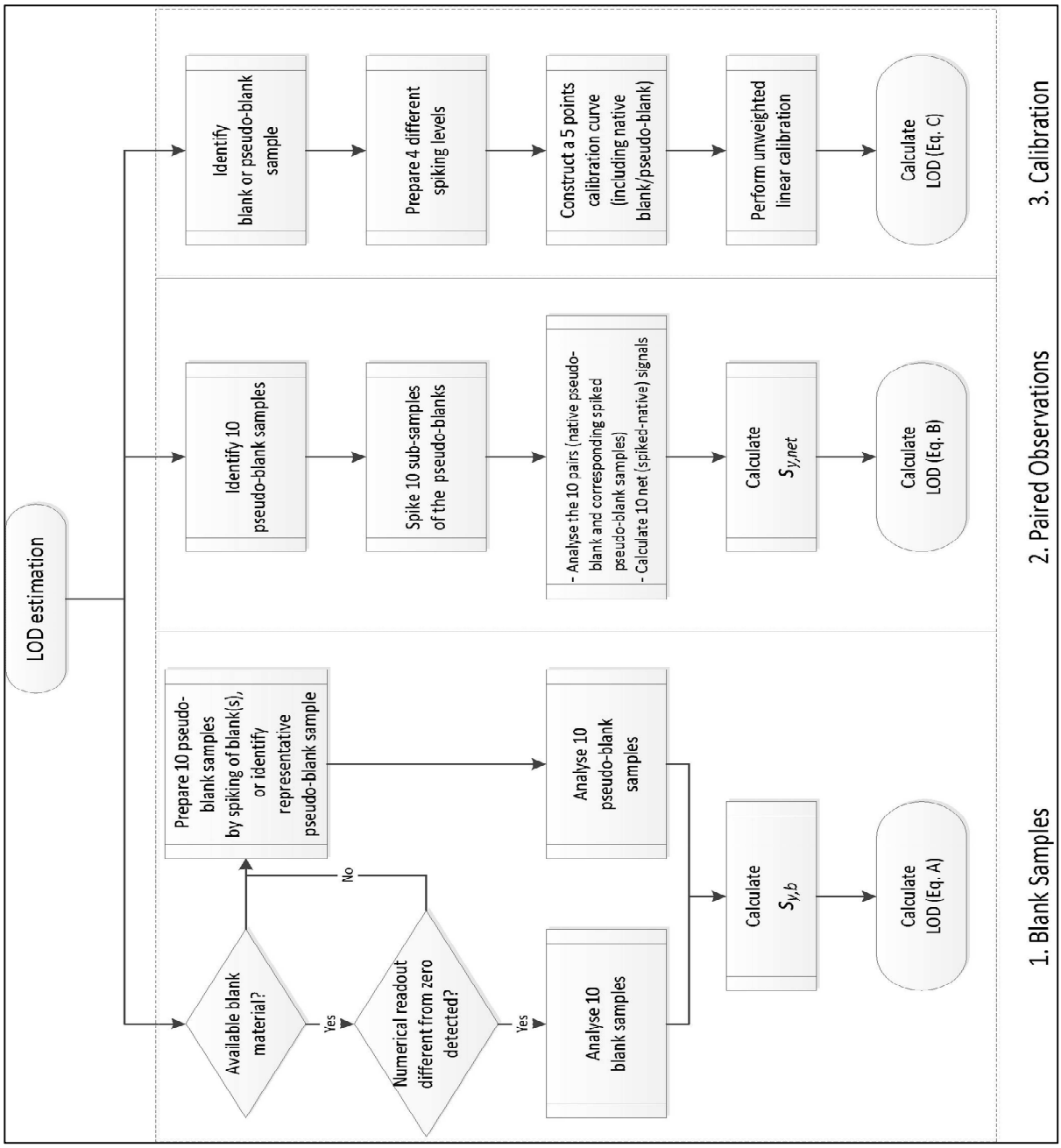
Different approaches may be applied for the estimation of LOD, such as calculation from the variability of blank signals or calibration experiments. If blank samples are not available or do not exist, pseudo-blanks or the paired observations approach may rather be applied.

Experiments for the estimation of LOD/LOQ are usually conducted under repeatability conditions unless high variability over time of blank values is expected. If so, LOD and LOQ estimates shall be derived under intermediate precision conditions.

In the field of PCDD/F and PCB analysis, results are calculated as sum-parameters based on concentrations and on LOQs only. Therefore, the emphasis is put on LOQs while LODs, within official control, do not carry any relevant information.

Figure 1 provides a flow chart for the estimation of the LOD in chemical elements, PAHs and mycotoxins analysis.

Figure 1: Flow chart for estimation of LOD, applied in the fields of heavy metal, mycotoxins, and PAH analysis



5.1. Estimation of LOD via Blank Samples

The variability of multiple analyses of representative matrix blank samples may be used to estimate the LOD of an analyte in the respective matrix. As a commonly accepted procedure for the estimation of LOD, 20 analyses of blank samples are certainly justified in cases of prohibited substances, where exceedance of LOD triggers immediate legal action. However, this is not the case for contaminants covered by the scope of this guidance document, for which no zero-tolerance applies. In the majority of cases, the respective legally binding maximum levels are significantly above LOD and LOQ as well.

Practical experience shows that 20 analyses of blank samples will result in slightly lower estimates of LOD if compared to performing less labour-intensive 10 analyses. Therefore, the four EURLs considered in their analytical fields a minimum of 10 analyses of blank samples under repeatability conditions sufficient and well-balanced with regard to experimental burden and expected gain in quality of the LOD.

Consequently, independent preparations of 10 blank samples, representing a particular, or, in cases where pooling of food matrices is possible and reasonable, different food matrices, have to be analysed each once. Where the measuring system does not provide suitable signals, a test portion of each blank is spiked with a low, but constant amount of analyte. The fortification level is preferably equal to the level of expected LOD, but not higher than five times the level of LOD. The native or spiked blanks (pseudo-blanks) are analysed each once under repeatability conditions.

If blanks do not exist a representative, naturally contaminated (native) pseudo-blank sample may be used instead. This sample will be analysed in ten replicates under repeatability conditions. However, this approach has only limited applicability as the derived method performance paramters (x_C, LOD, LOQ) are specific for this sample. They do not consider the variability of the test sample matrix. Hence, this approach is only recommended where the variability of a particular test sample matrix is low and does not influence significantly the variability of measurement results. If this condition is not met, the paired observations approach (see 5.2) may be chosen as an alternative.

The variability of signal values of the measuring instrument, expressed as standard deviation, obtained for the ten analyses of blanks, spiked blanks, or native pseudo-blank are used for the estimation of the critical value and of LOD and LOQ.

Note: The signals of the measuring instrument obtained for the analysed blank samples must be at levels, sufficiently high to allow the realisation of the full signal distribution. Blank (pseudo-blank) samples providing signal values of zero shall not be used for the estimation of the standard deviation of the blank (pseudo-blank) signals.

Calculation of LOD is performed according to *Equation A* under the condition of performing single analysis on the test sample, ten independent analyses of the blank(s), or pseudo-blank, and equal probabilities (α=ß=0.05) for false positive and false negative decisions.

$$x_{LOD} = 3.9 * \frac{s_{y,b}}{b} \qquad \textit{Eq. A}$$

x_{LOD}: Limit of detection
$s_{y,b}$: Standard deviation of the blank (pseudo-blank) signals
b: Slope of the calibration curve

Variable *b* in *Equation A* represents the slope of the calibration curve at concentration levels close to the expected LOD. The calibration model (e.g. external calibration, calibration by internal standardisation, matrix matched calibration) used for the analysis of routine samples has to be applied for deriving the slope of the calibration curve at concentration levels close to the expected LOD. The concentration levels of calibration standards are for this purpose preferably equidistant. The upper concentration level of calibration standards used for the estimation of the value of *b* should not exceed 10 times the expected LOD.

Equation A is only valid for the described experimental scenario. It shall be referred to Annex (A1.1), which presents the mathematical concept behind *Equation A* for estimating LOD according to different experimental scenarios (e.g. higher number of replicate analyses of the test sample, blank, or pseudo-blank).

Note: Appropriate equations for calculation of LOD in the case of background subtraction can be derived from the details provided in Annex (A1.2)

5.2. Estimation of LOD via Paired Observations

Many analyses suffer from the lack of blank samples due to the ubiquitous presence of the analytes. In such cases, the paired observation approach may be applied for the estimation of LOD. This approach has some merits if the estimated LOD is representative of a wider range of matrices (see the example given in A2.2), as it allows for the inclusion of different test samples (test sample matrices) in the experiments. The paired observation approach, allowing for the inclusion of different matrices, may reduce the effort necessary for estimating the LOD for analytical methods with a broad scope.

In the first step, 10 pseudo-blanks have to be identified. These samples represent one type or different types of food matrices. The analyte contents should be comparable, as portions of these samples will be spiked, each with the same small amount of analytes. The increase, by spiking, in analyte content levels is preferably equal to the level of expected LOD, but shall not exceed five times the expected LOD. In the next step, each portion of the native low contaminated samples and the respective spiked portion will be analysed under repeatability conditions.

Note: Different pairs may be analysed in different runs, i.e. under intermediate precision conditions. This may increase variability and therefore x_{LOD}.

The difference in signal abundances between the pairs of spiked portions and the corresponding native portions of each pseudo-blank is determined, and the variability of these differences is expressed as standard deviation. The standard deviation is used for calculation of LOD according to *Equation B*.

$$x_{LOD} = 5.2 * \frac{s_{y,net}}{b} \qquad \textit{Eq. B}$$

x_{LOD}: Limit of detection
$s_{y,net}$: Standard deviation of the net signals of paired observations
b: Slope of the calibration curve

Variable b in *Equation A* represents the slope of the calibration curve at concentration levels close to the expected LOD. The calibration model (e.g. external calibration, calibration by internal standardisation, matrix matched calibration) used for the analysis of routine samples has to be applied for deriving the slope of the calibration curve at concentration levels close to the expected LOD. The concentration levels of calibration standards are for this purpose preferably equidistant. The upper concentration level of calibration standards used for the estimation of the value of b should not exceed 10 times the expected LOD.

It should be stressed that *Equation B* is applicable only to the described experimental scenario. The mathematical concept of *Equation B* is presented in Annex (A1.2).

The statistics described for the paired observation approach also apply to measurements requiring background subtraction. Appropriate equations for calculation of LOD in the case of background subtraction can be derived from the details provided in Annex (A1.2).

5.3. Estimation of LOD via Calibration Approach

The experimental setup for estimating LOD from calibration data comprises for each matrix (or group of matrices) within the scope of the analytical procedure the analysis of representative blanks or pseudo-blanks spiked preferably at equidistant concentrations levels. For the purpose of this guide and in accordance with ISO 11843-2:2000 (ISO 2000), five concentration levels, including the zero spiking level shall be prepared in duplicate and analysed following the entire analytical procedure. The highest spiking level applied for the estimation of LOD should not exceed ten times the level of LOD. Additional calibration levels, with lower analyte concentration, shall be added to the calibration if it turns out that the highest calibrated level exceeded the threshold of ten times the level of LOD. The concentration levels exceeding the threshold will be not considered in the calculation of LOD.

Note: This approach is matrix specific, since it is based on 5 portions of the same blank/pseudo-blank sample (1 unspiked + 4 spiked ones).

Information on the expected LOD may not always be readily available. However, this problem can be solved by preliminary experiments resulting in a rough estimate of the expected level of LOD.

LOD is estimated according to *Equation C*.

$$x_{LOD} = 3.8 * \frac{s_{y,x}}{b} * \sqrt{1.1 + \frac{\overline{x}^2}{\sum_{i=1}^{n}(x_i - \overline{x})^2}} \qquad \textit{Eq. C}$$

x_{LOD}: Limit of detection
$s_{y,x}$: Standard deviation of the residuals
b: Slope of the calibration curve
$\overline{x}$: Mean calibration level
x_i: Content value of the analyte at calibration level i

ISO 11843-2:2000 acknowledges that different experimental designs may be reasonable and beneficial regarding costs. However, their mathematical treatment will be based on different assumptions and not necessary lead to the same equation reported here.

However, *Equation C* is applicable only to the experimental design comprising five calibration levels, duplicate analyses at each calibration level, single analysis of the test sample, and a probability level for false positive decisions of 0.05. Details on the mathematical model for estimating LOD based on a calibration design are provided in Annex (0).

If evidence is provided that the analyses of test samples are affected neither by matrix effects nor by interferences, pure calibration standards in solvent can be used for the experiments described above.

Note: The concentration range of the matrix calibration samples must not be too wide, as this will produce unrealistically high values for the estimates. A wide calibration range also favours the occurrence of heteroscedasticity of the measured signals. Ignoring heteroscedasticity may provide biased estimates, as leverage effects could come into play.

6. Limit of Quantification

Regulation (EU) 333/2007 (EU 2007) laying down the methods of sampling and analysis for the official control of the levels of several contaminants in foodstuffs, specifies the LOD as 3 times the standard deviation of the mean of blank determinations and LOQ as six or 10 times the standard deviation of the mean of blank determinations. Ten times the standard deviation of the blank corresponds, under the condition of constant standard deviations at low levels, to a relative standard deviation of 10 %. Depending on the definition of LOQ, ratios between LOQ and LOD of 2 or about 3.3 are obtained. For harmonising approaches, this guidance document specifies the relation between LOD and LOQ as follows:

$$x_{LOQ} = 3.3 * x_{LOD} \qquad \textit{Eq. D}$$

x_{LOQ}: Limit of quantification
x_{LOD}: Limit od detection

According to the IUPAC and Codex Alimentarius Commission recommendations [IUPAC 1995, Codex 2009], the analyst may set, based on experimental evidence, the level of LOQ to the analyte content level at which, in the absence of legislation specifying other levels of tolerable repeatability relative standard deviations (RSD_r), an analytical precision, of 10 % relative standard deviation (RSD) is achieved calculated on the results of at least six replicate analyses. This might be particularly relevant for mass spectrometry measurements, where mass spectra might be obtained with dominating base peak ions (usually used for quantitation) and low abundant qualifier ions. If the analytical protocol prescribes the qualification of peaks via one or more ion peak ratios, signals may be used for LOD estimations only when this provision is satisfied. Hence, peak identification via ion peak ratios, and consequently LOD estimations, might in such cases only be possible at levels at which the ions used for quantitation can already be measured with high precision. Consequently, the LOQ could be equal to the LOD. In these cases, identifying experimentally the analyte content level, at which an analytical precision of 10 % RSD, respectively the RSD_r value set in legislation, is provided, might avoid artificially high LOQ values, which would be derived by multiplying LOD values with the factor given in Eq. D.

This principle will be systematically implemented in the area of chemical elements, mycotoxins, and PAHs.

Table 2 provides a summary of equations applied for the estimation of LOD/LOQ according to the experimental designs earlier specified.

Table 2 Compilation of equations for estimation of LOD/LOQ

Approach	x_{LOD}	x_{LOQ}
Blank sample [*Eq. A*]	$3.9 * s_{y,b}/b$	
Paired observations [*Eq. B*]	$5.2 * s_{y,net}/b$	
Calibration [*Eq. C*]	$\mathbf{3.8} * \frac{s_{y,x}}{b} * \sqrt{\mathbf{1.1} + \frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}}$	
Reg. (EU) 333/2007 [*Eq. D*]		$3.3 * x_{LOD;}$ *or 10 % RSD, or* RSD_r *specified in legislation*

7. Limit of Quantification for the Analysis of Persistent Organic Pollutants, in particular, PCDD/F and PCBs, using Isotope Dilution Mass Spectrometry

Quantitative analysis of polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs) in a sample extract is frequently performed by gas chromatography coupled with high-resolution mass spectrometry (GC-HRMS) or tandem mass spectrometry (GC-MS/MS). Two or more characteristic ions of the relevant PCDD/F and PCB congeners, plus the ^{13}C-labelled ions of corresponding internal standards are monitored and quantified individually. Congener-based LOQs, above which quantitation is feasible with acceptable bias and precision are determined during the routine.

Besides the previously described approaches , congener-based LOQs can be estimated as described in EU regulations on analytical criteria (EU 2009, EU 2014). Two different approaches are presented, which take advantage of the quantitation of these compounds by isotope dilution mass spectrometry. One approach is based on the evaluation of the signal-to-noise ratios. The second approach is based on a calibration model proposed for low levels of noise.

Commission Regulations (EU) No 589/2014 (EU 2014) and No 152/2009 (EU 2009) define an "accepted specific limit of quantification of an individual congener in a sample" as "the lowest content of the analyte that can be measured with reasonable statistical certainty, fulfilling the identification criteria as described in internationally recognised standards […]."

"The limit of quantification of an individual congener may be defined as

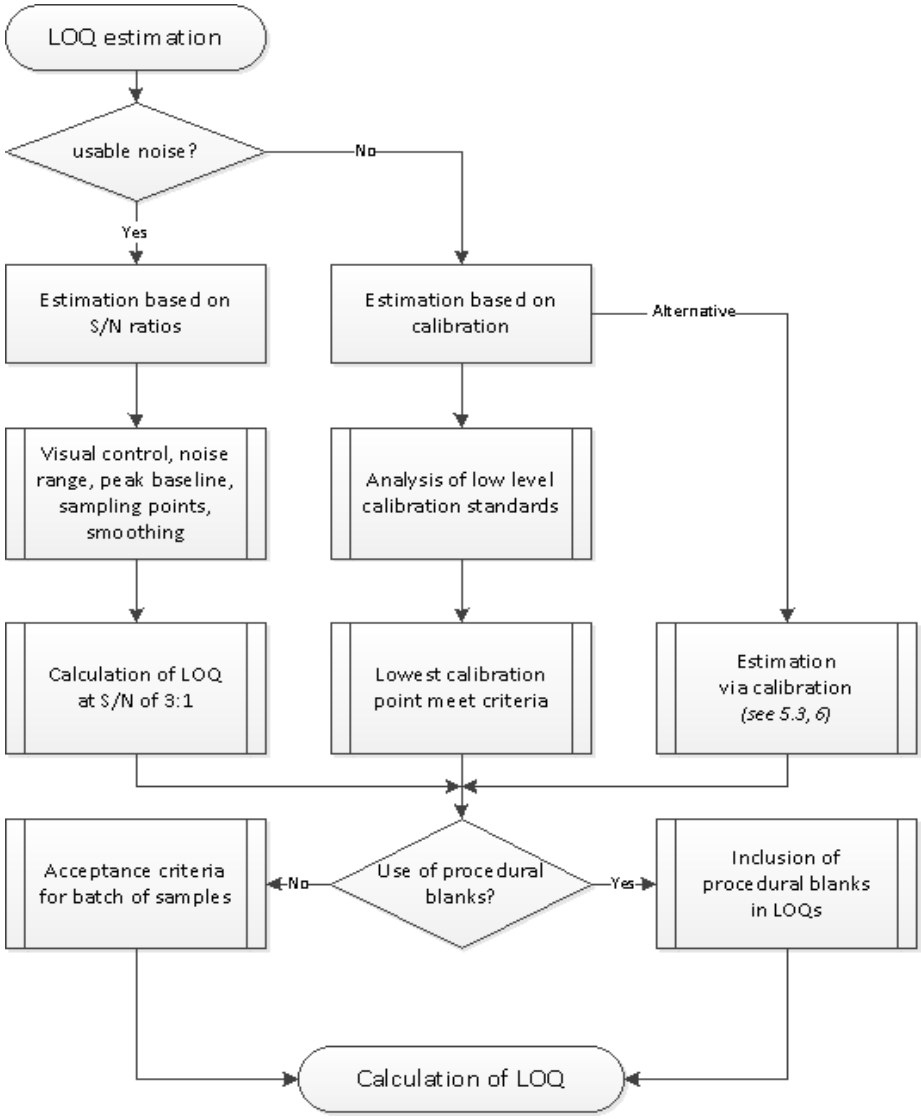
a) the concentration of an analyte in the extract of a sample which produces an instrumental response at two different ions to be monitored with a S/N (signal/noise) ratio of 3:1 for the less intensive raw data signal;

or, if for technical reasons the signal-to-noise calculation does not provide reliable results,

b) the lowest concentration point on a calibration curve that gives an acceptable (≤ 30 %) and consistent (measured at least at the start and at the end of an analytical series of samples) deviation to the average relative response factor calculated for all points on the calibration curve in each series of samples (The LOQ is calculated from the lowest concentration point taking into account the recovery of internal standards and sample intake.)."

More guidance on practical implementation is given in chapter 8.

Figure 2: Flow chart for LOQ estimation in the field of PCDD/Fs and PCBs



7.1. Estimation of LOQ from S/N Calculations

This approach identifies a specific LOQ for each congener and sample reflecting day-to-day method performance, related e.g. to the efficiency of extraction and clean-up, changes in sensitivity of the detection system over time, noise level, and recovery of internal standards. A congener-based LOQ is defined as the concentration of an analyte in the extract of a sample, which produces an instrumental response at two different diagnostic ion mass traces with a given signal-to-noise ratio (S/N) for both diagnostic ions. The respective higher LOQ value is used for further calculations.

LOQs are calculated for each congener in each sample of a series, from the signal-to-noise (S/N) ratios of the measured signals. Thereof derived estimates are corrected for recovery of the respective internal ^{13}C-labelled standards, and by taking into account contributions from the blank procedural sample of the series.

Commission Regulations (EU) No 589/2014 and No 152/2009 require a minimum S/N ratio of 3:1 for characteristic ion intensities versus the respective background noise(EU 2009, EU 2014). Therefore, within the scope of PCDD/F and PCB analysis, laboratories performing official feed and food control within the EU shall establish the S/N ratio preferably based on a noise height N of $2 \cdot \sigma_{noise}$ (σ_{noise} = standard deviation of the noise).

The LOQ is then calculated as the concentration corresponding to a signal (S), which is 3 times the noise height N (*Equation E*).

$$\boldsymbol{LOQ_{S/N=3} = 3 \cdot N = 6 \cdot \sigma_{noise}} \qquad \textbf{\textit{Eq. E}}$$

σ_{noise}: Standard deviation of the baseline noise
N: Noise height measured from the baseline: $N = 2 \cdot \sigma_{noise}$

Note: The approach to analytical noise chosen for determination of S/N may have a severe effect on the results. Many analysts apply the RMS algorithm, which downscales the noise level. For example, an S/N ratio derived from a particular chromatogram by the RMS algorithm might be several times higher than the S/N ratio calculated by the peak-to-peak algorithm from the same chromatogram, causing large differences in the deduced LOQ values.

Before a congener-LOQ is calculated, various procedures must be followed:

- visual check of chromatogram for the presence of signals and noise levels
- selection of the noise range
- setting of the peak baseline
- checking of the number of sampling points
- use of area/height transformation factor
- application of smoothing procedures
- use of procedural blanks

In addition, specific identification criteria apply. Requirements for relative ion intensities at and above the LOQ must be met. LOQ calculations are performed on both diagnostic ions. The respective higher LOQ value is used.

Specific LOQ values result for each congener in each sample, reflecting day-to-day variability of method performance, which might be related e.g. to

- changes in the efficiency of extraction and clean-up
- possible changes in sensitivity of the detection system over time
- the noise level (sample extraction, clean-up and instrument)
- the recovery of internal standards

Calculated LOQs may be verified with a sample matrix contaminated at low levels.

7.2. Estimation of LOQ from Calibration Standards

If the noise level is too small to perform a reliable signal-to-noise ratio calculation or no noise level is measurable at all, LOQ can be estimated according to the following approach, provided that matrix effects and interferences caused by the test sample do not contribute to variability and bias of the analytical results. Otherwise, matrix calibration is performed as described in chapters 5.3 and 6.

Note: The calibration range defines the working range of the analytical method. However, iLOQ may be even below this range. These iLOQs may then be approximated by measuring standard solutions with concentrations below the working range, followed by checking compliance with legal requirements

Congener-LOQs equal the lowest standard concentration meeting the following criteria as described in Commission Regulations (EU) No 152/2009 and No 589/2014 (EU 2009, EU 2014):

- retention time window (for all monitored ions),
- relative ion intensities (≤ 15 %),
- acceptable and consistent deviation (≤ 30 %, measured at least at the start and at the end of sample series) from the average relative response factor calculated at all points of the calibration curve.

Laboratories may use alternative approaches provided that identification and quantification criteria specified in Commission Regulations (EU) No 152/2009 and No 589/2014 are fulfilled.

Congener-LOQs are calculated from the iLOQs of the respective congeners by taking into account sample intake, final extract volume, and the recovery of the internal standard.

LOQ values may be verified with sample matrix contaminated at low levels.

8. Practical Implementation of LOQ Estimation in the Field of PCDD/F and PCB Analysis

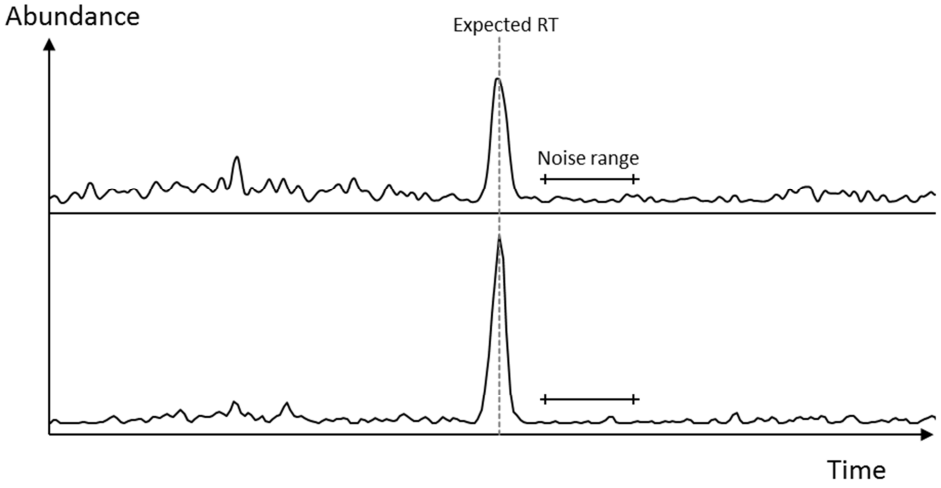
8.1. Estimation of Congener-based LOQs from S/N Calculations

This approach is recommended to be applied if chemical noise is present in the relevant mass traces of the individual congeners. The following criteria should be fulfilled:

A. Visual Control of the Noise Range

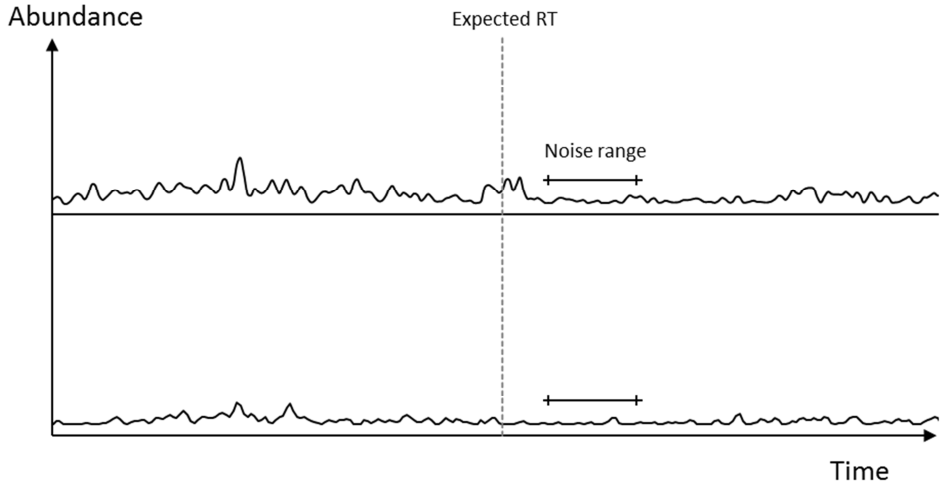
A.1 Peaks on both mass traces, fulfilling all identification criteria

In case peaks are present at the expected retention time in both mass traces, thus fulfilling the identification criteria for relative ion intensities, then a retention time window with a representative noise level prior or after the expected retention time is used to calculate LOQ. The concentration of the analyte is compared with the corresponding LOQ.



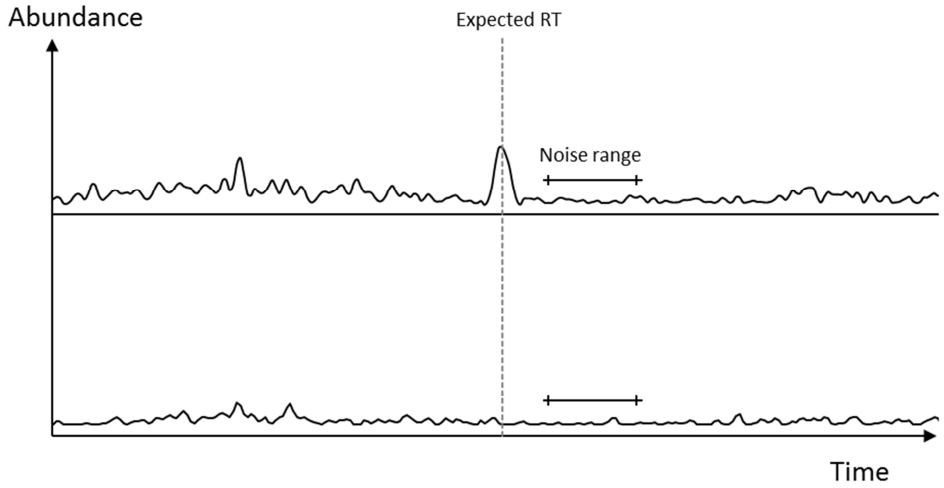
A.2 No peaks at expected retention time on both mass traces

In case peaks are not present at the expected retention time (identified by ^{13}C-labelled internal standards) on both mass traces above the respective noise level, the height of the noise around the expected retention time of the individual peak is applied for the calculation of the hypothetical S/N ratio.



A.3 Peak at expected retention time on 1 mass trace

In case a peak with abundance above the noise level is present at the expected retention time of the respective analyte on only one of the two mass traces, the height of the noise level around the expected retention time of the individual peak is applied for the calculation of S/N ratio for the mass trace without peak.

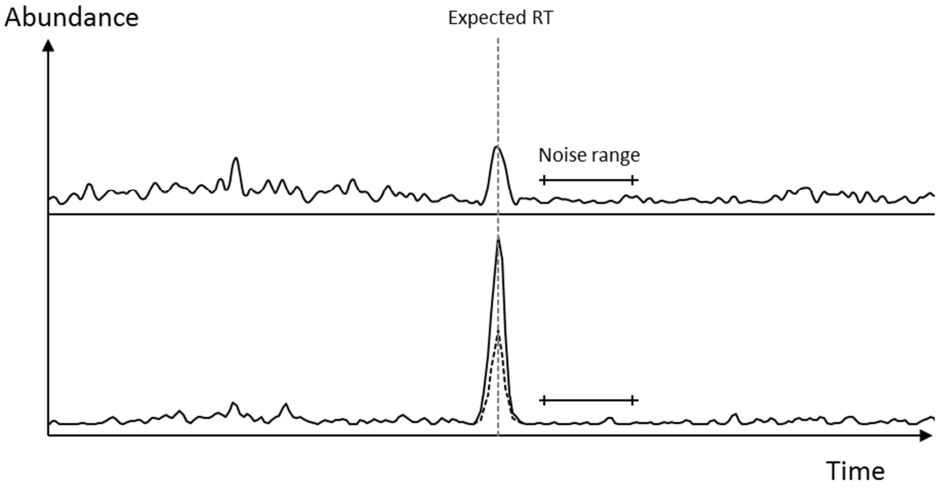


A.4 Peaks on both mass traces, not fulfilling all identification criteria

In case, a peak is present in both mass traces at the expected retention time, but the identification criteria for relative ion intensities are not fulfilled, the presence of the analyte of interest is still possible. It is assumed that interference affects, at least, one of the peaks. Various possibilities may explain this situation: 1., one or both signals of the potentially present analyte may be superimposed by interference, 2., peaks are out of ratio due to ion statistics, or 3., due to a bias of the instrument (e.g. not adequately corrected drift).

a) In the case of chemical interferences, repetition of the measurement using more suitable gas chromatographic conditions may lead to a better separation of possible interference from the target compound.

b) Alternatively, as a worst-case scenario, the analyst chooses the signal for LOQ estimation, which based on the expected relative ion intensity is assumed to contain the lower level of the (additive) interference. Compliance with the identification criterion for relative ion intensities is, thereby, assumed. The highest possible level of the analyte is then equal to or less than the level corresponding to this signal which is used to calculate LOQ.



B. Selection of the Noise Range/Segment

- A noise level reflecting the noise at the expected retention time of the respective peak (identified by the retention time of the respective ^{13}C-labelled standard) shall be used.
- The noise range selected in the sample for calculating S/N ratios shall be representative and as close as possible to the respective analyte peak.
- Because only narrow time windows can be used for data sampling, the width of the noise range may be as small as 5 times the peak width at half the height of the corresponding ^{13}C-labelled analogue, or the width of this peak if area units are used for noise estimation.

Note: The noise level determined off-peak is not necessarily identical with the noise level at the position of the peak. Substances eluting in the chosen "noise region", may positively bias the noise level, whereas interferences co-eluting with the analyte peak may remain undetected; hence falsifying the signal levels. Overcoming this problem by determining the noise level at the retention time of the peak in a blank sample is often not possible.

C. Setting of the Peak Baseline

In the evaluation of small peaks in the range of the LOQ, the setting of the peak baseline can profoundly influence the result. The peak height should be the top height of the peak from the baseline, which is determined by the average noise level. After a software-modeled baseline fit, a visual check shall be performed.

D. Number of Sampling Points

The data acquisition rate or sampling rate is an important detector parameter, which has a significant impact on the definition of a peak. Each peak shall consist of a minimum of 6 - 8 data points across the full peak.

E. Conversion Factor

Measurement of the S/N ratio is based on height values while quantification of analytes is based on peak areas values. The analyst must check if LOQs based on peak and/or noise heights can be used for comparison with analyte concentrations calculated from peak areas. If necessary, a suitable conversion factor must be applied.

F. Smoothing Procedures

The smoothing option should be used in a way that does not inappropriately influence or significantly alter peak heights or areas. However, if applied, it must be used consistently on all relevant mass traces recorded for all samples and calibration standards. In smoothing, the abundances of individual data points are modified so that signal abundances, which are higher than those of immediately adjacent data points are reduced, and signal abundances lower than those of adjacent points are increased. The commonly used *Savitzky-Golay* smoothing is based on least-squares fitting of polynomials to segments of the data. Compared to sliding-average smoothing, the *Savitzky-Golay* smoothing is less effective at reducing noise, but more effective at retaining the shape of the original signal.

G. Software-based S/N Calculations

Commercial software solutions estimating S/N ratios must ensure that results are in-line with the aspects discussed above.

NOTE: Modern instrument data processing software frequently offers the possibility to determine S/N ratios automatically. However, different instrument manufacturers do not necessarily use the same algorithms in their data processing software for calculating S/N ratios despite applying the same noise approach. Further, it would be desirable to enable the analyst to specify in the chromatogram both the position of the analyte signal and the retention time segment used for noise measurements. However, this option is not yet realized in most manufacturers' specific software solutions.

H. Techniques for improving the S/N Ratio

If smaller LOQs are intended to be achieved, various analytical and/or measurement techniques may be applied to increase the S/N ratio. In principle, they aim at either increasing the measurement signal or at reducing the measured noise or both. Resulting analyte signals must always comply with the above requirements. However, if such techniques are used, they must be used consistently for all samples and calibration standards.

8.2. Estimation of Congener- based LOQs from Calibration Standards

This approach may only be applied if matrix effects and interferences on congener results have proven to be negligible. As an indication, results from spiked samples should match results from measurement of the corresponding calibration standards.

The following criteria should be fulfilled for individual congeners:

- the working range of the instrumental calibration should correspond to the method working range
- the repeatability of results measured for a sample spiked at the level of interest should be comparable with the repeatability of the corresponding calibration standard

The iLOQ calculated from calibration, taking into account sample intake, final extract volume and the recovery of the internal standard, may then be used as LOQ of the individual congener in the respective sample series.

8.3. Use of Procedural Blanks

Procedural blanks are analyzed with every batch of test samples providing information on method performance, such as effects/interferences from the test method. They shall be monitored in QC charts and checked for acceptance of a batch of samples by comparing the measured blank with these charts. If acceptance criteria are met, LOQs calculated according to chapter 7.1., 7.2. or 5.3./6. are applied. In case these criteria are not met, the analyst must check, if the batch of samples has to be repeated.

Alternatively, if calculated LOQs or measured analyte contents of procedural blanks are higher than analyte contents in test samples of the same batch, the values estimated/measured in the procedural blanks are applied as LOQs for these test samples (taking into account sample intake and recovery of internal standards for the respective sample). If the estimated/measured values of procedural blanks are lower than the values of test samples, the values of the test samples are used for TEQ calculations.

9. Estimation of LOQs for Sum Parameters

In PCDD/F and DL-PCB analysis, legal limits (maximum levels and/or action levels) are only given for the sum-parameters (EC 2002, EU 2006, EU 2013) WHO-PCDD/F-TEQ, WHO-PCB-TEQ, and WHO-PCDD/F-PCB-TEQ. Therefore, the concentrations measured for individual congeners are converted into individual toxic equivalents (TEQs) by multiplication with the congener-specific toxic equivalency factor (TEF). Summing up the resulting toxic equivalents of each congener leads to the sum toxic equivalents.

The LOQ associated with a WHO-TEQ sum parameter must not exceed the respective target limit of quantification (target-LOQ), being approximately one fifth of the maximum level to be checked (EU 2009, EU 2014).

Following a practical approach, WHO-PCDD/F-TEQ, WHO-PCB-TEQ, and WHO-PCDD/F-PCB-TEQ values are calculated for the procedural blank representative for the respective series of samples. In case an individual congener cannot be quantified, the congener's LOQ is used (upper bound concept) for TEQ calculation. These WHO-TEQ values of the procedural blank are then used as LOQs, representing the laboratory's contribution (e.g. from the environment, chemicals, materials, glassware, apparatus, etc.) to blank signals.

10. References

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ANNEX

A1. Mathematical approaches

The mathematical background for the blank (pseudo-blank) and calibration approaches presented in this guidance document for estimation of LOD is primarily based on DIN 32645:2008-11. Data from the literature supplements this.

A1.1. Critical Value and LOD based on Blank

A1.1.1. General

For the purpose of this guidance document, ten blank samples have to be identified, representing a particular, or, in cases where pooling of food matrices is possible and reasonable, different food matrices. Where the measuring system does not provide suitable signals, a test portion of each blank is spiked with a low, but constant amount of analyte. The fortification level is preferably equal to the level of expected LOD, but not higher than five times the level of LOD. The native or spiked blanks (pseudo-blanks) are analysed each once under repeatability conditions.

If blanks do not exist a representative, naturally contaminated (native) pseudo-blank sample may be used instead. This sample will be analysed in ten replicates under repeatability conditions. However, this approach has only limited applicability as the derived method performance paramters (x_C, LOD, LOQ) are specific for this sample. They do not consider the variability of the test sample matrix. Hence, this approach is only recommended where the variability of a particular test sample matrix is low and does not influence significantly the variability of measurement results.

The variability of signal values of the measuring instrument, expressed as standard deviation, obtained for the ten analyses of blanks, spiked blanks, or native pseudo-blank are used for the estimation of the critical value and of LOD and LOQ.

A1.1.2. Critical Value

The critical value of the response variable (y_c) represents the signal level at which the probability of making false positive decisions (α) reaches a defined value.

Within the scope of this document, the error probability α is set to the value 0.05.

Besides, the magnitude of y_c depends on the estimated dispersion of the blank signals and also on the experimental design, which is reflected in the degrees of freedom and the expression under the square root.

It is defined by

$$\boldsymbol{y_c = \bar{y}_b + t_{\alpha;\upsilon} * \ s_{y,b} * \sqrt{\frac{1}{m} + \frac{1}{n}}} \qquad \textit{Eq. A 1}$$

y_c: Critical value of the response variable
$\bar{y}_b$: Mean of the blank signals
$t_{\alpha;\upsilon}$: Value from t-distribution for probability level α (one-sided test) and υ=n-1 degrees of freedom, with n being the number of experiments conducted for the estimation of the standard deviation of the blank
$s_{y,b}$: Standard deviation of the blank
m: Number of replicate analyses on the test sample
n: Number of replicate analyses on the blank sample

For 10 analyses of blank samples and single analysis per test sample *Equation A 1* becomes *Equation A 2*:

$$\boldsymbol{y_c = \bar{y}_b + 1.922 * s_{y,b}} \qquad \textit{Eq. A 2}$$

y_c: Critical value of the response variable
$\bar{y}_b$: Mean of the blank signals
$s_{y,b}$: Standard deviation of the blank

The critical value of the analyte content (x_c) is derived from the critical value of the response variable (y_c) via the slope (b) of the calibration function: $y_i = a + b * x_i$:

$$\boldsymbol{x_c = \frac{y_c - \bar{y}_b}{b} = \frac{s_{y,b}}{b} * t_{\alpha;\upsilon} * \sqrt{\frac{1}{m} + \frac{1}{n}}} \qquad \textit{Eq. A 3}$$

x_c: Critical value of the analyte content
$s_{y,b}$: Standard deviation of the blank
b: Slope of the calibration curve
$t_{\alpha;\upsilon}$: Value from t-distribution for probability level α (one-sided test) and υ=n-1 degrees of freedom, with n being the number of experiments conducted for the estimation of the standard deviation of the blank
m: Number of replicate analyses on the test sample
n: Number of replicate analyses on the blank sample

Equation A 3 becomes *Equation A 4* under the condition of 10 analyses of a suitable blank, single analyses per test sample, and a type I error probability of α=0.05.

$$\boldsymbol{x_c = 1.922 * \frac{s_{y,b}}{b}} \qquad \textit{Eq. A 4}$$

x_c: Critical value of the analyte content
$s_{y,b}$: Standard deviation of the blank
b: Slope of the calibration curve

A1.1.3. Limit of Detection

The LOD is derived from the critical value of the analyte content (accounting for the type I error), and the probability *ß* of committing a type II error (false negative decision). For the scope of this guide α=ß=0.05.

$$\boldsymbol{x_{LOD} = x_c + t_{ß;\,\upsilon} * \frac{s_{y,b}}{b} * \sqrt{\frac{1}{m} + \frac{1}{n}}} \qquad \textit{Eq. A 5}$$

x_{LOD}: Content level at LOD
x_c: Critical value of the analyte content
$t_{ß;\upsilon}$: Value from t-distribution for probability level ß (one-sided test) and υ=n-1 degrees of freedom, with n being the number of experiments conducted for the estimation of the standard deviation of the blank
$s_{y,b}$: Standard deviation of the blank measurements
b: Slope of the calibration curve
m: Number of replicate analyses on the test sample
n: Number of replicate analyses on the blank sample

As this guidance documents specifies the same level of probability for α and *ß* *Equation A 5* can be simplified to *Equation A 6*:

$$\boldsymbol{x_{LOD} = 2 * x_c} \qquad \textit{Eq. A 6}$$

x_{LOD}: Content level at LOD
x_c: Critical content level

Equation A 6 becomes *Equation A 7* under the condition of 10 analyses of blank samples and single analyses per test sample are performed, with a type I error probability of α=0.05.

$$\boldsymbol{x_{LOD} = 3.85 * \frac{s_{y,b}}{b} \approx 3.9 * \frac{s_{y,b}}{b}} \qquad \textit{Eq. A 7 (= Eq A)}$$

x_{LOD}: Content level at LOD
$s_{y,b}$: Standard deviation of the blank measurements
b: Slope of the calibration curve

A1.2. **Critical Value and LOD based on Paired Observations**

A1.2.1. General

The correction of the signal of a test sample by the signal of the unspecific background (blank) is performed in many types of analyses. In the simplest case, a constant background signal is subtracted from the signal of the test sample (spiked pseudo-blank). In a more complicated case, each test sample (spiked pseudo-blank) measurement is compensated by the measurement of a background/blank signal (y_{BL}), which is conducted directly before, after, or in parallel to the measurement of the test sample (spiked pseudo-blank). Both the results measured for the test sample (spiked pseudo-blank) and those measured for the background/blank are associated with uncertainties. Hence, the law of error propagation applies to the net signal, and the standard deviation of the net signal is corrected by a factor indicating the quality of the estimation of the standard deviations.

For the purpose of this guidance document, ten pseudo-blank samples have to be identified, representing a particular, or, in cases where pooling of food matrices is possible and reasonable, different food matrices. Two test portions are taken from each pseudo-blank sample. One test portion is analysed as it is, whereas the second test portion is spiked with a low amount of analyte. The fortification level is preferably equal to the level of expected LOD, but not higher than five times the level of LOD. The pairs of native and spiked test portions are analysed each once under repeatability conditions and the variability of the net signal corresponding to the spiked amount of analyte, expressed as standard deviation, is used for calculation of x_c, LOD, and LOQ.

A1.2.1. Critical value

The net signal is derived from *Equation A 8*:

$$\boldsymbol{y_{net} = y_j - y_{BL}} \qquad \textit{Eq. A 8}$$

y_{net}: Net signal in paired observations
y_j: Signal of the measurement system for the analyte in test sample *j*
y_{BL}: Signal of the measurement system for the analyte in the sample characterising the background (blank, pseudo-blank, or procedural blank sample)

Note: The symbol y_{BL} is used to avoid confusion with the signal of blank matrix samples (y_b) as this background/blank signal may be formed either from real blank matrix samples, from low contaminated samples (pseudo-blank), or, as practiced for some measurements, only from procedural blank samples.

The variance of the net signal is composed of:

$$\boldsymbol{s^2_{y,net} = s^2_y + s^2_{y,BL}} \qquad \textit{Eq. A 9}$$

$s^2_{y,net}$: Variance of the net signal
$s^2_{y:}$ Variance of the signal of the test sample (spiked low contaminated sample)
$s^2_{y,BL}$: Variance of the blank/background signal

The correction factor (η) accounting for the combined uncertainty is given by

$$\boldsymbol{\eta = \frac{n_y}{M} + \frac{n_{BL}}{K}} \qquad \textit{Eq. A 10}$$

η: Correction factor
n_y: Number of test sample measurements, included in a single correction
n_{BL}: Number of the background/blank measurements used for the correction of the test sample measurement
M: Number of replicate analyses of the test sample from which the mean signal forms a single data point for background correction
K: Number of replicate analyses of the background/blank sample from which the mean signal forms a single data point for background correction

Note: Presuming the correction of each test sample signal with an individual background measurement, both derived from single analyses on the respective samples, η assumes the value of two.

The critical value of the response variable is derived for paired observation from *Equation A 11*.

$$\boldsymbol{y_c = \bar{y}_{net} + t_{1-\alpha;\upsilon} * \ s_{y,net} * \sqrt{\eta}} \qquad \textit{Eq. A 11}$$

y_c: Critical value of the response variable
$\bar{y}_{net}$: Mean net signal of all pairs in paired observations
$t_{1-\alpha;\upsilon}$: Value from t-distribution for probability level 1-α (one-sided test) and υ=n-1 degrees of freedom, with n being the number of paired observations included in the estimation of the variance of the net signal
$s_{y,net}$: Standard deviation of the net signal of paired observations
η: Correction factor (see *Equation A 10*)

For paired observations the critical value of the analyte content is estimated in analogy to *Equation A 3* by *Equation A 12*, which takes into account the specific conditions of the applied experimental design.

$$\boldsymbol{x_c = \frac{s_{y,net}}{b} * t_{1-\alpha;\upsilon} * \ \sqrt{\eta}} \qquad \textit{Eq. A 12}$$

x_c: Critical value of the analyte content
$s_{y,net}$: Standard deviation of the net signal of paired observations
b: Slope of the calibration curve
$t_{1-\alpha;\upsilon}$: Value from t-distribution for probability level 1-α (one-sided test) and υ=n-1 degrees of freedom, with n being the number of paired observations included in the estimation of the variance of the net signal
η: Correction factor (see *Eq. A 10*)

A1.2.2. Limit of Detection

As for the blank approach, LOD is calculated according to *Equation A 6*

$$\boldsymbol{x_{LOD} = 2 * x_c} \qquad \textit{Eq. A 6}$$

x_{LOD}: Content value at the LOD
x_c: Critical level of the analyte content

Equation A 12 and *Equation A 6* can be combined to *Equation A 13*, considering the experimental conditions specified in this guidance document (n=10 paired observations, α=0.05), single analysis per test sample.

$$\boldsymbol{x_{LOD} = 5.185 * \frac{s_{y,net}}{b} \approx 5.2 * \frac{s_{y,net}}{b}} \qquad \textit{Eq. A 13 (= Eq B)}$$

x_{LOD}: Content value at the LOD
$s_{y,net}$: Standard deviation of the net signal of paired observations
b: Slope of the calibration curve

This approach is generally valid if analysis data are corrected for background levels. The experimental design of the background correction will be reflected in *Equation A 10* and consequently in the constant term in *Equation A 13*.

A1.3. **Critical Value, and LOD based on Calibration**

A1.3.1. General

For the purpose of this guidance document, the calibration function for the estimation of the parameters x_c, LOD and LOQ shall be based on at least five calibration levels, including the blank. All calibration levels have to be realized in the matrix by spiking of blanks. The calibration levels shall be equidistant and range from blank level (zero spiking level) up to a maximum of ten times the level of LOD. Low contaminated samples (pseudo-blanks) may be used if blanks are not readily available. At least two independent replicate analyses shall be performed at each calibration level, applying for each analysis the whole analytical procedure. In the following scenario one analysis of the test sample is to be performed.

Note: *ISO 11843-2:2000 recommends to perform on each calibration sample, including the blank, at least, two replicate analyses and to perform each instrumental measurement at least twice. The number of preparations per calibration sample should be identical with the number of preparations for routine samples.*

A1.3.2. Critical value

The critical value of the response variable is defined by:

$$\boldsymbol{y_c = a + \Delta a} \qquad \textit{Eq. A 14}$$

a: *Intercept of the calibration curve*
Δa: *Dispersion of a*

The dispersion of a, which defines the critical level of the response variable can be derived from the standard deviation of the residuals, as given in *Equation A 19*.

$$\boldsymbol{y_c = a + s_{y,x} * t_{\alpha;v} * \sqrt{\frac{1}{m} + \frac{1}{p*q} + \frac{\bar{x}^2}{Q_x}}} \qquad \textit{Eq. A 15}$$

a: Intercept of the calibration curve
$s_{y,x}$: Standard deviation of the residuals:

$$s_{y,x} = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_i - y_i)^2}{(p * q) - 2}}$$

$t_{\alpha;\upsilon}$: Value from t-distribution for probability level α (one-sided test) and $\upsilon=(p*q)-2$ degrees of freedom, with p being the number of calibration levels, and q the number of replicates at each calibration level
m: Number of replicate analyses of the test sample
p: Number of calibration levels
q: Number of replicate analyses per calibration level
$\bar{x}$: Content value corresponding to mean calibration level
x_i: Content value of the analyte at calibration level i
$\hat{y}_i$: Value derived via the calibration function for calibration level x_i ($\hat{y}_i=a+b*x_i$)
y_i: Signal value of the measurement system (e.g. peak area) for the calibration level i
Q_x: Sum of squared differences of the individual calibration levels from the mean calibration level:

$$Q_x = \sum_{i=1}^{n}(x_i - \bar{x})^2$$

The critical value of the analyte content is calculated from calibration data according to *Equation A 16*.

$$\boldsymbol{x_c = \frac{y_c - a}{b}} \qquad \textit{Eq. A 16}$$

x_c: Critical value of the analyte content
a: Intercept of calibration curve
b: Slope of calibration curve

Equation A 15 and *Equation A 16* can be combined to *Equation A 17*.

$$\boldsymbol{x_c = \frac{s_{y,x}}{b} * t_{a,v} * \sqrt{\frac{1}{m} + \frac{1}{p*q} + \frac{\bar{x}^2}{Q_x}}} \qquad \textit{Eq. A 17}$$

x_c: Critical value of the analyte content
$s_{y,x}$: Standard deviation of the residuals
b: Slope of the calibration curve
$t_{\alpha;\upsilon}$: Value from t-distribution for probability level α (one-sided test) and $\upsilon=(p*q)-2$ degrees of freedom, with p being the number of calibration levels, and q the number of replicates at each calibration level
m: Number of replicate analyses of the test sample
p: Number of calibration levels
q: Number of replicate analyses per calibration level
Q_x: Sum of squared differences of the individual calibration levels from the mean calibration level

Equation A 17 can be simplified for the specified experimental design to *Equation A 18*.

$$\boldsymbol{x_c = 1.86 * \frac{s_{y,x}}{b} * \sqrt{1.1 + \frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}}} \qquad \textit{Eq. A 18}$$

x_c: Critical value of the analyte content
$s_{y,x}$: Standard deviation of the residuals
b: Slope of the calibration curve
x_i: Content value of the analyte at calibration level *i*
$\bar{x}$: Content value corresponding to mean calibration level

A1.3.3. Limit of Detection

Under the assumption of homoscedasticity over the calibration range and with identical levels of tolerated type I and type II errors ($\alpha=\beta=0.05$), the level of LOD (x_{LOD}) is derived, in analogy to previous chapters, from *Equation A 6*.

$$\boldsymbol{x_{LOD} = 2 * x_c} \qquad \text{Eq. A 6}$$

x_{LOD}: Content value at the LOD
x_c: Critical level of the analyte content

Equation A 18 and *Equation A 6* can be transformed for the specified experimental design to *Equation A 19*.

$$x_{LOD} = 3.72 * \frac{s_{y,x}}{b} * \sqrt{1.1 + \frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}}$$

$$\approx 3.8 * \frac{s_{y,x}}{b} * \sqrt{1.1 + \frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}} \qquad \textit{Eq. A 19 (= Eq C)}$$

x_{LOD}: Content value at the LOD
$s_{y,x}$: Standard deviation of the residuals
b: Slope of the calibration curve
$\bar{x}$: Content value corresponding to mean calibration level
x_i: Content value of the analyte at calibration level i

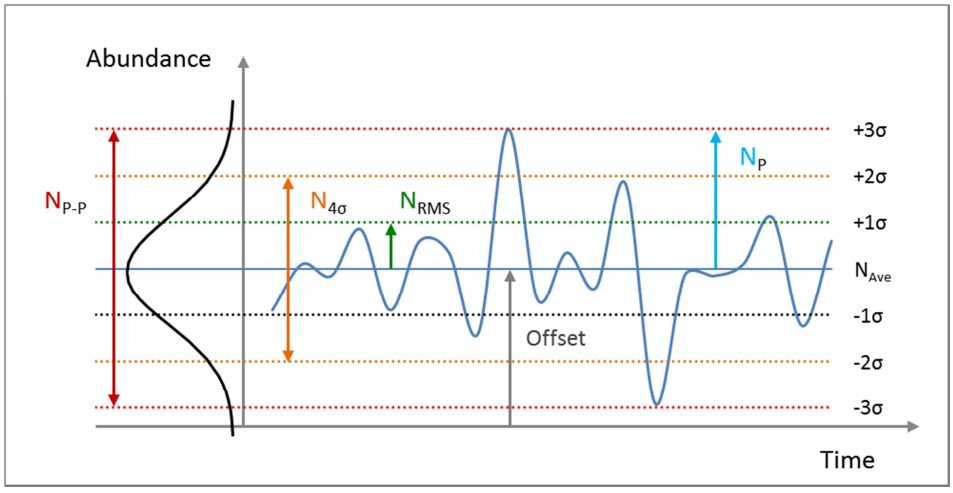
A1.4. **Signal-to-Noise Ratio (S/N)**

Analytical Signal Noise

Ideally, a chromatogram measured on a selected ion trace would be without offset and gain errors, as well as any noise. However, ion trace chromatograms often show both, offset and noise. While an offset can be equalized by establishing a representative averaged (signal) baseline, noise requires some further attention as it can be a critical factor in the analytical results. Noise will tend to make the measurement of analyte and baseline response less certain and limits the minimum signal that can be detected and/or processed.

The most common noise distribution in analytical measurements is the normal or Gaussian, distribution (Figure A-1). Gaussian noise is a random or almost random, time-dependent change in the instrument's output signal often composed of electronic, instrumental and chemical noise. While chemical noise is related to sample extraction and clean-up of the extract, it is not in any way associated with the response of the target compound.

Figure A-1: Segment of the baseline of a chromatogram. Schematic Gaussian noise probability density function, baseline offset and various measures of noise: peak-to-peak ($N_{P\text{-}P}$), 4 sigma ($N_{4\sigma}$), RMS (N_{RMS}), zero-to-peak (N_P)



The Gaussian probability density function (PDF) describes the distribution of an infinite number of random noise signals. It is in theory determined by an infinite number of noise signals, with the specifications of mean ($\bar{z}$) and variance (s^2). The mean represents the average noise offset, while the square root of the variance is the standard deviation (s), a measure of the RMS noise.

In practice, it is impossible to include even a larger number of noise signals. In assessing baseline noise, at least, a representative baseline segment must, therefore, be recorded. Next, the scale on the chromatogram should be expanded so that the noise is clearly visible. The baseline segment used for noise evaluation should be equivalent to at least five times the width of the peak of interest at half-height.

Average Noise ($N_{Average}$)

In Gaussian noise, the average of the amplitudes will be zero (or close to zero) since, over time, negative and positive values cancel each other out. If $N_{Average} > 0$, then another signal must be present and this needs to be considered. The positive/negative excursions that are largely averaging out make $N_{Average,}$ not a useful parameter.

$$\mathbf{N_{Average}} \approx \boldsymbol{0} \qquad \textit{Eq. A 20}$$

RMS Noise (N_{RMS})

To overcome the issue of cancelling out positive and negative noise values, squaring each amplitude makes all values positive. The square root of the average of the squared values returns an amplitude measure: the root-mean-square or RMS amplitude. The RMS value of the noise, also called the *noise power*, is the standard deviation (σ) of the noise distribution σ_{noise}:

$$\boldsymbol{\sigma_{noise}} = \sqrt{\frac{\sum_{i} \left(z_i - \bar{z}\right)^2}{r-1}} \qquad \textit{Eq. A 21}$$

z_i: *Value of noise signal i*
$\bar{z}$: *Average noise*
r: *Number of noise signals*

The RMS value of the noise is then calculated as:

$$\mathbf{N_{RMS} = 1 * \sigma_{noise}} \qquad \textit{Eq. A 22}$$

Zero-to-Peak Noise (N_P)

The zero-to-peak noise or peak noise is the difference between the maximum noise signal and the average noise within the selected representative baseline segment. It can be approximated by three standard deviations of the noise:

$$\mathbf{N_P = 3 * \sigma_{noise}} \qquad \textit{Eq. A 23}$$

4-Sigma Noise ($N_{4\sigma}$)

Two lines parallel to the baseline capturing most of the noise, ignoring the occasional outlying spike, envelope the "core noise". Core noise can be approximated by four standard deviations of the noise, for which reason it is also called 4-sigma noise:

$$\mathbf{N_{4\sigma} = 4 * \sigma_{noise}} \qquad \textit{Eq. A 24}$$

When calculating signal-to-noise ratios, it should be explicitly stated whether the full height corresponding to 4 * N_{RMS} or the half height of the core noise, being 2 * N_{RMS} is referred to:

$$\mathbf{N_{4\sigma,h/2} = 2 * \sigma_{noise}} \qquad \textit{Eq. A 25}$$

Peak-to-Peak Noise ($N_{P\text{-}P}$)

Peak-to-peak noise is the difference between the maximum and the minimum noise signal within the selected representative baseline segment. Strictly speaking, Gaussian noise does not have a defined peak-to-peak value because the tails of a Gaussian distribution reach to infinity, so any signal is possible. In practice, however, noise spikes beyond ±3x RMS are most unlikely to occur. An approximation of 6 times the RMS for the peak-to-peak value is therefore often used.

$$\mathbf{N_{P\text{-}P} = 6 * \sigma_{noise}} \qquad \textit{Eq. A 26}$$

Signal-to-Noise Ratio (S/N) Calculations

The signal-to-noise (S/N) ratio is one of the important performance indicators describing the quality of a particular analysis. Evaluating the power of the signal and comparing it to the power of the noise is essential when assessing the accuracy of a measurement and/or specifying the smallest signal level one can quantify (LOQ).

The S/N ratio strongly depends, among various other factors, on the chosen approach to noise (see the previous chapter) on the mathematical algorithm by which the noise is calculated, e.g. by a commercially available software on the width of the selected noise segment and its position in the chromatogram.

European Pharmacopeia S/N Ratios

The following is the S/N ratio definition as given in European Pharmacopoeia (EP) (EDQM 2016):

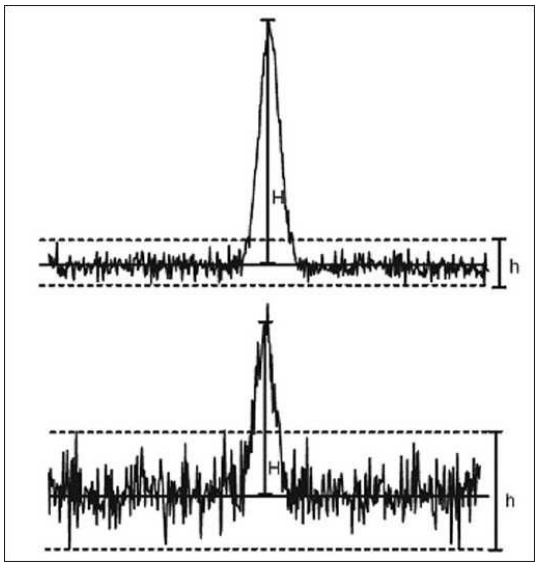
"The signal-to-noise ratio (S/N) influences the precision of quantification and is calculated from *Equation A27*:

$$\boldsymbol{S/N = \frac{2H}{h}} \qquad \textit{Eq. A 27}$$

H: Height of the peak corresponding to the component concerned in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to 20 times the width at half-height.

h: Range of the background noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to 20 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak could be found.

Figure A-2: S/N ratio examples using the European Pharmacopeia 2004 (EDQM 2016) method; 10:1 (top) and 3:1 (bottom)



Note: European Pharmacopoeia (EP) uses only half of the height of the maximum noise (h) equalling h/2, which can be approximated by zero-to-peak noise $N_P = 3\ N_{RMS}$.

When using the signal-to-noise method, the baseline noise close to the retention time of the analyte is measured. Subsequently, the concentration of the analyte that would yield a signal equal to a defined value of the S/N ratio is estimated. The noise power can be measured either manually on the chromatogram or with an automated process by the software of the instrument. A signal-to-noise ratio (S/N) of three is generally accepted for estimating LOD and a signal-to-noise ratio of 6 to 10, is used for estimating LOQ. This scheme is often applied in analytical methods that exhibit baseline noise.

Selected Approach to LOQ Estimation

As seen in Table A-1, different noise approaches lead to different values for LOQ, based on given S/N ratios.

Table A-1: Signal (S) calculated as multiple of the analytical noise (N) expressed as standard deviation (σ) from various approaches. The 4-sigma noise approach (N4σ,h/2) being most suitable and in accordance with EU legislation for PCDD/Fs and PCBs is highlighted.

Noise approach	N	S = 3 N
RMS	1 σ	3 σ
4 Sigma (h/2)	2 σ	**6 σ**
Zero-to-Peak	3 σ	9 σ
4 Sigma (h)	4 σ	12 σ
Peak-to-Peak	6 σ	18 σ

The RMS approach, which provides an S/N ratio equal or close to a LOD ($s = 3\sigma$) may lead to misunderstandings and even to underestimation of the LOQ, in an attempt to fulfill legal requirements.

Based on an S/N ratio of 3:1, neither of the zero-to-peak noise (N_P), full 4-sigma noise and peak-to-peak noise (N_{P-P}) shall be used, as they result in unrealistically high LOQs. Also, N_P and N_{P-P} estimates may be severely influenced by signal spikes.

Based on the requirement that LOQ shall be at least 6 times the standard deviation of the noise signals above the averaged baseline ensuring a high level of confidence, the 4-sigma noise approach ($N_{4\sigma,h/2}$) covering half of the noise height (h) is identified in Table 3 as being the most suitable approach. It is based on an S/N ratio of 3:1, and in full agreement with EU legislation for PCDD/Fs and PCBs.

Consequently, $LOQ_{4\sigma,h/2}$ (N covering half of the noise height h) is calculated according to *Equation A28*:

$$\boldsymbol{LOQ_{4\sigma,h/2} = 3 \cdot N_{4\sigma,h/2} = 6 \cdot \sigma_{noise}} \qquad \textit{Eq. A 28}$$

As mentioned above, additional criteria apply and must be verified before using the LOQ for any further calculations.

Data Acquisition and Data Processing

As seen from the previous sub-chapter, different approaches to analytical baseline noise (N_{RMS}, N_P, N_{P-P}, $N_{4\sigma,h/2}$, $N_{4\sigma}$) lead to different S/N ratios and subsequently to (largely) different LOQs. Nevertheless, even with a unified noise approach, S/N ratios will apparently fail to produce useable and/or comparable LOQs when there is low and high ion noise and a free choice of where to measure it. To render S/N ratios and ultimately LOQs, even more, comparable between laboratories, additional specifications are provided in chapter 8.1.

A2. **Examples**

This section provides examples for the estimation of LOD and LOQ according to procedures specified in chapters 5 and 6.
The respective measurements were conducted at the EU-RL PAH.
For better retracing of calculations, numerical values of parameters such as standard deviations, residuals, etc. were rounded to the displayed number of decimals. Further calculations were performed with the displayed (rounded) figures.

A2.1. **Estimation of LOD and LOQ based on the Analysis of Blank Samples**

LOD and LOQ were estimated for the determination of benzo[a]pyrene (BaP) in bread by GC-MS with internal standardisation, applying stable isotope labelled BaP. The experimental design was in accordance with the provisions given in 5.1. The GC-MS instrument was calibrated with standard solutions in toluene in the range between 0.03 µg/kg and 0.12 µg/kg. Standard solution concentrations were expressed as BaP contents in the bread test sample.
A commercial brown bread with low analyte content (pseudo-blank) was used for the experiments to estimate LOD and LOQ. Each test portion was spiked with a solution of $^{13}C_4$-BaP in toluene and extracted by pressurized liquid extraction followed by size exclusion chromatography clean up.

Equation A is applied for the calculation of LOD and *Equation D* for the calculation of LOQ.

$$x_{LOD} = 3.9 * \frac{s_{y,b}}{b} \qquad \textit{Eq. A}$$

x_{LOD}: Limit of detection
$s_{y,b}$: Standard deviation of the blank (pseudo-blank) signals
b: Slope of the calibration curve

$$x_{LOQ} = 3.3 * x_{LOD} \qquad \textit{Eq. D}$$

x_{LOQ}: Limit of quantification
x_{LOD}: Limit of detection

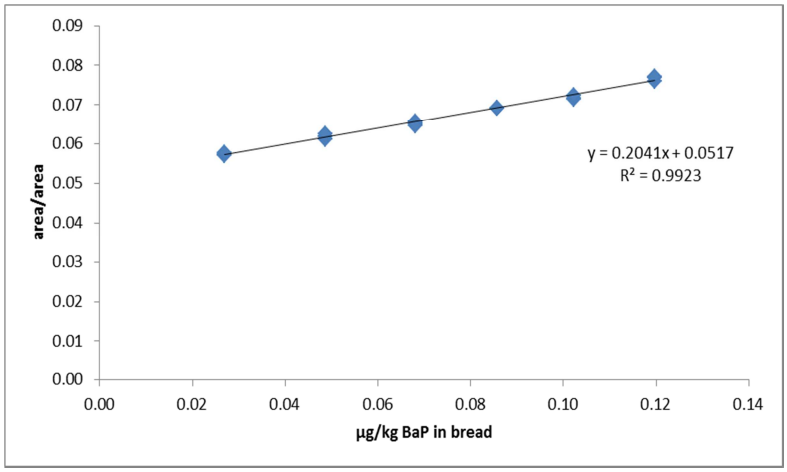
Table A-2 presents the measurement results of the replicate analyses.

Table A-2: Results of measurements (given as peak area ratio of native BaP peaks and ^{13}C-labelled BaP peaks) of 10 independent replicate analyses (B1-B10) of the bread test sample.

	Benzo[*a*]pyrene
	a/a_{ISTD}
B1	0.055
B2	0.056
B3	0.056
B4	0.058
B5	0.058
B6	0.054
B7	0.054
B8	0.057
B9	0.055
B10	0.056

The variable $s_{y,b}$ in *Equation A* corresponds to the standard deviation of the peak area ratios given in *Table A-2*, while *b* is the slope of the calibration curve (see Figure A-3).

Figure A-3: Calibration graph



Application of *Equation A* and *Equation D* results in the LOD and LOQ estimates given in *Table* A-3*.*

Table A-3: Compilation of parameter values for calculation of LOD and content values of LOD and LOQ

$s_{y,b}$		0.00145
b	kg/µg	0.2041
$s_{y,b}/b$	µg/kg	0.0071
x_{LOD}	µg/kg	0.0277
x_{LOQ}	µg/kg	0.0914

LOD and LOQ for the determination of BaP in brown bread were estimated via the blank approach at 0.03 µg/kg and 0.10 µg/kg, respectively.

A2.2. **Estimation of LOD and LOQ based on Paired Observations**

LOD and LOQ were estimated for the determination of benzo[*a*]pyrene (BaP) in bread. Contrary to the example given in A2.1, the scope of the analyses also comprised other bread types than brown bread. Therefore, it was considered appropriate to include breads with different compositions in the experiments. The measurements of the test sample extracts were performed by GC-MS with internal standardisation applying stable isotope labelled BaP. The experimental design was done following the provisions given in 5.2. The GC-MS instrument was calibrated with standard solutions in toluene in the range, between 0.03 µg/kg and 0.12 µg/kg (see Figure A-4). Standard solution concentrations were expressed as BaP contents in the bread test samples.

Ten different commercial bread samples with low, but varying analyte contents (pseudo-blanks) were used for the experiments to estimate LOD and LOQ. The bread samples comprised white breads, brown breads, as well as multi-grain breads and breads with special ingredients such as cereal flakes. Each bread sample was ground and homogenized. From each bread sample, one test portion was analysed directly and a second test portion was analysed after spiking with a BaP standard solution in toluene. The fortification level was 0.1 µg/kg. Before extraction, each test portion (both native and spiked) was spiked with a solution in toluene of $^{13}C_4$-BaP. All samples were extracted by pressurized liquid extraction followed by size exclusion chromatography clean up and finally measured by GC-MS.

Equation B is applied for the calculation of LOD, and *Equation D* for the calculation of LOQ.

$$\boldsymbol{x_{LOD} = 5.2 * \frac{s_{y,net}}{b}} \qquad \textbf{Eq B}$$

x_{LOD}: Limit of detection
$s_{y,net}$: Standard deviation of the net signals of paired observations (in this case, standard deviation of the signal differences between spiked and native pseudo-blank samples)
b: Slope of the calibration curve

$$\boldsymbol{x_{LOQ} = 3.3 * x_{LOD}} \qquad \textbf{Eq. D}$$

x_{LOQ}: Limit of quantification
x_{LOD}: Limit of detection

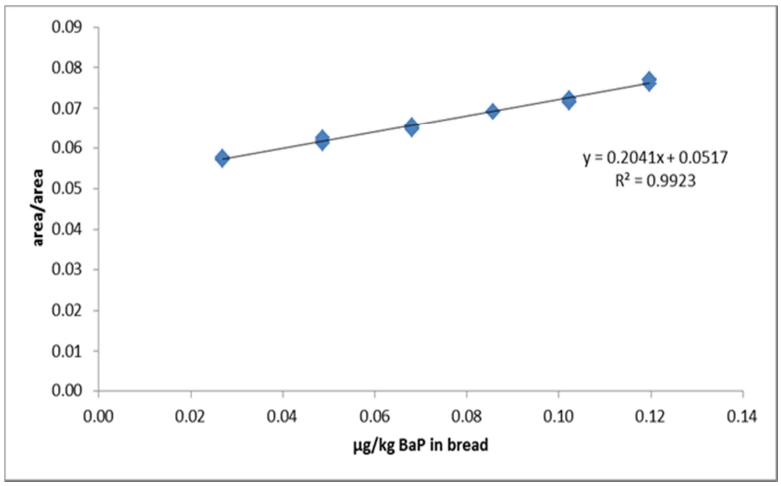
Table A-4 presents the measurement results of native and spiked bread test samples, as well as the difference (Δ) of area ratios between spiked and native test samples. This difference (net signal) corresponds to the spiked amount of BaP.

Table A-4: Results of measurements (given as peak area ratio of native BaP peaks and ^{13}C-labelled BaP peaks) of ten different native and corresponding spiked bread test samples, as well as the difference between both.

Sample	Native	Spiked	Δ
	a/a_{ISTD}	a/a_{ISTD}	a/a_{ISTD}
A	0.0550	0.0744	0.0194
B	0.0635	0.0897	0.0262
C	0.0604	0.0798	0.0194
D	0.0517	0.0690	0.0173
E	0.0533	0.0750	0.0217
F	0.0587	0.0790	0.0203
G	0.0593	0.0819	0.0226
H	0.0565	0.0756	0.0191
I	0.0790	0.1034	0.0244
J	0.0546	0.0733	0.0187

The variable $s_{y,net}$ in *Equation B* corresponds to the standard deviation of the difference of peak area ratios given in *Table A-4*, while *b* is the slope of the calibration curve (see Figure A-3).

Figure A-4: Calibration graph



Application of *Equation B* and *Equation D* results in the LOD and LOQ estimates given in *Table* A- 5

Table A- 5: Compilation of parameter values for calculation of LOD, and content values of LOD and LOQ.

$S_{y,net}$		0.00278
b	kg/µg	0.2041
$S_{y,net}/b$	µg/kg	0.0136
x_{LOD}	µg/kg	0.0709
x_{LOQ}	µg/kg	0.2341

LOD and LOQ for the determination of BaP in bread were estimated via the paired observation approach at 0.08 µg/kg and 0.24 µg/kg respectively.

A2.3. **Estimation of LOD and LOQ from Calibration Samples**

LOD and LOQ were estimated for the determination of benzo[*a*]pyrene (BaP) in brown bread. A commercial brown bread, containing both wheat and rye flour was used as test material for the experiments to estimate LOD and LOQ. The brown bread sample was spiked with aliquots of a standard solution of BaP in toluene. Fortification levels were equidistant between 0.05 µg/kg and 0.20 µg/kg. Additionally, each test portion was spiked with a solution of ^{13}C-labelled BaP. Each test sample (native and spiked samples) was analysed in duplicate, as described in 5.3. Initial calculations revealed that the highest spiking level exceeded the level of ten times the LOD. Therefore, the highest spiking level was replaced by another lower spiking level (0.08 µg/kg). Consequently, the mean calibration level was calculated for the concentration range 0 to 0.15 µg/kg.

Equation C is applied for the calculation of LOD:

$$x_{LOD} = 3.8 * \frac{s_{y,x}}{b} * \sqrt{1.1 + \frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}} \qquad \textit{Eq C}$$

x_{LOD}: Limit of detection
$s_{y,x}$: Standard deviation of the residuals
b: Slope of the calibration curve
$\bar{x}$: Mean calibration level
x_i: Content value of the analyte at calibration level i

Table A-6 presents the spiking levels and measurement results in the form of peak area ratios of BaP peaks and $^{13}C_4$-BaP peaks. Additionally, the average spiking level, as well as the squared differences of the respective spiking level to the average spiking level is given.

Table A-6: Spiking levels, response data, as well as average spiking level and squared differences of spiking levels to the average spiking level.

Spiking level	$(x_i - \bar{x})^2$	Benzo[*a*]pyrene
µg/kg	µg²/kg²	area/area$_{ISTD}$
0.000	0.006	0.0530
0.000	0.006	0.0550
0.050	0.001	0.0620
0.050	0.001	0.0660
0.080	0.000	0.0700
0.080	0.000	0.0720
0.100	0.001	0.0750
0.100	0.001	0.0750
0.150	0.005	0.0820
0.150	0.005	0.0860

$\bar{x}$	µg/kg	0.08
$\sum_{i=1}^{n}(x_i - \bar{x})^2$	µg²/kg²	0.025
$\sqrt{1.1 + \frac{\bar{x}^2}{\sum_{i=1}^{n}(x_i - \bar{x})^2}}$		1.154
$t_{\alpha,\vartheta}$		1.8595

The standard deviation of the residuals and the slope of the calibration curve are derived by linear regression from BaP content data and area ratios given in *Table A-6*.

In the following, the output of software based linear regression is presented. The "LINEST" function of MS Excel was used for that purpose. The standard deviation of the residuals ($s_{y,x}$) was calculated from the sum of squared residuals (ss(resid), in blue font, taking into account n-2 degrees of freedom. The slope of the calibration curve (for easier tracking highlighted in red) was also provided by the software.

y=a+b*x			
b	0.202236422	0.054230032	a
Seb	0.010544946	0.000959532	Sea
r2	0.978712958	0.001668636	SEy
F	367.8154905	8	df
ss(reg)	0.001024125	2.22748E-05	(ss(resid)

$s_{y,x}$	=sqrt(2.22748E-05/8)	0.001668636
$\frac{s_{y,x}}{b}$		0.008250916

Consequently *Equation C* becomes:

$$x_{LOD} = 3.8*0.00825*1.154 = 0.0362 \text{ µg/kg}$$

The LOQ is calculated applying *Equation D*:

$$x_{LOQ} = 3.3 * x_{LOD} = 0.1194 \text{ µg/kg}$$

LOD and LOQ for the determination of BaP in brown bread were estimated via the calibration approach at 0.04 µg/kg and 0.12 µg/kg respectively.