PRINCIPLES OF METHOD VALIDATION AND QUALITY CONTROL: COMPLIANCE WITH THE NEW EU CRITERIA FOR BIOANALYTICAL SCREENING OF FEED AND FOOD

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Introduction

For more than a decade, bioanalytical screening methods have effectively been applied in European official control of feed and food samples for elevated levels of polychlorinated dibenzo-p-dioxins (PCDDs), dibenzo-furans (PCDFs) and dioxin-like PCBs (dl-PCBs). Since requirements for application of these methods were first implemented in 2002[1,2], experience has grown in bioassay laboratories resulting in revised criteria[3,4,5,6] adopted by EU-legislation[7,8] in 2012. Each laboratory must demonstrate fitness-for-purpose of the bioanalytical method applied and observe specified run acceptance criteria while performing screening analysis. A total of 27 out of 78 requirements center around validation procedures, while a similar number of criteria were set each for quality control and evaluation of bioanalytical results. A particular focus is on cut-off concentrations for decisions of sample compliance with regulatory limits[9,10]. This paper outlines principles of bioanalytical method validation and quality control.

Materials and methods

Validation – a two-step procedure

Bioanalytical screening methods are performed to identify samples with significant levels of PCDD/Fs and dl-PCBs. By comparing the screening result expressed in bioanalytical equivalents (BEQs) to a cut-off level pre-established during validation, samples are classified as compliant or suspected to be noncompliant, requiring analysis by a confirmatory method. The cut-off is based on the respective EU maximum level (ML) or action level (AL), while taking into account GC/HRMS measurement uncertainty and the variability of bioanalytical results. It strongly depends on matrix properties and congener patterns, which may vary widely, even among samples of the same matrix. Therefore, the relationship between bioanalytical and GC/HRMS results (in TEQs) has to be established in a two-step validation procedure:

(1) Initial Validation: Basic method performance is evaluated by a formal validation process using spiked blank samples for calibration to prove the fitness-for-purpose of the method for each matrix/matrix group of interest. Variability of congener patterns and matrix properties such as lipid contents leading to more variability of results in routine screening are not taken into account. From the calibration data, an initial cut-off level is established.

(2) Re-validation: Method performance and the relationship between BEQs and TEQs shall be re-evaluated after the method was applied for some time in routine screening, by matrix-matched calibration experiments involving samples both compliant and noncompliant from GC/HRMS confirmatory analysis, to account for variability in congener patterns and matrix properties. Based on the within-laboratory reproducibility achieved with these samples, the bioassay cut-off is re-evaluated.

Continuous quality control

Being closely associated with validation, extensive quality control is the third pillar in ensuring reliable method performance and results. QC measures include, but are not limited to: careful selection of suitable reference

samples, inclusion of a procedure (or matrix) blank and a recovery control (reference) sample in each series, correction of results from unknown samples for procedure blank and apparent recovery, monitoring of QC data over time in QC charts and evaluation of QC results, GC/HRMS confirmation of all suspected samples and of 2 to 10% of the samples declared compliant, and continuous successful participation in interlaboratory proficiency test (PT) studies. Laboratories applying bioanalytical methods for official purposes therefore require a close cooperation with laboratories using a confirmatory method, for method validation and on-going quality control.

A QC database shall be established for each sample matrix/matrix group of interest, containing: (1) GC/HRMS results of confirmed suspected and compliant samples (minimum of 20 samples per matrix), to be used in (re-) evaluation of: the BEQ-TEQ relationship, method performance (re-validation), cut-off concentrations and actual false-compliant rates; (2) results from inter-laboratory PT studies < 2x ML (optional). Re-evaluation of results collected in the database shall be performed whenever sufficient new data for a certain sample matrix have been included, or for any other relevant reason (e.g. if sample results with unusual congener patterns were included, etc.), being an on-going process.

Method calibration

Initial validation: Confirmed blank samples are spiked around the respective level of interest, generating 6 series of samples e.g. with concentrations 0x, 0.5x, 1x and 2x ML, for each relevant sample matrix. Use of spiked instead of incurred materials ensures that the cell response is not significantly influenced by co-extracted non-regulated AhR-agonists but is mainly due to Ah-receptor active dl-compounds present in the sample extract. Samples are submitted to extraction, clean-up and measurement in 6 consecutive series, providing within-lab reproducibility conditions for the 6 repetitions on each level.

Re-Validation: Calibration experiments are performed for each relevant sample matrix involving a minimum of 20 confirmed routine samples contaminated in a range from the GC/HRMS-LOQ up to approx. 2x the level of interest, taken from the QC database. Samples are submitted to extraction, clean-up and measurement.

Linear regression: Bioanalytical results (BEQ) are plotted vs GC/HRMS results (TEQ), and the calibration (regression) line and its 95%-prediction interval are calculated.

Bioassay cut-off concentrations

In GC/HRMS confirmatory analysis, only those samples exceeding the ML after correction for measurement uncertainty U_{DL} (e.g. 15%) will be considered noncompliant. The bioassay cut-off is therefore based on the GC/HRMS decision limit (DL) and is calculated as lower endpoint of the distribution of bioanalytical results ($RSD_R \leq 25\%$, see below) corresponding to DL (figures 1 and 2). Mathematically, the cut-off is obtained from the lower band of the one-sided prediction interval at x_{DL}, as the BEQ-level above which 95% of the area under the Gaussian normal distribution curve of the response variables corresponding to x_{DL} are located:

$$\textit{Bioassay cut-off} = \bar{y}_{DL} - s_{y,x} * t_{\alpha,f=m-2} \sqrt{1/n + 1/m + (x_{DL} - \bar{x})^2 / Q_{xx}}, \quad \text{with } Q_{xx} = \sum_{j=1}^{m} (x_i - \bar{x})^2,$$

m: number of calibration experiments, n: number of replicates, $\bar{y}_{DL}$: mean y-value from n repetitions at x_{DL}, $s_{y,x}$: residual standard deviation, $t_{\alpha,f=m-2}$ (α = 5%, one-sided): student factor, the 95% level of confidence implying a false-compliant rate < 5%. With this approach, cut-off levels may be estimated both from initial and from re-validation data. Bioassay cut-offs may in practice often be close to 2/3 of the ML, i.e. they may be corresponding to the current AL (figure 2). Alternative methods for establishing cut-off concentrations are also available[3,4] however these do not provide full information on method performance as available from calibration experiments.

Restrictions to cut-off concentrations

During initial validation, the within-laboratory reproducibility (RSD_R) of the screening method may be below 25% leading to a cut-off (in BEQ) close to or even above the permitted maximum level (in TEQ). In those cases, a modified (lower) cut-off must be calculated based on a RSD_R of 25%, to account for increased variability of sample results expected in routine, due to the large variety of matrix properties and congener patterns potentially present in unknown samples. Only this modified cut-off may then be applied in routine screening to avoid false-compliant results. Cut-off concentrations should be re-evaluated in a re-validation procedure as soon as a minimum of 20 confirmed sample results covering a range up to approximately 2xML have become available. Bioassay cut-off levels are matrix/matrix group-related and to be applied during screening only to results of those sample matrices they were previously established for.

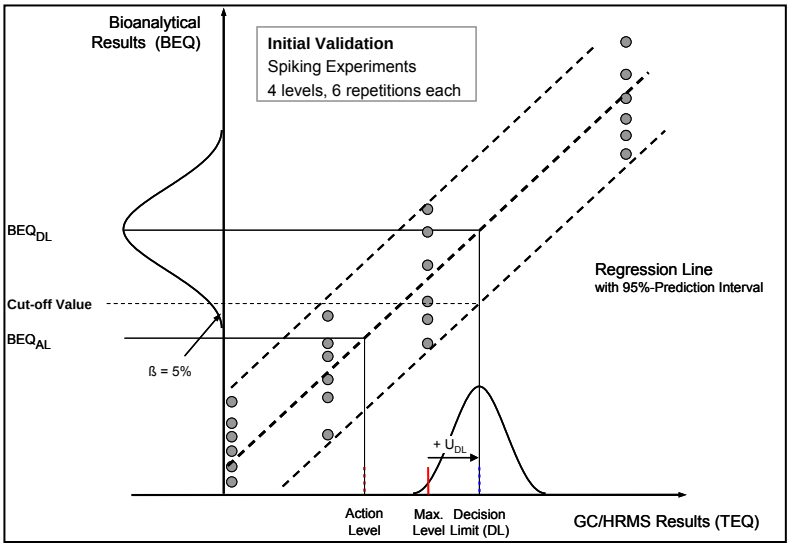

Figure 1. Initial validation: Exemplary calibration data (spiked samples), regression line, 95% prediction interval and preliminary bioassay cut-off concentration. U_{DL}: GC/HRMS expanded measurement uncertainty at DL

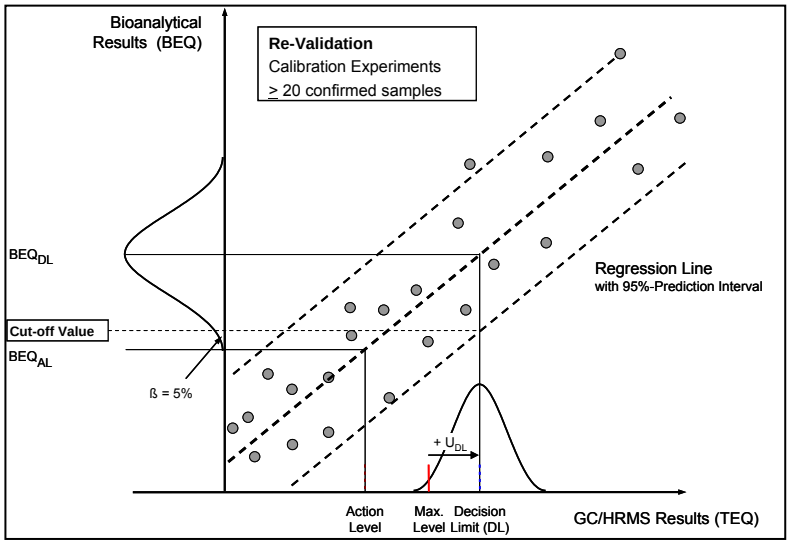

Figure 2. Re-validation: Exemplary calibration data (confirmed samples), regression line, 95% prediction interval and bioassay cut-off concentration. U_{DL}: GC/HRMS expanded measurement uncertainty at DL

Results and discussion

Verification of method performance criteria

Bioanalytical data included in method calibration during initial and re-validation must comply with the new run acceptance criteria. Restrictions related to assay performance (triplicate-CVs, standard curve, assay working range) apply. An estimate of TEQ-levels may be reported in BEQs only if they are at least by a factor of three above the procedure blanks. Estimated levels in unknown and recovery control samples must be corrected for the procedure (or matrix) blank value. Subsequently, sample results are corrected for the recovery estimated from the recovery control sample. For this, apparent recoveries must be within required ranges. Several required method performance criteria may then be verified from the calibration data sets acquired during initial validation

and re-validation, while some more general information on method performance may also be obtained:

Results from initial validation: Parameters of 6 individual calibration lines for each sample series (slope or sensitivity, y-intercept or mean matrix blank, coefficient of determination R^2), mean, recovery (corrected) and precision (RSD_R) on each concentration level.

Results from initial and re-validation: Parameters of the overall calibration line (slope/sensitivity of the method, y-intercept/mean matrix blank value, R^2), calibrated range, center of mass, variability of bioanalytical data: the relative residual standard deviation may be used (instead of RSD_R,) at any concentration within the calibrated range, correspondence between BEQ and TEQ results, cut-off level at the GC/HRMS-DL (based on ML, or on any other TEQ concentration), bioassay limit of detection (LOD) e.g. as in DIN 32645:2008. It may further be of interest to compare performance characteristics obtained from initial and from re-validation.

Actual false-compliant and false-noncompliant rates: Actual α- and ß- errors may be assessed by applying the cut-off concentration estimated during initial validation to the confirmed compliant and non-compliant results collected and/or used in re-validation. The fraction of false-compliant results must be below 5%. The fraction of false-noncompliant results may be calculated based on both the number of all screened samples and the number of samples suspected to be noncompliant.

After it could be demonstrated that the achieved performance meets all legal requirements, the bioanalytical method is ready for use in screening of feed and/or food samples according to European legislation.

Acknowledgements

We would like to thank the European Commission for the financial support of the work of the European Union Reference Laboratory for Dioxins and PCBs in Feed and Food (EU-RL), Freiburg, Germany

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