BIOANALYTICAL SCREENING METHODS (2): PRACTICAL EXPERIENCE WITH THE NEW EU PERFORMANCE CRITERIA

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Introduction

The use of well-characterized and fully validated methods is essential to generate reliable results that can be unambiguously interpreted. Bioanalytical methods are constantly undergoing changes and improvements, and as such are often at the cutting edge of the technology. Each bioanalytical method has its own characteristics, which may vary from analyte to analyte, and/or between groups of analytes. In response to this observation and based on a decade of experience with analytical requirements implemented in 2002[1,2], an EU-RL-headed expert group suggested to the European Commission specific performance criteria and validation requirements for screening of feed and food samples, which were adopted into legislation in March 2012[3,4].

Before these new criteria entered into force, bioanalytical screening results were directly compared to regulatory limits given in Toxic Equivalents (TEQs). However, correspondence between bioanalytical results expressed as Bioanalytical EQuivalents (BEQs) and results from GC/HRMS confirmatory analyses (expressed in TEQs), may not be in a one-to-one relationship. Observed differences between BEQs and TEQs are mainly due to recovery losses, differences between the Toxic Equivalency Factors (TEF) assigned to each of the 29 EU-regulated PCDD/F and DL-PCB congeners and their respective induced cell response expressed as RElative Potency (REP) factors, and the properties and suitability of the sample material used for recovery control[5]. This underlines the need to evaluate the BEQ-TEQ correspondence for each sample matrix of interest during an initial validation process. Before applying the method in routine screening, reliable BEQ-based cut-off concentrations must also be established above which a sample is declared to be suspected to exceed the respective legal limits. Each laboratory must demonstrate fitness-for-purpose of the bioanalytical method prior to use. During routine screening analysis, specified run acceptance and quality control criteria apply.

This paper outlines strategies followed and practical experience gained when performing bioanalytical method evaluation, validation and quality control at the European Union Reference Laboratory (EU-RL) for Dioxins and PCBs in Feed and Food, Freiburg (Germany).

Materials and methods

Bioanalytical screening methods compare the analytical result with a cut-off concentration, providing a yes/no-decision over sample compliance. In addition, an indication may be given of the summary levels of PCDD/Fs and/or DL-PCBs present in the sample, by expressing the result numerically as BEQ. Samples below the level of interest are classified as "compliant", while samples suspected to exceed EU maximum levels or action thresholds require further investigation by confirmatory gas chromatography / high resolution mass spectrometry (GC/HRMS) analysis. Naturally, this concept requires a close cooperation of the bioanalytical lab with the laboratory running the GC/HRMS confirmatory method, at the same time reducing the latter's workload by sieving out most of the compliant samples.

For various reasons mentioned above, bioanalytical results in BEQ can never fully equal TEQ values from GC/HRMS. However, as EU maximum levels and action thresholds are given in TEQs, it is essential to evaluate the extent of correspondence between results from both methods. As outlined in the new EU criteria for application of bioanalytical methods, the BEQ-TEQ relationship must be assessed prior to use by matrix-matched calibration experiments performed during the initial validation process, involving "blank" samples spiked around the level of interest. Matrix-related cut-off concentrations (in BEQs) must be established ensuring a false-compliant rate < 5%. Cut-offs in principle depend on the variability of BEQs, the BEQ-TEQ relationship or the sensitivity of the method, and the suitability of the recovery control material to compensate for apparent recovery losses[6].

Two alternative approaches developed at EU-RL for a statistically sound assessment of these important parameters form the core and center piece of recent revisions[3,4]. The first, more comprehensive approach is based on the correspondence between BEQ and TEQ values found for each matrix of interest during initial validation. Spiking levels are plotted vs. bioanalytical results obtained on these levels, corrected for blank and recovery. After fitting a linear regression model, significant parameters describing the method's performance at various concentrations within the working range including the cut-off concentration are calculated. The latter equals the lower branch of the prediction interval calculated around the regression line at the level of interest.

The second, alternative and abbreviated approach allows a simplified estimation of the cut-off as the lower endpoint of the distribution of bioanalytical results obtained from multiple analyses (under intermediate precision conditions) of a sample spiked at the level of interest.

At EU-RL, method performance is assessed in a two-step validation procedure[7]:

1. *Initial Validation:* Basic method performance is evaluated before application of the method in routine, by using blank samples spiked on various levels for calibration, to prove fitness-for-purpose for each matrix/matrix group of interest. Variability of congener patterns and matrix properties are not taken into account. An initial cut-off value and further performance parameters are statistically derived.
2. *Performance Re-evaluation:* Method performance, the relationship between BEQs and TEQs and the initial cut-off value are re-evaluated after the method has been applied for some time in routine screening. Matrix-matched calibration experiments are carried out with incurred samples, both compliant and noncompliant from GC/HRMS confirmatory analysis performed for QC purposes. Variability of congener patterns and matrix properties such as lipid contents, generally leading to more variability of bioanalytical results in routine screening are now taken into account. The actual false-compliant rate is also evaluated based on application of the initial cut-off to the confirmed samples, which may now be modified if required.

Being closely related to the re-evaluation process, *quality control* is the third pillar ensuring state-of-the-art performance and results. Laboratories applying bioanalytical methods for official purposes require a close cooperation with a laboratory performing the confirmatory method, for (1) method validation, (2) confirmatory analyses, and (3) on-going quality control. In full compliance with the legal requirements, EU-RL maintains a comprehensive QC system, based on, but not limited to:

- use of matrix-matched, fully GC/HRMS-characterized, representative "reference" samples
- inclusion of 1 procedure (or matrix) blank and 1 recovery control (reference) sample in each series
- verification of the acceptance criteria for the assay, and for the sample run
- monitoring and evaluation of quality control data over time in QC charts
- checking routine samples for the presence of AhR-active compounds lowering the cell response possibly leading to false-compliant results
- GC/HRMS confirmation of all suspected samples, and of a fraction of samples declared compliant
- maintenance of a QC-data base (BEQ/TEQ results) for re-evaluation of method performance
- verification of the maximum tolerable false-compliant rate
- continuous and successful participation in interlaboratory proficiency test (PT) studies

EU-RL further maintains a QC database for each sample matrix/matrix group of interest, including bioanalytical and GC/HRMS results of confirmed noncompliant and compliant samples, and results from inter-laboratory PT studies. Re-evaluation of method performance is carried out whenever sufficient new data for a certain sample matrix have been included, being an on-going process.

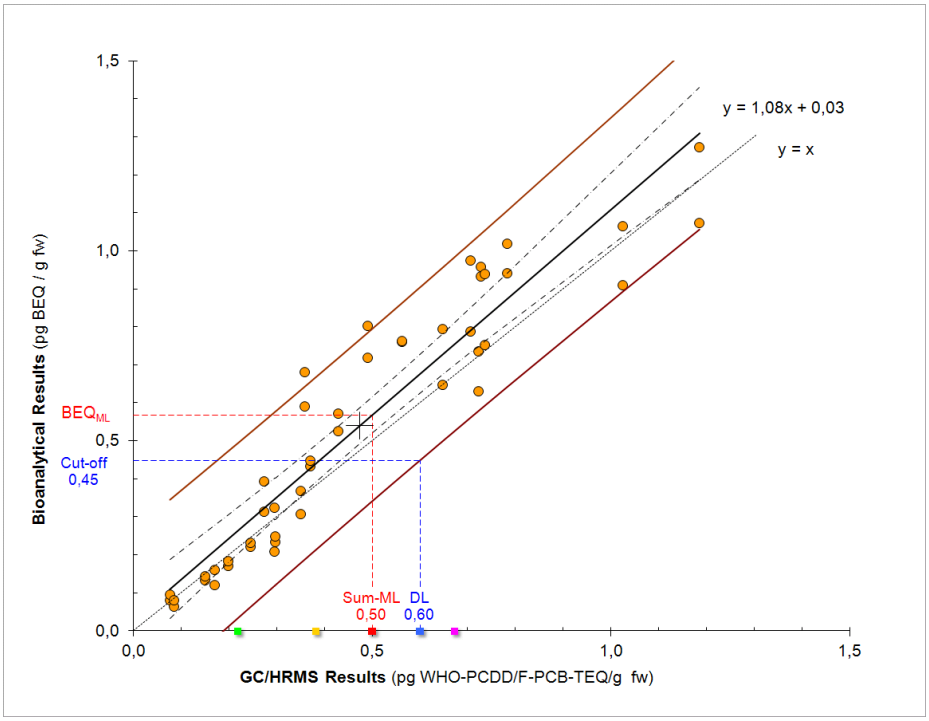
Actual α- and ß-errors are assessed by applying the initial (or previous) cut-off to confirmed compliant and noncompliant results collected in the QC data base. The fraction of false-compliant results must be below 5%. The fraction of false-noncompliant results should be reasonably small and may be calculated both based on the number of all screened samples, and based on the number of samples suspected to be noncompliant.

Results and discussion

Besides evaluating bioassay technology available on the European market, EU-RL now focuses on optimization and particularly new developments related to both assay conditions and sample extraction and clean-up procedures[8]. Changing a bioanalytical method that tests for the potency of dioxin-like compounds on the basis of molecular interactions requires extra care. A more sensitive method may respond to more impurities in the sample extracts leading to an increase or a decrease of the cell response. On the other hand, an increase in selectivity may, due to enhanced loss of target compounds, reduce the "apparent accuracy" significantly for individual congeners. Accuracy is one prime consideration, because any bias in results may lead to false-compliant decisions. Inaccurate results are therefore inacceptable within the scope of efficient consumer health protection as they may eventually lead to dramatic consequences. Last but not least, a reliable method must provide results with a precision ensuring acceptable reproducibility of the results.

A number of efficient bioanalytical screening methods were developed at EU-RL based on the new criteria and validated according to the procedures outlined above[3,4,7]. Comprehensive matrix-matched calibration experiments were carried out involving hundreds of GC/HRMS-confirmed food samples of interest, representing an array of physical properties (liquid, solid, powder, greasy, oily), lipid contents, extractability and congener patterns, including samples from various sources and years of collection. Bioanalytical methods are meanwhile established[6] for the following target analyte groups and sample matrix combinations:

1. total-BEQs; sample matrices: *fat* (bovine), *meat* (bovine), *liver* (bovine, sheep), *fish muscle tissue*, *fish oil*, *hen's eggs*, cow's *milk fat*, *human milk*, *cow's whole milk*, *vegetable oil*
2. PCDD/F- and DL-PCB-BEQs (analyzed separately); sample matrices: *fat* (bovine), *liver* (bovine, sheep), *fish oil*, *hen's eggs*, *cow's milk fat*, *human milk*, *cow's whole milk*



BEQ/TEQ-plot from calibration experiments involving *bovine liver* samples (analyzed in duplicate under intermediate precision conditions) contaminated in a range from 0.08 to 1.19 pg WHO-PCDD/F-PCB-TEQ/g fresh weight. Regression line: y=1.08x+0.03 (–) with confidence (--) and prediction intervals (–), EU maximum level (ML): 0.50 pg WHO-PCDD/F-PCB-TEQ/g fw, DL: GC/HRMS decision limit (ML + expanded measurement uncertainty 10%), Cut-off: 0.45 pg BEQ/g fw, BEQ_{ML} = 0.57 pg BEQ/g fw, r = 0.9409.

Exemplary, in the figure above a BEQ/TEQ-plot is shown as derived from calibration experiments involving bovine liver samples contaminated in a range from 0.08 to 1.19 pg WHO-PCDD/F-PCB-TEQ/g fresh weight.

Performance parameters fulfilling all criteria required by European legislation reflect fitness-for-purpose of EU-RL's methods ensuring reliability of bioanalytical results in routine screening. Cut-off concentrations were derived from the prediction intervals calculated around the regression lines at the levels of interest. These cut-offs are based on current maximum and action levels, and when applied to EU-RL's collected routine samples ensure ML-based false-compliant rates of generally 0% (except for bovine meat, sum-BEQ: ß=2.6%). False noncompliant rates are found in an acceptable 20 - 40 % range of those samples suspected to be noncompliant, implying a load removal from the GC/HRMS confirmatory laboratory by 80% of all routine samples under investigation. Expectedly, cut-offs for the various matrices and target analyte groups vary within certain ranges, predominantly depending on method precision achieved at the level of interest.

In conclusion, validation and QC data confirm that the new legal requirements for application of bioanalytical methods are achievable in practice. Moreover, these requirements proved very beneficial by representing a strong driving force for thorough step-by-step method optimization, thus providing a valuable framework for a high level of performance.

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