NEW CUT-OFF VALUES FOR APPLICATIONS IN BIOANALYTICAL SCREENING: DECISION OVER SAMPLE COMPLIANCE WITH LEGAL LIMITS SET BY THE EUROPEAN UNION FOR PCDD/Fs AND DIOXIN-LIKE PCBs

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Introduction

Daily analysis in a laboratory for control of polychlorinated dibenzodioxins and dibenzofurans (PCDD/Fs) and dioxin-like polychlorinated biphenyls (dl-PCBs) in feed and food often deals with concentration levels that are close to established regulatory limits, such as two of the main pillars of the current EU strategy on dioxins and dl-PCBs (1): Maximum levels (ML) were set to regulate compliance (2, 3), whereas action levels (AL) (4) were set as "early warning" for elevated levels to identify possible sources for contamination in these matrices. Samples exceeding the AL but not the ML are compliant with food or feed law and can be sold on the market but may require further action to discover the source of the contamination. MLs were set for PCDD/F-TEQ and the sum of PCDD/F- and dl-PCB-TEQs, whereas ALs were set separately for PCDD/F-TEQ and dl-PCB-TEQ.

The potential impact of elevated levels of those chemicals on consumer health and the environment and the possible economic consequences justify the need for optimum reliable routine analysis and interpretation of results. Therefore, strict analytical requirements have been established and documented in Commission Regulations (EC) numbers 1883/2006 for food (5) and 152/2009 for feed (6). The analytical criteria included in both documents were first proposed by a group of scientists in 2001 (7, 8) and then adopted into legislation. Among them are a number of key performance parameters to be met in routine screening and confirmatory analysis. However, a statistical evaluation of the correspondence between results from both methods, providing an indispensable frame for assessing cut-off values securely applicable in routine screening analysis, was not yet established.

European legislation permits the use of bioanalytical methods for screening of food and feed samples for PCDD/Fs and dl-PCBs. The purpose of such screening is to eliminate all samples below the level of interest, whether this is the compliance with the MLs as the decisive legal parameter or the indication that no elevated levels are present (samples below AL). In this way, those samples samples can quickly be identified which require further investigation by confirmatory gas chromatography – high resolution mass spectrometry (GC-HRMS) analysis. One promising screening method increasingly applied in official control during the past decade is the Chemical Activated LUciferase gene eXpression (CALUX) assay. It detects 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and structurally related halogenated aromatic hydrocarbons (HAHs) based on their ability to activate the aryl hydrocarbon receptor (AhR) signaling pathway. While GC-HRMS methods look for selected congeners, namely the 17 PCDD/Fs and 12 dl-PCBs to which individual toxic equivalency factors (TEF) have been assigned (9), CALUX detects all AhR-agonists present in a sample extract. Screening results may therefore be subject to interference from compounds structurally similar to the target analytes (10-13). As a consequence, some samples may be suspected to exceed MLs or ALs, respectively, from screening but turn out compliant from GC-HRMS confirmatory analysis. Correlation between the official TEF-values and the relative response of the CALUX cell system to the respective congeners, expressed in RElative Potency (REP) factors, is obvious. However, differences between TEF and REP values and the potential detection of AhR-active compounds not included in the TEQ-scheme suggest that a bioanalytical result can only provide an estimate of the TEQ-level measured by GC-HRMS. Considering these and other issues it seems appropriate to express bioanalytical results as Bioanalytical EQuivalents (BEQ).

According to Commission Regulations (EC) No 1883/2006 (food) and 152/2009 (feed), monitoring for the presence of dioxins and dioxin-like PCBs may be performed by a strategy involving a screening method in order to select those samples with levels of dioxins and dioxin-like PCBs that are less than 25 % below or exceed the maximum level. In practice this means that results from bioanalytical screening are directly to be compared to regulatory limits given in TEQs. However, correspondence between BEQ values and TEQ values from GC-HRMS analyses may not be in a one-to-one relationship, depending on sample matrix and congener patterns, and on the properties of the reference sample used for recovery control. This stresses the need to investigate this correspondence during validation of the bioanalytical method, and to evaluate cut-off values above which a sample is suspected to exceed the respective legal limits (MLs for compliance, ALs as warning indication for possibly elevated levels).

In this paper, we present two approaches for assessing matrix- and congener pattern-related BEQ-based cut-off concentrations suitable for routine bioanalytical screening, forming an important part of revisions recently proposed by an expert group established for re-evaluation of the current criteria for bioanalytical methods, based on gained experience of a number of National Reference Laboratories and of the European Union Reference Laboratory (14).

Materials and Methods

40 fish oil samples were prepared at 2 g each from material pre-analyzed by GC-HRMS, containing PCDD/Fs < LOQs and 3.86 pg PCB-TEQ per g fat. Samples were spiked with both 2,3,7,8-TCDD and PCB 126 at 0, 0.5x, 1x, 1.5x and 2x ML and AL, respectively (ML = 2 pg PCDD/F-TEQ per g fat, AL = 6 pg PCB-TEQ per g fat). Six replicate analyses were performed on each level, on different days. For recovery control, fish oil was chosen containing 2.0 pg PCDD/F-TEQ and 7.4 pg PCB-TEQ per g fat. Samples were cleaned-up on acidic silica followed by fractionated elution of dl-PCBs and PCDD/Fs from a column loaded with XCARB (1% activated carbon/celite) obtained from Xenobiotic Detection Systems (USA). In the bioassay, cells from a genetically modified mouse hepatoma cell line (15) (H1L6.1c3) were used, made available for research from Prof. M.S. Denison (University of California Davis, USA). After exposure of the cells to the sample extracts, luciferase activity was measured in a luminometer and transformed into a BEQ concentration using a 2,3,7,8-TCDD standard curve, taking into account sample intake and dilution of the extract in the incubation medium. Results were corrected for procedure blank and apparent recovery, and expressed as PCDD/F-BEQs and dl-PCB-BEQs.

Results and Discussion

The capability of a screening method to detect samples potentially exceeding the established legal limits is a fundamental performance characteristic. It is a function of the cut-off concentration, the evaluation of which must be based on certain key requirements and should be part of each validation study. Correspondence between BEQ and TEQ values must be taken into account, e.g. by performing matrix-matched calibration experiments involving samples spiked around the level of interest (approach 1). Spiking levels are ideally checked by GC/HRMS and plotted against bioanalytical results corrected for blank and recovery. Regression analysis is performed and from the prediction interval of the regression line the cut-off value is calculated. It shall be set in a way that screening results exceeding the cut-off concentration are likely to fall above the GC-HRMS decision limit (x_{DL}), taking into account the expanded measurement uncertainty (MU) of the GC-HRMS method (RSD_R<15%). From the lower band of the one-sided prediction interval at x_{DL}, the cut-off is estimated 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:

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

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, $t_{\alpha,f=m-2}$ = student factor (α = 5%, one-sided).

The 95% level of confidence implies a false-compliant rate < 5%. This seems appropriate especially from a practical point of view, although this requirement is currently set at 1% by EU legislation for MLs (but no requirement for ALs), while in other analytical fields 5% is more common. Current legislation further requires the within-laboratory reproducibility (RSD_R) in screening not to exceed 30%. Based on practical experience of a number of routine

laboratories it was suggested by the above mentioned expert group to tighten this requirement to a $RSD_R < 25\%$.

From the results for each series of samples, individual calibration lines were calculated for 2,3,7,8-TCDD and PCB 126 (figure 1). Figure 2 shows the overall calibration lines for 2,3,7,8-TCDD (y = 0.48x + 0.73), and for PCB 126 (y = 0.98x + 0.23) with their respective prediction intervals. Slopes represent the sensitivity of the method, while recovery depends on concentration whenever the line does not pass through the origin. Recoveries range from 117% to 67% (levels 1 - 4) for 2,3,7,8-TCDD, and are close to 100 % on each level for PCB 126, demonstrating that the reference material represents the calibration samples well with regard to physico-chemical properties, concentration and congener pattern. Y-intercepts representing the mean response from a blank matrix were 0.73 and 0.23 PCDD/F-BEQ / g fat, for 2,3,7,8-TCDD and PCB 126, respectively, indicating that other AhR-active compounds may be present in the PCDD/F-fraction, while the y-intercept of the PCB 126 calibration line is not significantly different from zero. Bioassay cut-off values were calculated based on 4 and on 5 levels, and on a GC-HRMS RSD_R of 10% and 15%, but no significant difference was observed. The cut-off (ML) for 2,3,7,8-TCDD was 1.7 pg BEQ/g fat, while the cut-off (AL) for PCB 126 was 6.2 BEQ/g fat, each based on 5 levels and a GC-HRMS RSD_R of 10%.

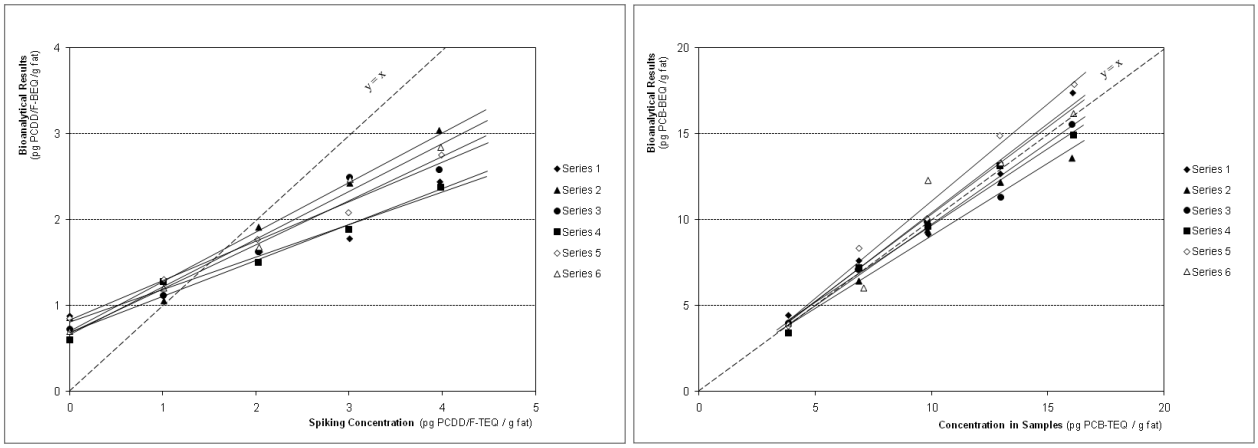

Figure 1. Individual calibration lines from 6 series of fish oil samples spiked with 2,3,7,8-TCDD and PCB 126

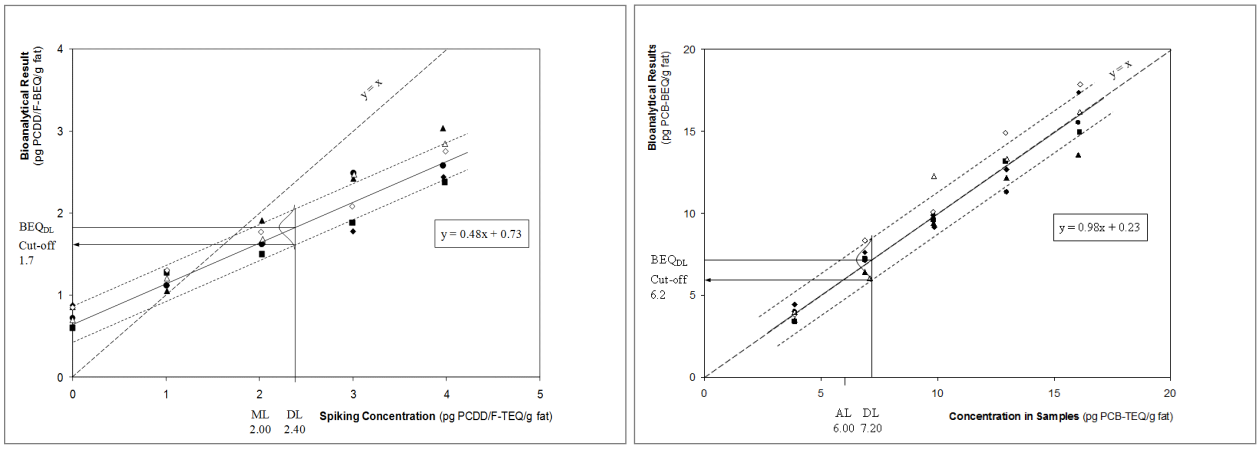

Figure 2. Bioassay cut-off values from the 95% prediction intervals for PCDD/Fs and for dl-PCBs in fish oil

The cut-off value may also be estimated as the lower endpoint of the distribution of BEQ results obtained from at least 6 repeated analyses of a sample spiked at the GC-HRMS decision limit (x_{DL}), above which 95% of the response variables are located (approach 2):

Bioassay cut-off value = $\bar{y}_{DL}$ − 1.64 * SD_R, with n ≥ 6, SD_R = reproducibility standard deviation

The Bioassay cut-off value calculated for 2,3,7,8-TCDD from 6 repetitions on level 2 was 1.5 pg BEQ/g fat, or 12% below the cut-off derived from the full calibration experiments (1.7 pg BEQ/g fat). This difference seems to be due to level 2 actually being 12% below the GC-HRMS decision limit, reflecting the sensitivity of the method. For PCB 126, the bioassay cut-off value calculated from 6 repetitions on level 1 and the cut-off value obtained from the full calibration experiments match perfectly, the first being 5.9 and the latter 6.2 pg BEQ/g fat.

Conclusions

New BEQ-based, matrix-related cut-off values designed for use in bioanalytical screening of food and feed samples were calculated for PCDD/Fs and dl-PCBs in fish oil according to the approaches 1 and 2 presented in this paper. Bioassay cut-off values derived from matrix-matched calibration experiments and those calculated from multiple analyses of samples contaminated at the GC-HRMS decision limit are comparable. For calibration experiments, 4 levels (0, 0.5x, 1.0x and 2.0x the level of interest) are sufficient. Practical experience at EU-RL and other laboratories applying bioassays shows that the cut-offs calculated are generally found between BEQ concentrations corresponding to the level of interest (ML or AL) and 2/3 of the level of interest (AL or 2/3xAL). With a given ß-error < 5%, this performance characteristic is almost solely a function of the relationship (ratio) between BEQ and TEQ values and of the within-laboratory reproducibility of bioanalytical results, if GC-HRMS expanded measurement uncertainty varies between 20 and 30% (RSD_R 10 - 15%) when using a coverage factor of 2 at 95% confidence level. Use of the BEQ value corresponding to the action level, being 2/3xML for most sample matrices, as a cut-off, as suggested by an expert working group as a fast alternative (14) is in principle supported. However, this may lead to increased rates of false noncompliant results.

Acknowledgements

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