VALIDATION OF THE CALUX BIOASSAY: QUANTITATIVE SCREENING APPROACH

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Introduction

Several papers and data demonstrating the suitability of the CALUX bioassay as a screening method have been published in recent years.[1-8]

The assay enables the detection of samples having unacceptably high levels of dioxins and dioxin-like PCBs and, consequently, the reduction of the amount of samples, which have to be confirmed by expensive HRGC-HRMS analysis. Moreover, the data obtained by the CALUX assay are usually highly correlated with the corresponding GC-MS data, indicating that CALUX results are good estimates of the TEQ contamination of samples. However, until present CALUX TEQ results have not been considered as quantitative TEQ results.

Objectives

The objective of this paper is to discuss the required performance characteristics to validate the CALUX method as a quantitative screening method. This implies that CALUX TEQ results are considered as quantitative results and that statistical evidence must determine the range of CALUX results requiring GC-HRMS confirmation. Our aim is to implement the proposed approach, using preliminary data obtained during the validation of the CALUX assay for liquid milk.

Results & Discussion

Screening approaches

Screening methods are used to detect the presence of a substance or class of substances at the level of interest. Our interpretation of what is meant by a qualitative screening is that the method determines whether the TEQ values of the investigated samples exceed the limit value or not.

One way to perform such a screening is to compare the response of samples to that of a reference sample at the level of interest. The level of the reference sample is such that a sample with a lower response is considered as negative, and a sample with a higher or equally high response as suspected, thus requiring GC-HRMS confirmation.[2] However, the uncertainty of the result for the reference sample is not demonstrated.

A quantitative screening method determines a TEQ value for an unknown sample and after appropriate statistical interpretation it is decided whether the sample should be confirmed by GC-HRMS analysis or not. This is the approach that we prefer to follow and which is outlined in the following sections.

Determination of the confirmation range for GC-HRMS

To be able to use the CALUX assay as a quantitative screening method we need to know which obtained results should be confirmed by GC-HRMS. It is clear that it is impossible to determine a

single CALUX TEQ value above which the result should be confirmed. Rather a range of TEQ values for which there is insufficient certainty to accept or reject the sample should be determined. This is the 'confirmation range'.

To determine the confirmation range, the concept of CCa and CCb (Figure 1) described in the draft revision of the EC directive 93/256/CE has been used.[9]

For substances with a maximum residue level (MRL, the limit TEQ level for dioxins), the results beyond the decision limit CCa are significantly larger than the MRL at a confidence level of 1-a. Although we can decide in the case of an obtained result above the CCa that the TEQ-value of the sample exceeds the MRL, we cannot guaranty with the same probability that samples with a true concentration larger than the MRL are found above the CCa. This can only be demonstrated for results above the detection capability (CCb). CCb eliminates the possibility of false negative results at a confidence level of 1-b.

Assuming that the data are normally distributed and that the variances at both the MRL and sample level are equal, CCb is the upper boundary and CCa* the lower boundary of the confirmation range (Figure 1). TEQ values beneath CCa* are significantly lower than the MRL.

Samples with a TEQ value within the confirmation range need to be confirmed. The use of this range reduces the likelihood that true negative samples or true positive samples are sent for confirmation to the GC-HRMS. In this way, expensive and time-consuming work can be avoided.

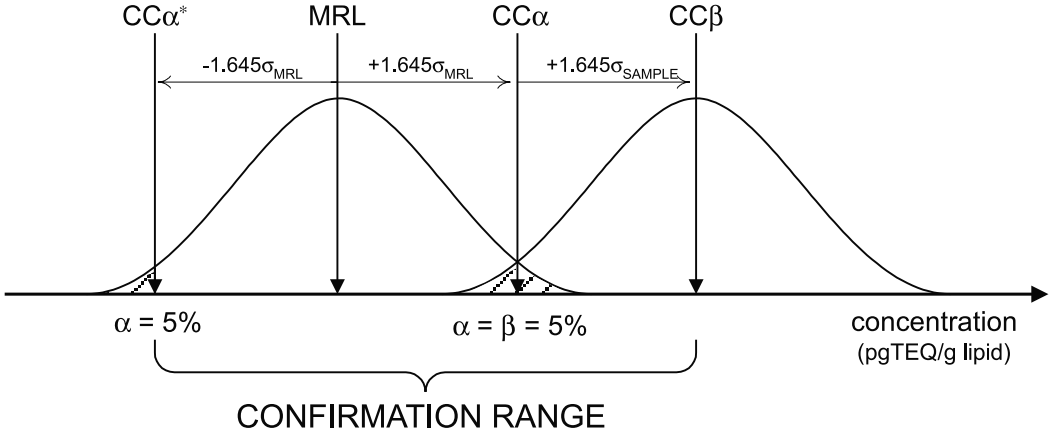

Figure 1. constructing the confirmation range

The CCa and the CCa* for the CALUX assay for milk are estimated by using CALUX results obtained for milk samples spiked at the MRL (i.e. 5 pg TEQ/g lipid.). When a=5 %, the values of CCa and CCa* are calculated by respectively adding or subtracting 1.64 times the s_{MRL} to/from the mean value (mean = 5.45 pg TEQ/g lipid, s_{MRL}=0.8 pg TEQ/g lipid, n=6). When b=5 %, the CCb is estimated by adding 1.64 x s_{SAMPLE} to CCa. Given the assumptions mentioned above, s_{MRL} can be substituted by s_{SAMPLE}.

The experimentally determined confirmation range reaches from 4.1 to 8.1 pg TEQ/g lipid. All samples leading to results within these boundaries need to be confirmed by GC-HRMS.

Determination of the detection limit

To estimate the detection limit (L_D) the same methodology with a- and b-error, as described above, can be applied (assuming normality and a=b=5 %). This methodology has also been described in the

IUPAC recommendations.[10]To calculate the L_D the solvent blank has been used as surrogate for real blank samples since real blank milk samples are not available. The solvent blank is a blank solution, which is taken through the whole procedure, from the pre-treatment up to the measurement. We also preferred the solvent blank and not the DMSO response because for solvent blanks TEQ values can be calculated. (mean=0.32 pg TEQ/well; s_{bl}=0.16 pg TEQ/well, n=25). First the critical value L_C is calculated. It is the critical level or decision limit above which a result may be reliably recognized as detected. When a=5 % L_C is calculated by adding .1.64s_{bl} to the mean blank result which gives L_C= 0.32+ 1.64 x 0.16 = 0.58 pg TEQ/well.

The L_D will be given by the following equation: $L_D = L_C + 1.64 \times s_{SAMPLE}$. L_D= 0.58 + 1.64 x 0.15 =0.83 pg TEQ/well.

When analysing milk samples with a TEQ of 5 pg TEQ/g lipid we measured 0.95 pg TEQ/well (s: 0.15, n = 12) considering the quantity of milk and purified extract used). This value is close to the calculated L_D.

By adjusting (increasing) the amount of purified milk extract it is possible for samples at the MRL to measure well above the L_D (results not shown). It needs to be confirmed if and to what extent the mean blank value and consequently L_C and L_D will change when more 'blank extract' is used.

It is advisable to calculate the L_D in this way for a certain procedure and matrix.

Otherwise, the L_D in the CALUX assay could be calculated for each plate and corresponding standard curve. In this case only the DMSO response can be used as a blank specific for that plate. We believe that the L_D expressed as TEQ is underestimated when the DMSO blank responses are used to calculate it. Moreover this approach hampers the comparison of calculated L_{Ds} between laboratories. We previously suggested calculating the L_D from the standard curve (described by the Hill equation) as the y-intercept plus 2.5 x s_{DMSO}.[11] Application of this formula resulted in L_{Ds} less than half of the above mentioned L_D of 0.83 pg TEQ/well.

In any case a calculated L_D should experimentally be demonstrated. This is not a simple task since blank samples are usually not available.

Conclusion

An approach to determine the range of CALUX data that need to be confirmed by GC-HRMS has been proposed. Secondly, a calculation model for the detection limit of a procedure has been illustrated.

When procedures for analysing other matrices are optimised during validation it must be checked and confirmed that detection limits fall sufficiently below the limit value for the matrix under study.

Acknowledgements

Financial support by the Office for Scientific, Technological and Cultural Affairs and from the Belgian Ministry of Public Health is gratefully acknowledged.

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