HARMONISED QUALITY CRITERIA FOR CHEMICAL AND BIOASSAYS ANALYSES OF PCDDs/PCDFs IN FEED AND FOOD PART 2: GENERAL CONSIDERATIONS, BIOASSAY METHODS

Peter A. Behnisch[1], Randy Allen[2], Jack Anderson[3], Abraham Brouwer[4], David J. Brown[5], T. Colin Campbell[6], Leo Goeyens[7], Robert O. Harrison[8], Ron Hoogenboom[9], Ilse Van Overmeire[7], Wim Traag[7] and Rainer Malisch[10]

[1] Life Science Research Laboratories, Kaneka Corporation, 1-8 Miyamae-Machi, Takasago, Hyogo 676-8688, Japan

[2] Hybrizyme Corp., 2801 Blue Ridge Rd., Raleigh, NC27607, USA

[3] Columbia Analytical Services, 1185 Park Center Drive, Vista CA 92083, USA

[4] Inst. for Environmental Studies, Vrije Universiteit, De Boelelaan 1115, Amsterdam 1081HV, The Netherlands

[5] Xenobiotic Detection Systems, 1601 E. Geer Street, Suite S, Inc. Durham, NC 27704, USA

[6] Paracelsian, and Cornell University, Ithaca, New York 14853, USA

[7] Scientific Institute of Public Health, J. Wytsmanstraat 14, Brussels B-1050, Belgium

[8] CAPE Technologies, L.L.C., 3 Adams Street S, South Portland ME 04106, USA

[9] State Institute for Quality Control of Agricultural Products (RIKILT), PO Box230, Wageningen, 6700AE Netherlands

[10] Chemisches und Veterinäruntersuchungsamt, Bissierstr. 5, D-79114 Freiburg, Germany

Introduction

Given the wide variation in methodology (different chemical analyses and bioanalytical methods) and uncertainty regarding quality assurance and control across laboratories, harmonisation of general acceptance criteria for dioxin TEQ analyses in feed/food is needed. Consistent quality assurance across these various methods will assist in comparing these state-of-the-art analytical methods. General principles should be valid not only for GC/MS methods, but also for bioassays. GC/MS methods are applied as quantitative assays which should provide exact qualitative and quantitative information on congeners of interest, whereas bioassays are predominantly intended as high throughput screening assays which should detect elevated levels. Therefore, if the same quality criteria (QCs) are used for bioassays, cost and time advantages that the bioassays provide would be reduced. However, harmonisation of acceptance criteria for dioxin analysis is needed to allow free trade if tolerances are developed. Therefore, in two parts quality criteria are presented: in part 1 for GC/MS methods, in part 2 for bioassays. In this expert round table, guidelines for cell culture and kit based bioassays for measuring dioxin-like compounds will be discussed to clarify specific technical questions associated with applying QCs for these biomethods.

Several review articles[1-7] and guidelines (reviewed in reference 2 and 3) have already been published regarding new biotechnologies applied for dioxin-TEQ measurement. Bioassays, like reporter gene assays (e.g. CALUX, P450HRGS)[4,5], Ah-immunoassay (AhIA)[7] or enzyme immunoassays (e.g. DF1)[7,8,9] provide a direct measure of the total TEQ of dioxin-like activity

present in a matrix, including possible interactive (synergistic or antagonistic) effects of all dioxin-like congeners in a complex mixture. However, bioassays as they operate presently do not discriminate among different PCBs, PCDDs and PCDFs congeners, although some assays can discriminate between PCBs and dioxins/furans (depending on the use of a more selective clean-up procedure). By using a Toxicity Identification Evaluation (TIE)[10] study design these biotechnologies are helpful in the risk assessment of the fraction responsible for dioxin-like effects in a complex mixture. Furthermore, they provide information about toxicological real-world effects since different toxicological endpoints (e.g. Ah receptor machinery based, or antibody based) are assessed. Therefore, bioassays are useful for toxicological evaluation and/or for "screening" purposes.

The reporter gene bioassays (e.g. CALUX or P450HRGS) and several enzyme-immunoassay (Delfia or DF1) have previously been applied, but only limited data are available to the public. One example of the application of the CALUX test for food samples is the study of Van Overmeire et al. (2000)[11]. Bovee et al. (1998)[12] and Hoogenboom et al. (2000)[4,5,13] confirmed the validation of the CALUX assay in the range of 5 pg TEQ/g fat for milk and 500 pg TEQ/kg feed, respectively. So far, RIKILT has been the only institute that used the assay during a crisis (e.g. citrus pulp and Belgian crisis) and now for official control and monitoring. In these cases, action limits were set at 5 pg TEQ/g in milk fat and 400 pg TEQ/kg for citrus pulp. The CALUX-test proved to be capable of identifying samples that contain elevated levels of dioxins, and, even more important, was able to indicate that dioxins were not present at levels above the regulatory limits. Further international validation studies are needed for a balanced evaluation of the performance of candidate test-methods.

Harmonised Quality Criteria (QCs) for cell based and kit based bioassays

1. General

Prior to beginning a measurement protocol of biological or chemical analyses the relevant quality control criteria should be well defined. The characteristics of these QCs will vary depending on the analytical approach being implemented. Three different analytical approaches could be performed using bioassays:

(A) The first one is a screening approach: the response of the samples is compared to that of a reference sample at the action limit. Samples with a response less than the reference are declared negative, those with a higher response are suspected. Requirements may be less strict than those for the quantitative method: (1) A blank and reference standard have to be included in each test series, which is extracted and tested at the same time under identical conditions; (2) the reference sample must show a clearly elevated response in comparison to a blank; (3) extra reference samples 0.5 and 2x action limit should be included to demonstrate the proper performance of the test in the range of interest for control of tolerances; (4) when testing other matrices, the suitability of the reference standards has to be demonstrated, preferentially by including samples shown by GC/MS to contain a dioxin level around that of the reference sample or a blank spiked at this level; (5) Tests on repeatability are very important to obtain information on the standard variation within one test series. Data should be provided that demonstrate the repeatability, e.g. the coefficient of variation could be regulated as being below 20% within a test series. (6) For bioassays it must be made clear what the assay identifies as target compounds, interferences, and what the level of

laboratory clean-up blank should be. (7) The determination of minimal detection limit and minimal quantitation limit should be reported in each test.

(B) The second is a quantitative approach, which requires a standard dilution series, duplicate or triplicate clean-up and measuring, blank and recovery controls. The result would be expressed as TEQ (pg/g), assuming that the compounds responsible for the signal correspond to the TEQ principle. This can be performed by using TCDD (or dioxin/furan standard mixture) to produce a calibration curve to calculate the WHO-TEQ level in the extract and thus in the sample. This is subsequently corrected for the WHO-TEQ level calculated for a blank sample (may include impurities from solvents and chemicals used), and a recovery (calculated from the WHO-TEQ level in a reference sample around the residue limit). It is essential to note that part of the apparent recovery differences may be due to differences between the relative effective potency (REP) values in the bioassays and the official TEF values set by WHO.

(C) The third design uses bioassays for toxicological studies with different toxic endpoints (e.g. AhR based or antibody-based bioassays). In this case it is preferable to use a wide range of effective concentrations to evaluate a full EC_{50}–curve and to obtain TEF or TEQ values from different measurement points (EC_{50}, EC_{10} or lowest data point closest to the minimal quantification limit).

2. General QCs for laboratories using bioassays

(1) Sampling, extraction, clean-up procedure and general validation should be done according to the guidelines for chemical analysis (e.g. EPA 8290 method), or specified for individual bioassays (e.g. EPA 4025 or 4425 method). (2) A Standard Reference Material (SRM) (containing PCBs, dioxins/furans and probably PAHs) should be included in every test. (3) Interferences of the test method should be defined as much as possible. (4) The Bioassay laboratory should be approved according to an ISO-Norm (e.g. 9001 or others) and/or Good Laboratory Practices (GLP). (5) Standard Operation Procedures (SOPs) must be established for extraction, clean-up, blank sample procedure, bioassay performance, data report and data handling. (6) Charts should be maintained to record the long time stability of the bioassay response for 2,3,7,8-TCDD (e.g. induction factor, EC_{50} value). If the response for a test is outside of 2 standard deviations from the long-term mean, the results from the test may be invalid and the samples should be re-tested. (7) Validation studies in inter- and intralaboratory studies must be performed. (8) An instrumental control chart should be reported. The sensitivity and linearity of the machine has to be tested at least monthly with a standard. (9) Routine quality control procedures associated with bioassays include the analysis of standards, samples, and both spiked and unspiked method and solvent blanks.

3. Special requirements for <u>all</u> bioanalytical detection methods

(1) Information on number of false-positive and false-negative results of a large set of samples below and above the residue limits, as determined by GC/MS. Actual false negative rates under 5% could be accepted (EPA Method 4025). The rate of acceptable false-positive results is more difficult to determine, since a positive result may be caused by a true Ah-receptor agonist that does not belong to classes of target compounds (in most cases dioxins and dioxin-like PCBs). However,

under normal circumstances the overall rate of positive samples should be low enough to make the use of a screening tool advantageous. (2) Positive results should be confirmed by HRGC/HRMS. Samples from a wide TEQ-range should be confirmed by HRGC/MS (approximately 2-10% of the total samples). (3) Standard testing procedures for use on environmental samples should first meet the criteria of national organisations (as e.g. USEPA Method 4425 or 4025; ASTM Guide E1853-98M or APHA Standard Method 8070)[2,3,14,15] or international standards.

4. Special requirements for cell based bioassays

(1) When performing a bioassay, every test run requires a reference concentration series of TCDD or a dioxin/furan mixture (full dose-response curve with a $R^2 > 0.95$ or with a minimal residue variance for nonlinear equations). However, for screening purposes an expanded low level curve for analysing low level samples could be used. (2) A TCDD reference concentration (about 3 x minimal detection limit) on a quality control sheet should be used for the outcome of the bioassay over a constant time period. (3) Requirements for the EC_{50} value of TCDD: QC charts for TCDD should be recorded and checked to make sure the outcome is in accordance with the stated guidelines. The EC_{50} of TCDD should be constant (e.g. >75%) over a longer time period (e.g. DR-CALUX: EC_{50} value ranging between 7.5-12.5 pM). (4) The induction of the sample dilution used must be within the linear portion of the response curve. Samples above that must be diluted and re-tested. Therefore, it is recommended that at least 3 dilutions have to be tested. However, the lower part of the dose-response curve is often useful for screening. Therefore, the minimal quantitation limit could be used for TEQ-measurement. (5) The percent standard-deviation should not be above 10% in a triplicate determination for each sample dilution and between three independent experiments not above a regulated percentage (e.g. 50%). (6) The limit of detection could be set as 3 times standard deviation of the solvent blank (cell based bioassays) or of the background response (kit based bioassays). Another approach is to apply a response that is clearly above background (induction factor 5 times the solvent blank) to the equation of the day from the calibration curve. (7) The final TEQ measurement could be analysed by using the EC_{50}, EC_{25} or $EC_{10\text{-}15}$ levels between TCDD and sample or a fixed effect level TEQ measurement (for review see reference 3).

5. Special requirements for kit based bioassays

Standard quality criteria requirements for kit based bioassays could be (see e.g. EPA Method 4025 or 4035): (1) Follow manufacturer's instructions for sample preparation and analyses. (2) Do not use test kits past their expiration date. (3) Do not use materials or components designed for use with other kits. (3) Use the test kits within the specified storage temperature and operating temperature limits. (4) An acceptable limit of detection traditionally for immunoassays is 10 times sigma of blank divided by the slope. (5) The comparable test for a immunoassay measurement machine would be 10 times sigma of a sample with dioxin/furan levels below detection. (6) Reference standards should be used for tests at the production facility and/or at the user to make sure that the responsiveness to the standard is within an acceptable range.

Essentially kits are screening assays and should follow many of the same QC guidelines suggested for the screening assays.

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