DETERMINATION OF DIOXINS AND PLANAR PCBs IN FISH USING THE GC\MS AND CALUX BIOASSAY

INTRODUCTION OF THE SCREENING APPROACH FOR CONTROL PURPOSES

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Introduction

During the Belgian dioxin crisis in 1999 it has once again been demonstrated that there is a strong need for rapid screening methods for dioxins and related compounds. Together with the Agricultural University in Wageningen and the University of California in Davis, RIKILT has been involved in the development and validation of the so-called CALUX bioassay, a reporter-gene assay for Ah-receptor agonists[1]. The assay is based on the increased production of the enzyme luciferase by hepatoma cells, following binding of *e.g.* dioxins to the Ah-receptor, transport of the complex to the nucleus and subsequent binding to a xenobiotic responsive element in the DNA. Exposure of the cells to TCDD will result in a dose-related formation of luciferase, which at the end of the exposure time can be measured in an enzyme assay by the production of light. The test has been shown to respond to the different dioxin and PCB-congeners in accordance to their TEF-values, although there is a tendancy for weaker agonists to give a relatively poor response[2]. The response factor for e.g. PCB 105 (TEF of 10^{-4}) was only 2.10^{-6}. Other compounds like certain polyaromatic hydrocarbons (PAHs), flavones and benzimidazoles may also cause a positive response, due to their capacity to bind to the Ah-receptor[3]. When aiming at the selective detection of dioxins and planar PCBs this can partly be overcome by the use of longer incubation periods (24 h), allowing the cells to metabolise certain agonists and to degrade the luciferase produced during the first part of the incubation period in response to the exposure[3]. An additional selection is obtained by the use of a clean-up procedure with acid silica (33% H_2SO_4) columns.

The combined approach, CALUX-cells and acid silica clean-up, has been validated for milk fat[2], animal blood[4] and citrus pulp. During the validation of the test for milk fat it became clear that suitable controls should be included for correction of results for 1) contaminants from the chemicals used in the clean-up, 2) deviations between WHO-TEFs and response factors in the assay (see below) and 3) recovery losses, especially since the test does not allow the use of internal standards. However, during the Brazilian citrus pulp crisis it turned out that any

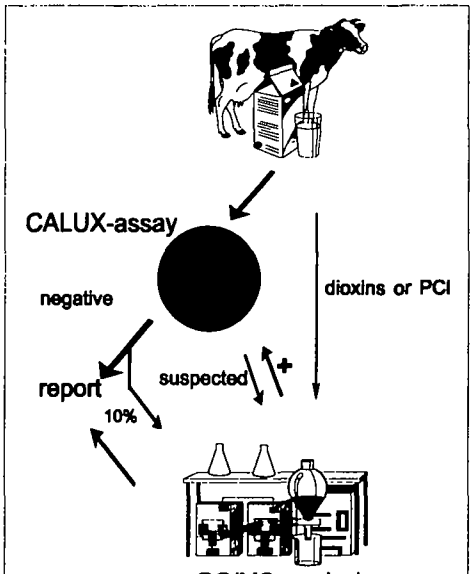

Figure 1 Procedure used for analysis of samples. Most samples were screened with the CALUX-assay. Samples showing a response lower than the reference sample were declared negative, those with a higher response suspected and requiring GC/MS confirmation. In addition 10% of the negative samples were analysed by GC/MS. Samples positive by GC/MS were routinely analysed by CALUX.

sample exceeding the residue limit still required GC/MS confirmation. Furthermore, quantification of dioxin levels in samples would not acknowledge the fact that the test is in principle a test for Ah-receptor agonists and not exclusively for dioxins or planar PCBs and that other agonists are not included in the TEQ-principle. For these reasons a more simple approach was chosen during last years crisis in which the response of the sample is compared with that of a reference sample included in the test. 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 response as suspected, thus requiring GC/MS confirmation. The test approach included testing 10% of the negative samples with GC/MS (see figure 1). The test and in particular the procedure was accreditated by the Dutch Sterlab system. The present paper will demonstrate that based on the very low chance on false-negative results, the test is especially suitable for pointing out samples with dioxin levels below the residue limit. Furthermore, regarding the possible action of unknown agonist, the test functions best when operated in combination with the GC/MS reference method, allowing rapid investigation of suspected samples.

Materials and methods

Fat samples (0.5 gram) were dissolved in hexane/diethyether (97/3) and eluted over a 33% sulphuric acid/silica column using hexane/diethylether 97/3 (v/v) as eluens. Citrus pulp (5 g sample) was mixed with water/methanol 15/85 (v/v) and extracted with hexane/diethylether 97/3 (v/v), prior to acid silica clean-up. The volume of the eluate was reduced using a Speed-Vac evapoator. The remaining 3-5 ml was transfered to a small tube, mixed with 20 µl DMSO and further dried under nitrogen. After addition of another 20 µl DMSO, the extract was transfered to 2 ml incubation medium. Each series of samples (1-26) contained one blanc sample and one or more reference samples. In the case of butter and animal fat a butter fat sample containing 2.7 pg WHO-TEQ dioxins and 2.3 pg WHO-TEQ PCBs per gram was used as reference, in the case of feed or feed components, a citrus pulp sample containing 430 pg WHO-TEQ dioxins/kg.

Transfected H4IIE-cells were plated in 48 well plates in 0.25 ml medium. After 24-36 h exposure was started by addition of 0.25 ml of medium containing the sample extract. Following exposure for 24 h, the cells were checked for cytotoxicity. Subsequently the medium was removed and the cells washed with PBS and lysed in 50 µl lysis buffer. An aliquot of the lysate was transfered to a 96 well-plate and used to quantify the amount of luciferase using a Luminometer.

Results and Discussion

Use of reference samples

In principle the CALUX assay can be used for a quantitative estimation of the levels of dioxins and planar PCBs in a sample, assuming that the response is completely caused by these compounds. When testing series of samples this requires inclusion of proper blanc and reference samples to correct for impurities from the used chemicals, recovery losses and the differences between WHO-TEFs and response factors in the test. When using a TCDD calibration curve for calculation of the dioxin levels, a major source for underestimation of the actual dioxin and planar PCB content, will be the differences between the WHO-TEF values and the relative response factors obtained in the CALUX-assay. In particular the recent adjustment of the TEF-value for

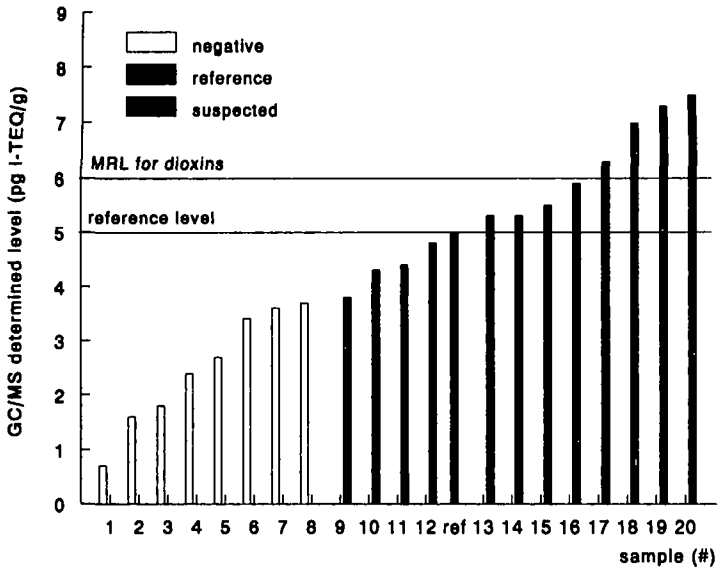

Figure 2. Screening of milk samples using the CALUX-bioassay. Samples were tested in comparison to a reference sample containing 2.7 pg TEQ dioxins and 2.3 pg TEQ PCBs. Samples showing a higher response were declared suspected, samples containing a lower response negative.

1,2,3,7,8-pentachloro-p-dioxin from 0.5 to 1 deviates remarkably from the response factor of 0.5 in the assay[2]. The relatively low response of *e.g.* 2,3,4,7,8-pentochlorofuran, PCB 126 and the hexachlorinated compounds, being about 70% of the WHO-TEF value, will further contribute to the difference. In practice, it can be calculated from field samples that the dioxin content obtained by using TCDD for calibration, would only be 70% of the actual figure expressed in WHO-TEQs. For the non-ortho PCBs a similar figure can be estimated, whereas for the mono-ortho PCBs the result would be dramatically low due to the non-responsiveness of these compounds in the test (response factors less than 5% of the WHO-TEFs). However, in most cases the latter compounds will not contribute significantly to the total TEQ-content. This problem can best be overcome by the use of either a standard mixture with the most important dioxins and PCBs or reference samples contaminated with a mixture of dioxins and PCBs representative for the contamination.

The screening approach

The screening approach used for testing of samples of various different matrices is exemplified in Figure 2, using a set of 20 milk samples from cows fed Brazilian citrus pulp[5]. Test samples were only analysed for dioxins, since it has been reported that planar PCBs represented only a very minor fraction of the total TEQ-level in the citrus pulp. Eight samples (1-8) showed a lower response than the reference sample and were declared negative, twelve samples (9-20) were declared suspected based on an elevated response. Four of the latter samples (9-12) actually contained a dioxin level slightly lower than the reference sample and could be regarded as false-positive. Similar is the case for an additional 4 samples which exceeded the level of the reference sample but not the residue limit (MRL) of 6 pg TEQ/g. All four samples with a dioxin level higher than the residue limit showed an elevated response, *i.e.* no false-negative results. Athough the fraction of false-positive samples is relatively high, it should be mentioned that this is primarily caused by the choice ofthe reference sample, aiming at the detection of any sample appoaching or exceeding the present MRL, and the relatively high levels in the samples. Regarding current background levels in the Netherlands below 3

pg TEQ /g (dioxins and PCBs) for milk and animal fat it is unlikely that this may actually cause problems in practice.

Evaluation of performance of the test during the crisis

By the end of September a total number of 1380 samples (see Table 2) samples had ben tested, revealing 1213 (88%) to be negative and 28 samples to be cytotoxic. About 10% (139 samples) gave a response higher than that of the reference sample and was declared suspected. Of the 81 negative samples tested by GC/MS only one gave a false-negative result, being a feed premix sample shown to contain 540 pg i-TEQ per kg and thus higher than the limit of 500 pg i-TEQ/kg. The effect may be explained by the poorer extraction efficiency of dioxins from the kaolinic clay containing matrix. For various reasons, only 48 of the 139 suspected samples were analysed by GC/MS. In particular at the start, it was shown that positive effects were caused by impurities from the solvents used by clients to isolate the fat. Reanalysis of new samples showed a negative result. Other samples were withdrawn based on the CALUX or PCB analysis. When focussing on the 48 suspected samples analysed by GC/MS for dioxins, 24 (50%) were shown to be true positives. Another 11 samples contained GC/MS determined dioxin levels close to the limits (> 4 pg i-TEQ/g) and being likely to exceed the limits if planar PCBs would have been included. The remaining 13 suspected samples could not be explained.

Table 2. Numbers of samples analysed with the CALUX bioassay in june-september 1999, including the number and fraction of GC/MS analysed negative, toxic and suspected samples.

Method	analysed	toxic	negative	suspected
CALUX	1380	28	1213	139
GC/MS	157	28	81	48
negative			80	24
positive			1	24

Based on the experience during the Belgian dioxin crisis it is concluded that the CALUX bioassay in combination with the acid silica clean-up procedure, is a very suitable screening method for dioxins and planar PCBs. Since in most cases a rapid investigation of suspected samples is required, the test functions best when operated in close combination with the GC/MS method.

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