

Short communication

## Importance of clean-up for comparison of TEQ-values obtained by CALUX and chemo-analysis

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Received 29 March 2004; received in revised form 12 May 2004; accepted 13 May 2004

Available online 2 July 2004

### Abstract

This paper presents Chemically Activated Luciferine gene eXpression (CALUX) TEQ-values obtained for nine plasma samples following two different purification procedures, one of them involving fractionation. CALUX results obtained for the dioxin (DX) and dioxin + PCB (DX + PCB) fractions were then compared to the GC-HRMS TEQ-values calculated for the 17 polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (17 PCDD/F) and 17 PCDD/F + 4 cPCB congeners, respectively. The overestimation of the CALUX (DX fraction) TEQ-values in comparison with the chemo-analyses of the 17 PCDD/F is mainly explained by the presence of other AhR agonists, like brominated compounds. Otherwise, the constancy of the CALUX (DX + PCB fraction) TEQ-value which compares to increasing the GC-HRMS (17 PCDD/F + 4 cPCB) TEQ results raises questions concerning (1) the significance of CALUX results obtained without fractionation as well as (2) the toxicological effect of a cocktail of contaminants on the human health.

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**Keywords:** CALUX; Fractionation; Human plasma; Dioxin-like compounds

### 1. Introduction

During the last decade the application of screening methods in environmental, toxicological and epidemiological studies [1–8] as well as in governmental control on food and feed safety, workplace conditions and others [9–13] received enhanced attention.

Chemically Activated Luciferine gene eXpression (CALUX) is a mechanistically based technique that detects all aryl hydrocarbon receptor (AhR) agonists [14]. The technique generates TEQ-values by comparison of the sample's signal to a dose-response curve of 2,3,7,8-tetrachlorinated dibenzo-p-dioxin (TCDD) stan-

dards. Consequently, CALUX TEQ-values correspond to an overall AhR activity, representing all AhR ligands of the extract, with their non-additive interactions. The CALUX technique does not identify them, however.

In contrast herewith, GC-HRMS TEQ-values invoke the additivity principle. They are based on analytical concentrations of 17 polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans (PCDD/F) and four coplanar polychlorinated biphenyls (cPCB), having an assigned WHO-TEF value [15].

Since CALUX results represent an overall AhR activity, extract purification as well as fractionation prior to exposure do largely affect the final result. In contrast, as long as there are no interferences, the clean-up does not affect the chemical measurements of PCDD/F and cPCB by isotope dilution. The present study illustrates the variability resulting from different clean-up procedures. Moreover, the paper

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discusses the data evaluation when plasma samples are analyzed in view of epidemiological investigations.

## 2. Material and methods

### 2.1. Samples

Nine plasma samples from an elaborate study on the impact of the Belgian dioxin crisis were submitted to different clean-up procedures for the current investigation. More details on sample collection, GC-HRMS and CALUX analyses are published by Van Wouwe et al. [16].

### 2.2. Chemo-analysis

Analyses of 21 dioxin-like congeners (17 PCDD/F + 4 cPCB) were performed by CART (Liège, Belgium). Details of the method have previously been described [17]. In summary, after addition of  $^{13}\text{C}$ -labeled internal standards, 30–60 mL of sample was mixed with formic acid and water (1:1:1). This mixture was loaded on a preconditioned Isolute C18 cartridge and target analytes were eluted with hexane. The extract was purified on a Power-Prep system with an automated multi-column clean-up using disposable silica, alumina and carbon. Purified extracts, including a recovery standard, were then injected on a Hewlett Packard 6890 serie Gas Chromatography-AUTOSPEC ULTIMA High Resolution Mass Spectrometer (Micromass, UK). TEQs of individual congeners were calculated, using human 2,3,7,8-TCDD TEFs reported by WHO [15].

### 2.3. CALUX analyses

Bio-analyses were performed by the Scientific Institute of Public Health (Brussels, Belgium). Dioxin-like contaminants were extracted from 10 mL of blood plasma with acetone and *n*-hexane. Extracts were dried on a Celite/ $\text{Na}_2\text{SO}_4$  column and transferred, afterwards, onto an acid Silica column in series with an activated carbon column (XCARB). After elution of the sample with *n*-hexane, the acid silica column was discarded and the XCARB column was differentially eluted to yield three fractions: (1) some toxic or interfering compounds eluted by a mixture of *n*-hexane/acetone, (2) the PCB fraction, which contains around 70% of the cPCBs and 30% of mono-ortho PCBs, eluted with a mixture of *n*-hexane/toluene/ethyl acetate, and (3) the PCDD/F (DX fraction) collected with 20 mL of toluene [18]. When using this three step clean-up, fractions 1 and 2 were discarded and only the DX fractions were used for bio-analysis.

For the collection of the combined PCB + DX fraction, the XCARB column was eluted with 20 mL of toluene directly after elimination of interfering substances with the *n*-hexane/acetone mixture.

The DX and DX + PCB fractions were evaporated and exposed to the mouse hepatoma H1L6.1 cell line developed

by Xenobiotic Detection System Inc. (Durham, USA). After an exposure time of 20h, cells were lysed and measurements were made with a Lucy 1 luminometer (Anthos Labtec, Austria).

## 3. Results and discussion

The qualitative description of the results is illustrated in Fig. 1. TEQ-values for the 17 PCDD/F as well as for the 17 PCDD/F + 4 cPCB congeners that were obtained by GC-HRMS, are compared with the CALUX TEQ-values for the DX and DX + PCB fractions, respectively.

Whereas the contributions of the PCBs to the dioxins and furans leads to higher TEQ results by chemo-analysis, the combination of the two fractions in the purification step for CALUX induces an opposite effect. Moreover, the CALUX TEQ-values for the DX fractions are consistently higher than the GC-HRMS TEQ-values for the 17 PCDD/F or the 17 PCDD/F + 4 cPCB congeners. For the PCB + DX fractions, however, TEQ results obtained with CALUX are below GC-HRMS TEQ-values.

To compare CALUX results with the corresponding ones obtained by chemo-analysis the data are graphically presented in Fig. 2. The comparison of CALUX TEQ (DX fraction) with GC-HRMS TEQ-values (17 PCDD/F) evidences that CALUX TEQ-values exceed largely the bisecting line, confirming herewith the earlier observed bias for plasma samples when comparing with TEQ-values for the 17 PCDD/F congeners, obtained by GC-HRMS [16]. This may be explained by three factors: (1) the use of lower bound values for the calculation of TEQ results by GC-HRMS, (2) the small differences between relative potencies (REP) and WHO-TEF values and (3) the plausible presence of other AhR agonists such as brominated compounds.

More striking is the comparison of CALUX TEQ-values for the DX + PCB fraction with corresponding GC-HRMS (17 PCDD/F + 4 cPCB) values (Fig. 2). All CALUX results are below the GC-HRMS TEQ-values; the regression trend line lays below the bisecting line. This is probably due to the antagonistic effect of some PCBs as well as other AhR ligands that may be present in the PCB fraction [19,21–23].

Moreover, the slope value of the regression line, which is not significantly different from 0 ( $P > 0.5$ ), indicates predominant constancy of the CALUX TEQ-values even when the corresponding GC-HRMS TEQ-values increase by a factor of three. The latter observation emphasizes that the CALUX TEQ-values for the DX + PCB fraction are no good alternatives of chemo-analytical data. When applying a clean-up protocol without separation of dioxins and PCB fractions, CALUX TEQ data obscure the information on the contamination level and can, therefore, not be used in view of developing or designing an appropriate approach for risk assessment. Similar conclusions must be considered for epidemiological statistic approaches. In the latter case, the CALUX TEQ-values for DX + PCB fractions are not accurate for

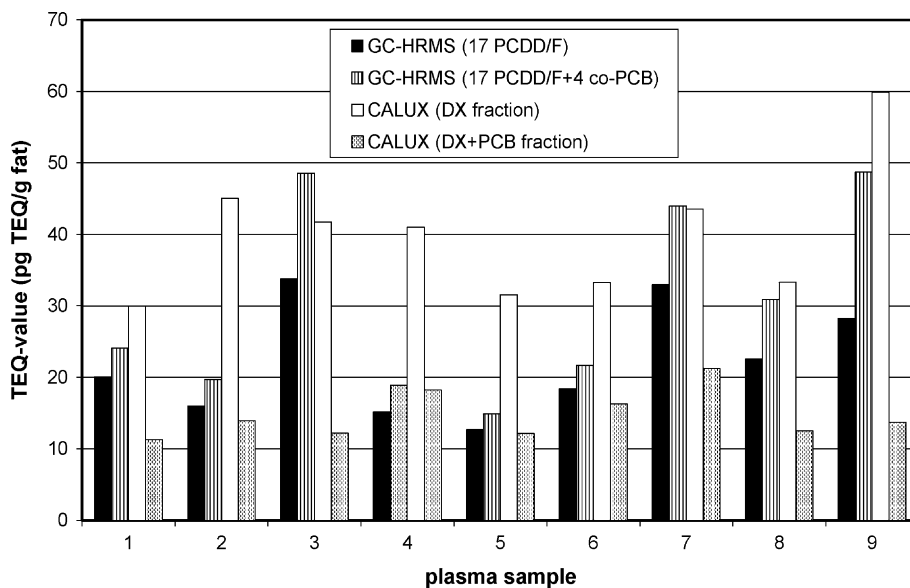


Fig. 1. Qualitative description of TEQ-values obtained for nine human plasma samples for the DX and DX + PCB fraction by CALUX bioassay and for the 17 PCDD/F and 17 PCDD/F + 4 cPCB congeners by GC-HRMS.

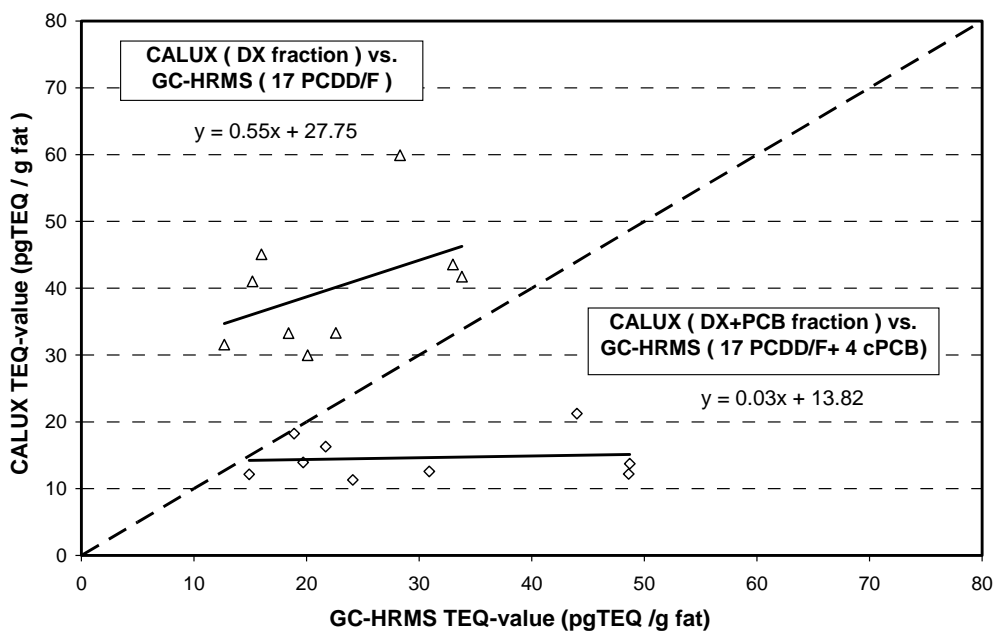


Fig. 2. Comparison of CALUX and GC-HRMS TEQ values obtained for nine plasma samples purified with or without fractionation.

the recognition of dioxin sources, epidemiological determinants of dioxins concentrations or cause effects, since they scatter around a constant average.

On the other hand, non-additive interactions occurring between PCB, PCDD/F and other AhR agonists present in the extract are taken into consideration in the CALUX TEQ-values (DX + PCB fraction) [19,21–23]. This gives emphasis to a fundamental question. Is it thinkable that higher contents of contaminants and, consequently, higher corresponding GC-HRMS TEQ-values for (17 PCDD/F + 4 cPCB) do not match measurably higher AhR activity be-

cause of a pronounced competition between the different compounds? This question can not be answered now, but deserves strong attention by the scientific community. Basically, the main interest of the CALUX test is to provide the laboratory with a tool, that estimates the AhR activity and, hence, the toxic potency of the samples under study [14].

Until now, three studies have focused on the comparison of CALUX and GC-HRMS responses observed for samples prepared with or without fractionation [4,12,20]. In those papers, the “dioxin” or “planar” fractions measured by CALUX yield higher [4,12] or similar [20] TEQ-values

when compared to those obtained with GC-HRMS. The comparison of bioassay data, obtained without separation into distinct fractions, with GC-HRMS values was more variable, on the other hand. For retail fish [12] and human adipose tissue samples [20], the CALUX TEQ-values obtained without fractionation were below the chemical results. This was mainly explained by the difference between REP and WHO-TEF for PCB. Since REP values for PCB are lower than the respective WHO-TEF values, the TEQ-values for PCBs analysed by CALUX are always inferior to the GC-HRMS TEQ-values, calculated with WHO-TEFs. Olsman et al. [20] have also pointed out the possible effect of the dioxin exposure on the metabolism of PCBs in CALUX cells to explain this difference [20]. For four milk samples, Laier et al. found CALUX results obtained without fractionation, that were higher or similar to the GC-HRMS values, however [4]. None of those studies show CALUX TEQ-values (PCB + DX fraction) lower than the CALUX TEQ-results (DX fraction) as observed in this investigation.

This difference between CALUX TEQ-values of both the DX and DX + PCB fractions must be taken into consideration when comparing different blood studies. Since the DX + PCB fractions, obtained in this paper, are very similar to purified sample extracts obtained with one single acid silica clean-up, the comparison of CALUX data obtained with or without fractionation must be carried out with extreme caution.

#### 4. Conclusion

The message of the presented study is twofold. Since CALUX bioassay gives TEQ-value representing the total AhR activity of sample extract, the purification step is a determining part of the analysis. Presently, there is a strong and generally accepted tendency to compare results obtained by screening technologies with those obtained by the golden reference GC-HRMS method. Regarding this objective, there can be no doubt that separation of DX and PCB fractions refines the results. Differences between TEQ-values obtained by CALUX for the dioxin fraction and by GC-HRMS for the 17 PCDD/Fs predominately indicate the presence of AhR agonists that are not detected or not searched for by the chromatographic techniques.

At first sight, it seems to be more informative for epidemiological survey to analyse the DX + PCB fraction, in order to take into account non additive interactions. But, on one hand, part of the interactions is lost since some compounds are discarded or degraded during the clean-up. The interactions measured are then not representative of the “global” non additive interactions present in the sample. On the other hand, rather constant CALUX TEQ-values of the DX + PCB fraction raise questions on mutual interactions between several contaminants of highly loaded samples. To improve the

sensitivity of the CALUX method, when comparing its results with those of chemo-analyses, fractionation into contaminant groups is a valuable tool.

The observations and conclusions are based on a limited number of data. They demonstrate relevant information on the significance of CALUX TEQ-values though. Moreover, they constitute a hint for caution in results’ interpretation when clean-up conditions are largely unknown.

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