INFLUENCE OF THE SOLVENT QUALITY USED FOR SAMPLE PREPARATION ON THE AhR MEDIATED PROCEPT® ASSAY MEASUREMENT OF DIOXIN AND DIOXIN-LIKE COMPOUNDS

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Abstract

The influence of solvent grade quality on the response of a DNA-binding AhR-mediated assay used for screening dioxins was investigated. Our results demonstrated a very critical impact of this parameter with both strong agonistic and antagonistic effects observed for any tested solvent lot. A small silver nitrate silica column partly removed these interfering compounds and then can be recommended as final purification step. Some preferable grades can be identified and selected in order to guarantee the best possible performances. However, it appears necessary to test every new lot, even if a grade previously appeared compliant.

Introduction

Most countries have implemented regulations aiming to reduce the impact of PCDD, PCDF and "dioxin-like" PCB (dl-PCB) on public health, and then appropriated survey control plans and monitoring programs dedicated to environmental matrices as well as foodstuff and biological samples. The extremely low residual concentration levels (down to sub-ppt) placed the isotopic dilution method with gas chromatography coupled to high resolution mass spectrometry on electromagnetic sector instruments as the reference analytical method. Since this approach remains time and cost-consuming[1], growing efforts of the scientific community have been focused on the development of analytical alternatives, especially for screening strategies. Various approaches have been described, including the use of (1) alternative GC-MS based instruments[2-5], (2) regression models[6-8], (3) immunoassays[9-11], or (4) aryl-hydrocarbon receptor-mediated assays including cell-based bioassays[12-15] and DNA-binding assays[16-19].

One DNA-binding assay, namely Procept®, measure the activated AhR by quantifying the bound DNA *in vitro*. More precisely, a rodent hepatic cytosolic fraction containing AhR and cofactors is mixed with the ligand extract and a synthetic DNA sequence containing the dioxin responsive element sequence. After activation of the AhR, the ligand/AhR/ARNT/DNA complex is trapped on the surface of a Quantitative Polymerase Chain Reaction (qPCR) well by a specific antibody. Thus, the bound DNA is amplified after removal of any excess reagents. A TCDD calibration curve allows to express the measured response as a bioanalytical equivalent quotient (BEQ), a TCDD equivalent correlated to the regulatory defined toxic equivalent quotient (TEQ)[20-22]. More details about the mechanism of action of dioxins can be found elsewhere[23-26].

Unfortunately, AhR ligands are highly diverse, including halogenated and polycyclic aromatic hydrocarbons and many other naturally occurring compounds[24]. Consequently, these AhR-mediated assays still require relatively substantial sample extraction and purification. Indeed, interfering compounds may come from the matrix but also from the analytical procedure[19,27]. Working with a cell-based bioassay, Windal et al. (2005) reported that in their lab, when just one column of acidic silica is used for the cleanup, the response of a spiked procedural blank is only about half of the expected response, indicating that some inhibitors occur in procedural consumables. Miniaturizing the sample preparation process might be helpful both for saving time/cost and improving performances, but the sensitivity usually achieved with cell-based and DNA-binding assays does not permit such reduction of sample size to a large extend. Consequently, procedural blanks responses are often higher than background level, in the linear

range of assays. This negatively impacts the detection limit by reducing the working range, and necessitates blank correction of the results.
Usually, high attention is paid to the clean-up of glassware and stationary phases, and to the quality of solvents as well. Some laboratories specialized in cell-based assays usually characterize all solvent lots (toluene, hexane, dichloromethane) since they identified it as possible inhibition source of signal inhibition and/or interference. However, this kind of study has to be conducted for each considered specific test. In the present work, the impact of different toluene grades on the Procept® DNA-binding assay was evaluated, taking in account both agonistic and antagonistic effects, for different target concentration levels.

Materials and Methods

Solvents
The six tested toluene grades were: LGC Promochem Picograde lot 811902 (Wesel, Germany), Biosolve Dioxins & PCB's Analysis lot 639871 and Biosolve Pesti-S lot 595921 (Leenderweg, The Netherlands), Carlo Erba Reactifs - SDS Atrasol lot D8M052248M, Carlo Erba Reactifs - SDS Pestipur lot D8G002088I (Val de Reuil, France) and JT Baker Ultra Resi-Analyzed lot 0829600025 (Deventer, The Netherlands).

TCDD solutions
TCDD was purchased from Wellington Laboratories (Guelph, Canada). For calibration curves, three times TCDD dilution series were prepared in *n*-heptane (Picograde, lot 701228, LGC Promochem) using dedicated syringes, from 59 049 fg/µL down to 27 g/µL.

Toluene concentration
All used glassware was heated at 400 °C for 4 hours just prior to use. 200 mL of each toluene grade was concentrated to ~2 mL using a Rotavapor®, transferred to a test tube using a Pasteur pipette and then concentrated to 500 µL under a gentle nitrogen stream (Class I, Air Liquide, Paris La Défense, France). The solvent volume was split into 4 equal aliquots in glass vials and evaporated to dryness under a gentle nitrogen stream. Dried extracts remained sealed one night and were resuspended in 25 µL of *n*-heptane or 25 µL of TCDD solution from the TCDD dilution series (81 fg/µL, 729 fg/µL or 6 561 fg/µL) just prior to AhR contact. These fortification levels corresponded to lower and medium part of linear range, and to a value close to the saturation plateau, respectively. These four toluene extracts with increasing TCDD concentrations can be directly compared to the assay calibration curve plots.

Silver nitrate treatment
Because a major negative impact of raw toluene extracts were initially observed on the assay, the addition of a purification step applied to the extract was considered, in order to remove most part of these interferences. Indeed, silver nitrate is known to degrade or retain numerous sensitive interfering compounds, including polycyclic aromatic hydrocarbons. Then, alternatively, 200 mL of each toluene grade was concentrated to ~2 mL using a Rotavapor®, transferred to a test tube using a Pasteur pipette and then evaporated to dryness under a gentle nitrogen stream. 150 mg of silica gel coated with 10% w/w of silver nitrate (Sigma-Aldrich, St Louis, USA) previously activated one night at 200 °C were packed in a glass column (7 mm i.d.) and pre-washed with 10 mL of *n*-hexane. The extract was loaded in 1 mL of *n*-hexane (Picograde, lot 808405, LGC Promochem) and eluted fractions of 6 mL (corresponding to 2,3,7,8-substituted PCDD/F and dl-PCB) were collected and submitted to the same concentration and split than previously described.

Procept® immuno-qPCR assay
The Procept® assay was performed according to the indicated instructions of use. All solution dispensing and measuring was performed using delivery pipets and plastic barrier pipet tips. Figure 1 summarizes the kit protocol. The templates included 8 duplicate wells for TCDD standard curve (including assay blank) and 8 duplicate wells for each tested solvent (blank and 3 TCDD spike levels, with/without $AgNO_3$ treatment).

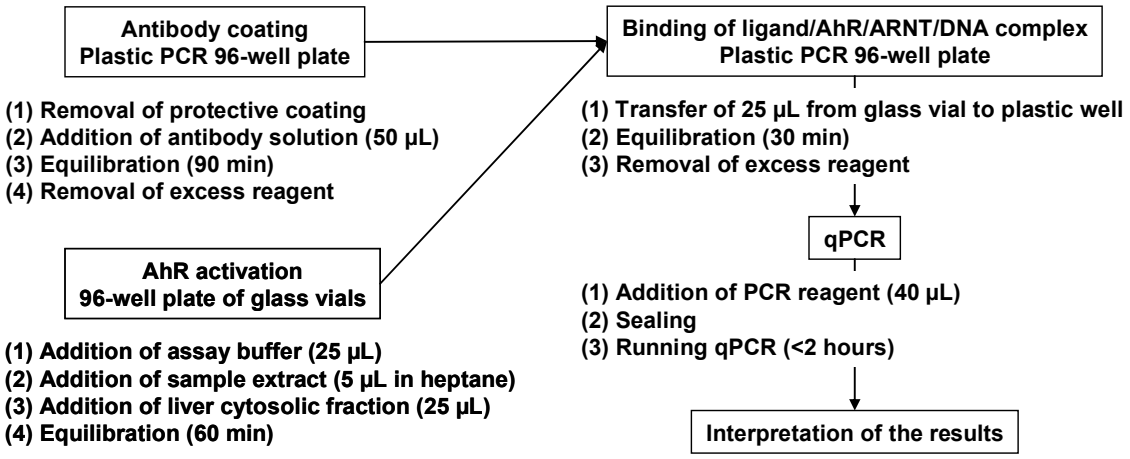

Figure 1: Scheme of the Procept® assay protocol.

Quantitative Polymerase Chain Reaction (qPCR)
PCR reagent was prepared according to the manufacturer's instructions by blending 40% Sterile water (Biosolve), 50% TaqMan 2X Universal PCR Master Mix (No AmpErase UNG, Applied Biosystems, Branchburg, USA) and 10% primer/probe solution (included in kit). 40 µL of PCR reagent were added to each well. The plates were sealed using optically clear adhesive film (Applied Biosystems) and placed in the real-time PCR instrument (Gene Amp 5700, Applied Biosystems). The PCR protocol included 2 min at 50 °C, 10 min at 95 °C and then 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The output from the PCR instrument for each sample is a threshold cycle (Ct). The Ct is the number of real-time PCR temperature cycles at which the fluorescence measurement exceeds a threshold level chosen in the exponential part of amplification curves. X-axis is usually set as logarithmic scale. Indeed, background level Ct appears higher than saturation plateau Ct, leading to a decreasing sigmoid curve.

Results and Discussion

TCDD calibration curve
Two 96-well plates were used in this study. The ΔCt between the background level (assay blank) and the saturation plateau (5.36 and 4.95 Ct) were compliant according to the manufacturer recommendations. The linear parts of the curves ranged from 135 to 10 935 fg/well (5 points, curve 1) or from 405 to 10 935 fg/well (4 points, curve 2). Coefficients of determination (R^2) were higher than 0.98, which appeared compliant with the European guidelines on screening bioassays for PCDD/F and DL-PCB. The obtained EC50 (concentration corresponding to the median the response between the background level and saturation plateau) were 1 214 fg and 1 811 fg, respectively, these values being considered as usual.

Background levels
Figure 2 shows the variation of ΔCt between toluene background level and TCDD calibration curve saturation plateau compared to ΔCt of the TCDD calibration curve, for each tested solvent. Four of the six toluene extracts (solvents A, B, C and D) gave positive responses, reducing the Ct range by 20% to 50%. Such result suggests that these toluene lots bring some agonistic compounds. Then, the sensitivity of the assay significantly decreases when toluene is used for sample preparation. Interestingly, solvent E showed the singularity to give a background level ~1.7 Ct higher than the calibration curve background. This could be interpreted as an inhibitory effect. Since the antibody is specific to the ARNT and that it is suspected that most of the background level is coming from some "auto-activated AhR" (e.g. AhR Repressor or naturally occurring agonist), this result suggests that what is affected is

the antibody binding mechanism. This hypothesis is supported by the fact that the resulting solvent E background level is corresponding to a response level obtained when no antibody is added in the plastic wells. In such case, the obtained response is coming from unwashed free DNA (adsorption). Finally, solvent F showed the best behavior since no significant deviation was observed.
When concentrated extracts were submitted to a further purification step using a silver nitrate treatment, the deviations of background levels significantly decreased for 3 of the solvent lots (solvents A, B and E). This purification step appeared to be partially efficient for selected grades, suggesting that agonistic compounds can differ from a grade to another one and that some are not sensitive to $AgNO_3$ treatment.

TCDD measurements
Figure 3 shows the obtained deviations of ΔCt between calibration curve background level and obtained value compared to reference ΔCt of TCDD calibration curve, for each solvent grade and spiked level. For spikes at 81 and 729 fg/µL, the reference values corresponded to the plot on linear regression curve and for spike at 6 561 fg/µL this was the obtained value. For most of the results, responses were decreased, up to -50%. For lower spike level, solvent A gave a higher response compared to calibration curve, in accordance to the background level (similar signal) which is also higher than the calibration curve for this TCDD level. The singularity of solvent E persisted for spiked levels, with obtained responses also ~1.7 Ct higher than the calibration curve background. This corroborates the fact that there is a negative effect of this grade on the assay response as if no complex with DNA was bound to the antibody and or captured to the surface of the well.
When concentrated extracts were submitted to the silver nitrate purification, the deviations decreased in most of the cases, but not completely. These results suggest that antagonistic compounds occurring in different toluene grades are generally sensitive to $AgNO_3$ treatment but are not completely removed.

Discussion
The present study investigated the occurrence of both agonistic and antagonistic compounds as impurities in various toluene grades. From this point of view, no toluene grade behaved like another one, suggesting that each manufacturing process lead to a distinct qualitative and quantitative profile in terms of impurities and interferences. One might suppose that involved compounds and their proportions also differ from one lot to another one. Comparing the results with the information available in analysis certificates didn't lead to pertinent trend concerning the identification of involved compounds in observed interferences.
Taking in account agonistic and antagonistic effects, the best studied solvent grade appeared to be toluene B (Figure 3). Starting from 50 mL and without silver nitrate silica column treatment, the working range was significantly reduced and a high inhibitory effect was observed. But after the silver nitrate silica column treatment, the diminution of ΔCt range (agonistic effect) and the inhibition effect on spiked levels appeared to be lower than 10%. This additional purification step appeared to be partially efficient for selected grades and should be adopted as final purification step of sample preparation just prior to assay measurement.
The present work shows that the performances of screening methods are not only depending on extraction/purification strategy but also to given solvent lots. Similar experiments were conducted with *n*-hexane and dichloromethane grades, with equivalent results and conclusions (data not shown). In order to obtain the best possible analytical performances, the control of each solvent lot before its use on "real" samples is highly recommended. Then, a question is what criteria should one respect to declare a lot compliant. A consensus should be to be defined by all the cell-based/DNA-binding assay and screening method experts.

Acknowledgments

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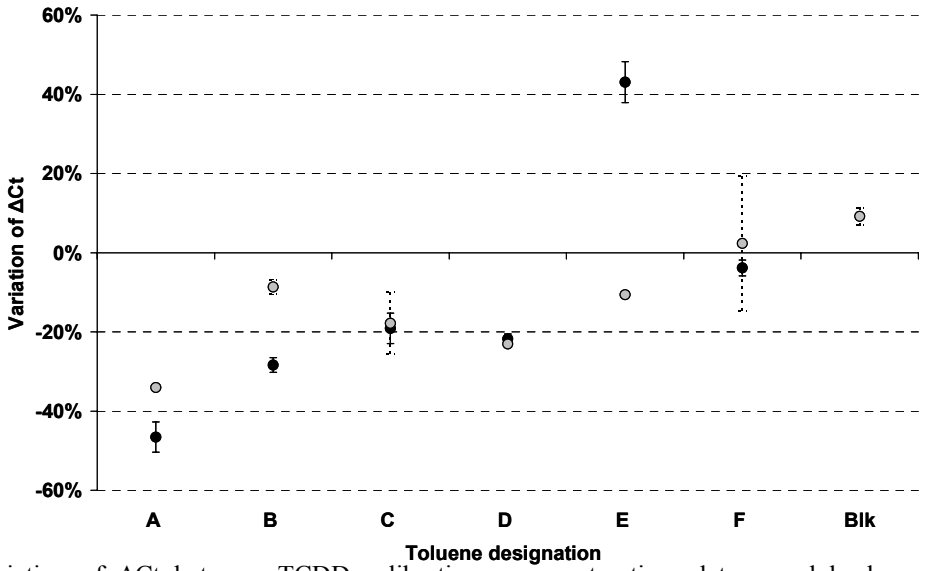

Figure 2: Variation of ΔCt between TCDD calibration curve saturation plateau and background level when concentrated toluene is reconstituted as background control, without (dark) or with (light) silver nitrate silica column purification, for each toluene grade. Standard deviation bars are from duplicate measurements. Blk: silver nitrate silica column procedural blank response.

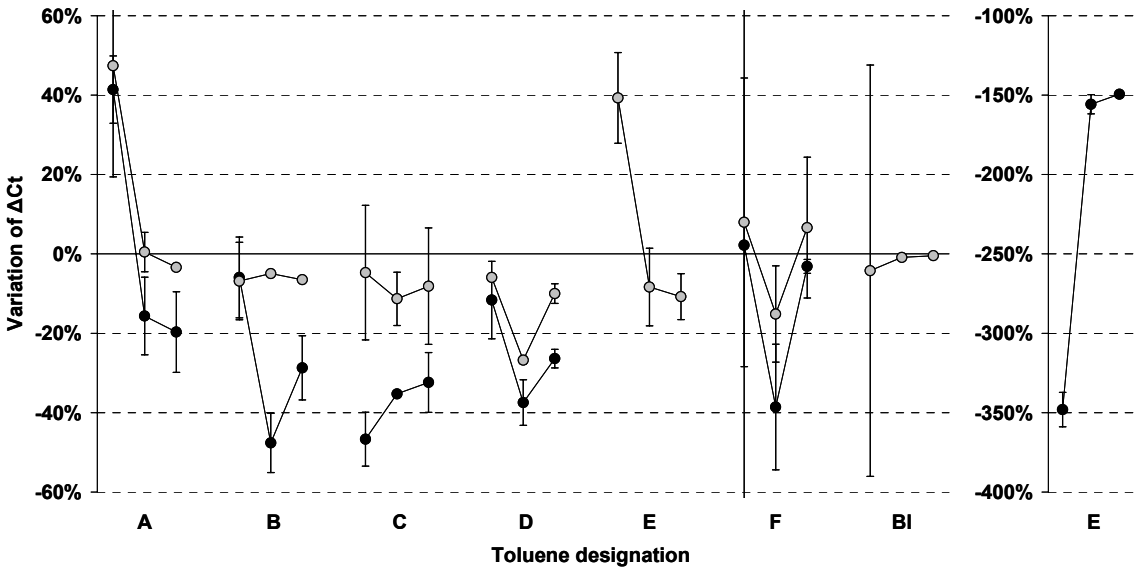

Figure 3: Variation of ΔCt between calibration curve background level and TCDD calibration curve responses at 3 different concentrations when concentrated toluene is reconstituted with TCDD solution, without (dark) or with (light) silver nitrate silica column treatment, for each toluene grade. Standard deviation bars are from duplicate measurements. For spikes at 81 and 729 fg/µL, the reference values corresponded to the plot on linear regression curve and for spike at 6 561 fg/µL this was the obtained value. Blk: silver nitrate silica column procedural blank response. Singular effect of solvent E without silver nitrate silica column treatment resulted in a distinct right side representation.

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