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Evaluation of the recombinant cell-based bioassay as a screening method for PCDD/Fs monitoring in fish tissue

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ABSTRACT

Among 210 congeners only 17 highly toxic, 2,3,7,8-chlorinated dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) are of toxicological concern. They exhibit high binding affinity to an intracellular aryl hydrocarbon receptor (AhR), causing harmful effects at exposure levels of thousands of times lower than most environmental toxicants. The Chemically Activated LUciferase gene eXpression bioassay (CALUX) utilizes recombinant cells that were transfected with a luciferase reporter gene, which responds to dioxin-like compounds with the induction of luciferase in a time-, dose-, AhR-dependent and chemical-specific manner. The bioassay evaluation concerning the European Community (EC) requirements for the PCDD/Fs determination for the official control of foodstuffs was performed on salmon tissue. In order to evaluate the bioassay performance characteristics, recovery range, limit of detection (LOD), limit of quantification (LOQ) and precision were determined. The results revealed that combining a dichloromethane: hexane extraction, an acid silica plus activated carbon clean-up provides reliable, reproducible (CV = 9-20%) measurements with acceptable recovery (78%) and sensitivity at the required ppt range. Due to the low cost and high throughput characteristics of the CALUX assay, food monitoring for PCDD/Fs may benefit from use of this bioassay as a prescreening tool to select and prioritize samples for subsequent analysis by high resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS). Although the bioassay may not be able to specify identity of the reactive substances, it may serve as a very useful tool for the evaluation of contamination sources.

INTRODUCTION

Chemicals known as persistent organic pollutants (POPs) endure in the environment, bioaccumulate through the food chain, and pose a risk of causing adverse effects to human health and the environment (Betianu and Gavrilescu 2006). The Stockholm Convention, which took effect in May 2004, focuses on eliminating or reducing release of POPs and aims to control production, disposal and use of these dangerous compounds. Polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) are included into the "dirty dozen" most toxic POPs (UNEP 2001). Among the 210 congeners of PCDD/Fs, only 17 compounds, chlorinated at 2, 3, 7 and 8- positions (Figure 1), are of toxicological concern (Van den Berg et al. 1998). They exhibit high binding affinity to an intracellular aryl hydrocarbon receptor (AhR), a transcription factor that regulates expression of multiple genes, important in development, physiologic function and adaptive responses to xenobiotics (Tijet et al. 2006). In recent years, much attention has also been paid to a few selected polychlorinated biphenyl (PCBs) congeners which possess toxicological properties and structural features similar to the most potent AhR agonist, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) (Van den Berg et al. 1998). The World Health Organization (WHO) has identified 12 PCBs (4 non-*ortho* and 8 mono-*ortho*) as being similar in toxicity to PCDDs and PCDFs (Figure 1). The role of the AhR in mediating the toxic and biological effects of the PCDD/Fs and dioxin-like PCBs (dl-PCBs) has been supported by numerous quantitative structure-activity relationship, biochemical, genetic, and targeted *Ahr* knockout studies (Fernandez-Salguero et al. 1996; Okey et al. 2005; Safe 1990; Waller and McKinney 1995).

Exposure to dioxin-like compounds produce a wide range of species- and tissue- specific toxic and biological effects including immune system suppression, reproductive and developmental toxicity, neurotoxicity and cancer (Giesy and Kannan 2002; Lindstrom et al. 2004; Schecter et al. 2006;

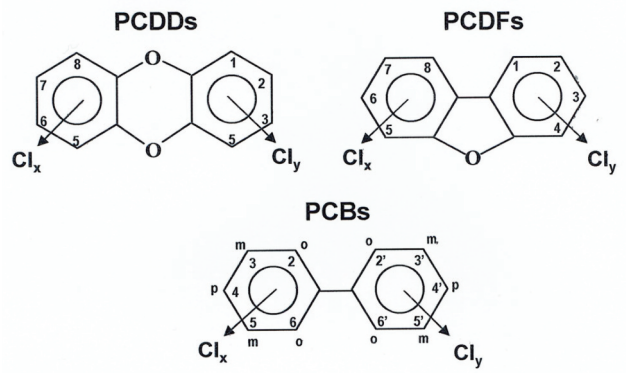

Figure 1. The general chemical structures of PCDD/Fs and PCBs; o, m, p denote ortho, meta and para positions, respectively. The possible number of chlorine atoms results in 75 PCDD congeners and 135 PCDF congeners (x = 1–4, y = 0–4), and 209 PCB congeners (x = 1–5, y = 0–5) (Fries 1995, Srogi 2008).

Steenland et al. 2004). Moreover PCDD/Fs and dl-PCBs act as endocrine disrupters (Birnbaum 1994; Safe 1998). Sending erroneous signals or blocking valid signals in the endocrine system dioxin-like compounds can cause thyroid disorders, diabetes, altered pulmonary function and serum testosterone level, eyelid pathology, skin rashes, loss of appetite, liver damage, elevated serum cholesterol and triglycerides (Clapp and Ozonoff 2000; Giesy and Kurunthachalam 2002; Safe 1993; Schecter et al. 2006). Dioxin-like compounds are still ubiquitous environmental pollutants. PCBs have been produced commercially since 1929 and used widely in industry as dielectric fluids, organic diluents, plasticizers, flame retardants, pesticide extenders, adhesives and dust-reducing agents (Giesy and Kannan 2002). Although many countries have severely restricted or banned the production of PCBs, further environmental contamination may occur from the disposal of old electrical equipment (Giesy and Kannan 2002). Unlike PCBs, PCDD/Fs have never been created intentionally.

Table 1. Toxic Equivalency Factors for dioxins and dioxin-like compounds used for TEQ calculation and reported CALUX-REP values.

Compound	WHO-TEF (Van den Berg et al. 1998)	CALUX-REP (Brown et al. 2001)
PCDD/Fs		
Chlorinated dibenzo-p-dioxins		
2,3,7,8-TCDD	1	1
1,2,3,7,8-PCDD	1	0.73
1,2,3,4,7,8-HxCDD	0.1	0.075
1,2,3,6,7,8-HxCDD	0.1	0.098
1,2,3,7,8,9-HxCDD	0.1	0.061
1,2,3,4,6,7,8-HpCDD	0.01	0.031
1,2,3,4,6,7,8,9-OCDD	0.0001	0.00034
Chlorinated dibenzofurans		
2,3,7,8-TCDF	0.1	0.067
1,2,3,7,8-PCDF	0.5	0.14
2,3,4,7,8-PCDF	0.5	0.58
1,2,3,4,7,8-HxCDF	0.1	0.13
1,2,3,6,7,8-HxCDF	0.1	0.14
1,2,3,7,8,9-HxCDF	0.1	0.11
2,3,4,6,7,8-HxCDF	0.1	0.31
1,2,3,4,6,7,8-HpCDF	0.01	0.024
1,2,3,4,7,8,9-HpCDF	0.01	0.044
1,2,3,4,6,7,8,9-OCDF	0.0001	0.0016
Dioxin-like PCBs		
Coplanar PCBs		
PCB 77	0.0001	0.00014
PCB 81	0.0001	0.00045
PCB 126	0.1	0.038
PCB 169	0.01	0.0011
Mono-ortho PCBs		
PCB 105	0.0001	0.000001
PCB 114	0.0005	0.00014
PCB 118	0.0001	0.000001
PCB 123	0.0001	0.0000003
PCB 156	0.0005	0.00014
PCB 157	0.0005	0.000003
PCB 167	0.00001	0.0000003
PCB 189	0.0001	0.000002

They are produced accidentally by a number of human activities like uncontrolled burning of residential waste, municipal waste incineration, manufacture and use of certain herbicides and chlorine bleaching of paper pulp (Fiedler 1996; Reiner et al. 2006). There are also natural sources for PCDD/Fs like forest fires and volcanic eruptions, but they contribute little to the current background dioxin levels (Fiedler 1996; Reiner et al. 2006).

Due to the fact that all dioxin-like compounds are considered to act by the common mechanism and because they exist in the environment as complex mixtures to simplify risk assessment and regulatory control, the concept of toxic equivalents (TEQs) has been introduced by an international WHO committee (Van den Berg et al. 1998). In this approach the toxic equivalency factors (TEFs) of 17 PCDD/Fs and 12 dl-PCBs congeners were determined based on *in vitro* and *in vivo* studies in relation to the most potent congener, 2,3,7,8-TCDD (TEF=1), (Table 1; Van den Berg et al. 1998). The toxicity of a mixture is assumed to be equal to the sum of the concentration of individual congeners multiplied by their TEFs:

$$TEQ=\sum_{i=1}^{7}(PCDD_i \times TEF_i)+\sum_{j=1}^{10}(PCDF_j \times TEF_j)+\sum_{k=1}^{12}(\text{dl-}PCB_k \times TEF_k)$$

(Van den Berg et al. 1998; Van Overmeire et al. 2004).

Although the original sources of dioxin-like compounds are mainly industrial, the general population route of exposure is predominantly through the food consumption (90%) (Bocio and Domingo 2007; Fries 1995; Srogi 2008). In a risk assessment of PCDD/Fs and dioxin-like PCBs in the diet, the European Commission's advisory Scientific Committee for Food (SCF) have recommended a Tolerable Weekly Intake (TWI) of 14pg WHO-TEQ·kgbody weight^{-1}·week^{-1} for these chemicals (SCF 2001). Fish and fishery products play an important role in dietary intake especially for the European population living close to the Baltic Sea (Kiviranta et al. 2002). For example, in Finland fish and fish products contribution to the PCDD/Fs intake was estimated to be responsible for 63% up to 94% of the daily intake of dioxins (Kiviranta et al. 2001, 2004). In the EC countries, the maximum permissible PCDD/Fs and dioxin-like PCBs levels for fish and fishery products were set at 4pg WHO-TEQ·$g_{\text{fresh weight}}^{-1}$ (·g_{fw}^{-1}) for PCDD/Fs, and 8pg WHO-TEQ·g_{fw}^{-1} for the sum of PCDD/Fs and PCBs (Commission Regulation (EC) No. 199/2006). The EC regulation No. 199/2006 also states that permanent monitoring of dioxins and dioxin-like PCBs in food should be performed across European Community (EC). In case of an abnormal increase in the level of those compounds, sources and/or pathways of contamination have to be identified to fulfil Community strategy to diminish harmful effect on human health (Community Strategy for Dioxins, Furans and Polychlorinated Biphenyls 2001; Van Overmeire et al. 2001). A necessary condition for effective control and monitoring mechanisms is the availability of appropriate measurement methods. Currently, recommended for PCDD/Fs and dl-PCBs determination in food are isotope dilution techniques utilizing HRGC/HRMS (Reiner et al. 2006; Schecter et al. 2006). However, their use has also limitations, such as high analysis costs and labor intensity that negatively affect their utility for large-scale screening (Hoogenboom 2002; Hoogenboom et al. 2006; Stypula et al. 2004). The effectiveness of routine instrumental HRGC/HRMS analysis in revealing PCDD/Fs and dl-PCBs contamination can be improved significantly by using *in vitro* biological tests based on the mechanism responsible for mediating toxicity of dioxin-like compounds (Figure 2). Bioassays may offer unique advantages in relation to not only providing a more efficient screening strategy but also doing so at a greatly reduced cost (Behnish et al. 2000; Denison et al. 2004; Jeong et al. 2005; Stypula et al. 2004). At present, the most promising for screening purposes are CALUX (Chemically Activated LUciferase gene eXpression) bioassays. They are based on genetically modified mouse or rat hepatoma cell lines, containing luciferase reporter gene under control of specific dioxin-responsive elements (DREs) that are able to respond to dioxin-like compounds in a time-, dose-, AhR-dependent and chemical-specific manner (Figure 3) (Denison and Heath-Pagliuso 1998; Han et al. 2002; Hoogeboom 2002; Murk et al. 1996). It was found that in the CALUX bioassays the relative potency (REP) values are dependent on AhR binding and activation and generally consistent with WHO-TEF values determined for 17 PCDD/Fs and 12 dl-PCBs (Table 1) (Brown et al. 2001). Also, high correlation was observed between the results obtained by the CALUX and HRGC-HRMS analysis (Hoogenboom 2002; Scippo et al. 2006; Tsutsumi et al. 2003). The CALUX bioassays have been widely used for screening of dioxin-like compounds in food and feed not only in the EC countries (Gizzi et al. 2005; Hoogenboom et al. 2006; Jeong et al. 2005; Murk et al. 1996; Scippo et al. 2006; Stypula-Trebas et al. 2005; Van Overmeire et al. 2004) but also outside the Europe (Choua et al. 2008; Hasegawa et al. 2007; Shaw et al. 2006; Tsutsumi et al. 2003). The CALUX reporter gene bioassay, patented by Xenobiotic Detection Systems (XDS, USA) is one them.

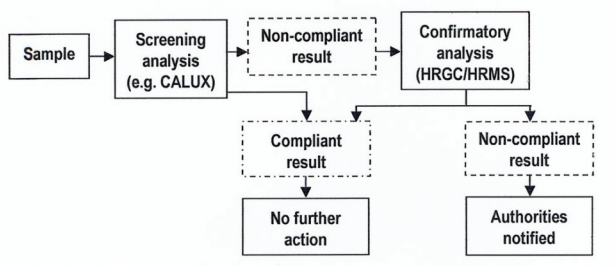

Figure 2. The current PCDD/Fs and dl-PCBs testing scheme within the European Community (Denison et al. 2004).

The aim of the study was to examine the performance parameters and applicability of the CALUX bioassay as a screening tool for PCDD/Fs monitoring in fish tissue samples. We have chasen salmon tissue since this matrix type frequently contains the high PCDD/Fs concentrations (Isosaari et al. 2006; Piskorska-Pliszczynska et al. 2004; Szlinder-Richert et al. 2009).

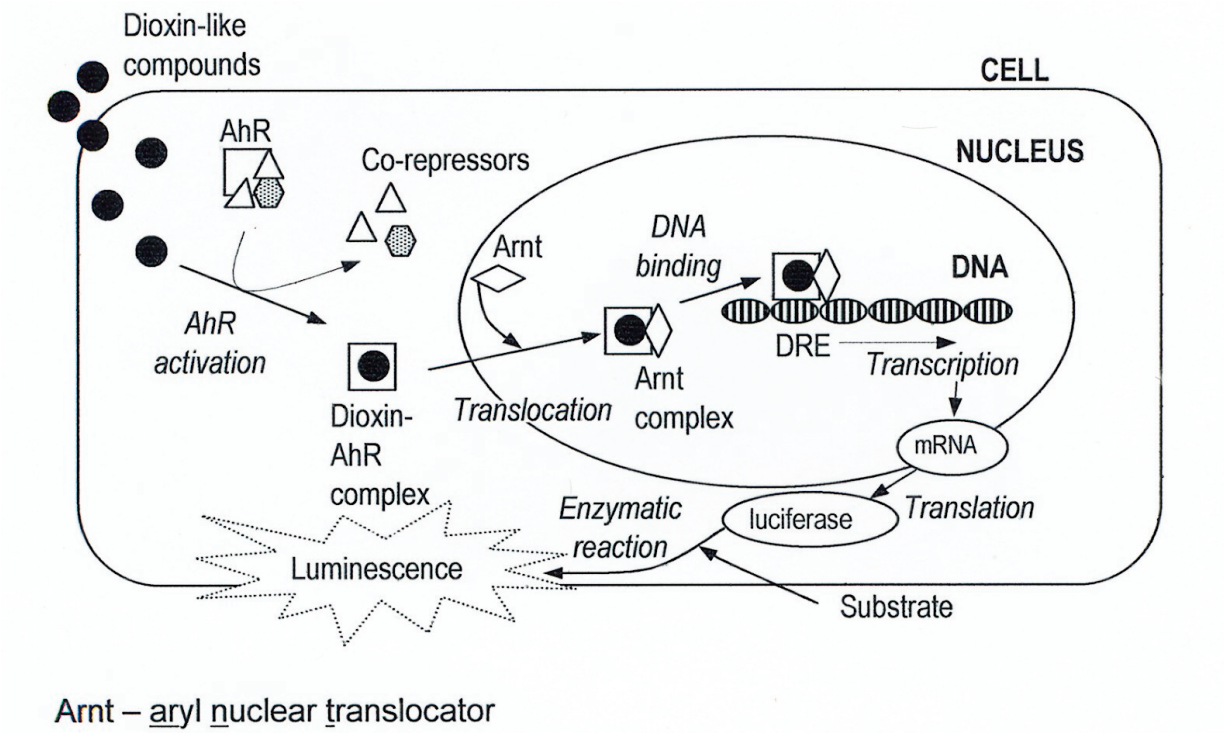

Figure 3. Mechanistic model of AhR-mediated *in vitro* CALUX bioassay used in this study (U.S. EPA 4435 2008; Stypula et al. 2004).

MATERIALS

Reagents

All chemicals used were pesticide or HPLC grade. Cell culture media were purchased from GIBCO (UK) and Hyclone (USA). Activated carbon (1% XCARB/Celite®) was obtained from Xenobiotic Detection Systems (XDS, USA). PCDD/Fs standard solutions (50μg·ml^{-1}) were purchased from Wellington Laboratories Ltd. (Canada) and AccuStandard (USA). The Luciferase Assay System was purchased from Promega Corp. (USA). Fortification solution (FS) for precision and recovery experiments was prepared as a mixture of 17 PCDD/Fs in DMSO. The confirmed concentration of PCDD/Fs in FS was 4.225ng WHO-TEQ·ml^{-1} (HRGC/HRMS). All congeners in the FS were in equal concentrations (1.25ng·ml^{-1}).

Recombinant cell line

Mouse hepatoma H1L6.1c3 cells which were stably transfected with AhR-regulated, dioxin-inducible luciferase expression vector pGudLuc6.1 were obtained from XDS (USA) (Han et al. 2002). This vector contains the firefly luciferase gene under control of specific regulatory elements (DREs). Induction of luciferase in H1L6.1c3 cells occurs in a AhR- and dose-dependent manner. The conditions for cell culture have been described in detail elsewhere (Shaw et al. 2006; US EPA Method 4435). Briefly, the cells were maintained in 75cm^2 cell culture flasks, in a RPMI-1640 medium (Gibco, UK) supplemented with 8% fetal calf serum (Hyclone, USA), and 1% penicillin/streptomycin solution (Gibco, UK) at 37°C and in atmosphere of 5% CO_2.

Fish samples

Salmon (*Salmo salar*) originated from the Baltic Sea was purchased during 2004-2005 from a supermarket in Lublin, Poland. The heads, guts and skin were removed from all fishes. Fillets in slices of 150-200g were grinded using a food mincer. Minced muscle portions of 450-500g were mixed thoroughly, minced and stored at -20°C until analysis.

METHODS

Validation procedure

Validation studies and performance characteristics were done according to the European regulations: Commission Decision 2002/657/EC, Council Directive 2002/69/EC and Commission Directive 2004/44/EC, concerning the performance of analytical methods and interpretation of results, sampling methods, and the methods of analysis for the official control of dioxins in foodstuffs. Performance features described functional quality and attributes of the tested analytical method. In this case it was linearity, sensitivity, detection capability, repeatability, reproducibility and recovery. Precision study was performed by three analysts.

Quality control

Monitoring of solvents used in the sample preparation was conducted by testing each lot of solvent for activity in the bioassay prior to use. The solvent was accepted if RLU induction was less than two times of a background RLU value. Except the eleven calibration points, each plate contained additional quality control points including four DMSO controls and one PCDD/Fs mixture standard (FS, 8.37pg WHO-TEQ·well^{-1}). The RLU value obtained for the 6.25pg WHO-TEQ·well^{-1} point on the standard curve (response near the middle of the curve) was compared to the RLU value for the FS standard. The plates with calculated $TCDD_{6.25}$/FS ratios in the range 0.55-0.95 were accepted. One solvent blank was included to each batch of samples to monitor RLU activity from solvents and materials used in sample preparation. Samples that exceeded 75% of 2,3,7,8-TCDD maximal response (response in upper part of the curve) or were below the limit of detection were reanalyzed using appropriate sample dilutions. A reference material was prepared from pooled salmon muscles that were analyzed 20 times by the bioassay to determine the average PCDD/Fs concentration (mean CALUX TEQ = 2.1±0.6pg·g_{fw}^{-1}) and the initial control chart limits. The reference sample was analyzed for each set of 15 samples and the calculated CALUX TEQ concentration was entered into the QC chart. If reported values differed by more than two standard deviations from the average, samples were reanalyzed.

Measurement uncertainty

Measurement uncertainty expressed as an expanded uncertainty and a decision limit (Ccα) was evaluated following the official European regulations and recommendations (Commission Decision 2002/657/EC, Commission Directive 2004/44/EC; Commission Regulation (EC) No. 1883/2006; EURACHEM/CITAC 2000). All details have been described previously (Stypula-Trebas and Piskorska-Pliszczynska 2005). Briefly, after identifying sources of uncertainty, its estimation was obtained from validation data (i.e. precision and recovery studies) as well as additional sources (i.e. calibration and purity certificates, manufacturers' specifications). Individual uncertainty estimates were combined to give standard and expanded uncertainties for the method. The expanded uncertainty was calculated using a coverage factor of 2, which gives a level of confidence of 95%. The decision limit was determined by analysing 20 blank salmon samples fortified with the PCDD/Fs at the permitted limit (4pg WHO-TEQ·g_{fw}^{-1}). The Ccα was calculated as the concentration at the permitted limit plus 1.64 times the corresponding standard deviation.

Preparation of fortified fish muscle samples

Salmon muscles with known background concentration of dioxins (1.23±0.1pg WHO-TEQ·g_{fw}^{-1}, HRGC/HRMS) were fortified with FS standard at concentrations of 2, 4 and 6pg WHO-TEQ·g_{fw}^{-1}, corresponding to 0.5, 1.0, and 1.5 times of the maximum permitted level for fish tissue. Analysis was performed with six replicates at each level. All analytical series contained three sample blanks. The percentage recoveries were determined by measuring sample extract activity in the CALUX assay in comparison to the activity of the same reference compound placed directly into DMSO. Concentration values in spiked samples were corrected by subtraction of blank sample PCDD/Fs levels determined by the CALUX bioassay (1.67±0.20pg WHO-TEQ·g_{fw}^{-1}). Altogether seventy seven samples were analyzed.

Extraction and clean up procedure

Samples (10g) of minced fish were immersed in acetone (15ml) and shaken at 250 rpm, for 10min on an orbital shaker (Heidolph). After this treatment fat from salmon was extracted three times by shaking with 10ml of dichloromethane/n-hexane (1:2, v/v) on the orbital shaker (250 rpm, 30min), followed by centrifugation (1000 rpm, 5min, Sigma). The upper layer of the supernatant was collected and passed over the chromatographic column with celite (2g) and anhydrous Na_2SO_4 (5g). Collected elutes were evaporated to dryness under stream of nitrogen and the lipid content was estimated gravimetrically (U.S. EPA Method 1613B). The clean-up procedure was performed on two columns: acid silica gel to remove PAHs and activated carbon column with 1% XCARB/Celite®, that binds halogenated dioxins/furans and biphenyls (Brown et al. 2002; Clark and Chu 2004; U.S. EPA Method 4435 2008). These chemical classes were differentially eluted first with the mixture of toluene/hexane/ethyl acetate (8:1:1 v/v/v), (PCB fraction) and with toluene (PCDD/F fraction) from activated carbon column (U.S. EPA Method 4435 2008). Extracts were concentrated to dryness in a vacuum centrifuge and brought up in hexane for analysis in the bioassay.

CALUX bioassay

The bioassay was performed using the genetically modified mouse hepatoma H1L6.1c3 cell line (Han 2002). The details of the CALUX bioassay procedure (Figure 4) have been described elsewhere (Shaw et al. 2006). Briefly, when cells reached 80-90% confluence they were harvested with trypsin (Gibco, UK), resuspended in fresh culture medium at a density of $7.5 \cdot 10^5$ cells·ml^{-1} and seeded into 96-well culture plates (200μl cell suspension·well^{-1}). The cells were incubated at 37°C and in the presence of 5% CO_2 for 24-48h. Dosing solutions were prepared by diluting extracts in 1ml of hexane and brought up in 4μl DMSO by evaporation of hexane in a vacuum centrifuge. Samples were then diluted in 400μl of culture medium. Dosing of cells was initiated by replacing cell culture media covering cells in 96-well plates with 190μl of the media containing the purified sample extracts or standards. The final concentration of DMSO in each well was 1%. To determine a standard curve, 2,3,7,8-TCDD was used at concentrations of 0.10, 0.20, 0.39, 0.78, 1.56, 3.13, 6.25, 12.50, 25.00, 50.00 and 100.00pg·well^{-1}. The cells were exposed to extracts and to standard TCDD solutions during 24-26h to allow optimal luciferase gene expression. Following the exposure, cells were examined microscopically for cytotoxicity of the sample and lysis solution (30μl·well^{-1}) was added to each well. The plates were shaken for 15-20min to allow complete cell lysis. The induced luciferase activity was determined with a luciferase assay system kit (Promega), using microplate luminometr (Orion II, Berthold, USA) and expressed as relative light units (RLU). After addition of the substrate containing luciferine, ATP and Mg^{2+} (30μl·well^{-1}), the luminescence was measured for 15s in each well. The RLU values were corrected by subtraction of blank RLU values. The luciferase activity of an extract was estimated from 2,3,7,8-TCDD standard curve using a four-parameter Hill equation using a least squares algorithm (Van Overmeire et al. 2004):

$$f = y_0 + \frac{mx^n}{o^n + x^n}$$

where f is a observed RLU response and x is an logarithm of analyte concentration. The four parameters are: an intercept parameter (y_0), a maximal response (m), a parameter that describes the sigmoidal shape of the curve (n) and a dose at which the response is 50% of the maximum (o).

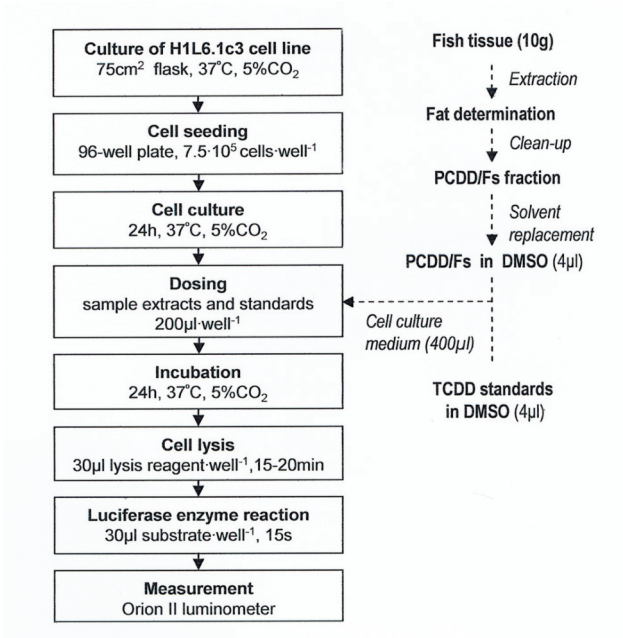

Figure 4. Flowchart of the CALUX bioassay.

RESULTS

Standard curve and linearity range

Figure 5 shows the standard curve of the CALUX bioassay, obtained from five individual assays performed in the intralaboratory reproducibility conditions. The curves of eleven calibration points were highly reproducible. A mean concentration of 2,3,7,8-TCDD that resulted in 50% of the maximal response (i.e. median effective concentration, EC_{50}) estimated from dose-response curves was 3.64pg·well^{-1}, with CV = 1%. The determination coefficient (R^2) which expresses the fitness of the standard points to the curve was always above 0.990.

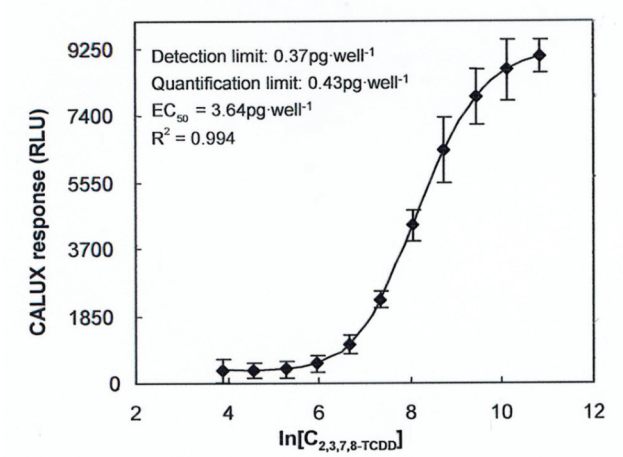

Figure 5. The calibration curve for the CALUX bioassay, obtained from five separate measurements in the intralaboratory reproducibility conditions. The curve points are the mean ± SD RLU values. The measured RLU values were corrected by subtraction of blank (no TCDD).

The linear working range of the 2,3,7,8-TCDD calibration curve was set between 0.98 and 31.10pg·well^{-1}. For the linear range not only standard deviation (SD) but also difference between theoretical and measured 2,3,7,8-TCDD concentration were significantly lower (0.00-0.03 %) than for the higher 2,3,7,8-TCDD concentrations (0.050-0.036%). This suggests that the most accurate results will be obtained from the lower part of the calibration curve. The others reported that CALUX assay would be more reliable when the responses for PCDD/PCDF were less than 75% of the maximal 2,3,7,8-TCDD response because some of dioxin isomers could not reach the maximal value (Brown et al. 2001).

Detection and quantification limits

The CALUX bioassay detection and quantification limits defined as three and five standard deviations above the mean background RLU value for the zero standards, were 0.37 and 0.47pg WHO-TEQ·well^{-1}, respectively. The 0.37pg WHO-TEQ·well^{-1} detection limit (LoD) corresponded to 0.82pg WHO-TEQ·g_{fww}^{-1} of the fish sample tested. Analogously the quantification limit (LOQ) for 10g of salmon tissue was 1.16pg WHO-TEQ·g_{fww}^{-1}.

Precision study

The repeatability and intra-laboratory reproducibility of the CALUX quantification, expressed as a percent of the relative standard deviation (RSD), also known as coefficient of variation (CV), were both determined from repeated blank samples (i.e. salmon samples, that were not fortified with the PCDD/F mixture) (Table 2). Blank samples were analyzed in two runs with six replicates, performed by two analysts on different days. The repeatability and reproducibility were both below critical value 30%. The repeatability and reproducibility were also determined for 36 salmon samples fortified at 2, 4 and 6pg WHO-TEQ·g_{fw}^{-1} (0.5, 1.0, 1.5 times the maximum required limit) (Table 3).

Recovery

Recoveries for the fortified salmon tissues were 58-109% (mean 78%) with CVs below 30%. These recovery levels and CVs were satisfactory, which suggested that under these clean-up procedures the matrix did not interfere significantly with the assay.

Table 2. Precision study - results for blank salmon samples (N=12).

Parameter	Repeatability		Intralaboratory reproducibility
	Run 1 n=6	Run 2 n=6	
Mean (pg·g_{fw}^{-1})	1.64	1.71	1.67
SD (pg·g_{fw}^{-1})	0.153	0.252	0.197
CV (%)	9	15	12

Table 3. Precision study – results for fortified salmon samples. Data were corrected for the blank salmon.

Fortified concentration (pg·g_{fw}^{-1})	Repeatability				Intralaboratory reproducibility	
	Run 1		Run 2			
	Mean±SD (pg·g^{-1}), n*	**CV** (%)	**Mean±SD** (pg·g^{-1}), n*	**CV** (%)	**Mean±SD** (pg·g^{-1}), n*	**CV** (%)
2	1.77±0.30 n = 6	17	1.61±0.17 n = 6	11	1.69±0.34 n = 12	20
4	2.85±0.25 n = 7	9	2.90±0.27 n = 7	9	2.85±0.35 n = 12	9
6	4.15±0.67 n = 6	16	4.07±0.49 n = 6	12	4.11±0.45 n = 12	11

n* - number of analyzed samples

Measurement uncertainty

The evaluated uncertainty considers the full range of variability likely to be encountered during application of the method. This includes PCDD/Fs concentrations, sample matrix and experimental parameters (temperature, extraction and equipment settings). The expanded uncertainty was 33% (coverage factor k=2, 95% confidence). The decision limit, defined as the limit at and above which it can be concluded with an error probability of α=5% that a sample is non-compliant was set as 4.21pg WHO-TEQ$\cdot g_{fw}^{-1}$.

Quality Control

All of the calculated TCDD6.25/FS ratios were within acceptable limits (0.55-0.95) of the QC chart. The average TCDD6.25/FS ratio, calculated for the 5 plates was 0.78±0.15 with a CV of 19%. The observed CV can be seen as a reproducibility among plates. All of the determined concentrations for reference samples were within acceptable limits (0.9-3.3pg WHO-TEQ$\cdot g_{fw}^{-1}$) of the QC chart.

Using CALUX bioassay for dioxin determination in fish meat tissues the detection and quantification limits were found at ppt level. Repeatability and intralaboratory reproducibility were much below required value (30%) and were set at 12% and 13%, respectively. The biotest has met all performance parameters, required by European Community law concerning official dioxin control.

DISCUSSION

Current control actions to enforce EC regulations are based in the majority of cases on the detection and identification of pollutants at unacceptably high concentrations. However, conventional instrumental chemical dioxins analysis applied to the monitoring of contaminants is expensive and rather slow. The limitation of classical monitoring could be overcome by the development of appropriate faster high throughput screening procedures that determine dioxins in food. Reporter gene assays provide such a screening tool. The rapidity and lower cost of the CALUX bioassay are attractive for the high sample numbers required for epidemiological monitoring and ensuring the lack of contamination in the food chain by dioxin-like chemicals. The key application role of the bioassay is screening and prioritization of samples.

Reporter genes have already been used in many cases to develop test systems that facilitate the detection of drugs, chemicals or contaminants. In this study luciferase reporter assay was used to detect contaminants with dioxin-like activity. In combination with an appropriate extraction and clean up technique, this biotest provides a screening tool to detect unwanted dioxin-like activities in food of animal origin and can be applied to the fish tissue. Within European Community, the appropriate permitted level in fish tissues intended for human consumption has been established. The analysis of fish samples by reporter assays provides a practical screening method to control compliance with current EC regulations. The bioassay possesses some advantages but also some weak points (Windal et al. 2005). One of the advantages is that it provides a test system that is able to screen for dioxin-like activity without knowledge of the chemical identity of the compounds of interest. More than ten samples can be analyzed in parallel thereby providing high throughput necessary to process a large number of samples. Taking into account determined performance parameters, the method is compliant with EC requirements for screening tools and "fit for purpose" according to ISO/IEC 17025 standard requirements (EN ISO/IEC 17025:2005). Only positive results need to be confirmed by more specific but laborious HRGC/HRMS method which lead to identification of the chemical nature of the contaminants. Because cell-based assays are sensitive to all known dioxin-like compounds able to activate the Ah receptor, results considered as positive after the cell–based screening assay, can become negative after the HRGC/HRMS confirmatory step. Results from CALUX as well as HRGC/HRMS method are only the approximation of the overall biological/toxicological sample potency. The concentration measurement by HRGC/HRMS is accurate, but expressed in toxicity equivalents (TEQ) remains also an approximation. Even with some limitations, the CALUX is currently one of the best biological tools for estimating relative TCDD equivalents (TEQ) of an unknown sample extract. Future efforts should be made to improve detection capabilities of the luciferase reporter gene assay and to extend the range of food and feed matrices that can be analyzed.

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