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Quantification of PCDD/Fs and dioxin-like PCBs in small amounts of human serum using the sensitive H1L7.5c1 mouse hepatoma cell line: Optimization and analysis of human serum samples from adolescents of the Flemish human biomonitoring program FLEHS II

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ABSTRACT

Since the CALUX (Chemically Activated LUciferase gene eXpression) bioassay is a fast and inexpensive tool for the throughput analysis of dioxin-like compounds in a large number of samples and requires only small sample volumes, the use of this technique in human biomonitoring programs provides a good alternative to GC–HRMS. In this study, a method for the separate analysis of PCDD/Fs and dioxin-like PCBs (dl-PCBs) in human serum with the new sensitive H1L7.5c1 mouse hepatoma cell line was optimized.

Sample dilution factors of 5 and 2.4 were selected for routine analysis of respectively the PCDD/Fs and dl-PCBs. The validation studies showed that repeatability and within-lab reproducibility for the quality control (QC) standard were within the in-house criteria. A long-term within-lab reproducibility of 25% for the PCDD/F fraction and 41% for the dl-PCB fraction for the analysis of pooled serum samples, expressed as pg BEQ/g fat, was determined. CALUX recoveries of the spiked procedural blanks were within the acceptable in-house limits of 80–120% for both fractions and the LOQ was 30.3 pg BEQ/g fat for the PCDD/Fs and 14.5 pg BEQ/g fat for the dl-PCBs. The GC–HRMS recovery of a C13-spiked pooled serum sample was between 60 and 90% for all PCDD/F congeners and between 67 and 82% for the non-ortho PCBs. An adequate separation between both fractions was found. The CALUX/GC–HRMS ratio for a pooled serum sample was respectively 2.0 and 1.4 for the PCDD/Fs and the dl-PCBs, indicating the presence of additional AhR active compounds. As expected, a correlation was found between human serum samples analyzed with both the new H1L7.5c1 cell line and the more established H1L6.1c3 cell line. The geometric mean CALUX-BEQ values, reported for the adolescents of the second Flemish Environment and Health Study (FLEHS II) recruited in 2009–2010, were 108 (95% CI: 101–114) pg CALUX-BEQ/g fat for the PCDD/Fs and 32.1 (30.1–34.2) pg CALUX-BEQ/g fat for the dioxin-like PCBs.

1. Introduction

Although emissions of PCDD/Fs and PCBs have decreased during recent years, these compounds are still environmental pollutants of concern: (1) since PCDD/Fs and dioxin-like PCBs are persistent in the environment, accumulate in fat tissue and in the food chain, have hormone disrupting properties and are carcinogenic [1,2] and (2) because emissions in certain locations in Flanders are still high

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[3]. Therefore, it is important to include the analysis of these compounds as exposure biomarkers in human biomonitoring programs.

In 2007, a second cycle of the Flemish Environment and Health Study (FLEHS II) started and more than 40 biomarkers of exposure (i.e., metals, persistent organic pollutants, perfluorinated compounds, . . .) and 10 effect markers (i.e., hormones) were measured in 650 samples, recruited from 14 to 15 year-old adolescents (n = 200), adults between 20 to 40 years (n = 200) and mother-child pairs (n = 250) [4]. Since only a small amount of serum (5 mL) was available for the PCDD/F and dioxin-like PCB determination, screening of these samples by GC–HRMS analysis was not possible, since the individual congeners would be below the quantification limit when using such low sample volumes. The CALUX (Chemically Activated LUciferase gene eXpression) bioassay provided a good alternative, since it requires only a small amount of serum to analyze the total amount of dioxin-like compounds in the extract.

This publication presents an optimized method for the separate analysis of PCDD/Fs and dioxin-like PCBs in human serum with the newly developed and more sensitive third generation CALUX (H1L7.5c1) mouse hepatoma cell line [5,6]. The H1L7.5c1 cell line was specially designed to analyze low concentrations of PCDD/Fs and PCBs in small sample volumes. With the less sensitive H1L6.1 cell line, which was commonly used in previous biomonitoring studies [7–9] and food/feed analysis [10,11], only a single-point analysis of the whole extract was often used and it was not possible to measure the dioxin-like PCB fraction, since most samples were below the quantification limit (LOQ) [9]. In this study, for the first time, dioxin-like PCBs could be measured in serum samples with the improved H1L7.5c1 cell line with a high percentage of the samples above the LOQ. Dose-response analysis using different dilutions of serum sample extracts allowed determination of an optimal dilution factor to facilitate screening analysis and to minimize sample volumes needed for analysis. The use of this new H1L7.5c1 cell line will also allow optimization of CALUX protocols for the analysis of both PCDD/Fs and dioxin-like PCBs in various matrices, especially those with low concentrations and/or small sample volumes like food and feed or human samples (i.e., blood and milk).

2. Materials and methods

2.1. Chemicals and standards

Hexane (for dioxins and PCBs, minimum 96%), acetone (Pesti-S grade, minimum 99.9%) and toluene (for dioxins and PCBs, minimum 99.8%) were purchased from Biosolve (The Netherlands). Ethyl acetate pestanal and silica gel 60 for column chromatography were purchased from Sigma–Aldrich (Germany). Sulphuric acid (95–97%, ACS reagent), Celite 545 (0.02–0.1 mm) and DMSO were obtained from Merck (Germany). Anhydrous sodium sulphate was purchased from Boom (The Netherlands) and the X-CARB from XDS (USA). The standard solution of 2,3,7,8-TCDD (50 ng/mL) was purchased from Campro Scientific (The Netherlands).

2.2. Analytical procedure

2.2.1. Extraction and clean up

The extraction and clean up procedure for the analysis of PCDD/Fs and dioxin-like PCBs was based on the protocol used by Schroijen et al. and Van Wouwe et al. [12,13] with some small adaptations. Briefly, 5 mL of human serum was weighted and mixed with 15 mL of acetone for 2 min in order to denature the proteins. PCDD/Fs, PCBs and other lipophilic compounds were extracted 3 times with each time 5 mL of hexane using shaking for 2 min, followed by centrifugation for 2 min. This hexane solvent phase was filtered upon a pre-conditioned celite column, filled with 0.5 g (1.3 cm^3) of celite and 6.5 g (4.3 cm^3) of anhydrous sodium sulphate and conditioned with 30 mL of hexane. The hexane fractions containing serum lipids were collected in a glass tube and the celite column was then washed with 10 mL of hexane, which was also collected in the tube, to elute all lipids from the column. After extraction, the samples were gently evaporated at 40 °C under a flow of pure air until only the serum lipids remained. The amount of fat was weighted and the extract was redissolved in 5 mL hexane and cleaned up on a pre-conditioned multi-layer silica column coupled in series with a carbon column.

The silica gel column (25 mL) was filled from bottom to top with glass wool, 1.9 g (1.3 cm^3) sodium sulphate, 3.0 g (4.3 cm^3) of 33% (w/w) sulphuric acid silica gel and 1.9 g (1.3 cm^3) sodium sulphate. The carbon column (10 mL) was filled with glass wool, 0.7 g (0.5 cm^3) sodium sulphate, 0.34 g (1 cm^3) X-CARB and 0.7 g (0.5 cm^3) sodium sulphate. Before loading the sample extract, the silica column was rinsed with 30 mL hexane, while the carbon column was conditioned with respectively 5 mL acetone, 20 mL toluene, 10 mL hexane. After sample addition, the glass tube containing the serum lipids was rinsed with 2 times 5 mL hexane and finally the column was eluted with 15 mL hexane. After full elution, the upper acidic silica column was removed and the carbon column was eluted with 8 mL of a hexane/acetone (90/10) mixture. Because this fraction was toxic to the cells, it was discarded [14]. The coplanar PCBs were eluted from the carbon column with 15 mL of hexane/ethyl acetate/toluene (80/10/10), followed by elution of the PCDD/Fs with 20 mL toluene. The PCDD/F and PCB fractions were evaporated until dry (40 °C) and redissolved in a defined volume hexane. The samples were stored at room temperature until CALUX analysis.

2.2.2. CALUX bioassay

The CALUX (Chemical Activated LUciferase gene eXpression) assay is a reporter gene mammalian cell bioassay. The recombinant cells used in the bioassay contain a stably transfected AhR-responsive firefly luciferase reporter gene, which responds by the induction of luciferase. The cell lines used in the bioassay were the recombinant mouse hepatoma cell lines H1L6.1c3, stably transfected with pGudLuc 6.1 containing 1 dioxin responsive domain (1 DRD), and the more sensitive H1L7.5c1 mouse hepatoma cell line, stably transfected with pGudLuc 7.5 and containing 5 dioxin responsive domains (5 DRDs) [5,6]. Cell treatment and measurement were based on the protocols described by Windal et al. [14] and the XDS method 4435 from 2008. Briefly, the cells were grown in cell culture plates containing 15 mL RPMI 1640 supplemented with 8% FCS and 1% penicillin/streptomycin (Gibco, UK). After trypsinization, the cells were counted and diluted to a concentration of 55–65 E4 cells/mL and each well of the 96-well plate was seeded with a 200 μL cell suspension in RPMI. After 24 h of incubation (37 °C, 5% CO_2), the 96-well plate was seeded with the standard solutions and sample extracts. Therefore, the target compounds in the sample extracts were transferred to 4 μL DMSO by evaporation of hexane at 40 °C using a vacuum centrifuge. Then, 400 μL RPMI medium was added and the mixture was dosed to the cell lines in duplicate (2 wells, 188 μL/well). When 188 μL of a standard solution, blank or sample extract in RPMI with 1% DMSO was added to every well, the plate was again incubated for 20–24 h. Afterwards, the medium was removed, the wells were rinsed with 75 μL PBS buffer pH 7.4 (Gibco, UK) and the cells in each well were visually inspected under the microscope in order to evaluate if the extract was toxic to the cells. If not, 50 μL lysis reagent (Promega, USA) was added to each well and the plate was shaken for 5 min. After a 10 min incubation period in the luminometer (Glomax, Promega, USA), 50 μL luciferin reagent (Promega, USA) was injected and the light output was given in relative light units (RLUs) (integration

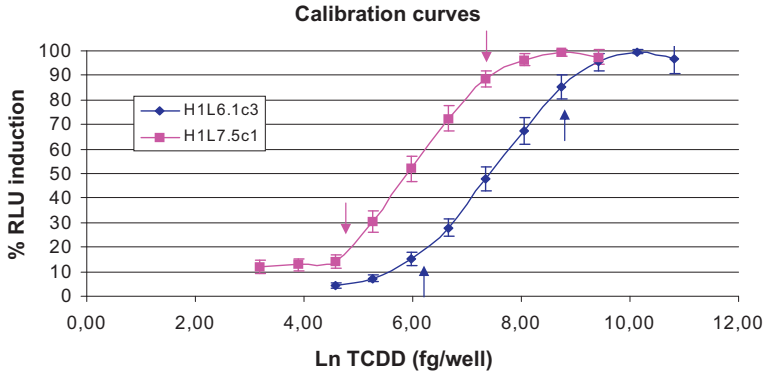

Fig. 1. 2,3,7,8-TCDD standard calibration curves for the mouse hepatoma H1L6.1c3 and the H1L7.5c1 cell lines. The working range is indicated by the arrows.

time 5 s for the H1L6.1c3 and 3 s for the H1L7.5c1, lag time of 5.6 s for both cell lines). A four parameter Hill-function was used to fit a sigmoid curve through the standard solutions. The measured luminescence in relative light units of an unknown sample was converted into a bioassay toxic equivalency value (CALUX-BEQ) by comparison of the response of the sample to the 10-point sigmoid dose-response curve obtained with 2,3,7,8-TCDD standards (concentration range of 50,000–97.7 fg/well for H1L7.5c1 and 12,500–24.4 fg/well for the H1L6.1c3). Three quality control solutions (i.e., a standard solution of TCDD corresponding to a RLU induction of around 50%) and 3 DMSO blanks were added in duplicate to every 96-well plate as an internal control. The PCB fractions were measured with the sensitive H1L7.5c1 cell line, since the concentrations were very low. The PCDD/F fractions could be measured using either the H1L6.1c3 or the H1L7.5c1 cell line. However, the % RLU induction is very low with the H1L6.1c3 cell line. And therefore the H1L7.5c1 cell line, which gave % RLU inductions near the EC50 value for the pooled samples, is preferably used for both the analysis of PCDD/Fs and dl-PCBs in small volumes of human serum samples.

3. Optimization and validation parameters

3.1. Quantification limit and EC50 calculation

A detection limit (LOD) shows that the signal is significantly higher than the blank (background value). To calculate the concentration of chemical in a sample, the signal produced by that concentration needs to be quantifiable. The limit of quantification is defined as the compound concentration which gives a luminescent signal that is equal to the DMSO blank plus 6 times the SD of that mean DMSO blank signal (6 wells/plate). However, when using a sigmoid calibration curve, the LOQ is normally defined as the lower limit of the working range (indicated by the arrows in Fig. 1). Using this definition, the LOQs for the H1L6.1c3 and H1L7.5c1 cell lines were 0.30 and 0.10 pg CALUX-BEQ/well, respectively. Occasionally, in case of high DMSO concentrations and/or high variability between the wells (high SD), the calculated LOQ (DMSO + 6 × SD) could be higher than the here above defined LOQ of 0.30/0.10 pg CALUX-BEQ/well. Serum samples below the calculated LOQ values should in this case be reanalyzed with the CALUX assay for an adequate BEQ-quantification. For the more sensitive H1L7.5c1, the LOQ of 0.10 pg CALUX-BEQ/well corresponded to 14.5 pg CALUX-BEQ/g fat for the PCBs and 30.3 pg CALUX-BEQ/g fat for the PCDD/Fs, taking into account a mean amount of fat in the serum sample (5 mL) of 0.0165 g for adolescents and a total dilution factor of 2.4 for the dl-PCBs and 5 for the PCDD/Fs. This means that the H1L7.5c1 cell line was three times more sensitive than the H1L6.1c3 cell line. At the EC50 value, which was defined as the concentration corresponding to the minimum induction level plus half of the difference between the maximum and minimum induction levels (% RLU of the EC50 = $Min_{\%\,RLU} + (Max_{\%\,RLU} - Min_{\%\,RLU})/2$), the H1L7.5c1 cell line was even four times more sensitive than the H1L6.1c3 cell line (mean EC50 values of 438.6 and 1804.5 fg/well respectively).

3.2. Full dose-response curves of dioxin-like compounds in human serum samples

When analyzing serum samples, it is important to determine the optimum dilution factor. The most efficient way to do this is by analysis of different sample dilutions from the same sample or the same pool of samples.

In this study, dilutions curves were obtained for pooled human serum samples from 300 adults (50–65 years old). Dilutions of 1.2, 1.5, 2, 4, 5, 8, 31 and 40 for the PCDD/F fraction and dilutions of 1.2, 1.5, 1.67, 2.4 and 4 for the dl-PCB fraction, obtained from the same pooled serum sample, were analyzed with the H1L7.5c1 cell line.

Fig. 2 shows two full dose-response dilution curves for the PCDD/F fraction, measured with the H1L7.5c1 cell line. The samples were from the same pool, but extraction, clean up and bioassay measurements were done independently for both curves. Table 1 gives an overview of the CALUX-BEQ in pg/g fat for the PCDD/F fraction for the different dilution points from the full dose curves in Fig. 2. The CALUX-BEQ was calculated for each dilution factor using the solver function on the TCDD standard calibration curve and taking into account the amount of fat and the used dilution factor.

From Fig. 2 and Table 1 it is clear that the optimum dilution factor (df) is in the range of 4, 5 and 8, with induction levels between 70 and 49% and BEQ-values ranging from 66.2 to 102.3 pg CALUX-BEQ/g fat. When incubating cells with more diluted samples (df

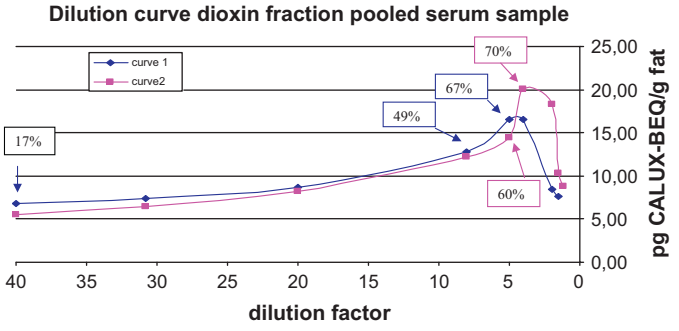

Fig. 2. Full dose-response curves for the PCDD/F fraction from the same pooled serum samples, measured with the H1L7.5c1 cell line (experiment in duplicate). The percentage induction (RLUs relative to the maximum of the TCDD standard curve) is shown at the positions indicated by the arrows.

Table 1
CALUX-BEQ values in pg/g fat for the PCDD/F fraction of two full dose response curves from pooled serum samples. The numbers in bold indicate the optimum dilution range that can be used for quantification. The results were not corrected for blank responses.

Dilution factor	pg CALUX-BEQ/g fat PCDD/F fraction	
	Curve 1	Curve 2
1.2		10.6
1.5	11.7	15.9
2	16.9	36.7
4	**66.2**	**80.3**
5	**82.5**	**71.9**
8	**102**	**97.7**
20	174	165
31	226	199
40	271	219

20–40), the induction levels were rather low, which resulted in an overestimation of the CALUX-BEQ. Also, serum samples with low PCDD/F levels would be below the quantification limit. When dosing more concentrated samples (df 1.2, 1.5 or 2), the % RLU induction was again lower than the curve maximum (past the top of the curve) and the sample extracts were in the so called "toxic range" of the calibration curve. This resulted in very low CALUX-BEQ values (Table 1) and thus an underestimation of the PCDD/F concentration. Since no cell death was observed in these cells, there were probably interfering compounds from the serum matrix that suppressed the CALUX signal and/or the induction response. More research is needed to identify these interfering compounds.

Measurements of the PCDD/F fraction with the H1L6.1c3 cell line gave similar dose-response curves with the same curvature, but the maximum induction level was only 12.5% (compared to 70% with the H1L7.5c1 cell line) at df 4. For the PCB fraction, dilution factors 1.2, 1.67, 2.4, 3.3, 4 and 5 were tested. The optimal dilution factors for the H1L7.5c1 cell line were between 1.2 and 2.4; dilution factors 3.3, 4 and 5 yielded responses below the quantification limit. In contrast to the PCDD/F fraction, no "toxic effects" were observed for the PCB fraction. When using the less sensitive H1L6.1c3 cell line, most PCB extracts were below quantification limits (data not shown). From this experiment, it was decided to use the more sensitive H1L7.5c1 cell line with a dilution factor of 5 for the PCDD/F fraction (0.8 mL out of 2 mL hexane extract, dosed in duplicate) and a dilution factor of 2.4 for the PCB fraction (1 mL out of 1.2 mL hexane extract, also dosed in duplicate).

3.3. Repeatability and within-lab reproducibility

Repeatability and within-lab reproducibility of the luciferase induction of the cell bioassay were calculated by analysis of the QC TCDD-standard on different days. On every 96-well plate, the QC standard solution was analyzed 3 times in duplicate (6 wells/plate) and the concentration (0.25 pg/well or 125 fg/μL) was chosen such that it would produce an induction response near the EC50 value (i.e., in the middle of the working range).

Table 2 gives an overview of the repeatability of the QC standard on a 96-well plate (SD_r and RSD_r), the within-lab reproducibility for analysis on different days (SD_{RW} and RSD_{RW}) and the CALUX recovery.

From Table 2 it is clear that all parameters of the bioassay analysis were within the quality criteria in our lab: RSD_r and RSD_{RW} were lower than 15% and the recovery was between 80% and 120%.

The long-term within-lab reproducibility of the whole procedure (extraction, clean up and bio-assay analysis) was calculated for pooled human serum samples from adults, analyzed on different days (between September 2009 and February 2011) with the H1L7.5c1 cell line. Table 3 gives an overview of the results for

Table 2
Overview of the repeatability, within-lab reproducibility and apparent recovery for the QC standard, measured with the H1L7.5c1 cell line. SD is standard deviation; RSD is the relative standard deviation, also often called coefficient of variation (CV). SD_r and RSD_r describe the repeatability; SD_{RW} and RSD_{RW} show the within-lab reproducibility.

	H1L7.5c1
Spike QC standard (pg/well)	0.250
Number of plates/days	30
Mean QC value (pg/well)	0.245
SD_r (pg/well)	0.016
RSD_r (%)	6.7
SD_{RW} (pg/well)	0.028
RSD_{RW} (%)	11.4
Recovery (%)	98

the PCB and the PCDD/F fractions, expressed in pg BEQ/g fat and in pg BEQ/g serum. For the PCB fraction a dilution factor of 2.4 was used, while for the PCDD/Fs a dilution factor of 5 was analyzed. The mean CALUX-BEQ values were 20.0 pg BEQ/g fat for the dl-PCB fraction and 83.3 pg BEQ/g fat for the PCDD/F fraction. As expected, the % RSD was higher for the samples than for the QC standards, since for the serum samples also extraction and clean up contribute to the variability.

3.4. Procedural blanks

To assess the specificity and to account for interferences that may derive from the solvents used and sample treatment methods, procedural blanks were analyzed during every run. Therefore, 5 mL ultra pure water was extracted, cleaned up and analyzed with the CALUX assay according to the previously described protocol. To detect possible antagonistic and synergetic effects, the blank extract from both the PCB and PCDD/F fraction was spiked with the QC TCDD-standard (Section 3.3). The recovery of the spiked blank was compared with the recovery of the 3 QC standards on the CALUX 96-well plate and should be between 80% and 120% [15]. For the PCB fraction, the mean recovery from 9 spiked procedural blanks was 114% (RSD 5.1%), while for the PCDD/F fraction ($n = 12$) a mean recovery of 111% (RSD 9.1%) was found. In both fractions, a slightly elevated recovery was found, which could be due to impurities originating from the used solvents or column fillings. In later experiments, new batches of solvents, silica and X-CARB were used and no elevations of the recovery were found. During all experiments, no cell death was observed in the samples and the procedural blanks.

3.5. Recovery and correlation with GC–HRMS

The recovery was calculated from a C13 spiked pooled serum sample, that was measured with GC–HRMS (VITO, Unit Environmental Analysis and Technology, Mol, Belgium). Therefore, 8 serum samples (5 mL) from the same sample pool were extracted and cleaned up with separation of the PCDD/F and dl-PCB fraction, according to the normally used CALUX protocol, and then pooled together for analysis by GC–HRMS. The serum pool was the same

Table 3
Long-term within-lab reproducibility of a pooled serum sample, analyzed with the H1L7.5c1 cell line.

	PCB fraction		PCDD/F fraction	
	pg BEQ/g fat	pg BEQ/g serum	pg BEQ/g fat	pg BEQ/g serum
Mean	20.0	0.09	83.3	0.40
SD	8.3	0.04	20.8	0.14
% RSD	41	42	25	34
n	35	35	51	51

Table 4
Ratio between CALUX and GC–HRMS for the PCDD/F and PCB fractions of a pooled serum sample, where *n* equals the number of analysis. TEF-values were from 2005 [16]. Only non-ortho PCBs were analyzed with GC–HRMS.

	PCDD/Fs	PCBs	Sum PCDD/Fs and dl-PCBs
GC–HRMS (pg WHO-TEQ/g fat)	41.7	14.7	56.4
CALUX (pg BEQ/g fat)	83.3 (n = 51)	20.0 (n = 35)	103.3
Ratio CALUX/GC–HRMS	2.0	1.4	1.8

as the one used to analyze the long-term within-lab reproducibility of the CALUX assay (Table 3).

Table 4 provides an overview of the CALUX and GC–HRMS results for both the PCDD/Fs and the dioxin-like PCBs in the sample extracts. For the GC–HRMS analysis, the WHO-TEF values of 2005 [16] were used for both PCDD/Fs and non-ortho PCBs. The mono-ortho PCBs were not analyzed by GC–HRMS.

The CALUX-BEQs were higher than the GC–HRMS results. For the PCDD/F fraction a CALUX/GC–HRMS ratio of 2.0 was found, while for the PCB fraction the CALUX response was 40% higher compared to the GC–HRMS TEQ-values. Most authors report CALUX PCDD/F fraction levels commonly higher than those of GC–HRMS [13,15,17–19], while for the dl-PCB fraction, CALUX values are often lower than those obtained by GC–HRMS [17–19]. However, in this study, only the non-ortho PCBs were measured by GC–HRMS, while the CALUX assay can detect any PCB (including non-ortho and mono-ortho) that can bind to and activate the AhR. Although mono-ortho PCBs have low TEF/REP values, this could maybe partly explain the higher BEQ values found for the PCB fraction with the CALUX-bioassay compared to GC–HRMS.

The concentrations of all PCDD/F and non-ortho PCB congeners (pg WHO-TEQ/sample) and the recoveries of all the C13 standards (%), measured with GC–HRMS, are presented in Table 5. Spiking of the serum samples with the C13 standards was done before extraction of the sample via the CALUX procedure. The % recovery in Table 5 therefore reflects the recovery of the total CALUX sample pre-treatment method (extraction and clean up) after measurement with GC–HRMS.

All congeners could be quantified by GC–HRMS in the pooled serum sample. The recovery of the C13 spiked PCDD/Fs was between 60% and 90% for all congers and between 67% and 82% for the C13 spiked non-ortho PCBs. Also, an adequate separation between both fractions was obtained during the clean up of the sample extract. In the PCB fraction, not more than 13% of the dioxin congener TCDF (WHO 2005-TEF of 0.1) was found which corresponds to 0.01 pg CALUX-BEQ/g fat and 9.4% of the dioxin congener TCDD (WHO 2005-TEF of 1), which corresponds to 0.81 pg BEQ/g fat. In the PCDD/F fraction not more than 14% of the PCB congener 169 (WHO 2005-TEF of 0.03) or 0.83 pg CALUX-BEQ/g fat was detected, while for the most potent PCB congener, PCB 126 with a WHO 2005-TEF value of 0.1, 8.6% or 1.0 pg BEQ/g fat was found in the PCDD/F fraction (Table 5).

3.6. Quantification of PCDD/Fs and dioxin-like PCBs in human serum samples from adolescents

Five millilitre serum samples obtained from 173 14–15 year-old adolescents of the general population, recruited via the school system in Flanders, were analyzed using the optimized CALUX procedure described above. The dl-PCB and PCDD/F fraction were measured separately for all samples with the H1L7.5c1 cell line, using dilution factors for these fractions of respectively 2.4 and 5. For these dilution factors, quantification of the sample extracts could be carried out on 100% and 98.8% of the PCDD/F and dl-PCB fractions respectively. The PCDD/F fraction of 45 out of 173 samples were also analyzed using the H1L6.1c3 cell line (also at df 5) to compare the two cell lines. The % RSD was lower than 20% for 42 out of 45 samples (see supplementary info: Table 7).

The geometric mean PCDD/F and dioxin-like PCB concentrations in the total study population (n = 173) were respectively 108 (95% confidence interval (CI): 101–114) pg CALUX-BEQ/g fat and 32.1 (95% CI: 30.1–34.2) pg CALUX-BEQ/g fat. The levels of both biomarkers were significantly higher for samples from boys (124.9 pg CALUX-BEQ/g fat for PCDD/Fs and 36.7 pg CALUX-BEQ/g fat for dl-PCBs) than for those of girls (92.6 pg CALUX-BEQ/g fat for PCDD/Fs and 27.9 pg CALUX-BEQ/g fat for dl-PCBs) (ANOVA: $p < 0.001$). The PCDD/F CALUX-BEQ value increased with a lower education level ($p = 0.02$) and was significantly higher in subjects consuming self-caught fish ($p = 0.02$). For dl-PCBs only a non-significant increasing trend was seen for some food factors

Table 5
GC–HRMS congener concentrations (pg WHO-TEQ/sample) and C13 standard recoveries (%) from CALUX sample extraction and clean up for PCDD/Fs and dl-PCBs. TEF-values were from 2005 [16]. nd is not detected.

GC–HRMS	Dioxin fraction (pg WHO-TEQ)	Dioxin fraction (% recovery)	PCB fraction (pg WHO-TEQ)	PCB fraction (% recovery)
PCDD/Fs				
2,3,7,8-T4CDF	0.0080	60.6	nd	13
1,2,3,7,8-P5CDF	0.0051	80.5	nd	0.7
2,3,4,7,8-P5CDF	1.7	89.7	nd	2.5
1,2,3,4,7,8-H6CDF	0.16	79.8	nd	0.6
1,2,3,6,7,8-H6CDF	0.17	75.1	nd	0.4
2,3,4,6,7,8-H6CDF	0.045	74.6	nd	0.1
1,2,3,7,8,9-H6CDF	0.0080	63.3	nd	0.2
1,2,3,4,6,7,8-H7CDF	0.021	69.2	nd	0.1
1,2,3,4,7,8,9-H7CDF	0.00070	77.6	nd	0.1
O8CDF	0.00012	60.8	nd	0.1
2,3,7,8-T4CDD	0.99	67.5	nd	9.4
1,2,3,7,8-P5CDD	2.0	88.8	nd	1.3
1,2,3,4,7,8-H6CDD	0.15	79.9	nd	0.3
1,2,3,6,7,8-H6CDD	1.5	75	nd	0.8
1,2,3,7,8,9-H6CDD	0.14	–	nd	
1,2,3,4,6,7,8-H7CDD	0.11	71.8	nd	0.2
O8CDD	0.039	59.5	nd	0.1
dl-PCBs				
PCB 81	nd	4.4	0.00011	68.9
PCB 77	nd	4.9	0.00021	67.2
PCB 126	nd	8.6	1.7	82.1
PCB 169	nd	13.7	0.75	72.5

Table 6
Literature overview for the PCDD/F concentrations from human biomonitoring studies in different countries.

Reference	Country	Period	Population	*N*	Calculation	Unit	Value	Method
This study	Flanders	2008–2009	Students (14–15 years old), general population	173	GM (95%CI)	pg CALUX-BEQ/g fat	108 (101–114)	UDC-CALUX, H1L7.5c1 PCDD/F
	Flanders	2008–2009	Students (14–15 years old), general population	172	GM (95%CI)	pg CALUX-BEQ/g fat	32.1 (30.1–34.2)	UDC-CALUX H1L7.5c1, dl-PCB
Van Wouwe et al. [13]	Belgium	2000	Adults, men and women	341	GM	pg CALUX TEQ/g fat	41.8	XDS-CALUX, H1L6.1c2 PCDD/F
	Belgium	2000	Adults, men and women	341	GM	pg WHO-TEQ/g fat	25.7	GC–HRMS, PCDD/F
Long et al. [1]	Greenland	2002–2004	Adults, men	75	Median	pg CALUX TEQ/g fat	197	UCD-CALUX, Hepa1.12cR
	Poland	2002	Adults, men	99	Median	pg CALUX TEQ/g fat	312	UCD-CALUX, Hepa1.12cR
	Sweden	2002	Adults, men	78	Median	pg CALUX TEQ/g fat	428	UCD-CALUX, Hepa1.12cR
	Ukraine	2002	Adults, men	86	Median	pg CALUX TEQ/g fat	337	UCD-CALUX, Hepa1.12cR
Koppen et al. [22]	Flanders, Peer	1999	Adults, women, 50–65 years old	22	Mean (SD)	pg CALUX TEQ/g fat	37.2 (13.1)	BDS-CALUX, sum PCDD/F and dl-PCB
	Flanders, Antwerp	1999	Adults, women, 50–65 years old	25	Mean (SD)	pg CALUX TEQ/g fat	35.0 (16.5)	BDS-CALUX, sum PCDD/F and dl-PCB
	Flanders, Peer	1999	Adults, women, 50–65 years old	22	GM (95%CI)	pg WHO-TEQ/g fat	70.9 (65.3–76.9)	GC–HRMS, sum PCDD/F and dl-PCB
	Flanders, Antwerp	1999	Adults, women, 50–65 years old	25	GM (95%CI)	pg WHO-TEQ/g fat	78.9 (72.7–85.6)	GC–HRMS, sum PCDD/F and dl-PCB
Kayama et al. [23]	Japan	2002	Female farmers, 55,5 years old (average)	1407	Mean (SD)	pg CALUX TEQ/g fat	32.3 (12.1)	XDS-CALUX, PCDD/F
Todaka et al. [24]	Japan	2002–2005	Mothers	119	Mean (SD)	pg WHO-TEQ/g fat	11(4.2) PCDD/F 5.5 (2.5) dl-PCB	GC–HRMS
Wittsiepe et al. [25]	Germany	2000–2003	Pregnant women, 19–42 years old	169	Mean	pg WHO-TEQ/g fat	16.79 PCDD/F 11.57 dl-PCB	GC–HRMS
Burns et al. [26]	Russia	2003–2005	Children 8–9 years old	482	Median	pg WHO-TEQ/g fat	21.1	GC–HRMS
Ayotte et al. [27]	Canada	na	Adults, men and women, 25-75 years old	40	Median (min–max)	pg CALUX TEQ/g fat	102 (37–287)	BDS-CALUX, sum PCDD/F and dl-PCB
Warner et al. [9]	Italy	1999	Women, 20–49 years old	22	Mean (min–max)	pg CALUX TEQ/g fat	30.8 (1.6–67.3)	XDS-CALUX, PCDD/F

such as eating self-caught fish ($p = 0.12$) and local eggs ($p = 0.17$) and being breastfed as newborn ($p = 0.07$).

4. Conclusions and discussion

An optimized method was validated for the quantification of PCDD/Fs and dioxin-like PCBs in small amounts of human serum samples. With the newly developed sensitive H1L7.5c1 cell line, it was possible to detect both dioxin like-PCBs and PCDD/Fs in serum samples of adolescents and adults.

First, full dose-response dilution curves of a pooled sample were established and hence, an optimum dilution range of the sample matrix for analysis with the CALUX cell line was obtained. The choice of an appropriate dilution factor is very important, since matrix effects can occur when dosing concentrated sample extracts and this could result in inaccurate BEQ determinations. Normally, it should be expected that all dilutions yield the same CALUX-BEQ result. However, when the slope and the maximum of the sample curve are not the same as those of the standard TCDD calibration curve, a wide spread in calculated BEQ values can be found for different dilutions (Table 4). Therefore, it should be investigated which dilution range yields comparable CALUX-BEQ values. Often this optimum dilution range is around the EC50 value, when using the mouse hepatoma cell lines. For the serum samples dilution factors of 2.4 for dl-PCBs and 5 for PCDD/Fs were selected for routine analysis, since these dilutions yielded CALUX-BEQ results with a same order of magnitude that were around the EC50 value. All individual serum samples were then analyzed with these dilution factors.

These results show the need to establish a full dose curve before starting routine analysis to determine the optimum dilution range. A single point analysis can not provide the needed information, since one does not know if this point is in the "toxic range" of the full dose curve (i.e., past the top of the curve) or not. When the optimum dilution range for the (biological) sample matrix is known, a single point analysis can be done for time-saving and economic reasons and/or if not enough sample is available for measuring multiple dilutions, which may be the case for human serum samples which should be used sparingly.

The validation studies showed that the relative standard deviation for the repeatability and within-lab reproducibility for the QC standard were lower than the in-house quality control criteria limits of 15% and 20%. For the pooled serum samples a % RSD for long-term within-lab reproducibility of 41% for the dl-PCB fraction and 25% for the PCDD/F fraction, both expressed as pg BEQ/g fat, was found. The mean recovery of the procedural blanks was for both fractions within the accepted limits of 80–120%. The LOQ was 14.5 pg BEQ/g fat for the dl-PCBs and 30.3 pg BEQ/g fat for the PCDD/Fs, taking into account a mean fat amount in the 5 mL serum sample of 0.0165 g for adolescents and the used total dilution factors. The mean EC50 values for the H1l7.5c1 and H1L6.1c3 cell lines were respectively 438.6 and 1804.5 fg/well. The recovery, measured by analysis of a C13-spiked pooled serum sample with GC–HRMS, was between 60% and 90% for all PCDD/F congeners and between 67% and 82% for the non-ortho PCBs. The separation between both fractions was also adequate: a maximum of 13% of PCDD/F congeners was found in the PCB fraction and maximum 14% of PCB congeners was found in the PCDD/F fraction. CALUX values from the pooled serum samples were higher than concentrations calculated with GC–HRMS, with a CALUX/GC–HRMS ratio of respectively 2.0 and 1.4 for the PCDD/Fs and the dl-PCBs. As expected, given the similarity in the two recombinant cell lines, a good correlation was found between the samples of adolescents analyzed using both the H1L7.5c1 and the H1L6.1c3 cell lines.

The CALUX-BEQ values, reported for the adolescents in this study, were 108 (95% CI: 101–114) pg CALUX-BEQ/g fat for the PCDD/Fs and 32.1 (30.1–34.2) CALUX-BEQ/g fat for the dioxin-like PCBs. These results will be used as a reference value for Flanders and will allow the researchers to determine the pollution pressure in specific hot spots and to follow up the concentration levels in adolescents over a period of time. Comparison of the CALUX-BEQ values with the results of other instrumental and bio-analytical studies will be more difficult, since the biological response of the CALUX assay is strongly dependent on the cell type and the measurement conditions.

The PCDD/F values in this study were relatively high compared to most values from human biomonitoring studies that have been reported in literature (Table 6), but it is not always known which sample analysis protocol was followed in the other studies. Often different techniques are used (CALUX rat cells, CALUX mouse cells, GC–HRMS and analysis with or without separation of PCDD/Fs and dioxin-like PCBs), which makes clear interpretation and comparison of the results difficult. Also differences between clean up methods [19], the used dilution factor employed, different methods for quantification using CALUX standard curves [20] and the choice of TEF/REP values for quantification with GC–HRMS [21] all have an important influences on the quantification of the final result. Therefore, it is difficult to compare different studies with different protocols unless a correlation between the two methods has been established. This can be done by analyzing the same pooled sample(s), with a concentration(s) in the expected range of the unknown samples, multiple times with both methods.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2011.07.103.

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