CALUX DETERMINATION OF PCDD/F'S AND DIOXIN-LIKE PCB'S IN SMALL AMOUNTS OF HUMAN MILK FROM THE RURAL AREAS OF FLANDERS (BELGIUM) USING THE H1L7.5C1 MOUSE HEPATOMA CELL LINE

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Introduction

In the first Flemish Environment and Health survey run by the Flemish Centre of Expertise on Environment and Health (FLEHS I 2002-2006) increased concentrations of PCBs, dioxin-like substances and chlorinated pesticides (a metabolite of DDT and hexachlorobenzene) were observed in cord blood of newborns and in blood of 14-15 year-old adolescents and 50-65 year-old adults living in low populated rural communities of East and West Flanders and Flemish Brabant compared to other Flemish regions. Due to the health concern associated with chlorinated compounds follow-up of pollutant levels in this area is of importance. Therefore, human breast milk from mothers living in these regions was collected (2009-2010) for analysis of different POPs.

For the quantification of PCDD/Fs and/or dioxin-like PCBs in (human) milk samples both GC-HRMS and the CALUX bioassay are used in routine analysis. However, most CALUX methods use high amounts of milk (varying between 10 and 60 mL)[1, 2]. Since for the Flemish human breast milk survey in the rural areas not only PCDD/F and dioxin-like PCBs were analyzed, but also other POPs like DDE, PBDEs, marker PCBs, HCB, HCH, perfluorinated compounds, etc., the amount of milk available for the CALUX bioassay was limited.

Therefore, a new method was developed for the analysis of PCDD/Fs and dioxin-like PCBs in only 5 mL milk.

Materials and methods

In this study, 5 mL human breast milk samples obtained from 84 18-35 year-old breastfeeding mothers residing in the rural areas of Flanders (24 low-populated rural communities in East and West Flanders), were analyzed for PCDD/Fs and dl-PCBs with a new sensitive CALUX mouse hepatoma cell line. Additionally, from each of the 84 individual samples, 10 mL was taken to compose a pooled sample, which was analyzed with both the CALUX assay (VUB, ANCH, Brussels, Belgium) and by GC-HRMS (WHO reference laboratory, State Institute for Chemical Analysis and Veterinary of Food, Freiburg, Germany). Details about inclusion criteria and characteristics of the study population are described by Colles et al., 2011[3].

Since the matrix of human breast milk is very similar to that of human serum[4], the same CALUX protocol was used for the analysis of the individual human milk samples, but with a higher amount of acid silica due to the higher fat content of milk compared to blood. Briefly, the milk samples were extracted with 15 mL acetone and 3 times 5 mL hexane and the extract was purified on a celite column. The fat extract was weighted and redissolved in hexane. The sample was then further purified on an acid silica column coupled in series with a carbon column.

The cell line used in the bioassay was the sensitive H1L7.5c1 recombinant 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. (2005)[7] and the XDS method 4435 from 2008. A four parameter Hill-function was used to fit a sigmoid curve through the standard solutions. The measured luminescence in relative light units (RLU) of an unknown sample was converted into a bioassay toxic equivalency value (CALUX-BEQ) by comparison of the response of the sample to the sigmoid dose-response

curve obtained with 2,3,7,8-TCDD standards. Three quality control (QC) 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.
Methods used for analysis of the pooled milk sample by the WHO reference laboratory (State Institute for Chemical Analysis of Food) in Freiburg, Germany, are described elsewhere[8, 9,10].

Results and discussion:
When analyzing a new sample matrix with the CALUX bioassay, it is important to determine the optimum dilution factor by analysis of different sample dilutions from the same sample or the same pool of samples. In this study, dilutions curves were obtained for human breast milk samples from 1) a volunteer and 2) from a pool of human breast milk from 5 volunteers.
For the PCB fraction, dilution factors of 1.2; 1.54; 2.4 and 4 were analyzed. Dilution factor 1.2 gave the highest results for the pooled milk samples, but for the volunteer a maximum response was found for dilution factor 1.54. Dilution factors 2.4 and 4 yielded low % RLU inductions, around the LOQ. Dilution factor 1.54 also gave the best repeatability (curves 1 and 2 from pool in Figure 1) and therefore, it was decided to use this dilution factor for further experiments.

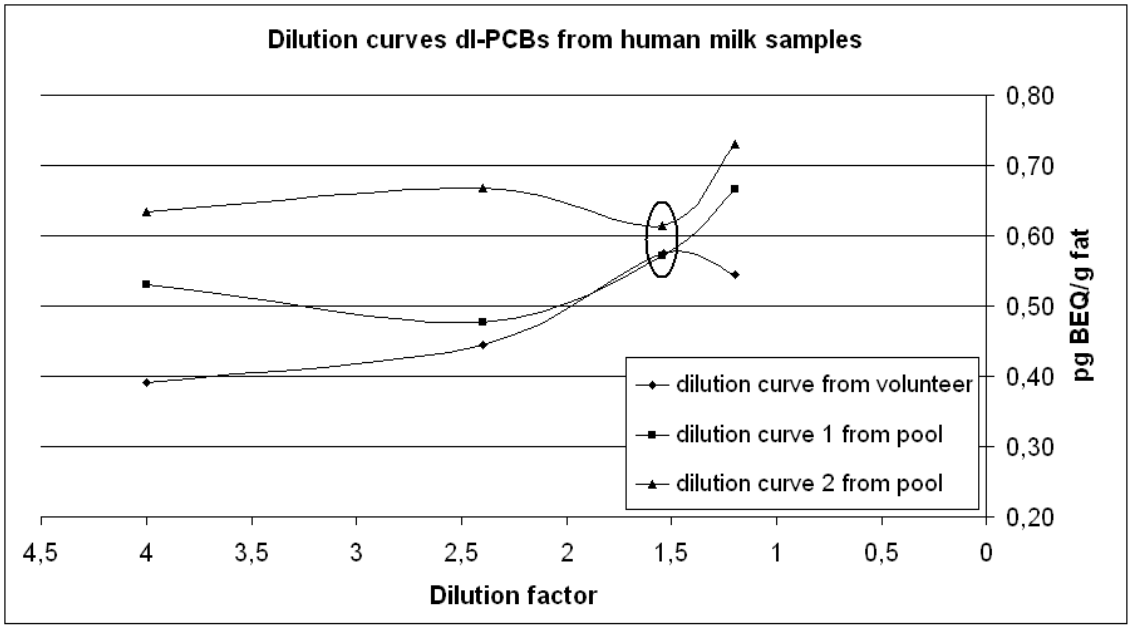

Figure 1: Full dose-response curve for the dl-PCB fraction from pooled milk samples, measured with the H1L7.5c1 cell line. The circle indicates the optimum dilution factor.

For the PCDD/F fraction, 4 full dose-response curves with dilution factors 1.2; 1.54; 2; 4; 8; 20 and 40 were analyzed. For the pooled human milk samples (curves 1 and 2 from pool on Figure 2), dilution factors 2 and 4 gave % RLU inductions around the EC50 value (inductions between 63.4 and 46.8%). For the dilution curves of the volunteer (curves 1 and 2 in dashed line), the optimum dilution factor was 2 (% RLU induction close to the EC50 value and good agreement between the two sample extracts).
Since for the PCDD/F fraction the optimum dilution factor is dependent on the sample load and thus can differ from one person to another, it was decided to analyze two dilutions for every sample (df 2.5 and 4). For heavily contaminated samples dilution factor 4 gave values around the EC50 value, while for less contaminated samples dilution factor 2.5 was better. When the EC50 value was between the % RLU inductions measured for dilution factor 2.5 and 4, an extrapolation to the EC50 was made.

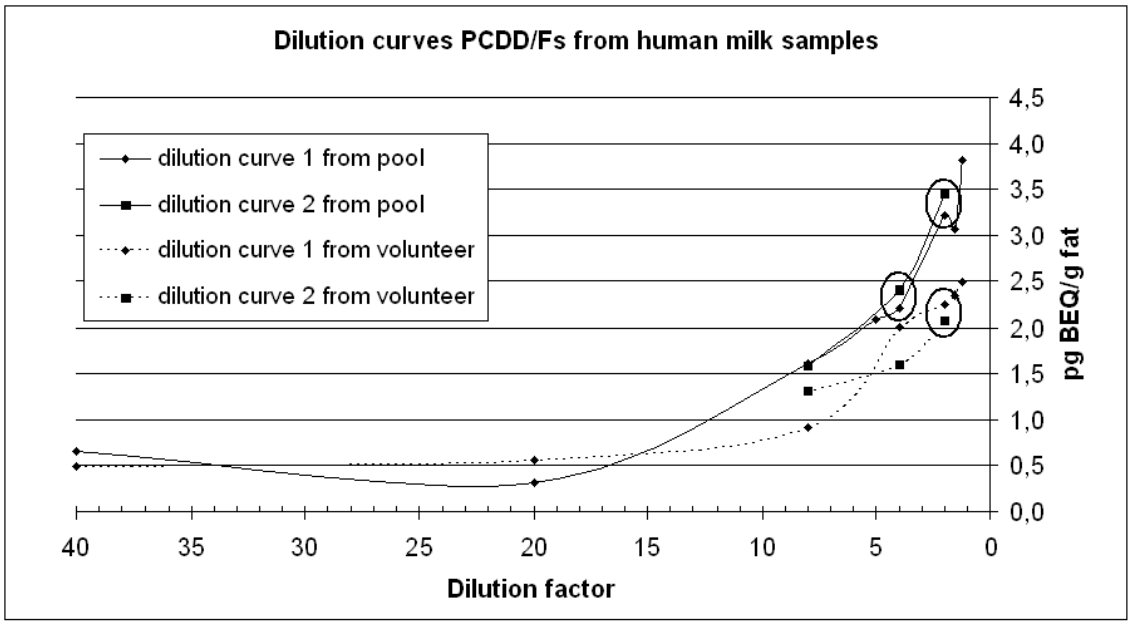

Figure 2: Full dose-response curve for the PCDD/F fraction from pooled milk samples, measured with the H1L7.5c1 cell line. The circles indicate the optimum dilution factors

The geometric mean of the individual samples in the total study group (n=84) was 10.4 (95% CI: 9.4-11.4) pg CALUX-BEQ/g fat for the PCDD/Fs and 1.73 (1.57-1.91) pg CALUX-BEQ/g fat for the dl-PCBs. PCDD/F concentrations were significantly higher for mothers who lost weight compared to mothers who recently gained weight (p=0.012) and also for the consumption of local eggs (p=0.023). For both PCDD/Fs and dl-PCB, a significant increase in BEQ was found with a higher amount of breast milk fat (p=12E-5 for PCDD/Fs and p=0.007 for dl-PCBs).

The CALUX-BEQ values on the pooled breast milk sample were in good agreement with the GC-HRMS data for both the PCDD/F and the dl-PCB fractions (Table 1). The PCDD/F BEQ values were 32 % and 60 % higher with CALUX compared to GC-HRMS, when using respectively the 1998 and 2005 TEF scheme. For the dl-PCBs a better agreement was found between the two techniques with the 2005 TEF scheme (a CALUX/GC-HRMS ratio of 0.58 compared to 0.37 for the 1998 TEFs).

pg WHO TEQ/g fat and pg BEQ/ g fat	**GC-HRMS (TEF 1998)**	**GC-HRMS (TEF 2005)**	**Mean CALUX-BEQ (n=2)**	**Ratio: mean CALUX/GC-HRMS (TEF 1998/2005)**
PCDD/Fs	8.41	6.95	11.09	1.32/1.60
dl-PCBs	5.80	3.77	2.17	0.37/0.58
Total dl-compounds	14.21	10.72	13.26	0.93/1.24

Table 1: The CALUX-BEQ and GC-HRMS WHO-TEQ values, expressed per g of fat, for the PCDD/F and dl-PCB fractions from a pooled human milk sample. TEF values from 1998 and 2005 were used.

The GC-HRMS TEQ values on the pooled human milk sample (Table 1) were lower than the Belgian result from the 4th WHO-coordinated survey of human milk for persistent organic pollutants (2006). In this WHO-study respectively 10.31 and 7.02 pg WHO-TEQ/g fat (TEF 1998) was found for the PCDD/F and dl-PCB fractions[11].

The concentration of dl-PCBs in human milk was much lower than these of the PCDD/Fs, which was also found in former Belgian studies. The PCDD/F levels were comparable to the concentrations found in German studies

from Wittsiepe et al., 2007 (2000-2003, 13.30 pg WHO-TEQ/ g fat)[12] and Raab et al., 2007 (2005: 9.91 pg WHO-TEQ/g fat)[13] and to the European mean concentration found in the 3rd and 4th WHO human breast milk survey (8.9 pg WHO-TEQ/g fat, 2001 and 2006). When comparing the Belgian PCDD/F levels to the results from other European countries participating the 4th WHO human milk survey from 2006 (Czech Republic, Slovakia, Sweden, Norway, Finland and Hungary), the Belgian levels were still higher despite the declining trend[3, 14]. The dl-PCBs levels were lower in Flanders than in other European studies, where values of 13.00 (200-2003)[12], 9.92 (2005)[13] and 9.4 pg WHO-TEQ/ g fat (mean concentration from 3rd and 4th WHO human breast milk campaign, 2001 and 2006) were found.

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