Biological screening of Ah receptor agonist activity in butter fat and coconut oil by means of chemical-activated luciferase expression in a genetically engineered cell line (CALUX)

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1. Introduction

Polyhalogenated aromatic hydrocarbons (PHAHs), such as polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and biphenyls (PCBs) represent a class of widespread environmental contaminants. Their lipophilic nature and persistence contribute to their high bioaccumulation potential and their biomagnification in higher tropic levels of the food chain [1]. Exposure to specific PHAHs results in a wide variety of species- and tissue-specific toxic and biological effects, including: birth defects, immunotoxicity, lethality, tumor promotion and enzyme induction [2,3].

Many of these effects are mediated by the ability of certain PHAHs to bind to the soluble intracellular aromatic hydrocarbon receptor (AhR). This is followed by a transformation of the PHAH:AhR complex and translocation of the transformed complex to the nucleus, where it binds with high affinity to DNA on a specific dioxin responsive element (DRE) [4]. Normally this results in an increased transcription of genes coding for enzymes, like cytochrome P450 IA and glucuronyl transferase.

Biological tests for detecting the presence of dioxins and dioxin-like compounds in food, or environmental samples are a promising alternative for the often expensive and time-consuming analytical methods. Recently it was shown that newly developed cell lines showing Ah receptor-mediated chemical-activated luciferase gene expression (CALUX) provide a reliable biomarker for AhR-mediated effects [5].

The aim of the present study was to investigate whether the CALUX-bioassay can be used for screening of food products. Therefore, a comparison was made between levels of dioxins and planar PCBs in butter fat samples, as determined by the CALUX-bioassay and gas chromatographic/mass spectrometric (GC/MS) analysis. Using oil prepared from smoked coconuts, we also studied the possible use of the bioassay for the determination of carcinogenic polycyclic aromatic hydrocarbons (PAHs) in food products. In these studies, special attention was paid to the development of relatively simple and fast procedures for the preparation of suitable extracts for the bioassay.

2. Materials and methods

Materials
The pGudluc 1.1-transfected H4IIE cells were obtained from the Department of Toxicology, Agricultural University, Wageningen. Fetal calf serum (FCS) was purchased from Gibco BRL (Breda, The Netherlands), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) from Schmidt BV (Amsterdam, The Netherlands), α-MEM, penicillin/streptomycin, bovine serum albumins from Sigma (St. Louis, MO, U.S.A.), dimethylsulfoxide (Uvasol), n-hexane (p.a.), diethyl ether (p.a.), cyclohexane (p.a.), sodium sulphate anhydrous (p.a.) and dried for 16 hours at 150 °C, silica gel 60 (70-230 mesh) from Merck (Darmstadt, F.R.G.), ethylacetate for HPLC from Acros (Geel, Belgium), activated carbon (Anderson AX 21), benzo[a]pyrene 98% (B(a)P) from Aldrich (Steinheim, F.R.G.). Standards of PCB#77, PCB#126 and PCB#169 (in iso-octane) and two dioxin mixtures containing respectively 2.0 and 0.2 pg each of the 17 different 2,3,7,8 congeners of PCDD/F per µl (in toluene) were prepared from standards obtained from Cambridge Isotope Laboratories (Woburn, MA, U.S.A.). Native butter fat came from a batch with a dioxin content of 6 pg I-TEQ/g, used at RIKILT-DLO as a control sample in the analysis of dioxins.

Cleaning of native butter fat with activated carbon and subsequent spiking
At 50°C 200 g fat was dissolved in 200 ml distilled n-hexane with 4 g activated carbon and stirred for 1 hour. To remove the carbon the solution was filtered four times, twice over a Whatman 41 Ashless filter (ϕ 9 cm) and twice over a Whatman 41 Ashless filter with anhydrous sodium sulphate. The solution was evaporated to dryness under vacuum and subsequently under nitrogen. The fat was divided over 5 batches. Batch A was not spiked. For preparation of batch B, 800 µl of the solution containing 2.0 pg dioxins per µl was added to 36.6 g fat dissolved in ethylacetate/cyclohexane (1:1, v/v) and mixed thoroughly. For preparation of batch C, 400 µl of the solution containing 0.2 pg dioxins per µl, as well as 2 ml of solutions containing 1 ng/ml of PCB#77, PCB#126 and PCB#169 in iso-octane were added to 31.2 g butter fat dissolved in ethylacetate/cyclohexane. Using I-TEFs for the PCDDs and PCDFs and TEFs 0.0005, 0.100 and 0.010 for respectively PCB#77, #126 and #169 [6] resulted in calculated contents for batch B and C of 125.8 and 14.5 pg TEQ/g fat.

Clean-up of butter fat and coconut oil for GC/MS analysis
Samples of the three batches butter fat were extracted with a gel-filtration/graphitized carbon clean-up procedure used for GC/MS analysis at RIKILT-DLO [7]. Contents of the 17 PCDD/Fs and 3 planar PCBs were determined by GC/MS. In a parallel clean-up the planar fraction was taken to dryness and dissolved in DMSO for exposure in the CALUX-bioassay. However, the samples in this parallel clean-up were not spiked with ^{13}C-labeled standards which is necessary for GC/MS analysis to correct for recovery losses.
Samples of coconut oil were extracted using the gel-permeation column and contents of 14 different polycyclic aromatic hydrocarbons (PAHs) were determined by GC/MS analysis.

Clean-up of acid stable PHAHs in spiked butter fat using silica/33% H_2SO_4 columns
Samples of 0.50 g fat of batch A, B and C were dissolved in 2 ml n-hexane/diethyl ether (97:3, v/v). Columns of 10 g silica/33% H_2SO_4 were packed in glass (ϕ 0.8 cm) with glass mineral wool at the bottom and some sodium sulphate on top. The columns were washed with 10 ml n-hexane/diethyl ether (DEE) and the 2 ml samples were quantitatively transfered to the column. The compounds of interest were eluted with 28 ml of n-hexane/diethyl ether. All solvent eluting from the column was collected starting from the moment of sample application and subsequently taken to dryness and dissolved in 30 µl DMSO for exposure in the CALUX-bioassay.

In a second experiment samples of 0.10 g fat of batch A, B and C were dissolved in 1 ml n-hexane/DEE and these samples were cleaned on 4 g silica/33% H_2SO_4 columns (ϕ 1.2 cm). The 15 ml of eluate was taken to dryness and dissolved in 30 µl DMSO for exposure in the CALUX-bioassay. The reliability of the latter column was confirmed by an additional radioactivity recovery study, using ^{14}C-labeled 2,2',4,4',5,5'-hexachlorobiphenyl [8], which was kindly provided by Prof. Åke Bergman, Wallenberg Laboratory, Stockholm University.

Emulsifying of coconut oil
A sample of 50 mg of coconut oil was added to 2 ml of incubation medium containing 0.5% (w/v) lecithine and 0.5% (v/v) DMSO. The sample was sonicated three times for one minute with an ultrasonic microtip to obtain a stable emulsion. After sonicating, the sample was added to the cells (0.5 ml/well).

Exposure of cells
The pGudluc 1.1-transfected cells were cultured in α-MEM supplemented with 10% (v/v) FCS, 50 IU/ml penicillin and 50 µg/ml streptomycin. Cells were seeded in 24-multi well plates at a density of about 10^5/well. Exposure of the cells to the samples and standards was started at confluency by replacing the medium with 0.5 ml sample. The final concentration of DMSO, which was used to dissolve the fat extracts, TCDD and B(a)P standards was 0.5% (v/v). Exposure time to TCDD standards and butter fat extracts was 24 hours. Exposure time to B(a)P standards and coconut oil samples was 4 hours. All samples and standards were tested in triplicate and from most samples 2 or 3 dilutions were used.

Determination of luciferase activity (CALUX)
Following exposure, cells were homogenized and the luciferase activity and protein content in these homogenates were determined as described previously [5].

3. Results

Exposure of pGudluc 1.1-transfected H4IIE cells to TCDD resulted in a dose related increase in luciferase activity (data not shown). After fitting the data of the TCDD standard curve, the 'dioxin' contents of the butter fat samples could be calculated from their luciferase activity. From the results shown in Table 1 it is clear that there is a very good agreement between spiked levels and GC/MS determined levels in the three batches of butter fat. The CALUX content of the planar fraction (Cb), obtained from the carbon column in spiked batches B and C is in the same

Table 1. Chemical and CALUX determined TEQ contents of spiked butter fat.

Butter fat	Spiked amount [pg TEQ/g]	GC/MS content [pg TEQ/g]	CALUX content [pg TEQ/g] Cb[1]	S1[2]	S2[3]
Batch A	0.0	0.1	3.3	1.1	3.1
Batch B	125.8	124.7	35.2	78.7	134.9
Batch C	14.5	13.7	9.6	5.2	29.0

[1] The toluene fraction of the porous graphitized carbon column (Cb) containing the planar PCBs and dioxins.
[2] The eluate of the 10 g silica/33% H_2SO_4 column (S1).
[3] The eluate of the 4 g silica/33% H_2SO_4 column (S2).

Table 2. Chemical and CALUX determined contents of PAHs in coconut oils.

Sample	GC/MS content		CALUX content
	total [1] PAHs [ng/g]	mutagenic [2] PAHs [ng/g]	[ng 'B(a)P'/g]
sample#4	515	33	< 10
sample#5	332	24	< 10
sample#6	239	20	< 10
sample#3	2213	150	541
sample#9	1753	81	23
sample#23	534	54	27
sample#7	9298	943	1232
sample#19	4038	447	99
sample#21	7870	632	487

[1] Total PAHs content: sum of the contents of Acenaphtene, Fluorene, Phenanthrene, Anthracene, Fluoranthene, Pyrene, Benz(a)anthracene, Chrysene, Benzo(b)fluoranthene, Benzo(k)fluoranthene, Benzo(a)pyrene, Indeno(123cd)pyrene, Dibenz(ah)anthracene and Benzo(ghi)perylene.

[2] Total of the toxicologically relevant compounds (mutagenic) Benzo(a)pyrene, Benz(a)anthracene, Dibenz(a,h)anthracene and Chrysene (contents of Dibenz(a,c)anthracene and Benzo(e)pyrene were not determined), which contribute to the CALUX content.

order of magnitude but lower and the blank batch A still contains some Ah agonist activity. Using a clean-up with silica/H_2SO_4, there is a reasonable agreement between the CALUX-bioassay and the GC/MS analysis (Table 1).

Exposure of pGudluc 1.1-transfected H4IIE cells to B(a)P, B(e)P, B(a)A and Chrysene resulted in dose related increases in luciferase activity and yielded, compared to B(a)P with a 'TEF' 1, preliminary 'TEFs' of 0.0001, 1 and 5 for B(e)P, B(a)A and Chrysene respectively. Exposure to B(a)P for 24 instead of 4 hours resulted in a lower sensitivity. The non-mutagenic PAHs pyrene, phenantrene and fluoranthene and mixtures of these three congeners (tested at concentrations as high as those observed in the highest contaminated sample) had no synergistic or antagonistic effect on the CALUX test result of benzo(a)pyrene or mixtures of benzo(a)pyrene, benz(a)anthracene and chrysene (data not shown). After fitting the data of the B(a)P standard curve, the 'B(a)P' content of the coconut oil samples could be calculated from their luciferase activity (in ng 'B(a)P'/g fat). The results shown in Table 2 demonstrate that at low concentrations of mutagenic PAHs a low CALUX content is determined while at higher concentrations an increase in luciferase activity is observed.

4. Discussion

The fact that in three batches of butter fat there is almost no difference between the spiked amounts and the GC/MS determined levels, demonstrates that the GC/MS method is very reliable. Regarding the very efficient removal of planar compounds with activated carbon, the small Ah agonist activity in blank batch A is likely to be caused by other planar compounds than the ones determined by GC/MS analysis, or by some unknown Ah receptor agonists. The latter possibility is supported by data from an experiment in which a soy infant sample was extracted and determined by GC/MS analysis, revealing a total of 0.1 pg TEQ/g fat due to planar PCBs

and dioxins, while exposure of the planar carbon fraction (Cb) in the CALUX-bioassay resulted in a content of 8.5 pg TEQ/g fat.
The discrepancies between GC/MS and CALUX-bioassay contents in the planar carbon fractions (Cb) are likely to be caused by recovery losses during the very extensive clean-up (up to 50%). The CALUX content determined in both silica fractions (S1 and S2) shows a reasonable agreement with GC/MS analysis. The latter (S2) column was eluted with relatively more solvent resulting in higher recoveries and therefore showed a better agreement. The results of these experiments indicate that the CALUX-bioassay in combination with a clean-up on a silica/H_2SO_4 column is a promising alternative for the expensive and laborious GC/MS analysis of PHAHs in butter fat.
The CALUX determined contents in the coconut oil samples show a reasonable agreement with the sum content of the toxicologically relevant (mutagenic) PAHs determined with GC/MS analysis. However, more samples need to be tested. The results also indicate that PAHs are the major contaminants in the coconut oil samples studied. The sensitivity of the CALUX-bioassay based upon the toxicologically relevant compounds is in the order of 30 ng/g fat, which corresponds with about 1 ng B(a)P, while *e.g.* the sensitivity of the Ames-test is about 1 µg B(a)P (data not shown). The lower sensitivity for B(a)P after exposure for 24 hours instead of 4 hours is probably caused by biodegradation of this PAH during the incubation procedure.
These experiments demonstrate the potential use of the CALUX-bioassay to screen coconut oil samples for their PAH content, without any sample clean-up. Unfortunately this simple method was not applicable to butter fat.
In conclusion, the CALUX-bioassay can be a useful pre-screening tool for detection of Ah agonist activity in butter fat and PAHs in coconut oil. However more experiments must be performed to establish a quantitative relationship between the CALUX-bioassay and the GC/MS analysis.

Acknowledgements

This study was financially supported by grants from the Dutch Ministry of Agriculture, Nature management and Fisheries, the Dutch Technology Foundation (STW) and from the EU-research program Life Sciences and Technologies for Developing Countries (STD 3).

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