

DR- AND ER-CALUX ASSAYS AS TOOLS TO DIRECT TOXICITY IDENTIFICATION AND EVALUATION OF ENDOCRINE DISRUPTING CHEMICALS

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Introduction

Nowadays, the presence of endocrine disrupting chemicals (EDCs) in the environment is a topic of great concern. Although instrumental analysis can be used to identify and quantify known EDCs, hazard evaluation based on chemical monitoring is complicated, because EDCs are structurally highly distinct compounds, mixture interactions have to be taken into account and compounds responsible for estrogenic activity in sediment samples are still mainly unknown¹.

Dioxin responsive (DR-) and Estrogen responsive (ER-) CALUX-assays (Chemical Activated Luciferase Gene Expression)^{2,3} were recently developed as mechanism-based, rapid and extremely sensitive *in vitro* reporter gene bioassays to assess dioxin-like and estrogenic activity. They provide useful information about the total dioxin-like and estrogenic potentials of complex mixtures of chemicals in environmental samples and are thus able to account for both unknown active compounds and mixture interactions in a sample. Their application is especially valuable if combined with instrumental analytical approaches. In Toxicity Identification and Evaluation (TIE) bioassays are used to direct fractionation and chemical analysis in order to elucidate what compounds are responsible for the toxic activity found in a sample.

In this report we present our progress regarding the development of a TIE method for dioxin-like and estrogenic compounds in sediment, directed by responses generated in DR- and ER-CALUX assays. The suitability of Gel Permeation Chromatography as clean up method in TIE studies to remove sulphur and most matrix constituents present in the extract was investigated. Second, a method to separate compounds according to log K_{ow} with reversed phase high pressure chromatography (RP-HPLC)⁴ was adapted for sample fractionation after GPC clean up. Finally, the developed TIE-method was applied to investigate the log K_{ow} distribution of dioxin-like and estrogenic components in a sediment sample.

Methods and Materials

Gel Permeation Chromatography clean up

A standard mixture of 6 poly chlorinated biphenyls (PCBs) and 20 organo chlorinated pesticides (OCPs), with individual concentrations around 250 ppb, was injected on a GPC system (PL-gel, 10µm, 50A, 300 x 25 mm, Polymer Laboratories, 2 columns in serial connection, with 10 ml/min dichloromethane as eluents). Eluate was collected in fractions of 1 minute, evaporated and dissolved in isooctane. Recovery and elution profile of each standard compound were estimated using gas chromatography with electron capture detection (GC-ECD). In addition to the standard mixture sediment extract obtained by Accelerated Solvent Extraction (hexane: acetone 9:1 v/v, 100 °C, 2000 psi) was cleaned on GPC. Eluate was collected in fractions of 1 minute, evaporated and dissolved in dimethylsulfoxide (DMSO) for CALUX measurements or isooctane for chemical analysis. CALUX activity was expressed as dioxin equivalents and estradiol equivalents per gram dry weight and

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compared to the activity measured in extract not treated with GPC. RP-HPLC-fractionation: A standard mixture of poly aromatic hydrocarbons (PAHs), PCBs, chloro benzenes and several pesticides differing in log Kow was composed and injected on HPLC (C18, Vydac TP254, 5 μ m, 4.6 x 250 mm at 22 °C with a mobile phase initially of 50 % methanol and 50 % water (1 ml/min) linearly changing to 100 % methanol in 50 min⁴). Retention times were correlated to log Kow values to obtain a standard curve from which fraction borders could be derived. Elution profile of the fractionation procedure was estimated with the same mixture as used for GPC validation. Application of the method to sediment extract: Sediment was extracted, cleaned with GPC and fractionated with HPLC. CALUX-activity was measured in the fractions and compared to activity in unfractionated extract.

Results and Discussion

Method development

Gel Permeation Chromatography clean up: The elution profile and recovery of several PCBs and OCPs after GPC-clean up were estimated (Table 1). Tested compounds eluted between 18 and 23 minutes after injection. The presence of matrix did not cause components to elute outside this window. Previous studies with sediment extracts showed that matrix components elute between 12 and 18 minutes and sulphur after 24 minutes indicating that GPC-treatment enables the separation of matrix from target compounds⁵. For GC-ECD analysis no further clean-up of GPC-treated extracts was necessary. Average recovery over all compounds was 90 %. To assess the elution profile of the total CALUX-active fraction, sediment extract was treated with GPC and each fraction was tested in both DR- and ER-CALUX (Figure 1). Estrogenic activity eluted between 18 and 23 minutes. Estrogenic activity summed over all fractions was equal to 96 % of the activity measured in untreated extract. Only 75 % of dioxin-like activity eluted within the collection window. The other part of the activity eluted between 24 and 60 minutes. Remarkably, the sum of the dioxin-like activity found in GPC-fractions was more than twice as high as found in untreated extract. This may have been caused by non-specific or antagonistic components in the untreated extract, which may have been separated from aryl hydrocarbon receptor (AhR) agonists during GPC treatment, thereby increasing the response induced by individual fractions. This underlines the necessity of sufficient clean up measures to obtain a relevant extract. For both bioassays measured activity was distributed over different fractions. Because for standard test compounds elution of at least 95 % of the compound always was confined to at most two fractions, more than one compound is likely to be responsible for the biological activities measured.

Table 1. Elution profile of a standard mixture of PCBs and OCPs after GPC-treatment. Recoveries are summed over all fractions and shown as percentage of total theoretically present amounts.

Compounds	Fraction (min)						Total recovery (%)
	17-18	18-19	19-20	20-21	21-22	22-23	
PCB							99
DDs							78
DRINs							89
HCH (α + γ)							98
HCH (β)							99
HEPO (cis + trans)							84
HCB							106
Heptachlor							85
Octachlorostyrene							87

Abbreviations: DDs: dichloro diphenyl dichloro ethane, -dichloro ethylene and trichloro ethane (all in o,p'- as well as p,p' isomer); DRINs: aldrin, dieldrin, endrin, isodrin, telodrin; HCH: hexachloro hexane; HEPO: heptachloro epoxide; HCB: hexachloro benzene.

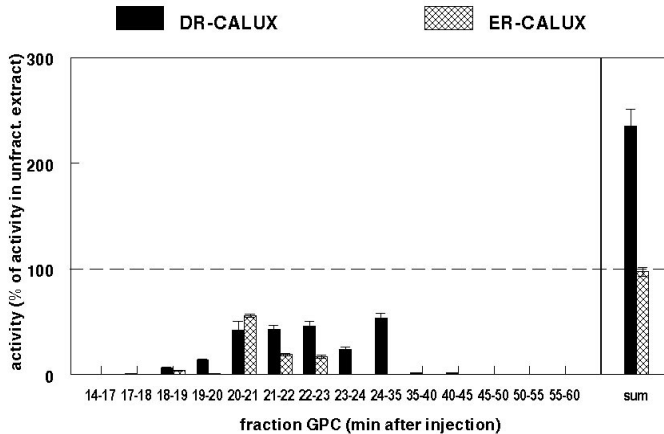


Figure 1. Elution profile of dioxin-like and estrogenic activity in DR- and ER-CALUX assays of sediment extract after extract clean up with Gel Permeation Chromatography.

RP-HPLC-fractionation: The relationship between retention time and log Kow was estimated using a mixture of chloro benzenes, PAHs, PCBs and several pesticides (Figure 2). The strong correlation found ($r^2 = 0.94$) enabled the collection of fractions of sediment extract according to decreasing polarity. This was confirmed for environmentally relevant concentrations in an experiment in which PCB/OCP standard mixture was fractionated (Figure 3).

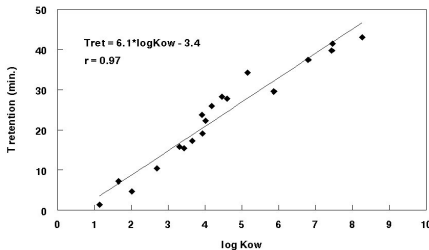


Figure 2. Relationship between retention time and log Kow of various pesticides, PAHs and PCBs used to calibrate RP-HPLC elution conditions for fractionation of environmental sample extracts in TIE analysis.

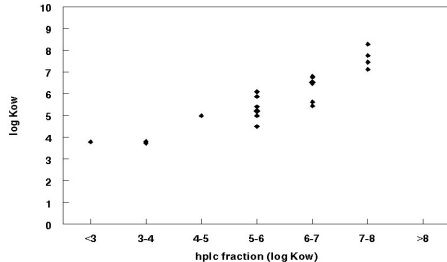


Figure 3. Confirmation of relationship found between retention time and log Kow. A mixture of PCBs and OCPs was used for RP-HPLC fractionation at environmentally relevant concentrations (250 ppb). For each compound log Kow is shown as function of the fraction in which the compound eluted.

Application to sediment sample

As a first test of applicability the method was applied to a standard sediment sample available at our laboratory. During the fractionation dioxin-like and estrogenic activities were separated into almost completely different fractions (figure 4). Although some estrogenic activity was found in the non polar fraction (log Kow > 8), most estrogenic activity was found in a fraction of low log Kow (fraction 2-3), suggesting the presence of relatively polar estrogenic compounds in sediment. The natural estrogenic

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hormones possess log Kow values (between 2.45 for estriol to 4.01 for 17- β -estradiol) in the same range as in which most of the estrogenic activity in the sediment sample was found. However, because chemical analysis of estrogenic compounds in this sample has not been performed, their possible involvement in the observed estrogenicity is unknown. In accordance with chemical properties of known persistent AhR agonists, dioxin-like activity in this sample was mainly found in the non polar fraction. Further steps in TIE, involving chemical analysis, will be performed to identify the compounds responsible for the observed estrogenic and dioxin-like activities.

In conclusion, the combination of CALUX assays with analytical separation and fractionation techniques is a promising tool for the elucidation of dioxin-like and estrogenic compounds in sediment samples. GPC treatment is a useful method for the clean up of sediment extracts, in which there is no loss of acid unstable compounds as in e.g. clean up with sulphuric acid silica. RP-HPLC can be used to separate compounds in complex mixtures according to log Kow. In this study, fractionation of sediment extract led to the almost complete separation of fractions with estrogenic and dioxin-like activity. The method can be used to generate extract fractions of high relevance and of far reduced complexity. These are necessary first steps towards the chemical identification of unknown active compounds in Toxicity Identification and Evaluation of EDC in sediment.

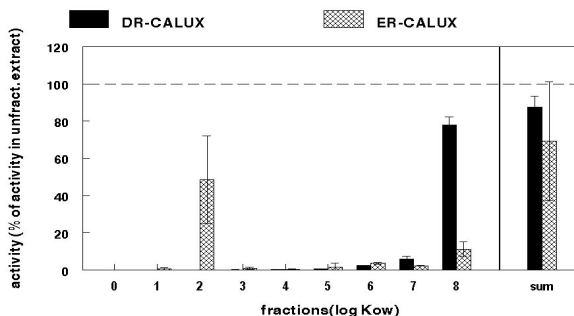


Figure 4. DR- and ER-CALUX activity in different fractions of sediment extract fractionated according to log Kow. Activities are expressed as percentage of activities in unfractionated extract. On the right, activity of all fractions is summed. Obviously DR- and ER-CALUX-active compounds (in this extract) are confined to certain areas of log Kow and can be separated very well by fractionation.

Acknowledgement

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