DETERMINATION OF PBDES, ORGANOCHLORINE PESTICIDES AND PYRETHROIDS INCLUDING THEIR METABOLITES IN LIMITED AMOUNTS OF BIOLOGICAL SAMPLES

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Introduction

A number of children with learning and developmental disorders (i.e. attention deficit hyperactivity disorder ADHD, autism spectrum disorders, anxiety disorders) is increasing, currently 4-7% of children are reported to have ADHD[1]. The European project DENAMIC "Developmental Neurotoxicity Assessment of Mixtures in Children" investigates the relationship between the presence of pesticides and other environmental pollutants in mother and child and their neurotoxic effects. An important part of the DENAMIC project is to develop sensitive, miniaturized and robust methods for the determination of polybrominated diphenyl ethers (PBDEs), organochlorine pesticides (OCPs) and pyrethroids including their metabolites. The matrices to study are exposed rat tissues (perinatal fat, liver, brain) from *in vivo* experiments and human breast milk which can pose the risk of transfer of toxicants from the mother to the child. In both cases the amount of the sample is limited; therefore the methods have to assure the detection of ultratrace levels of the above mentioned compounds. For the sample clean-up, non-destructive methods for fat removal have to be used to avoid the decomposition of some OCPs and non-persistent pyrehtroids.

PBDEs and OCPs are both persistent lipophilic compounds with many adverse effects on human health. Their production and use has already been banned in Europe. However, the residues of OCPs are still present in different environmental and human matrices and PBDEs are present in textiles, fabrics and flexible polyurethane foams, as well as in electric appliances and electronic devices[2]. Pyrethroid insecticides are (unlike PBDEs and OCPs) not persistent and are rapidly metabolized in humans. Recent studies showed neurotoxic effects of these compounds.

Materials and methods

Sample preparation procedure for test animal tissues

Rat brain and liver were mixed with 1 or 2.5 g of anhydrous sodium sulphate. This mixture was transferred into a glass extraction cartridge on a layer of anhydrous Na_2SO_4, ^{13}C-internal standards were added and a piece of glass wool was placed on the top. The extraction was carried out in a mini-Soxhlet apparatus with 15 ml dichloromethane for about 2 hours. The solvent was evaporated to dryness and the amount of lipids was weighted. 2 ml of acetonitrile were added to the lipid residue and sonicated for 10 min. SPE with a C_{18} cartridge (Agilent Bond Elut, 200 mg) and additional 1 g of basic alumina (deactivated with 5% water, w/w) was used for sample clean-up. After sorbent conditioning, the extract was passed through the cartridge and 2 ml of fresh acetonitrile completed elution[3]. The eluate was evaporated to dryness, and solvent was exchanged for *n*-nonane. These samples were prepared for GC-HRMS analyses (scheme of the procedure is in Fig. 1).

Sample preparation procedure for whole milk

10 ml of whole milk was extracted (liquid-liquid extraction, LLE) with cyclohexane and isopropanol (water:2-propanol:cyclohexane=11:8:10) – twice with 30 ml of organic phase[4]. The extract containing lipids and compounds of interest was concentrated to approximately 0.5 ml, placed in a drying oven at 103°C for 1 hour and the residue was weighed. Two clean-up techniques for fat removal were compared: gel permeation chromatography (GPC) and freezing-lipid filtration (FLF). In GPC, Waters Envirogel columns were used (two columns, 19 x 150 mm and 19 x 300 mm, connected in series with 25 x 40 mm BioBeads SX-3 guard column) with DCM and a flow-rate of 5 $ml.min^{-1}$. In FLF, 40 ml of acetonitrile were added to a dry lipid residue, in two repetitions. After 15 min of ultrasonication, the extract was placed in a freezer at -24°C for 40 min to allow the precipitation of lipids which can then easily be removed by filtration. The final purification step was identical to

that applied for the rat tissue samples (see above) where the sorbent amount and elution solvent volume were adjusted to the amount of lipids processed.

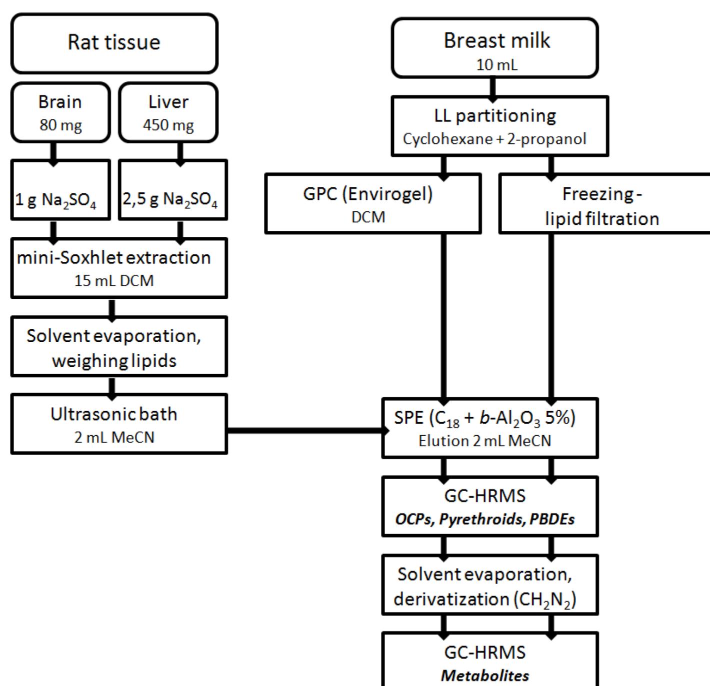

Figure 1. Scheme of tissue and milk sample preparation

Instrumental analysis tests
Pyrethroid and OCP were analyzed by GC-HRMS DFS (Thermo Scientific) using a 60 m × 0.25 mm × 0.25 um SGE HT8 column or DB5MS column of identical dimensions. For measurement of native PBDEs and methyl derivatives of hydroxylated PBDE metabolites and pyrethroid ones, an Agilent GC 7890A fitted with a 15 m × 0.25 mm × 0.1 um Restek Rtx-1614 column coupled to a Micromass AutoSpec Premier HRMS (Waters) was used. A Sigma Aldrich miniaturized generator was used for safe and fast diazomethane preparation. A Waters UPLC Acquity coupled to a TQ-S Xevo MS/MS and an Agilent GC 6890 coupled to a Waters Quattro Micro MS/MS fitted with a 60 m × 0.25 mm × 0.25 um Restek Rxi-5Sil column were also used and tested for the optimization of PBDE and pyrethroid metabolites separation and detection.

Results and Discussions

Tissue samples

Due to the *in vivo* exposure experiments performed with selected OCPs, pyrehtroids and their metabolites, 10 ng of the analyte mixture was added to rat brain and liver samples to calculate the overall recoveries of the method.

Table 1. Recoveries (%) of OCPs, pyrethroids and their metabolites in rat brain and liver samples

	Pyrethroids	OCPs	Pyrethroid metabolites
Brain	59 - 99	79 - 95	69 - 147
Liver	61 - 95	77 - 90	41 - 119

Milk samples

The choice of an LLE method for the extraction of lipids was made from previous comparison of LLE partitioning and an accelerated solvent extraction method (ASE). As to the ASE, it was found that the amount of extracted lipids depended on methanol content in the mixture of n-hexane:DCM The optimal composition of extraction solvent was found to be hexane:DCM:MeOH = 5:2:1 (v/v/v) and optimal temperature 30°C. But due to coextracted substances causing problems with evaporation to dryness, LLE partitioning was chosen. Moreover, this method provides several advantages compared to previously reported LLE methods (i.e. Bligh and Dyer, Röse Gottlieb) as it uses non-chlorinated solvents with low toxicity, non-explosive properties and is robust.

As regards lipid clean-up methods, GPC removed 75% and FLF 85% of extracted lipids. Recoveries after SPE clean-up varied from 86 to 134% for PBDEs, from 60 to 136% for OCPs and from 66 to 118% for pyrethroids.

Instrumental analysis
Analyses of PBDEs and OCPs are routinely performed using GC-HRMS in our trace analytical laboratories. The details including IQL (instrumental quantification limits responding to S/N ratio 10 expressed as the amount injected) are reported below. For the analysis of pyrethroids LC-MS/MS, GC-MS/MS and GC-HRMS were compared. The analysis of OH-BDE and pyrethroid metabolites was performed on LC-MS/MS and GC-HRMS (after derivatization) and the chromatograms were compared in terms of separation efficiency and sensitivity.

Pyrethroid separation using LC-MS/MS (BEH C18 column, 1.8 um, 2.1 mm × 100 mm) was less efficient, for co-elution of peaks and broad peaks were observed. The electrospray ionization of pyrethroids was not sufficient as regards sensitivity – bifenthrin and fenvalerate did not show any response. On the contrary, gas chromatography provided better separation of individual isomers. Additionally, GC-EI-MS/MS and GC-HRMS were compared for pyrethroid analysis. However, the sensitivity of MS/MS was lower, so finally GC-HRMS with the lowest IQLs has been selected for analyte separation and detection (IQLs in Table 2).

Two isomers of OH-tri-BDEs and OH-hexa-BDEs and six of OH-tetra-BDEs and OH-penta-BDEs were analysed, therefore it was necessary to find a suitable chromatographic system for their good separation and different MRM transitions for individual isomers since the MS spectra of product ions depend on the position of OH-groups. Several UPLC and HPLC chromatographic columns were tested (BEH C18, X-Terra C18, Cortecs UPLC C18, Ascentis RP-Amide) and finally Acclaim Surfactant column (3 um, 2.1 mm × 150 mm)[5] showed a good chromatographic separation. GC-HRMS was used as another separation and detection method after the methylation of the OH-BDE metabolites with diazomethane. Comparison of both methods and an example of chromatographic separation of OH-tetra-BDEs is shown in the Figure 2.

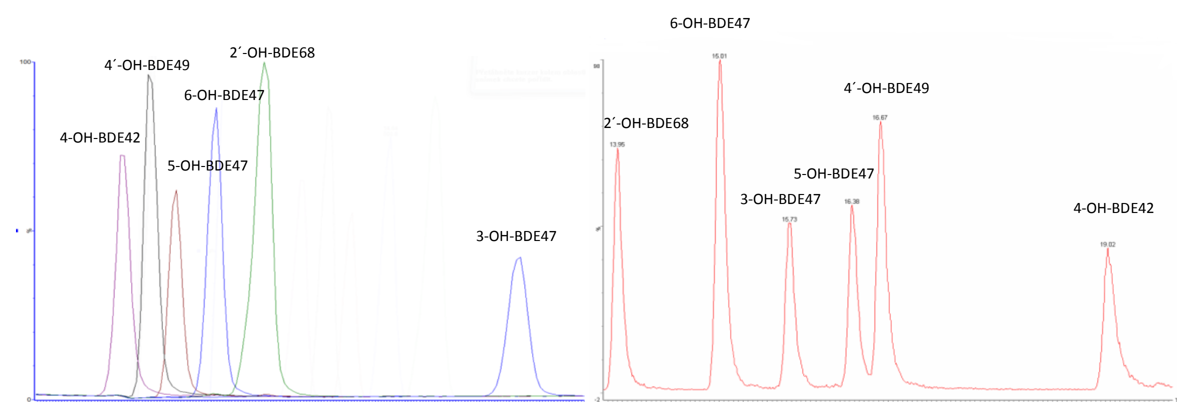

Figure 2. Comparison of chromatographic separation of OH-tetra-BDE isomers using LC-MS/MS (left) and GC-HRMS (after methylation, right)

Table 2. Comparison of IQLs (pg injected) in GC-HRMS, GC-MS/MS and LC-MS/MS method for determination of PBDEs, OCPs, pyrethroids and their metabolites

	Native			*Metabolites*	
	Pyrethroids	**PBDEs**	**OCPs**	**Pyrethroid**	**OH-BDEs**
GC-HRMS	**0.3-9**	**0.04-0.4**	**0.1-1**	**20-60**	**30-500**
GC-MS/MS	10-40	1-12	20-45	-	-
LC-MS/MS	170-700	-	-	5000-80 000	0.15-0.85 (OH-tri-penta-BDE) 2400 (OH-hexa-BDE)

Conclusions
Analytical methods for the determination of PBDEs, OCPs, pyrethroids and their metabolites were developed. LC-MS/MS did not show good separation efficiency at low concentrations in real samples, satisfactory robustness (OH-BDEs) or sufficient limits of detection (pyrethroids and pyrethroid metabolites). An isotope dilution GC-HRMS method showed the best sensitivity and separation efficiency for all the groups of compounds. After the validation of these methods they can be used for the analysis of exposed rat tissues and for the case-control study of breast milk samples from European cohorts within the DENAMIC project.

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