HAIR AS A BIOMARKER OF HUMAN EXPOSURE TO PFRs. CASE STUDY ON A MOTHER-CHILD COHORT.

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Introduction

Phosphate flame retardants (PFRs) are commonly used as plasticizers and flame retardants (FRs) in a wide range of materials and products. The use of PFRs has recently increased due to the phase-out and ban of Polybrominated Diphenyl Ethers (PBDEs) and other brominated flame retardants (BFRs). In consequence, recent studies have reported widespread environmental occurrence and human exposure to PFRs[1,2]. Unfortunately, information about potential health risks of these compounds is lacking. Human biomonitoring is a valuable tool that can provide important information about impact and relevance of exposure[3]. For ethical and practical reasons, non-invasive matrices, such as hair, nails, saliva are preferred. Hair seems to be a very promising matrix as it has many advantages, such as sample stability, large window of detection, information on short to long term exposure, etc. Its lipid content (2 - 4 %) also makes this matrix useful for measurement of lipophilic chemicals, such as Persistent Organic Pollutants (POPs). Two routes of contamination are relevant for hair, namely external by deposition from air and dust and internal through uptake from blood at the hair follicle/root[4]. Because distinction between external and internal contamination is practically impossible, recent research suggests hair as a biomarker that provides information on retrospective and integrative exposure[5]. In the present study, we measured PBDEs and PFRs in human hair samples collected from mothers and their children (6-12 years) living in the Greater Oslo area, Norway. The hair samples were analyzed according to a method that was previously developed and validated and that allows to measure both PBDEs and PFRs from a single sample (intake)[6]. Since PBDE levels were below the method limit of quantification (LOQ_m) in most samples, only the following PFR compounds were considered for further investigations: tris(2-ethylhexyl) phosphate (TEHP), tris(2-butoxyethyl) phosphate (TBEP), tri-phenyl phosphate (TPhP), tris(1,3-dichloro-*iso*-propyl) phosphate (TDCIPP), 2-ethyl-hexyldiphenyl phosphate (EHDPP), tri-isobutyl phosphate (TiBP), tri-n-butyl phosphate (TnBP), tris(2-chloroethyl) phosphate (TCEP), tri-cresyl-phosphate (TCP). Levels of PFRs found in hair from mothers were compared to those found in hair of their children. Further, PFR levels found in the human hair were compared to those found in house dust and air samples collected simultaneously in the same households[7].

Materials and methods

Hair samples collection: A set of 95 human hair samples from mothers (n = 45) and children (n = 50) were collected according to the criteria defined by the DEMOCOPHES procedure[8]. Samples were wrapped in a paper envelope and a plastic bag and stored at -20 °C until analysis. Before analysis, hair straws were cut into small pieces (1-2 mm) with stainless steel scissors and homogenized by shaking for 10 min.

Hair extraction and clean-up: Around 200 mg of hair were accurately weighed, spiked with Internal Standards (ISs) (BDE-77, TPP-d15, TCEP-d12, TBEP-d6, TDCPP-d15, and TnBP-d27) and ultrasonically extracted at 25 °C for 25 min with 4 mL of 10 % HNO_3 and 4 mL of Hex:DCM (4:1; *v:v*). Solid-liquid extraction with 2 x 4 mL of Hex:DCM (4:1; *v:v*) was assisted with 1 min vortexing. After each extraction cycle, hair extracts were centrifuged at 3500 rpm for 6 min. The supernatants were transferred to clean glass tubes. The combined fractions of organic solvent were evaporated to near dryness under a gentle nitrogen stream and re-dissolved in 1 mL of Hex. Prior to fractionation, SPE cartridges (filled up from the bottom with 1 g of Florisil and 250 mg of anhydrous Na_2SO_4) were conditioned with 10 mL of EtAc and 6 mL of Hex. The extracts were quantitatively transferred and eluted with 10 mL of Hex and 6 mL of Hex:DCM (6:1; *v:v*) (Fraction A–FA) and 10 mL of EtAc (Fraction B–FB). The second fraction was evaporated to dryness and reconstituted in 500 µL of MeOH and measured on LC-MS/MS. The first fraction (FA) was evaporated near to dryness and reconstituted in 1 mL of Hex. The SPE cartridge filled with acidified silica and anhydrous Na_2SO_4 was pre-washed with 2 mL of

Hex:DCM (1:1; *v:v*) and 2 mL of Hex. FA was quantitatively transferred onto the cartridge and eluted with 9 mL of Hex and 3 mL of DCM. The extract was evaporated until dryness under a gentle nitrogen stream and reconstituted in 100 µL of toluene and analyzed on GC-ECNI-MS.

GC-MS conditions: Analysis of FA containing PBDEs was performed with a Hewlett Packard HP 6890 Series GC system coupled to an Agilent 5975C MS operated in ECNI mode. One µL of cleaned extract was injected on a DB-5MS column (20 m x 0.18 mm x 0.18 µm) using pulsed splitless injection. The injection temperature was set at 300 °C with a 10 psi pulse until 1.25 min. and purge flow to split vent 70.0 mL/min after 1.25 min. The GC temperature program was started at 110 °C, held 1.25 min, ramped at 30 °C/min to 230 °C, held 4 min, ramped at 40 °C/min to 300 °C and held 10 min. Helium was used as a carrier gas with a flow rate of 1.0 mL/min. The mass spectrometer was operated in selected ion monitoring (SIM) mode. Dwell time was set at 40 ms. The ion source, quadrupole and interface temperatures were set at 230 °C, 150 °C and 300 °C, respectively and the electron multiplier voltage was 2271 V. Methane was used as moderating gas.

LC-MS/MS conditions: FB containing PFRs was analyzed by Ultra Performance Liquid Chromatography (UPLC)-tandem mass spectrometry (MS/MS). The Waters Acquity UPLC system (Waters, Milford, MA, USA) consisted of an Acquity binary solvent manager, an Acquity sample manager and an Acquity column heater. The PFRs were separated on an Acquity UPLC BEH C18 column (2.1 mm x 100 mm, 1.7 µm) with a Van Guard Acquity UPLC BEH C18 precolumn (2.1 mm x 5 mm, 1.7 µm). The column temperature was kept at 40 °C. Optimum separation was obtained with a binary mobile phase constituted of ultrapure water (solvent A) and MeOH (solvent B), both solvents acidified with 0.1 % formic acid. The mobile phase flow rate was set 0.4 mL/min and the following gradient was used: 0-0.25 min 95 % A, 0.25-0.75 min 30 % A, 0.75-5.50 min 0 % A held 2 min, 7.50 – 7.60 min 95 % A (return to initial conditions) and held 2.40 min. Five µL of extract was injected into the LC system. The UPLC system was coupled to a Waters Quattro Premier XE Micromass tandem mass spectrometer that was operated in positive electrospray ionization mode (ESI+). The parameters of the mass spectrometer were as follows: electrospray source block and desolvation temperature 120 °C and 350 °C, respectively; argon collision gas flow 0.22 mL/min; cone and desolvation nitrogen gas flow 50 L/h and 800 L/h, respectively. A capillary voltage of 3.20 kV was used for all compounds. The cone voltage and collision energy were compound-dependent and optimized as such. Parent and daughter ions were selected in the multiple reaction monitoring (MRM) mode.

QA: Procedural blank samples were run with each batch of samples (usually 3 procedural blanks per 10 human hair samples). As a blank sample, 50 mg of human hair sample washed in methanol was used. For statistical analysis, the concentrations below LOQ_m (calculated as 3 times the standards deviation (SD) of the mean of the blank measurements or when non detects in blanks 3 times SD of the instrumental limit of detection – Table 1) was set to zero.

Results and discussion

In most of the samples PBDEs were not detected or below LOQ_m (LOQ_m for each PBDE congener - BDE-28, 47, 99, 100, 153, 154, 183, set as 0.8 ng/g). They are therefore not further discussed here. On the contrary, all investigated PFRs were found with high frequencies as shown on Figure 1. The highest detection frequency, namely 100 % (both, in hair of mothers and children) were found for EHDPP, TPhP, TiBP, TBEP, TnBP. TEHP was found in 93 % of the analyzed hair samples whereas TDCIPP was found in 92 % of them. Only TCP and TCEP have lower frequency of detection, 69 % and 21 %, respectively.

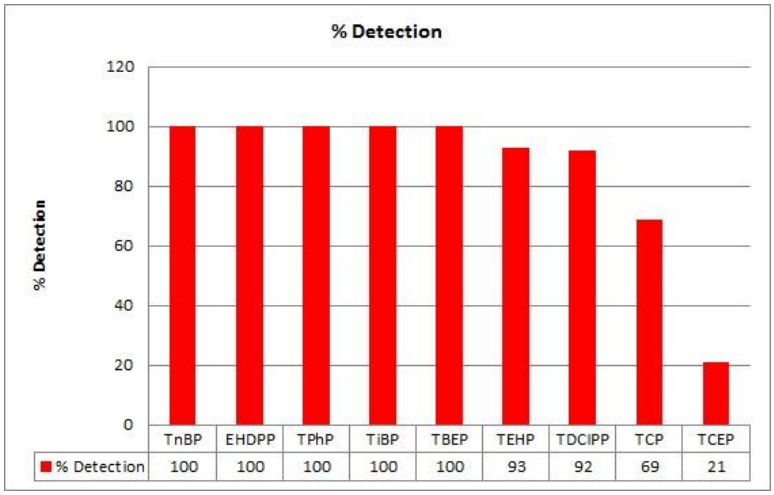

Figure 1. Percentage of detection of PFRs in human hair samples (mothers and children)

The hair concentration of PFRs in mothers and children were similar and in the range of 2 – 3744 ng/g. The integrated concentrations (from mothers and children) for each compound are shown in Table 1. TBEP, TnB, TPhP were the most abundant compounds in the investigated population. The chlorinated PFR, TDCIPP a suspected carcinogen[1], was present in the samples with high frequency at relatively high levels. Although we have not found any strong correlations between levels of PFRs in hair of mothers and the corresponding child/children, we observed the same profile of compounds in the hair samples.

Table 1. Levels of PFRs in human hair samples

	Levels of PFRs in Human Hair Samples (ng/g)								
	TnBP	**EHDPP**	**TPhP**	**TiBP**	**TBEP**	**TEHP**	**TDCIPP**	**TCP**	**TCEP**
Median	14	23	56	17	146	10	30	8	65
Min	3	2	5	5	14	2	9	2	35
Max	672	346	1256	82	2411	114	3744	134	163
LOQm	2	2	4	4	5	1	9	2	33

In addition, differences between median concentration of PFRs in hair from mothers and their children were evaluated by Man-Whitney test. This is the case for TiBP, TEHP, TnBP and TBEP. Remarkably, only TBEP level was higher for children, the others showed higher concentrations for the mothers.

In the present study, levels of PFRs in human hair are also compared with levels found in dust and air samples collected in the houses of the study population. Again, the same profile of compounds was observed in hair and air/dust. The frequency of detection of EHDPP, TPhP and TBEP was 100 % in human hair and dust samples (Figure 3). TnBP which is the most volatile compound among investigated PFRs was detected in only 58 % of the dust samples. Volatilization from the dust and rapid distribution is proven by higher detection of TnBP in the air samples (80 %)[7]. On the other hand, TnBP was detected in all of the human hair samples which demonstrates that using only dust sample for an exposure assessment of this particular compound, might lead to underestimations.

Furthermore, a number of statistically significant or suggestive associations between human hair samples and air/dust samples collected from the same households were found.

Figure 3. Comparison of detection of PFRs in hair, dust and air from the same households

Statistical analysis using non-parametric tests was performed showing moderate correlations between levels of TBEP in hair samples of mothers and children ($r = 0.428$, $p < 0.05$). Stronger correlations were observed between levels of TBEP in mothers' hair and dust ($r = 0.526$, $p < 0.05$) than children's hair and dust ($r = 0.414$, $p < 0.05$). The strongest and significant correlations were found between levels of TBEP in children hair and air ($r = 0.561$, $p < 0.05$). Further, moderate correlations were observed mainly between levels of EHDPP and TDCIPP in children hair and dust ($r = 0.355$, $r = 0.347$, respectively at $p = 0.05$). TnBP and TEHP showed correlation of 0.457 and 0.413 (at $p < 0.05$), respectively between levels in mothers hair and air. Furthermore, an interesting negative correlation between the living area 'in m^2 and TBEP levels in children hair was observed (- 0.348, $p < 0.05$). This could be explained by the fact that a larger area gives a greater dilution of compounds in the air and in consequence lower levels found in human hair samples.

We can conclude that hair might be a good indicator of exposure to PFRs and supplemental to dust or air, especially when we consider a general population with varying life style, living surroundings, habits, etc. In general, PFR levels in human hair are lower than in dust but they might reflect the most relevant pathways of exposure to the investigated compounds. The lack of strong correlations between levels of selected PFRs found in human hair and air/dust collected in the houses might be explained by the fact that during a day the humans are staying at many different places other than at home (work place, schools, cars etc.) Human hair samples might therefore be used as a passive sampler, "attached" to the human body 24h/day thus integrating the exposure from the environment. This approach might meaningfully contribute to human biomonitoring and exposure assessment.

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