Using hair for biomonitoring studies: analytical challenges to be addressed

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Introduction

Human biomonitoring is a scientific technique allowing to assess whether and to what extent various environmental chemicals have entered human bodies and how exposure may be changing over time. Under certain conditions potential health risks might be identified. Parent compounds, their metabolites and/or other ´markers´ are measured for this purpose in a suitable biological fluid such as blood, urine, and breast milk, or tissues, hair, nails, fat, and bone have been occasionally used. As regards the reporting value of obtained data, blood (or component of, such as plasma), might seem to be an ideal matrices due to its contact with different organs and tissues, however, the occurrence of targeted contaminants and/or their forms is highly dynamic, thus, the discovery of robust biomarkers is rather limited. In addition to this limitation, blood collection has several other drawbacks including complications such risk of hematomas and pain, moreover, an invasive sampling might be rather difficult in some population groups represented e.g. by children, elderly, or chronically ill people. For these reasons, the interest in samples obtained by a non-invasive way mainly represented by hair has been increasing. Among advantages associated with these matrices, easiness and cost effectiveness of sampling together with ethical acceptability (medical staff´s support not required) are the most important. Contrary to biotic fluids and some tissues, no special storage conditions are needed, hair samples are stable under ambient temperature for a long time period, since all the components of interest are well preserved. The memory-effect of hair, due to accumulation of chemicals in this matrix and possibility of retrospective analysis, also account for popularity of its application in solution of various forensic scenarios such as drug-facilitated crime evidence or assessment of the drug history consumption in addiction treatment. Moreover, hair can provide information about short-term and long-term exposure (months or even years), which is not always possible for biological fluids (blood, plasma, urine) [**1,2,3**].

One of the most recurrent criticisms concerning hair analysis lies is a possibility of external deposition of chemicals on a hair surface. Chemicals concentration in hair is interpreted as representing internal doses that an individual undergone during the period in which the hair sample has grown. Although the biological mechanisms of respective substances incorporation into hair are still debated, it is admitted that they are mainly transferred from blood into living cells in the hair bulb [**4**]. It is generally believed that these biologically incorporated molecules are located inside hair shafts (cortex and medulla), while external contamination is likely to remain on the surface of hair scales (i.e. cuticle). Nevertheless, no standardized ´versatile´ procedure for the ´decontamination´ of hair surface before analysis has been set up to date, in most studies the individual approaches are selected with the respect to the target analytes.

The main aim of the present study was to implement and validate the multi-analyte methods enabling quantitative analysis of multiple contaminants occurring in hairs collected within the human biomonitoring study.

The targeted compounds were persistent perfluoroalkylated substances (PFASs), metabolites of phthalates, monohydroxylated metabolites of polycyclic aromatic hydrocarbons (OH-PAHs) and residues of ´modern´ pesticides. For analysis, ultra-high performance liquid chromatography coupled to tandem mass spectrometry (MS/MS) with triple quadrupole mass analyser was used, for non-target fingerprinting, high resolution mass spectrometry (HRMS) was employed. Extensive experiments focused on a hair sample preparation prior to target / non-target analysis were realized, special attention was paid to a selection of suitable solvent for a washing step aimed the removal of possible external contamination. Fingerprints of small molecules (less than 1,200 Da) isolated from hair strands by washing solvents were compared with those obtained from the analysis of extracted disintegrated washed hairs with the aim to assess extractability of various components.

Materials and method

Analytical standards and chemicals: High purity pesticide standards were supplied by Dr. Ehrenstorfer GmbH (Augsburg, Germany) and Sigma–Aldrich (Taufkirchen, Germany. Standards of PFASs were obtained from Wellington Laboratories (Ontario, Canada). Standards of OH-PAHs and metabolites of phthalates were supplies by Toronto Research Chemicals (Toronto, Canada). Acetonitrile, analytical grade ethyl-acetate for pesticide residue analysis, formic acid (99%) and HPLC grade ammonium formate (99.99%) were supplied from Sigma-Aldrich (Taufkirchen, Germany). Methanol for LC-MS was obtained from Honeywell (Germany) and iso-propanol from Merck (Darmstadt, Germany). Anhydrous magnesium sulphate ($MgSO_4$) was from Fluka (Buchs, Germany) and sodium chloride (NaCl) was obtained from Penta (Chrudim, Czech Republic). Water purified by a Milli-Q® Integral system supplied by Merck was used throughout the study.

Samples: Human hairs were collected from volunteers of general population living in Prague, Czech Republic. Hair samples (0.3-1.0 g) were cut as closely to the scalp as possible, wrapped in aluminium foil, placed into paper envelops, and stored in the dark at room temperature. Before analysis, the hair strand was washed by various solvents, then was air-dried at room temperature and cut into small pieces (< 1 mm) with scissors. A part of the samples was homogenized by liquid nitrogen to get fine particles with a high surface area, enabling more effective extraction.

Extraction procedure: Three extraction solvents, water, acetonitrile and ethyl-acetate, were tested for the removing of external contamination of hair during the washing step. Approximately 300 mg of hair sample were sonicated with 20 ml of each solvent. After removing of hair, the acetonitrile and ethyl-acetate were evaporated to dryness and reconstituted in 200 µl of acetonitrile. In the case washing by water, QuEChERS like-extraction followed (analytes after adding inorganic salts transferred into 15 ml acetonitrile. After centrifugation (5 min, 10 000 rpm), 12 ml of upper layer was placed into flask, evaporated to the dryness and reconstituted in 200 µl of acetonitrile. These extracts were used for target analysis. For the non-target, fingerprinting analysis, 1500 mg of hair strand was successively washed using a series of solvents with a decreasing polarity: water : methanol (1:1, *v/v*), methanol, ethyl-acetate, each washing by particular solvent (40 ml) was performed two times. Hairs after final washing were cooled down to -80 °C and powdered. Then, successive extraction procedure was repeated.

Instrumental analysis: The target analysis of pesticides residues was performed using an Acquity Ultra-Performance LC system (Waters, USA). Analytes were separated on an Acquity UPLC HSS T3 analytical column (100 mm × 2.1

mm i.d., 1.8 µm particle size, Waters). The mobile phase consisted of (A) 5 mM ammonium formate and 0.1% formic acid in Milli-Q water and (B) 5 mM ammonium formate and 0.1% formic acid in methanol. The U-HPLC system was coupled to a triple quadrupole mass spectrometer Xevo TQ-S (Waters, USA) with electrospray ionization in both positive and negative ionization modes (ESI+/ESI-). The instrument was operated in MRM mode. Regarding the non-targeted fingerprinting, sample separation was performed on Acquity BEH C18 column (100 mm × 2.1 mm i.d., 1.7 µm particle size, Waters) column. The mobile phase consisted of (A) 5 mM ammonium formate and 0.1% formic acid in water:methanol mixture (95:5, *v/v*) and (B) 5 mM ammonium formate and 0.1% formic acid in iso-propanol:methanol:water mixture (65:30:5, *v/v*). The instrument was an Agilent 1290 Infinity LC system coupled to 6560 Ion Mobility Q-TOF (Agilent Technologies, USA).

Results and discussion

In the first part of study, a set of hair samples was screened for 402 pesticide residues; in total, 56 representatives of various classes were detected and quantified in preliminary washed hairs. As expected, a considerable variability was observed among individual samples, the concentrations of detected residues ranged from 0.1-300 ng/g of hairs. Benzyl alkonium chlorides (BACs) with various even-numbered alkyl chains (C8-C18) and dialkyl dimethyl ammonium chlorides (DDACs), compounds which are mainly used as antimicrobials and disinfectants, were found at highest levels. Based on the obtained data, comparing the amount of these substances transferred to washing solvent (external contamination) and then isolated from the homogenized hair sample (due to the better accessibility of internal part of hairs, isolation of incorporated compounds is expected), the need for follow up studies explaining distribution of these compounds was clearly documented. In general, the washing by water showed a less extensive removal of pesticides deposit on hair surface, and consequently, larger spectrum of residues was detected in hair extract. However, there is a question, if using of less polar methanol-water, methanol and ethyl-acetate would penetrate into internal parts of hair thus extracting also incorporated residues. In any case, other relatively abundant pesticides that were detected, imazalil, azoxystrobin and thiabendazol, i.e. fungicides commonly occurring in food commodities, probably indicated dietary exposure.

In addition to pesticide residues, also priority pollutants represented by PFASs were screened together with metabolites of phthalates and OH-PAHs. The investigation of metabolites of parent compounds represented one of possibilities how to exclude the possibility that the detected contaminant is due to a potential external contamination. This is because metabolites produced during biological processes and supposed to be absent in the external environment. Using described extraction procedure, a number of typical primary metabolites of phathalate diesters, monoethyl phthalate (MEP), mono-isobutyl phthalate (MiBP), mono-n-butyl phthalate (MnBP) and mono-2-ethylhexyl phthalate (MEHP) were detected, what might indicate internal intake. No other targeted contaminants were found in the current study, therefore, the possibility to process a higher sample amount and to miniaturize the analytical system will be searched to further improve limits of detection. As mentioned above, in some cases, it is rather difficult to recognize contamination pathway. Therefore, in the next phase, extensive washing experiments were performed, employing various solvents differing in polarity: methanol-water (1:1, *v/v*), methanol, and ethyl-acetate. The aim was to evaluate their effectiveness in removal of compounds located at the external part of hair coticule. The non-targeted analysis of washed and unwashed hairs was assumed to provide relevant information in

this context. The U-HPLC-HRMS fingerprints obtained on all the washing solvents showed that large number of compounds, dominating by lipids, was extracted in each washing step. An example of fingerprints of methanolic extract from not disintegrated hair strand and hairs that were extracted by the same solvent after a thorough washing and powdering is illustrated in **Figure 1**. As shown here, most of lipids were extracted during washing process before hairs disintegration, (the number of ´features´ i.e. detected ions was almost 8,000), the spectrum of lipids extracted from washed powdered hairs was rather different (also the number of features was lower, less than 4,000). The results obtained in this study not only documented how complicated matrix hairs are when considered for biomonitoring study, but also suggestions for further challenging research in the field of sample preparation were addressed.

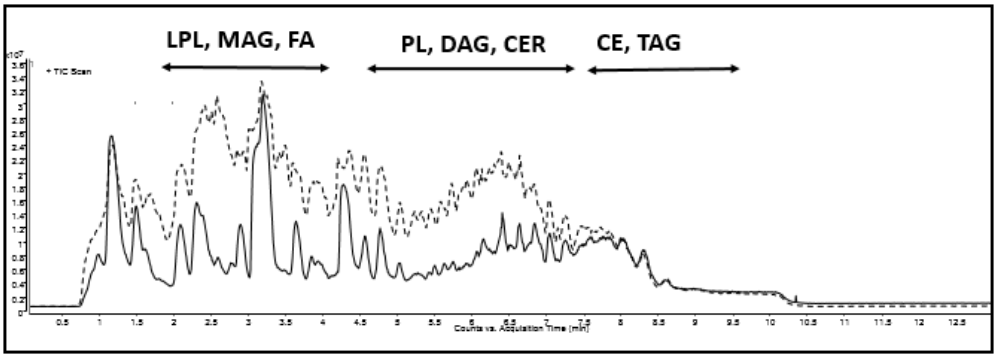

Figure 1. UPLC-HRMS fingerprints of extracts obtained by second washing of hairs by methanol (dotted line) and second extraction of washed powdered hairs by methanol (full line); elution zones corresponding to: LPL - lysophospholipids, MAG - monoacylglycerols, FA - fatty acids, PL - phospholipids, DAG - diacylglycerols, CER - ceramides, CE - cholesterol esters, TAG - triacylglycerols)

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