A new analytical framework for verification of biomarkers of exposure to chemicals combining human biomonitoring and water fingerprinting

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Abstract

Molecular epidemiology approaches in human biomonitoring are powerful tools that allow for verification of public exposure to chemical substances. Unfortunately, due to logistical difficulties and high cost, they tend to evaluate small study groups and as a result might not provide comprehensive large scale community-wide exposure data. Urban water fingerprinting provides a timely alternative to traditional approaches. It can revolutionise the human exposure studies as urban water represents collective community-wide exposure. Knowledge of characteristic biomarkers of exposure to specific chemicals is key to the successful application of water fingerprinting. This study aims to introduce a novel conceptual analytical framework for identification of biomarkers of public exposure to chemicals via combined human metabolism and urban water fingerprinting assay. This framework consists of: Step 1 - In vitro HLM/S9 assay; Step 2 – In vivo pooled urine assay; Step 3 - In vivo wastewater fingerprinting assay; Step 4 - Analysis with HR-MSMS; Step 5 - Data processing and Step 6 - Selection of biomarkers. The framework was applied and validated for PCMC (4-chloro-m-cresol), household derived antimicrobial agent with no known exposure and human metabolism data. Four new metabolites of PCMC (hydroxylated, sulphated/hydroxylated, sulphated PCMC and PCMC glucuronide) were identified using the in vitro HLM/S9 assay. But only one metabolite, sulphated PCMC, was confirmed in wastewater and in urine. Therefore, our study confirms that water fingerprinting is a promising tool for biomarker selection and that *in vitro* HLM/S9 studies alone, although informative, do not provide high accuracy results. Our work also confirms, for the first time, human internal exposure to PCMC.

Introduction

Antimicrobials are extensively used as additives in a broad range of personal care and consumer products to preserve the integrity of the products against biological agents, although their effectiveness against the potential hazard has been questioned [1]. In particular, antimicrobials are added to soaps, cosmetics and disinfectants to protect against the growth of microorganisms, including bacteria, viruses and fungi. Some of these chemicals, their metabolites and/or their degradation products have been reported to be potentially bioaccumulative[2], endocrine disrupting[3], ecotoxic in aquatic ecosystems[4] and leading to microbial resistance[5,6]. However very little is known about actual human exposure to antimicrobials in personal care products and therefore about the possibility to cause long term health effects. Even though available information concerning the percutaneous absorption of antimicrobials in humans is still scarce, it is known that some of them can be absorbed through the skin[7], suggesting that exposure results mostly from topical application of personal care products. However, ingestion of contaminated food and water [8,9] and inhalation of indoor dust[10] represent other important indirect/environmental sources of exposure. Antimicrobials can be metabolised in humans followed by excretion of parent compound and their metabolites primarily with urine. Because the presence of those compounds in blood, serum and urine has been demonstrated[11–15] and their environmental persistence and widespread use documented, it is unsurprising that they can be found in wastewater and in the receiving environment[16,17]. Their omnipresence, potential for bioaccumulation and possible synergistic effects of mixtures have raised public concern regarding their possible effects on human health as well as their role in the development of antimicrobial resistance[18]. There is therefore the need to consider a greater range of factors contributing to potential health effects of combined exposures within the risk assessment process. Risk assessment of mixtures is known to be difficult due to complexity of contributing factors when compared to the assessment of single chemicals[19]. New approaches towards

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risk assessment and evaluation of public exposure to antimicrobial agents in personal care products are therefore critically needed.

By comparing community levels of environmental stressors (both external and internal) with observed health effects, conclusions could be drawn as to whether elevated levels of certain chemicals could be linked with particular diseases. Such epidemiological studies are currently being undertaken via traditional approaches which use simple tools including case histories, questionnaires, or molecular epidemiology, which combines the above with sensitive laboratory techniques. These approaches monitor biological responses, rather than diseases in human populations through the usage of biomarkers[20]. However, a limitation of molecular epidemiology, due to logistical difficulties and high cost, is the restricted size of study groups and inability to gather comprehensive information on the complexity of combined (and cumulative) exposure to mixtures of chemicals and their effects. Therefore the community lacks robust measures that can be used to gather real-time information on community-wide health.

Urban water fingerprinting for human metabolic biomarkers is a new approach in epidemiological exposure studies that can revolutionise the way we estimate public exposure to chemicals. This approach is also known wastewater based epidemiology (WBE). WBE is a new concept that aims to overcome the above limitations and to provide spatial and temporal near-real time estimation of community-wide exposure to wide range of chemicals. This unique approach assumes that epidemiological information can be retrieved from wastewater via the analysis of human metabolic biomarkers. Although still in its infancy, it is currently used to determine illicit drug use trends at the community level through the analysis of urinary biomarkers in wastewater[21–23]. This approach can be also extended to make a real time assessment of population health status[24]. WBE postulates that specific human metabolic biomarkers (e.g. characteristic metabolites of toxicants or pollutants) excreted with urine and faeces, and resulting from exposure to certain chemicals, are pooled by the urban wastewater system providing evidence of the amount and type of toxicants or pollutants to which a population contributing to the analysed water, has been exposed. Urban water fingerprinting can therefore provide anonymous and comprehensive estimation of the community-wide health status in near-real time.

The selection of unique metabolic biomarkers that are characteristic for each individual chemical and route of exposure is a critical step in order to verify public exposure to these chemicals via WBE, e.g. in order to distinguish between internal and external exposure, and to account for direct disposal, since many sources contribute to chemicals being discharged into wastewater. Unfortunately, in the case of many chemicals, especially those that are not intended for human consumption (e.g. antimicrobials), there is no public knowledge of characteristic metabolic biomarkers that could be utilised in WBE. Nevertheless, due to their extensive use in personal care and consumer products[25] dermal absorption is considered to be one of the main routes of human exposure. Understanding toxicokinetic process, including metabolism, is therefore crucial in the determination of toxicological effects and potential for bioaccumulation of these chemicals, as well as in the identification of biomarkers of exposure. Still, there are only a few studies which reported their *in vivo* or *in vitro* biotransformation. Wu, Liu and Cai (2010)[15] investigated the metabolism of triclosan *in vivo* and *in vitro*. They observed both oxidative and phase II metabolites and identified glucuronidated triclosan as the major metabolite. Schebb et al. (2011)[25] reported that the 0.6% circa of the amount of triclocarban present in bar soaps (70 ± 15 mg) was absorbed through the skin and that the 25% of total amount was excreted in urine almost exclusively as N-glucuronides. Unfortunately, most antimicrobials still remain hardly investigated.

We are proposing a novel conceptual framework for identification of metabolic biomarkers via combined human metabolism and urban water fingerprinting assays. In this study, we identified, for the first time, human specific metabolites of the antimicrobial agent, 4-chloro-3-methylphenol (PCMC), as potential biomarkers of community-wide exposure to PCMC via WBE. This antimicrobial agent, also known as 4-chloro-*m*-cresol, is a phenolic compound that has been proven to have an estrogenic activity determined by an *in vitro* yeast bioassay[26]. PCMC is also known to have an effect on Ca^{2+} homeostasis being a strong activator of the ryanodine receptors in the endoplasmic reticulum[27] and to interfere with the thyroid hormone functions[28]. To the authors' knowledge, there is no published data on metabolic pathways of PCMC in humans.

Experimental section

Reagents and analytical standards

Pooled human liver microsomes (HLM), S9 fraction pooled from human liver, β-nicotinamide adenine dinucleotide 2′-phosphate reduced (β-NADPH ≥ 95%), Uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA 98-100%), alamethicin from *Trichoderma viride* (≥ 98%), 3′-phosphoadenosine 5′-phosphosulphate lithium salt (PAPS ≥ 60%), 4-chloro-3-methylphenol (p-chlorocresol), potassium phosphate monobasic tetrasodium salt hydrate (KH_2PO_4), magnesium chloride hexahydrate ($MgCl_2$), were purchased from Sigma-Aldrich (Gilligam,UK). The internal standard: 4-chloro-3-methylphenol-2,6-d2, was purchased from QMX Laboratories Ltd.

Solvents were of HPLC purity and were purchased from Sigma-Aldrich (Gilligam, UK). Stock standard solutions were prepared in methanol and stored in the dark at -20°C. 24h volume-proportional (100 mL every 15 minutes) composite wastewater influent samples were collected in PTFE bottles from a local wastewater treatment plant (WWTP) serving 70000 inhabitants on the 8th of June 2015. They were then transported to the laboratory in cool boxes packed with ice blocks and filtered through GF/F 0.7 µm glass fibre filter (Whatman, UK).

In vitro assays for verification of metabolic profile of PCMC in humans

Two *in vitro* assays were selected in this study: HLM and combined HLM and S9 fraction. Currently HLM represents the most commonly used *in vitro* model, providing an affordable way to give a good indication of the cytochrome P450 (CYP) and uridine 5'-diphospho-glucuronosyltransferase (UGT) metabolic profile [29]. Unfortunately, the absence of other enzymes such as N-acetyltransferase (NAT), glutathione S-transferase (GST) and sulphotransferase (ST) implies, as a result, an incomplete range of metabolites being formed. A valid alternative to the use of HLM is the liver S9 fraction which contains both microsomal and cytosolic fractions (phase I and phase II metabolic enzymes) that lead to the formation of a range of metabolites giving, as a result, more representative metabolic profile when compared to HLM only. However, the overall amount of metabolites formed is lower due to lower enzyme activity in the S9 fraction when compared to microsomes. This might result in minor metabolites to remain unnoticed[30]. Therefore, in this paper, method development included different subcellular fractions (HLM and a combination of HLM and S9 fraction).

In vitro HLM assay for verification of metabolic profile of PCMC. 10 µL of a phosphate buffer (50mM KH_2PO_4, pH 7.4, 5mM $MgCl_2$), 10 µL of analyte solution (50 µM) were mixed with 10 µL human liver microsomes spiked with 1 µL of an alamethicin solution 12.5 mg/mL and 10 µL of a 100µM UDPGA solution. The reaction was initiated by addition of 10 µL of a 10 mM NADPH solution followed by incubation at 37°C for 1.5 h. After 1.5 h of incubation 10 µL of a 100µM PAPS solution were added and the incubation continued under the same conditions for 1.5 h. The negative controls with either no analyte or no HLM were incubated as described above to exclude all the non-enzymatic reactions. Each specific incubation was performed in duplicate. The reaction was quenched with 100 µL of acetonitrile ice cold, followed by centrifugation at 10000 rpm for 10 min (Centrifuge 5418, Eppendorf). The supernatant was removed and transferred to a new eppendorf tube and gently dried down by a stream of nitrogen at 40°C using TurboVap evaporator (Caliper, UK). The resulting residue was reconstituted with 50 µL of a 80:20 H_2O:MeOH solution containing the internal standard (100 ng/mLM) and transferred into a polypropylene vial for analysis.

All analyses were undertaken using a Dionex Ultimate 3000 HPLC (Thermo Fisher UK Ltd.) coupled with a Bruker Maxis HD Q-TOF (Bruker) equipped with an electrospray ionization source. Nitrogen was used as nebulising gas at a flow rate of 11 L/min at a temperature of 220°C and at a pressure of 3 Bar. Capillary voltage was set at 4500 V and End Plate offset was set at 500 V. The analyses were performed in both positive and negative modes and acquisition was performed in both full scan mode (MS) and broadband CID acquisition mode (MS/MS). HyStar™ Bruker was used to coordinate the LC-MS system. Chromatographic separation of the metabolites formed was achieved by using a WATERS ACQUITY UPLC BEH C18 column (50 mm x 2.1 mm, 1.7 µm) and the following mobile phase composition: 1 mM ammonium fluoride in water (A) and methanol (B). The gradient elution both in

ESI positive and negative mode was as follows: 5% B (0 -3 min) - 60% B (3 - 4 min) - 60% B (4 -14 min), - 98% B (14 - 17 min) - 5% (17.1 - 20 min). The flow rate was kept constant at 0.4 ml/min and the column temperature was set at 40 °C. The source and operating parameters were optimized as follows: capillary voltage, 4500 V; dry gas temperature, 220 °C (N_2); dry gas flow 12 L h^{-1} (N_2); quadrupole collision energy, 4 eV; collision energy, 7 eV MS (full-scan analysis) and 20 eV MS/MS (bbCID mode). Nitrogen was used as the nebulising, desolvation and collision gas. The method was fully quantitatively validated for PCMC (intra-day, accuracy 120.2%, precision 2.4%; inter-day, accuracy 120.2%, precision 3.5%; IQL, 22 ng/L; IDL, 6.6 ng/L; linearity range, 0.07-27.5 mg/mL; R^2 0.9987; MDL, 0.013 ng/L; MQL, 0.045 ng/L).

In vitro combined HLM/S9 fraction assay for verification of metabolic profile of PCMC Two incubation mixtures were prepared in duplicate by mixing 10 µL of phosphate buffer (50mM KH_2PO_4, pH 7.4, 5mM $MgCl_2$), 10 µL of analyte solution (50µM), 10 µL of the100µM UDPGA solution and 10 µL of HLM spiked with 1 µL of an alamethicin solution 12.5 mg/mL. The reaction was initiated by addition of 10 µL of a 10 mM NADPH solution followed by incubation at 37°C. The incubation was carried out for 3 h under the same conditions for three of the four samples. At 3 h 10 µL of S9 fraction and 10 µL the 100µM PAPS solution were added to the samples to be incubated for six h and incubation was continued. The negative controls with either no analyte or no enzymes were prepared as well for each time point. After quenching the reaction with 100 µL of acetonitrile ice cold, samples were prepared for analysis as described above.

In vivo pooled urine assay

Seven pooled urine samples were collected from a UK festival event. They came from five different urinals sampled on three different days. Solid phase extraction (SPE) was performed on pooled urine samples using HLB Oasis® cartridges Water, UK) to reduce the matrix effect and to concentrate each sample by 4-fold. SPE procedure was as follows: 2 mL of pooled urine were loaded onto Oasis HLB cartridges, which were preconditioned with 2 mL MeOH followed by 2 mL H_2O. After loading, the cartridges were dried for 30 min and analytes were eluted with 4 mL MeOH. Extracts were then dried under a gentle nitrogen stream using a TurboVap evaporator (Caliper, UK, 40◦C). Dry extract was then reconstituted in 500 µL 80:20 H_2O:MeOH, transferred to polypropylene vials and analysed using Dionex Ultimate 3000 HPLC coupled with a Bruker Maxis HD Q-TOF according to the procedure described above.

Wastewater fingerprinting assay

Raw wastewater samples collected from local wastewater treatment works, were filtered using GF/F glass microfibre filter 0.75 µm (Fisher Scientific, UK) followed by a solid phase extraction (SPE) using HLB Oasis® cartridges Water, UK) to reduce the matrix effect and to concentrate each sample by 400-fold. SPE procedure was as follows: 100 mL of filtered wastewater were loaded onto Oasis HLB cartridges, which were preconditioned with 2 mL MeOH followed by 2 mL H_2O. After loading, the cartridges were dried for 30 min and analytes were eluted with 4 mL MeOH. Extracts were then dried under a gentle nitrogen stream using a TurboVap evaporator (Caliper, UK, 40◦C). Dry extract was then reconstituted in 250 µL 80:20 H_2O:MeOH, transferred to polypropylene vials and analysed using Dionex Ultimate 3000 HPLC coupled with a Bruker Maxis HD Q-TOF according to the procedure described above.

After analysis, data extracted from the Bruker system were processed with MetID software (Advanced Chemistry Development, Inc., ACD/Labs, UK) in order to predict metabolite structures. However, the software predicts a large number of possible metabolites, of which a rather small number is actually observed in *in vitro* experiments. We therefore developed a systematic workflow as presented in Figure 1 to limit false positive measurements.

Results and discussion

In vitro assays

The *in vitro* metabolism of PCMC catalysed by CYP and SULT enzymes has been investigated using a combination of pooled HLM an S9 fraction tests. Hydroxylation of un-substituted carbon atoms was expected to be the major biotrasformation reaction catalysed by CYPs whilst conjugations with phase II cofactors were expected to be the major reactions catalysed by UGT and ST. Phase II conjugations were expected to occur directly or following mono- and/or di-hydroxylation phase-I biotransformations.

In vitro HLM assay. After incubating PCMC with HLM a number of peaks were detected using LCMS. Initial analysis of samples, performed using ACDLabs software, identified two potential metabolites. A representative extracted ion chromatogram (XIC) of PCMC metabolites detected are reported in Figs. S1 and S2. All samples were analysed in negative and in positive ionisation modes. However, all the potential metabolites had better intensity in the negative ionization mode.

Incubation of PCMC produced a metabolite (m/z 157.0057) with elemental composition of the deprotonated molecule denoting C7H6ClO- (-3.6 ppm mass error) and a second one (m/z 317.0422) with elemental composition of the deprotonated molecule denoting C13H14ClO7- (-3.8 ppm mass error). ACDLabs analysis led to their identification as mono-hydroxylated metabolite (Fig S1b) and glucuronide conjugated (Fig. S2b). PCMC hydroxylate did not provide a distinctive fragmentation pattern in bbCID mode which necessitated MS/MS analysis. Fragmentation of ions with m/z 157.0062 +/- 0.005 at 31 eV led to the formation of a fragment 121.0284 which corresponded with the loss of a chlorine moiety from the precursor ion (Fig. S1c). PCMC glucoronate instead produced in bbCID mode a fragment ion at m/z 141.0108 (C7H6ClO-, + 3.5 ppm mass error) that was assigned to [$C_6H_8O_6$] loss, and was related to the presence of a glucuronate group (Fig. S2c, bottom). The fragments obtained confirmed the chemical structure of the metabolites. Additionally, two chlorine isotope peaks at m/z 158.0086 and m/z 159.0024 (Fig. S1d) and at m/z 318.0452 and m/z 319.0390 (Fig. S2d) were observed. The peaks had small mass errors (<5 ppm) and their relative heights match those expected from a compound with one chlorine within 5% of the predicted abundance.

PCMC metabolites have not been previously documented in literature, therefore the results of this study are of considerate importance. However, sulphate metabolites that were initially thought to be suitable as a biomarker were not detected in the *in-vitro* HLM assay. This could be due to two main factors. Firstly, the incubation time may not have been sufficiently long to allow detectable amounts of metabolites to be formed, as well as also not allowing the higher number of metabolites to be produced. Secondly this could be due to the lack of phase II enzymes being used such as sulphotransferases, of which HLM are deficient. To account for this, HLM/S9 fraction assay was undertaken (see below).

In vitro combined HLM/S9 fraction assay. The *in vitro* combined HLM/S9 fraction assay included verification of quantitative and qualitative changes of metabolic profile in two time intervals (3 and 6 h). Moreover, due to the addition of the S9 fraction to the incubation mixture, further metabolites (sulphate conjugated) were expected to be produced. Indeed, an incubation of PCMC with pooled HLM/S9 fraction produced two further metabolites: sulphated PCMC and mono-hydroxylated sulphated PCMC (Fig. 2 and S3).

It can be seen in Fig. 2 that the *in vitro* test leads to the formation of a metabolite with retention time denoting 6.4 min (Fig. 2b, dark peak). This chromatographic peak was absent in the blank control (Fig. 2a). Spectral analysis performed using ACDLabs software identified the compound to be a sulphated metabolite (m/z 220.9684). Elemental composition of the deprotonated molecule of the sulphated metabolite was assigned as C7H6ClO4S- (+ 1.3 ppm mass error). The fragment ion at m/z 141.0117 (C7H6ClO-, + 3.6 ppm mass error) was assigned to [O_3S] loss, and was related to the presence of a sulphate group (Fig. 2c, bottom). To further confirm that the fragment ion originates from the suspected metabolite its chromatogram was extracted. The resulting XIC produced a peak whose elution time matched perfectly with that of the suspected metabolite (Fig. 2b, light peak). Additionally, the presence of two chlorine isotope peaks at m/z 221.9713 and m/z 222.9653 (Fig. 2d) was observed. The peaks

had small mass errors <5 ppm and their relative heights match those expected from a compound with one chlorine within 5% of the predicted abundance.

The *in vitro* HLM/S9 fraction assay led to the formation of another PCMC metabolite with retention time of 6.3 min (Fig S3b, dark peak). This is the same chromatographic peak that was absent in the blank control (Fig. S3a). Spectral analysis performed using ACDLabs software identified the compound to be the sulphated and hydroxylated metabolite (m/z 236.9632). Elemental composition of the deprotonated molecule of the metabolite was assigned as C7H6ClO5S- (+ 1.3 ppm mass error). The fragment ion at m/z 157.0065 (C7H6ClO2-, + 1.9 ppm mass error) was assigned to [O_3S] loss, and was related, as previously, to the presence of a sulphate group (Fig. S3c, bottom). To further confirm that the fragment ion originates from the suspected metabolite its chromatogram was extracted. The resulting XIC produced a peak whose elution time matched perfectly with that of the suspected metabolite (Fig. S3b, light peak). Also, as above, two chlorine isotope peaks at m/z 237.9664 and m/z 238.9601 (Fig. S3d) were observed. The peaks had small mass errors <5 ppm and their relative heights matched those expected from a compound with one chlorine within 5% of the predicted abundance.

Phase II cofactor (PAPS) was added after 3 h to the incubation mixture to permit all the possible phase I metabolites to form before conjugation with sulphate took place. This approach attempts to replicate what happens in a living cell, where generally (but not necessarily) phase I minor biotransformations occur in preparation for successive phase II conjugation. Results are summarised in Fig. S4. It can be seen from Fig. S4 that hydroxylated metabolites are preferentially formed after 3 h of incubation time (88.7% against 11.3% conjugation with glucuronic acid). The hydroxylated PCMC was still the most abundant biotransformation product (40% of the total metabolites produced circa) after 6 h of incubation time, although at this sampling point phase II metabolites accounted for 59.8% of all the metabolites produced. In particular amongst the three phase II biotransformation observed after 6 h direct sulphation seemed to be the preferential conjugation route accounting for more than 25% of total biotransformation.

In summary, both HLM and HLM-S9 fraction assays allowed for the identification of metabolites that have not been previously documented in literature, although the latter assay allowed the identification of a higher number of metabolites due to the addition of the S9 fraction resulting in a more efficient sulphation. Moreover a two-step approach, which entails the addition of phase II enzymes and sulphation cofactor after 3 h permits the identification of all the phase I and II metabolites and conjugated metabolites, providing a wider range of biotransformation products. The formation of PCMC sulphate conjugates means also that a more efficient sulphate conjugation takes place in the HLM-S9 fraction assay, when compared to the HLM assay. All the identified metabolites are presented in Tab. 1. The table reports also elemental composition and the mass accuracy measured in the two *in vitro* assays and in a wastewater sample from a local wastewater treatment plant (WWTP) (see discussion below).

In vivo pooled urine assay

The *in vivo* pooled urine assay led to identification of only one metabolite of PCMC, sulphated PCMC (Tab. 1 and Fig. 3). Interestingly, hydroxylated and glucuronated metabolites were not observed in analysed pooled urine samples. This is in contrast with *in vitro* assays where glucuronated, sulphated and hydroxylated metabolites were identified.

In vivo wastewater fingerprinting assay

The aim of the two *in vitro* assays was to select potential biomarkers of exposure to PCMC. However, as the ultimate goal of this study was to verify community-wide exposure to these chemicals, analysis of untreated wastewater samples serving large community of 70 thousand people was undertaken. The identification of biomarkers was based on the systematic workflow presented in Fig. 1. The compounds detected in wastewater are summarised in Tab. 1. As expected, given the complexity of the matrix, mass accuracy measured was lower than that measured in *in vitro* studies but still within set limits, with mass error values between 5 and 10 ppm (Tab. 1).

In vivo wastewater fingerprinting assay resulted in the detection and identification of only one metabolite of PCMC, sulphated PCMC, in wastewater (Fig. 4). The loss of [O_3S] deduced by TOF MS spectra has been crucial for justifying and suggesting possible chemical structures. Interestingly, hydroxylated and glucuronated PCMC were not observed in analysed wastewater samples. This is in line with results obtained for *in vivo* pooled urine assay and it confirms that *in vitro* studies, although informative, cannot serve as the only tool intended for selection of biomarkers of exposure.

Conclusions

This study proved that combined human metabolism and wastewater fingerprinting assay is a powerful tool to investigate human exposure to chemicals present in personal care products and a wider-group of chemicals that are not intended for human consumption and therefore lack comprehensive risk assessment data. We have proposed a robust systematic workflow that enables fast and comprehensive selection of characteristic biomarkers of public exposure to chemical substances (Fig. 1). The workflow consists of several steps: Step 1: *In vitro* HLM/S9 assay; Step 2: *In vivo* pooled urine assay; Step 3: *In vivo* wastewater fingerprinting assay; Step 4: Analysis with HR-MSMS; Step 5: Data processing and Step 6: Selection of biomarkers. In Step 4, after the establishment of a list of suspected metabolites using ACDLab software (Step 4a), in order to avoid false positives, their accurate mass, retention time and fragmentation pattern are examined (Step 4b,c,d). Finally the structure of the suspects is confirmed by investigating the MS/MS fragmentation pattern in bbCID mode (Step 4e). For those metabolites that do not provide an optimal MS/MS fragmentation pattern in bbCID mode, a further confirmation step performing a data-dependent MS/MS acquisition is required (Step 4f), i.e. an MS/MS analysis is triggered if a compound from a target ion list is detected. In contrast to targeted screening, non-target screening starts without any a priori information on the compounds to be detected. However, this study falls in between these two categories, since the chemically meaningful structures which can be assigned to an unknown peak are limited to structures showing a close relationship with the parent compound.

Four new possible metabolites of PCMC (hydroxylated, glucuronidated, sulphated and hydroxylated & sulphated PCMC) were identified after *in vitro* HLM/S9 studies and were proposed as biomarkers of exposure. The absence of phase I metabolites in the presence of phase II cofactor PAPS suggested that sulphation was the preferential metabolic pathway for this compound. Only one of these metabolites (PCMC sulphated) was confirmed in wastewater and in urine suggesting human internal exposure to PCMC despite the fact that this compound is utilised in products meant for external use. Consequently to the results obtained in this present work it seems evident that the impact of the exposure to PCMC and other chemicals not intended for human consumption might need to be reconsidered. Also in a realistic overview of its impact on the aquatic ecosystem its identified metabolite should be also investigated to verify their potential environmental impact.

The aim of this paper was to introduce a new assay for identification of new metabolic biomarkers in WBE. Further work will be undertaken to verify utility of selected biomarkers in a large urban water catchment monitoring campaign.

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ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website

Supporting Information includes the following:

Figure S1 XIC of hydroxylated PCMC metabolite produced with HLM. XICs at m/z 157.0062 (0.005-Da mass-window width) for analyte-sample (b), blank control (a), fragmentation pattern of the metabolite obtained in MRM mode (c) and XIC at m/z 157.0049, 158.0079 and 159.0017 for PCMC and the two chlorine isotope peaks (top), and mass spectra (bottom).

Figure S2 Detection and identification of PCMC glucuronate metabolite by UHPLC-QTOF-MS following in-vitro HLM assay (3 hour time point). XICs at m/z 307.0646 and 227.1078 (0.005-Da mass-window width) for analyte-sample (b) and control-sample (a). (c) (top) Low-energy (full-scan analysis) and (bottom) high-energy (bbCID mode) spectra of the metabolite and fragment ion observed. (d) XIC at m/z 317.0422, 318.0452 and 319.0390 for PCMC glucoronate and the two chlorine isotope peaks (top), and mass spectra (bottom).

Figure S3 Detection and identification of sulphated and hydroxylated PCMC by UHPLC-QTOF-MS following in-vitro HLM/S9 assay. XICs at m/z 236.9630 and 157.0062 (0.005-Da mass-window width) for analyte-sample (b) and control-sample (a). (c) (top) Low-energy (full-scan analysis) and (bottom) high-energy (bbCID mode) spectra and structures of the metabolite and fragment ion observed. (d) XIC at m/z 236.9632, 237.9660 and 238.9601 for PCMC hydroxylate & sulphate and the two chlorine isotope peaks (top) and mass spectra (bottom).

Figure S4 Distribution of PCMC metabolites obtained with in-vitro HLM and HLM/S9 fraction assay over a 3 and 6 h incubation time.

Report 1 Detection and identification of PCMC metabolites by UHPLC-QTOF-MS following *in-vitro* HLM assay.

- Sample Name 4-Cl-3-Me_1_neg and PCMC_10ul_enz_B_Neg_2 XIC and mass spectrum of PCMC hydroxylated, PCMC glucuronidated, PCMC and relative isotopes following *in-vitro* HLM assay for verification of metabolic profile of PCMC.
- Sample Name 4-Cl-3-Me_2_neg and PCMC_10ul_enz_B_Neg_2 XIC and mass spectrum of PCMC hydroxylated, PCMC glucuronidated, PCMC and relative isotopes following *in-vitro* HLM assay for verification of metabolic profile of PCMC (duplicate sample)
- Sample Name 4-Cl-3-Me_Blank_neg and PCMC_blank_Neg XIC and mass spectrum of PCMC hydroxylated, PCMC glucuronidated, PCMC and relative isotopes following *in-vitro* HLM assay for verification of metabolic profile of PCMC (blank control)

Report 2 Detection and identification of PCMC metabolites by UHPLC-QTOF-MS following *in-vitro* HLM/S9 assay.

- Sample Name S9_4-Cl-3-Me_A_6_Hour_Neg and 4_Cl_6hA_Neg XIC and mass spectrum of PCMC hydroxylated, PCMC glucuronidated, PCMC sulfated, PCMC sulfated and hydroxylated, PCMC and relative isotopes (including bbCID fragmentation pattern for phase II metabolites), following *in-vitro* HLM/S9 assay (6 hour sampling point) for verification of metabolic profile of PCMC.
- Sample Name S9_4-Cl-3-Me_B_6_Hour_Neg and 4_Cl_6hB_Neg XIC and mass spectrum of PCMC hydroxylated, PCMC glucuronidated, PCMC sulfated, PCMC sulfated and hydroxylated, PCMC and relative isotopes (including bbCID fragmentation pattern for phase II metabolites), following *in-vitro* HLM/S9 assay (6 hour sampling point) for verification of metabolic profile of PCMC. (duplicate sample)
- Sample Name S9_4-Cl-3-Me_Blank_6_Hour_Neg and 4_Cl_6hBlank_Neg XIC and mass spectrum of PCMC hydroxylated, PCMC glucuronidated, PCMC sulfated, PCMC sulfated and hydroxylated, PCMC and relative isotopes (including bbCID fragmentation pattern for phase

II metabolites), following *in-vitro* HLM/S9 assay (6 hour sampling point) for verification of metabolic profile of PCMC. (blank control)

Report 3 Detection and identification of PCMC metabolite by UHPLC-QTOF-MS following urine analysis.

- Sample Name Urine_141_A neg XIC and mass spectrum of PCMC sulfated (including bbCID fragmentation pattern) and relative isotopes, following direct *in-vivo* urine profiling assay.
- Sample Name Urine_141_B neg XIC and mass spectrum of PCMC sulfated (including bbCID fragmentation pattern) and relative isotopes, following direct *in-vivo* urine profiling assay.

Report 4 Detection and identification of PCMC metabolites by UHPLC-QTOF-MS following wastewater analysis.

- Sample Name Inf day 1A neg XIC and mass spectrum of PCMC and PCMC sulphated (including bbCID fragmentation pattern) and relative isotopes.

Report 5 MRM fragmentation pattern of PCMC standard solution.

- Sample Name MRM_4Cl3MPox_Met2_STD_5 MRM fragmentation pattern of PCMC standard solution

References

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455 Table 1 PCMC and their metabolic biomarkers.

			In-vitro HLM assay		*In-vitro HLM/S9 fraction assay*		*In-vivo pooled urine assay*		*In-vivo wastewater fingerprinting assay*	
Compound	**Elemental composition $[M-H]^-$**	**Exact mass (m/z)**	**Peak top mass (m/z)**	**Mass error (ppm)**	**Peak top mass (m/z)**	**Mass error (ppm)**	**Peak top mass (m/z)**	**Mass error (ppm)**	**Peak top mass (m/z)**	**Mass error (ppm)**
PCMC	$C_7H_6ClO^-$	141.0113	141.0118	+3.6	141.0116	+2.1	-	-	141.0122	+6.0
PCMC hydroxylated	$C_7H_6ClO_2^-$	157.0062	157.0049	-8.2	157.0061	-0.6	-	-	-	-
PCMC glucuronidated	$C_{13}H_{15}ClO_7^-$	317.0434	317.0422	-3.8	317.0442	+2.5	-	-	-	-
PCMC sulphated	$C_7H_6ClO_4S^-$	220.9681	-	-	220.9684	+1.3	220.9670	- 5	220.9695	+6.4
PCMC hydroxylated & sulphated	$C_7H_6ClO_5S^-$	236.9630	-	-	236.9632	+0.9	-	-	-	-

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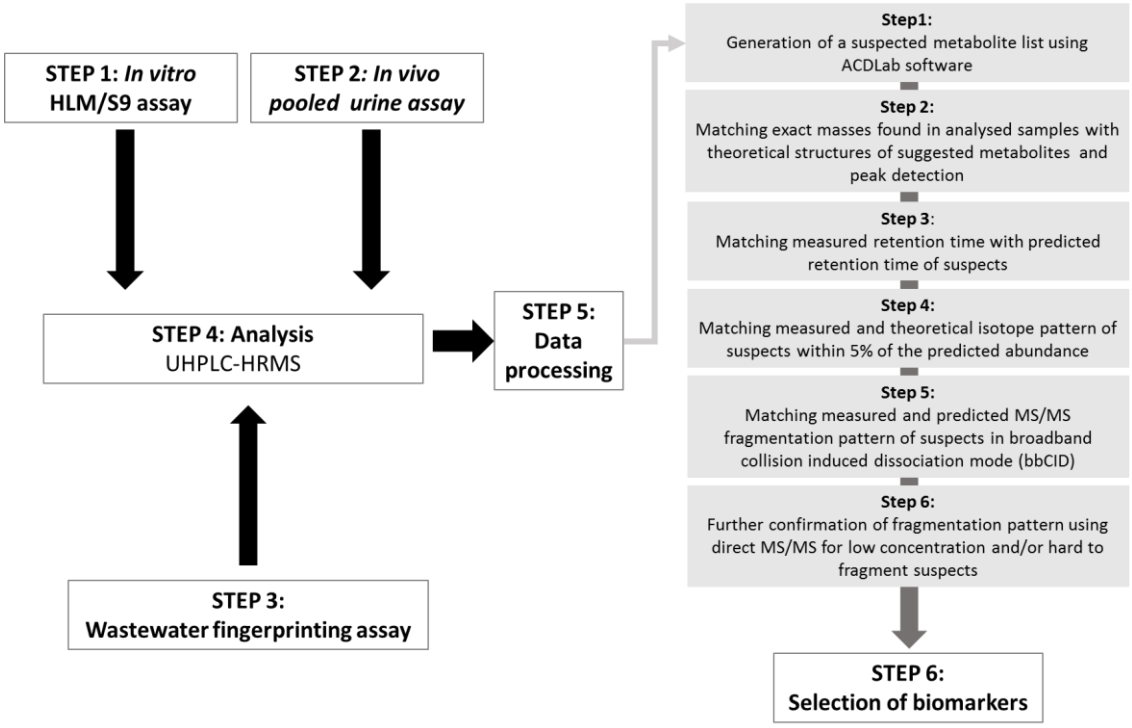

Figure 1 A systematic workflow for verifying human exposure to chemicals via combined *in-vitro* HLM/S9 and *in-vivo* pooled urine and wastewater profiling assay

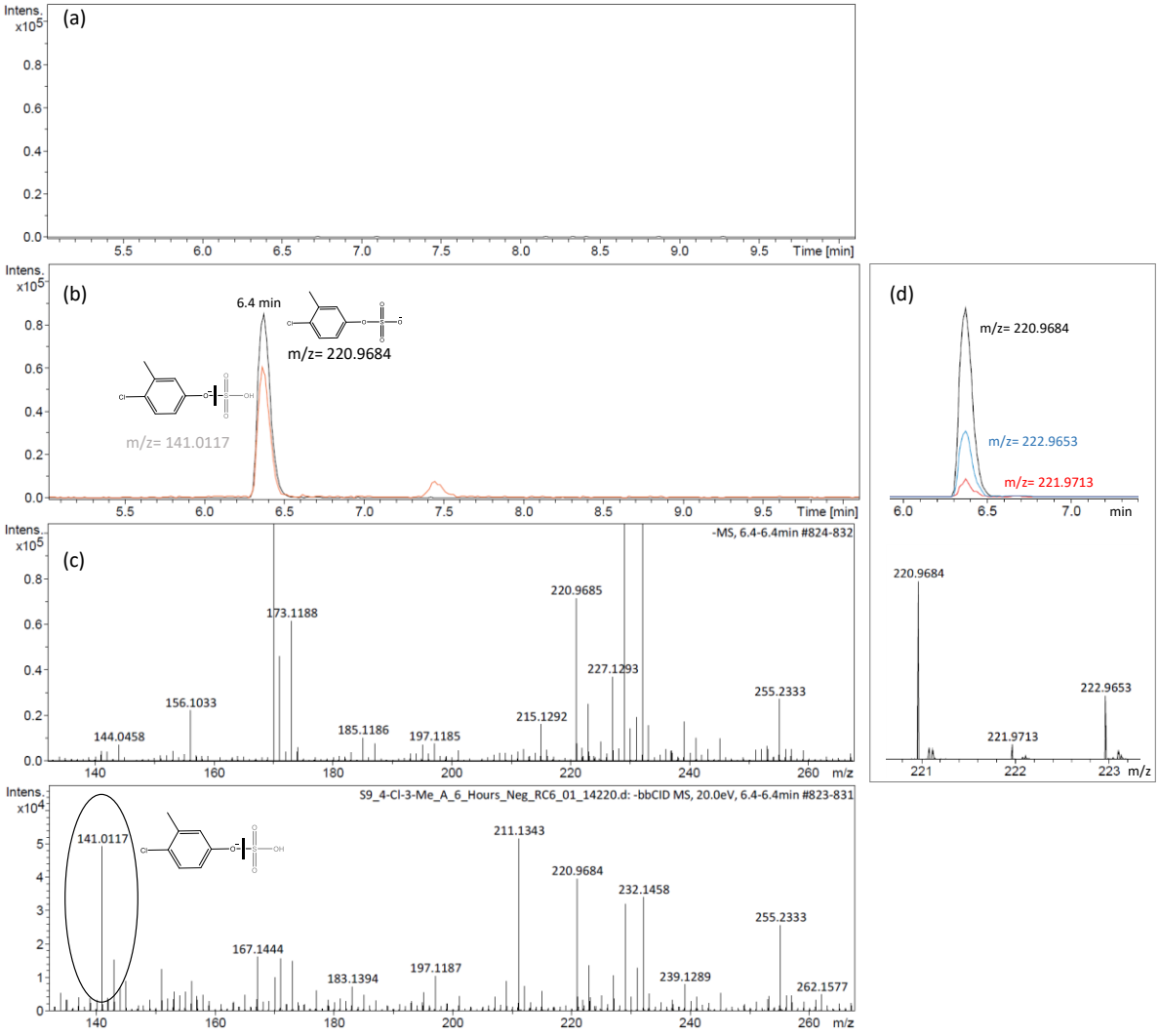

Figure 2 Detection and identification of sulphated PCMC by UHPLC-QTOF-MS following *in-vitro* HLM/S9 assay. XICs at m/z 220.9681 and 141.0113 (0.005-Da mass-window width) for analyte-sample (b) and control-sample (a). (c) (top) Low-energy (full-scan analysis) and (bottom) high-energy (bbCID mode) spectra and structures of the metabolite and fragment ion observed. (d) XIC at m/z 220.9684, 221.9713 and 222.9653 for PCMC sulphate and the two chlorine isotope peaks (top) and mass spectra (bottom).

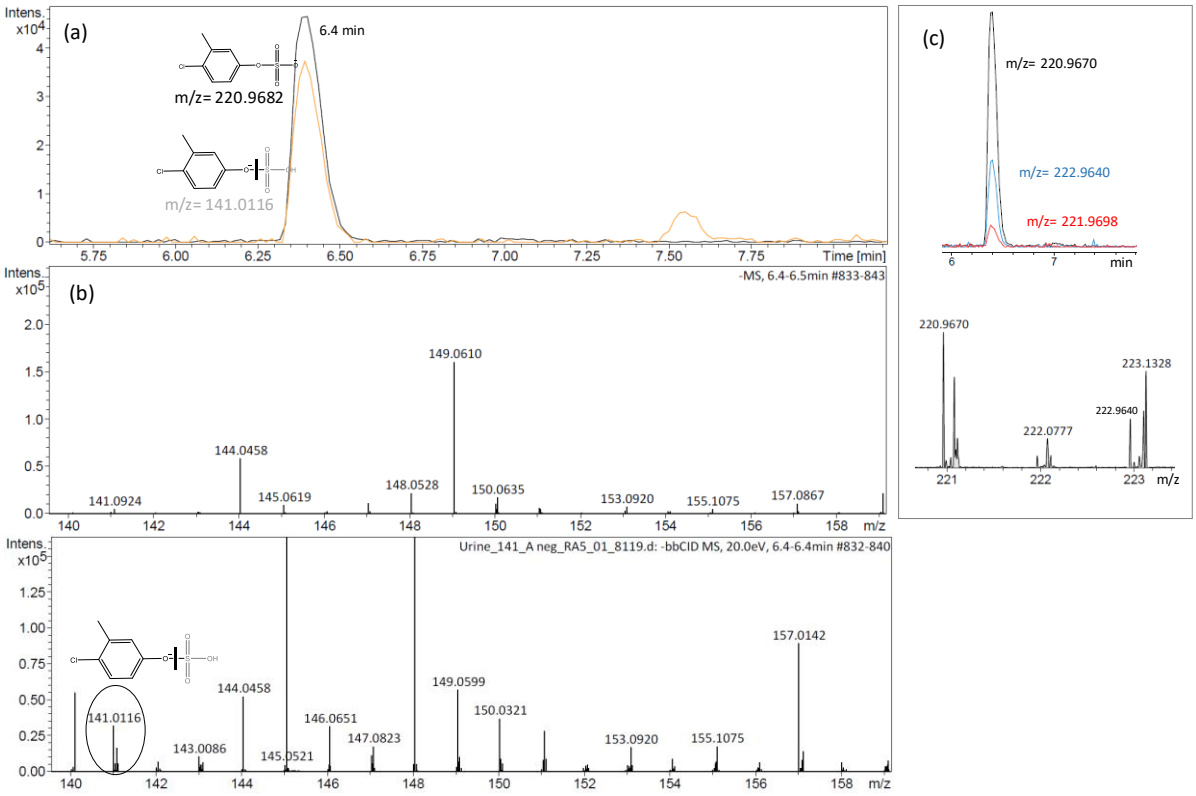

Figure 3 Detection and identification of sulphated PCMC by UHPLC-QTOF-MS following *in-vivo* poled urine assay. (a) XICs at m/z 220.9681 and 141.0113 (0.005-Da mass-window width). (b) (top) Low-energy (full-scan analysis) and (bottom) high-energy (bbCID mode) spectra and structures of the metabolite and fragment ion observed. (c) XIC at m/z 220.9670, 221.9698 and 222.9640 (0.005-Da mass-window width) for PCMC and the two chlorine isotope peaks (top) and mass spectra (bottom).

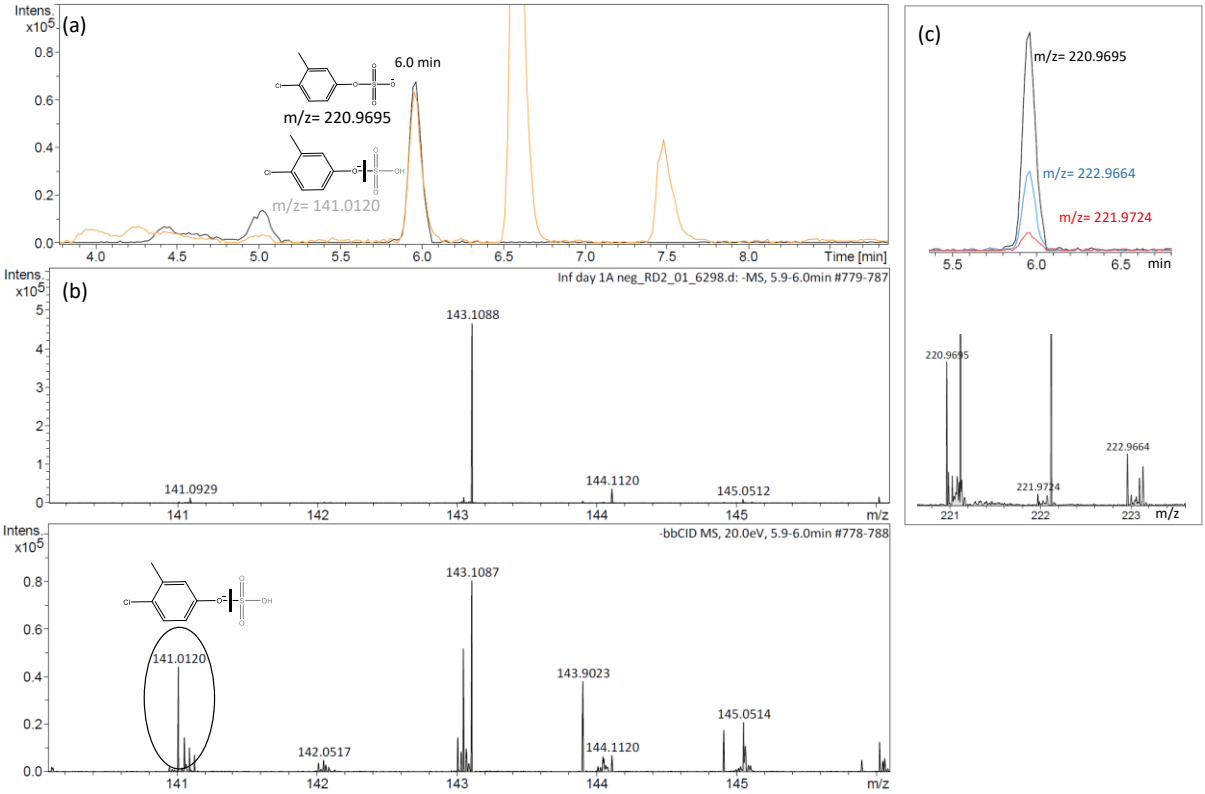

Figure 4 Detection and identification of sulphated PCMC by UHPLC-QTOF-MS following *in-vivo* wastewater profiling assay. (a) XICs at m/z 220.9681 and 141.0113 (0.005-Da mass-window width). (b) (top) Low-energy (full-scan analysis) and (bottom) high-energy (bbCID mode) spectra and structures of the metabolite and fragment ion observed. (c) XIC at m/z 220.9695, 221.9724 and 222.9664 (0.005-Da mass-window width) for PCMC and the two chlorine isotope peaks (top) and mass spectra (bottom).