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Extending analysis of environmental pollutants in human urine towards screening for suspected compounds

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ABSTRACT

Today there is a large difference in the number of chemicals of commerce and the number of chemicals being monitored in environmental and human samples. During the last decades suspect screening methods have been developed to increase the number of monitored analytes. Peaks detected during high resolution mass spectrometry full scan measurements are compared to a list of suspect chemicals with known exact masses. These methods, however, have so far focused on environmental samples. Thus we present a method development for a suspect screening of human urine samples. The sample preparation techniques and instrumental analysis were tested by target chemicals with a wide range of properties. A combination of direct injection and QuEChERS extraction followed by liquid chromatography coupled to high resolution mass spectrometry was able to detect 33 of the 40 spiked target compounds at 30-120% absolute recovery. For suspect evaluation peaks were deconvoluted and aligned with the software MZmine followed by R script processing. Comparing detected and *in-silico* fragmentation, nine suspect chemicals could be tentatively identified in a pooled human urine sample and four of these were confirmed by a reference standard.

1. Introduction

A large number of chemicals is currently in commercial use and many of these may be intentionally or unintentionally present in food and consumer products [1] or can be released into the environment. As a consequence, humans are exposed to these chemicals via dermal, oral or inhalative uptake. Human exposure to chemicals can be assessed by monitoring of human fluids and tissues [2]. Urine is the most important pathway for excretion of environmental chemicals and metabolites thereof. Thus, urine is among the most commonly analyzed matrices and can be obtained by non-invasive sampling in relatively large quantities. However, only relatively polar compounds are excreted with urine, more hydrophobic and persistent chemicals are stored in fat or blood after uptake or are being metabolized [3]. Thus, in many cases metabolites instead of the parent compounds have to be analyzed in urine. In case of conjugates (phase II metabolites) these can be analyzed directly or after a deconjugation step [4].

Several contaminants and metabolites thereof have been detected in human urine, for example aromatic amines [5], flame retardants [6], parabens [7], phthalates [8] and UV filters [9]. The relatively small number of compounds analyzed so far stands in contrast to the large number of chemicals humans are exposed to. Typical human biomonitoring methods specifically focus on a rather small number of analytes with similar properties, due to the complex matrix craving sample preparation or high dilution factors and quality control for quantification methods. Solid phase extraction (SPE) or liquid–liquid extraction (LLE) are besides direct injection (often including dilution) the most frequent sample preparation methods used [10]. These methods are validated for a certain group of chemicals, although they might be applicable towards other analytes as well. In the field of drug monitoring and doping control, multi-target methods for human urine have been described, containing up to several hundred analytes [11,12].

In food and water analysis multi-compound or screening methods have been put forward to simultaneously analyze a large number of compounds with a wide range of properties [13,14]. Particularly suspect and non-target screening methods based on liquid chromatography-high resolution mass spectrometry (LC-HRMS) have gained popularity [15]. Their main aim is not an accurate quantification, but the detection of as many contaminants

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as possible present in the samples, including compounds not detected previously. Generic sample preparation methods are thus essential, followed by analysis using LC-HRMS. Subsequently, chromatograms can be searched for peaks with specific masses or isotope patterns to identify suspected chemicals beyond the target analytes for which the method has been validated. Tentatively identified contaminants are verified by reference standards. A detailed explanation of target, suspect and non-target screening approaches is given in Krauss et al. [15]. Suspect chemicals are substances that due to their production, usage and properties are likely to end up in the samples of interest. Thus, a list of chemicals is compiled containing exact masses that can be searched for in LC-HRMS chromatograms.

Using a validation with 45 target chemicals, Moschet et al. [16,17] identified 144 pesticides and transformation products in river water from five different sites in Switzerland. Another study by Hug et al. [18] was able to identify six suspect and five non-target chemicals present in wastewater samples from an industrial region in Germany. However, most suspect and non-target screening methods focused on environmental samples, with a few exceptions addressing human samples [19,20].

As a step towards a more comprehensive assessment of human exposure to contaminants we developed a LC-HRMS-based suspect screening method for the analysis of human urine samples for a broad range of contaminants. Our main focus was the evaluation of different generic sample preparation methods with the aim to cover a large compound domain, which was based on a wide range of target analytes. Both, direct injection of urine and the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) extraction method were tested. The latter has evolved as a widely-used method in pesticide residue analysis of food items [21,22], relying on a phase separation of acetonitrile-water mixtures induced by the addition of salts. While most pesticides partition into the acetonitrile-rich phase followed, which can be further cleaned-up by dispersive solid phase extraction, many hydrophilic biomolecules such as amino acids and peptides interfering with the analysis are likely to remain in the water-rich phase [23]. Thus, this approach seems to be promising also for the extraction of urine samples.

The LC-HRMS method and parts of the data evaluation methods were adapted from a previous approach for water samples [18]. The suitability of the developed method was demonstrated for a suspect screening of a pooled urine sample.

2. Experimental

2.1. Chemicals

Representatives from different groups of chemicals that have been detected or suspected in urine (among these allergenic substances, aromatic amines, hydroxy-PCBs, hydroxy-PAHs, different industrial chemicals, pesticides, PFASs, plasticizers, preservatives and UV filters) were selected from a list of 1500 suspect chemicals that resulted from a thorough literature survey (more detailed description see Supplementary data, the full list of suspects can be found in the Excel spreadsheet provided as Supplementary data). Based on this approach, a list of 40 compounds covering a wide range of properties was used for method development (Table 1 and Table S1, Supplementary data). Additionally, two glucuronides and two sulfates were used to test the applicability of the method including a deconjugation step. A standard mixture containing all analytes at 5 μg/mL (exceptions due to low sensitivity of the LC-HRMS for these compounds are marked in Table S1, Supplementary data) was prepared from individual stock solutions (1 mg/mL).

2.2. Urine sample

Morning urine from male and female members of the department was collected and pooled in a 5 L aluminum container, followed by aliquotation (10 mL) into 50 mL polypropylene (PP) tubes. These aliquots were stored at −20 °C until analysis after thawing at room temperature. This sample was used for method development and demonstration of the suspect screening.

2.3. Deconjugation

The deconjugation was tested with glucuronic acid and sulfate conjugates of 4-methylumbelliferone and estrone. Different amounts (~300 units/mL and ~600 units/mL) of β-glucuronidase (Type H-1 from *Helix pomatia*, Sigma–Aldrich) dissolved in 1 M ammonium acetate buffer (pH 5) were added to spiked urine, followed by incubation at 37 °C for 19 h. For comparison a spiked sample was analyzed without the deconjugation step. In addition, three urine blanks were analyzed with 0, 300 and 600 mg/L β-glucuronidase. During analysis also the formation of 4-methylumbelliferone was monitored, while it was not possible to monitor the formation of estrone with the instrumental method used. The deconjugation step was not included in the recovery experiments, but was used during the final target and suspect analysis.

2.4. Direct injection (DI)

Urine samples were centrifuged (4000 × g, 10 min) and 10% of methanol was added to the supernatant. For comparison and calculation of recoveries bidistilled water was handled the same way. For recovery experiments samples were spiked at different levels before centrifugation.

2.5. QuEChERS extraction

Extraction by a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method followed in general the method developed by Anastassiades et al. [21]. Briefly, 10 mL of urine were thoroughly mixed with 10 mL of acetonitrile. Subsequently, 4 g of $MgSO_4$ and 1 g of NaCl were added for phase separation, followed by centrifugation (4000 × g, 10 min). Three mL of the supernatant, the acetonitrile-rich phase, were concentrated under a N_2 stream to 300 μL to test the liquid–liquid extraction (LLE) step. Another five mL of the supernatant were transferred to a 15 mL PP tube for further clean-up by dispersive solid phase extraction (dSPE) using 125 mg of primary secondary amine-modified silica gel (PSA) and 750 mg of $MgSO_4$. The tube was vortexed for 30 s, centrifuged (4000 × g, 10 min) and three mL of the supernatant were concentrated under a N_2 stream to 300 μL. All extractions were conducted at 22 ± 2 °C. It should be noted here that the polarity of both the water-rich and acetonitrile-rich phases depends on the temperature and concentration of salts added, thus an accurate dosing of salts and temperature control is necessary.

Urine samples were spiked at different levels prior to the extraction, additionally blank urine and matrix spikes were conducted for both extraction steps (LLE and dSPE). Recoveries were calculated against external standards prepared in acetonitrile. Method blanks were conducted by using 10 mL bidistilled water followed by the described extraction procedure.

2.6. Instrumental Method

The instrumental method was adapted from Hug et al. [18]. Samples were injected onto a Kinetex C18 column (100 mm × 3 mm, 2.6 μm, Phenomenex) controlled by an Agilent 1200 LC system.