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LC-HRMS based method for suspect/non-targeted screening for biomarkers of chemical exposure in human urine

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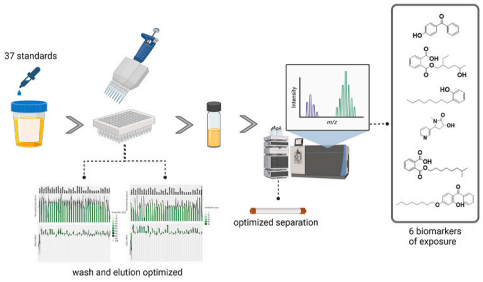
HIGHLIGHTS

- Enzymatic hydrolysis of phase II metabolites is the vital sample preparation step.
- The method supports high-throughput HBM analysis of urine samples.
- The method demonstrates low retention time drifts and high mass accuracies.
- Limits of detection are relevant for HBM exposure studies.
- 80% of the spiked standards were identified following the non-targeted workflow.

GRAPHICAL ABSTRACT



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ABSTRACT

Every day we are exposed to a cocktail of anthropogenic compounds many of which are biologically active and capable of inducing negative effects. The simplest way to monitor contaminants in a population is via human biomonitoring (HBM), however conventional targeted approaches require foreknowledge of chemicals of concern, often have compound specific extractions and provide information only for those compounds. This study developed an extraction process for human biomarkers of interest (BoE) in urine that is less compound specific. Combining this with an ultra-high resolution mass spectrometer capable of operating in full scan, and a suspect and non-targeted analysis (SS/NTA) approach, this method provides a more holistic characterization of human exposure. Sample preparation development was based on enzymatically hydrolysed urine spiked with 34 native standards and extracted by solid-phase extraction (SPE). HRMS data was processed by MzMine2 and 80% of standards were identified in the final data matrix using typical NTA data processing procedures.

1. Introduction

In the last century the advent of mass chemical production including pesticides, industrial compounds and flame retardants led to many compounds becoming ubiquitous in the environment. However, knowing compounds may be in the environment does not mean that they find their way into the human body. Due to the difficulties with obtaining human samples, the instrumental matrix effects, limitations on volume, metabolism of target chemicals and ethical considerations, human biomonitoring (HBM) has lagged in development of non-targeted

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monitoring methods.

However, understanding the body burden and trends in exposure are essential to formulate strategies to mitigate health impacts. HBM is a vital tool to provide the measure of individual and population exposure to chemicals from different source pathways and estimate potential risks associated with exposure (Ganzleben et al., 2017). Traditionally, HBM provided only quantifiable exposure data through targeted analysis of compounds of concern in various biological matrices such as blood and urine (Dennis et al., 2017). However, the number of emerging compounds of concern is increasing while legacy contaminants still require monitoring.

It is estimated that every year the number of new chemicals created increases by 4.4% (Llanos et al., 2019), with some 700 new compounds approved for use annually, while just a handful of existing compounds are removed from global production. Monitoring for these emerging contaminants and their metabolites becomes an impossible task if traditional targeted mass spectrometry approaches are used. The latest generation of ultra-high resolution mass spectrometers may provide a solution, as they are capable of acquiring accurate mass detection across a wide mass range, with sensitivity comparable to some targeted approaches. Non-targeted (NT) analysis is agnostic in nature and in ideal cases it provides information about 'every' compound in a sample that may be detected using the instrumental and extraction methods. NT potentially affords the opportunity to detect known-knowns (target), known-unknowns (suspect) and unknown-unknowns (unrecorded in databases or never determined).

In this regard NT screening is a starting, hypothesis-generating tool, and it has been widely applied in various fields such as in environmental and water quality monitoring and forensics (Caballero-Casero et al., 2021). However even in established fields of study the approaches for instrumental detection and identification of known-unknowns and unknown-unknowns is still evolving to remove false negative and positive reporting. In contrast, methods for NT screening for biomarkers of exposure (BoE) in human biological samples are even more limited (Pourchet et al., 2021).

To enable comprehensive screening for BoE, careful sample preparation and analysis procedures should be applied, however NT screening for BoEs faces several challenges, including that most synthetic chemicals are several orders of magnitude less abundant than some endogenous compounds. While the physical and chemical properties of many endogenous compounds overlap with BoEs making it difficult to exclude endogenous compounds without losing targets of interest. A further complication arises in that parent compounds may undergo phase I and phase II metabolism, producing chemically distinct metabolites. Phase I metabolism involves reactions such as oxidation, hydroxylation and hydrolysis, while phase II reactions involve binding of very polar groups, such as glucuronide, sulphate or glutathione, rendering the compounds more polar and facilitating their excretion via urine (James, 2021).

Some of these challenges can be addressed with optimized sample preparation procedures, which in NT context aim for a balance between removal of matrix and compound preservation (Hajeb et al., 2022). To date, there has been no universally accepted sample preparation procedure. Both liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are commonly used for biological samples. However, the choice of solvents, sorbents and clean-up is often selected to provide compounds that fit within the hypothesis of the research rather than a catch all approach.

Another key factor in HBM is which bio-material to sample with the most common being blood and urine. While blood reflects an equilibrium with the whole body, its collection is invasive, requiring qualified collection personnel, and often is volume limited. On the other hand, the collection of urine is non-invasive (Khamis et al., 2017), it is easily available and suitable for groups where blood samples are difficult to obtain, such as children. There are some limitations in that urine contains excreted compounds at low abundance which are often bio transformed. All in all, urine remains one of the few bio-fluids that may be used in large scale cohort studies.

The aim of this study was to develop workflow for NT screening in human urine, that includes sample preparation, an instrumental method, and data analysis. The development was founded upon urine matrix fortified with reference standards of chemicals of emerging concern. The sample preparation was based on enzymatic hydrolysis followed by concentration and purification using SPE with instrumental analysis on U(H)PLC with HRMS detection. The method was validated by detection and identification of the spiked standards in the data matrix obtained from processing of HRMS data with MzMine2. The study presents a high-throughput procedure for NT screening of urinary BoE applicable for small and large scale HBM studies.

2. Materials and methods

2.1. Chemicals and reagents

Analytical standards (n = 34, minimum purity ≥99%) were selected to cover a wide range of compound classes with a broad mass range (122–753 Da) and differing physical and chemical properties (see Table SI-1 for full details). The solvents used for sample preparation and chromatographic separation were LC-MS grade and were purchased from J.T. Baker (Deventer, the Netherlands). Oasis, SPE 96 well plates (60 mg) were purchased from Waters (Milford, USA).

2.2. Test sample preparation

A fortified human urine sample, was prepared by enzymatic digestion to validate the extraction method, clean-up and chromatographic separation. β-glucuronidase (Abalone, purified) was added to 1 mL of urine (to achieve 250 U/mL of enzyme in sample) and incubated by gently mixing for 18 h at 37 °C. Then, the deconjugated urine was spiked with the mixture of reference standards to the final concentration of 10 μg/L. Their full names, logP-values, elemental compositions reflecting their functionalization and monoisotopic masses are presented in Table 1. Ethical permissions were granted by Republic of Slovenia National Medical Ethics Committee (65/09/14 and 0120-118/2017/3).

2.3. Development of SPE for NT screening

Current trends in HBM involve large numbers of samples and low sample volumes making SPE with well plates an efficient option as opposed to larger SPE cartridges or LLE. HLB 60 well plates were selected as a broad-coverage sorbent for extraction, clean-up and concentration of urine samples. The sorbent was preconditioned with 1 mL of acetonitrile (ACN), methanol (MeOH) and water, respectively. Test sample (1 mL) was loaded to the sorbent, and allowed to pass through under gravity, the sorbent was then washed using 1.5 mL of a wash solvent. The sorbent was then dried under a gentle stream of nitrogen and each well was eluted with 1.5 mL of an elution solvent. Finally, the eluate was dried at 35 °C under a gentle stream of nitrogen and reconstituted in 100 μL 5% ACN in water creating a ten-fold concentration step. The selection of both wash solvent and eluent was optimized for this study.

To cover for the wide polarity of analytes, the elution solvent was selected based on a binary mixture of MeOH and ACN, from 10 to 100 v/v % of MeOH in ACN in increment steps of 10%. For each mixture we evaluated two parameters, the elution efficiency and the matrix effects. Elution efficiency was monitored as absolute abundance of eluted analyte in test samples. Matrix effects were calculated for each mixture as a ratio of the absolute abundance of analyte in hydrolysed urine versus the absolute abundance in pure water. Optimal elution mixture was regarded as one with maximum elution efficiency and minimal matrix effects for the majority of analytes. In this study 10 v/v % MeOH in ACN was deemed as the optimum elution solvent.

For the wash solvent the aim was to enable the maximal detection of

Table 1
Full names of compounds for the test mix, with their names and abbreviations as used throughout the text and in the plots, with their corresponding logP-values, elemental formulas and monoisotopic masses.

Name/Abbreviation	Compound	logP[a]	Formula	Monoisotopic mass	RT (min)	Ionization mode[b]
AdipicAcid/AdipicA.	Adipic acid	0.08	C6H10O4	146.0579	0.96	ESI(−)
Erythritol/Eryth.	Erythritol	−2.29	C4H10O4	122.0579	1.32	ESI(−)
Acesulfame/Acesulf.	Acesulfame	−1.33	C4H5NO4S	162.9939	1.70	ESI(−)
Saccharin/Sacchar.	Saccharin	0.91	C7H5NO3S	182.9990	2.24	ESI(−)
Cyclamate/Cyclam.	Cyclamate	−1.61	C6H13NO3S	179.0616	2.54	ESI(−)
Omethoate/Ometh.	Omethoate	−0.74	C5H12NO4PS	213.0225	2.63	ESI(+)
Biphenol	Biphenol	2.8	C12H10O2	186.0681	3.31	ESI(+)
DemetonSS/DemSS	Demeton S Sulfone	0.07	C8H19O5PS2	290.0412	4.39	ESI(−)
Sucralose/Sucral.	Sucralose	−1	C12H19Cl3O8	396.0146	4.20	ESI(−)
Aspartame/Aspart.	Aspartame	−0.1	C14H18N2O5	294.1216	4.26	ESI(−)
Alitame	Alitame	−0.37	C14H25N3O4S	331.1566	5.30	ESI(+)
Mecoprop/Mecopr.	Mecoprop	3.13	C10H11ClO3	214.0397	6.53	ESI(−)
MeP	Methyl paraben	1.96	C8H8O3	152.0473	7.02	ESI(−)
BPS	Bisphenol S	1.65	C12H10O4S	250.0300	7.06	ESI(−)
Pravastatin/Pravast.	Pravastatin	0.59	C23H36O7	424.2461	7.72	ESI(−)
NDHH	Neohesperidin dihydrochalcone	0.2	C28H36O15	612.2054	8.13	ESI(−)
Acridone/Acrid.	Acridone	1.69	C13H9NO	195.0684	8.22	ESI(+)
EtP	Ethyl paraben	2.47	C9H10O3	166.0630	8.91	ESI(−)
Ketoprofen/Ketopr.	Ketoprofen	3.12	C16H14O3	254.0943	10.01	ESI(+)
Carbamazepine/Carbam.	Carbamazepine	2.77	C15H12N2O	236.0950	9.31	ESI(+)
Naproxen/Naprox.	Naproxen	3.18	C14H14O3	230.0943	10.57	ESI(−)
Aminoanthraquinone/AAQ	Aminoanthraquinone	3.74	C14H9NO2	223.0633	10.96	ESI(+)
BPA	Bisphenol A	3.32	C15H16O2	228.1150	11.32	ESI(−)
Diclofenac/Diclofen.	Diclofenac	4.51	C14H11Cl2NO2	295.0167	13.58	ESI(+)
DHBP	Dihydroxybenzophenone	2.96	C13H10O3	214.0630	11.73	ESI(−)
BuP	Butyl paraben	3.57	C11H14O3	194.0943	12.70	ESI(−)
BzP	Benzyl paraben	3.7	C14H12O3	228.0786	12.94	ESI(−)
BP8	Benzophenone 8, dioxybenzone	3.82	C14H12O4	244.0736	13.51	ESI(−)
TPP	Triphenylphosphate	4.59	C18H15O4P	326.0708	20.16	ESI(+)
Fenamiphos/Fenam.	Fenamiphos	3.32	C13H22NO3PS	303.1058	14.62	ESI(+)
NaDBS	Sodium dodecylbenzensulfonate	4.78	C18H30O3S	326.1916	17.93	ESI(−)
Chlorophene/Chlorop.	Chlorophene	3.6	C13H11ClO	218.0498	16.96	ESI(−)
Coumaphos/Coumap.	Coumaphos	4.13	C14H16ClO5PS	362.0145	20.12	ESI(+)
Phoxim/Phox.	Phoxim	4.39	C12H15N2O3PS	298.0541	34.78	ESI(+)

[a] Values obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/, accessed 21.10.2021).
[b] Many compounds are ionizable in both modes. The table shows the mode which produced better results.

low-level BoEs, while removing very polar excess matrix constituents such as salts, sugars and small charged molecules. To remove very polar compounds, milli-Q water (MQ) is a natural choice for wash step, and to increase removal of less polar matrix a percentage of MeOH was added. We studied binary mixtures of 5, 10, 15, 20, 35 and 50 v/v % of MeOH in MQ. The wash step optimization was performed with 1.5 mL of 10 v/v % MeOH in ACN as the optimum elution solvent. More detailed information on the optimization of elution and wash solvents are provided in the results and discussion.

2.4. Instrumentation and chromatographic separation

2.4.1. NT method development on ESI(±)-UHPLC-QTrap-MS/MS

An ultra-high-performance liquid chromatography (UHPLC) separation was performed on Shimadzu Nexera X2. The standards were separated using the Waters Acquity HSS-T3 (2.1 × 100 mm, 1.8 μm) column with MQ (A) and ACN (B) as the mobile phases. Waters Acquity HSS-T3 column used for separation of polar and non-polar compounds. To maximize separation efficiency 100 mm column was used. MQ and ACN were selected as mobile phases and no modifiers were used in order to maximize detectability of standards in both, positive and negative ionization modes. Optimal separation was studied by varying elution program, solvents and column temperature. Using best parameters, standards in test samples were efficiently separated.Their RTs are presented in Table 1. The optimal elution gradient was: 5–15% B (0.01–1 min), 15–25% B (1–5 min), 25–40% B (5–8 min), 40–60% B (8–18 min), 60–75% B (18–22 min), 75–85% B (22–24 min), 85–100% B (24–28 min), 100-5% B (28–30 min), 5% B, 30–35 min. Flow rate was 0.3 mL/min, while heating the column to 40 °C. The injection volume was 1 μL. The UHPLC was coupled to quadrupole-linear ion trap mass spectrometer (Sciex QTrap 4500). Electrospray ionization (ESI) was used as ionization source at the spray voltage of 4500 V and −3500 V and vaporizer temperature at 500 °C with curtain gas, gas 1 (GS1) and gas 2 (GS2) at 40,0 psi. Compounds were detected in multiple-reaction monitoring (MRM) mode. The MRM transitions and compound-specific MS parameters are presented in Table SI-1.

2.4.2. Method validation and proof-of-concept using ESI(+)-UHPLC-Q-IT-Orbitrap-MS/MS

The developed NT method was first applied to the sequence of 10 test samples in full-scan (FS) mode and then to 5 actual urine samples in order to test its suitability for NT screening in real samples. For the UHPLC separation the parameters were kept the same as those used for target analysis. The MS detection was performed on Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific Inc., Waltham, MA), in ESI(+) FS mode. Heated electrospray ionization (HESI) was used as the ionization source, at the spray voltage of 4500 V, sheath gas flow 40 L/min, nebulizer auxiliary gas 15 L/min, and sweep gas 2 L/min. The ion transfer capillary was heated to 350 °C. The full-scan mass acquisition covered the mass range of 100–900 *m/z* and was performed at the resolution of 120 000 FWHM (full width at half maximum at *m/z* 200), while the MS2 data was acquired with the resolution of 60 000 FWHM. The automatic gain control (AGC) was 5×10^5 ions and maximum injection time 50 ms. Cycle time was 0.8 s.

2.5. Data processing and compound identification

HRMS FS data was processed using MzMine 2.53 (Pluskal et al., 2010) in the original Thermo.raw file format. Processing parameters were optimized to detect the features corresponding to spiked standards

in test samples. Processing parameters are presented in SI-2. Spiked standards were identified as features with matching MH^+ at the maximum error of 5 ppm and the maximum retention time variation of 0.5 min.

For proof-of-concept, the compounds in real urine samples were identified by submitting all MS2 spectra within the feature list to Sirius-CSI:FingerID (Dührkop et al., 2019). Elemental formula match required to be 100%, while candidate match score was required to be $\geq$ 60% using PubChem, KEGG and CheBI compound databases. Candidates achieving the criteria were then filtered for xenobiotics using T3DB (Wishart et al., 2015), Exposome Explorer (Neveu et al., 2017) and Chemistry Dashboard (Williams et al., 2017).

2.6. Validation and quality control procedures

Several quality control (QC) measures were taken. During extraction and instrument runs several blanks were generated. First, LCMS grade water (n = 3) was processed as test samples served and as procedural blanks in order to identify contaminants. Deconjugated urine blanks (n = 3) were used in order to monitor possible presence of the fortified compounds in urine. Instrumental solvent blanks, (injected every 5 samples) were used to monitor any possible carryover from injections on the HPLC system.

The limits of detection (LOD) of fortified standards in FS mode were determined by serial dilution of test samples with LC-MS grade water. Samples were prepared in concentrations 1000, 500, 250, 100, 50, 25, 10, and 0.1 μg/L. LOD was determined as lowest concentration at which features corresponding to standards were detected above 3 times the baseline. All features identified less than the LOD were excluded from the data set.

Retention time stability was assessed by monitoring retention time drifts for each identified standard across test samples. Stability of mass accuracy was monitored by following mass errors for MH+ of identified standards across test samples.

Non-specific activity of deconjugation enzyme was checked by spiking 1 mL of synthetic urine (CDC, 2009) with native standards and incubating them under the same conditions as those used for urine deconjugation. Spontaneous degradation was controlled by concurrently incubating the standards without the presence of the enzyme. Deconjugation stability was checked at the beginning of incubation and after 2, 8, 24, 30 and 48 h, while spontaneous degradation was monitored as a difference in abundance at time zero and at the end of the experiment. After incubation, the deconjugation samples were treated the same way as other samples.

3. Results and discussion

3.1. Test compounds and their chromatographic separation

Test samples used in the analytical method development were prepared by spiking enzymatically hydrolysed urine with native standards. The native standards served as model compounds and were selected in order to cover a wide range of polarities (described with partition coefficient, logP), molecular masses and functional groups (Table 1 and Fig. 1). The native standards included pharmaceuticals, artificial sweeteners, flame retardants, bisphenols, parabens, benzophenones, surfactants and pesticides, and were variously functionalized, with moieties including oxygen, nitrogen, phosphorus, sulphur and chlorine. Compound masses ranged from 122.0579 to 612.2054, and logP from 0.96 to 4.51. Post-extraction labelled standards were included to adjust for any variations during the sample preparation and instrumental analysis. Chromatographic separation was studied by varying elution

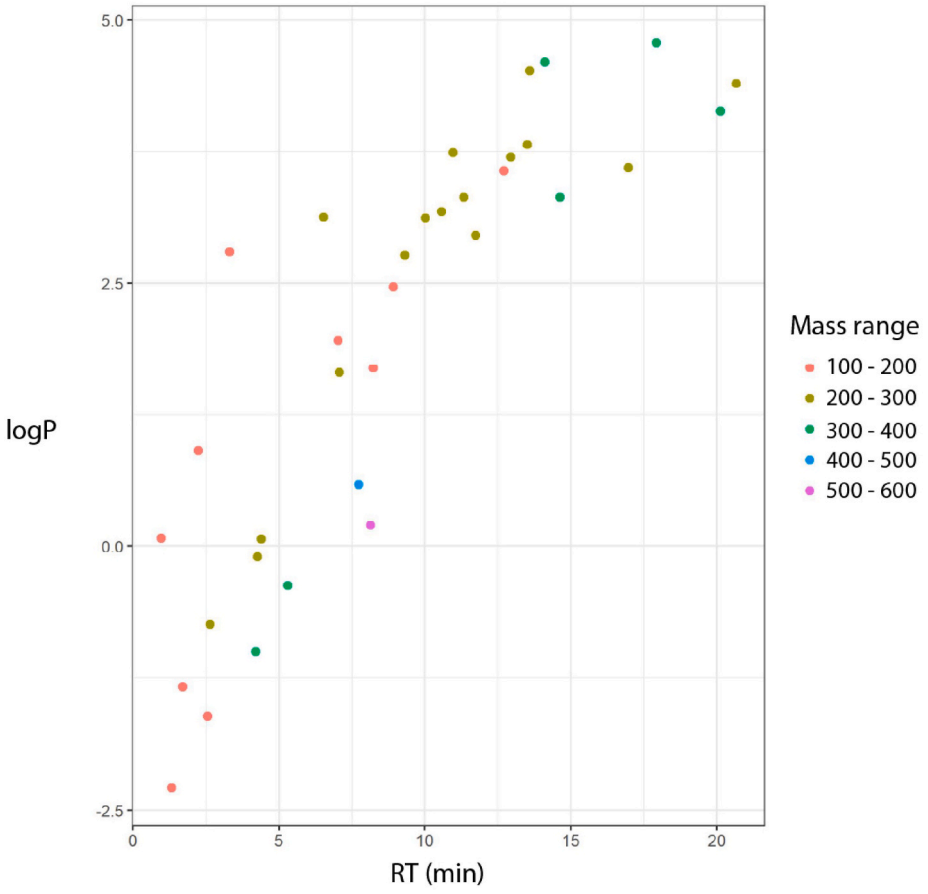

Fig. 1. Separation of native and labelled standards used for NT method development according to their logP and molecular mass.

gradients and solvents. The optimal separation program, as described in section 2.4 produced chromatograms with no overlapping peaks, evenly distributed throughout chromatographic run (Fig. 1). Even distribution is essential to avoid local chromatographic crowding of compounds, producing clearer chromatograms and enhancing the chance of detecting low-level BoEs, which can overlap with high-abundance endogenous metabolites.

3.2. Deconjugation specificity

Xenobiotics can undergo phase I and II metabolism when introduced into the organism. Phase I reactions occur mostly in the liver and are driven by cytochrome P450 group of enzymes (James, 2021). They include oxidation, reduction and hydrolysis reactions to attach or transform into moieties suitable for the phase II metabolism. The latter involves formation of conjugates with, among others, glucuronic acid, sulfuric acid and glutathione, which renders xenobiotics more polar and hence easier to excrete via urine.

The deconjugation step, often part of a sample preparation procedure for urine, is in principle the opposing reaction to the phase II metabolism. It cleaves the bond between a xenobiotic and a polar conjugate molecule, releasing a parent compound or phase I metabolite, depending on which was conjugated in the first place (James, 2021). The deconjugation step can offer several advantages, which we implemented in our NT analytical method. First, increased efficiency of sample clean-up, as more non-polar compounds bind more strongly to reversed phase SPE sorbent and with that a higher proportion of the more polar matrix can be removed. Second, the efficiency of a reversed phase chromatographic separation can be increased, since more polar compounds elute at shorter retention times, while the more non-polar ones demonstrate stronger retention on the stationary phase, which results in a more efficient separation. This further benefits in the higher chance of detection of low-abundance compounds due to reduced coelution with more polar high-abundance endogenous metabolites. Thirdly, the deconjugation process reduces molecular complexity which is very beneficial for compound identification. Also, from the MS perspective, phase II conjugated metabolites would be more prone to electrospray negative ionization, which generally yields a weaker response and less informative mass spectra. On the contrary, the electrospray positive ionization is however expected to be a more viable alternative for deconjugation products, i.e. the phase I metabolites and parent compounds, also supporting the detection of low-abundance compounds.

Despite the several advantages offered by deconjugation, our concern was that the enzymatic hydrolysis might change the chemical structure of the analytes due to reported non-specific activity. For example, lipase activity has been reported, which causes hydrolysis of ester bonds and was observed for phthalates (Blount et al., 2000). To check the analyte integrity after deconjugation, we incubated the standards with the deconjugation enzyme, β-glucuronidase at the activity levels used for sample deconjugation (Fig. 2, tabulated numeric results are reported in SI-Deconjugation_stability.xlsx). The abundances were normalized to an average for each standard in order to plot on the same scale. Locally estimated scatterplot smoothing (LOESS) curves were plotted to visualize trends.

Abundances of the majority of compounds have not significantly changed through the duration of the experiment, in fact final abundance did not differ more than 10% for the majority of the tested compounds (31 out of 34). Exceptions were benzophenone 8, chorophene and coumaphos, for which the abundances reduced to 52%, 46% and 40% of the starting abundance, respectively. However, we observed similar trends in the control experiments with no enzyme, where for the majority of samples abundances did not decrease for more than 20%, while the abundances for benzophenone 8, chlorophene and coumaphos reduced to 42%, 23% and 66%, respectively. This shows that degradation of these compounds is due to spontaneous breakdown processes rather than on account of the non-specific activity of β-glucuronidase.

The deconjugation stability experiment was conducted on a group of compounds with various functional groups, including esters, amides, ketones, ethers, phosphates, sulfones, polyols, phenols, carboxylic acids and so on. This increases the confidence that once the NT analytical method is applied to real urine samples, BoEs will not chemically change during urine deconjugation due to non-specific activity of β-glucuronidase.

3.3. Development of SPE for NT screening

We selected reversed-phase (RP) m-divinylbenzene N-vinylpyrrolidone copolymer sorbent Waters Oasis HLB, which absorbs both polar and non-polar compounds, and is not biased for acid, base or neutral compounds. This sorbent has been most widely used SPE sorbent across a variety of NT as well as targeted applications (Hajeb et al., 2022). Steps for SPE involve sorbent conditioning, sample loading, washing to remove high abundance endogenous matrix, drying and elution of analytes from the sorbent. As the first step we selected the elution solvent in order to recover the maximum of spiked standards with lowest eluted matrix, followed by selection of the wash solvent. Comparison of elution volumes showed that elution of 2.0 mL, 1.8 mL and 1.5 mL do not differ significantly in absolute abundance of compounds in the eluted extract, therefore we opted for the lowest volume, 1.5 mL.

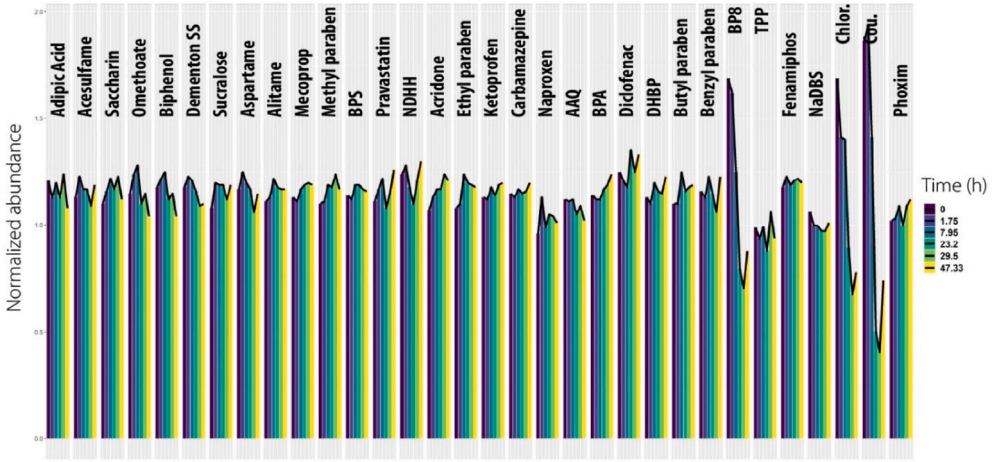

Fig. 2. Abundance of standards when incubated with β-glucuronidase for different time periods.

3.3.1. Selection of the elution solvent

As Oasis HLB retains polar and non-polar compounds, we opted for a binary mixture of a weak (MeOH) and stronger elution solvent (ACN). The elution efficiency was monitored through trends in abundance of eluted standards, while the amount of eluted matrix was monitored through the trends of matrix effects for each spiked standard (Fig. 3, Numeric results are presented in table SI-Selection_Elution_Solvent.xlsx.).

From the elution efficiency (Fig. 3a), it is evident that with increasing MeOH, the abundance of early eluting polar compounds such as adipic acid, erythritol, acesulfame, saccharin, aspartame and alitame is increased. However, the late eluting/non-polar, compounds, such as aminoanthraquinone, fenamiphos, chlorophene, coumaphos and phoxim are retained on the sorbent, both events clearly in agreement with compounds' polarities. The matrix effects affect the instrumental sensitivity for the majority of compounds (Fig. 3b), while adipic acid displays signal enhancement in the extracted urine as compared to water. The trend indicates that greater MeOH does not have a significant effect on matrix effects for any of the more polar, semi polar or non-polar compounds, and with that does not favour any group of compounds in terms of increased ionization and detectability. Therefore, according to elution efficiency, a lower percentage of MeOH in ACN is in favour of greater abundance of the more non-polar compounds, while not significantly influencing the matrix effects. Based on that, we identified 10 v/v % MeOH in ACN as the elution solvent of choice.

3.3.2. Selection of the wash solvent

After selecting the elution solvent, we studied the pertinence of wash solvents aiming to remove high abundance endogenous matrix constituents at the minimum loss of the test compounds. Accordingly, the wash step needed to be performed by a solvent mixture with low eluting power for more non-polar compounds, while eluting unwanted matrix constituents such as salts and highly charged species. Based on that, different mixtures of MeOH in MQ were tested. The level of matrix removal through matrix effects was monitored, and analyte loss through abundance of eluted analyte was measured for mixtures of 5, 10, 15, 20, 35 and 50 v/v % MeOH in MQ (Fig. 4, numeric results are presented in table SI-Selection_wash_solvent.xlsx). The elution was done with the previously selected solvent, 10 v/v % of MeOH in ACN.

Increasing MeOH percentage in MQ causes significant loss of more polar compounds such as adipic acid, erythritol, acesulfame, saccharine, omethoate and aspartame (Fig. 4a). Abundances of more non-polar compounds, such as BPS, chlorophene, coumaphos and phoxim are first improved with increasing since the matrix is being washed out, but the compounds are then themselves eluted from the sorbent when MeOH content exceeds 15 v/v %. Certain compounds, such as saccharine, alitame, diclofenac, DHBP and BuP show reduction in matrix effects, with increasing MeOH leading to lower LODs. However, at the same time, this would cause systematic loss of abundance of more polar compounds, and at a certain point also non-polar compounds. As a compromise, 5% v/v MeOH in MQ was selected as wash solvent.

3.4. Proof-of-concept and method performance

Detection and identification of fortified standards in the test mix acquired in FS mode and prepared according to the above described sample preparation procedure served as a proof-of-concept and confirmation of the workflow's suitability. By application of the developed workflow, 12 out of 15 standards ionizable in the electrospray positive mode (Table 1) were identified using FS HRMS. Three compounds, BPA,

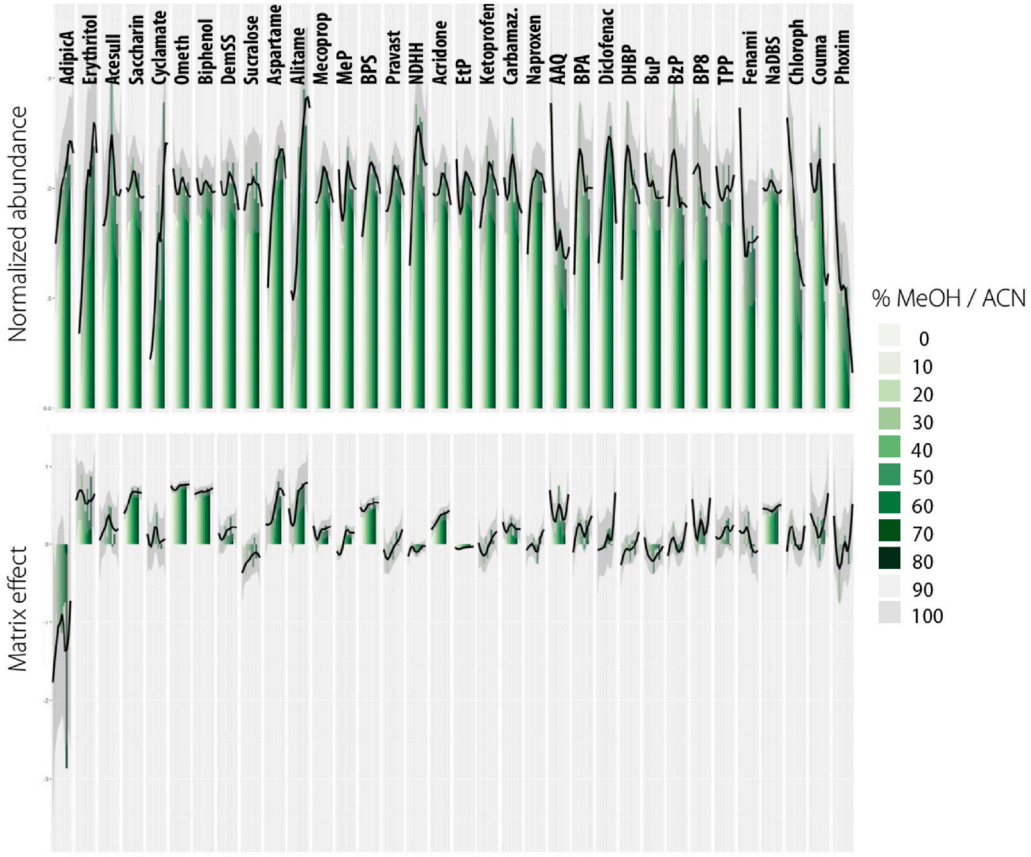

Fig. 3. Elution efficiency (a) of native standards by mixture of MeOH and ACN. Standards are ordered according to their RT. Absolute abundances were scaled by normalization to average abundance for each compound. Matrix effects (b) of native standards when eluted by mixture of MeOH and ACN. LOESS curves have been added to visualize trends.

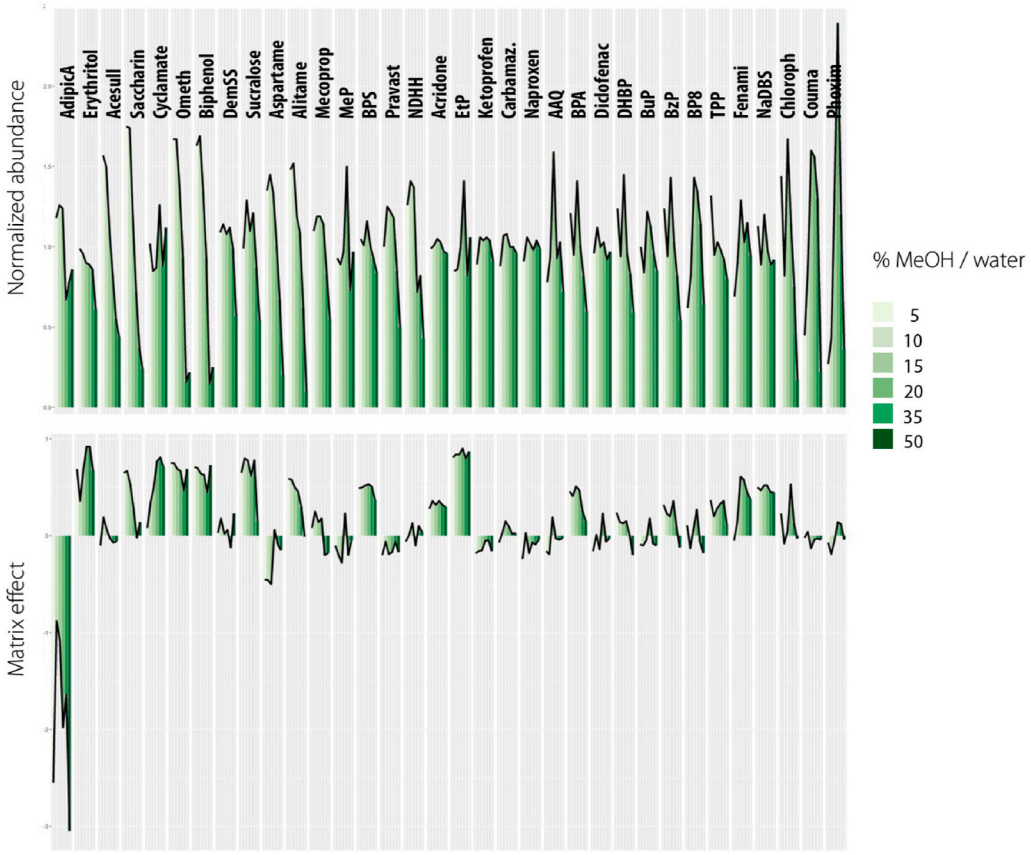

Fig. 4. (a) Abundance of native standards when washed by mixture of MeOH and MQ. Standards are arranged from left to right according to their RT. Absolute abundances were scaled by normalization to average abundance for each compound. (b) Matrix effects of native standards when washed by mixture of MeOH and MQ. LOESS curves have been added to visualize trends.

demeton SS and biphenol were not detected. This is likely due to ion-suppression by matrix, subsequently lowering the signal abundance below the LOD. For the remainder of 12 standards, the corresponding features were successfully annotated. A feature was annotated as a molecular ion if the mass error compared to theoretical mass did not exceed 5 ppm and retention time did not differ for more than 0.5 min from that of a standard. Furthermore, for 11 of 12 successfully annotated standards, MS2 spectra were acquired and were used for further validation by comparing their MS2 spectrum to the MS2 spectrum of standards, (details of which are presented in table SI-MS2_matching.xlsx). The successful identification of 11 standards spiked into deconjugated urine at the expected level of xenobiotics, 10 μg/L served as a proof-of-concept and confirmed the method's suitability for NT screening of BoEs.

Table 2
Estimated method performance parameters based on identified standards.

Test compound	LOD (μg/L)	SD[a] of RT (±min)	Max. mass error (ppm)
Omethoate/Ometh.	10	0.10	0.47
Alitame	10	0.09	0.90
Acridone/Acrid.	10	0.05	1.02
Ketoprofen/Ketopr.	10	0.08	3.53
Carbamazepine/Carbam.	1	0.05	0.42
Aminoanthraquinone/AAQ	10	0.06	0.45
Diclofenac/Diclofen.	10	0.07	0.68
TPP	1	0.06	0.61
Fenamiphos/Fenam.	1	0.07	0.33
Coumaphos/Coumap.	10	0.06	0.83
Phoxim/Phox.	10	0.31	−0.33

[a] SD- Standard deviation.

Estimation of method performance parameters (Table 2), based on analysing the features corresponding to spiked standards, showed the method's capability of achieving LODs in FS mode at as low as 1 μg/L, indicating that large number of BoEs present in urine could be detected when applied to samples used in HBM. However, it should be noted that LOD was determined by serial dilutions of the test sample with water, where along with the analytes matrix was diluted and so matrix effects were reduced and the effective LOD might differ. Retention times proved stable, with maximum deviation of ±0.31 min, which was observed for pesticide phoxim, which was eluted from the column at the near end of chromatographic separation. This information is useful for setting processing parameters, especially for algorithms for retention time alignment during data processing. Large drifts in RT can cause doubling of features in the data matrix and increases the noise in the data. Furthermore, the instrumental method showed consistently low mass errors, with maximal mass error of 3.53 ppm for ketoprofen (Table 2, Retention time drifts and mass errors for each sample are tabulated SI-RT_drifts_mass_errors.xlsx and presented in SI-3).

High mass accuracy is immensely important for finding the correct elemental composition and for reducing the number of candidates in the identification challenge. This is crucial when identifying BoEs to xenobiotics, as the chemical space (Milman and Zhurkovich, 2017) for xenobiotics is incredibly large and often the search for candidates is done through general databases such as PubChem, which contains data on more than 96 million unique chemical structures. This in turn produces a high number of potential hits.

Achieving good LODs and mass accuracies for spiked standards, the method was further applied to the preliminary NT study involving 5 real urine samples. With application of the developed workflow, two UV-

filters, 4-hydroxybenzophenone and octabenzone, a surfactant residue octylphenol, two phthalate metabolites, monoethylhydroxyhexyl phthalate and monocarboxyisooctyl phthalate, and BoE of smoking, hydroxycotinine were tentatively identified at Schymanski confidence level 3 (Schymanski et al., 2014). The MS2 spectra and identification scores for the compounds are presented in SI-4. The number of identified compounds could be significantly enhanced by application of orthogonal identification methods and analysis of larger HBM cohorts, as exposure to certain chemicals is highly individual and therefore chance of detecting them lower. Nevertheless, this unambiguously demonstrates the method's effectiveness in identifying low level BoE and it's potential for application in large scale HBM studies. In spite of developing an optimized sample preparation and analysis procedure. compound annotation and subsequent identification are still the major bottlenecks in implementation of NTA to HBM, especially due to the high number of potentially relevant compounds. To circumvent this problem, the efforts to compile BoE, have been made through databases such as The Toxic Exposome Database or T3DB (Wishart et al., 2015), Exposome Explorer (Neveu et al., 2020), Chemistry Dashboard (https://comptox.epa.gov/dashboard/) and recently launched PubChemLite (Schymanski et al., 2021), a PubChem subset containing exposome-related entries. Creation of relevant databases and libraries along with *in-silico* identification methods, such as Sirius-CSI:FingerID (Dührkop et al., 2013, 2015), Metfrag (Ruttkies et al., 2016) and CFM-ID (Allen et al., 2014) aid in improvement of the confidence of BoE identification, and with that promote rapid real-time identification of contaminants.

4. Conclusions

There is a clear need for the development of a less target-specific extraction and detection methods for BoEs in human urine. Thus, a UHPLC-MS/MS method and sample preparation procedure suitable for a wide range of compounds as presented here for non-targeted analysis of biomarkers of exposure in urine is essential to keep up with emerging compounds.

Deconjugation of phase II metabolites is a vital step in detection of parent or phase I molecules but concerns in stability and loss of molecules of potential interest have been considered for NT screening. In this study it is clear that some compounds may be affected during deconjugation, however this is due to spontaneous processes rather than non-specific enzymatic action. The elution for non-targeted screening is often a balance between compounds of interest and matrix interferences. The logical testing of 34 compounds as presented for differing elution strategies and washes as presented here provides a clear understanding of which potential compromises between recovery and ion-suppression may be considered and an ideal method option was developed. The method was applied to a preliminary batch of samples and six biomarkers of exposure successfully identified, demonstrating the methods suitability for large scale HBM studies.

Credit author statement

ŽT, TK: Conceptualization; ŽT: Data curation; ŽT: Formal analysis; JK, MH: Funding acquisition; ŽT, GC: Investigation; ŽT, GC, TK: Methodology; JK, MH, TK: Project administration; JK, MH, TK: Resources; ŽT: Software; TK: Supervision; ŽT: Validation; ŽT, TK: Visualization; ŽT: Writing – original draft; ŽT, GC, TK: Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.134550.

References

Allen, F., Pon, A., Wilson, M., Greiner, R., Wishart, D., 2014. CFM-ID: a web server for annotation, spectrum prediction and metabolite identification from tandem mass spectra. Nucleic Acids Res. 12, 94–99. https://doi.org/10.1093/nar/gku436.

Blount, B.C., Milgram, K.E., Silva, M.J., Malek, N.A., Reidy, J.A., Needham, L.L., Brock, J. W., 2000. Quantitative detection of eight phthalate metabolites in human urine using HPLC-APCI-MS/MS. Anal. Chem. 72, 4127–4134. https://doi.org/10.1021/ac000422r.

Caballero-Casero, N., Belova, L., Vervliet, P., Antignac, J.P., Castaño, A., Debrauwer, L., López, M.E., Huber, C., Klanova, J., Krauss, M., Lommen, A., Mol, H.G.J., Oberacher, H., Pardo, O., Price, E.J., Reinstadler, V., Vitale, C.M., van Nuijs, A.L.N., Covaci, A., 2021. Towards harmonised criteria in quality assurance and quality control of suspect and non-target LC-HRMS analytical workflows for screening of emerging contaminants in human biomonitoring. TrAC Trends Anal. Chem. (Reference Ed.) 136. https://doi.org/10.1016/j.trac.2021.116201.

CDC, 2009. Laboratory Procedure Manual for Bisphenol A and Other Enviromental Phenols and Parabens in Urine 1–12.

Dennis, K.K., Marder, E., Balshaw, D.M., Cui, Y., Lynes, M.A., Patti, G.J., Rappaport, S. M., Shaughnessy, D.T., Vrijheid, M., Barr, D.B., 2017. Biomonitoring in the era of the exposome. Environ. Health Perspect. 125, 502–510. https://doi.org/10.1289/EHP474.

Dührkop, K., Fleischauer, M., Ludwig, M., Aksenov, A.A., Melnik, A.V., Meusel, M., Dorrestein, P.C., Rousu, J., Böcker, S., 2019. SIRIUS 4: a rapid tool for turning tandem mass spectra into metabolite structure information. Nat. Methods 16, 299–302. https://doi.org/10.1038/s41592-019-0344-8.

Dührkop, K., Scheubert, K., Böcker, S., 2013. Molecular formula identification with SIRIUS. Metabolites 3, 506–516. https://doi.org/10.3390/metabo3020506.

Dührkop, K., Shen, H., Meusel, M., Rousu, J., Böcker, S., 2015. Searching molecular structure databases with tandem mass spectra using CSI:FingerID. Proc. Natl. Acad. Sci. Unit. States Am. 112, 12580–12585. https://doi.org/10.1073/pnas.1509788112.

Ganzleben, C., Antignac, J.P., Barouki, R., Castaño, A., Fiddicke, U., Klánová, J., Lebret, E., Olea, N., Sarigiannis, D., Schoeters, G.R., Sepai, O., Tolonen, H., Kolossa-Gehring, M., 2017. Human biomonitoring as a tool to support chemicals regulation in the European Union. Int. J. Hyg Environ. Health 220, 94–97. https://doi.org/10.1016/j.ijheh.2017.01.007.

Hajeb, P., Zhu, L., Bossi, R., Vorkamp, K., 2022. Sample preparation techniques for suspect and non-target screening of emerging contaminants. Chemosphere 287, 132306. https://doi.org/10.1016/j.chemosphere.2021.132306.

James, M.O., 2021. Drug metabolism: phase II enzymes. In: Reference Module in Biomedical Sciences. Elsevier. https://doi.org/10.1016/B978-0-12-820472-6.00123-7.

Khamis, M.M., Adamko, D.J., El-Aneed, A., 2017. Mass spectrometric based approaches in urine metabolomics and biomarker discovery. Mass Spectrom. Rev. 36, 115–134. https://doi.org/10.1002/mas.

Llanos, E.J., Leal, W., Luu, D.H., Jost, J., Stadler, P.F., Restrepo, G., 2019. Erratum: exploration of the chemical space and its three historical regimes. In: Proceedings of the National Academy of Sciences of the United States of America (2019) 116 (12660–12665) DOI: 0.1073/pnas.1816039116, vol. 116. Proc. Natl. Acad. Sci. U. S. A., p. 14779. https://doi.org/10.1073/pnas.1910465116

Milman, B.L., Zhurkovich, I.K., 2017. The chemical space for non-target analysis. TrAC Trends Anal. Chem. (Reference Ed.) 97, 179–187. https://doi.org/10.1016/j.trac.2017.09.013.

Neveu, V., Moussy, A., Rouaix, H., Wedekind, R., Pon, A., Knox, C., Wishart, D.S., Scalbert, A., 2017. Exposome-Explorer: a manually-curated database on biomarkers of exposure to dietary and environmental factors. Nucleic Acids Res. 45, D979–D984. https://doi.org/10.1093/nar/gkw980.

Neveu, V., Nicolas, G., Salek, R.M., Wishart, D.S., Scalbert, A., 2020. Exposome-Explorer 2.0: an update incorporating candidate dietary biomarkers and dietary associations

with cancer risk. Nucleic Acids Res. 48, D908–D912. https://doi.org/10.1093/nar/gkz1009.

Pluskal, T., Castillo, S., Villar-Briones, A., Orešič, M., 2010. MZmine 2: modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. BMC Bioinf. 11, 1–11. https://doi.org/10.1186/1471-2105-11-395.

Pourchet, M., Narduzzi, L., Jean, A., Guiffard, I., Bichon, E., Cariou, R., Guitton, Y., Hutinet, S., Vlaanderen, J., Meijer, J., Le Bizec, B., Antignac, J.P., 2021. Non-targeted screening methodology to characterise human internal chemical exposure: application to halogenated compounds in human milk. Talanta 225. https://doi.org/10.1016/j.talanta.2020.121979.

Ruttkies, C., Schymanski, E.L., Wolf, S., Hollender, J., Neumann, S., 2016. MetFrag relaunched: incorporating strategies beyond in silico fragmentation. J. Cheminf. 8, 1–16. https://doi.org/10.1186/s13321-016-0115-9.

Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., Hollender, J., 2014. Identifying small molecules via high resolution mass spectrometry: communicating confidence. Environ. Sci. Technol. 48, 2097–2098. https://doi.org/10.1021/es5002105.

Schymanski, E.L., Kondić, T., Neumann, S., Thiessen, P.A., Zhang, J., Bolton, E.E., 2021. Empowering large chemical knowledge bases for exposomics: PubChemLite meets MetFrag. J. Cheminf. 13, 1–15. https://doi.org/10.1186/s13321-021-00489-0.

Williams, A.J., Grulke, C.M., Edwards, J., McEachran, A.D., Mansouri, K., Baker, N.C., Patlewicz, G., Shah, I., Wambaugh, J.F., Judson, R.S., Richard, A.M., 2017. The CompTox Chemistry Dashboard: a community data resource for environmental chemistry. J. Cheminf. 9, 1–27. https://doi.org/10.1186/s13321-017-0247-6.

Wishart, D., Arndt, D., Pon, A., Sajed, T., Guo, A.C., Djoumbou, Y., Knox, C., Wilson, M., Liang, Y., Grant, J., Liu, Y., Goldansaz, S.A., Rappaport, S.M., 2015. T3DB: the toxic exposome database. Nucleic Acids Res. 43, D928–D934. https://doi.org/10.1093/nar/gku1004.