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The role of sample preparation in suspect and non-target screening for exposome analysis using human urine

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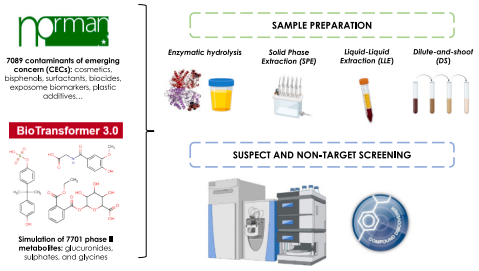
HIGHLIGHTS

- SPE allows the identification of more suspects in SNTS than LLE, SALLE, and DS.
- *In-silico* tools are necessary for simulating structures of phase II metabolites.
- Screening metabolites in non-hydrolysed samples can help in decoding the exposome.

GRAPHICAL ABSTRACT



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ABSTRACT

The use of suspect and non-target screening (SNTS) for the characterization of the chemical exposome employing human biofluids is gaining attention. Among the biofluids, urine is one of the preferred matrices since organic xenobiotics are excreted through it after metabolization. However, achieving a consensus between selectivity (i.e. preserving as many compounds as possible) and sensitivity (i.e. minimizing matrix effects by removing interferences) at the sample preparation step is challenging. Within this context, several sample preparation approaches, including solid-phase extraction (SPE), liquid-liquid extraction (LLE), salt-assisted LLE (SALLE) and dilute-and-shoot (DS) were tested to screen not only exogenous compounds in human urine but also their phase II metabolites using liquid-chromatography coupled to high-resolution tandem mass spectrometry (LC-HRMS/MS). Additionally, enzymatic hydrolysis of phase II metabolites was evaluated. Under optimal conditions, SPE resulted in the best sample preparation approach in terms of the number of detected xenobiotics and metabolites since 97.1% of the total annotated suspects were present in samples extracted by SPE. In LLE and SALLE, pure ethyl acetate turned out to be the best extractant but fewer suspects than with SPE (80.7%) were screened. Lastly, only 52.5% of the suspects were annotated in the DS approach, showing that it could only be used to detect compounds at high concentration levels. Using pure standards, the presence of diverse xenobiotics such as parabens, industrial chemicals (benzophenone-3, caprolactam and mono-2-ethyl-5-hydroxyhexyl phthalate) and chemicals related to daily habits (caffeine, cotinine or triclosan) was confirmed. Regarding enzymatic hydrolysis, only 10 parent compounds of the 44 glucuronides were successfully annotated in the

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hydrolysed samples. Therefore, the screening of metabolites in non-hydrolysed samples through SNTS is the most suitable approach for exposome characterization.

1. Introduction

The extensive use of chemicals present in our daily lives leads to constant exposure of the population to potentially harmful substances (Caballero-Casero et al., 2021a). With the increasing concern about the characterization of chemical exposome (i.e. all the chemical exposures suffered through our life course), monitorization programs, such as The Human Biomonitoring for Europe (HBM4EU) have emerged as tools for policy making (Ganzleben et al., 2017). For that aim, those programs are dedicated to the detection of chemicals in human biofluids, including urine, blood, breast milk or even placenta and follicular fluid (Pourchet et al., 2020). From those biofluids, urine is preferentially used due to its sampling feasibility and the larger volumes available compared to other fluids (Hon and Motiwala, 2022; Saito et al., 2014). Additionally, organic exogenous compounds (i.e. xenobiotics) are mainly excreted through urine after metabolization (i.e. phase I and II metabolites) and they could act as biomarkers for exposome characterization (Janov and iller, 2012; Steckling et al., 2018).

High-resolution mass spectrometry (HRMS) has enhanced our analytical capacity to identify chemicals through non-targeted methodologies (i.e. suspect and non-target screening (SNTS)) (Tolani et al., 2021) in comparison to targeted approaches that are more intended to quantify a few selected compounds (Krauss et al., 2010). Regarding sample preparation, it limits the analytical coverage and is crucial for data quality. Many SNTS approaches use non-selective sample preparation protocols to ensure the widest analytical coverage, but the complexity of human biofluid matrices and/or the low concentration levels of the exogenous chemicals might require the use of specific sample preparation methods to reduce matrix effect and gain sensitivity (Hajeb, 2022). Consequently, one of the biggest challenges in SNTS is to achieve a consensus between selectivity and sensitivity (Pourchet et al., 2020).

For the comprehensive coverage of chemical exposure using human urine samples, centrifugation and/or dilution (i.e., 'dilute-and-shoot' (DS)) have been used as non-discriminatory sample preparation strategies (Christia et al., 2022; Díaz et al., 2012). However, signal suppression and decreased sensitivity are commonly reported as drawbacks (Plassmann et al., 2015). To gain sensitivity, solid-phase extraction (SPE) using polymeric cartridges (Musatadi et al., 2022; Tkalec et al., 2022b) and liquid-liquid extraction (LLE) based protocols have been applied. For the latter, simplified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) procedures are reported in the literature, performing a salt-assisted LLE (SALLE) without further clean-up steps (López et al., 2016; Roca et al., 2014). Methods for lipid and/or protein removal (Caballero-Casero et al., 2021b) and online extraction techniques (López-García et al., 2019) have been proposed as alternatives as well.

A key step in sample preparation procedures involving human samples is the deconjugation of phase II metabolites. In most works, enzymatic hydrolysis is performed for the deconjugation of the metabolites into the parent compounds (Hashimoto et al., 2013; Musatadi et al., 2022; Plassmann et al., 2015), although acid (Feng et al., 2016) and basic (Ekman et al., 2013) hydrolyses have been reported as well. With that, univocal confirmation of tentatively identified suspects and their further quantification is possible with standards, since very few standards of phase II metabolites are available (Huber et al., 2021). Nevertheless, the identification of metabolites through SNTS could provide additional information for further risk assessment and a comprehensive study of the chemical exposome (Gao et al., 2022).

The bottleneck for the identification of phase II metabolites through SNTS is the generation of possible candidates for building the annotation library or suspect list (Pourchet et al., 2020). In the literature, several approaches have been used to assist in the identification of metabolites but without any harmonisation, such as manual addition of conjugate groups according to the molecular formula (Huber et al., 2022b; Kim et al., 2022), *in-vitro* incubation (Gys et al., 2018; Huber et al., 2021) and *in-silico* prediction (Djoumbou-Feunang et al., 2019; Meijer et al., 2021). In that context, the present work aimed to evaluate several sample preparation procedures (i.e. SPE, LLE, SALLE and DS with and without enzymatic hydrolysis) for the analysis of the exposome using ultra-high-performance liquid chromatography (UHPLC) coupled to HRMS. To that end, a SNTS workflow has been employed for annotating simultaneously xenobiotics and their *in-silico* simulated phase II metabolites excluding endogenous substances that could act as false positives.

2. Materials and methods

2.1. Reagents and solutions

A wide variety of xenobiotics in terms of usage and physicochemical properties were selected to test the sample preparation protocols. Precisely, 165 analytes consisting of 40 industrial chemicals and personal care products (PCPs), 63 biocides (mainly herbicides, fungicides and insecticides) and 52 diverse pharmaceuticals were chosen. The list of the analytes is included in Table S1 in the Supplementary Information (SI), together with additional information on the commercial suppliers, characteristics of the compounds and UHPLC-qOrbitrap conditions. The reagents, solvents and solutions are listed in the SI.

2.2. Samples and sample preparation

In this work, synthetic and real urine samples were employed. For the preparation of the former, the instructions provided by B.A. Rocha and co-authors were followed (Rocha et al., 2016), while for the latter urine samples of 5 volunteers from the research group were collected. Briefly, the first-morning urine samples (~40 mL) were collected in glass vials, stored at 4 °C in the fridge, and treated within 24 h from collection. Informed consent was obtained from each volunteer and the samples were handled according to the indications of the Ethics Commission for Research and Teaching of the University of the Basque Country (CEISH-UPV/EHU, BOPV 32, February 17, 2014 M10 2021 124 and CEIAB-UPV/EHU, BOPV 32, 14/2/14, M30 2021 158).

In the sample preparation step, enzymatic hydrolysis using β-glucuronidase was first studied employing conditions previously optimized in the research group (Musatadi et al., 2022). After hydrolysis, SPE, LLE, SALLE, and DS were tested. In the case of SPE, a procedure previously optimized by the research group for the analysis of endocrine-disrupting compounds and other exposome-related xenobiotics in urine using Oasis HLB (6 mL, 200 mg, 30 μm, Waters) polymeric cartridges was used with minor modifications (Musatadi et al., 2022). In LLE, ethyl acetate (EtOAc) and methyl *tert*-butyl ether (MTBE) were tested as extractants. In SALLE, acetonitrile (ACN) was also employed besides EtOAc and MTBE and it was carried out by the addition of QuEChERS salts to urine samples before the extraction. Lastly, three dilution ratios (i.e. 1:2, 1:10 and 1:20, expressed as V_{urine}:V_{total}) were tested in DS. The details of the procedures are described in SI.

2.3. UHPLC-qOrbitrap analysis

Analyses were performed in a Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific) coupled to a high-performance Q Exactive

Focus Orbitrap (qOrbitrap, Thermo Fisher Scientific) mass analyser with a heated electrospray ionization source (HESI, Thermo Fisher Scientific). The chromatographic separations were carried out at pH 2.5 and 10.5 by injecting 7 μL of the extract in an ACE UltraCore 2.5 SuperC18 (2.1 mm × 100 mm, 2.5 μm) column with an UltraCore Super C18 UHPLC precolumn (both purchased from Avantor) at 35 °C and using a constant flow of 0.3 mL/min. The gradient elutions, the HESI parameters and qOrbitrap conditions for the Full Scan - data dependent MS2 (Full

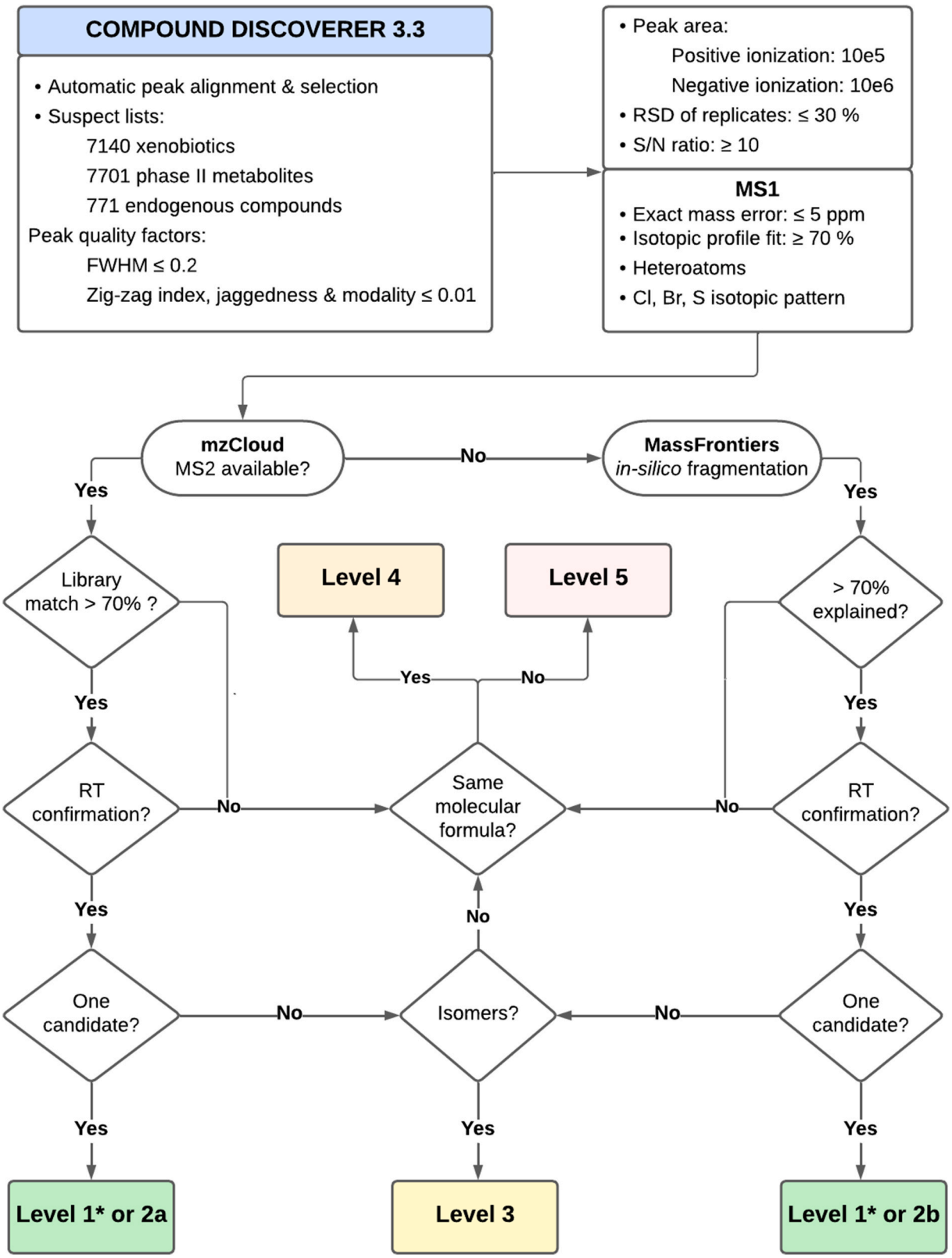

Fig. 1. Suspect and non-target screening workflow for the annotation of xenobiotics and phase II metabolites in human urine. Level 1 is only confirmed with a pure standard while the Retention Time Indices Platform (http://rti.chem.uoa.gr/) is used for retention time (RT) confirmation.

MS-ddMS2) discovery acquisition mode for both positive and negative ionization are described elsewhere (Musatadi et al., 2022).

2.4. Data analysis

The data obtained from UHPLC-qOrbitrap was processed using (i) target analysis and (ii) SNTS.

2.4.1. Target analysis

Target analysis was carried out to define the best conditions for LLE, SALLE and DS and to compare results get using all sample-treatment protocols including SPE. For that, synthetic urine samples spiked with the target 165 xenobiotics were employed. For compounds' quantification, Trace Finder 5.1 (Thermo Fischer Scientific) software was used, which contains a database including the retention time, molecular formula and characteristic fragments of each analyte (see Table S1). The retention time (RT) window was set to 0.2 min, a maximum mass error of 5 ppm was deemed acceptable for both monoisotopic mass and fragments, and a 70% fitting for the experimental and theoretical isotopic pattern was permitted.

To compare each protocol, recoveries and matrix effects were studied (Matuszewski et al., 2003). In the cases of SPE, LLE, and SALLE, 1 mL urine samples were spiked with 20 ng of all analytes (A) before and (B) after extraction. Besides, (C) a reference standard was prepared in a matrix-free solvent with 20 ng of all targets. The evaluation parameters were calculated using chromatographic peak areas as follows: (i) extraction recoveries (%): (A/B) x 100, (ii) absolute recoveries of the entire method (%): (A/C) x 100 and, (iii) matrix effect at the detection (%): ((B/C)-1) x 100, being negative values indicative of signal suppression and positive values of signal enhancement.

Those quality parameters were calculated differently in the case of DS since there are no potential losses or extraction deficiencies. In DS extracts, the signal difference between the measured and standard should be attributed to the matrix effect. To calculate it, 0.5 mL synthetic urine samples were spiked after dilution according to the tested dilution ratios (i.e., 1:2, 1:10 and 1:20) to have a nominal concentration of 20 ng/mL. However, to assess the risk of sample dilution in terms of quantification limits, other assays were run with urine samples spiked at the same initial concentration (20 ng/mL) and diluted afterward. The chromatographic signals obtained from each set of experiments were compared with standards at the same concentration, as previously stated.

2.4.2. Suspect and non-target screening

For SNTS of xenobiotics and phase II metabolites in real human urine samples, a previously validated workflow (Musatadi et al., 2022) was employed using Compound Discoverer (CD) 3.3 software (Thermo--Fischer Scientific) with minor changes. Suspect lists were obtained from the Norman Network (NORMAN Suspect List Exchange (norman-network.com)) and included cosmetics, bisphenols, surfactants, biocides, exposome biomarkers, potential persistent and mobile toxicants, neurotoxicants, plastic additives, European food safety priority substances and chlorination by-products. The merged and curated suspect list contained 7089 xenobiotics, which are included in Table S2 in the SI. In addition, the tentative phase II metabolites of all the previous compounds were simulated using the BioTransformer 3.0 software (https://biotransformer.ca/), obtaining a suspect list of 7701 phase II metabolites (see Table S3 in the SI). Fig. 1 shows the workflow followed for annotation and the criteria used for assigning levels 1–5 of identification according to the Schymanski scale (Schymanski et al., 2014). A brief explanation of the workflow is included in SI.

2.5. Quality control/Quality assurance (QC/QA)

The quality of the data and observations found in this work was assured with several measures. Regarding data acquisition, blank solvents (i.e., MeOH) were injected every 6 injections to avoid any carryover effect and a standard solution at 75 ng/mL was injected every 12 injections to get information about retention time drifts and chromatographic signal variabilities along the sequence. Moreover, the mass analyser was externally calibrated every three days using Pierce LTQ ESI (Thermo Fisher Scientific) calibration solutions.

In the case of targeted analysis, procedural blanks (i.e., non-spiked synthetic urine samples) were processed and the signals in blanks were subtracted from the corresponding signals detected in spiked samples. All samples were processed in triplicate and a maximum relative standard deviation (RSD) of 30% was accepted for quantification. Only recovery and matrix effect values within the 10%–190% and (-90%) – 90% ranges, respectively, were accepted to get conclusions during the assessment of sample preparation approaches.

In SNTS, replicates with RSD values above 30% and a ratio with respect to the procedural urine blanks lower than 10 were discarded to minimize the identification of artifacts. Additionally, several peak quality factors were implemented to discard features with unacceptable chromatographic peak shapes:

(i) Full width at half maximum (FWHM), which compares the peak width at half-maximum height to the peak width at its base, less than or equal to 0.2
(ii) Zig-zag index, which captures the quality of the chromatographic peak shape by measuring the normalized variance between each point on the peak trace and its immediate neighbour on either side, less than or equal to 0.01
(iii) Jaggedness, which captures the quality of the chromatographic peak shape by calculating the number of changes in direction over the length of the intensity vectors, less than or equal to 0.01
(iv) Modality, which measures the biggest unexpected change in direction of intensity to detect splitting and integration of multiple chromatographic peaks, less than or equal to 0.01.

Moreover, if the candidates did not contain at least an O, Cl, N, Br, S, Si and/or F atom in their structure besides C and H atoms were also eliminated, and if the chemical formula contained halogens, the characteristic isotopic pattern of those elements was checked in the MS1. Besides, if the suspect compound was identified as a possible phase II metabolite, a neutral loss corresponding to the chemical group should be present in the MS2 fragmentation spectra.

Furthermore, to discard natural endogenous compounds from the data collected, a list of 769 endogenous urine metabolites was obtained from Human Metabolome Database (HMDB, https://hmdb.ca/, see Table S4 in SI). All the criteria for annotation were also considered for the endogenous suspects to avoid false positives. When for a given feature, if an endogenous candidate passed all the criteria, that feature was discarded even if an exogenous candidate for that feature passed the criteria as well.

3. Results and discussion

3.1. Multitarget analysis

3.1.1. Optimization of LLE and SALLE

LLE and SALLE procedures were optimized using synthetic urine samples spiked with the 165 xenobiotics. The extraction recoveries and the matrix effects at detection of the LLE and SALLE procedures using the different solvents tested are summarized in Fig. 2. Using pure EtOAc and MTBE as extractants rendered the best results in terms of recoveries and salt addition caused a considerable salting-in effect as can be seen in Fig. 2a. Although the addition of salts is prone to improve the extraction of polar compounds (i.e., salting-out effect), the salting-in effect has also been described for compounds with log D values higher than 3.5 for other extraction techniques (Prieto et al., 2010).

Different hypotheses have been proposed to explain the observed

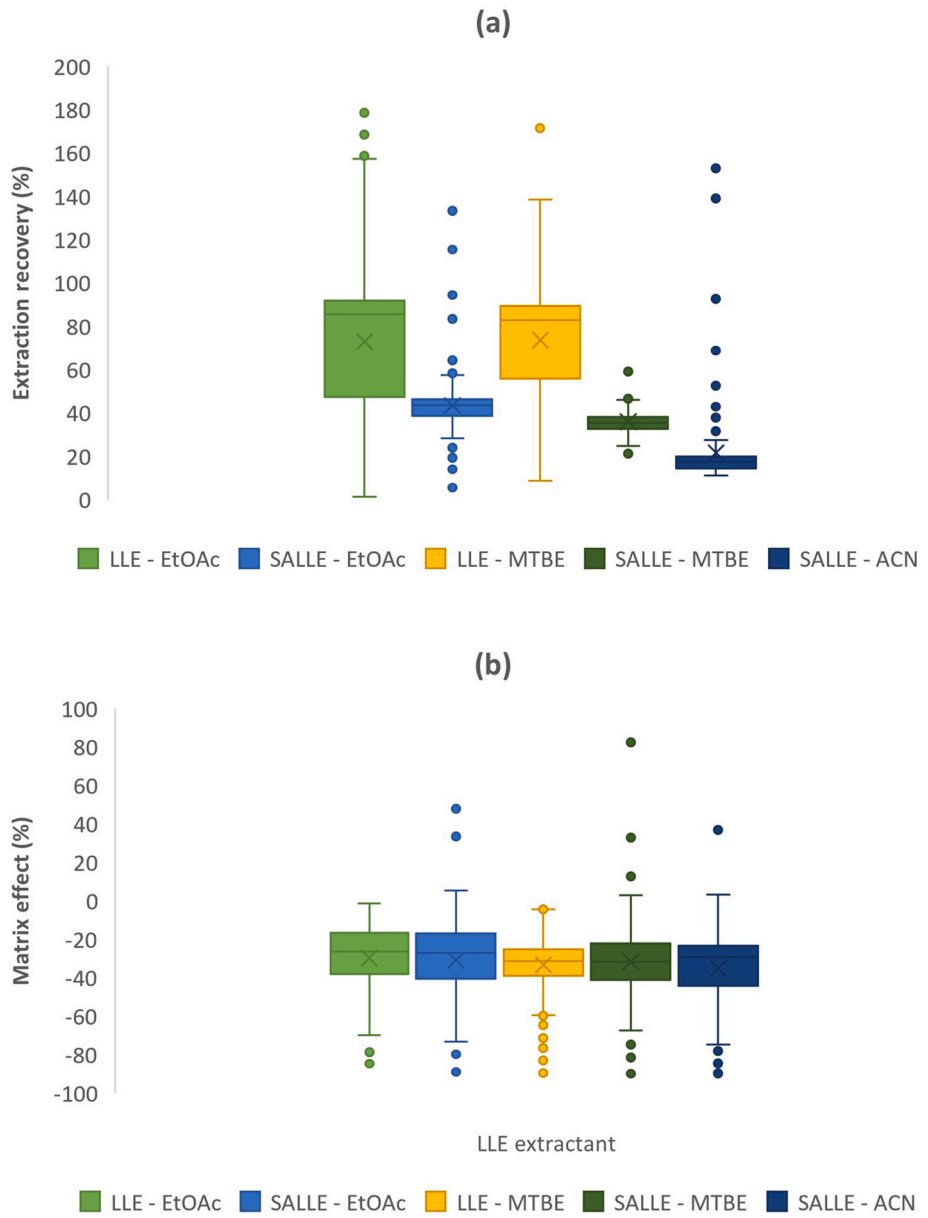

Fig. 2. Boxplots of the results obtained during LLE and SALLE optimization: (a) extraction recovery (%) and (b) matrix effect (%) for each of the tested conditions using spiked synthetic urine. The "x" indicates the mean value, the bar the median and the dots outside the boxes are outliers.

salting-in effect: (i) the occupation of the superficial area of the acceptor phase with salt ions, which decreases the superficial area available to interact with the analytes, and (ii) the reduction of the extraction efficiency of analytes due to electrostatic or ion-pairing interactions between the solutes and the salt, which reduce the ability of the analytes to move. Regarding the matrix effects shown in Fig. 2b, although negative matrix effects were observed regardless of the technique or organic solvent used, the ion suppression was not very significant ((−50)-(-25) % range).

In terms of the detected compounds, EtOAc rendered better results than MTBE since a larger number of compounds were detected (i.e., 80% and 72% of the spiked target compounds, respectively). However, several compounds were only detected using MTBE or ACN, or adding salts to urine samples, such as plasticizers (monobutyl phthalate, mono-(2-ethyl-5-hydroxyhexyl) phthalate and 2,6-di-*tert*-butyl-4-(dimethylaminomethyl) phenol), several biocides (benzothiazole, fenpropidin, propamocarb, quinoxyfen and spiroxamine) and pharmaceuticals (azelastine, glimepiride, irbersartan, pyrantel and sulfamethoxazole).

Lastly, some xenobiotics were not detected with any of the protocols tested due to extraction deficiencies and/or strong matrix effects, precisely, industrial chemicals and PCPs (benzyl paraben, PFOSA and cotinine), biocides (bentazone, crotamiton, methiocarb, pirimicarb, prosulfocarb and quinmerac) and pharmaceuticals (atenolol, bupropion, eprosartan, gabapentin, metformin, pindolol, ranitidine, sotalol and valsartan).

3.1.2. *Optimization of DS*

DS approach was tested as a non-selective extraction protocol to check whether more compounds could be detected although a larger influence of the interferences present in the matrix is expected. Based on the matrix effects detected in diluted synthetic urine samples spiked at 20 ng/mL, signal suppression was observed in all dilution ratios due to the abundant salts in urine that interfered with the ionization (Figure S1 in the SI). However, that effect was less evident with the highest dilution ratio since the matrix effects were in the (−8) – (−33) %, (−13) – (−36) % and (−24) – (−47) % ranges for the 1:20, 1:10 and 1:2 dilutions,

respectively.

Nevertheless, high dilution of urine samples may result in the detection of compounds found at very low concentrations in urine. In fact, when urine was spiked at 20 ng/mL and diluted afterward, only 72% of the target xenobiotics were quantified in the less diluted urine samples (1:2 ratio), while less than 10% of the compounds were detected in the higher tested dilution ratios (1:10 and 1:20). Based on all those observations, DS approach was limited only for determining compounds at relatively high concentrations in urine samples.

3.1.3. Comparison of SPE, LLE and DS

The absolute recoveries get using the optimum conditions for LLE (i.e. EtOAc as extractant) and DS (i.e. 1:2 dilution ratio) were compared with those get using the more selective SPE sample treatment. As can be seen in Fig. 3, while DS showed the highest absolute recoveries due to the simplicity of the sample treatment, SPE rendered the best results in terms of the number of detected compounds, which is key for exposome analysis. Precisely, some industrial chemicals (MEHHP, PFOSA, 2,6-di-*tert*-butyl-4-(dimethylaminomethyl)phenol and benzothiazole), biocides (quinoxyfen and spiroxamine) and pharmaceuticals (atenolol, azelastine, eprosartan, irbersartan, ranitidine and sulfamethoxazole) were only recovered with SPE.

Even though SPE using Oasis HLB cartridges might seem the most adequate sample preparation method for SNTS of similar exposome-related xenobiotics in urine samples, some compounds were not detected using SPE as sample treatment but only using LLE and DS. In fact, 16 analytes (2-ethylhexyl-4-dimethylaminobenzoate, 2-isopropylthioxanthone, chloridazon, fenthion, imazalil, metazachlor, prosulfocarb, pyrazophos, gabapentin, glycitin, indomethacin, metformin, sulfathiazole, telmisartan, terbinafine and valsartan) were only detected with LLE using EtOAc and/or DS.

3.2. Suspect and non-target screening

3.2.1. Annotation of xenobiotics and phase II metabolites

To get the widest analytical coverage in human exposome assays, non-spiked human urine samples (n = 5) were also measured following a non-targeted approach. That non-targeted strategy includes monitoring 7089 xenobiotics and 7701 phase II metabolites from the built suspect list as explained in section 2.4.2.

Besides the procedures used in the targeted analysis (i.e., SPE, LLE using pure EtOAc and DS with the 1:2 ratio), the use of SALLE with EtOAc and ACN was also considered in the non-targeted analysis approach since real urine samples contain compounds (e.g. phase II metabolites) that could be more polar than the tested compounds in target analysis. DS was only applied to non-hydrolysed urine samples, but the rest of the sample preparation methods were evaluated for both non-hydrolysed and hydrolysed urine samples.

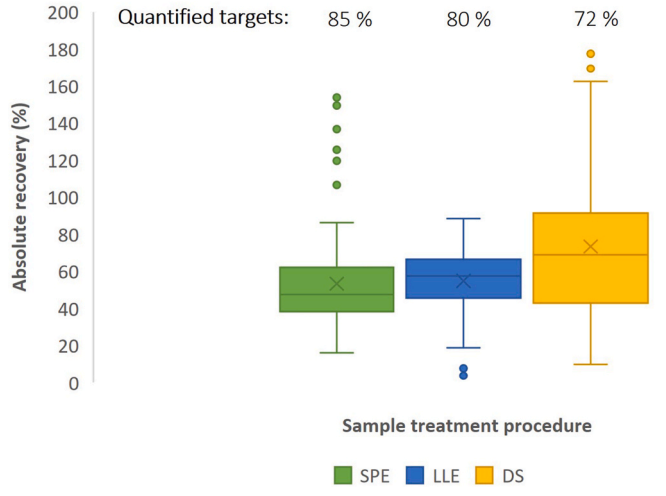

Fig. 3. Boxplots of the absolute recoveries obtained for SPE, LLE and DS in spiked synthetic urine. The "x" indicates the mean value, the bar the median and the dots outside the boxes are outliers.

Features annotated following the previously validated SNTS workflow (Musatadi et al., 2022) (Fig. 1) are included in Tables S5 and S6 in SI for positive and negative ionization, respectively, together with the information used for annotation, confidence-level (1–4) and their presence or absence in 5 urine samples treated with the 9 different sample treatments.

Overall, 70% of the total annotated features were screened at positive ionization specially because of the 220 parent xenobiotics annotated at that mode compared to only 17 at negative mode. Standards were only used for identification purposes, and not for quantification, as this work aimed to evaluate the adequacy of sample pre-treatment strategies that allow the largest chemical coverage. As examples of identified chemicals using standards, parabens (methyl, ethyl and butylparaben), industrial chemicals (benzophenone-3, caprolactam and mono-2-ethyl-5-hydroxyhexyl phthalate) and chemicals related to daily habits (caffeine, cotinine or triclosan) could be highlighted.

Regarding phase II metabolites, glucuronides (74) and sulphates (46) were mostly annotated in the samples. In addition, glycine by-products (26) were also screened since amino acid conjugation is also a form of elimination of xenobiotics containing carboxylic groups (Janov and iller, 2012). For some of the phase II metabolites annotated at level 4, when all the candidates, despite not being isomers, contained the same specific chemical group (i.e., glucuronide, sulphate or glycine) allowed us to define the type of phase II metabolite. An example of the identification of each type of metabolite is included in Figures S2 – S4in the SI. In the case of the glucuronide example, the neutral loss corresponding to glucuronic acid is also included(Figure S2b).

Unlike in positive ionization mode, negative ionization allowed the annotation of almost twice (97) as many metabolites as in positive mode (49) due to the higher number of glucuronides and especially sulphates screened. In fact, only 1 sulphate was annotated at positive ionization at level 4 against 45 in negative (23 at level 2b). That outcome is linked to the fact that the acidic conjugate groups are fully deprotonated at pH 10.5 providing an intense signal at the ESI.

Moreover, the *in-silico* simulation of the metabolites for building the suspect list turned out to be compulsory for their annotation due to the absence of those types of metabolites in spectral libraries. Consequently, from all the annotated metabolites at level 2, only 1 sulphate in the negative mode was identified at level 2a using the mzCloud library, with the rest (45) annotated at level 2b. Despite the *in-silico* prediction has been less used in the literature (see Table 1), it is a much more straightforward approach since it does not require individual interpretation of the xenobiotics to evaluate their suitability for metabolization.

3.2.2. Sample preparation for SNTS

To perform a comprehensive discussion about which sample preparation approach allowed the widest analytical coverage, the SNTS data was analysed using Principal Component Analysis (PCA). PCA was performed with the features annotated in levels 1–4 considering the samples as scores, and mean centred and scaled chromatographic areas as loadings. Fig. 4a and b include the scores and loadings plots, respectively, for the first two principal components (PC1 vs PC2) using all the suspects annotated using the positive ionization mode.

Observing the score plot in Fig. 4a, all samples treated by SPE tended to group in the negative side of PC1, separated from the rest of the protocols due to the larger number of suspects with higher areas annotated (see loadings plot in Fig. 4b, where each dot corresponds to an annotated suspect). In fact, almost 35% of the whole variance was explained by PC1. Regarding PC2, it explained the differences among volunteers but it was not further investigated and discussed since it was out of the scope of the present work.

Table 1
Summary of the suspect and non-target screening (SNTS) works in the literature for human urine.

Suspects	Sample preparation		Analysis	Metabolites in the suspect list	Reference
	Enzymatic hydrolysis	Extraction/Clean-up			
Xenobiotics	Yes (β-glucuronidase) No	SPE (Oasis HLB 200 mg) LLE (EtOAc) SALLE (EtOAc, ACN) DS	UHPLC-HESI-Q-Orbitrap (+ and -)	*In-silico* prediction of phase II metabolites (glucuronides, sulphates, glycines) using BioTransformer 3.0	This work
Chemicals of emerging concern	No	Captiva Non-Drip Lipids	UHPLC-ESI-Q-TOF (+ and -)	Manual addition or subtraction of groups: Phase I (oxidation, reduction hydrolysis) and phase II (glucuronidation, acetylation, methylation, sulfonation and glutathione addition) metabolites	Caballero-Casero et al. (2021b)
Chemicals of emerging concern	No	Captiva Non-Drip Lipids	UHPLC-ESI-Q-TOF (+ and -)	Manual addition or subtraction of groups: Phase I (hydroxylation) and phase II (glucuronidation, methylation) metabolites	Roggeman et al. (2022)
Biomarkers	Yes (β-glucuronidase)	SPE (Oasis HLB 60 mg 96-well plates)	UHPLC-HESI-IonTrap-Orbitrap (+ and -)	Phase I metabolites from Harmonized SPECIMEN study	(Tkalec et al., 2022a, 2022b)
Pesticides	No	SPE (Oasis HLB 60 mg 96-well plates)	UHPLC-HESI-Q-Orbitrap (+ and -)	Phase I metabolites from literature and manual addition of phase II metabolites (sulphates, glucuronides)	(Huber et al., 2022b; Ottenbros et al., 2023)
Environmental pollutants	Yes (β-glucuronidase)	Direct injection QuEChERS (SALLE with ACN)	UHPLC-HESI-IonTrap-Orbitrap (+ and -)	Not included	Plassmann et al. (2015)
Pesticides	No	Dilution	UHPLC-ESI-IonTrap-Orbitrap (−)	Transformation products and phase II metabolites from the literature	Bonvallot et al. (2021)
Endocrine disruptors	Yes (β-glucuronidase)	SPE (Oasis HLB 200 mg)	UHPLC-HESI-Q-Orbitrap (+ and -)	Not included	Musatadi et al. (2022)
Pesticides	No	SPE (Strata-X 40 mg 96-well plates)	UHPLC-HESI-Q-Orbitrap (+ and -)	Phase I metabolites from the literature and *in-silico* prediction using BioTransformer	Huber et al. (2021)
All glucuronides (non-target)	No	SPE (Strata-X 40 mg 96-well plates)	UHPLC-HESI-Q-Orbitrap (+) UHPLC-ACPI-Q-TOF (+)	Not included but parents identified by neutral loss screening, spectral modification and spectral library search	Huber et al. (2022a)
Plasticizers	No	Dilute and shoot	UHPLC-ESI-Q-TOF (+ and -)	Phase I metabolites from in-vitro experiments and *in-silico* simulation of phase II metabolites (sulphates, glucuronides) using Meteor Nexus	Christia et al. (2022)
Pesticides	Yes (β-glucuronidase-arylsulfatase)	QuEChERS (SALLE with ACN)	UHPLC-HESI-Q-Orbitrap (+ and -)	Pesticide phase I metabolites from the literature and previous datasets	López et al. (2016)
Persistent and mobile chemicals and per- and poly-fluoroalkyl substances	No	LLE (ammonia addition)	UHPLC-ESI-Q-TOF (+ and -)	Manual generation of phase I and phase II metabolites	Kim et al. (2022)

Abbreviations: SPE: solid phase extraction, LLE: liquid-liquid extraction, EtOAc: ethyl acetate, SALLE: salt-assisted LLE, ACN: acetonitrile, DS: dilute-and.-shoot, HESI: heated electrospray ionization, UHPLC: ultra-high-performance liquid-chromatography, Q: quadrupole, +: positive ionization, -: negative ionization, QuEChERS: Quick, Easy, Cheap, Effective, Rugged and Safe.

A similar pattern was observed for the features annotated at levels 1 to 4 in the negative mode (see Figure S5). Furthermore, the same PCA approach was run but only considering phase II metabolites. The same trend was also observed in the scores and loadings plots in Figures S6a and S6bfor positive ionization, respectively since PC1 is related to the features of the largest chromatographic areas in urine samples treated with SPE. In the case of negative ionization, PCA was not performed with only metabolites since 85% of the total annotated features were phase II metabolites so the PCA would hardly differ from the one shown in Figure S5.

Overall, 91% and 93% of the total annotated features could be screened with SPE in the non-hydrolysed and hydrolysed samples, respectively. The second-best extraction protocols were LLE using EtOAc in non-hydrolysed samples and SALLE employing ACN in hydrolysed samples. However, only 74% of the total annotated features were annotated in both cases. Moreover, detection frequencies of the features were calculated for each sample treatment with and without hydrolysis. In the non-hydrolysed samples, 40.5% of the total features were detected at least in 80% of the samples extracted with SPE, followed by 32.4% in LLE using EtOAc. In the rest of the cases, less than 14% were annotated at that frequency. In the samples submitted to hydrolysis, a similar pattern was followed since 49.1% of features were frequently present (>80%) in urine samples processed with SPE. In the rest of the procedures, only 16.4% or less were present in most samples.

Considering all the mentioned outcomes, and specially owing to the large amount of the compounds detected (97.1% of the total features considering hydrolysed and non-hydrolysed samples), SPE using Oasis HLB was shown to be the most adequate pre-treatment for SNTS of xenobiotics in human urine. The hybrid nature of the SPE cartridge allows the retention of a wider range of analytes including polar metabolites. In the literature (see Table 1), SPE using polymeric cartridges has also been successfully applied for SNTS in urine for a diversity of xenobiotics, especially in 96-well plates to process a large number of samples (Huber et al., 2021, 2022a; Tkalec et al., 2022a, 2022b). Nevertheless, SALLE

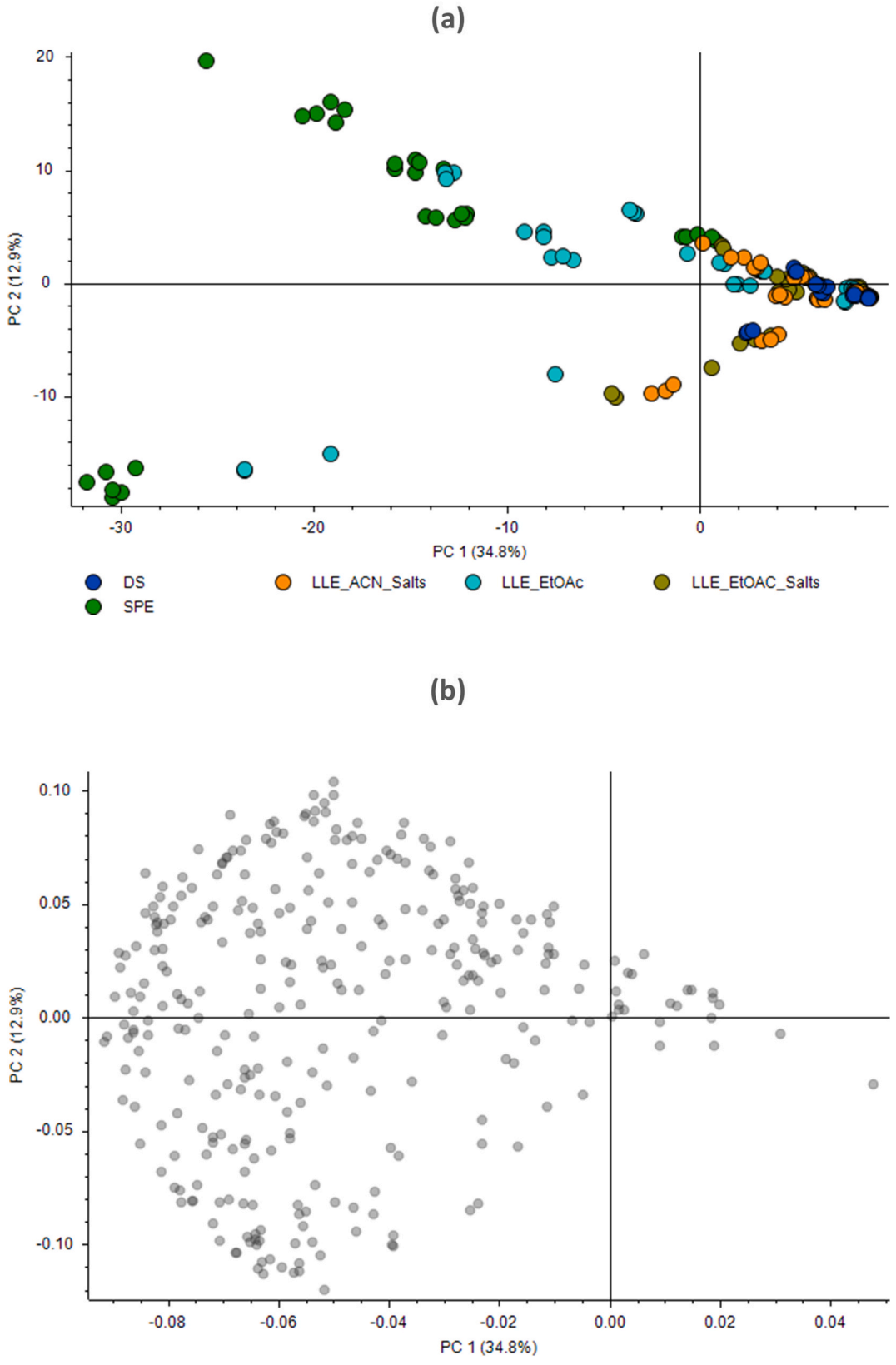

Fig. 4. PCA results considering the features annotated at levels 1–4 in the positive ionization mode: (a) scores and (b) loadings for PC1 vs PC2.

using ACN (López et al., 2016; Plassmann et al., 2015), LLE with ammonia addition (Kim et al., 2022), DS (Bonvallot et al., 2021; Christia et al., 2022; Plassmann et al., 2015) or even Captiva Non-Drip Lipid filters for phospholipids removal (Caballero-Casero et al., 2021b; Roggeman et al., 2022) have been employed in SNTS in the urine although no comparisons have been made from a SNTS perspective.

3.2.3. Evaluation of enzymatic hydrolysis

To evaluate the complementarity of hydrolysed and non-hydrolysed sample pre-treatment approaches, the results were further investigated using PCA. While PC1 vs PC2 plots explained the separation between extraction protocols as discussed in the 3.2.2 section, PC1 vs PC3 described the effect of the hydrolysis step in the compounds detected in each sample, as can be observed in Fig. 5 for positive ionization mode. In the case of the negative ionization mode, that grouping pattern was not observed. However, it should be mentioned that besides fewer features annotated at negative mode, especially parent compounds (17 out of 114), a high number of sulphates was screened (45) and the enzyme used only contained glucuronidase activity and not sulfatase (see Tables S6 in the SI).

In the literature, both hydrolysis and non-hydrolysis approaches have been carried out in SNTS works as compiled in Table 1. In target analysis, despite total and free concentrations of xenobiotics being commonly provided (González et al., 2019), no work has shown the complementarity of the analysis of hydrolysed and non-hydrolysed samples from a SNTS view and parent/metabolite identification. In this work, the parent compounds of the annotated metabolites in the non-hydrolysed samples were searched in the corresponding hydrolysed samples. To that end, the exact mass of the conjugate group (176.03209 for glucuronic acid loss, 79.95682 for sulphate loss and 57.02146 for glycine loss) was subtracted to the corresponding metabolite and the feature of that exact mass was searched in the feature list provided by the software before the application of the SNTS workflow. Only features at levels 1 to 3 were considered for discussion.

From the total 44 glucuronides annotated at levels 2b and 3, only 10 corresponding parent xenobiotics were identified in the hydrolysed samples. Those compounds were 1,2,3,4-tetrahydronaphthalenol, 3-hydroxy-4,7,7-trimethylbicyclo[2.2.1]heptan-2-one, 1-(2,6,6-Trimethyl-2-cyclohexen-1-yl)butane-1,3-dione, chrysin, daidzein, genistein, propylparaben, O-desmethylnaproxen, triclosan, benzophenone-3 and (3Z)-hex-3-en-1-yl salicylate. For the rest of the parent compounds, several scenarios occurred, such as, (i) the peak could not be detected (13 suspects), the molecular formula could not be calculated (4 suspects), MS2 match was lower than 70% (14 suspects) and retention time did not correspond with the parent (3 suspects) considering that the polar metabolite elutes earlier than the respective parent compound.

In some cases, the enzymatic hydrolysis was not quantitative since glucuronide residues were also screened in hydrolysed samples. That observation suggests that enzymatic hydrolysis needs to be further studied for SNTS approaches instead of optimizing the reaction variables for a selected and very reduced number of glucuronides. In other cases, although the deconjugation was quantitative, the parent xenobiotic was not annotated. Those cases are closely related to the poor signal obtained for some parent compounds, especially for phenolic compounds. Consequently, the quality of the MS1 and/or MS2 was not good enough to elucidate the final structure. In the cases of their metabolites, the addition of the ionizable group provided a higher signal at the ESI and, therefore, the good quality of the MS1 and MS2 allowed the proper annotation of the suspect. An example of that observation is included in Fig. 6 for the satisfactory annotation of 2,6-di-*tert*-butyl-4-(hydroxymethyl)phenol glucuronide (pK_a 3.8) but not for the parent xenobiotic (pK_a 9.6).

In the case of the 16 glycine metabolites annotated at level 2b in the positive ionization mode, 6 corresponding parents were annotated at levels 1, 2a and 2b, and, for the rest, not even the peak could be detected. On the contrary, for the 32 sulphates annotated in the negative ionization mode, only 1 parent was screened. In those cases, the non-detection of the parent xenobiotics should be attributed to the enzyme since it only contained glucuronidase activity. All in all, the non-simulation of the metabolites may result in an important information loss in human exposome studies, and in that sense, the screening of metabolites in non-hydrolysed samples seems more suitable for exposome evaluation. Consequently, semi-quantification strategies without pure standards should be addressed in future works for phase II metabolites.

4. Conclusions

From the multi-target analysis assays, it was concluded that SPE using Oasis HLB cartridges was the best extraction technique for the simultaneous detection of 165 diverse xenobiotics. In the cases of LLE, SALLE and DS, each sample preparation technique showed different

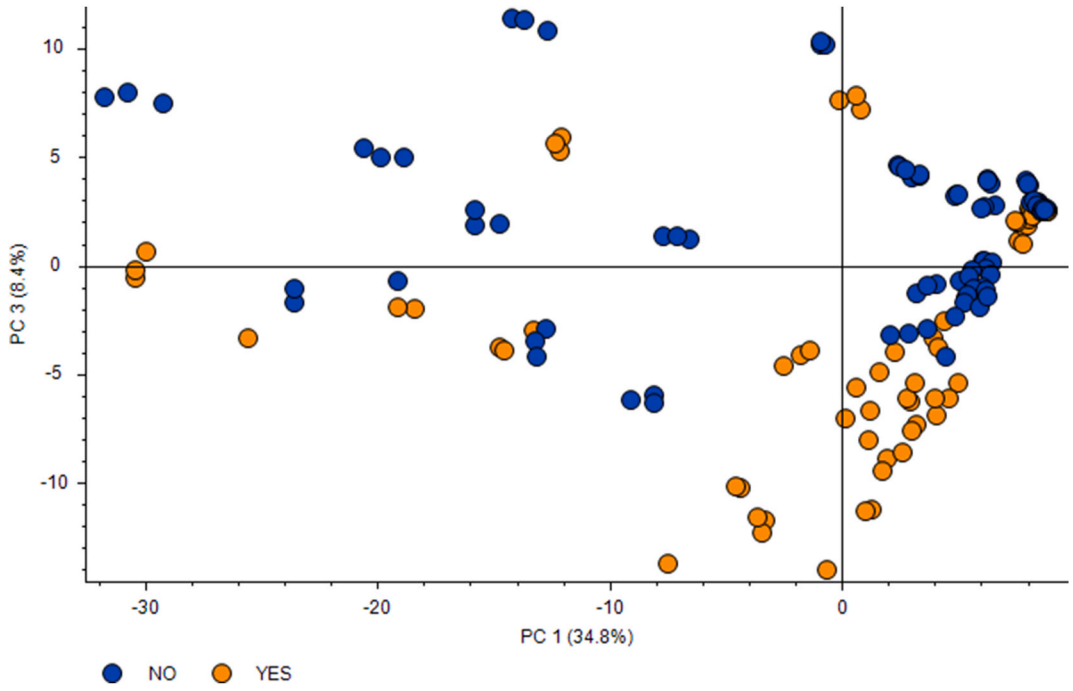

Fig. 5. Scores diagram for PC1 vs PC3 considering the features annotated at levels 1–4 in the positive ionization mode. Blue dots in the scores diagram corresponds to non-hydrolysed samples (NO), while orange dots are samples submitted to hydrolysis (YES).

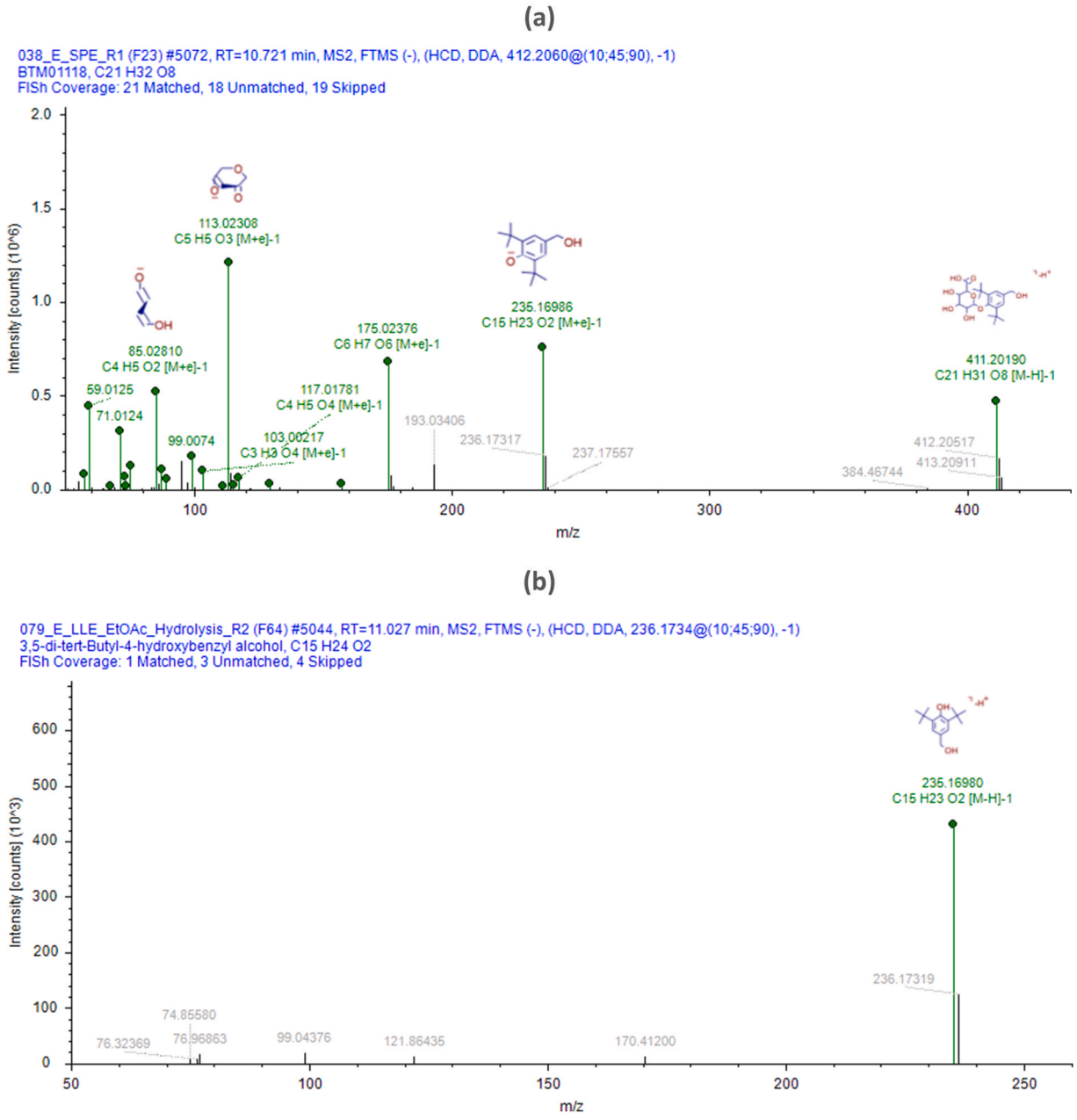

Fig. 6. MS2 spectra of (a) 2,6-di-*tert*-butyl-4-(hydroxymethyl)phenol glucuronide annotated at level 2b and (b) 2,6-di-*tert*-butyl-4-(hydroxymethyl)phenol not explained by *in-silico* fragmentation due to poor intensity and fragmentation.

limitations in terms of analytical coverage of low-concentration level xenobiotics. The same tendency was observed when xenobiotics and their respective phase II metabolites were screened in real urine samples treated with SPE, LLE, SALLE and DS in a SNTS approach. In fact, PCA analysis demonstrated that samples treated with Oasis HLB SPE cartridges differed from the rest because of the higher number of suspects with larger chromatographic areas. In terms of enzymatic hydrolysis, the annotation of metabolites in non-hydrolysed samples was performed with ease in comparison to parent xenobiotics in hydrolysed samples. It was concluded that the ionizable nature of the conjugate group resulted in a higher signal in ESI, and consequently, good-quality spectra were obtained that permitted the elucidation of the molecular structure of the metabolites. In that sense, SNTS of non-hydrolysed samples seems more suitable for exposome analysis. For further studies, since the screening of metabolites using Oasis HLB cartridges in non-hydrolysed urine samples seems the most suitable strategy for exposome analysis, semi-quantification techniques of the metabolites could be considered. Additionally, the quantitativity of the hydrolysis should be evaluated for SNTS approaches to unequivocally identify the suspect, and subsequently quantify it using pure standards if aimed.

Credit author statement

Mikel Musatadi: Methodology, Software, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. **Asier Andrés-Maguregi**: Software, Validation, Formal analysis, Investigation, Writing - Original Draft. **Francesca De Angelis**: Methodology, Software, Writing - Original Draft. **Ailette Prieto**: Resources, Supervision, Project administration. **Eneritz Anakabe**: Data Curation, Investigation. **Maitane Olivares**: Methodology, Supervision, Data Curation, Writing - Review & Editing. **Nestor Etxebarria**: Resources, Project administration, Funding acquisition. **Olatz Zuloaga**:

Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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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.2023.139690.

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