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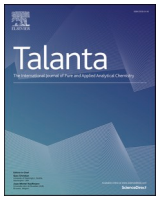

Sample preparation for suspect screening of persistent, mobile and toxic substances and their phase II metabolites in human urine by mixed-mode liquid chromatography

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ABSTRACT

Persistent, mobile and toxic substances have drawn attention nowadays due to their particular properties, but they are overlooked in human monitorization works, limiting the knowledge of the human exposome. In that sense, human urine is an interesting matrix since not only parent compounds are eliminated, but also their phase II metabolites that could act as biomarkers. In this work, 11 sample preparation procedures involving pre-concentration were tested to ensure maximum analytical coverage in human urine using mixed-mode liquid chromatography coupled with high-resolution tandem mass spectrometry. The optimized procedure consisted of a combination of solid-phase extraction and salt-assisted liquid-liquid extraction and it was employed for suspect screening. Additionally, a non-discriminatory dilute-and-shoot approach was also evaluated. After evaluating the workflow in terms of limits of identification and type II errors (i.e., false negatives), a pooled urine sample was analysed. From a list of 1450 suspects and *in-silico* simulated 1568 phase II metabolites (i.e. sulphates, glucuronides, and glycines), 44 and 14 substances were annotated, respectively. Most of the screened suspects were diverse industrial chemicals, but biocides, natural products and pharmaceuticals were also detected. Lastly, the complementarity of the sample preparation procedures, columns, and analysis conditions was assessed. As a result, dilute-and-shoot and the Acclaim Trinity P1 column at pH = 3 (positive ionization) and pH = 7 (negative ionization) allowed the maximum coverage since almost 70 % of the total suspects could be screened using those conditions.

1. Introduction

Contaminants of emerging concern (CECs) have been the main focus in recent years due to their continued emission and potential ecotoxicological threats [1]. Despite their occurrence in environmental samples and human matrices, most monitorization studies rarely include very polar compounds which tend to be mobile (organic carbon-water coefficient, log K_{oc}, over the pH range of 4–9 less than 4.0 [2]) in aqueous compartments [3,4]. In addition, some mobile compounds could be persistent due to their elevated degradation half-life times and could pose potential risks to not only biological systems but also to humans [5]. Those persistent, mobile and toxic substances (PMTs) can also accumulate in the water cycle eventually reaching drinking water as reported in the literature [6,7]. Among PMTs, chemicals from all classes and application fields can be found, for instance, pharmaceuticals, pesticides, herbicides, industrial chemicals, and even their transformation products [8].

In the context of decoding the human exposome, the analysis of human urine could have great potential for assessing the exposure to PMTs since it is the main biofluid for excreting polar compounds (including metabolites) from our organism [9–11]. However, several gaps concerning PMTs have been stated, such as modelling, monitoring, analytical, treatment, or regulatory ones, that are all linked to the very polar nature (octanol-water distribution ratio, log D_{ow}, values below 0) of those chemicals [12,13].

Well-established analytical methods based on reverse-phase liquid-

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chromatography (RPLC) for water-soluble organic contaminants cannot retain very polar and/or ionic molecules [14]. Consequently, the need for comprehensive analytical methods for the simultaneous analysis of PMTs has emerged. In that sense, other LC-based techniques, such as hydrophilic interaction LC (HILIC) [15,16], ion exchange chromatography (IEC) [17,18], supercritical fluid chromatography (SFC) [19,20] or even mixed-mode LC (MMLC) [21–23] have been employed as alternatives to RPLC. Recently, MMLC has been used for the separation of more than 33 PMTs in urine, including neutrals, acids, bases, and zwitterions [24].

Mass spectrometry (MS) is usually the selected detector in those works although it can be prone to matrix effect, especially for PMTs that are expected at low ng mL^{-1} levels in urine. Therefore, adequate sample treatment procedures are mandatory for their enrichment in a matrix-free solvent [25]. In the case of PMTs, common extraction/clean-up techniques, such as solid phase extraction (SPE) or liquid-liquid extraction (LLE) might fail to achieve the required objective [26]. In that context, alternative SPE sorbents and LLE variations have been tested to extract very polar organic compounds from aqueous samples. Regarding SPE, polymeric sorbents, ion exchangers for mixed-mode (MM), or even graphitized carbon black (GCB) based materials have been employed in mono- or multi-layer SPE approaches [27–29]. In other studies mostly related to the analysis of amino acids and proteins, HILIC-SPE has been the selected option [30,31]. In LLE extraction, a QuEChERS-based extraction approach, which is fundamentally a salt-assisted LLE (SALLE) [32] or even the addition of acids or bases to fix pH [33] have been used to enhance the transfer of the polar compounds into the organic phase. Alternatively, dilution and subsequent analysis of urine (i.e. 'dilute-and-shoot' (DS)) has been the selected option to avoid losses during sample treatment [24,34,35], although enhanced matrix effect and sample dilution could result in high detection limits [36].

In the analysis step, the power of high-resolution MS (HRMS) could have an added value in the study of the exposome. Concretely, it allows the screening of a wide range of chemicals through suspect and non-target screening (SNTS) initially without standard references [33]. Therefore, the screening of metabolites is also possible. However, the compromise between selectivity and sensitivity, and the presence of endogenous substances that could act as false positives need to be carefully evaluated in SNTS methods using biofluids [37].

Despite the potential adverse effects of PMTs on human health status, few studies can be found in the literature focused on the analysis of such compounds in human biofluids due to the mentioned challenges [33]. Therefore, the present work aimed to develop a comprehensive analytical method based on MMLC-HRMS to screen PMTs and their metabolites in human urine. To that end, up to 12 sample preparation approaches were investigated including 11 protocols based on SPE and LLE, and also DS a non-selective procedure.

2. Materials and methods

2.1. Chemicals and reagents

The list of the 37 PMTs used in this work is compiled in Table S1 as Supplementary Information A (SI-A) together with their vendors and properties. The PMTs selected were diverse in terms of chemical nature (i.e. neutrals, acids, bases, and zwitterions) with log D_{ow} values in the 3 and -7.2 range considering both pH = 3 and pH = 7. The list of the reagents and solvents is also included in SI–B.

2.2. Sample preparation procedures

Several sample preparation procedures based on SPE and LLE were studied to select the procedure that allowed the maximum PMT coverage. Regarding the SPE assays, numerous sorbents were employed in mono- and multi-layer approaches. For the former, HILIC-SPE, RP-SPE, and GCB-SPE were tested, while RP/GCB-SPE and MM-SPE were evaluated for the latter. In LLE, a QuEChERS-based SALLE procedure and the addition of an acid or base for pH control were assessed. In total, 11 different protocols involving preconcentration were tested and are detailed in the SI–B.

The optimal procedure consisted of a combination between the SPE protocol using Oasis HLB (500 mg, 6 mL, Waters, Milford, Massachusetts, USA) cartridges and the SALLE procedure. The 1 mL urine sample was diluted with 2 mL Milli-Q water and loaded into the cartridge previously conditioned using 4 mL of methanol:acetone (50:50, v/v) and 4 mL of Milli-Q water. The permeate was collected and the cartridge was dried under vacuum before eluting the analytes using 4 mL of methanol: acetone (50:50, v/v). Then, the SALLE procedure was applied to the SPE permeate. To that end, 0.5 g Na_2SO_4, 0.1 g NaCl and 3 mL acetonitrile were added to the permeate collected from SPE and the mixture was vortexed using a Multi Reax Mixer agitator from Heidolph (Schwabach, Bavaria, Germany) for 10 min at 2000 rpm. After phase separation, the organic phase was quantitatively withdrawn and combined with the SPE eluate. The combined extract was evaporated to dryness using N_2 and re-dissolved in 200 μL UHPLC-water:acetonitrile (75:25, v/v).

In addition, a non-selective DS method was also employed, which is ideally a procedure intended for SNTS. Briefly, 1 mL UHPLC-water: acetonitrile (1:1, v/v) was added to 1 mL urine and the mixture was vortexed for 1 min and centrifuged at 15 000 rpm for 10 min at 4 °C. Then, 250 μL of the supernatant was recovered and frozen at - 20 °C overnight before analysis [38].

2.3. MMLC-MS/MS

The separation of the PMTs was carried out by a 1290 Infinity II UHPLC system (Agilent Technologies) with an ACE UltraCore 5 SuperC18 (2.1 mm × 30 mm, 5 μm, Avantor) column placed between the loop and the mobile phase line to delay interfering artefacts coming from the LC system. An electrospray ionization (ESI) interface was used for mobile phase evaporation and a 6430 Triple Quadrupole (QqQ) tandem mass detector (Agilent Technologies) for the detection. The samples were only analysed with the Acclaim Trinity P2 (50 × 2.1 mm, 3.0 μm, Thermo Scientific, Waltham, Massachusetts) column that combines HILIC with a strong anion exchanger (SAX) and a weak cation exchanger (WCX). The chromatographic conditions concerning gradient separations, the ESI parameters, and Dynamic Multiple Reaction Monitoring (DMRM) mode conditions were optimized in our previous work [24] and are detailed in the SI-A in Table S2.

2.3.1. Target analysis

The data collected from the MMLC-QqQ system was only used for target analysis of the PMTs for the calculation of recoveries during method optimization. Besides the number of detected analytes, recoveries were also studied using synthetic urine [39]. To that end, samples were spiked with 50 ng of all targets before the extraction, and chromatographic areas were compared to reference standards of the corresponding final concentration depending on the preconcentration factor. In the 11 protocol comparison experiments, 33 PMTs were employed while the target list was extended to 37 PMTs in the evaluation of the optimum protocol (see Table S1 in the SI-A).

For quantification, the MassHunter Quantitative Analysis software (version 10.0 from Agilent) was used. The criteria were that (i) both *m/z* quantitative and qualitative transitions should be detected in the spiked sample, (ii) the ratio of the abundances of both transitions in the spiked samples should be no larger than 30 % in comparison to the pure standard, and (iii) the experimental retention time should be within ±0.2 min of the pure standard.

2.4. MMLC-HRMS/MS

The samples were also analysed in a Dionex UltiMate 3000 UHPLC

coupled to a Q Exactive™ Focus quadrupole-Orbitrap mass spectrometer (qOrbitrap) equipped with a heated ESI source (HESI) all from Thermo Fisher Scientific. Besides the Acclaim Trinity P2 column, the complementary Acclaim Trinity P1 (50 × 2.1 mm, 3.0 μm) column that contains a RP base with a weak anion exchanger (WAX) and a strong cation exchanger (SCX) was also employed. For the P2 column, the same chromatographic conditions as in the MMLC-QqQ system were used, while the gradient elutions used for the P1 column are described in Table S2 of the SI-A. Regarding the HESI and the Full Scan MS – data-dependent MS2 acquisition mode parameters, they were based on the research group's experience [40] and are fully described in the SI-A (Table S3).

2.4.1. Suspect screening

Suspect screening was carried out using the data acquired from MMLC-qOrbitrap and Compound Discoverer (CD) software (version 3.3 from Thermo). Suspect lists containing PMTs and drinking water contaminants (S5, S36, S63, S64, and S82) were obtained from the Norman Network (NORMAN Suspect List Exchange (norman-network.com). Those lists were merged and curated, and the final version containing 1450 suspects is included in Table S4 in the SI-A. Moreover, the tentative phase II metabolites of all the suspects were *in-silico* simulated using BioTransformer 3.0 (https://biotransformer.ca/) [41], obtaining a suspect list of 1568 phase II metabolites including glucuronides, sulphates, and glycines which are also compiled in Table S5 in the SI-A.

For suspect annotation, the CD software performed an automatic peak picking [42] and only retrieved those features that matched with the suspects included in the annotation list if the minimum peak area was 10^5, the error in the exact mass error lower than 5 ppm, and an isotopic profile fit higher than 70 %. To reduce the number of features with bad peak shapes or signals close to noise, several filtering parameters were applied. Only peaks with relative standard deviation (RSD) values below 35 %, a signal 3 times higher than urine blanks, a minimum of one heteroatom (O, Cl, N, Br, S, and/or F) in the molecular formula, and an available MS2 fragmentation spectrum were further considered. All peaks were manually evaluated and the ones not following a Lorentzian peak shape [43–45] were discarded.

For MS2 evaluation, a similarity-match higher than 70 % between the experimentally acquired MS2 and the one collected in the mzCloud library was required. If the suspect was not included in the library, *in-silico* fragmentation was performed using Mass Frontier 8.0 implemented in CD. For the *in-silico* MS2 match, 70 % of the most characteristic fragments should be explained as well. In the cases of glucuronides and sulphates, the neutral loss corresponding to the respective conjugate group should be present in the MS2 spectra. Finally, if the pure standard of the tentatively annotated suspect was available, it was used for confirmation. Lastly, annotation confidence levels were assigned from 1 to 5 based on Schymanski et al., 2014 scale [46].

2.5. Methods' performance and quality control/quality assurance (QC/QA)

The suspect screening workflow was evaluated in terms of instrumental and procedural limits of identification (iLOI and pLOI, respectively) and type II errors (i.e., false negatives) using the 37 PMTs [47]. To that end, a different set of samples was analysed by MMLC-HRMS/MS (section 2.4).

From the calibration curves, instrumental limits of identification (iLOIs) were established as the minimum concentration level that passed all the mentioned criteria for suspect screening [48,49]. From those iLOIs, corresponding procedural limits of identification (pLOIs) were estimated considering each procedure's preconcentration/dilution factor. Additionally, synthetic urine samples spiked at 50 ng mL^{-1} were processed with both the SPE and LLE combination procedure and the non-selective DS to evaluate type II errors. These errors were defined as the number of the non-annotated spiked suspects following the workflow [47].

In order to widen the chemical space and study the most suitable approach for suspect screening, 10 volunteers from the research group provided urine samples. Informed consent of all individual participants who provided the samples was obtained before conducting the experiments that 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, 17/2/2014 M10 2021 124 and CEIAB-UPV/EHU, BOPV 32, 14/2/14, M30 2021 158). Briefly, first-morning urine samples (30–40 mL) were collected in glass vials, anonymized, and pooled using equal volumes. The pooled sample was stored at 4 °C and analysed within 24 h with both methods. Procedural blanks were also processed with every set of analyses and all experiments were performed in triplicate.

Besides the previously explained filtering criteria, a list of endogenous compounds that can be found in real urine was obtained from Human Metabolome Database (HMDB, https://hmdb.ca/) and their phase II metabolites were also simulated by BioTransformer 3.0. The combined list contained 2549 endogenous suspects and was employed as an inclusion list to reduce false positives (Table S6 in the SI-A). Briefly, if an endogenous candidate passed all the criteria, that feature was discarded even if an exogenous candidate for that feature also passed the criteria.

In the analytical sequences, blank UHPLC-water:acetonitrile (75:25) samples were introduced every 6 samples to check for possible carryover or background contamination, while the 100 ng mL^{-1} calibration point was also injected throughout the sequence every 12 samples to study RT shifts and signal intensity stability. The QqQ and qOrbitrap mass analyzers were externally calibrated every 3 days using ESI-L Low Concentration Tuning Mix (Agilent Technologies) and Pierce LTQ ESI (Thermo Fisher Scientific) calibration solutions, respectively.

3. Results and discussion

3.1. Comparison of sample preparation procedures

Recoveries and the number of detected PMTs were assessed to set the optimum sample preparation procedure that allowed the maximum PMT coverage using 33 PMTs (see Table S1 in the SI-A). The extracts were only analysed in the Acclaim Trinity P2 column at MMLC-QqQ (pH = 3 and 7) and no internal standard correction was applied since suspect screening was the ultimate goal.

3.1.1. Mono-layer SPE

Considering the mono-layer SPE sample preparation procedures (see Fig. 1), HILIC-SPE rendered the worst results in terms of detected analytes (27 %). In fact, only very polar PMTs (mean log D_{ow} −2.3 at pH = 7) were detected with low recoveries (17–36 %) probably due to the

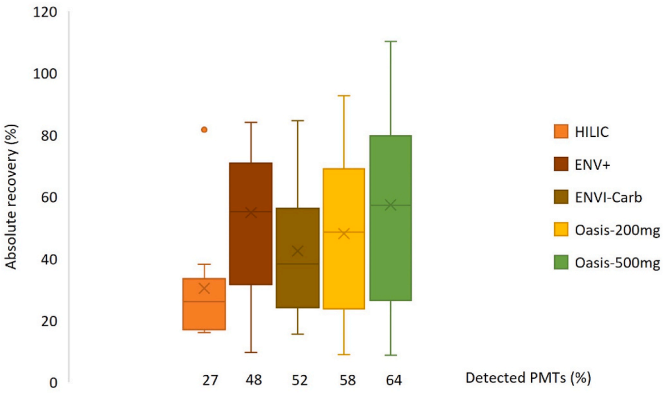

Fig. 1. Recoveries and detected PMTs (n = 33) in mono-layer SPE experiments.

retention of the polar interferences in the HILIC cartridge. In the literature, HILIC-SPE has been successfully used in proteomics for the analysis of amino acids or zwitterions (i.e., molecules with simultaneous positive and negative charges) [50–52], but it is not adequate for a wide range of PMTs in urine as shown in this study.

The other mono-layer SPE approaches provided more promising results in terms of the number of detected analytes and recoveries. Exactly, 48 % and 58 % of the target PMTs were detected with the polymeric ENV+ and Oasis HLB (200 mg) cartridges, respectively. With the GCB-based ENVI-carb cartridge, 52 % of the targets were quantified. Unlike in HILIC-SPE, most polar PMTs were lost and only the ones with higher log D_{ow} (−1.6 – 0.8 range at pH = 7) values were recovered with mean recoveries around 30–60 %. Finally, 500 mg Oasis HLB allowed the detection of 64 % of the compounds with higher recoveries (32–80 %) in comparison to the previous procedures as can be seen in Fig. 1. Nevertheless, some of the most polar targets (e.g. metformin, melamine, and chlormequat) were not recovered.

3.1.2. Multi-layer SPE

Three multi-layer SPE approaches were evaluated to study the retention of all PMTs (see Fig. 2). First, the efficiency of the multi-layer SPE consisting of Oasis HLB, WAX, and WCX was evaluated. When salty aqueous samples are treated using cartridges with ionic exchangers, they can get saturated as pointed out in the literature [53]. Therefore, two elution methods were tested (see the detailed procedure in the SI–B). In the first case, it was assumed that analytes also got retained in the ion exchangers and their elution was controlled with the pH of the eluent using ammonia and formic acid in the elution solvents. In that case, a clean-up step was also introduced. Regarding the other case, no additives were used in the elution solvent assuming that analytes only got retained in the Oasis HLB polymer and not in the ion exchangers due to their saturation with inorganic ions coming from urine. Therefore, no clean-up step was introduced.

Observing Fig. 2, higher recoveries (mean value 49 %) were obtained in the first case in comparison to the second approach (mean recovery 37 %). However, fewer PMTs were recovered in the first approach using additives in the elution solvent (61 % vs 67 %). That observation confirms the hypothesis that the exchangers were saturated with interfering inorganic ions from urine. As a consequence, the clean-up step eluted some of the most ionic PMTs that could not get retained in the exchangers alongside the interferences. Therefore, the cleaner extracts obtained resulted in higher recoveries due to less ion suppression at the expense of some analytes. In the last multi-layer approach that combined Oasis HLB with GBC, no improvements were observed since the results obtained were similar to the mono-layer SPE procedures as can be observed in Fig. 2.

3.1.3. LLE and SALLE

In the SALLE and LLE protocols (see Fig. 3), the highest number of detected PMTs was obtained with the SALLE protocol (70 %). In fact, it was the procedure with the highest percentage of detected analytes among all tested procedures. In the protocols involving pH adjustment, instead, only half of the targets could be detected. At pH = 2, acidic PMTs such as 6-chloropyridine-3-carboxylic acid, bisphenol S or phosphates were successfully extracted, while they were not recovered at pH = 12. However, the opposite effect was seen at pH = 12 since basic PMTs (i.e. N,N-dimethylbenzylamine and N,N,N′,N'-tetrakis(2-hydroxypropyl)ethylenediamine) were only quantified at pH = 12. For the simultaneous analysis of PMTs, both pH conditions should be combined but still, some analytes (i.e. ionic or neutral PMTs that were only extracted with SALLE) could not be detected.

3.2. Optimal sample preparation evaluation

Taking into consideration the results discussed in section 3.1, no sample preparation procedure ensured the simultaneous detection of all PMTs due to their complicated nature and the complexity of the urine matrix. Nevertheless, the SALLE protocol and the Oasis HLB (500 mg) cartridge showed the best results overall. In fact, complementarity was observed in the results of both extraction protocols. Despite 18 out 33 targets matched between the two methods, some PMTs that were not detected with the Oasis HLB cartridge (3-phenoxybenzoic acid, acrylamide, chlormequat, melamine, metformin, N,N-dimethylbenzylamine) were extracted with SALLE, while other targets (2-acrylamido-2-methyl-1-propanesulfonic acid, dibutyl phosphate, O,O-diethyl thiophosphate, omethoate) were only recovered with Oasis HLB. Therefore, a procedure combining SPE and SALLE was evaluated in terms of detected analytes and recoveries using both trimodal Acclaim Trinity P1 and P2 columns for the target 37 PMTs (see section 2.2). A similar procedure has been previously employed in the literature to extract polar metabolites of pesticides from urine [54].

The Acclaim Trinity P1 column allowed the quantification of 78 % of the PMTs, while 73 % were detected in the P2 column. Regarding recoveries, similar ranges were obtained. For the P1 column, absolute recoveries between 16 and 73 % were achieved, while the P2 column rendered slightly lower recoveries (14–66 %). Complementarity of the columns was also observed since some targets were only quantified in the P1 column (i.e. 2-imidazolidinthione, benzenesulfonamide, melamine, monomethyl phthalate, and phthalic acid), and others (i.e.

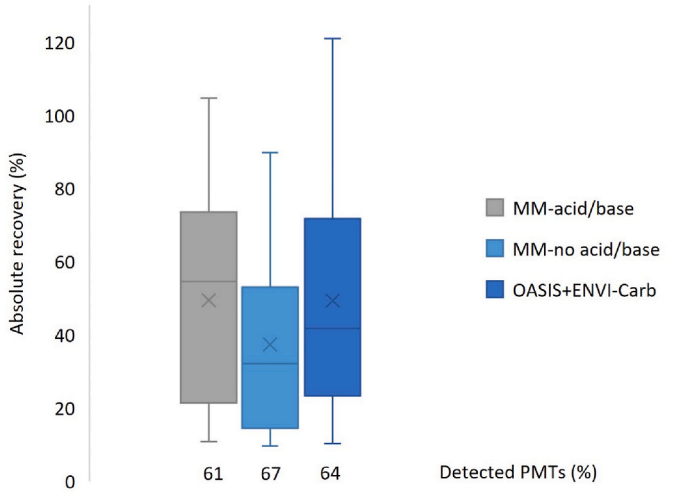

Fig. 2. Recoveries and detected PMTs (n = 33) in multi-layer SPE experiments.

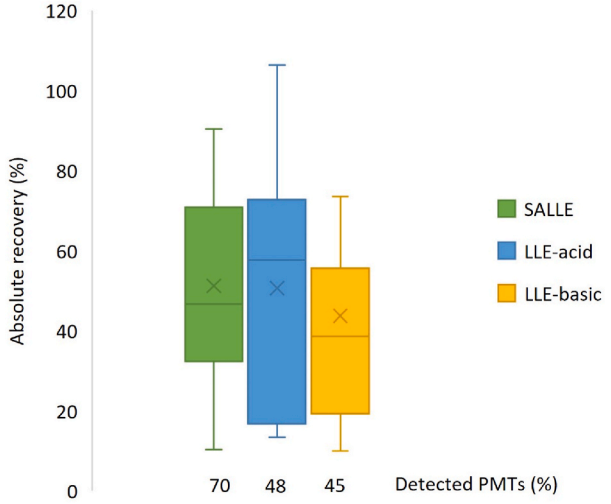

Fig. 3. Recoveries and detected PMTs (n = 33) in LLE and SALLE experiments.

diethyl phosphate, omethoate, and saccharin) in the P2 column. However, no selectivity in terms of the analyte's nature (basic, acidic, neutral or zwitterionic) was observed for the columns. In the cases of acephate, ammonium glufosinate, cytarabine, diethanolamine, and dimethyl phosphate, they were not detected at any of the conditions showing the extraction procedure developed in this work is not suitable for those compounds at that spiking concentration.

In comparison with our previous work using DS [55], the 5 PMTs not quantified in this work were successfully detected. In that sense, the non-discriminatory DS procedure might show a major analytical coverage, although 2-imidazolidinthione, benzenesulfonamide, monomethyl phthalate, and phthalic acid were not included in that work so no strict comparison can be performed. Consequently, both DS and SPE-SALLE were further extended for suspect screening.

3.3. Suspect screening workflow evaluation

The suspect screening workflow was evaluated using calibration curves built between 0.1 and 500 ng mL^{-1} and a 50 ng mL^{-1} spiked synthetic urine sample processed through the SPE-SALLE protocol and DS. All the results concerning the evaluation of the suspect screening workflow are compiled in Table S7 in the SI-A.

Regarding the iLOIs, 89 % and 76 % of the PMTs passed the criteria established for suspect screening at the P1 and P2 columns with mean values of 136 and 155 ng mL^{-1}, respectively. That result comes in accordance with the wider coverage shown by the P1 column in section 3.2. In the cases of 3-phenoxybenzoic acid, benzenesulfonamide, and phthalic acid, they were not identified at any condition despite they were detected in the MMLC-QqQ system. Moreover, a difference of two orders of magnitude between iLOIs calculated in this work and iLOQs calculated in our previous study [24] was observed. Those outcomes are related not only to the higher sensitivity of the DMRM mode in comparison to the Full Scan MS – data-dependent MS2 [40], but also to the stricter criteria established for defining iLOIs than iLOQs [56].

As for the pLOIs, the mean values for the SPE-SALLE protocol at both columns were 23 and 31 ng mL^{-1} due to the enrichment factor, respectively. For DS instead, average pLOIs were 272 and 309 ng mL^{-1} since a 1:2 dilution of the sample was applied. That difference of one order of magnitude can be appreciated in Fig. 4 in which PMTs are gathered by their nature. Regarding the nature, the main differences that can be observed are for acidic PMTs since not only much higher pLOIs are obtained in DS, but also the P2 column rendered worse values than P1.

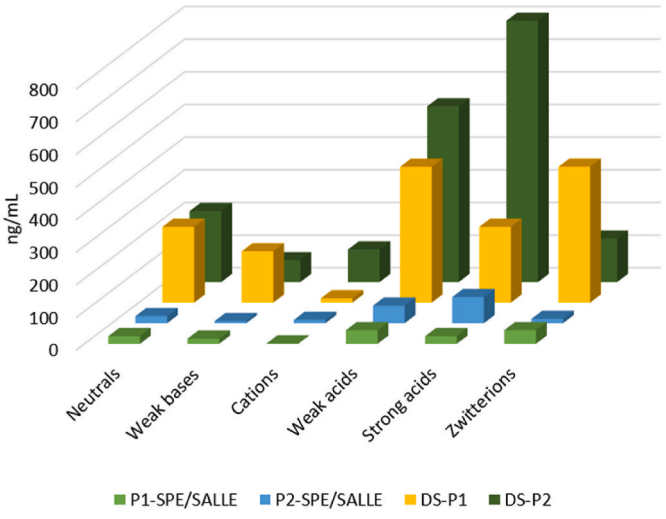

Fig. 4. Procedural limits of identification (pLOIs, ng mL^{-1}) obtained using DS and SPE-SALLE at both trimodal P1 and P2 columns.

In the spiked urine sample, 73 % of the PMTs could be screened using the method developed in this work. In the case of DS, only 46 % were identified at that spiking level (50 ng mL^{-1}) due to the high pLOIs. Therefore, despite DS showing major analytical coverage in the target approach using QqQ detection, preconcentration of the compounds seems to be compulsory for suspect screening to obtain MS2 spectra since most of the spiked PMTs were not identified due to their lack of fragmentation. A similar outcome was obtained in our previous work that aimed to screen contaminants of emerging concern and their metabolites in human urine [57]. In that work, more suspects were identified with sample preparation procedures involving preconcentration in comparison to DS even if no potential analyte losses occur with the latter.

3.4. Pooled real urine analysis

The pooled urine sample (n = 10) submitted to both DS and SPE-SALLE procedures was analysed by MMLC-qOrbitrap using both trimodal columns (P1 and P2) to screen PMTs (1450) and their phase II metabolites (1568). The annotated features at all 8 conditions (two protocols x 2 mixed mode columns x 2 pHs) with their confidence level (1–4) following the workflow presented in section 2.4.1 are presented in Table 1.

In total, 58 suspects were annotated, 14 of them corresponding to phase II metabolites. Examples of the annotated suspects are shown in Figs. S1–S7 in the SI–B. At level 1, 7 PMTs included in this work were identified. Additionally, other 4 PMTs were confirmed at level 1 because the pure standards were available in the laboratory although those suspects were not included during the optimization. Regarding the usage, the PMTs confirmed could be divided into industrial chemicals (i.e. diethanolamine, caprolactam, melamine, and tetrapropylammonium), substances related to personal habits (i.e., caffeine, aspartame, and nicotine), and biocides (i.e. acephate and omethoate). Additionally, transformation products and metabolites were also identified, such as nicotine's metabolite cotinine and terbufos insecticide's transformation product diethyl dithiophosphoric acid. Since the aim of the work was to find a method for suspect screening of diverse PMTs in human urine, the identified suspects were not quantified.

Regarding the rest of the levels, 28 of the suspects were annotated using Mass Frontier software for simulating their fragmentation (level 2b), and only 5 suspects were annotated at level 2a using the mzCloud library. At level 3, 7 positional isomers were annotated from which 6 turned out to be phase II metabolites. From those 40 suspects annotated at levels 2a, 2b and 3, 18 turned out to be industrial chemicals used for diverse purposes, which is the most concerning chemical class from the human health point of view. As examples, glue components (ethyl cyanoacrylate), ingredients in fragrances (acetophenone), polymerizing agents (1,6-diisocyanatohexane) or even a chelating agent (HEDTA) can be mentioned. Moreover, some natural products and pharmaceuticals were also detected.

As for the phase II metabolites, they were divided into 4 glucuronides and glycines, and 5 sulphates. Similarly as with the parent compounds, most metabolites corresponded to chemicals used in the industry for different purposes, such as, substances used in resins (ethyl phenol glucuronide and sulfate, or benzyl alcohol sulfate metabolite), a dying agent (amino phenol as both N- and O-glucuronides) and solvents (the sulfate of dihydroxytoluene). However, metabolites of pharmaceuticals (the sulfate of the analgesic paracetamol), biocides and plant-related natural chemicals were also screened in the pooled sample as it can be seen in Table 1. Lastly, the structure could not be elucidated for 7 suspects so level 4 was assigned since only one molecular formula was possible.

Only one work in the literature performed suspect screening of PMTs in human urine [33]. However, that work was not focused only on PMTs but also analysed per-and poly-fluoroalkyl substances, and, thus, sample preparation was optimized for extracting both classes of substances from

Table 1
Suspect screening of PMTs in the pooled human urine sample using both sample preparation approaches (SPE-SALLE and DS) and both trimodal columns (Acclaim Trinity P1 and P2).

Feature number	Name	Usage	log D_{ow} (pH = 7)	Formula	Exact mass	Annotation	Functional group	RT (min) P1/P2	Acclaim Trinity P1				Acclaim Trinity P2			
									pH = 3		pH = 7		pH = 3		pH = 7	
									DS	SPE-SALLE	DS	SPE-SALLE	DS	SPE-SALLE	DS	SPE-SALLE
1	Diethanolamine	Industrial chemical	−3.4	C4 H11 N O2	105.07922	1	Alcohol	8.26/ 5.97	✔	x	x	x	✔	x	x	x
2	Caprolactam	Industrial chemical	0.3	C6 H11 N O	113.08426	1*	Amide	0.92/ 1.70	✔	✔	x	x	x	✔	x	x
3	Melamine	Industrial chemical	−2.5	C3 H6 N6	126.06541	1	Amine	11.19/ 8.43	✔	✔	x	x	✔	✔	x	x
4	Nicotine	Stimulant	−0.4	C10H14 N2	162.11569	1*	Amine	16.28/ 26.29	✔	✔	x	x	✔	✔	x	x
5	Cotinine	Metabolite of nicotine (stimulant)	0.2	C10H12 N2 O	176.09482	1*	Amide	7.85/ 8.81	✔	✔	x	x	✔	✔	x	x
6	Acephate	Biocide	−0.3	C4 H10 N O3 P S	183.01173	1	Amide	0.72/ 0.76	✔	x	x	x	x	x	x	x
7	Tetrapropylammonium	Industrial chemical	−0.5	C12H27 N	185.21412	1	Ammonium salt	7.87/ 11.16	x	✔	x	x	x	✔	x	x
8	Caffeine	Stimulant	−0.5	C8 H10 N4 O2	194.08025	1*	Amide	1.19/ 2.15	✔	✔	x	x	✔	✔	x	x
9	Omethoate	Biocide	−0.5	C5 H12 N O4 P S	213.02234	1	Amide	0.73/ 0.93	✔	x	x	x	x	x	x	x
10	Aspartame	Artificial sweetener	−2.2	C14H18 N2 O5	294.12130	1	Carboxylic acid	7.86/ nd	x	✔	x	x	x	x	x	x
11	Diethyl dithiophosphoric acid	Metabolite of organophosphate insecticides	0.5	C4 H11 O2 P S2	185.99295	1	Dithiophosphoric acid	8.04/ nd	x	x	x	✔	x	x	x	x
12	δ-Valerolactam (2-piperidone)	Industrial chemical	−0.1	C5 H9 N O	99.06860	2a	Amide	0.78/ 1.18	✔	✔	x	x	✔	✔	x	x
13	4-Aminophenol	Industrial chemical	0.8	C6 H7 N O	109.05300	2a	Alcohol	9.53/ 9.45	✔	x	x	x	✔	x	x	x
14	Acetophenone	Industrial chemical/ Ingredient in fragrances	1.5	C8 H8 O	120.05757	2a	Ketone	10.37/ nd	✔	✔	x	x	x	x	x	x
15	Quinoline	Industrial chemical	2.2	C9 H7 N	129.05781	2a	Imine	6.59/ nd	x	✔	x	x	x	x	x	x
16	Aminosalicylic acid	Antibiotic	−1.4	C7 H7 N O3	153.04246	2a	Carboxylic acid	0.88/ 1.52	✔	x	x	x	✔	✔	x	x
17	Ethyl cyanoacrylate	Industrial chemical	1.1	C6 H7 N O2	125.04778	2b	Ester	nd/ 12.13	x	x	x	x	✔	✔	x	x
18	1-Butyl-2-pyrrolidinone	Industrial chemical	1.0	C8 H15 N O	141.11529	2b	Amide	8.56/ nd	x	✔	x	x	x	x	x	x
19	1,4-Diazabicyclo[2.2.2]octane-2-methanol	Industrial chemical	−1.4	C7 H14 N2 O	142.11051	2b	Alcohol	0.75/ nd	✔	✔	x	x	x	x	x	x
20	Estragole	Natural product	2.9	C10H12 O	148.08874	2b	Ether	5.17/ nd	✔	✔	x	x	x	x	x	x
21	Dimethylaminoethyl methacrylate	Industrial chemical	−0.1	C8 H15 N O2	157.11014	2b	Ester	1.58/ 2.01	✔	✔	x	x	✔	x	x	x
22	1,6-Diisocyanatohexane	Industrial chemical	1.2	C8 H12 N2 O2	168.08974	2b	Cyanate	0.84/ 1.00	✔	✔	x	x	✔	✔	x	x
23	Salsolinol	Natural product	−0.2	C10H13 N O2	179.09444	2b	Alcohol	8.64/ nd	x	✔	x	x	x	x	x	x
24	Pilocarpine	Pharmaceutical	0.8	C11H16 N2 O2	208.12126	2b	Ester	nd/ 1.15	x	x	x	x	✔	x	x	x

(*continued on next page*)

Table 1 (*continued*)

Feature number	Name	Usage	log D_{ow} (pH = 7)	Formula	Exact mass	Annotation	Functional group	RT (min) P1/P2	Acclaim Trinity P1				Acclaim Trinity P2			
									pH = 3		pH = 7		pH = 3		pH = 7	
									DS	SPE-SALLE	DS	SPE-SALLE	DS	SPE-SALLE	DS	SPE-SALLE
25	Zeatin	Natural product	−0.2	C10H13 N5 O	219.11178	2b	Alcohol	8.53/ nd	✔	✔	x	x	x	x	x	x
26	Pentobarbital	Pharmaceutical	1.8	C11H18 N2 O3	226.13162	2b	Amide	nd/ 1.06	x	x	x	x	✔	x	x	x
27	Acetamidophenyl hydrogen sulfate	Metabolite of paracetamol (pharmaceutical)	−1.9	C8 H9 N O5 S	231.01988	2b	Hydrogen sulfate (phase II)	14.62/ nd	✔	✔	x	x	x	x	x	x
28	Diethylene glycol dimethacrylate	Industrial chemical	2.4	C12H18 O5	242.11513	2b	Ester	6.54/ nd	✔	x	x	x	x	x	x	x
29	2-methoxycarbonylamino-3,3-dimethylbutanoyl glycine	Metabolite of 2-((Methoxycarbonyl) amino)-3,3-dimethylbutanoic acid (antiviral)	−2.9	C10H18 N2 O5	246.12151	2b	Glycine (phase II)	6.42/ 1.78	✔	✔	x	x	✔	x	x	x
30	(7-oxabicyclo[4.1.0]heptan-3-yl) methyl 7-oxabicyclo[4.1.0] heptane-3-carboxylate	Industrial chemical	1.6	C14H20 O4	252.13579	2b	Ester	6.39/ nd	✔	x	x	x	x	x	x	x
31	2,2′-Dihydroindigo	Industrial chemical	3.0	C16H12 N2 O2	264.08977	2b	Alcohol	nd/ 15.53	x	x	x	x	✔	✔	x	x
32	4-(2-acryloyloxyethoxy)-4-oxobutanoyl glycine	Metabolite of 4-(2-acryloyloxyethoxy)-4-oxobutanoic acid (industrial chemical)	1.4	C11H15 N O7	273.08457	2b	Glycine (phase II)	7.45/ nd	✔	✔	x	x	x	x	x	x
33	2-Ethylhexyliminodipropionic acid	Industrial chemical	−2.9	C14H27 N O4	273.19381	2b	Carboxylic acid	nd/ 8.64	x	x	x	x	✔	✔	x	x
34	Aminophenol N-glucuronide	Metabolite of aminophenol (industrial chemical)	−4.2	C12H15 N O7	285.08469	2b	Glucuronide (phase II)	nd/ 2.23	x	x	x	x	✔	✔	x	x
35	Aminophenol O-glucuronide	Metabolite of aminophenol (industrial chemical)	−4.3	C12H15 N O7	285.08469	2b	Glucuronide (phase II)	nd/ 2.23	x	x	x	x	✔	✔	x	x
36	Lycopsamine	Natural product	−0.8	C15H25 N O5	299.17299	2b	Ester	7.83/ nd	x	✔	x	x	x	x	x	x
37	14-((carboxymethyl)amino)-14-oxotetradecanoic acid	Metabolite of tetradecanedioic acid (natural product)	−1.9	C16H29 N O5	315.20401	2b	Glycine (phase II)	7.82/ nd	✔	x	x	x	x	x	x	x
38	2-((tert-Butoxycarbonyl)amino)-2-(3-hydroxyadamantan-1-yl)acetic acid	Pharmaceutical	−1.2	C17H27 N O5	325.18864	2b	Carboxylic acid	nd/ 8.92	x	x	x	x	✔	✔	x	x
39	N-Dodecyl-N,N-bis(2-carboxyethyl)amine	Industrial chemical	−1.2	C18H35 N O4	329.25601	2b	Carboxylic acid	7.74/ nd	✔	✔	x	x	x	x	x	x
40	Fiboflavin	Natural product	−1.9	C17H20 N4 O6	376.13758	2b	Amide	1.49/ nd	✔	x	x	x	x	x	x	x
41	1-phenoxypropan-2-yl hydrogen sulfate	Metabolite of 1-phenoxy-2-propanol (industral chemical)	−0.8	C9 H12 O5 S	232.04012	2b	Hydrogen sulfate (phase II)	13.30/ nd	x	x	✔	x	x	x	x	x
42	Sulfobenzenedicarboxylic acid	Industrial chemical	−8.1	C8 H6 O7 S	245.98316	2b	Carboxylic acid	13.20/ nd	x	x	✔	x	x	x	x	x
43	2-methoxy-4-(3-oxoprop-1-en-1-yl)phenyl hydrogen sulfate	Metabolite of coniferyl aldehyde (natural product)	−1.3	C10H10 O6 S	258.01962	2b	Hydrogen sulfate (phase II)	12.67/ nd	x	x	✔	x	x	x	x	x

(*continued on next page*)

Table 1 (*continued*)

Feature number	Name	Usage	log D_{ow} (pH = 7)	Formula	Exact mass	Annotation	Functional group	RT (min) P1/P2	Acclaim Trinity P1				Acclaim Trinity P2			
									pH = 3		pH = 7		pH = 3		pH = 7	
									DS	SPE-SALLE	DS	SPE-SALLE	DS	SPE-SALLE	DS	SPE-SALLE
44	2-Amino-2-ethyl-1,3-propanediol	Industrial chemical	−3.6	C5 H13 N O2	119.09472	2b	Alcohol	nd/ 5.89	x	x	x	x	✔	x	x	x
45	5,5-Dimethylhydantoin	Industrial chemical	−0.5	C5 H8 N2 O2	128.05871	3	Amide	7.69/ 2.09	✔	x	x	x	✔	x	x	x
46	N-(2-Hydroxyethyl) ethylenediaminetriacetic acid (HEDTA)	Industrial chemical	−10.5	C12H21 N3 O8	335.13262	3	Glycine (phase II)	0.94/ 0.89	✔	x	x	x	✔	x	x	x
47	X-Ethylphenyl hydrogen sulfate	Metabolite of ethylphenol (industrial chemical)	−0.2	C8 H10 O4 S	202.02929	3	Hydrogen sulfate (phase II)	10.51/ nd	x	x	✔	✔	x	x	x	x
48	X-hydroxy-X-methylphenyl hydrogen sulfate	Metabolite of dihydroxytoluene (industrial chemical)	−1.0	C7 H8 O5 S	204.00851	3	Hydrogen sulfate (phase II)	7.86/ nd	x	x	x	✔	x	x	x	x
49	(X-amino-X-hydroxybenzoyl) glycine	Metabolite of aminohydroxy benzoic acid (biocide)	−3.2	C9 H10 N2 O4	210.06336	3	Glycine (phase II)	3.69/ nd	x	x	✔	x	x	x	x	x
50	Benzyl alcohol-glucuronide	Metabolite of benzyl alcohol (industrial chemical)	−3.5	C13H16 O7	284.08965	3	Glucuronide (phase II)	nd/ 7.46	x	x	x	✔	x	x	x	x
51	Ethylphenol-glucuronide	Metabolite of ethylphenol (industrial chemical)	−2.6	C14H18 O7	298.10514	3	Glucuronide (phase II)	nd/ 7.89	x	x	✔	✔	x	x	x	x
52	–			C8 H11 N	121.08914	4	–	8.78/ nd	✔	✔	x	x	x	x	x	x
53	–			C10H14 O2	166.09927	4	–	5.17/ nd	x	✔	x	x	x	x	x	x
54	–			C8 H10 O5 S	218.02435	4	–	7.52/ 8.45	x	x	x	✔	x	x	✔	x
55	–			C8 H10 O5 S	218.02426	4	–	12.94/ nd	x	x	✔	✔	x	x	x	x
56	–			C8 H8 O6 S	232.00370	4	–	7.84/ nd	x	x	x	✔	x	x	x	x
57	–			C8 H8 O6 S	232.00366	4	–	13.11/ nd	x	x	✔	x	x	x	x	x
58	–			C9 H10 O6 S	246.01956	4	–	13.48/ nd	x	x	✔	x	x	x	x	x

Abbreviations: PMTs: Persistent, Mobile, and Toxic substances, RT: retention time, DS: Dilute-and-Shoot, SPE: Solid Phase Extraction, LLE: Liquid-Liquid Extraction, nd: not detected.
Superscripts: *: Not included in the sample preparation comparison experiments.

human urine. In that sense, the extract was submitted to LLE with ammonia addition and a C18 column was used in the analysis. Therefore, only 9 PMTs were identified in those urine samples. In the present work, the MMLC columns allowed retaining much more suspects due to their trimodal nature. In fact, the log D_{ow} values (at pH = 7) of the suspects screened in this work range from – 10.5 to 3 (see Table 1), much wider that other RPLC-based methods that usually analyse substances with positive log D_{ow} values.

As a limitation of the work, only a pooled sample was analysed since the aim was to study the best approach to perform suspect screening of PMTs in human urine, focusing on both the sample preparation step and the analysis. Therefore, neither the detection frequency of each suspect in the samples nor the semi-quantitative data is provided.

3.4.1. *Complementarity of the methods*

To evaluate which condition was the most suitable for suspect screening of PMTs in human urine, their complementarity was assessed (see Fig. 5). Out of the 58 suspects, DS-P1-pH = 3 allowed the annotation of 28 (48.3 %), followed by SPE/LLE-P1-pH = 3 which was able to screen 23 (39.7 %) PMTs. The reason why the P1 column showed a greater analytical coverage than the P2 column at pH = 3 is related to the functional groups of the trimodal columns. While at pH = 3 both groups of the P1 column (i.e. WAX and SCX) are activated, only the SAX group is activated in the P2 since the WCX is neutralized. That group activation is especially crucial with PMT compounds that are very polar and the ionic retention mechanism is compulsory for retention.

In the case of pH = 7, only the Acclaim Trinity P1 trimodal column was adequate for screening the suspects since 14 PMTs were annotated in comparison to the unique suspect annotated using the Acclaim Trinity P2 column. In those cases, however, both ionic exchangers are theoretically activated, but, since the details about neither the ionic exchangers nor the RP and HILIC phases are provided, no further discussion is possible.

For future human monitorization studies, the conditions that allowed the maximum analytical coverage of PMTs with viable laboratory work and chromatographic runs were studied. To that end, DS-P1-pH = 3 was selected since it was the condition that allowed the maximum annotation of PMTs, and the complementarity of the rest of the conditions towards that method was studied. As a result, both DS-P2-pH = 3 and DS-P1-pH = 7 allowed the annotation of 9 additional suspects. However, with DS-P1-pH = 7 more phase II metabolites were added including 1 glucuronide, 1 glycine, and 3 sulphates, while with the other approach only 2 glucuronides were screened. Therefore, the analysis of the pooled human urine using DS-P1 at both pHs for positive and negative ionization screened 63.8 % of all suspects (69.2 % of the phase II metabolites). In the case of SPE-SALLE-P1 at both pHs, instead, only 52.6 % and 46.2 % of the suspects and metabolites were annotated, respectively.

Taking everything into consideration, although the SPE-SALLE protocol performed better with a reduced number of spiked analytes in synthetic urine (37), the DS allowed the annotation of more suspects. That could be attributed to its non-selective nature, despite the absence of preconcentration. Additionally, matrix effects could have played a significant role, as real urine may differ from the artificial one.

4. Conclusions

A comprehensive method for suspect screening of PMTs in human urine was developed in this work using MMLC. In the sample preparation step, several SPE and LLE approaches (11) were tested as procedures involving enrichment. Under optimal conditions, a combination of SPE using Oasis HLB cartridge and LLE with salt addition was chosen since it allowed maximum PMT coverage. In addition, a DS approach was also evaluated as a non-discriminatory procedure for suspect screening. The adequacy of the suspect screening workflow was assessed using a synthetic urine sample spiked with 37 PMTs. As a result, 73 % of the PMTs were detected with the SPE-SALLE method, while only 46 % of the targets were identified with DS due to the higher LOIs obtained. To get a wider view of the protocols for suspect screening, a pooled urine sample was analysed to screen 1450 PMTs and *in-silico* simulated 1568 phase II metabolites. In that sample, 44 PMTs and 14 metabolites (i.e. glucuronides, sulphates, and glycines) were annotated at different confidence levels. Concerning the detected compounds, a wide variety of substances were highlighted, including industrial chemicals, biocides, natural products, and even pharmaceuticals. In contrast to the spiked experiments, the use of the DS with the Acclaim Trinity P1 column at both pH = 3 for positive ionization and pH = 7 for negative ionization emerged as the most effective strategy for suspect screening of PMTs in real urine. Specifically, this combination enabled the coverage of the maximum number of PMTs (63.8 %) and metabolites (69.2 %) compared to the procedure using SPE and LLE with the Acclaim Trinity P2 column.

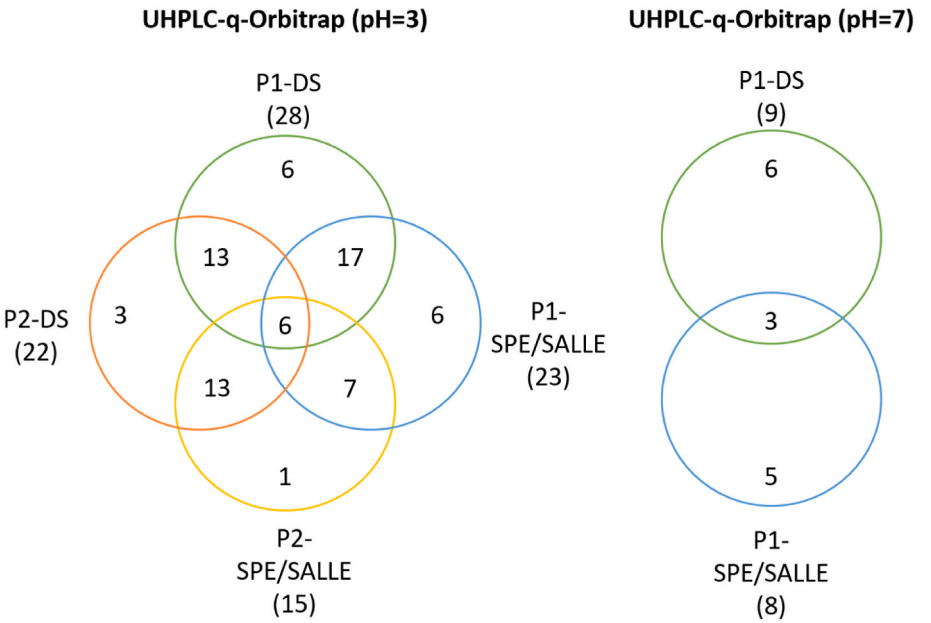

Fig. 5. Annotated suspects in a pooled (n = 10) urine sample using DS and SPE/LLE with Acclaim Trinity P1 and P2 trimodal columns at positive (pH = 3) and negative (pH = 7) ionization. The results for Acclaim Trinity P2-pH = 7 are not included since only one suspect was annotated level 4 using DS.

CRediT authorship contribution statement

Mikel Musatadi: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. **Iker Alvarez-Mora:** Data curation, Formal analysis, Investigation, Software. **Ines Baciero-Hernandez:** Conceptualization, Investigation, Software, Validation. **Ailette Prieto:** Project administration, Resources, Supervision. **Eneritz Anakabe:** Formal analysis, Investigation. **Maitane Olivares:** Data curation, Methodology, Writing – review & editing. **Nestor Etxebarria:** Funding acquisition, Project administration, Resources. **Olatz Zuloaga:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, 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.

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.talanta.2024.125698.

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