Development and evaluation of a comprehensive workflow for suspect screening of exposome-related xenobiotics and phase II metabolites in diverse human biofluids

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HIGHLIGHTS

- A comprehensive suspect screening workflow was developed for 4 human biofluids.
- The "peak rating" parameter discarded bad peaks avoiding peak area thresholds.
- Type I errors were noted for bisphenol Z and benzyl paraben.
- Type II errors occurred for 14 xenobiotics in standards and 29 in spiked biofluids.
- An inclusion mass list of endogens was selected over an exclusion mass list.

GRAPHICAL ABSTRACT
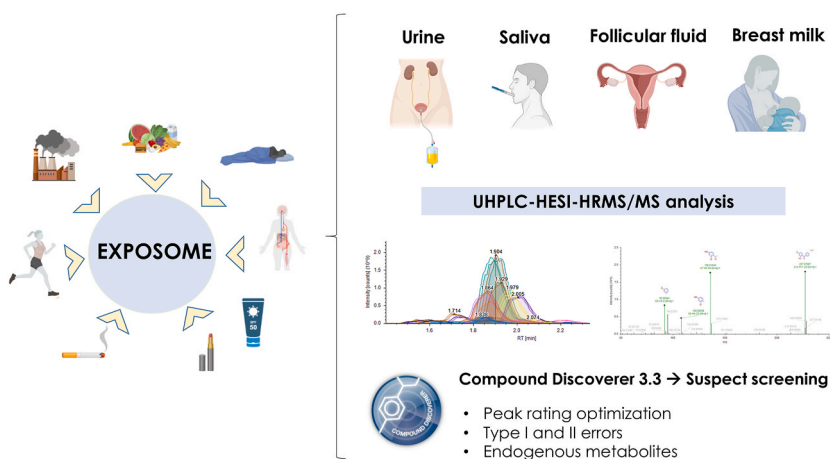
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ABSTRACT

Suspect and non-target screening (SNTS) methods are being promoted in order to decode the human exposome since a wide chemical space can be analysed in a diversity of human biofluids. However, SNTS approaches in the exposomics field are infra-studied in comparison to environmental or food monitoring studies. In this work, a comprehensive suspect screening workflow was developed to annotate exposome-related xenobiotics and phase II metabolites in diverse human biofluids. Precisely, human urine, breast milk, saliva and ovarian follicular fluid were employed as samples and analysed by means of ultra-high performance liquid chromatography coupled with high resolution tandem mass spectrometry (UHPLC-HRMS/MS). To automate the workflow, the "peak rating" parameter implemented in Compound Discoverer 3.3.2 was optimized to avoid time-consuming manual revision of chromatographic peaks. In addition, the presence of endogenous molecules that might interfere with the annotation of xenobiotics was carefully studied as the employment of inclusion and exclusion suspect lists. To

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evaluate the workflow, limits of identification (LOIs) and type I and II errors (i.e., false positives and negatives, respectively) were calculated in both standard solutions and spiked biofluids using 161 xenobiotics and 22 metabolites. For 80.3 % of the suspects, LOIs below 15 ng/mL were achieved. In terms of type I errors, only two cases were identified in standards and spiked samples. Regarding type II errors, the 7.7 % errors accounted in standards increased to 17.4 % in real samples. Lastly, the use of an inclusion list for endogens was favoured since it avoided 18.7 % of potential type I errors, while the exclusion list caused 7.2 % of type II errors despite making the annotation workflow less time-consuming.

1. Introduction

In the year 2005, Christopher Paul Wild raised the idea that the human genome should be complemented with the "exposome" (i.e., life-course exposures from the prenatal period onwards) to identify the origin of chronic diseases (Wild, 2005). In that context, human bio-monitoring emerged as a tool to understand the risk of chemicals through their analysis in human biofluids (Ganzleben et al., 2017).

Suspect and non-target screening (SNTS) methods based on high-resolution mass spectrometry (HRMS) provide an added value in the rough task of understanding the exposome since a wide range of chemicals (including metabolites and/or transformation products) can be screened (Pourchet et al., 2020). Nevertheless, the abundant and complex data obtained in SNTS approaches by data-dependent or in-dependent acquisition modes (DDA and DIA, respectively) requires automation of data processing (Chen et al., 2022; Samanipour et al., 2018). To solve that issue, both commercial and open-access software that automatically perform peak detection, alignment, grouping (i.e., generation of unique "features") and integration before structure elucidation are available (Chen et al., 2022; Hollender et al., 2023).

Although a wide variety of tools are useful for structural elucidation, such as spectral libraries (e.g., mzCloud, METLIN, MassBank) and *in-silico* tools (e.g., Mass Frontier, MetFrag), previous feature filtering or prioritization is crucial. Some strategies include common actions, such as mass errors, isotopic pattern fits, peak area/intensity thresholds, or signal-to-noise ratios (S/N). However, more specific information can be used depending on the used instrumentation (e.g., collision cross section (CCS) values in ion-mobility) or characteristics of the suspects (e.g., neutral losses, mass defect, relevant fragments) (González-Gaya et al., 2021a; Hollender et al., 2023). Nevertheless, peak picking and the assurance of Lorentzian peak-shaped features are still unsolved and tedious bottlenecks within SNTS workflows. Besides, when commercial software such as Compound Discoverer (Thermo Fisher Scientific) is used, some steps like *in-silico* fragmentation are manually applied for each candidate of every feature to be annotated. In that sense, peak revision is necessary to discard unacceptable peaks that should not be submitted to MS2 interpretation (González-Gaya et al., 2021a). Conse-quently, parameters that discard low-quality peaks have been recently implemented and are promising to speed up time-consuming manual work but are still infra-studied (Musatadi et al., 2023).

Moreover, regarding the annotation workflows, some specific chal-lenges of SNTS in biofluids have been poorly addressed in the literature since the screening of xenobiotics in human samples has been less studied in comparison to environmental and food matrices (Hajeb et al., 2022). On the one hand, comprehensive workflows are required since several human biofluids can be employed for exposome evaluation, such as, urine (Huber et al., 2022; Tkalec et al., 2022b), blood/plasma (Bandow et al., 2020; Sunyer-Caldú et al., 2023), breast milk (Alcala and Phillips, 2017; Baduel et al., 2015), saliva (Moscoso-Ruiz et al., 2022; Mullangi et al., 2009) or even ovarian follicular fluid (Hallberg et al., 2021; Li et al., 2023). On the other hand, endogenous substances, which are at higher concentrations in such matrices, could interfere with the identification of xenobiotics and need to be carefully considered. Nevertheless, few works take into account the presence of endogenous substances using different strategies, for instance, inclusion or exclusion lists and even manual elimination of endogenous candidates (Musatadi

et al., 2023; Plassmann et al., 2015; Roggeman et al., 2022). Further-more, organic xenobiotics undergo metabolism in the human body and are more prominently present as metabolites. Those metabolization re-actions are phase II (i.e., oxidation, dealkylation) and phase II (conju-gation reaction to obtain glucuronides, sulphates and glycine) reactions so the presence of those metabolites in human samples should be also addressed (Meijer et al., 2021).

A harmonised procedure is hard to implement in the data pre-processing step but the establishment of common quality control/qual-ity assurance (QC/QA) measures in the SNTS annotation workflows is necessary (Pourchet et al., 2020). Besides the above-mentioned priori-tization criteria, type I and II errors (false positives and negatives, respectively) and the limits of identification (LOIs) should be also re-ported in SNTS methods besides the transparency in communicating annotation confidence (Hollender et al., 2023).

All in all, this work aimed to develop a comprehensive suspect screening workflow to screen exposome-related chemicals (including phase II metabolites) in diverse human biofluids, such as urine, saliva, breast milk and follicular fluid. To fulfil that objective, the optimization of several parameters that facilitate compound annotation, the calcu-lation of LOIs, type I and II errors, and the study of the best strategy to consider endogenous substances were set as sub-objectives.

2. Materials and methods

2.1. Sample preparation and UHPLC-HRMS/MS analysis

Human urine, saliva, follicular fluid, and breast milk were employed as biofluids. Informed consent was obtained from each subject before conducting the experiments and the samples were handled according to the indications of (i) The Committee on Ethics for Research on Biological Agents and Genetically Modified Organisms (CEIAB-UPV/EHU, BOPV 32, February 17, 2014, M30-2021-158, M30-2022-311, M30-2022-327, M30-2023-136) and (ii) The Committee on Ethics for Research Involving Human Subjects (CEISH-UPV/EHU, BOPV 32, February 17, 2014, M10-2020-230, M10-2021-124, M10-2023-135) of the University of the Basque Country (UPV/EHU).

All the specific details regarding sample preparation and analysis by ultra-high performance liquid chromatography coupled to high resolu-tion tandem mass spectrometry (UHPLC-HRMS/MS), including quality control/quality assurance (QC/QA), are detailed elsewhere (Bacier-o-Hernández et al., 2024). Briefly, urine and saliva samples were treated with solid phase extraction (SPE) using Oasis HLB cartridges, while milk and follicular fluid samples were extracted with salt-assisted liquid--liquid extraction (SALLE) followed by protein precipitation at low temperatures. In addition, follicular fluid extracts were centrifuged to ensure maximum clogging of proteins, while an additional clean-up step using Captiva EMR-Lipid filters was performed with the milk extracts. No hydrolysis was performed in order to screen possible phase II me-tabolites in the samples. For the UHPLC-HRMS/MS analysis, a Dionex Ultimate 3000 UHPLC coupled to a high-performance Q Exactive Focus Orbitrap (qOrbitrap, Thermo Fisher Scientific) mass analyser with a heated electrospray ionization (HESI) source was used. The analyses were performed at the Full Scan – data dependant MS^2 (Full MS – $ddMS^2$) discovery acquisition mode at positive and negative ionization, so two runs were performed per sample using an ACE UltraCore 2.5

SuperC18 column (2.1 mm × 100 mm, 2.5 μm) column at acidic (pH = 2.5) and basic (pH = 10.5) mobile phase conditions, respectively.

2.2. Suspect screening workflow

The suspect screening workflow was designed using the Compound Discoverer 3.3.2 software (Thermo Fisher Scientific) based on the research group's experience (González-Gaya et al., 2021b; Musatadi et al., 2022) with adaptations to simultaneously screen xenobiotics and phase II metabolites in human samples. First, the software pre-processed the UHPLC-HRMS/MS data and consisted of retention time alignment, compound detection, compound grouping (e.g., composition prediction, pattern scoring, neutral loss search, library search, mass list search), gap filling and background subtraction. Those parameters are detailed in Table S1 of the Supplementary Information (SI).

Then, with the data retrieved by the software, the suspect screening workflow was applied. For compound annotation, an in-house mass list of 7055 exposome-related xenobiotics obtained from NORMAN (https://www.norman-network.com/?q=suspect-list-exchange) and 7670 phase II metabolites *in-silico* simulated by BioTransformer 3.0 (http://biotransformer.ca/) were employed. The full mass lists containing the name, molecular formula, exact mass and chemical structure (i.e., Simplified Molecular-Input Line-Entry System (SMILES)) for each suspect can be found in Table S2 in the SI. In addition, the information about the phase II reaction and the precursor is indicated for the metabolites. Regarding phase I metabolites, only phase I metabolites of phthalates were included (hydrolytic monoesters) since the rest of suspects would likely undergo phase II metabolization due to their polar functional groups. Moreover, another in-house mass list of 707 human endogenous substances obtained from the Human Metabolome Database (HMDB, https://hmdb.ca/metabolites) and 1739 phase II metabolites of those endogens simulated by BioTransformer 3.0 was also employed (see Table S3 in the SI). The mass list of a total of 2446 endogenous molecules was used either as an inclusion list or as an exclusion list (see section 3.4).

From the initial list of features (each with one or more potential candidates from the mass lists) retrieved by Compound Discoverer after the initial pre-processing, the peak rating parameter and the minimum peak area were optimized to reduce the number of features that should be manually revised to submit to MS2 evaluation. Additionally, only features that contained a heteroatom in their molecular formula and a mass error lower than 5 ppm were considered. The peak rating filter allows the removal of low-quality chromatographic peaks without setting an overly restrictive minimum peak intensity threshold. To that end, the peak rating parameter considers peak quality factors (Musatadi et al., 2023), relative peak area, and the relative standard deviation (RSD). For more detailed information, see the Compound Discoverer User Guide for LC Studies (Software Version 3.3 SP2).

The fragmentation spectra were evaluated using the mzCloud mass spectral database (https://www.mzcloud.org/), setting a 70 % match threshold between the experimental MS2 spectra and the ones collected in the library. If the MS2 spectrum was not available for a candidate, *in-silico* fragmentation was simulated using Mass Frontier 8.0 software implemented in Compound Discoverer 3.3.2. For a satisfactory match, 70 % of the major fragments should be explained when three or more fragments were present in the MS2. When only one or two major fragments were present in the MS2, one of them should be explained (Hollender et al., 2023). In the case of glucuronides, the glucuronide loss should be explained, and if the molecule contained Cl, Br or S atoms, the MS1 should be explained. For the latter, the SFit parameter (i.e., the spectral similarity score between defined and measured isotope pattern) value higher than 50 % and the pattern coverage (i.e., matched intensity percentage of the theoretical pattern) higher than 70 % were established.

Lastly, the retention time (RT) was considered. If possible, pure standards were used and a ±0.2 min error was accepted. If not, the

Retention Time Indices (RTI) platform (http://rti.chem.uoa.gr/) was employed. In that case, the measured RT and the experimental RT provided by the RTI model built with RTI calibrants (see Table S4 in the SI) should be comparable (box1 and box2 levels were accepted) (Aalizadeh et al., 2021). With all the information mentioned above, the annotation confidence levels were defined based on the Schymanski scale (Schymanski et al., 2014).

2.3. Quality control/quality assurance (QC/QA)

To evaluate the workflow, type I errors (i.e., false positives) and type II errors (i.e., false negatives) were calculated in both standard solutions and spiked samples. In the case of the standard solutions, 15 concentration points between 0.1 and 200 ng/mL were prepared for 183 exposome-related xenobiotics (see Table S4 in the SI). Type I errors were defined as the percentage of xenobiotics wrongfully identified using the workflow, while the percentage of xenobiotics that could not be annotated was defined as type II errors.

Concentration levels below 50 ng/mL were injected in triplicate, and limits of identification (LOIs) were established from the lowest concentration point in which the compound could be correctly annotated in at least two replicates with the workflow (Vergeynst et al., 2015). For comparison, limits of detection (LODs) were also calculated in the standard solutions as the lowest concentration point injected in triplicate with an acceptable peak shape and a relative standard deviation (RSD) below 30 % using TraceFinder 5.1 software (Thermo Fischer Scientific) for peak integration (Musatadi et al., 2023).

Type I and II errors were also estimated in the 4 biofluids to consider potential extraction deficiencies and matrix effects. For each fluid, a pooled sample (n = 5) was spiked with the 183 xenobiotics in order to obtain a 100 ng/mL concentration in the final extract, and processed with their respective sample preparation procedure (Baciero-Hernández et al., 2024).

In order to study the influence of endogenous metabolites on the identification, the use of the in-house generated endogenous suspect list (including phase II metabolites) was considered in two approaches in the data post-processing: (i) inclusion list and (ii) exclusion list (see section 3.4 for details). The study was not limited to the spiked compounds but other compounds present in the samples were considered to get a wider view of the interferences in the annotation workflow caused by endogens.

Lastly, a signal-to-noise (S/N) ratio higher than 10 was established as compulsory QC/QA criteria to avoid the identification of artefacts. In the standard solutions, chromatographic areas of the solutions containing the xenobiotics (S) and areas of pure injection solvent (N) were considered, while in the biofluids, the areas of spiked samples (S) and non-spiked synthetic biofluids (N) were used. In the spiked samples, (iv) a RSD lower than 30 % within the three replicates of each biofluid was also required.

3. Results and discussion

3.1. Optimization of the suspect screening workflow's parameters

The peak rating parameter introduced in Compound Discoverer 3.3.2 aims to filter out low-quality chromatographic peaks since an appropriate intensity or peak area threshold is difficult to estimate for untargeted experiments. In that sense, the parameter was optimized testing values in the 2–8 range, with higher values meaning stricter criteria. The contribution of each parameter included in the "peak rating", indicated as the proportion of the contribution compared to other contributors, was selected according to the vendor specification and is detailed in Table S1.

To optimize the peak rating parameter, spiked human biofluids were used instead of pure standard solutions to consider the effect that the matrices might have on the peak shapes. In that sense, several measures

and steps explained hereinafter were defined. With the initial pre-processing (Table S1), a total of 25,801 and 27,483 molecular features were obtained in the positive and negative modes, respectively.

Then, features without MS2 and heteroatoms, S/N ratios lower than 10, and mass errors higher than 5 ppm were discarded. As a starting point, the chromatographic peak areas were set to values that provided a viable number of features to be studied individually. Consequently, the area threshold was set to 10^7 (positive mode) and 10^6 (negative mode) and the number of features was reduced to 2026 and 1597 in the positive and negative modes, respectively. Then, all the features were individually evaluated and classified as "acceptable" or "non-acceptable" based on their chromatographic peak shape, which should ideally be Lorentzian-shaped (Caballero et al., 2002). Considering both modes, a total of 2343 peaks (64.6 %) were considered "non-satisfactory". To avoid individual time-consuming revision, peak rating values (2–8) were studied to maximize the filtering of features with non-acceptable peak shapes while minimizing the loss of features with acceptable Lorentzian peak shapes. The results for both ionization modes are summarized in Fig. 1(a–b).

In the positive mode, the optimum value was set to '5' since it preserved 91 % of features with acceptable peak shapes while 50 % of the features with non-acceptable peak shapes were discarded (601 features). Although a peak rating value of '6' in the positive ionization mode would increase the elimination of undesirable features (up to 68 %), the loss of Lorentzian peak-shaped features would increase to 21 % so it was not selected. In the negative mode, a peak rating value of 4 turned out to be the best value in the consensus of the preservation of acceptable peaks (84 % preserved) and the elimination of non-acceptable peaks (646 features).

With the optimum peak rating values, the chromatographic areas were minimized so as not to discard features with Lorentzian shapes but low abundance. In the positive mode, a maximum of 28 features were recovered minimizing the area from 10^7 to 10^6. However, no more features were recovered setting the area below 10^6. In the negative mode, 12 additional features were gained lowering the area from 10^6 to $5 \cdot 10^5$. Although those areas seem to be relatively high, the peak rating
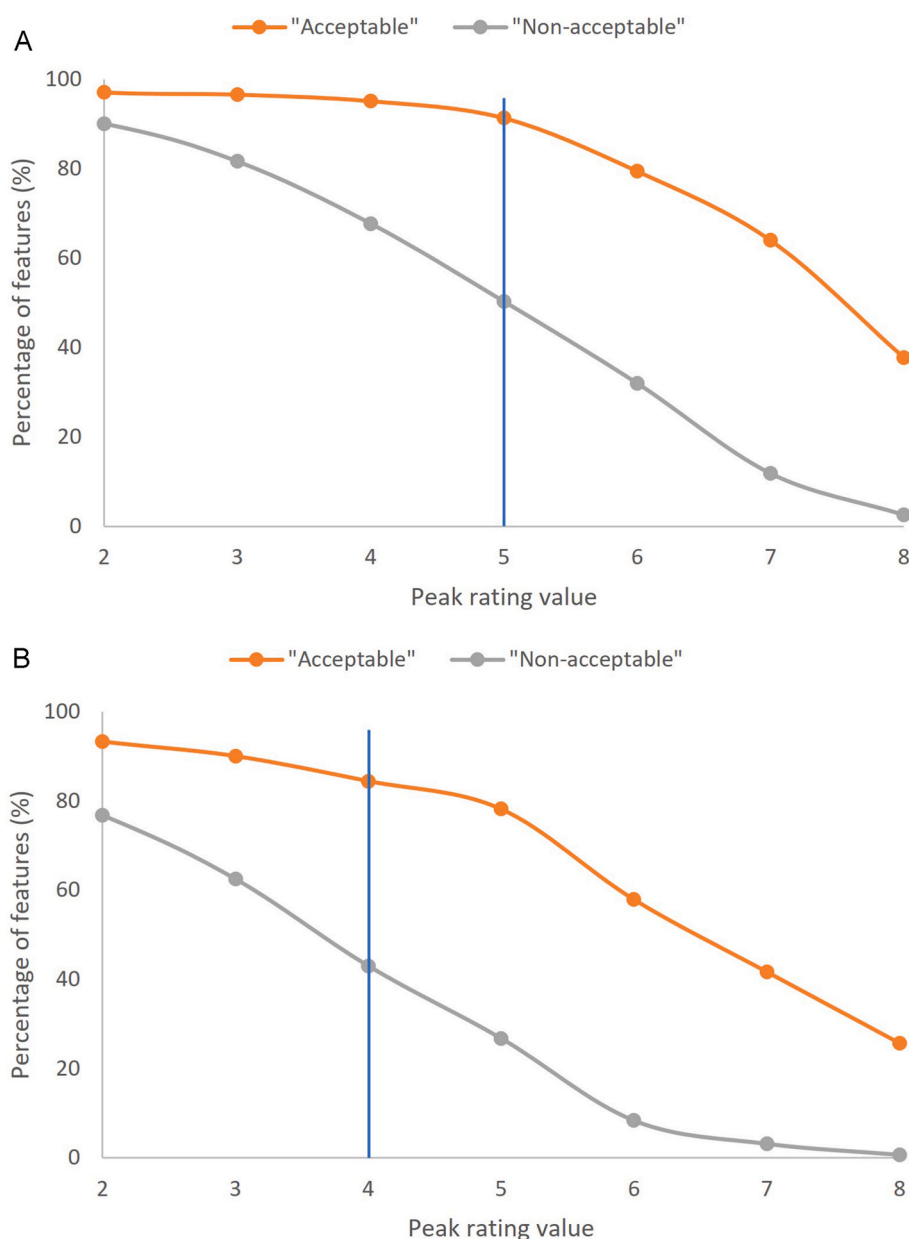

Fig. 1. "Acceptable" and "non-acceptable" peak shapes at several "peak rating" values (2–8) at the (a) positive and (b) negative mode.

parameter showed that no more acceptable features in terms of peak shape were retrieved below those orders of magnitude. In fact, similar peak area threshold values (10^5–10^7) are established in other SNTS works using qOrbitrap detection (López et al., 2016; Musatadi et al., 2021). Consequently, the peak area/intensity threshold criteria can be removed by the implementation of the adequate "peak rating" value avoiding the removal of low intensity peaks that could be interesting from the exposomic point of view.

3.2. Evaluation of type I and type II errors

Type I errors or false positives, correspond to wrongly annotated substances that are not present in the sample. Nevertheless, those errors are difficult to assess and they are not reported in SNTS works in the literature, especially using human fluids. Type II errors or false negatives instead, are substances present in the sample that are not reported in the final list. Nevertheless, those losses can occur at different points in the analytical process, including the sample preparation step (e.g., extraction deficiencies), LC-HRMS/MS analysis (e.g., co-elution, poor ionization, matrix effects at detection) or data processing (e.g., filters, the inadequacy of *in-silico* tools, limited annotation lists, selection of MS2 databases) (Hollender et al., 2023). In this study, type I and II errors were evaluated in the (i) calibration solutions and (ii) spiked real biofluids using the suspect screening workflow as if the compounds spiked were unknowns. All the results are gathered in Table S5 in the SI.

In the calibration solutions, 167 analytes were successfully annotated from the total list of 183 (91.3 %). In most cases, the candidates were included in the mzCloud library and for the rest *in-silico* fragmentation was simulated. It should be mentioned that most phase II metabolites are not included in spectral libraries (Baduel et al., 2015) as it happened with the metabolites included in this work (i.e., bisphenol A glucuronide, bisphenol A sulfate, 4-methylumbelliferyl glucuronide, 1-hydroxypyrene glucuronide, and 8:2 fluorotelomer alcohol glucuronide) that were screened via *in-silico* fragmentation. Therefore, the generation of mass lists including the structures of metabolites is critical for their annotation. In the cases of phase II metabolites, the neutral loss of the conjugate group can be used for further annotation criteria. The neutral loss corresponding to the glucuronide ($C_6H_8O_6$, 176.03209 Da) is implemented in the software, while the neutral loss corresponding to the sulfate loss (as SO_3, 79.9568 Da) needs to be manually checked and should be introduced in upcoming versions.

However, type I errors were identified for benzyl paraben and bisphenol Z (BZP) in the calibration solutions. Benzyl paraben was annotated as 2-hydroxy-4-methoxybenzophenone or 4-(benzyloxy)benzoic acid, which both have the same molecular formula ($C_{14}H_{12}O_3$, 227.07094 Da) as benzyl paraben but the structures are different. 4-(benzyloxy)benzoic acid is included in the mzCloud library and the MS2 match against it was 83.9 % (Fig. 2a). In the cases of 2-hydroxy-4-methoxybenzophenone and benzyl paraben, they are not included in the library so *in-silico* fragmentation was simulated (Fig. 2b and c) with Mass Frontier explaining 4 and 3 fragments out 5, respectively (80 % and 60 % MS2 match). Consequently, benzyl paraben should be discarded according to the established criteria and a wrong name would be provided for that feature.

In the case of BZP, three suspects were possible: BZP, diethylstilbestrol and equilin, which have the same exact mass (268.14632 Da) and molecular formula ($C_{18}H_{20}O_2$). Although BZP was spiked, the feature was annotated as diethylstilbestrol. From the proposed 3 candidates, only diethylstilbestrol was included in mzCloud so *in-silico* fragmentation was simulated for BZP and equilin. Although the fragmentation was poor, diethylstilbestrol passed the mzCloud match criteria (83.7 % match), while *in-silico* fragmentation was not able to explain the MS2 spectra if the structures of BPZ and equilin were considered (see Fig. 3). A previous work of the research group also showed weaknesses of the *in-silico* fragmentation tool, especially for compounds ionized in the negative mode due to poor MS2 spectra

(Musatadi et al., 2022).

In the specific cases of acetamiprid-N-desmethyl, carbofuran phenol, ethyl 4-dimethylaminobenzoate, naproxen and tramadol, other possible candidates that were not structural isomers passed the criteria besides the spiked target. However, for those candidates, the RT was confirmed with the RTI model, which has an uncertainty ($R^2 = 0.965$ for the positive mode and $R^2 = 0.921$ for the negative mode). Therefore, they could be discarded against the candidate confirmed with the pure standard but in its absence, they would become type I errors as well.

In addition to the type I errors, some suspects were not detected in the standard solutions (type II errors) due to different scenarios. For dimethyl phosphate, 6-chloronicotinic acid, bendiocarb, 3-(2-chloro-3,3,3-trifluoroprop-1-en-1-yl)-2,2-dimethylcyclopropanecarboxylic acid, 2,6-di-*tert*-butyl-4-(dimethylaminomethyl)phenol, 2-ethylhexyl 4-(dimethylamino)benzoate, pendimethalin, bisphenol P and fipronil desulfinyl, only the molecular formula could be elucidated since the MS2 match was lower than 70 %. Regarding ethylparaben, mecoprop, 2,4-diethyl-9H-thioxanthen-9-one, chloroxuron and diphenhydramine-N-glucuronide, instead, not even the molecular formula could be elucidated even at the most concentrated calibration solution (200 ng/mL). Therefore, considering the analytes with bad MS1 (5) and MS2 (9) as unsatisfactory, 7.7 % of type II errors were identified in the standard solutions.

In spiked and extracted samples, the same type I errors occurred for benzyl paraben and BZP. However, no other type I error was identified so it can be concluded that the sample treatment procedures or the different matrices did not have a significant impact in terms of false positives (only 2 out of 183). On the contrary, type II errors were incremented in the spiked biofluids. From the 167 analytes that were correctly screened in the standard solutions, 29 could not be screened in the extracted biofluids (17.4 %). For 14 spiked analytes (acetaminophen, gabapentin, propamocarb, 2-(4-chlorophenyl)-3-methylbutyric acid, hydroxypyrene, atenolol, alachlor, sotalol, triclosan, desloratadine, ranitidine, chlorpyrifos-methyl, ambroxol, bisphenol A glucuronide), poor peak shapes were obtained so they were discarded after applying the peak rating parameter. In the cases of acetamiprid and genistin, although they were not discarded with the peak rating parameter, the quality of the MS1 spectra was not good enough to elucidate the molecular formula.

In the particular cases of perfluorooctanesulfonamide (PFOSA) and perfluorooctanesulfonic acid (PFOS), which are perfluorinated molecules, they could be properly annotated by optimizing the workflow's hydrogen-to-carbon atom ratio (H/C) parameter. In the suspect screening workflow employed in this work, the H/C ratio was established in the 0.1–3.5 range (default conditions). To elucidate molecular formulas of per- and poly-substituted chemicals, the ratio should be decreased since the software prioritizes molecular formulas with higher ratios. However, that might result in more possible molecular formulas for each feature and, therefore, an increase in type I errors. Consequently, that parameter should be carefully evaluated in future works aimed to screen per- and poly-substituted chemicals alongside other strategies like the mass defect (Oró-Nolla et al., 2023; Rehnstam et al., 2023).

For 4-hydroxybenzophenone, benzophenone-1, dichlorvos, benzophenone-3, methyl 3,5-di-*tert*-butyl-4-hydroxybenzoate, fenthion and triadimenol, the MS2 match was lower than the established threshold. For metformin, instead, no MS2 spectrum was acquired due to its non-retention in the non-polar column and co-elution with polar molecules. Lastly, methylparaben, triethyl phosphate and dodemorph were also type II errors because the S/N ratio was lower than 10 showing blank problems.

All in all, extraction deficiencies and/or matrix effects alongside the type II errors in standard solutions summed up to 23.5 % of type II errors or false negatives (43 suspects in total). Regarding the differences observed among the biofluids, 95 % (131 out of 138) of spiked suspects passed all the criteria in the follicular fluid, while the percentage
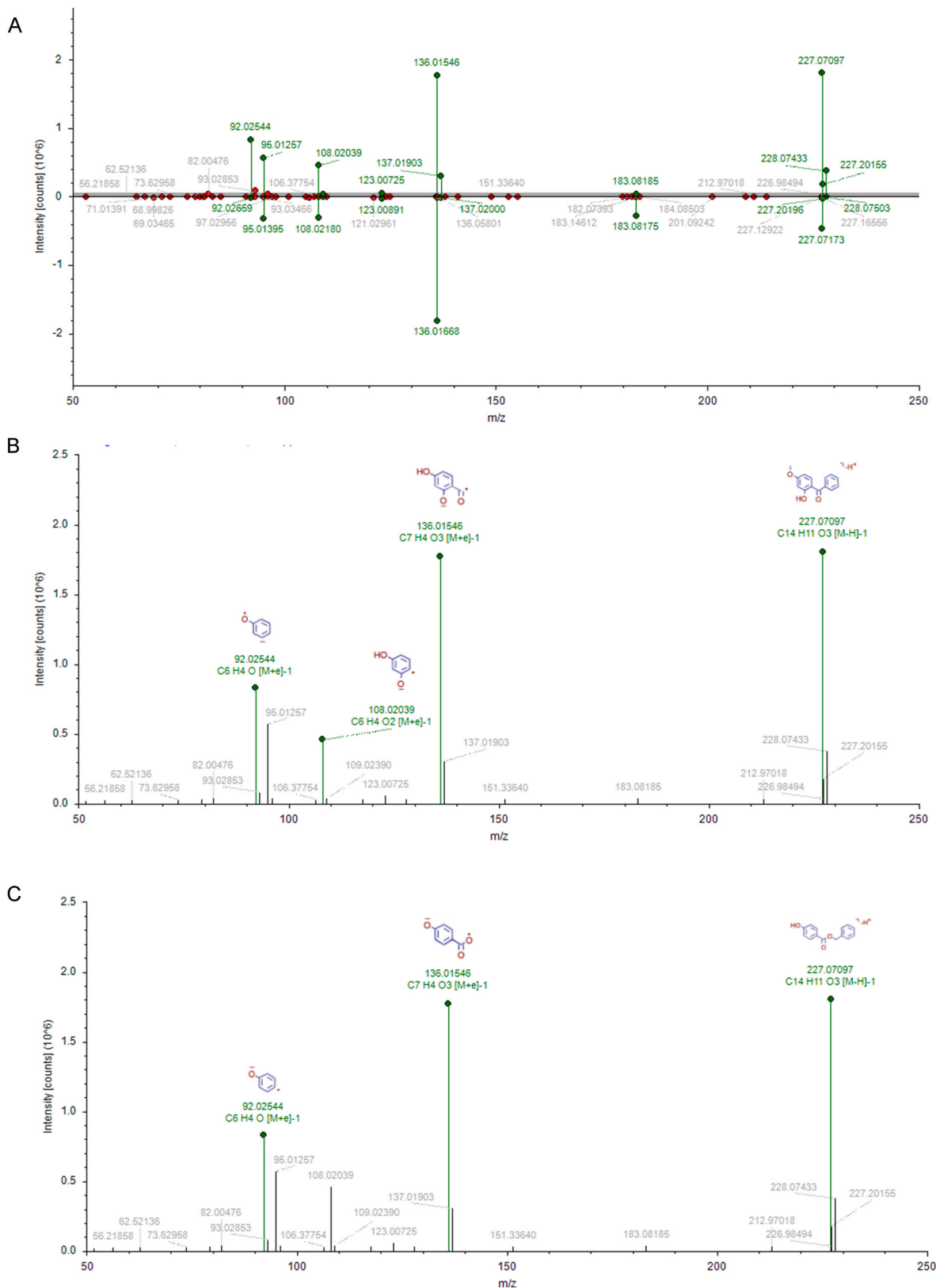
Fig. 2. MS2 match of possible candidates for the feature $C_{14}H_{12}O_3$ ([M − H]⁻ = 227.07094 Da, RT = 7.3 min): (a) 4-(benzyloxy)benzoic acid using mzCloud (the spectrum above is the experimental one while the spectrum below is the one included in the library), (b) 2-hydroxy-4-methoxybenzophenone *in-silico* simulation and (c) benzyl paraben *in-silico* simulation. Green dots indicate fragments explained by *in-silico* simulation.
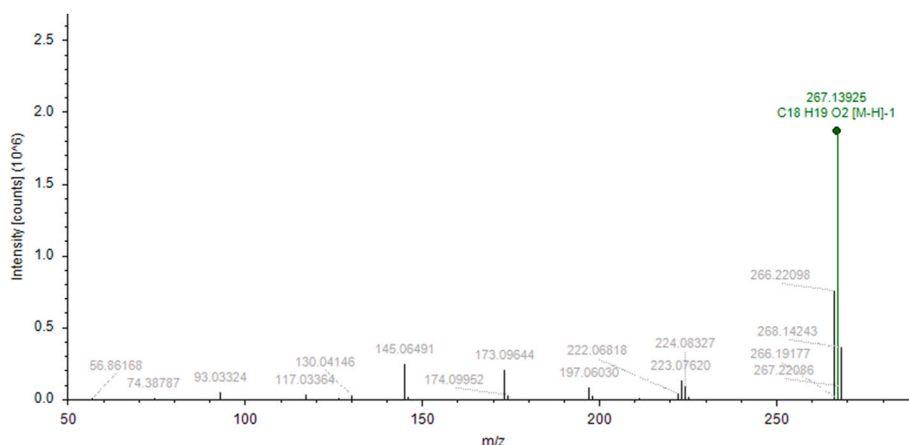
Fig. 3. A poor MS2 match using *in-silico* fragmentation for the feature $C_{18}H_{20}O_2$ ([M − H]⁻ = 267.13916 Da, RT = 12.3 min) for BPZ and equilin as candidates. Green dots indicate fragments explained by *in-silico* simulation.

decreased to 78 % and 71 % in breast milk and urine, respectively. Lastly, only 60 % of the xenobiotics were screened in the pooled saliva sample, suggesting that sample preparation should be studied in detail in the future for that matrix. No further discussion was made regarding the particularities of each matrix and their role in decoding the exposome, since the present work was focused on the data processing part rather than sample preparation and/or exposome-related conclusions.

With respect to the SNTS studies in the literature, there are no defined acceptance criteria for type I and II errors as for other QC/QA parameters (Caballero-Casero et al., 2021) and should be more frequently calculated to assess the performance of non-targeted methods. Due to the laborious SNTS workflows, a threshold of 25–30 % of errors could be considered as satisfactory. In addition, internal standards (IS) could be used to individually spiked all samples and detect errors in large monitorization works aiming to analyse a considerable number of samples since they are not naturally present in the samples. In fact, the use of IS is promoted in the SNTS works aiming to homogenise QC/QA parameters (Hollender et al., 2023). However, for the purpose of this work, 183 substances that might be present in real samples were employed to get a wider view of type I and II errors.

3.3. Limits of identification (LOIs)

LOIs were defined from the lowest concentration calibration solution where the suspect could be annotated. In the cases where the analyte was annotated at both ionization modes (e.g., 4-hydroxybenzophenone, propanil, valsartan), the lower LOI value was provided.

In the literature, few works that mostly focus on environmental samples define LOIs (Dasenaki et al., 2015; Liu et al., 2019; Segura et al., 2019). In some cases, the limits of detection (LODs) are provided (Getzinger and Ferguson, 2021; Picardo et al., 2020; Tkalec et al., 2022a) although they do not fit the purpose. As a consequence, the lack of homogeneity in QC/QA measures of SNTS is enhanced. As a comparative, instrumental LODs were also calculated (see section 2.3) for the analytes included in this work to contrast with their respective LOIs. All the specific values are included in Table S5 in the SI for each suspect.

As can be seen in Fig. 4, 164 analytes (89.1 %) provided LOD values below 5 ng/mL. Precisely, the average value was 1.9 ng/mL. In the case of LOIs, only 75 compounds (41.0 %) provided values below 5 ng/mL, while a similar percentage (39.3 %) was in the 5–15 ng/mL range. Only in the cases of benzophenone-2 and mono-benzyl phthalate, the LOD values turned out to be higher than the respective LOI. It should be taken into account that the LOD definition is stricter in terms of precision. Lastly, for 16 analytes LOIs were not calculated since they were either type I (2) or type II (14) errors (section 3.2).

Consequently, the usage of LODs could lead to misleading outcomes about the performance of the method, and therefore, an important step towards the homogenisation of SNTS methods might be the usage of
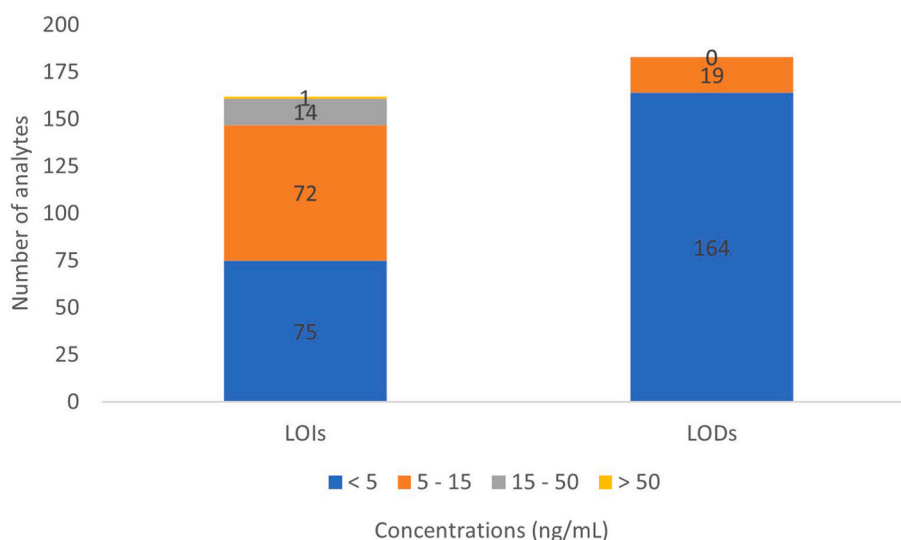

Fig. 4. Instrumental limits of detection (LODs) and identification (LOIs) of the 183 xenobiotics.

LOIs. However, if the ultimate aim is to quantify after confirming the presence of a suspect, a fully validated target method should be employed in which reliable limits of quantification need to be defined (González et al., 2014).

3.4. Endogenous substances

The influence of endogenous substances that are found in human biofluids should be carefully considered in SNTS works to avoid type I errors (i.e., annotating an exogenous substance that could also be an exogenous chemical) or type II errors (i.e., not annotating the xenobiotic due to the presence of endogenous molecule). To address that issue, a mass list of endogenous substances and their respective phase II metabolites (section 2.2) was used in this study both as an inclusion and exclusion list but always after data acquisition. One of the nodes in Compound Discoverer 3.3.2 allows including suspect lists of compounds that can be used during the post-processing of the data. In that sense, apart from the suspect list of exogenous and their phase II metabolites, a list of endogenous metabolites was included. In this approach, during the post processing of the data, the software retrieved only the features with a match in the exogenous suspect list. However, we could know whether there was also a candidate in the endogenous list apart from the exogenous candidate. Unlike in the previous sections, all the features detected in the biofluids besides the spiked suspects were considered.

3.4.1. Inclusion list

Using the mass list of exogenous substances as an inclusion list does not discard any feature until the final suspect list is provided. In that approach, all endogenous substances were considered as possible candidates for the feature and, thus, their MS2 and RT were also evaluated when applicable. If an endogenous metabolite candidate passed all the established criteria for a given feature, the feature was removed in the final step even if a xenobiotic compound passed the criteria as well avoiding a potential type I error.

All of the features that fulfilled the initial criteria for both positive and negative modes were submitted to the MS2 evaluation of all their tentative candidates, no matter whether they were xenobiotics or endogenous (see Fig. 5 for the whole annotation procedure).

In the positive mode, 627 features were manually discarded and set to MS2 evaluation, from which 380 features passed the criteria. The discarded features were directly set to levels 4 or 5. Finally, a total of 274 suspects were annotated at levels 1–3 with a satisfactory RT evaluation. In the negative mode, 495 were manually removed, 120 passed the MS2 match and finally, a total of 84 suspects were annotated at levels equal to or above 3 due to satisfactory RT evaluation.

In total, 358 suspects were annotated at levels 1–3 without considering endogenous substances. But when the inclusion list was included, the presence of the endogenous candidate(s) in the final list allowed the removal of 60 suspects in the positive and 7 in the negative mode. The full lists are included in Tables S6 and S7 in the SI for both ionization modes. Therefore, 291 suspects were finally annotated considering both ionization modes. Despite being a relatively large number of suspects, it should be bear in mind that 183 analytes were spiked to the samples and the total number of unique features were accounted gathering the 4

human biofluids.

Considering the abovementioned, the inclusion list allowed the discarding of 18.7 % level 2a/2b–3 suspects (67 out of 358). In the absence of pure standards for all the putative exogenous and endogenous molecules, it cannot be assured that those compounds eliminated were univocally endogenous, but the lack of this inclusion list of endogenous suspects could lead to potential type I errors. Lastly, it was observed that phase II metabolites of endogenous substances were also screened in the samples so they have to be considered in future SNTS works using human samples.

Considering the final list of 291 xenobiotics annotated at levels 1–3 using the inclusion list approach, 202 suspects were screened in the pooled urine sample, followed by the follicular fluid (185). In the cases of breast milk and human saliva, fewer compounds were annotated (145 and 116, respectively). Without considering the spiked analytes, 146 unique suspects were screened from which 103 were present in urine. In the rest of the biofluids, considerably fewer suspects were detected, since 49 were identified in follicular fluid, 36 in breast milk and 32 in saliva. Nevertheless, each biofluid can be relevant for exposome analysis depending on the aim despite fewer suspects being detected. As reported in the literature, the presence of exogenous chemicals in follicular fluid or breast milk for instance, can be related to decreased fertilization rate (Petro et al., 2012) or breast cancer (Koual et al., 2020), respectively.

3.4.2. Exclusion list

In this approach, the endogenous suspect list was used to filter any feature with at least one candidate in the list. Therefore, the use of the exclusion list automatically discarded a feature if at least one of the possible candidates was included in the endogenous mass list. That action involves a considerable reduction in the number of features that are submitted to manual revision making the process much more straightforward.

In the positive ionization mode, from the 1397 features that passed the compulsory criteria (see section 3.1), the inclusion list reduced the number to 1001 features (see Fig. 5). Those features were submitted to MS2 and further RT study to eventually annotate 197 suspects at levels 1–3. In the negative mode, the initial list of 891 features was reduced to 644 before MS2 and RT evaluation, from which eventually 73 were annotated at confidence levels above 3. That means that 643 features were discarded in total before studying their fragmentation spectra due to their presence in the exclusion list. In that sense, the exclusion list made the data processing less time-consuming.

On the negative side, some of the compounds annotated using the inclusion list approach were missed when using the exclusion list. From the 291 suspects annotated at levels 1–3 with the inclusion list of endogens, 21 were missed using the exclusion list. As an example, for the feature with 177.10222 Da exact mass detected in the positive mode, the software calculated the molecular formula $C_{10}H_{12}N_2O$, and two candidates were possible, (i) cotinine as an exogenous compound for being the metabolite of the psychoactive substance nicotine, and (ii) serotonin as an endogen. With the exclusion list, the feature was directly discarded without further consideration. However, with the inclusion list, the MS2 spectra of both candidates were evaluated. In both cases, the mzCloud library contained the spectra and the experimental MS2 acquired in the
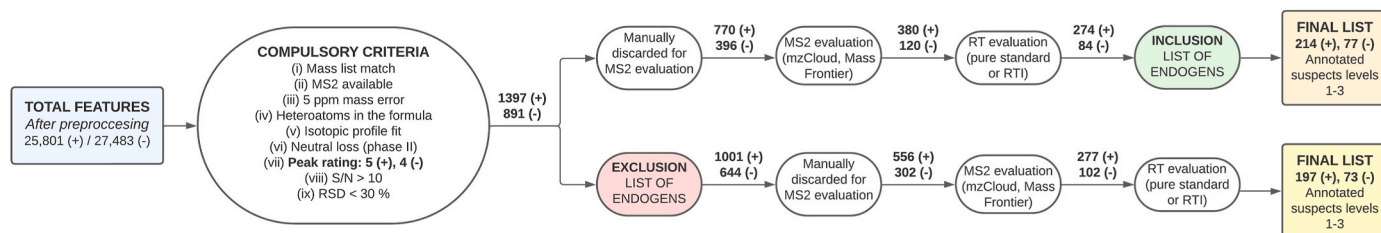

Fig. 5. The annotation workflow followed by considering the endogenous substances as (i) inclusion list or (ii) exclusion list.

spiked samples and the ones available in the library matched 95.7 % and 41.5 % for cotinine and serotonin, respectively (see Fig. 6). Consequently, serotonin was discarded and cotinine was eventually confirmed with the pure standard.

All in all, despite the exclusion list eliminating a considerable number of features (643) before their MS2 and RT evaluation, 7.2 % of type II errors (21 out of 291) were identified in the final suspect list in comparison to the inclusion list (see cotinine's case). Consequently, the inclusion list of endogenous substances seems a more suitable approach for discarding natural compounds and preserving relevant xenobiotics as exposome biomarkers in SNTS of xenobiotics in human samples.

4. Conclusions

A comprehensive workflow was designed and applied to screen exposome-related xenobiotics in a variety of human biofluids (urine, saliva, breast milk and ovarian follicular fluid). The "peak rating" parameter implemented in Compound Discoverer software, allowed removing automatically a considerable number (1247) of not-acceptable chromatographic peaks that would have to be manually revised otherwise. In addition, the minimum peak area threshold could be avoided, which is a controversial parameter in SNTS workflows. Moreover, type I and II errors were calculated in standard solutions and spiked human biofluids using 183 diverse xenobiotics. BPZ and benzyl paraben were identified as type I errors in both cases since the name of other suspects was wrongly provided for those spiked analytes (i.e., false positives). In terms of type II errors (i.e., false negatives), 7.7 % and 17.4 % of spiked suspects could not be screened in the standard solutions and spiked biofluids due to several reasons (e.g., peak shape, MS1 or MS2

errors), respectively. Lastly, a critical aspect of the SNTS methods in biofluids was studied, which is the presence of endogenous substances. Using an exclusion list of endogens in the annotation workflow, 643 features were directly discarded but type II errors were identified since 21 features were erroneously removed. In the case of the inclusion list, although the annotation process was a bit more time-consuming, no type II errors were committed. Moreover, it ensured avoiding type I errors since in the absence of the inclusion list of endogens, 67 probable endogens would be annotated as xenobiotics.

CRediT authorship contribution statement

Mikel Musatadi: Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft. **Inés Baciero-Hernández:** Formal analysis, Investigation, Methodology, Software, Validation, Writing – review & editing. **Ailette Prieto:** Conceptualization, Resources, Writing – review & editing. **Maitane Olivares:** Conceptualization, Data curation, Writing – review & editing. **Nestor Etxebarria:** Funding acquisition, Project administration, Resources. **Olatz Zuloaga:** Conceptualization, Funding acquisition, 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.
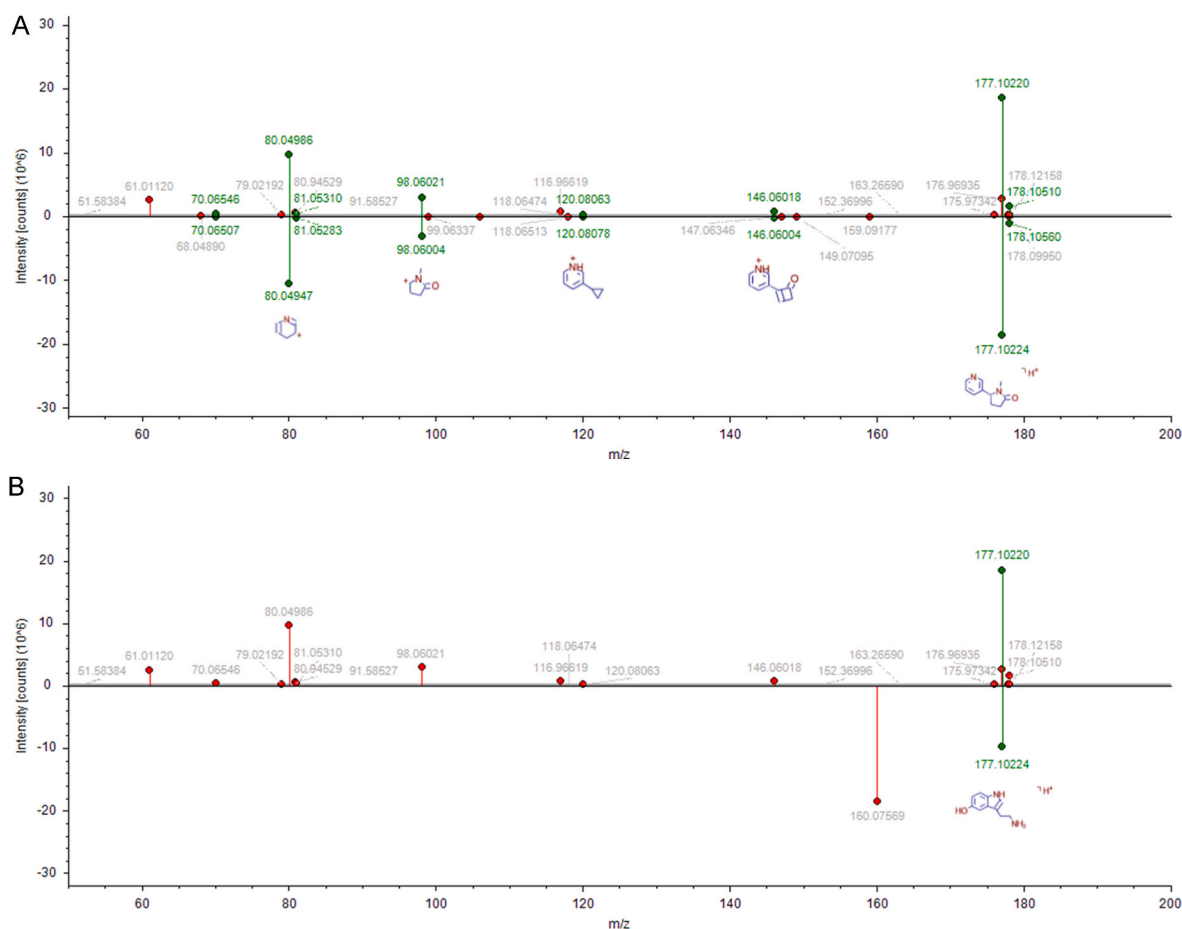

Fig. 6. MS2 match of possible candidates for the feature $C_{10}H_{12}N_2O_3$ ([M+H]$^+$ = 177.10222 Da, RT = 1.0 min) using mzCloud library: (a) satisfactory (95.7 %) match for cotinine and (b) unsatisfactory (41.5 %) match for serotonin.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2024.141221.

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