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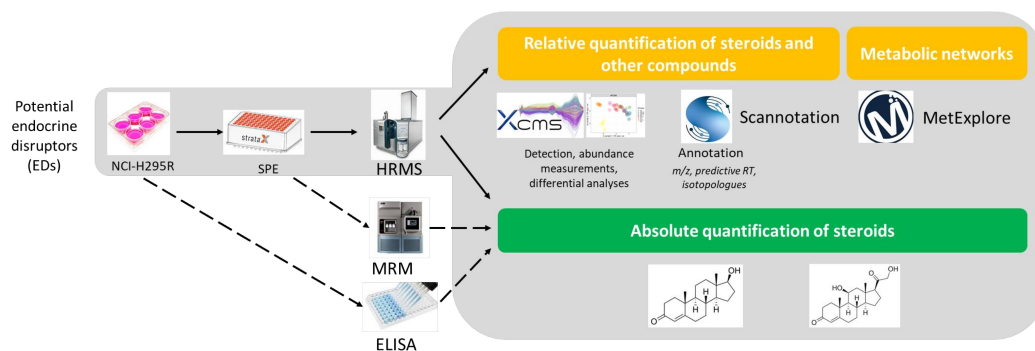
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Journal Pre-proof

High-throughput screening to identify endocrine disruptors: contribution of low resolution tandem MS and high-resolution MS

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Abbreviations

BPA: bisphenol A, ED: endocrine disruptor, ESI: electrospray, HRMS: high-resolution mass spectrometry, LAC: lowest amount on column, LOD: limit of detection, LOQ: limit of quantification, MRM: multiple reaction monitoring, MS/MS: tandem mass spectrometry, PCA: principal component analysis, sPLS-DA: sparse partial least squares discriminant analysis, TA: targeted analyses, ToF: time of flight.

Abstract

Background: Considering the large diversity of chemicals present in the environment and the need to study their effects (alone or as mixtures), the development of high-throughput *in vitro* assays in line with the Replacement, Reduction, Refinement (3R) strategy is essential for chemical risk assessments.

Results: We developed a robust analytical workflow based on both low resolution tandem mass spectrometry (MS/MS) and high-resolution mass spectrometry (HRMS) to quantify 13 steroids in NCI-H295R cell culture medium, human plasma and serum. The workflow was validated by screening media from the NCI-H295R cell line exposed in dose-response experiments to 5 endocrine disruptors (EDs) such as bisphenol A, prochloraz, ketoconazole, atrazine and forskolin. Absolute quantifications of the 13 steroids performed on a triple quadrupole (QqQ) MS/MS demonstrated that the performances obtained were in line with OECD recommendations. HRMS (MS1-HRMS) provided measurements nearly as sensitive and as reproducible as those obtained using multiple reaction monitoring (MRM) and ELISA. A bioinformatics workflow, using HRMS, was implemented to detect and annotate disrupted metabolites. HRMS allowed to detect disruptions in pathways associated to fatty acids, purines and amino acids metabolisms after exposure to the EDs tested, in addition to that linked to steroidogenesis.

Significance: We developed a robust MS1-HRMS workflow, from sample preparation to compound quantification or annotation, compatible with absolute steroid quantification, to screen NCI-H295R cell media exposed to potential EDs. Using only 200 μ L of medium, the method integrates MS/MS and HRMS analyses, 96-well plate solid-phase extraction for high throughput, and automated pre-annotation for cost efficiency. This optimized workflow identifies EDs in cell assays by detecting disruptions in steroidogenesis and other biological pathways.

Keywords: High-resolution mass spectrometry; Steroidogenesis; NCI-H295R; Human plasma and serum; MRM; ELISA; Environmental exposure; Chemical safety.

1. Introduction

The burden of diseases attributable to chemicals is high; in particular, chemical pollution is responsible for 1.8 million deaths each year globally, which is probably an underestimate [1]. The presence of many toxic compounds (including endocrine disruptors (EDs)) in the environment is reflected by the growing sizes of databases listing hazardous compounds such as the Hazardous Substances Data Bank (HSDB) with more than 5600 entries [2], the Food and Drug Administration (FDA)'s Endocrine Disruptor Knowledge Base (EDKB) including 1800 EDs [3], the European Union (EU)'s Endocrine Disruptor lists [4], or French Agency for Food, Environmental and Occupational Health & Safety (ANSES)'s ED list including 906 potential EDs [5]. Not all potential ED compounds can be tested; steps for prioritizing potential EDs are generally carried out in parallel to the development of appropriate biological models and high-throughput tests [6]. Many compounds can also be only partially evaluated due to non-intended uses or to the narrow scope of screening tools. To address this challenge, robust, rapid and exhaustive screening tools are needed to assess the toxicity or ability of contaminants to be EDs and to prioritize them for risk assessment [7].

The human adrenocortical cell line NCI-H295R [8] possesses all the genes coding for the enzymes of steroidogenesis and, according to the Organisation for Economic Co-operation and Development (OECD), thus represents a good model for *in vitro* steroidogenesis tests [9-11]. This cell model can be used to detect chemical compounds that affect steroidogenesis and in turn for xenobiotic prioritization purposes [12]. This cell model was initially developed to measure 17 β -estradiol (E2) and testosterone (T) production, but was recently extended to other steroid compounds [13, 14]. The H295R steroidogenesis assay can also be used to validate the

cell model and cell culture conditions using EDs with steroidogenesis action such as prochloraz, forskolin, bisphenol A, ketoconazole and atrazine to determine the influence of other compounds on steroidogenesis. The mechanisms of these known steroid inducers or inhibitors have been partially described and can help to identify signalling pathways for new potential EDs.

There are already many analytical methods available to determine steroid concentrations in biological fluids, organs or cells [15]. Among them, immunoassays such as enzyme-linked immunosorbent assays (ELISA) are routinely used to quantify steroids, but lack specificity and are little amenable to multiplexing in comparison to gas chromatography–mass spectrometry (GC-MS) [16] and liquid chromatography–mass spectrometry (LC-MS) experiments, and are limited to one chemical compound. LC-MS is increasingly used because, in contrast to GC-MS, it does not require derivatization to make steroids volatile and analyse them. LC-MS generally relies on a targeted method (i.e. tandem mass spectrometry (MS/MS)) and focuses on specific molecules (drugs, metabolites) for which non-labelled and labelled standards are available for absolute or relative quantification. Targeted analyses (TA) using triple quadrupole mass spectrometers (QqQ) reportedly offer great sensitivity, accuracy and specificity [16], because these analysers can carry out a fast double selection of precursors and fragments. However, the main limitation of MS/MS-based targeted analyses lies in the restricted number of molecules that can be measured during the analysis relative to the large number of endogenous chemicals that can be present in *in vitro* cell line media (including any potential metabolites of xenobiotics). Toxicity assays would clearly benefit from the use of new technologies that cover a broader spectrum of biological disturbances to characterize complex effects without compromising absolute quantification.

To counter the limitations of targeted analyses, high-resolution mass spectrometry (HRMS) (e.g., quadrupole time-of-flight (Q-ToF) or Orbitrap mass spectrometry (MS)) coupled to ultra-

high-performance liquid chromatography (UHPLC) are increasingly used to separate and detect tens of thousands metabolites present in samples, directly from precursor MS spectra (and MS2 data for structural identification). Today, HRMS workflows can handle absolute quantification in MS1 or MS2 as well as relative quantification of other metabolites in MS1 in the same experiment, and in addition provide additional data for risk assessment (including by detecting phase I/II metabolites of xenobiotics during exposure). Data treatment for HRMS is complex and requires a number of steps that include data preprocessing with peak detection, total chromatogram alignment, normalization of data, automation of statistical analysis with unsupervised (e.g., PCA) or supervised analyses (e.g., sPLS-DA,) [17] to discriminate between differential compounds accurately. Recent improvements in dedicated metabolomics software [18] such as the open-source XCMS or Mzmine tools, or commercial software such as MarkerView, Progenesis-QI, Metaboscape, Compound Discoverer [19], have facilitated these workflow steps. Enrichment in tools and functionalities and in specific and general compound databases [20] has recently made it possible to handle the most complex steps of analyses such as annotation [21]. Indeed, many tools facilitating annotation such as tools dedicated to predicting retention time (RT) [22] or isotopologue abundances or to matching MS/MS with spectral databases (e.g. MetFrag, Metlin) are now available [23]. We recently developed a new annotation tool to identify xenobiotics detected using HRMS [24]. This suspect screening tool, named Scannotation, based on precise mass measurements, RT prediction and isotopologue ratio fidelity can also be used for annotations of endogenous metabolites [25].

In line with a Replacement, Reduction, Refinement (3R) strategy, we developed a high-throughput analytical workflow using 96-well plates and both MS/MS and HRMS to quantify 13 steroids and detect simultaneously disrupted metabolites after exposure to endocrine disruptors (EDs) in low volumes of media from NCI-H295R cell line. We assessed the contributions of MS/MS, HRMS and ELISA (testosterone only for the last) for steroids

quantification in NCI-H295R cell line and studied the balance between data exhaustivity (i.e., the additional information provided with HRMS), sensitivity and accuracy. The method was also assessed to quantify steroids in human plasma and serum in addition to culture media of the NCI-H295R cell line using recovery experiments and LOD/LOQ determinations. Finally, the workflow was validated by screening media from the NCI-H295R cell line exposed to bisphenol A, prochloraz, ketoconazole, atrazine and forskolin in dose-response experiments following the OECD recommendations. A bio-informatics and statistical workflow using R were implemented to detect and annotate metabolites disrupted after exposures in the HRMS datasets. The suspect screening tool Scannotation was used for a rapid pre-annotation of discriminated features.

2. Material and methods

2.1. Reagents and human blood samples

Native certified standards of atrazine, bisphenol A, prochloraz and forskolin, 1-deoxycorticosterone, 11-deoxycortisol, 11-ketotestosterone, 17 α -estradiol, 17 β -estradiol, 17-hydroxypregnenolone, 17-hydroxyprogesterone, 2-hydroxyestradiol, 2-hydroxyestrone, 4-hydroxyestradiol, 4-hydroxyestrone, aldosterone, allopregnanolone, androstenediol, androstenedione, cholesterol, corticosterone, cortisone, dehydroepiandrosterone (DHEA), dihydrotestosterone, estriol, estrone, hydrocortisone/cortisol, pregnenolone, progesterone, prostaglandin D2, prostaglandin E2, prostaglandin F2 α , testosterone, hydroxytestosterone, leukotriene B4 and deuterated certified standards cortisol d4, androstenedione-d3, cortisone-d8, estrone-d4, leukotriene B4-d4, pregnenolone-d4, progesterone-d9, prostaglandin E2-d4, testosterone-d3 were purchased from suppliers Bertin, LGC, Sigma Aldrich and VWR and were stored at -20°C. Ultrapure water was produced using a Millipore Milli-Q Gradient system.

UPLC-MS-grade acetonitrile and formic acid were purchased from Biosolve (Dieuze, France). UPLC-MS-grade methanol was purchased from Carlo Erba (Val-de-Reuil, France). Human blood plasma and serum bags used for method development were acquired from the French blood donation agency (Etablissement Français du Sang, EFS).

2.2. Cell culture

NCI-H295R cells were purchased from the ATCC collection (Manassas, VA, USA). Cells were grown at 37°C, 5% CO₂ in recommended medium: DMEM/F1 (Sigma D6421) supplemented by 10% of fetal bovine serum (FBS), 1% of 2.5% NuSerum (Corning 355100), 1% insulin-transferrin-selenium (ITS) +Premix (Corning 354352), 1% L-glutamine (Sigma G7513), 1% antibiotics (Sigma P4333). The complete medium contained approximately 70 pg/mL testosterone. Cells were plated in 96-well plates (30 000 cells/well for 96-well plates and 300 000 cells/well for 24-well plates).

2.3. NCI-H295R cell exposure

Twenty-four hours after seeding, cells were exposed to chemicals in complete cell culture media (with serum). The following concentration ranges were tested for each ED compound: atrazine and bisphenol A (0, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 µM), prochloraz and ketoconazole (0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 µM), forskolin (0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 µM); all tests were performed in biological quadruplicates. After a 48 h incubation in medium supplemented with serum, cell culture media were collected and frozen at -80°C.

2.4. Testosterone ELISA assay

After exposure to EDs, cells were washed in PBS and fixed by cooled 90% ethanol/5% acetic acid/5% ultrapure water for 20 minutes for cell counts based on nuclei stained with Hoechst 33342 (B2261 Sigma). Image acquisition and analysis were performed using a Cellomics

ArrayScan VTI/HCS Reader (Thermo Scientific). Secreted testosterone was assayed using a commercial ELISA kit (Testosterone high sensitivity ELISA Kit ADI-900-176, Enzo) according to the manufacturer's instructions. Testosterone measurements were normalized relative to cell number. The complete cell culture medium contained approximately 70 pg/mL of testosterone.

2.5. Sample preparation for LC-MS analyses

After exposure, 200 μ L of NCI-H295R culture medium were collected and extracted using StrataX polymeric reversed-phase cartridges (Phenomenex, Le Pecq, France) according to the same conditions used in a previous study (12). Briefly, labelled standards were added before solid-phase extractions (SPE) at 25 ng/mL (final concentration in the injection mix). SPE cartridges were conditioned with 1 mL of methanol, then washed with 1 mL of ultrapure water. Then, cartridges were loaded with 200 μ L of culture medium (40 μ L for plasma and serum to limit the amount of endogenous steroids) diluted in 1 mL of water with 5% methanol. Labelled standards were added at this step except for recovery experiments to determine absolute recoveries. Cartridges were then washed twice with 1 mL of ultrapure water with 5% methanol. Steroids and other metabolites were eluted from the cartridge with 1 mL of 100% methanol. For plasma and serum, SPE procedures were identical with the exception of an additional precipitation step using 100% methanol after SPE elution to eliminate any remaining proteins. To do so, eluates were frozen for 30 min at -20°C and then centrifuged at 13300 rpm for 20 minutes and supernatants were kept. Eluates were evaporated under a speed vacuum, and then reconstituted in 20 μ L of H_2O /acetonitrile (ACN) (90:10, v/v) with 0.01% formic acid (FA), and 2 μ L was injected onto the LC-HRMS. Before MRM injections, 5 μ L (from the 20 μ L) was diluted in 50 μ L of H_2O /ACN (90:10, v/v) with 0.01% FA and 5 μ L were injected on the LC-MS/MS. Labelled standards were at 25 ng/mL final concentration in the final extracts for LC-HRMS and 2.5 ng/mL in the final extracts for LC-MS/MS MRM injections.

2.6. Spiking the NCI-H295R medium and human blood for recovery experiments

The culture medium, human plasma and serum samples were spiked with steroid standards at three concentrations (0.1, 0.5 and 2.5 ng/mL). To calculate absolute recoveries, HRMS signals for steroids in these samples were compared with matrix-match calibrations points (i.e., standards were spiked after SPE) at five concentrations (0.1, 0.5, 2.5, 5 and 10 ng/mL). Labelled standards were added at this step at a final concentration of 25 ng/mL to correct for analytical drift. The protocol is described in section 2.5. Recovery for each steroid and concentration are expressed in percentages and relative standard deviations (RSD) were calculated.

2.7. UHPLC-ESI-HRMS acquisitions

Samples (2 μ L) were injected on a X500R Q-ToF coupled to a micro-HPLC Exion system (both from Sciex). Chromatographic separations were performed using the following parameters: column ACQUITY UPLC HSS T3 (1.8 μ m, 1 mm x 150 mm, 100 \AA), 100 μ L.min⁻¹ flow, gradient rising from 90% mobile phase A (100% ultrapure water, 0.01% FA) to 20% mobile phase B (100% acetonitrile, 0.01% FA) in 2 min, then to 30% mobile phase B in 10 min, and to 45% mobile phase B in 9 min, to 100% mobile phase B in 3 minutes, isocratic flow at 100% mobile phase B during 5 min followed by a column regeneration at 10% mobile phase B for 6 min giving a total run time of 35 min. Samples were first acquired in full scan mode (ESI positive). Other parameters of the HRMS mass spectrometer are listed in a previous study [24]. Samples remaining after MS1 screening were acquired in data-dependent mode to facilitate the steps involved in annotating compounds with MS/MS data. The Q-ToF mass spectrometer was calibrated before each sample sequence. Samples were also randomized within the sequence. Extraction blanks were also considered to identify background noise from sample preparation.

2.8. Absolute quantification of steroids by UHPLC-ESI-HRMS

Steroids were quantified in the cell culture media of NCI-H295R cells exposed to five ED compounds (cf. Section 2.3) using a calibration curve and labelled standards corrections. Sciex OS software (version 1.2) was used to determine the absolute quantification of steroids. Precursor masses and RTs obtained using pure standards are given in File S1. Peaks corresponding to steroid compounds (m/z < 5ppm and matching RT) were integrated and reviewed manually. Ten matrix-matched calibrations points (from 0.05, 0.1, 0.5, 2.5, 5, 10, 20, 50, 100 to 300 ng/mL) in culture medium (using the same SPE protocol as the samples) were used to obtain standard curves. The calibration curves were used for HRM determination of absolute steroid concentrations in NCI-H295R media. Standard curves were validated if their coefficient of determination (R^2) was greater than 0.99. The sensitivity of the MS1-HRMS quantification methods was compared with those obtained with the LC-MSMS instrument using the quantity of compounds injected on the column. Injected quantities on columns ranged from 1 to 6000 pg for MS1-HRMS quantification.

2.9. Relative quantification using MS1-HRMS

Wiff2 files were converted into mzML files (full scan) using MS-convert software from Proteowizard [26]. An in-house R workflow using the XCMS (version 3.12) and mixOmics [27] (version 6.14.1) packages, was used to detect, quantify and discriminate the metabolites of interest found in the cell media. For peak detection, the deconvolution algorithm centWave was used with the following parameters: m/z tolerance of 10 ppm, signal on noise threshold of 5, a prefilter between 3 and 100, 50 for noise and peak width between 10 and 50. RTs were corrected using the AdjustRtime function with a locally weighted smoothing (LOESS) regression. Peaks were grouped with the GroupChromPeaks function and areas of missing peaks were integrated using the FillChromPeaks function. Differentially expressed features were determined using the Diffreport function of the XCMS package with Student's t -tests for comparison group by

group and ANOVA p-values for multiple groups. P-value thresholds were set to 0.01. The Camera package was used to annotate adducts and isotopologues in the peaklist.

2.10. Compound annotation for HRMS data using the Scannotation suspect screening tool

The annotation step was based on MS1 m/z measurements, predicted RT using the partition coefficient of a solute between octanol and water (logP) or else XlogP extracted from Pubchem, and isotopologue ratio fidelity measurements was performed according to Chaker et al. [24]. We used a database containing 745 compounds including steroids, steroid metabolites and endogenous metabolites. LogP values were the only RT predictor used in this study. We used 100 experimental RTs corresponding to 19 steroids, 12 labelled steroids and 76 pesticides to construct a linear regression between their RT and logP values. Based on this regression, our tool tested the hits detected using the m/z measurements and the database (File S2). The isotopologue ratio fidelity measurements, which relied on the MIDAs tool [28] integrated in our Scannotation tool, were also used to validate annotation hits. When possible, chemical identity was confirmed from accurate mass, isotopic fit and fragmentation data obtained from standard compounds (highest level of confidence). Additionally, pre-annotation of compounds by Scannotation were validated using MS2 data and the joint use of the MetFrag web tool [29] and the KEGG and Pubchem databases. The scoring system from Schymanski et al. [30] was used for all provided annotations. The Scannotation tool can be downloaded at <https://github.com/scannotation>. Annotated molecules whose abundances were impacted (p -value<0.01) by EDs exposure (maximum concentration) were represented in functional metabolic networks using the webtools MetExplore [31] and the Recon3D human database [32]. Over-represented pathways including annotated molecules were set according to a Right tailed Fisher Exact Test (p -value<0.05).

2.11. UHPLC-ESI-MS/MS acquisitions

Extracts (5 μL) were injected and steroids were quantified using a Xevo QqQ XS equipped with an ESI source and coupled to an Acquity-LC system (all devices are from Waters). Chromatographic separation of steroids was carried out using the following parameters: Acquity UPLC HSST3 column (1.8 μm , 2.1x150mm, 100 \AA), 300 $\mu\text{L}/\text{min}$ flow, gradient rising from 75% mobile phase A (ultrapure water, 0.01% formic acid) to 40% mobile phase B (100% acetonitrile, 0.01% formic acid) in 1 minute, to 45% mobile phase B in 3 minutes, to 55% mobile phase B in 9 minutes, to 100% mobile phase B in 1 minute, to 65% mobile phase A in 3 minutes for a total time of 20 minutes. MRM transitions of steroids, associated MRM parameters (retention times, cone voltage, collision energy) are reported in File S1. The QqQ mass spectrometer was calibrated before each sample sequence. Samples were also randomized within the sequence. Extraction blanks were also considered to identify background noise from sample preparation.

2.12. Absolute quantification of steroids using LC-MS/MS MRM

Steroids were quantified in cell culture medium of NCI-H295R cells exposed to five ED compounds (cf. 2.3). Standard curves and ISTD corrections were used to quantify steroids. TargetLynx (Waters) was used to carry out data processing and determine the absolute quantifications of steroids. The same spiked samples as those used for the absolute quantification of steroids in MS1-HRMS were used. Ten matrix-matched calibrations points (from 0.05, 0.1, 0.5, 2.5, 5, 10, 20, 50, 100 to 300 ng/mL) in culture medium samples (using the same SPE protocol as the samples) were used to obtain standard curves. Injected amounts ranged from 0.025 to 1500 pg for MRM quantification. Standard curves were validated if their coefficient of determination (R^2) were greater than 0.99.

2.13. Determination of limits of detection and quantification and lowest amount on column

Lowest amounts on column (LAC), limits of detection (LOD) and limits of quantification (LOQ) values were determined for each fluid using MS1-HRMS and LC-MS/MS MRM experiments using respectively Q-ToF and QqQ MS. The LOQ value was defined as 3.3 times the LOD value. Steroids were added at concentrations of 0.005, 0.01, 0.025, 0.050, 0.1, 0.25, 0.5, 1, 2, 4 ng/mL in 200 μ L of cell culture medium. For plasma and serum samples, steroids were added at concentrations 0.025, 0.05, 0.125, 0.25, 0.5, 1.25, 2.5, 5, 10, 20 ng/mL in 40 μ L of plasma and serum, both diluted in 200 μ L of water. The concentration of injected amounts of spiked steroids ranged from 0.1 to 80 pg and 0.025 to 20 pg for MS1-HRMS and MRM experiments, respectively.

2.14. Data availability

The complete sets of MS1-HRMS data are available as .mzML files in the Metabolights repository [33] under identification number MTBLS9547 (www.ebi.ac.uk/metabolights/MTBLS9547). The non-targeted analysis Reporting Tool (NTA-SRT) was used to report on methods and results (Supplementary File S3) [34].

3. Results

3.1. General workflow

A workflow based QqQ and HRMS instruments were developed for NCI-H295R cell culture media, human plasma and serum for the determination of steroid concentrations and detection of additional biological pathways disrupted. We also compared the sensitivity of these MS methods using the lowest amount on column (LAC) values obtained with spiked standards. Recovery of spiked steroids were also determined (Supplementary Table S1). Moreover, xenobiotics (atrazine, bisphenol A, ketoconazole, forskolin and prochloraz) known as EDs were tested on NCI-H295R cells to validate the developed workflow. The ability of the MRM and MS1-HRMS methods to quantify endogenous steroids was evaluated and compared with each

other and with an ELISA assay (for testosterone only). The contribution of MS1-HRMS to detect and determine relative quantities of steroids, xenobiotic metabolites and additional endogenous metabolites in addition to the absolute quantities of steroids were evaluated. To do so, we used a workflow based on the XCMS R package and on an in-house annotation tool (Fig. 1). Confident annotations were obtained with predictors such as the m/z , RT prediction and the fidelity of the isotopologue ratio between theoretical and experimental detected compounds. Candidates were then all manually curated and confirmed using MS2-HRMS. The list of studied steroids with their m/z values observed in MS1-HRMS, their experimental RTs and chosen MRM transitions are given in File S1.

3.2. Sensitivity of the MRM and MS1-HRMS methods in the detection of steroids in NCI-H295R cell culture medium and human plasma and serum

Before determining the sensitivity of the methods, spike-and-recovery experiments were carried out using MS1-HRMS experiments and NCI-H295R culture medium, human plasma and human serum. Recovery values were expressed as a percentage and RSD values were provided. (Supplementary Table S1). To determine the sensitivity of the developed MS1-HRMS and MRM methods, we compared the LAC of steroids spiked in the three different matrices (NCI-H295R culture medium, human plasma, human serum) obtained by MS1-HRMS and MRM (Table S1). LAC values for the cell culture medium ranged from 0.125 to 2.5 pg and 1 to 20 pg for MRM and MS1-HRMS, respectively. LAC values were on average lower for MRM by a factor of 8.52, 1.42 and 1.25 for cell culture medium, plasma and serum, respectively (Fig. 2A). Comparisons between LAC values for the different matrices (culture medium, plasma and serum) showed a heterogeneous response according to steroid type. Nevertheless, LAC values were generally lower for MRM in cell culture medium than in plasma and serum. This contrast among matrices was less marked for MS1-HRMS analyses (Fig. 2A, Table S1). LOD/LOQ in (ng/mL) were also determined (Table S1). LOQ values obtained for each steroid varied with

the biological fluid, with sensitivity clearly dependent on the nature of the matrix used. LOQ ranges for cell culture medium, plasma and serum samples were respectively 0.03-1, 0.25-40, 0.25-40 ng/mL for MRM and 0.1-2, 0.08-20, 0.08-20 ng/mL for MS1-HRMS (Fig. 2B). LOD/LOQ values were clearly lower for the culture medium regardless of the method used, probably due to higher matrix interferences in blood samples. The MRM method gave the lowest LOQ values compared with HRMS for the NCI-H295R cell culture medium. For human blood samples (plasma and serum) (Table S1, File S4), LOQ were the lowest for MS1-HRMS (Fig. 2B). However, this was due in part to the use of 40 μ L of plasma and serum, but 200 μ L of cell culture medium during the process.

3.3. Comparison of absolute endogenous steroid concentration and their variability obtained using HRMS, MRM and ELISA in response to xenobiotic exposure in NCI-H295R cell culture medium

Steroid concentrations were measured with Q-ToF (MS1-HRMS) and QqQ (MRM) instruments in the NCI-H295R medium after exposure to different xenobiotics (atrazine, bisphenol A, prochloraz, ketoconazole and forskolin) at different doses. MS1-HRMS and MRM experiments generally showed similar variations in absolute steroid concentrations for the forskolin, atrazine and ketoconazole xenobiotics and the steroids (testosterone, corticosterone and progesterone) (Fig. 3A). Concentrations obtained with all other steroids and other xenobiotics are given in File S4. A concentration measurement shift was observed between the absolute values of corticosterone concentration obtained using MS1-HRMS and MRM for the basal and lowest concentration points (Fig. 3A). The ELISA assay was performed and compared with MS1-HRMS and MRM experiments only for testosterone (Fig. 3A, File S5). The lowest steroid concentrations showed large variations among the three methodologies (Fig. 3B). Coefficients of variation (CVs) associated with the absolute concentrations obtained for each steroid at each concentration and for all xenobiotics were compared between the three

methodologies used (Fig. 3C). Overall, 89.5% (563 values) and 83.8% (511 values) of measured concentrations showed CVs below 20% for the MS1-HRMS and MRM analyses, respectively (File S4) and were considered acceptable. MS1-HRMS gave slightly lower CVs than MRM for all steroids and all xenobiotics, with average CVs of 12.23 and 13.13%, respectively. For testosterone, average CVs were 11.62, 13.42 and 11.05% for MS1-HRMS, MRM and ELISA, respectively (Fig. 3C). Although the mean CVs obtained with the three methodologies were consistent for testosterone and the other steroids, MS1-HRMS seemed to produce fewer CV values greater than 20% than MRM when considering all steroid CVs (Fig. 3C). Low concentrations of steroids favoured high CVs in all three methods (Fig. 3D). Average CVs were similar for steroids with concentrations below 0.2 ng/mL, with CV values of 10.0 and 11.9% for respectively MS1-HRMS and MRM, whereas median CVs were 14.7 and 26.9%, respectively. High CV values were also more frequently observed for xenobiotics that decrease steroid abundance such as prochloraz, bisphenol A and ketoconazole (Fig. 3D). For example, 10.5 and 16.2% of 'high' CVs (i.e. >20%) were observed respectively for MS1-HRMS and MRM over all steroids. For testosterone, measurements gave 19.05, 19.04 and 11.9% of relatively high CV values for MS1-HRMS, MRM and ELISA, respectively. CVs for MS1-HRMS and MRM were also more similar to each other than those obtained using ELISA (Fig. 3D).

3.4. Effects of model xenobiotics on NCI-H295R steroidogenesis based on absolute quantification of steroids using MS1-HRMS

As a validation step, we then measured the absolute steroid concentration using MS1-HRMS to study the alterations in the steroid signalling pathway in response to known EDs such as atrazine, bisphenol A, ketoconazole, forskolin and prochloraz (Fig. 4). As expected, testosterone concentrations increased following exposure to atrazine (T (30 μ M):DMSO ratio=1.42) and forskolin (T (10 μ M):DMSO ratio=1.82). Likewise, we observed expected

decreases in testosterone concentration following exposure to ketoconazole (T (1 μ M):DMSO ratio=0.013), BPA (T (30 μ M):DMSO ratio =0.044) and prochloraz (T (1 μ M):DMSO ratio=0.055). The OECD guidelines indicate a minimal increase of testosterone concentration with a factor of 1.5 for forskolin (10 μ M) and a minimal decrease with a factor of 0.5 following prochloraz (1 μ M) exposure [10]. Therefore our culture conditions met the performance criteria listed in the OECD guidelines for testosterone compounds. An overall increase in steroid concentration was observed with increasing doses of atrazine compounds, with a more marked increase for corticosteroids. Similar variations were observed with increasing doses of forskolin, whereas an increase in progestogens and a decrease in androgen and corticosteroid concentrations were observed in response to exposure to prochloraz. An overall inhibition of steroid concentration was also measured using MS1-HRMS following exposure to increasing BPA and ketoconazole doses, except for progesterone exposed to ketoconazole.

3.5. Detection of steroids and endogenous molecules using the MS1-HRMS-based workflow

In addition to absolute concentrations of steroids, HRMS at the MS1 level can also detect and compare relative abundances of other metabolites between the control and the different doses of exposure (Fig. 5A, File S6). Detection and abundance measurements were carried out using the XCMS package. No variation in the intensity of the total ion currents (TIC) was observed during the different LC-MS1-HRMS analyses, demonstrating that no analytical drift occurred during the experiment (Fig. S1). No batch effects were detected by PCA (Fig. S1). sPLS-DA was carried out to select the most differential molecules using the mixOmics package (Fig. S1) and indicated a clear separation between concentrations. We used the `diffreport` function of the XCMS package to calculate p-values for the *t*-tests and ANOVAs. The numbers of features detected with the XCMS parameters (cf. Section 2.8) were 16453, 17160, 18329, 16111 and 17023 for prochloraz, ketoconazole, forskolin, BPA and atrazine, respectively (Supplementary File S6). Volcano plots highlighted that a large number of metabolites other than steroids were

differentially abundant according to increasing concentrations of the five ED compounds (Fig. 5A, Fig. S2). Comparison of testosterone abundances measured with XCMS with the absolute measurements of testosterone obtained with Sciex OS using the same MS1-HRMS acquisitions showed that both MS1-HRMS-based relative and absolute quantifications provided similar testosterone fold-changes following exposure to prochloraz and forskolin (Fig. 5B, Supplementary File S4). For example, fold-changes between the highest concentrations of xenobiotics and control were respectively 0.036 (relative) and 0.044 (absolute) for prochloraz and 1.66 (relative) and 1.56 (absolute) for forskolin. The average RSD values were similar between absolute testosterone concentration ratios and relative abundance ratios with 6.91% (absolute) and 6.61% (relative) values for forskolin and 15.32 % (absolute) and 11.75% (relative) for prochloraz (Supplementary File S4).

3.6. Annotation of steroids, xenobiotics and their metabolites and endogenous molecules using the MS1-HRMS-based workflow

In addition to the relative quantification step, the MS1-HRMS-based workflow can annotate different compounds using a suspect screening approach relying on a library with a 746 compounds of interest and a scoring system (Fig. 6A) for compound annotation based on the precision of MS1 m/z measurements, predicted RT using logP, and isotopologue ratio fidelity measurements [24]. The suspect screening tool named Scannotation was described in a precedent study [25]. Three groups were defined according to annotation confidence. G2 indicated that one predictor—either predicted RT (score >0.3) or isotopologue ratio fidelity—was acceptable in addition to m/z precision (score >0.7). G3 indicated that all three predictors were correct for the annotation. The metascore is a synthesis of the three predictor scores and must be greater than 0.7. The closer the molecule's metascore is to 1, the higher the level of confidence in the annotation is. The model of RT prediction was constructed with 100 experimental RT (RT_{exp}) of various molecules (steroids, labelled steroids and pesticides)

obtained in the same LC-HRMS conditions and their corresponding logP. The correlation ($R^2 = 0.62$) between these logP and RT_{exp} (Fig. 6B), was used to predict the RTs of molecules for which no RT_{exp} has been compiled, from their logP. This suspect screening tool made it possible to annotate studied steroids, other steroids or modified steroids, the xenobiotics and some of their metabolites, and other metabolites present in the cell culture medium or secreted by the cells. All pre-annotated molecules derived from datasets with xenobiotic exposure were classified in three groups (G2, G3a and G3b) and sorted according to their metascore (Fig. 6C). Individual scores are provided in Supplementary File S7. A total of 210 molecules were pre-annotated in all the datasets with 40.5% (85) common to the five datasets (Supplementary Fig. S3). Steroids (endogenous) and labelled steroids (spiked) fell mostly in group G3 (the group with the highest confidence scores) indicating the relevance of the scoring system. Additionally, pre-annotated compounds by Scannotation were validated using MS2 data and the MetFrag web tool (Supplementary File S8). To assess whether Scannotation is able to pre-annotate compounds correctly, we took pre-annotated compounds with a G3 score (best metascores with m/z, retention time and isotopologue fidelity) for atrazine and BPA datasets and counted compounds that could be considered annotated either by using standards, labeled standards but also using MS2 data and the MetFrag online tool (Supplementary File S8). This resulted in 28/33 (84.8%) and 30/36 (83.3%) annotated compounds for the atrazine and BPA datasets respectively (Supplementary Fig. S4).

3.7. The MS1-HRMS workflow allowed to monitor abundance of tested EDs and their metabolites

In addition to identifying steroids and xenobiotics, MS1-HRMS was able to monitor that the signals obtained for the tested xenobiotics were consistent across the different doses tested (Fig. 7). In addition, it was also possible to monitor the kinetics of formation of the different xenobiotic metabolites from phase I such as 2,3 dehydro-ketoconazole, 2,2-bis(4-

hydroxyphenyl)propanal (derived from BPA) or deethylatrazine compounds derived from atrazine (Fig. 7). Phase 2 metabolites were also detected and quantified such as the BPA-glucuronide compound (Fig. 7). Abundances of all these metabolites increased with increasing doses of native xenobiotics.

3.8. The MS1-HRMS workflow highlighted that the metabolism of fatty acids, purines and amino acids was affected by the ED compounds tested

Abundances of metabolites not derived from xenobiotics or steroids were also measured by the MS1-HRMS workflow. Abundance variations of annotated metabolites were presented in the supplementary File S7. MetExplore was used to determine metabolic networks in which differentially abundant metabolites were present. Impacted networks (p-value <0.05) were represented in a metabolic pathway analysis plot and showed that steroid metabolism (p-value=7.60E-27), purine catabolism (p-value=1.80E-07), protein degradation (p-value=1.09E-08) and protein formation (p-value=9.27E-05) were altered according to EDs exposures (Fig. 8A, Supplementary File S9). The molecules with the highest differential abundances for maximum ED concentrations are shown in a heatmap (Fig. 8B). Deoxycarnitine (100 μ M:control ratio=16.94, adjusted p-value= 3.65E-03) and adenine (100 μ M:control ratio=4.91, adjusted p-value=2.54E-04) abundances increased following exposure to atrazine and BPA, respectively (Fig. 8C). Abundance of cis-4-decenoylcarnitine decreased with increasing doses of atrazine (100 μ M:control ratio=0.52, adjusted p-value=5.48E-03) and prochloraz (10 μ M:control ratio=0.57, adjusted p-value=2.90E-01), whereas its abundance increased with increasing doses of BPA (100 μ M:control ratio=5.58, adjusted p-value= 2.54E-03) and forskolin (30 μ M:control ratio=32.3, adjusted p-value= 5.18E-05) (Fig. 8C). Abundance of hypoxanthine decreased following BPA (100 μ M:control ratio=0.34, adjusted p-value= 2.2E-02) and ketoconazole exposures (10 μ M:control ratio=0.37, adjusted p-value= 3.0E-02) (Fig. 8C). Abundance of N-acetylputrescine decreased following BPA (100 μ M:control ratio=0.77,

adjusted p-value=1.4E-02), ketoconazole (10 μ M:control ratio=0.73, adjusted p-value=9.0E-02) and forskolin (30 μ M:control ratio=0.70, adjusted p-value= 3.3E-02) exposures (Fig. 8C).

4. Discussion

In line with the 3R strategy, we designed a complete workflow using MS1-HRMS on NCI-H295R cells, a model for steroidogenesis studies [10], to develop a high-throughput assay to test if specific compounds disrupt the steroidogenesis (and other metabolite pathways). MS1-HRMS-based methodologies have been successfully applied to measure abundances of steroids [35] or quantify them [36] using the NCI-H295R cell line model. In this study, we compared an MS1-HRMS-based workflow to MRM acquired on the UHPLC-ESI-QqQ and to the ELISA assay for testosterone and demonstrated that absolute quantifications of steroids were similar in terms of accuracy and sensitivity. Comparisons of LAC values indicated that MS1-HRMS is almost as sensitive as MRM for the absolute quantification of steroids. These results are in line with the comparison of steroid concentrations measured with MS1-HRMS and QqQ performed in meat [37]. A comparison of MS1-HRMS using ToF-MS with QqQ quantification in steroid-spiked plasma [38] indicated less sensitivity and a wider linear range for HRMS, but nonetheless performed satisfactorily. Pozo et al. reported that QqQ sensitivity is necessary to test for specific steroids in doping controls, but conclude that HRMS can also be used on non-targeted steroids [39].

Compared with the literature, we observed an overall consistency of the variations in absolute steroid concentration following exposure to EDs. Previous studies report similar variations in steroid abundances in the NCI-H295R cell line consecutive to exposure to prochloraz and forskolin [40, 41] as well as ketoconazole [41]. Overall increases in steroid concentrations, as measured using MS1-HRMS, consecutive to forskolin and atrazine exposure are consistent with their respective functions as an adenylate cyclase activator and a phosphodiesterase inhibitor,

respectively [42]. The effects of ketoconazole on steroids obtained using MS1-HRMS are consistent with its inhibitor activity on cytochrome P450 enzymes Cyp11 and Cyp17 [43]. The androgen receptor antagonistic activity of prochloraz was also reflected in the high specific progesterone concentrations observed in our data. Our findings also uncovered the anti-androgenic activity of BPA [44], which binds at high concentrations to androgen receptors as an antagonist.

Moreover, our MS1-HRMS-based workflow offers an additional advantage by providing relative comparisons of steroids between the different conditions tested, but also unexpectedly affected metabolites (including the metabolites of xenobiotics) during the same experiment without influencing absolute steroid quantification. Moreover, specific metabolite profiles obtained for each of the five ED compounds can be used independently of metabolite annotation to determine the possible mode of action of the screened xenobiotics. The Scannotation suspect screening tool [25] incorporated in the workflow allowed annotations of compounds based on accurate m/z , by comparing isotopologue profiles with theoretical profiles and predicting RTs based on logP, using an appropriate database containing steroids, xenobiotics and other metabolites secreted from cells or present in the cell culture medium. Increasing doses of EDs induced increased abundance of their known metabolites for BPA (2,2-bis(4-hydroxyphenyl)propanal, BPA-glucuronide), atrazine (deethylatrazine) and ketoconazole (2,3-dehydro-ketoconazole). This response thus illustrates that MS1-HRMS can be used for drug monitoring, providing a fairer picture of xenobiotic exposure, and incorporating xenobiotic metabolites in the search for their effects, while allowing for the assay of steroids. Metabolomics on NCI-H295R cell culture media showed that purine and amino acid metabolism were mainly affected by the EDs used in addition to steroids. We observed also specifically that abundance of decenoylcarnitine increased following exposure to BPA and forskolin, but decreased slightly following exposure to prochloraz and atrazine pesticides. To

note, a protective role of carnitines against hepatotoxicity associated to BPA exposure was described in rats [45] indicating a potential cellular response of NCI-H295R cells to counter BPA exposure. Therefore, the use of MS1-HRMS showed that EDs have specific effects on fatty acid import and fatty acid metabolism, given that carnitines were reported to be implicated in fatty acid transport within cells and to promote mitochondrial fatty acid oxidation (FAO) [46]. Adenine levels in the cell culture medium increased following BPA exposure, which is concordant with intracellular losses of adenine nucleotide pools reported in rat hepatocytes following BPA exposure [47] but also following prochloraz, ketoconazole and atrazine exposures. This is concordant with the fact that purine metabolism is affected following BPA exposure as observed in the MCF-7 human breast cancer cell line [48]. The abundance of the purine derivative hypoxanthine abundance was particularly reduced after exposure to ketoconazole and BPA. This decrease could be induced by the fact that BPA was capable of activating the xanthine oxidase and then converting hypoxanthine to xanthine in vitro in rat liver [49].

5. Conclusions

Our study highlighted the contribution of MS1-HRMS to measure absolute concentrations of steroids as precisely as ELISA or MRM experiments and can simultaneously provide additional information on the relative abundances of other molecules. We designed a MS1-HRMS workflow, from sample preparation to annotation of the detected compounds with the Scannotation tool, and applied it successfully to describe the response of the model adrenocortical cell line NCI-H295R to exposure to five EDs. Future developments should make it possible to include ion mobility values and MS2 measurements in the Scannotation tool's scoring system to improve the annotations of the detected compounds. Moreover, we have shown that carnitines and the metabolism of purines and amino acids are particularly disrupted by exposure to these EDs.

CRedit authorship contribution statement

Thibaut Léger: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. **Rémy Le Guevel:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Resources; Software; Supervision; Validation; Visualization. **Hélène Solhi:** Formal analysis; Investigation; Methodology; Resources; Software; Validation. **Bertrand Evrard:** Conceptualization; Investigation; Methodology. **Thomas Darde:** Conceptualization; Methodology. **Christèle Desdoits-Lethimonier:** Methodology; Resources. **Philippe Glorennec:** Conceptualization; Writing - review & editing, **Nathalie Bonvallot:** Conceptualization; Writing - review & editing. **Frédéric Chalmel:** Conceptualization; Funding acquisition; Investigation; Project administration; Resources; Validation; Visualization; Writing - review & editing. **Arthur David:** Conceptualization; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing - review & editing

Declaration of competing interest

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

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Figure legends

Figure 1: Experimental design. Sensitivity and reproducibility of HRMS and MRM for the determination of absolute concentrations of steroids in human plasma, serum and NCI-H295R cell culture medium were compared by considering lowest amount on column (LAC) and LOD/LOQ values obtained on steroid-spiked samples. Variations of absolute steroid concentrations in the NCI-H295R cell culture medium following exposure to EDs (prochloraz, bisphenol A, ketoconazole, atrazine and forskolin) in dose-response experiments were assessed with HRMS, MRM and ELISA (only for testosterone) experiments and compared. Additional metabolites other than steroids and obtained by HRMS were quantified (relatively) using the XCMS R package and identified using the Scannotation tool based on mass accuracy, RT predictions and isotopologue ratio fidelity. **B:** Boxplot showing LAC values for all quantified steroids obtained using MRM and HRMS and according to the type of biological fluid used (cell culture medium, plasma, serum). **C:** Boxplot showing LOQ values for all quantified steroids obtained by MRM and HRMS and according to the type of biological fluid used (cell culture medium, plasma, serum). Detailed LAC and LOD/LOQ values are reported in Table S1.

Figure 2: Sensitivity of analytical instrumentations and workflows for steroids detection and quantification. Boxplot showing LAC values for all quantified steroids obtained using MRM and HRMS and according to the type of biological fluid used (cell culture medium, plasma, serum). **C:** Boxplot showing LOQ values for all quantified steroids obtained by MRM and HRMS and according to the type of biological fluid used (cell culture medium, plasma, serum). Detailed LAC and LOD/LOQ values are reported in Table S1.

Figure 3: Assay of endogenous steroids and the comparison of the absolute quantification and coefficient of variations (CVs) obtained from HRMS, MRM experiments and ELISA assay in response to xenobiotic exposure, in the H295R cell culture medium. **A:** Boxplots

representing absolute concentrations of testosterone (top left panel), corticosterone (top right panel), testosterone (bottom left panel) and progesterone (bottom right panel) obtained from HRMS, MRM and ELISA experiments after forskolin, ketoconazole, atrazine and ketoconazole exposure, respectively. EDs concentrations are indicated on the horizontal axis. **B**: Scatter plots of absolute steroid (left panel) and testosterone (other panels) concentrations induced by all xenobiotics at all concentrations and obtained by HRMS, MRM and ELISA. **C**: Scatter plots representing comparisons of CVs associated to steroids (top left panel) and testosterone (other panels) concentration determination according to HRMS, MRM and ELISA experiments. **D**: Boxplots of CVs associated with steroids (top panel) and testosterone (bottom panel) concentrations determined from HRMS, MRM and ELISA experiments. All steroids and all xenobiotic concentrations were used to compile CVs.

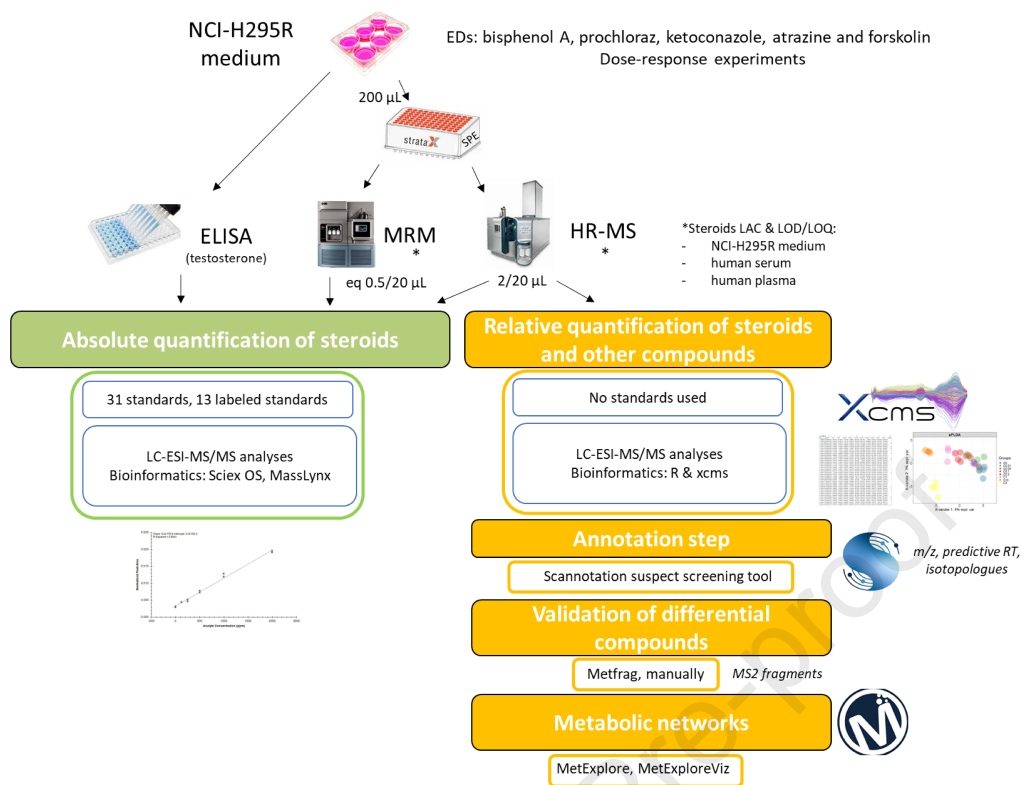
Figure 4: Variations in steroid concentrations in NCI-H295R cell culture medium, measured using HRMS, induced by atrazine, prochloraz, ketoconazole, forskolin and bisphenol A exposures. Absolute steroid concentrations were measured by HRMS (in positive ionization) in NCI-H295R cell culture media in response to different xenobiotic concentrations. For forskolin, the concentration range was 0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30 μM . For prochloraz and ketoconazole concentration ranges were 0, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 10 μM whereas for BPA and atrazine concentration ranges were 0, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100 μM . Biological quadruplicates were used for each concentration.

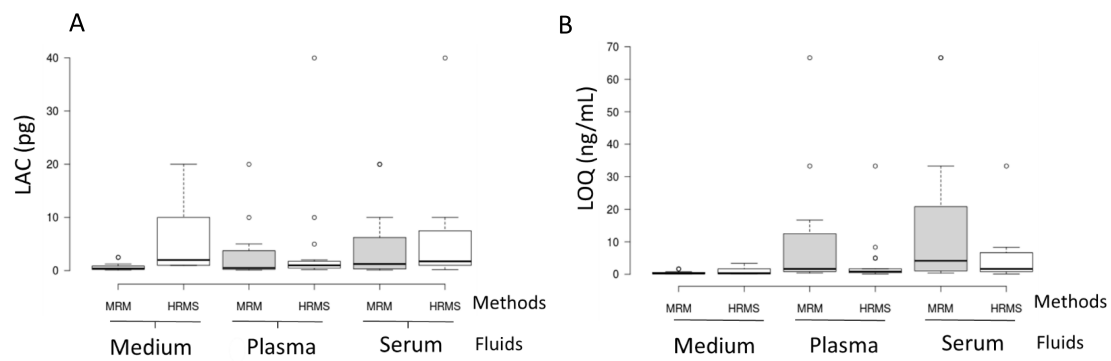
Figure 5: Relative quantification of additional features detected using HRMS. **A**: Variation in relative metabolite abundances induced by prochloraz (10 μM), ketoconazole (10 μM), BPA (100 μM), atrazine (100 μM) and forskolin (30 μM) and measured using HRMS and the MS1-HRMS workflow. Volcano plots representing abundance ratios of detected metabolites (steroids and other endogenous) as a function of calculated p-values. The XCMS R package was used to measure relative abundances of steroids and other metabolites obtained by HRMS (in positive ionization) in NCI-H295R media in response to the different endocrine disruptors. Volcano plots corresponding to other concentrations are shown in supplemental Figure S2. **B**: Scatter plot of absolute testosterone concentrations determined with Sciex OS software and their relative abundances obtained using our MS1-HRMS workflow and the XCMS R package following prochloraz exposure. Each concentration measurement comes from the same samples and HRMS datasets.

Figure 6: Annotations of compounds detected using the MS1-HRMS workflow with the Scannotation suspect screening tool. **A**: Principles of the annotation steps of the MS1-HRMS workflow. Annotation scoring was done with the suspect screening tool Scannotation. A metascore was generated according to m/z precision, isotopologue ratio fidelity and predicted retention time (RT) based on $\log P$ values. A database including suspect molecules such as steroids, labelled steroids, xenobiotics (used in this study) and their known metabolites, pesticides (with known RT) and endogenous metabolites was used (File S2). **B**: Correlation between experimental RT of 103 compounds with their corresponding $\log P$ values. This correlation was used to predict RT in the M1-HRMS workflow to pre-annotate compounds detected by HRMS. **C**: Bar chart representing the distribution of metascores for each pre-annotated molecule in each dataset corresponding to xenobiotic exposure.

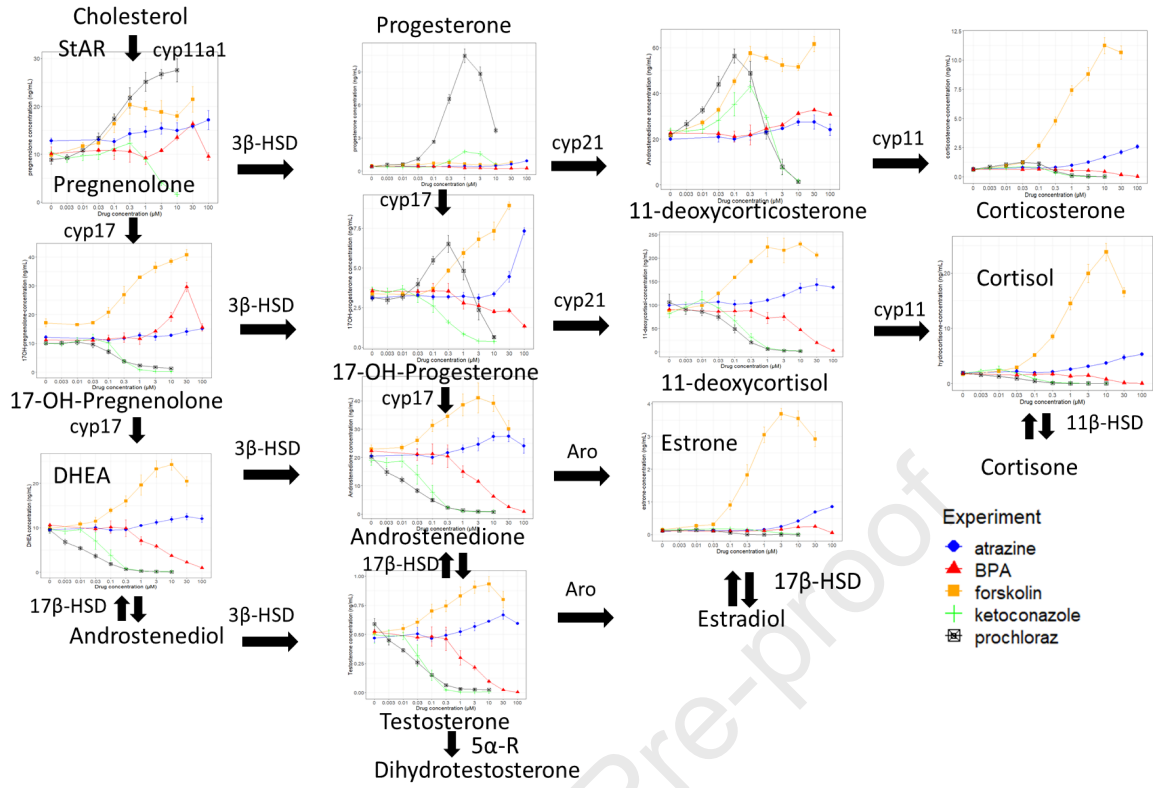
Figure 7: Monitoring endocrine disruptors and their metabolites using the MS1-HRMS workflow. Scatter plot representing examples of variations in abundance of tested endocrine disruptors (right panel) and their metabolites (left panel) obtained using HRMS and annotated using the Scannotation tool.

Figure 8: Endogenous molecules and metabolic networks impacted by EDs exposures and determined using the MS1-HRMS workflow. **A:** Metabolic pathway analysis plot of over-represented pathways implicating in EDs exposures determined by the webtool MetExplore. Metabolic pathways representing the discriminative metabolites were set using a Right tailed Fisher Exact test and Bonferroni corrections (p -values < 0.05). The database Recon 3D containing *Homo sapiens* metabolites was used. **B:** Heatmap representing ED/CTRL abundance ratio (maximum ED concentration) of differentially abundant endogenous metabolites annotated with the Scannotation tool and obtained from HRMS following different EDs exposures. All ratios have adjusted p -values of less than 0.05. Up-regulated and down-regulated molecules are shown in red and green respectively. Metabolism type is indicated in colour. Confidence level is indicated (Schymanski classification). To note, all steroids observed obtained a confidence level of 1 (use of standards) and were validated also at the level 2 using MS2 data. MetFrag outputs and MS2 data of annotated compounds were presented in the supplementary File S8. **C:** Trendline charts representing examples of variations in abundance of the most significant metabolites, excluding steroids, annotated with the in-house annotation tool and obtained from HRMS following ED exposures.

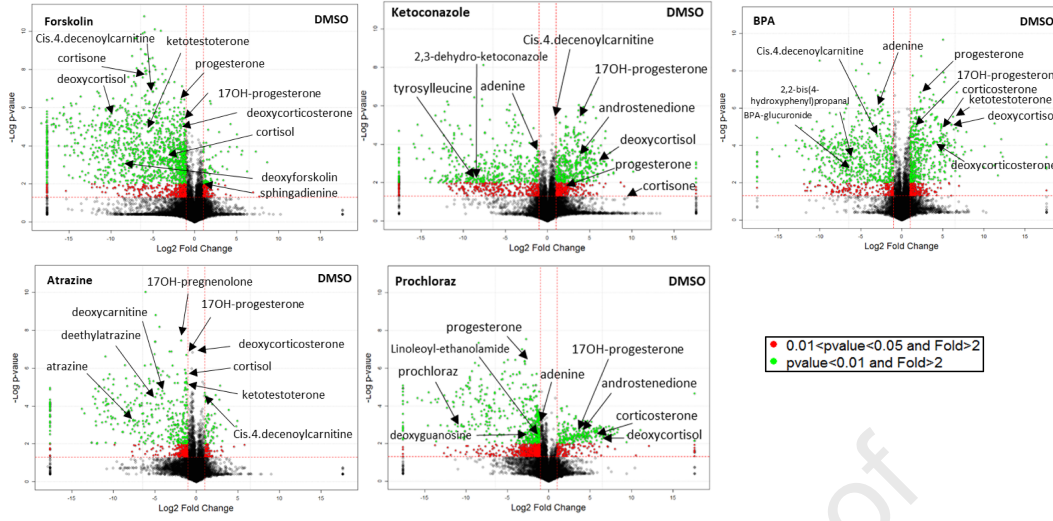




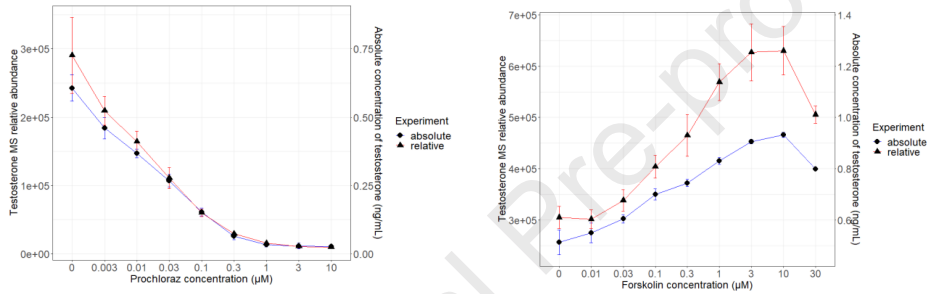
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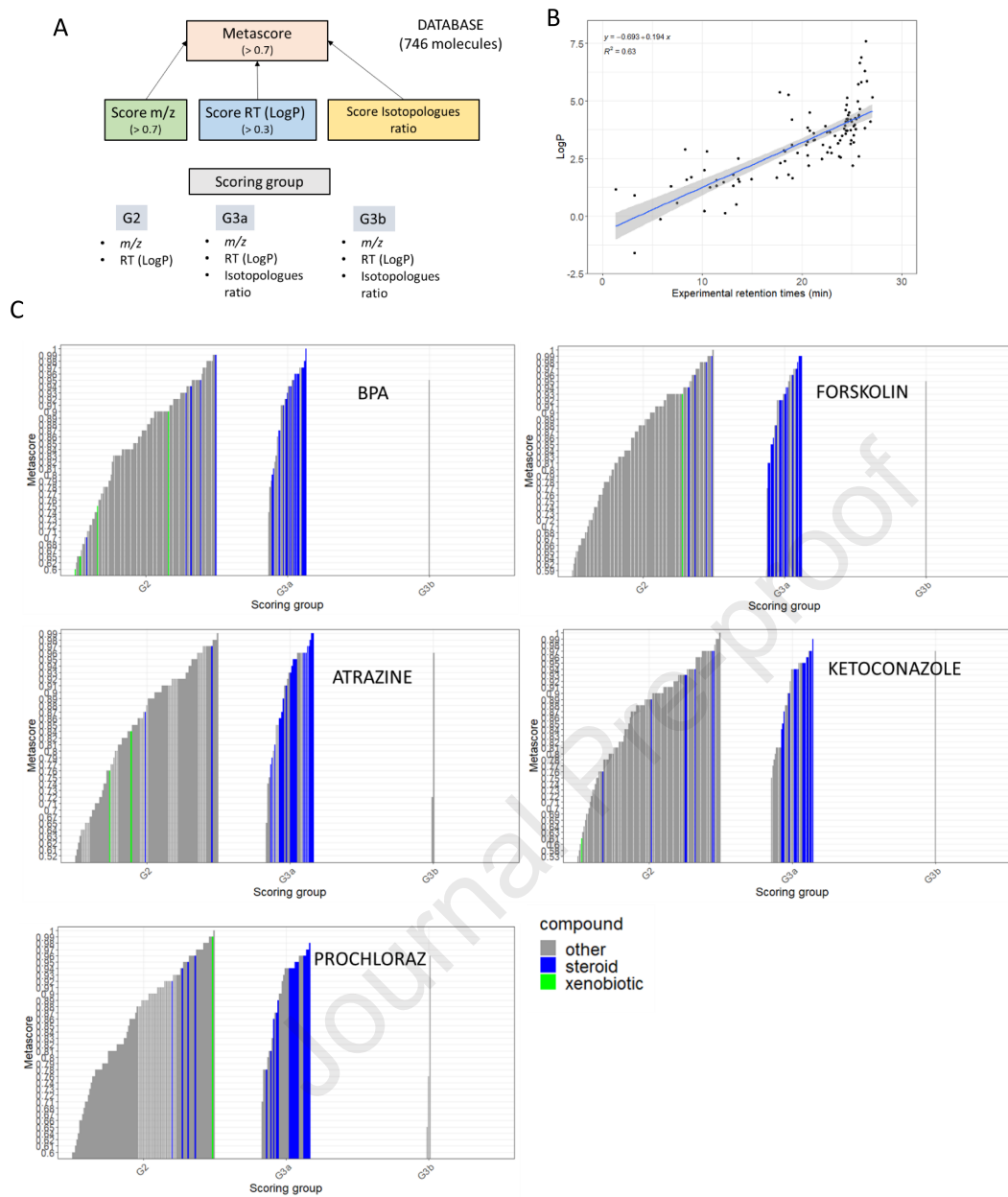


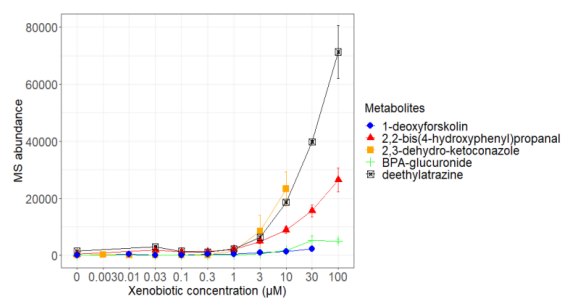
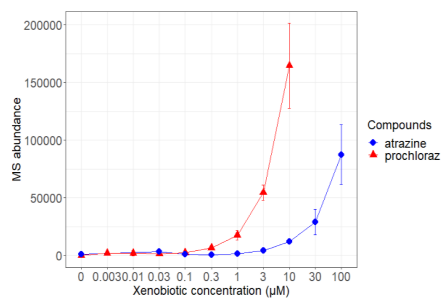
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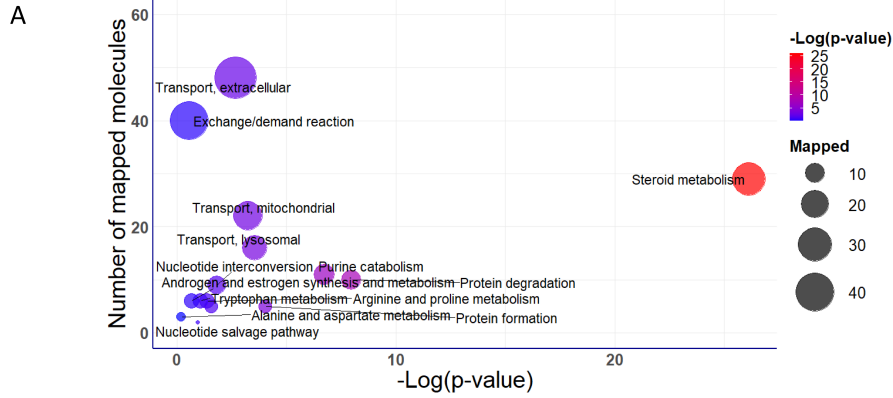
B







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B

	Prochloraz	atrazine	ketoconazole	forskolin	BPA	Confidence levels
adenosine	4.62		3.71			2
adenine	1.86	1.31	1.95		4.91	2
deoxyguanosine	4.65		3.70			2
deoxyinosine	0.52		0.82		0.27	2
hypoxanthine	0.53	0.34	0.38	2.95	0.33	2
sphingadienine				0.45		2
Hexadecaspinganine					0.25	3
sphinganine					0.82	2
5-methylthioadenosine		1.47		0.77		2
Nacetylputrescine		0.76	0.75	0.64	0.75	2
deceoylcarnitine	0.57		0.51	32.3	5.58	2
deoxycarnitine		16.95				3
Myristoleoylcarnitine				0.83		2
tryptophan	2.55					2
kynurenate			0.59		0.49	3
proline		0.79		0.62		3
Leucine/isoleucine		0.78				2
glycylproline			0.56			3
oxoproline		1.64	1.88			2
Phenylalanine				2.00		2
Phenylalanylleucine		0.72				3
tyrosylleucine		2.18	342			2
alanine			2.00			3
Nacetylphenylalanine		1.20				2
Arginylphenylalanine				0.61		3
Tryptophylvaline			0.76	1.54	0.75	3

● purine

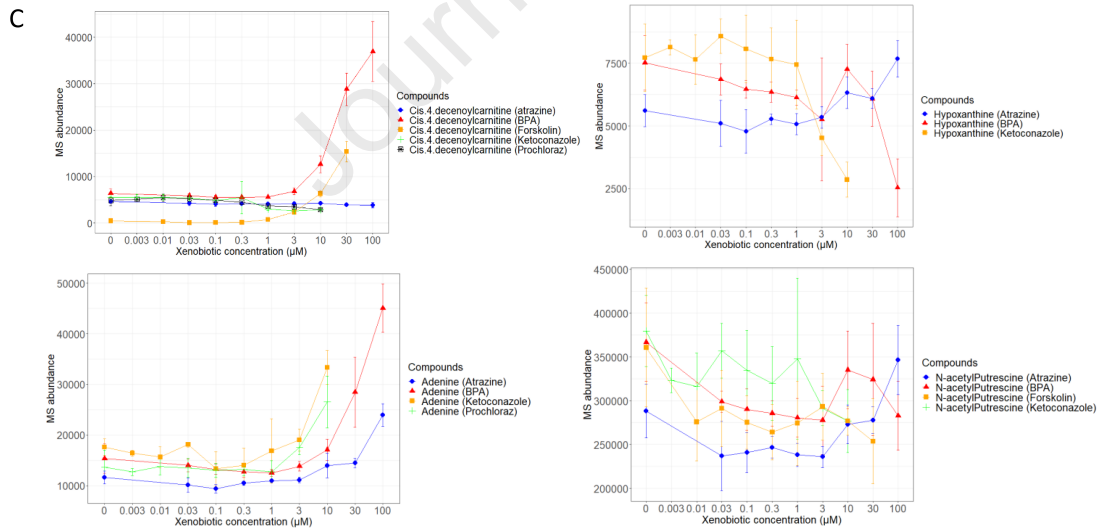
● carnitine

● tryptophan

● amino acids

● sphingolipids

● spermidine



- HRMS showed sensitivity for steroid quantification similar to that of MRM and ELISA in three biological fluids
- Steroids, xenobiotics and their metabolites were simultaneously quantified or detected using HRMS
- The HRMS workflow on NCI-H295R cells allowed high-throughput screening of endocrine disruptors
- In addition to steroidogenesis, the metabolism of fatty acids, purines and amino acids was particularly affected by the various EDs.

Declaration of interests

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.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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