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Sample preparation for suspect and non-target screening of xenobiotics in human biofluids by liquid chromatography—High resolution tandem mass spectrometry

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ABSTRACT

The concept of exposome covers all the exposures an individual suffers from conception to death, which can be partially assessed through the monitoring of human biofluids. In there, target analytical approaches tend to focus on a limited set of xenobiotics, whereas exposomic studies need broad scopes in search of a full understanding. Given the issue, suspect and non-target screening are feasible alternatives. However, adequate sample preparation procedures should minimize interferences without significantly reducing the number of xenobiotics. Within this context, the present article aims to describe comprehensive sample preparation procedures for suspect or non-target screening of organic xenobiotics in several human biofluids, all coupled to unified separation and detection conditions based on ultra-high performance liquid chromatography-high resolution tandem mass spectrometry (UHPLC–HRMS/MS). The referred biofluids consist of human urine, breast milk, saliva and ovarian follicular fluid.

- Analytical methods for untargeted analysis of a wide range of xenobiotics in human biofluids are fully described in order to ensure reproducibility.
- The sample preparation procedures balance selectivity and sensitivity.
- Unified analysis conditions allow simultaneous analysis of diverse biofluids.

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Method details

Context & aim

Environmental exposure is the main cause of several chronic diseases, whereas genetic predisposition is only responsible for 10 % of the risks [4]. In addition, half of the worldwide deaths are attributable to environmental factors, including lifestyle habits (e.g., smoking, alcohol consumption) [5,6]. In 2005, the concept of “exposome” was coined, which comprehends every exposure an individual suffers throughout their lifetime, involving internal processes and external interactions [7,8]. An approach to evaluate the latter is by linking the potential exposures to the presence of endogenous and/or exogenous substances (i.e., xenobiotics) in the human body [9]. Nonetheless, the scope of targeted biomonitoring assays is limited to a few hundred compounds at a time [10], which contrasts with the over 193 million compounds registered in the Chemical Abstracts Service (CAS) by 2023 [11].

Suspect and non-target screening offer a wide-scoped approach, complementary to the more limited targeted analysis. Still, samples need to be pre-processed to remove interferences or pre-concentrate the potential suspect xenobiotics, among others. In that sense, the chemical coverage aimed at exposomic studies is reduced, in pursuit of minimizing matrix effects [9].

In view of the issue, comprehensive sample preparation procedures need to reach the equilibrium between the chemical scope and previous treatment requirements. Within that context, this article details sample treatment procedures and UHPLC–HRMS/MS analysis conditions that allow the screening of a wide range of organic xenobiotics and their phase II metabolites in four human biofluids (i.e., urine, breast milk, saliva and ovarian follicular fluid), to perform suspect or non-target analysis of LC-amendable compounds.

Materials, reagents and equipment

Along sample preparation, anhydrous sodium sulfate (Na_2SO_4, 99 %, Merck), anhydrous magnesium sulfate ($MgSO_4$, 99.5 %, Alfa Aesar) and sodium chloride (NaCl, 100 %, PanReac AppliChem) are employed in extraction steps. Besides, necessary pH adjustments are performed either with ammonium acetate buffer (1 mol/L, pH 5.5), prepared with ammonium acetate (NH_4OAc, 98 %, Sigma-Aldrich) and glacial acetic acid (AcOH, 100 %, Merck); or phosphate buffer (0.1 mol/L, pH 2.0), prepared with orthophosphoric acid (H_3PO_4, 85 %, PanReac AppliChem) and sodium hydroxide (NaOH, 98 %, Honeywell Fluka). For enzymatic hydrolysis, $\beta\beta$-glucuronidase/sulfatase enzyme solution from Helix pomatia is used (Merck), which has a specific glucuronidase activity of 5.5 U/mL and an arylsulfatase activity of 2.6 U/mL both at 38 ˚C using phenolphthalein-β-glucuronide and phenolphthalein disulfate as substrates, respectively.

For further extract purification, when needed, Oasis HLB cartridges (200 mg, 6 mL, 30 µm, Waters) and Captiva EMR-Lipid filters (300 mg, 3 mL, Agilent Technologies) are used. Final extracts of all the procedures are filtered before analysis (Clarify-PP, 0.22 µm, Phenomenex).

UHPLC-quality water (H_2O, UHPLC-MS quality, Scharlau) and dimethyl sulfoxide (DMSO, Honeywell Fluka) are employed in the final extract reconstitution. Remaining solvents include acetone (99.5 %, Avantor Performance Materials), Milli-Q water (from a Millipore 185 water purification system, <0.05 µS/cm, Millipore) and HPLC-grade acetonitrile (ACN, ChromAR, Macron Fine Chemicals, Avantor,) and methanol (MeOH, ChromAR HPLC quality, Macron Fine Chemicals, Avantor). Mobile phase constituents are UHPLC quality methanol (MeOH, 99.9 %, Scharlau) and the previously mentioned UHPLC-water, both adjusted to the desired pH adding either optima grade formic acid (HCOOH, 98 %, Fisher Scientific) or optima grade ammonia in solution (NH_4OH, 20–22 %, Fisher Scientific). Synthetic biofluids (breast milk, urine, saliva and follicular fluid) used for quality control/quality assurance were purchased from Biochemazone.

Regarding laboratory equipment, samples are homogenized in a Multi Reax Shaker (Heidolph) and phase separation is promoted in 5804R (when at 4 ˚C) or 5424 (separation at room temperature) centrifuges by Eppendorf. Besides, extract concentration is performed in a TurboVap LV Evaporator (Caliper Life Sciences) under a gentle nitrogen stream (N_2, 99.999 %, Air Liquide).

Sample preparation procedures

All biofluids can be processed with enzymatic hydrolysis if the aim is to screen only parent xenobiotics and further quantify them if intended, or without enzymatic hydrolysis to annotate simultaneously phase II metabolites and parent xenobiotics. Then, urine and saliva samples are extracted and cleaned up by solid phase extraction (SPE), while breast milk and follicular fluid are extracted with salt-assisted liquid-liquid extraction (SALLE) and further cleaned up by different protein precipitation strategies explained hereinafter.

- ***Enzymatic hydrolysis***

1 mL urine (first morning) and breast milk, and 0.5 mL of saliva (non-induced) and ovarian follicular fluid (obtained at oocyte retrieval) are first room tempered. Urine samples are placed in glass test tubes, saliva samples in 1.5 mL polypropylene Eppendorf vials and breast milk and follicular fluid in 15 mL polypropylene Falcon tubes. The pH adjustment is done using ammonium acetate buffer for optimum enzyme performance. For urine and breast milk, 200 µL are used, while 100 µL are added for saliva and follicular fluid. Then, 25 µL (for saliva and follicular fluid) or 50 µL (for urine and breast milk) of the $\beta\beta$-glucuronidase/sulfatase enzyme solution is added, the mixtures are vortexed for 30 min and incubated at 37 ˚C for a minimum of 2 h. In the cases where enzymatic hydrolysis is not performed, the addition of the enzyme and the incubation steps are skipped.

- ***Urine***

2 mL of the phosphate buffer are added to both hydrolysed or non-hydrolysed 1 mL urine samples. Then, Oasis HLB cartridges are used for SPE. The cartridges are activated with 4 mL of MeOH:acetone (1:1, v/v) and subsequently equilibrated with 4 mL of the phosphate buffer. The urine samples are loaded into the cartridge and 5 mL of H_2O:MeOH (95:5, v/v) are added for clean-up purposes. Before elution, the SPE cartridges are fully dried under vacuum. Finally, the elution is carried out by adding 5 mL of MeOH:acetone (1:1, v/v) and the eluate is collected in a silanized test tube.

- ***Saliva***

The hydrolysed or non-hydrolysed saliva samples are centrifuged at 15,000 rpm for 10 min. The supernatant is collected and diluted with 2 mL of phosphate buffer. Then, the diluted saliva sample is treated with the same SPE protocol as the urine samples.

- ***Breast milk***

For SALLE, 3 mL of cold ACN (4 ˚C) are added into the hydrolysed or non-hydrolysed samples, followed by 0.5 mg Na_2SO_4 and 0.1 g NaCl. To ensure a homogeneous mixing, the tubes are hand-shaken first and then vortex-mixed (5 min, 2000 rpm). Next, samples are centrifuged (8000 rpm, 5 min, 4 ˚C) and supernatants are quantitatively recovered and concentrated to 1 mL under a gentle nitrogen stream (35 ˚C). The extracts are transferred to 1.5 mL-Eppendorf vials and kept at −20 ˚C overnight (12 h) to promote protein precipitation. After that, the supernatants are centrifuged (15,000 rpm, 10 min) and kept at −20 ˚C for an additional hour. Finally, 250 µL of Milli-Q water is added to the clean supernatants and they are further purified employing Captiva EMR Lipid-filters. To that end, extracts are passed through the filters and an additional 250 µL ACN:H_2O (80:20, v/v) are used to maximize the recovery of less polar compounds retained.

- ***Follicular fluid***

In the case of hydrolysed and non-hydrolysed follicular fluid samples, a similar SALLE procedure as described for breast milk is employed. Briefly, 1.5 mL of cold ACN, 0.25 mg $MgSO_4$ and 0.1 g NaCl are added to the sample and the mixture is agitated for 5 min at 2000 rpm. Then, the mixture is centrifuged (8000 rpm, 10 min, 4 ˚C), and the upper organic phase is quantitatively recovered and kept in a freezer at −20 ˚C for 12 h to induce protein precipitation. After protein precipitation, the clean supernatant is recovered in 1.5-mL Eppendorf vials and further centrifuged (15,000 rpm, 10 min) to ensure maximum precipitation. Finally, the protein-free supernatant is quantitatively recovered.

- ***Extract´s reconstitution***

Before the analysis, all extracts are concentrated and reconstituted in DMSO:H_2O (1:4, v/v) as the final solvent. In the cases of urine, follicular fluid, and saliva, 40 µL of DMSO are added to the organic extracts as an evaporation keeper and the mixtures are evaporated to 40 µL using a gentle stream of N_2 at 35 ˚C. Then, 160 µL UHPLC grade H_2O is added to obtain the final proportion. For milk samples, instead, 80 µL of DMSO are added and the mixture is concentrated up to 380 µL. Next, 20 µL of UHPLC-grade water is added to obtain the desired proportion. Eventually, all four biofluids' extracts are filtered with 0.22 µm polypropylene syringe filters and kept at −20 ˚C until analysis.

UHPLC–HRMS/MS analysis

A Dionex Ultimate 3000 UHPLC (Thermo Fisher Scientific) coupled to a high-performance Q Exactive Focus Orbitrap (qOrbitrap, Thermo Fisher Scientific) mass analyser with a heated electrospray ionization source (HESI, Thermo Fisher Scientific) is used for the analysis of the xenobiotics and phase II metabolites. The separation is carried out using an ACE UltraCore 2.5 SuperC18 (2.1 mm × 100 mm, 2.5 μm, Avantor, Symta) chromatographic column (working pH range of 1.5 – 11), equipped with an UltraCore Super C18 UHPLC guard precolumn placed in an ACE UHPLC guard holder (both purchased from Avantor, Symta). The column temperature is maintained at 35 ˚C and 7 μL of the extracts are injected.

Measurements are performed at negative and positive ionization modes in the Full scan - data dependent MS^2 (Full MS-ddMS^2) discovery acquisition mode. To that end, two chromatographic runs are performed per sample. For the mobile phases, UHPLC grade water (A-line) and MeOH (B-line) are used at pH 2.5 (0.1 % HCOOH in both lines, v/v) for the positive ionization mode, and 10.5 (0.05 % NH_4OH in both lines, v/v) for the negative ionization mode. At a flow rate of 0.3 mL/min, different gradient elutions are used for each ionization mode: in the positive ionization mode, it starts at 10 % B (1 min), with a following increase up to 65 % B (1–8 min) and a maximum B proportion of 95 % (8–15 min), which is further maintained (15–20 min). In the negative ionization mode, instead, the initial conditions at 10 % B are kept for 1.5 min, to then increase the organic modifier up to 95 % B (1.5–15 min) and uphold it until the end of the analysis (15–20 min). Eventually, for both cases, the column is re-equilibrated to initial conditions in 5 min (20–25 min).

Regarding the HESI source and HRMS/MS detection parameters, the working conditions are displayed in Table 1 for both ionization modes.

Table 1
HESI source working conditions and Full MS-ddMS^2 detection parameters for positive and negative ionization.

	Positive ionization	Negative ionization
HESI source parameters		
Sheath gas flow rate	40	
Aux. gas flow rate	15	
Sweep gas flow rate	1	
Spray voltage (kV)	3.20	2.90
Capillary temperature (˚C)	320	
S-lens RF level	55.0	
Aux. gas heater temperature (˚C)	310	
Overall Full MS-ddMS^2 method settings		
Use lock masses	–	
Lock mass injection	off	
Chrom. peak width (FWHM) (s)	6	
Method duration (min)	25	
Lock Masses	–	
Inclusion	–	
Exclusion	–	
Dynamic Exclusion	–	
Full MS		
Polarity	Positive	Negative
dd-MS^2	Discovery	
In-source CID	–	
Resolution	70,000	
# Scan ranges	1	
Scan range	70 to 1050 m/z	
AGC target	10^6	
Maximum IT	Auto	
Microscans	1	
Spectrum data type	Profile	
dd-MS^2 Discovery		
Resolution	17,500	
Isolation window	3.0 m/z	
Isolation offset	–	
Stepped normalized collision energy (NCE)	10, 30, 70	10, 45, 90
Fixed first mass	50.0 m/z	
Default charge state	1	
AGC target	$5 \cdot 10^4$	
Maximum IT	Auto	
Loop count	3	
Minimum AGC target	$8 \cdot 10^3$	
Intensity threshold	Auto	
Appex trigger	–	
Dynamic exclusion	Auto	
Charge exclusion	–	
Exclude isotopes	–	
Spectrum data type	Profile	

Table 2
Validation parameters for the selected 134 compounds.

Compound	Sensitivity (iLOQ, ng/mL)[a]	Linearity (upper limit, ng/mL)	R^2	Trueness range (%)[b]	Precision range (RSD,%)[c]	Matrix effect range (%)[d]
1H-Benzotriazole	0.7	147	0.9987	65–79	7–24	28–96
2,4-Dinitrophenol	5.9	134	0.9989	52–63	4–8	28–105
3-(2-chloro-3,3,3-trifluoro-1-propenyl)−2,2-dimethylcyclopropanecarboxylic acid	0.7	168	0.9455	37–73	8–9	47–86
4-Hydroxybenzophenone	0.6	140	0.9968	37–80	8–32	39–105
4-Methylbenzophenone	0.6	118	0.9865	21–44	6–33	36–56
Acetaminophen	12.7	125	0.9930	15–79	7–23	30–78
Acetamiprid	3.5	146	0.9911	36–75	6–16	36–73
Alachlor	0.6	145	0.9998	37–45	5–13	50–66
Ametryn	8.1	145	0.9992	23–48	7–35	41–58
Atrazine	3.3	145	0.9981	35–61	7–31	57–64
Azoxystrobin	0.6	125	0.9790	26–64	3–16	47–79
Bendiocarb	3.1	136	0.9865	35–67	8–16	29–101
Bentazone	3.1	144	0.9990	44–83	8–12	53–105
Benzophenone-1	0.6	117	0.9962	30–71	4–11	38–95
Benzophenone-2	3.3	114	0.9779	27–72	7–25	28–39
Benzophenone-3	11.1	143	0.9983	21–78	2–17	37–95
Benzophenone-8	1.2	144	0.9953	31–72	8–35	37–94
Benzothiazole	3.3	140	0.9985	34–62	10–19	52–104
Benzyl paraben	0.5	148	0.9565	23–78	3–11	40–95
Bezafibrate	0.5	147	0.9982	24–63	8–19	32–72
Bisoprolol	1.2	148	0.9998	23–76	8–27	39–87
Bisphenol AF	0.5	143	0.9655	14–85	1–12	62–91
Bisphenol AP	1.1	144	0.9605	10–82	11–17	49–104
Bisphenol Z	1.1	141	0.9645	14–77	3–11	53–102
Boscalid	1.1	150	0.9998	35–48	5–15	44–66
Bosentan	0.6	147	0.9978	35–146	3–6	10–67
Butylparaben	1.2	127	0.9995	31–73	3–8	45–95
Caffeine	3.9	127	0.9900	57–68	17–24	33–180
Caprolactam	3.9	136	0.9996	61–143	5–35	34–110
Carbaryl	3.3	116	0.7855	26–56	7–34	42–85
Carbendazim	3.3	199	0.9863	29–66	7–21	39–75
Carbofuran	0.8	143	0.9997	52–54	8–30	39–86
Celecoxib	1.7	191	0.9875	18–113	1–11	56–85
Cetirizine	1.7	214	0.9943	20–50	9–21	36–77
Chloridazon	0.9	142	0.9907	31–64	7–22	35–82
Chloroxuron	0.6	141	0.9979	31–51	2–13	44–68
Chlorpyrifos	5.6	127	0.9870	10–26	14–20	13–79
Chlortoluron	14.4	142	0.9991	40–51	7–29	39–85
Clomazone	0.6	151	0.9997	39–56	7–28	43–73
Cotinine	5.8	105	0.9655	–	–	–
Crotamiton	5.8	130	0.9989	28–52	6–29	43–75
Diazepam	11.4	138	0.9958	31–42	5–34	44–74
Dichlorvos	3.1	134	0.9585	36–41	3–15	42–77
Diclofenac	3.1	152	0.9990	12–53	8–14	26–58
Diethyl phosphate	3.5	168	0.9995	36–46	6–10	70–83
Diethyl Toluamide	0.1	147	0.9939	54–60	7–28	38–87
Diflufenican	3.3	111	0.9845	15–46	4–9	23–69
Dimethachlor	3.3	130	0.9997	45–58	6–31	40–79
Dimethoate	0.5	146	0.9985	44–59	4–27	35–85
Dimethyl phosphate	1.2	167	0.9993	21–52	3–32	61–82
Diuron	1.2	138	0.9996	39–58	8–30	49–73
Ethion	3.0	111	0.9900	11–22	4–15	13–79
Ethyl 4-dimethylaminobenzoate	8.3	132	0.9997	17–46	6–31	42–66
Ethyl Paraben	3.0	133	0.9988	49–83	4–7	30–105
Fenoxycarb	0.6	113	0.9810	26–46	5–16	49–57
Finasteride	3.2	146	0.9995	29–79	5–11	40–77
Fipronil	0.6	137	0.9865	21–79	7–9	55–73
Fipronil desulfinyl	0.6	145	0.9755	22–66	2–8	57–66
Fipronil sulfone	0.6	141	0.9835	11–69	7–11	49–68
Fluconazole	0.6	145	0.9992	57–65	4–10	40–84
Genistein	3.6	128	0.9870	22–73	1–10	21–70
Genistin	12.9	183	0.9870	35–59	1–10	54–84
Glibenclamide	3.3	114	0.9665	25–51	3–17	37–52
Glimepiride	0.9	185	0.9760	37–58	3–12	34–60

(*continued on next page*)

Table 2 (*continued*)

Compound	Sensitivity (iLOQ, ng/mL)[a]	Linearity (upper limit, ng/mL)	R^2	Trueness range (%)[b]	Precision range (RSD,%)[c]	Matrix effect range (%)[d]
Glycitin	0.9	225	0.9994	30–69	6–11	23–81
Hexazinone	9.1	146	0.9923	53–59	6–24	37–84
Imidacloprid	0.6	138	0.9805	30–55	6–12	47–64
Indometacin	1.1	136	0.9785	43–51	0–22	24–53
Irbersartan	1.1	163	0.9988	39–62	9–32	40–75
Isoproturon	0.7	123	0.9868	52–66	8–30	37–85
Ketoprofen	3.6	148	0.9999	24–55	7–11	40–70
Lidocaine	0.6	121	0.9900	21–63	8–22	41–91
Linuron	0.6	138	0.9983	34–59	7–32	43–65
Lorazepam	3.5	147	0.9938	44–55	10–30	36–74
Losartan	1.2	156	0.9999	21–62	10–26	38–90
Mebendazole	0.2	192	0.9635	35–49	6–35	39–64
Mecoprop	0.2	138	0.9952	20–62	6–10	43–99
Mefenamic acid	0.6	211	0.9996	43–63	7–20	38–100
Metalaxyl	0.1	132	0.9924	52–66	9–30	38–87
Metamitron	0.5	194	0.9809	40–58	6–16	40–85
Metazachlor	0.8	141	0.9936	52–55	8–33	37–76
Metconazole	1.1	140	0.9996	28–32	4–9	45–58
Methyl 3,5-dihydroxybenzoate	5.4	141	0.9305	16–59	3–31	47–77
Methyl 3,5-di-tert-butyl-4-hydroxybenzoate	3.6	109	0.9957	15–34	10–28	31–73
Metolachlor	1.1	139	0.9998	37–44	5–33	48–63
Metribuzin	0.6	140	0.9988	35–62	7–9	36–105
Mexacarbate	3.2	142	0.9923	30–47	6–30	35–76
Mono-(2-ethyl-5-hydroxyhexyl) phthalate	0.6	131	0.9705	13–60	11–13	30–95
Mono-2-ethyl-5-oxohexyl phthalate	0.1	131	0.9705	29–64	6–12	46–119
Mono-2-ethylhexyl phthalate	0.5	113	0.9720	17–58	9–12	32–66
Mono-benzyl phthalate	8.5	121	0.9675	15–63	7–29	46–96
Mono-butyl phthalate	0.6	131	0.9740	20–77	4–36	44–114
Myclobutanil	0.6	136	0.9997	45–52	2–12	44–58
Naproxen	6.0	132	0.9540	26–57	3–23	38–58
N-Desmethylacetamiprid	6.0	165	0.9920	61–73	7–18	37–102
Oryzalin	0.7	139	0.9979	19–102	1–12	55–100
Perfluorobutanesulfonic acid	0.1	118	0.9830	51–91	1–12	38–101
Perfluorooctanesulfonamide	5.9	111	0.9860	29–113	4–7	12–96
Perfluorooctanesulfonic acid	0.6	110	0.9895	25–76	9–14	63–158
Perfluorooctanoic acid	0.6	119	0.9715	39–90	1–11	41–90
Phthalic acid	3.8	153	0.9555	49–63	5–31	43–49
Pindolol	3.8	146	0.9899	28–49	4–15	49–88
Pirimicarb	0.6	140	0.9997	50–56	5–31	40–93
Pirimiphos-methyl	0.5	115	0.9900	23–38	1–14	44–62
Prochloraz	0.5	137	0.9998	18–119	1–16	32–108
Propachlor	1.1	141	0.9940	46–59	8–27	38–78
Propanil	1.1	140	0.9961	34–73	10–29	38–89
Propiconazole	5.6	145	0.9999	22–55	7–13	38–60
Propranolol	0.6	146	0.9994	17–74	8–29	41–82
Propyphenazone	0.6	143	0.9980	46–53	8–30	40–91
Propyzamide	0.1	145	0.9957	37–59	9–31	40–65
Prosulfocarb	0.6	121	0.9900	19–40	2–21	31–65
Pyrazophos	0.6	147	0.9895	22–37	3–4	31–144
Quinmerac	0.6	100	0.9705	17–40	3–10	29–80
Rimexolone	3.2	140	0.9991	23–27	4–8	42–134
Simazine	0.6	110	0.9730	44–57	8–27	37–70
Sulfadiazine	0.7	159	0.9860	76–77	2–10	79–79
Sulfamethazine	0.7	155	0.9706	39–70	3–20	55–76
Sulfamethoxazole	0.6	157	0.9749	25–59	4–14	43–68
Sulfapyridine	0.6	155	0.9634	39–59	3–13	38–73
Sulfathiazole	0.6	146	0.9892	51–74	4–19	12–85
Tebuconazole	1.2	141	0.9995	31–57	7–15	50–60
Terbuthylazine	0.6	139	0.9998	19–43	3–34	35–49
Terbutryn	0.1	144	0.9994	15–47	1–12	34–60
Thiabendazole	0.1	145	0.9926	38–68	8–27	36–79
Thiacloprid	0.6	140	0.9968	38–58	7–27	30–64

(*continued on next page*)

Table 2 (*continued*)

Compound	Sensitivity (iLOQ, ng/mL)[a]	Linearity (upper limit, ng/mL)	R^2	Trueness range (%)[b]	Precision range (RSD,%)[c]	Matrix effect range (%)[d]
Thiamethoxam	1.4	127	0.9600	38–72	2–13	55–56
Tramadol	1.4	135	0.9971	29–72	7–21	39–94
Triadimenol	0.5	137	0.9993	44–47	5–11	45–59
Triclocarban	0.6	136	0.9730	14–49	5–10	35–77
Triclosan	1.1	135	0.9320	14–42	16–18	22–48
Triethylphosphate	1.1	140	0.9991	19–45	4–19	56–82
Triphenylphosphate	0.6	136	0.9988	12–100	5–15	36–99
Valsartan	0.5	150	0.9994	21–55	8–16	37–83

[a] The lowest calibration point where (i) the relative standard deviation (RSD) of repeated injections is below 30 % and the calculated concentration has a systematic error below 30 % in respect to the real value.

[b] Recoveries of the selected analytes at a spiking level of 20 ng/mL in the artificial biofluids expressed as the ratio between the concentration obtained from the calibration-curve and the real spiked concentration.

[c] Relative standard deviation (RSD) of procedural replicates (n = 3) of spiked matrices at 20 ng/mL.

[d] Matrix effect at detection spiking the extracted artificial biofluids at 50 ng/mL prior analysis. Matrix effects are expressed as the ratio between the concentration obtained from the calibration-curve and the real spiked concentration. A value below 100 % indicates signal suppression, while a value above 100 % shows signal enhancement.

Quality control/quality assurance (QC/QA)

As procedural blanks, synthetic urine, follicular fluid, breast milk and saliva samples are processed alongside the real biofluid samples in order to consider the presence of potential interferences created during the sample preparation steps. Three replicates ($n = 3$) of procedural blanks for every sample preparation batch should be processed. In the case of the real samples, they should also be processed in triplicate although a single extraction and injection in triplicate can be performed depending on the sample volume available or the number of samples to be analyzed.

Since the aim of the method is suspect screening, the evaluation of potential losses during sample preparation and matrix effects at detection might be challenging since no targets are selected beforehand. However, two strategies can be considered. On the one hand, the samples can be fortified with a selection of isotopically labeled compounds that cover a wide range of polarities (covering the whole chromatogram) and have diverse physicochemical properties can be used. Ideally, one analogue per chemical family that it is intended to screen in the sample should be used. On the other hand, QC-extraction samples can be prepared by fortifying the artificial biofluids with a wide selection of xenobiotics and phase II metabolites. The QC-extraction samples should be processed in triplicate every sample preparation batch. Moreover, an aliquot of every real sample is pooled in order to obtain a QC-pool sample per biofluid, extracted in triplicate and intended to be used for signal correction during the data post-processing step. The QC-pool sample should also be processed every sample preparation batch.

During UHPLC-qOrbitrap acquisition, blank solvent (i.e., MeOH) injections are performed, five before the analysis sequence, and then every 8–10 sample injections to avoid carryover. In addition, QC-pool and/or QC samples should also be injected every 8–10 samples. Finally, another five blank solvents are injected at the end of the analysis sequence and the column is cleaned and storaged following the vendor´s instructions.

Besides, the qOrbitrap mass analyzer is routinely calibrated every three days using Pierce LTQ ESI (Thermo Fisher Scientific) calibration solutions and Xcalibur 4.4 software (Thermo Fischer Scientific) is used for external instrumental control.

Methods' performance evaluation

In the present work, the methods have been evaluated using diverse 134 analytes to demonstrate their usefulness to screen a wide range of xenobiotics. As validation parameters, sensitivity, linearity, trueness, precision and matrix effect are shown for each xenobiotic in Table 2.

As can be observed in Table 2, a diversity of compounds can be simultaneously screened in the biofluids. It is worth mentioning that since the aim of the methods is untargeted analysis, their capability to detect a wide range of compounds is preferred over sensitivity and trueness. In that sense, recovery correction using isotopically labeled analogues is not performed. In addition, the example of cotinine (metabolite of nicotine) can be highlighted. Despite it could not be recovered with the proposed methods at the spiked concentration, it can be detected at real samples due to its original high concentration.

Ethics statements

Informed consent was obtained from each subject before conducting the experiments and the samples were handled according to the (i) The Committee on Ethics for Research Biological Agents and Genetically Modified Organisms (CEIAB, BOPV 32, 17/02/2014, M30-2021-158, M30-2022-311, M30-2022-327, M30-2023-136) and (ii) The Committee on Ethics for Research Involving Human Subjects (CEISH, BOPV 32, 17/02/2014, M10-2020-230, M10-2021-124, M10-2023-135) from the University of the Basque Country (UPV/EHU).

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.

CRediT authorship contribution statement

I. Baciero-Hernández: Methodology, Investigation, Writing – original draft, Visualization. **M. Musatadi:** Methodology, Investigation, Writing – original draft, Visualization. **M. Olivares:** Conceptualization, Methodology, Writing – review & editing. **A. Prieto:** Conceptualization, Resources, Writing – review & editing. **N. Etxebarria:** Resources, Project administration, Project administration. **O. Zuloaga:** Conceptualization, Supervision, Writing – review & editing, Project administration, Project administration.

Data availability

Data will be made available on request.

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