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A Generic Liquid Chromatography−Tandem Mass Spectrometry Exposome Method for the Determination of Xenoestrogens in Biological Matrices

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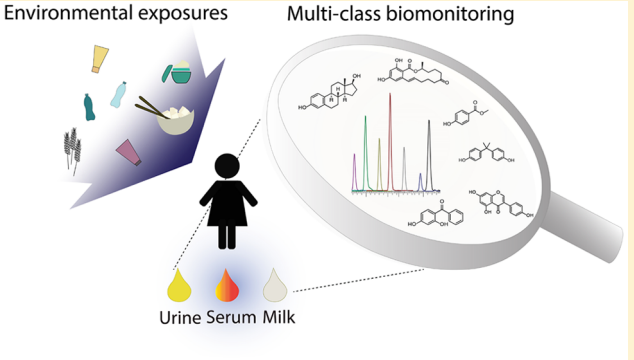
Supporting Information

ABSTRACT: We are constantly exposed to a variety of environmental contaminants and hormones, including those mimicking endogenous estrogens. These highly heterogeneous molecules are collectively referred to as xenoestrogens and hold the potential to affect and alter the delicate hormonal balance of the human body. To monitor exposure and investigate potential health implications, comprehensive analytical methods covering all major xenoestrogen classes are needed but not available to date. Herein, we describe a liquid chromatography−tandem mass spectrometry (LC−MS/MS) method for the simultaneous determination of multiple classes of endogenous as well as exogenous estrogens in human urine, serum, and breast milk to enable proper exposure and risk assessment. In total, 75 analytes were included, whereof a majority was successfully in-house validated in the three matrices. Extraction recoveries of validated analytes ranged from 71% to 110% and limits of quantification from 0.015 to 5 μg/L, 0.03 to 14 μg/L, and 0.03 to 4.6 μg/L in urine, serum, and breast milk, respectively. The applicability of the novel method was demonstrated in proof-of-principle experiments by analyzing urine from Austrian individuals and breast milk from Austrian and Nigerian individuals. Thereby, we proved the methods' feasibility to identify and quantify different classes of xenoestrogens simultaneously. The results illustrate the general importance of multiclass exposure assessment in the context of the exposome paradigm. Specifically, they highlight the need for estimating total estrogenic burden rather than single analyte or chemical class measurements and its potential impact in endocrine disruption and hormone related diseases including cancers.



The human endocrine system is an important signaling system which regulates organ communication and behavior. In recent years, attention has been drawn to the so-called endocrine disrupting chemicals (EDCs), compounds not produced by the body itself that can disturb the hormonal balance in various ways.[1] One class of EDCs are xenoestrogens, small molecules imitating or interfering with endogenous estrogens, the most important female sex hormones.[2] Xenoestrogens are a highly diverse group of chemicals and can be produced either naturally, e.g., by plants (phytoestrogens) and fungi (mycoestrogens), or synthetically as pesticides, plasticizers, personal care product additives, and other industrial chemicals.[3] In the past, research efforts typically focused on single representatives such as bisphenol A (BPA) and substance classes including parabens or phthalates. As a result, these chemicals may be banned and replaced by less investigated analogues for which similar or even more potent toxicological effects cannot be ruled out.[4]

A suspected adverse health effect of xenoestrogens is reduced fertility. It has been shown that high urinary concentrations of BPA, parabens, and phthalate metabolites reduce positive outcomes of *in vitro* fertilization.[5] A high uptake of phytoestrogens has been associated with negative effects on male fertility in some studies,[6−8] while not in others.[9,10] These controversial outcomes might be caused by differing time windows of exposure or the applied, rather targeted, single class human biomonitoring (HBM) approaches which did not consider mixture effects. Exposure to xenoestrogens may also be a risk factor for hormone-related

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cancers including those of the breast and the reproductive tract, especially during the sensitive, early developmental stages.[11−13]

The proper assessment of combinatory effects of multiple xenoestrogens are a current key challenge. Numerous studies investigating the potency of individual compounds or compound classes have been published, while data on estrogenic mixtures is scarce. There is a growing body of evidence that xenoestrogen combinations, even at very low concentrations, can have potentiating[14−17] and/or antagonizing effects.[18] Because of the variety of different structural classes, routes of exposure are diverse; they may be inhaled through the ambient air, ingested via contaminated food, or absorbed by the skin. Due to these multiple exposure scenarios, HBM is the best option for proper exposure assessment and should lead toward a more comprehensive, exposome-scale assessment of environmental risk factors in the etiology of chronic disease.[19,20]

To correlate exposure with potential health implications, advanced analytical assays for a simultaneous measurement of the different classes of xenoestrogens are essential yet not readily available. To date, the majority of multianalyte methods focus on single classes,[21−23] and the few existing multiclass methods typically contain no more than two different classes of xenoestrogens.[24−28] The development of a method covering multiple classes of xenoestrogens is a challenging task due to the diverse chemical properties and low biological concentrations of such exposures. Single analyte/class methods are not practical in large-scale epidemiological studies involving the measurement of thousands of samples as proposed for future exposome-wide association studies (EWAS). A targeted multiclass method constitutes a feasible approach for the implementation of this challenging task. Advantages of the targeted approach, compared to nontargeted high resolution LC−HRMS screening, are the increased sensitivity and the improved ability to perform quantitative measurements. This work addresses the need for a broad, quantitative LC−MS/MS method covering a multitude of estrogenic compounds in three relevant biological fluids. Following in-house validation, the method was applied to biological samples for assessing early life xenoestrogen exposures in first proof-of-principle experiments.

EXPERIMENTAL SECTION

Chemicals and Reagents. In this study, 75 analytes, 61 xenoestrogens and 14 endogenous estrogens, were included. Detailed information regarding analytes and suppliers are provided in Table S1 and Figures S2−S6.

Sample Collection. For validation experiments of human serum, pooled male AB plasma derived serum was used (USA origin; Sigma-Aldrich, Vienna, Austria). For urine, a pooled sample was obtained from a female volunteer who avoided foodstuff and beverages stored in plastic containers, foods rich in phytoestrogens, and cosmetics containing parabens for 2 days prior to sample collection. For breast milk validation experiments, anonymized pooled breast milk was provided by the Semmelweis Women's Clinic in Vienna, Austria, as previously reported.[29] Urine and breast milk were stored at −20 °C, serum at −80 °C until analysis.

To evaluate the applicability of the developed method, four urine samples obtained from an Austrian mother−infant pair as well as two samples from a female Austrian volunteer were analyzed. Moreover, five randomly selected breast milk samples from a previously reported HBM study focusing on mycotoxin exposure in Nigerian mothers,[29] as well as four breast milk samples from two Austrian mothers were analyzed. All samples were stored at −20 °C until analysis and the studies approved by the respective ethic committees in Austria (University of Vienna, No. 00157) and Nigeria (Babcock University, No. BUHREC294/16).

Sample Preparation. A volume of 200 μL of urine or serum was spiked with 10 μL of internal standard solution and extracted with 790 μL of ACN/MeOH (1/1) by sonication (10 min, 4 °C). After precipitating proteins in a freeze-out step (2 h, −20 °C), the samples were evaporated and resolved in 200 μL of ACN/H_2O (1/9). For breast milk extraction, the protocol of Braun et al. (2018)[29] was slightly modified. Further details are reported in the Supporting Information.

LC−MS/MS Analysis. Measurements were performed on a Dionex Ultimate 3000 UHPLC system coupled to a TSQ Vantage triple quadrupole mass spectrometer, operated with a heated electrospray ionization source (Thermo Scientific, Vienna, Austria). An Acquity HSS T3 column (1.8 μm, 2.1 mm × 100 mm) equipped with a VanGuard precolumn (1.8 μm; Waters, Vienna, Austria) served to achieve chromatographic separation. The column compartment and the autosampler were maintained at 40 °C and at 10 °C, respectively. LC−MS grade water with 0.3 mM ammonium fluoride (NH_4F) as additive (eluent A) and acetonitrile (eluent B) were used as mobile phases. Aqueous ammonium fluoride solutions have to be handled with care as HF may outgas. Although, the NH_4F concentration in the eluent was very low, contact with acids (e.g., from previously used eluent additives in the waste) was prevented. To protect the analytical column and expand its lifetime, the column was rinsed with acetonitrile after each sequence. During the development, validation, and application of the method, no deleterious effects on column lifetime or performance were experienced as a result of the additive. The system was operated at a flow rate of 0.4 mL/min with the following gradient: 0−1 min, 5% B; rise to 18% B until 1.8 min and to 35% B until 4.2 min; rise to 48% B until 13 min and to 90% B until 14 min; flush with 98% B from 15.8 min to 17.6 min; re-equilibrate with 5% B from 17.7 min to 20 min. Multiple reaction monitoring (MRM) experiments were performed in positive and negative electrospray ionization (ESI) mode using fast polarity switching. The detailed settings are described in the Supporting Information.

Quantification and Data Evaluation. To account for matrix effects and potential retention time deviations in the different matrices, matrix-matched reference standards were prepared by resolving extracted and evaporated nonspiked pooled samples of urine, serum, and breast milk with 200 μL (urine and serum) or 250 μL (breast milk) with solvent standards of six different concentrations. For quantification, the fragment ion with the highest signal-to-noise ratio was chosen. A second fragment ion was obtained for confirmation and ion ratio determination (Table S2). Methyl-, ethyl-, propyl-, butylparaben, genistein, mono-*n*-butyl phthalate (MBP), perfluoroctanoic acid (PFOA), and perfluoroctanesulfonic acid (PFOS) were evaluated by internal standards in all matrices. Zearalenone (ZEN) and estradiol (E2) were evaluated by internal standard calibration in urine and serum. 4-*tert*-Octylphenol (4*t*OP) was evaluated by internal standard calibration in serum, and mono-2-ethylhexyl phthalate (MEHP) was evaluated by internal standard calibration in urine. Data evaluation was carried out with

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TraceFinder software (version 4.1, Thermo Scientific). Linear calibration curves were created using $1/x$ weighing. In the case of matrix contamination (not all pooled samples for validation purpose were true blanks as the high sensitivity of the developed method allowed the identification of some trace level contamination), all concentrations were corrected.

Validation. In-house method validation was performed according to the Commission Decision (EC) No. 657/2002[30] and evaluated in terms of selectivity, linearity, matrix effects (signal suppression or enhancement, SSE), extraction recovery (R_E), intermediate precision (interday precision, RSD_R), repeatability (intraday precision, RSD_r), and limits of detection (LOD) and quantification (LOQ). Experiments were carried out three times with independent sample preparation, calibration, and measurements. Due to the absence of a suitable certified reference material, validation experiments were performed by spiking pooled matrix samples. These were spiked in triplicate at two levels ("low" and "high") by addition of 20 μL (for urine and serum) or 25 μL (for breast milk) of a 10× concentrated multianalyte stock. Spiking levels aimed for 3× and 30× the LOQ values obtained in pre-experiments. In addition, three matrix "blanks" (nonspiked pooled matrix samples) and three system blanks (LC−MS grade water instead of matrix) were extracted for each matrix. Measurement sequences consisted of external quality control samples at the beginning and end of a sequence to evaluate the general performance of the system. Before and after each matrix batch, solvent and matrix matched calibration sets were measured. Intermediate precision (RSD_R) was determined by evaluation of the three validation batches (n = 9 for each spiking level). Repeatability (RSD_r) was determined by evaluation of the repeated measurement of one validation batch (n = 6 for each spiking level). Since the Commission Decision (EC) No. 657/2002[30] provides no RSD_R limits for spikes with a concentration below 100 μg/L, the limit was set to 25%. Equally, the RSD_r limit for spiked concentrations below 10 μg/L was set to 25%. To investigate selectivity, nonspiked pooled matrix samples were manually inspected for potentially interfering peaks. Linearity was obtained by determination of the matrix matched calibration regression coefficient (R^2) as well as by a manual inspection of the calibration curves. Signal suppression/enhancement (SSE) was determined as the ratio of the slope of the matrix matched calibration curve and the slope of the solvent calibration curve in percent. LOD and LOQ values were determined for each matrix by a signal-to-noise ratio of 3 and 10, respectively.

RESULTS AND DISCUSSION

To the best of our knowledge, this is the first LC−MS/MS method covering a vast number of known and suspected xenoestrogens including the most relevant endogenous estrogens. The 75 evaluated analytes included 13 phytoestrogens, 12 mycoestrogens, 7 parabens, 5 UV-filters, 7 bisphenols, 5 plasticizers, 5 industrial side products, 3 pesticides, 2 perfluorinated alkylated substances, 1 antimicrobial, and 1 pharmaceutical as well as 14 endogenous estrogens, including precursors, phase I and II metabolites.

LC−MS/MS Method Development. To identify analytes suitable for a multiclass xenoestrogen method, the scientific literature, EDC priority lists of the United Nations (UN), the European Food Safety Authority (EFSA), the U.S. Environmental Protection Agency (EPA), and the European Commission (EC) were screened. Analytes were selected based on their proven or strongly suspected estrogenicity, feasibility for LC−MS analysis, and the availability of reference standards. In addition, precursors of estrogenic compounds (e.g., matairesinol, xanthohumol, and isoxanthohumol) and in-house synthesized biotransformation products (phase II metabolites of ZEN) were included. *N*-Butylbenzenesulfonamide, 2-naphthol, and 2-*tert*-butylphenol were selected based on the first application of cognitive computing/artificial intelligence (AI) in exposome research from a list of xenobiotics, ranked by similarity to known estrogen receptor agonists.[19] These compounds were selected as of their high scoring and (to date) no experimentally reported estrogenicity.

Compound specific mass spectrometric parameters were optimized by direct flow injection of a single solvent standard (5 mg/L) utilizing a T-piece. All analytes have been tested thereafter in the respective matrices in order to identify the best suited qualifier and quantifier ion (Table S2). Different eluent additives were tested in order to increase sensitivity which was a major objective: ammonium acetate, acetic acid, and ammonium fluoride. Overall, ammonium acetate resulted in the lowest signal intensities while acetic acid resulted in decent ionization for most analytes. However, neither enabled a satisfying ionization of endogenous estrogens. Previous studies reported signal enhancement of steroids by addition of NH_4F to the aqueous mobile phase.[31−33] It has been proposed that the basicity of the fluoride ion draws protons from the steroid and produces, by forming HF molecules, $[M - H]^-$ species.[34] In this study, we detected a vast improvement in steroid ionization and signal enhancement of various other substance classes like bisphenols and phytoestrogens by adding ammonium fluoride, hence this modifier was selected as most appropriate to achieve ultimate sensitivity.

Chromatographic gradient optimization was essential due to several structural similarities of compounds with exactly the same mass to charge ratio $[m/z]$ and similar fragmentation patterns (e.g., zearalenol (ZEL) and zearalanol (ZAL) isomers, 16-/17-epiestriol and estriol, 2-/4-methoxy-estrogens, *iso*butyl- and butylparaben). For all analytes but *iso*butylparaben (iBP) and butylparaben (BP), baseline separation was achieved. For iBP and BP, a resolution factor R_S of 0.7 was obtained. Although no baseline separation was possible, two clearly separable peaks and linear calibration curves were generated (see Figure 2, Table S2). The lowest acceptable retention time, according to EC No. 657/2002, is 2 times the retention time of the columns void volume. In this method, analytes eluting after 1.75 min were accepted. Due to its hydrophilic character, the paraben metabolite *p*-hydroxybenzoic acid (*p*HBA)[35] elutes early in reversed phase chromatography and, despite highly aqueous starting conditions (5% B), only in breast milk stable retention times above 1.75 min were achieved for *p*HBA. All other analytes did not elute before 4 min.

Sample Preparation and Optimization. The majority of the few existing multianalyte methods, measuring more than one class of estrogenic compounds in biological matrices, rely on derivatization methods to increase sensitivity.[27,36,37] We, however, aimed for a simple and quick sample preparation protocol without time intensive derivatization or sample dilution which would decrease sensitivity but nevertheless acceptable matrix effects and low LOD values. For exposome-scale studies involving the measurement of samples in a high-throughput manner, a time and cost-effective sample preparation is a prerequisite. These requirements were met with our optimized protocol for serum and urine (Table 1 and

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Table 1. Extraction Efficiencies (R_E), Intermediate Precision (RSD_R), Repeatability (RSD_r), and Limits of Detection (LOD) and Quantification (LOQ) in Urine, Serum, and Breast Milk of Analytes That Have Been Successfully Validated in at Least One Biological Matrix[a]

	urine			serum			breast milk				
	R_E[b] ± RSD_R[c] LL[d] [%]	R_E ± RSD_R HL[e] [%]	RSD_r[f] LL/HL [%]	R_E± RSD_R LL [%]	R_E± RSD_R HL [%]	RSD_r LL/HL [%]	R_E± RSD_R LL [%]	R_E± RSD_R HL [%]	RSD_r LL/HL [%]	LOD[g] (U/S/M)[h] [μg/L]	LOQ[i] (U/S/M) [μg/L]
Phytoestrogens and Metabolites											
8-prenylnaringenin	98 ± 21	103 ± 7	19/5	94 ± 15	76 ± 11	9/8	29 ± 77	74 ± 18	37/20	0.05/0.1/0.2	0.15/0.2/0.6
coumestrol	96 ± 10	99 ± 6	14/2	86 ± 6	87 ± 7	3/5	83 ± 9	91 ± 3	10/10	0.15/0.4/0.3	0.5/1.3/1.0
daidzein	112 ± 15	103 ± 6	15/4	90 ± 13	90 ± 6	9/4	86 ± 16	93 ± 4	12/9	0.15/0.1/0.05	0.5/0.4/0.15
enterodiol	99 ± 12	101 ± 5	5/3	89 ± 7	91 ± 10	8/3	54 ± 28	56 ± 34	76/32	0.15/0.05/0.06	0.50/0.15/0.20
enterolactone	102 ± 9	104 ± 3	6/2	85 ± 13	88 ± 13	8/5	88 ± 12	91 ± 6	10/5	0.15/0.2/0.1	0.50/0.7/0.4
equol	103 ± 9	102 ± 6	5/4	94 ± 10	89 ± 12	5/7	70 ± 15	78 ± 9	14/10	0.2/0.2/0.1	0.5/0.7/0.4
formononetin	105 ± 9	102 ± 7	7/3	90 ± 4	92 ± 5	3/2	87 ± 4	89 ± 3	7/8	0.05/0.015/0.02	0.15/0.050/0.06
genistein	100 ± 21	94 ± 10	9/10	79 ± 17	88 ± 12	12/14	85 ± 13	84 ± 12	13/7	0.2/0.15/0.09	0.5/0.60/0.30
glycitein	103 ± 13	105 ± 6	13/1	90 ± 12	94 ± 4	8/4	91 ± 13	92 ± 6	9/8	0.2/0.15/0.01	0.5/0.6/0.03
isoxanthohumol	112 ± 20	102 ± 7	13/2	98 ± 9	94 ± 10	8/3	94 ± 12	83 ± 8	11/8	0.005/0.015/0.010.009	0.015/0.050/0.03
matairesinol	102 ± 10	103 ± 9	13/6	86 ± 13	88 ± 10	22/5	90 ± 15	93 ± 5	15/6	1.0/0.6/0.75	3.0/2.0/2.50
resveratrol	99 ± 22	107 ± 4	19/6	126 ± 21	81 ± 36	33/20	n.d.	11 ± 80	n.d./63	2.0/4.8/7.5	3.0/16.0/25.0
Mycoestrogens and Metabolites											
alternariol	101 ± 12	100 ± 5	13/7	88 ± 10	85 ± 8	6/4	77 ± 19	93 ± 3	20/8	0.5/0.6/0.35	1.5/2.0/1.20
alternariol monomethyl ether	107 ± 10	105 ± 4	9/2	97 ± 12	96 ± 10	8/7	72 ± 21	78 ± 20	17/7	0.05/0.05/0.25	0.15/0.15/0.8
α-zearalanol (ZAL)	106 ± 8	100 ± 6	5/2	92 ± 8	94 ± 10	11/3	71 ± 13	80 ± 12	6/12	0.25/0.2/0.35	0.70/0.7/1.2
β-ZAL	108 ± 9	102 ± 7	6/2	95 ± 12	92 ± 7	9/3	73 ± 7	79 ± 4	10/9	0.25/0.2/0.2	0.70/0.6/0.6
α-zearalenol (ZEL)	105 ± 11	104 ± 7	9/3	95 ± 7	90 ± 10	4/3	70 ± 29	80 ± 20	6/10	0.3/0.3/1.0	0.8/1.0/3.0
β-ZEL	98 ± 16	104 ± 11	14/4	91 ± 17	93 ± 7	14/7	76 ± 17	79 ± 10	8/12	1.0/0.55/0.55	2.5/1.80/1.80
α-ZEL-14-glucuronide (GlcA)	136 ± 32	103 ± 6	8/6	92 ± 13	88 ± 7	7/10	77 ± 14	73 ± 15	37/22	12/2.7/0.2	40/9.0/0.6
β-ZEL-14-GlcA	n.d.	98 ± 12	n.d./6	90 ± 16	90 ± 5	11/5	79 ± 29	77 ± 15	35/18	45/2.4/0.3	140/8.0/1.0
zearalanone	107 ± 9	102 ± 7	8/3	88 ± 9	87 ± 11	8/4	64 ± 31	82 ± 12	25/11	0.15/0.25/0.6	0.5/0.8/2.0
zearalenone (ZEN)	98 ± 11	98 ± 11	11/7	90 ± 9	90 ± 7	14/7	109 ± 30	117 ± 28	34/26	0.15/0.25/0.5	0.5/0.8/1.5
ZEN-14-GlcA	n.d.	101 ± 12	n.d./7	95 ± 19	90 ± 10	6/11	86 ± 22	87 ± 4	16/8	40/2.4/0.25	120/8.0/0.8
ZEN-14-sulfate	101 ± 7	100 ± 5	5/2	93 ± 4	92 ± 5	3/4	36 ± 67	38 ± 74	37/44	0.4/0.1/0.15	1.2/0.3/0.45
Personal Care Product Ingredients, Pharmaceuticals, and Metabolites											
benzophenone 1	110 ± 11	101 ± 5	14/5	90 ± 7	92 ± 3	5/6	84 ± 15	89 ± 6	27/7	0.05/0.1/0.05	0.10/0.2/0.15
benzophenone 2	100 ± 11	100 ± 6	6/3	91 ± 14	96 ± 16	9/6	87 ± 20	92 ± 4	10/6	0.1/0.1/0.06	0.3/0.3/0.2
benzylparaben	94 ± 12	99 ± 5	21/4	89 ± 11	93 ± 14	16/6	55 ± 36	87 ± 13	29/13	0.05/0.03/0.08	0.10/0.09/0.25
butylparaben	95 ± 5	94 ± 2	7/2	91 ± 6	91 ± 3	5/4	82 ± 13	86 ± 6	21/13	0.05/0.03/0.06	0.15/0.10/0.20
ethylparaben	97 ± 13	97 ± 7	11/7	94 ± 9	93 ± 3	10/12	82 ± 12	82 ± 8	9/11	0.05/0.05/0.02	0.15/0.15/0.08
*iso*butylparaben	97 ± 7	96 ± 2	5/3	90 ± 7	93 ± 10	8/3	83 ± 7	85 ± 6	9/8	0.05/0.03/0.05	0.15/0.10/0.20
methylparaben	95 ± 9	99 ± 3	8/3	93 ± 5	92 ± 6	8/2	78 ± 12	77 ± 11	7/7	0.3/0.1/0.1	1.0/0.4/0.3
propylparaben	96 ± 8	97 ± 4	6/3	88 ± 7	94 ± 5	10/5	82 ± 11	84 ± 4	4/4	0.06/0.06/0.06	0.3/0.2/0.2
ethinylestradiol	101 ± 13	100 ± 4	8/3	90 ± 25	87 ± 19	6/7	79 ± 17	82 ± 9	20/6	0.5/0.6/1.7	1.5/2.0/5.9

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Table 1. continued

	urine			serum			breast milk				
	R_E[b] ± RSD_R[c] LL[d] [%]	R_E ± RSD_R HL[e] [%]	RSD_r[f] LL/HL [%]	R_E± RSD_R LL [%]	R_E± RSD_R HL [%]	RSD_r LL/HL [%]	R_E± RSD_R LL [%]	R_E± RSD_R HL [%]	RSD_r LL/HL [%]	LOD[g] (U/S/M)[h] [μg/L]	LOQ[i] (U/S/M) [μg/L]
Plastizicer/Plastic Components and Metabolites											
bisphenol A (BPA)	102 ± 12	100 ± 6	12/2	95 ± 13	91 ± 18	9/4	86 ± 16	90 ± 4	11/9	0.2/0.6/0.3	0.7/2.0/0.9
BPAF	108 ± 12	107 ± 5	11/4	96 ± 19	87 ± 26	10/8	76 ± 9	79 ± 13	8/6	0.5/0.3/0.45	0.5/1.1/1.5
BPB	99 ± 7	102 ± 3	3/4	90 ± 12	94 ± 22	14/5	84 ± 9	90 ± 6	14/9	0.2/0.3/0.3	0.5/0.9/0.9
BPC	104 ± 13	101 ± 6	7/3	103 ± 16	90 ± 24	9/7	79 ± 19	85 ± 11	9/13	0.5/1.5/1.2	1.5/5.0/4.0
BPF	104 ± 9	100 ± 5	12/3	92 ± 15	90 ± 17	17/11	91 ± 20	90 ± 8	18/6	0.5/1.1/0.4	1.5/3.5/1.3
BPS	97 ± 20	102 ± 5	10/6	87 ± 14	92 ± 5	13/5	87 ± 10	91 ± 4	15/6	0.05/0.03/0.01	0.15/0.10/0.04
mono-*n*-butyl phthalate	93 ± 8	94 ± 5	9/5	158 ± 13	165 ± 16	9/9	85 ± 13	97 ± 8	12/10	0.8/0.3/0.4	2.2/1.0/1.3
mono-2-ethylhexyl phthalate	96 ± 9	97 ± 5	7/6	180 ± 33	96 ± 10	17/5	56 ± 58	64 ± 33	44/13	1.5/n.d./12	4.0/n.d./40
N-butylbenzenesulfonamide	102 ± 7	97 ± 3	6/3	98 ± 10	92 ± 5	11/4	82 ± 16	90 ± 4	7/7	0.8/1.1/1.0	2.2/3.7/3.5
Perfluorinated Alkylated Substances											
perfluoroctanoic acid	101 ± 9	96 ± 6	5/2	91 ± 9	93 ± 4	8/2	12 ± 7	11 ± 6	7/2	0.1/0.09/0.06	0.3/0.30/0.20
perfluoroctanesulfonic acid	84 ± 9	92 ± 7	6/5	84 ± 22	97 ± 15	18/11	80 ± 15	84 ± 16	20/12	2.5/2.4/1.4	2.5/8.0/4.6
Industrial Side Products and Pesticides											
2-naphthol	85 ± 9	87 ± 4	6/2	94 ± 11	92 ± 16	6/8	74 ± 17	81 ± 8	28/12	0.25/0.1/0.7	0.80/1.4/2.4
methiocarb	102 ± 9	96 ± 8	5/5	62 ± 11	51 ± 16	15/2	73 ± 18	82 ± 10	10/9	0.8/3.6/2.7	2.5/12.0/9.0
prochloraz	103 ± 10	99 ± 6	17/3	92 ± 17	79 ± 12	18/12	71 ± 48	98 ± 73	146/36	0.01/0.01/0.1	0.03/0.03/0.3
Endogenous Estrogens											
estrone (E1)	101 ± 7	102 ± 5	6/3	91 ± 10	86 ± 14	9/5	82 ± 8	85 ± 9	9/11	0.1/0.15/0.2	0.3/0.50/0.6
estradiol (E2)	105 ± 11	104 ± 9	11/6	90 ± 23	86 ± 14	18/11	92 ± 24	84 ± 12	27/14	0.5/1.0/1.4	1.5/3.2/4.6
E2-17-GlcA	n.d.	111 ± 11	n.d./47	88 ± 12	87 ± 4	14/3	81 ± 19	90 ± 6	19/11	60/4.2/1.2	200/14.0/3.5
E2-3-sulfate	106 ± 16	103 ± 7	20/3	89 ± 2	90 ± 5	3/3	49 ± 24	50 ± 44	13/6	1.5/0.2/0.1	5.0/0.5/0.3
estriol (E3)	90 ± 14	103 ± 7	13/6	94 ± 14	92 ± 9	11/7	82 ± 19	77 ± 10	19/8	1.0/0.3/0.9	3.0/1.0/3.0
16-epiestriol	99 ± 11	101 ± 8	11/4	95 ± 8	88 ± 8	8/6	48 ± 50	48 ± 49	15/30	0.8/0.9/1.4	2.4/3.0/4.8
16-α-hydroxyE1	107 ± 8	99 ± 5	12/3	82 ± 13	83 ± 8	10/6	86 ± 14	85 ± 6	23/10	0.15/0.2/0.1	0.50/0.6/0.3
17-epiestriol	102 ± 12	98 ± 7	10/4	95 ± 6	88 ± 9	11/7	54 ± 30	57 ± 54	11/46	1.0/0.9/1.1	3.5/2.8/3.8
2-methoxyE1	99 ± 6	99 ± 5	6/2	91 ± 7	86 ± 11	6/1	74 ± 12	83 ± 9	9/8	0.2/0.3/0.6	0.7/0.9/1.9
2-methoxyE2	103 ± 5	99 ± 7	6/5	86 ± 8	87 ± 12	9/5	63 ± 14	76 ± 14	12/13	0.3/0.4/0.6	1.0/1.2/2.0
4-methoxyE1	99 ± 6	100 ± 4	5/5	90 ± 11	87 ± 15	5/8	76 ± 11	84 ± 9	3/10	0.05/0.05/0.10	0.15/0.15/0.30
4-methoxyE2	101 ± 8	98 ± 4	6/1	91 ± 6	90 ± 12	4/5	75 ± 13	80 ± 12	14/10	0.15/0.1/0.2	0.50/0.3/0.6

[a]Analytes not successfully evaluated in any matrix are reported in Table S3. n.d. stands for not determined due to either insufficient ionization or extraction recovery, too high background noise, or invalid calibration caused by disturbing blank matrix contamination. [b]Extraction efficiency. [c]Intermediate precision. [d]Low spiking level. [e]High spiking level. [f]Repeatability. [g]Limit of detection. [h]Values in the following order: urine/serum/breast milk. [i]Limit of quantification.

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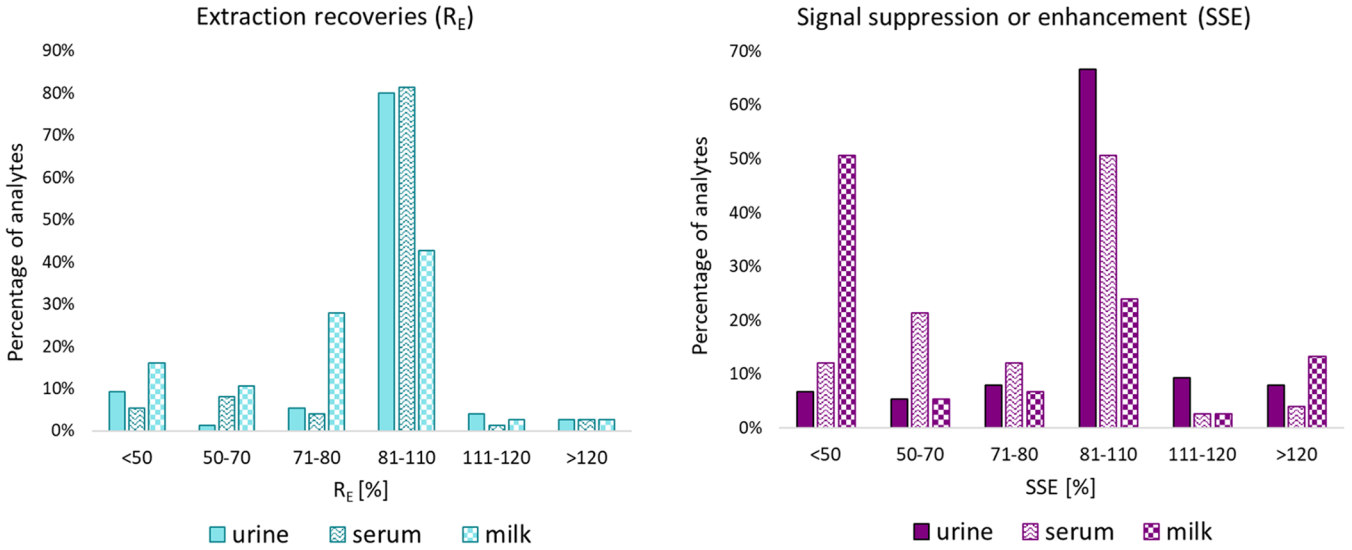

Figure 1. Extraction efficiency (R_E) and matrix effect (SSE) distribution of all 75 tested analytes in urine, serum, and breast milk.

Figure 1. For the particularly challenging matrix of breast milk, we adapted the protocol of Braun et al. (2018),[29] which was originally optimized for the extraction of mycotoxins including the estrogenic ZEN and its metabolites α-/β-ZEL. We reduced the sample volume and exchanged the "filtering" to a "dry down and resolve" step in order to overcome sample contamination with plastic components from the filter material. This modifications led, at least for ZEN and α-/β-ZEL, to comparable extraction efficiencies (70−117% vs 82−106% in Braun et al. (2018)[29]) but a higher variance. The reduced volume resulted in a relatively larger surface area in contact with the reaction tube and the added salts, which potentially bind to or react with the analytes. The evaporation in turn resulted in a difficult to dissolve pellet containing one small fat drop, which could not be resolved again. We assume that this is a reason why less than 50% of the analytes yielded extraction recoveries above 80% in breast milk (see Figure 1).

Method Validation and Limitations of the Method. Overall, 55 and 53 out of 75 analytes were successfully validated in urine and serum and 31 out of 75 in breast milk. Selectivity was carefully evaluated throughout method validation but also later during the application of the method to naturally contaminated samples.

No interferences in a retention time window of 0.1 were detected. However, due to the high sensitivity of the developed assay, we detected a number of xenoestrogens in the nonspiked samples. In the nonspiked serum, 2-naphthol (approximately 9.6 μg/L), PFOA (<LOQ), and MEHP (heavily contaminated, no matrix matched calibration feasible) were determined. The nonspiked pooled urine (from one volunteer) was contaminated with 2-naphthol (2.7 μg/L), enterolactone (<LOQ), MBP (<LOQ), and pHBA (~337 μg/L). In the nonspiked pooled breast milk, we detected 2-naphthol (~6.2 μg/L), BPA (<LOQ), bisphenol S (<LOQ), methylparaben (~0.6 μg/L), ethylparaben (<LOQ), propylparaben (<LOQ), MBP (2.1 μg/L), genistein (<LOQ), PFOA (<LOQ), and pHBA (<LOQ).

Our LC−MS/MS system appeared to be heavily contaminated with dibutyl phthalate and nonylphenol, which occurred in every sample including the solvent blanks. One previous study also reported background contamination with 4-nonylphenol due to laboratory air contamination.[38] We also observed a minimal system contamination of bisphenol AF. 4-Hydroxyestradiol exhibited carry over after highly concentrated standards across several injections. All successfully validated compounds displayed a R^2 value higher than 0.98. R_Es of successfully validated analytes ranged from 94% (PFOS) to 110% (benzophenone 1) in urine, 76% (8-prenylnaringenin) to 103% (bisphenol C) in serum, and 71% (α-zearalanone) to 97% (MBP) in breast milk.

In urine, the SSEs of the successfully validated analytes ranged from 66% (bisphenol S) to 263% (MBP). In serum and breast milk the SSE of validated analytes ranged from 57% (BPA) and 31% (2-methoxyestrone) to 145% and 144% (estradiol-17-glucuronide), respectively. SSE values are reported in Table S4. Interestingly, 51% of all analytes in milk (compared to 7% in urine and 12% in serum) experienced strong signal suppression with a SSE < 50% (Figure 1). Whereas, 17% and 16% of all analytes in urine and milk (7% in serum) experienced signal enhancement with a SSE above 110%.

All analytes with R_E, RSD_R, and RSD_r values which did not meet the criteria of the Commission Decision (EC) No. 657/2002[30] were regarded as not successfully validated (see Table 1, Tables S3 and S5). In some cases (e.g., α/β-zearalenol-14-glucuronide in urine), the lowest spiking level was below the LOQ value and could not be properly evaluated in all three matrices. Some R_E values slightly exceeded the validation criteria in one spiking level (e.g., daidzein in urine and β-ZAL in breast milk). However, for these analyte/matrix combinations, at least a semiquantitative evaluation is feasible and constitutes a valuable tool for combined exposure assessment.

Although the ionization efficiency of endogenous estrogens is very weak and derivatization is typically required,[27,37] we yielded acceptable LODs by the addition of ammonium fluoride to the aqueous mobile phase. LOD values of the successfully validated endogenous estrogens ranged from 0.05 μg/L (4-methoxyestrone) in urine and serum and 0.1 μg/L (16-α-hydroxyestrone and 4-methoxyestrone) in milk to 1.5 μg/L (E2−3-sulfate) in urine, 4.2 μg/L (E2−17-glucuronide) in serum, and 1.4 μg/L (E2) in milk. Levels of E2, the most prevalent endogenous estrogen, reach maximum concentrations around 0.4 μg/L in serum of ovulating premenopausal women,[39] which are below our LOD value (0.5 μg/L, 1 μg/L, and 1.1 μg/L in urine, serum, and breast

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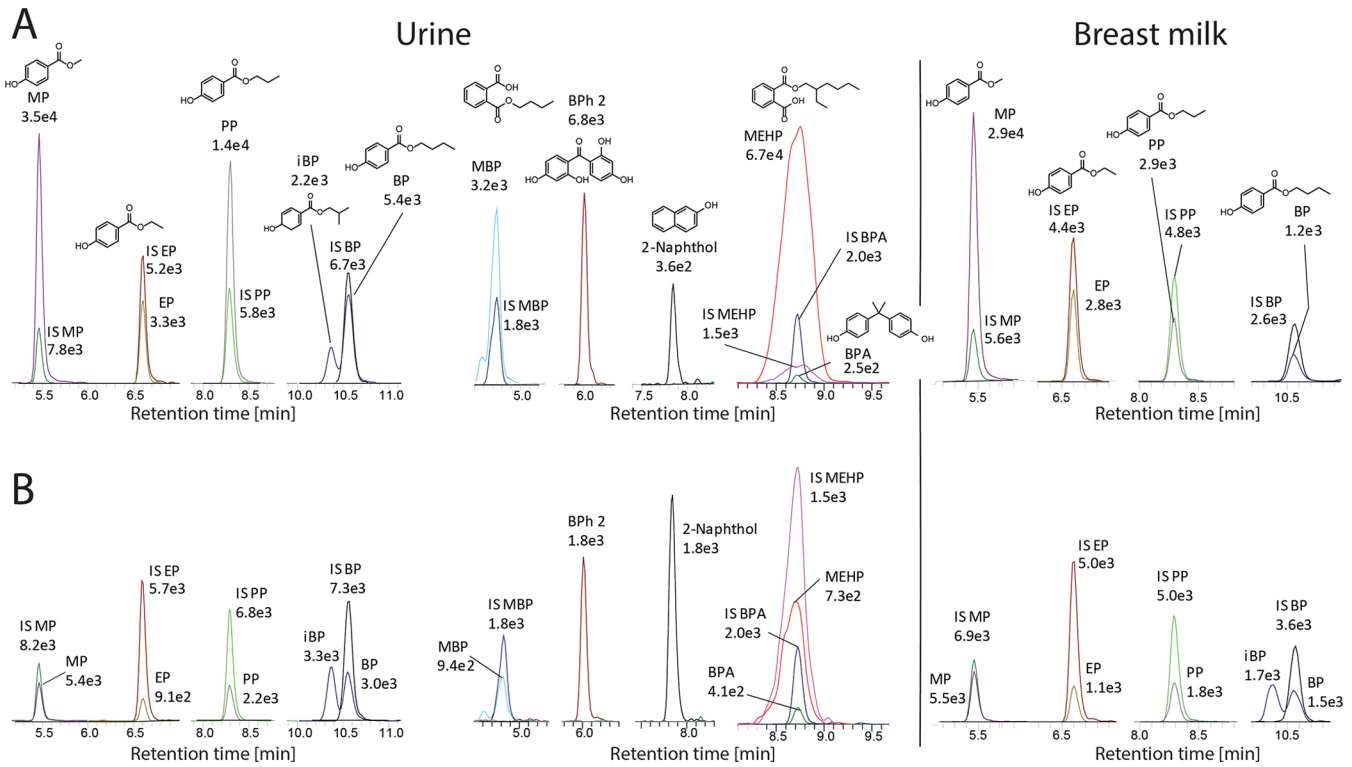

Figure 2. MRM-chromatograms demonstrating combined xenoestrogen exposure in (A) an infant urine and a breast milk sample compared to (B) matrix matched calibration standards. Numbers represent intensity; the following transitions and concentrations (in the samples displayed in part A) were observed: methylparaben (MP) m/z 151.0 → 91.9, urine, 23.4 μg/L; breast milk, 28.3 μg/L; isotopic labeled MP (IS MP) m/z 157.0 → 97.9; ethylparaben (EP) m/z 165.0 → 91.9; urine, 5.5 μg/L; breast milk, 4.9 μg/L; IS EP m/z 171.0 → 97.9; propylparaben (PP) m/z 179.0 → 91.9, urine, 11.7 μg/L; breast milk, 2.8 μg/L; IS PP m/z 185.0 → 97.9; *iso*butylparaben (iBP) and butylparaben (BP) m/z 193.0 → 92.0, urine, 1.1 μg/L and 2.8 μg/L; breast milk, n.d. and 1.7 μg/L; IS BP m/z 199.0 → 98.0; mono-*n*-butyl phthalate (MBP) m/z 221.0 → 77.0, 24.9 μg/L; IS MPB m/z 225.0 → 79.0; benzophenone 2 m/z 244.9 → 134.9, 0.6 μg/L; 2-naphthol m/z 143.0 → 115.0, 1.5 μg/L; mono-2-ethylhexyl phthalate (MEHP) m/z 277.1 → 133.9, >200 μg/L; IS MEHP m/z 281.1 → 136.9; bisphenol A (BPA) m/z 227.0 → 132.9, 1.6 μg/L; IS BPA m/z 239.0 → 224.0

milk). Estrone (maximum values of about 0.2 μg/L in serum)[40] might be detectable. While rather sensitive for a nontailor made method without chemical derivatization, the method may not be feasible for the determination of endogenous estrogens in women not pregnant or without any medical condition. However, in pregnant women, E2 reaches concentrations above 30 μg/L and estriol spikes to about 15 μg/L, both clearly above the respective LOQ values.[40,41] Higher concentrations of endogenous estrogen metabolites are also expected during human gestation. The primary purpose of the developed method will be the investigation of critical exposure windows including pregnancy and breast feeding, as these early life exposures are likely to be of special relevance in the etiology of chronic disease later in life.[13] The majority of all other included and successfully validated compounds yielded LOD values below 1 μg/L which were generally deemed fit-for-purpose for the first of its kind multianalyte method.

Application to Biological Samples. For proof-of-principle experiments in urine, six samples from three individuals were analyzed. Four samples resembled a mother−infant pair. We did not detect any contamination source due to sample preparation (clear system blank). The following compounds have been detected in at least one urine sample: 2-naphthol, alternariol monomethyl ether, benzophenone 1 and 2, BPA, BPS methyl-, ethyl-, propyl-, butyl-, and *iso*butylparaben, daidzein, enterodiol, enterolactone, equol, formononetin, genistein, glyctein, isoxanthohumol, *p*HBA, MBP, and MEHP. Concentrations are reported in Table S6. Interestingly, adult females showed a lower contamination of parabens, with an averaged sum of 2.6 μg/L than the two infant samples with an averaged sum of 36.8 μg/L in urine. Parabens are metabolized in the liver via esterase hydrolysis and glucuronidation.[35]

Infants have a lower plasma protein concentration and a reduced metabolic performance resulting in a limited capacity of drug detoxification via protein binding or glucuronidation (reviewed by Lu and Rosenbaum (2014)[42]). Therefore, it is of particular importance to monitor and control environmental exposures of un- and newborn babies. However, we want to point out that only a very limited number of samples were investigated in this preliminary work, and the obtained coexposure data needs to be interpreted with caution and followed up in larger studies. In this study, we did not include a deconjugation step since we intentionally assessed sulfates and glucuronides (e.g., estradiol-3-sulfate or zearalenone-14-glucuronide) directly. In future studies, deconjugation of samples will be considered and likely result in an even higher number of positive samples.

Interestingly, we did not detect any phytoestrogens in infant urine although the mothers' urine contained reasonable concentrations (averaged sum 62.4 μg/L). This leads to the assumption that the phytoestrogen uptake of infants via breast milk is moderate to low (detection of enterolactone, genistein,

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and glycitein below LOQ in breast milk). To confirm this hypothesis, more samples have to be analyzed, especially because phytoestrogen concentrations strongly depend on the diet and can vary significantly even in a single individual.[43] Our findings of personal care product additives and bisphenols are in line with previous reports in urine, although some of our samples contained slightly higher amounts of methylparaben, benzophenone-2, and bisphenol S.[24,26] One infant urine sample exhibited high concentrations of MEHP (>200 μg/L) and MBP (24.9 μg/L). MEHP and MBP are metabolites of diethylhexyl phthalate (DEHP) and di-*n*-butyl phthalate, respectively,[44,45] and it has been reported in a previous study that children typically have a higher DEHP intake than adults.[46]

Figure 2 shows chromatograms of a naturally contaminated urine and breast milk sample. To demonstrate the biological feasibility of breast milk analysis, five Nigerian samples and four breast milk samples from two Austrian mothers were analyzed. The following compounds have been detected in at least one breast milk sample: 2-naphthol, benzophenone 2, BPS, methyl-, ethyl-, propyl-, and butylparaben, enterolactone, genistein, glycitein, MBP, MEHP, PFOA, and *p*HBA. BPA was detected in trace amounts in all breast milk samples but also in the system blank, which leads to the assumption that the samples were possibly contaminated with BPA during sample preparation. Consistent with our findings, parabens, phthalate metabolites, some phytoestrogens, and PFOA have been identified in breast milk before.[24,47−50] Interestingly, a previous study found more BPF, which was not detected at all in our samples, than BPS in breast milk.[51] Nigerian samples did not contain parabens but methylparaben.

Every single biological sample contained 2-naphthol, which is a metabolite of the carcinogenic naphthalene.[52,53] The mean concentration in urine was 1.9 μg/L which is slightly lower than in a previous study of Korean individuals.[54] In breast milk, a mean of 7.7 μg/L was detected in our samples. Together with 1-naphthol, 2-naphthol is usually used to estimate polycyclic aromatic hydrocarbon exposure.[52] However, it has to be considered that 2-naphthol showed only a low-abundance qualifier fragment due to its low molecular mass (2% of quantifier intensity). Therefore, not in all measured biological samples qualifier ions were detected.

CONCLUSION AND OUTLOOK

In this study, a novel method for the identification of endogenous and exogenous estrogens in the three human matrices urine, serum, and breast milk was developed. For the first time, it is now possible to analyze these classes of estrogenic compounds simultaneously and thereby estimate the total estrogenic burden at an individual level. This is of particular interest since xenoestrogens often appear at concentrations in which a single compound itself has no significant toxicological effect; however, exposure to a mixture of several low-dose xenoestrogens may lead to adverse outcomes as demonstrated *in vitro* and *in vivo*.[14−17] The analysis of a limited number of real-world samples indicated that different classes of xenoestrogens can co-occur and may be found simultaneously in one sample of urine or breast milk. These findings clearly demonstrate the need for large-scale epidemiological studies investigating potential correlations between exposure to xenoestrogens and health effects including the development of uterine or breast cancer. Further possible applications include the investigation of placental transfer and metabolism of estrogenic compounds as well as correlation studies between total xenoestrogen exposure and early onset of puberty or infertility.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b02446.

Detailed description of the sample preparation protocol, additional tables and figures, including chemicals and reagents with structures, LC−MS/MS parameters, validation summary, and a table of all quantitative measurements in the biological samples (PDF)

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Notes

The authors declare no competing financial interest.

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