Endocrine Disruptors and Estrogens in Human Prostatic Tissue

Jana VITKU[1], Tereza SKODOVA[1], Anezka VARAUSOVA[1], Lukas GADUS[2,3], Ludmila MICHNOVA[4], Lenka HORACKOVA[1], Lucie KOLATOROVA[1], Marketa SIMKOVA[1], Jiri HERACEK[2,3]

[1]Institute of Endocrinology, Department of Steroids and Proteofactors, Prague, Czech Republic, [2]Military University Hospital Prague, Department of Urology, Prague, Czech Republic, [3]Charles University, First Faculty of Medicine, Department of Urology, Prague, Czech Republic, [4]Military University Hospital Prague, Department of Pathology, Prague, Czech Republic

Received August 12, 2023
Accepted September 26, 2023

Summary

Endocrine disruptors (EDs) are ubiquitous substances both in the environment and everyday products that interfere with the hormonal system. Growing evidence demonstrates their adverse effects on the organism, including the reproductive system and the prostate, owing to their (anti)estrogenic or antiandrogenic effects. Since EDs can interact with steroid hormone actions on-site, understanding the levels of intraprostatic EDs in conjunction with steroids may hold particular significance. The aim of this study was to develop and validate a method for determining estrogens, various groups of EDs (bisphenols, parabens, oxybenzone and nonylphenol) and phytoestrogens in their unconjugated and conjugated forms in prostate tissue by liquid chromatography-tandem mass spectrometry, and subsequently analyze 20 human prostate tissue samples. The method enabled 20 compounds to be analyzed: estrogens (estrone, estradiol, estriol), bisphenols (bisphenol A- BPA, BPS, BPF, BPAF, BPAP, BPZ, BPP), parabens (methyl-, ethyl-, propyl-, butyl-, benzyl- paraben), oxybenzone, nonylphenol and phytoestrogens (daidzein, genistein, equol) with LLOQs between 0.017-2.86 pg/mg of tissue. The most frequently detected EDs in prostate tissues were propylparaben (conjugated and unconjugated forms in 100 % of tissues), methylparaben (unconjugated in 45 % and conjugated in 100 %), ethylparaben (unconjugated in 25 % and conjugated in 100 %), BPA (unconjugated in 35 % and conjugated in 60 %) and oxybenzone (both forms in 45 %). To the best of our knowledge, this is the first study detecting EDs, phytoestrogens and estriol conjugate (E3C) in the prostate. E3C was the most abundant estrogen in prostatic tissue. This highlights the need for further explorations into estrogen metabolism within the prostate.

Key words

Endocrine disruptor • Phytoestrogen • Estrogen • LC-MS/MS • Prostate tissue

Corresponding author

Jana Vitku, Institute of Endocrinology, Department of Steroids and Proteofactors, Narodni 8, 116 94 Prague, Czech Republic. E-mail: jvitku@endo.cz

Introduction

The incidence of prostate cancer (PCa) has been on the rise over the past 40 years [1], and it now stands as one of the most frequently diagnosed cancers in men in the Czech Republic [2] as well as in other countries [3,4]. Apart from factors like age, race, inflammation, and genetic background, environmental factors are also suspected to play a role in the development of PCa. Among these are diet, smoking, and exposure to certain endocrine-disrupting chemicals (EDs). EDs can interfere with the hormonal system in the body through various mechanisms [5]. Given that the prostate gland relies heavily on hormones throughout life, it is expected that these chemicals may alter prostate physiology [6,7].

In fact, a majority of both human and animal studies have reported an increased risk of PCa associated with developmental and adult exposure to EDs (reviewed in [6]). The most frequently examined EDs included pesticides, bisphenol A (BPA), polychlorinated biphenyls, diethylstilbestrol, vinclozolin and dioxins. In addition, other well-known EDs include bisphenols, parabens, phthalates, per- and poly-fluorinated compounds (PFAS), polybrominated diphenyl ethers (PBDE) and other phenolic substances such as triclosan, alkylphenols and oxybenzone. They are present in

personal care products (parabens, triclosan, oxybenzone, phthalates), food and drink packaging (bisphenols, phthalates, PFAS), common household products (PBDE, PFAS), and other materials such as thermal receipts (bisphenols), toys (phthalates), outdoor equipment (PFAS) and pesticides. Humans are exposed to EDs through ingestion, inhalation and dermal contact throughout their life, including intrauterine development [5,8].

Phytoestrogens are compounds of natural origin and can be found in soy beans and many soy-derived products [9]. They exhibit both estrogenic and antiestrogenic activities depending on the concentration of endogenous estrogens in the body, since they compete for binding sites on the estrogen receptor (ER). When endogenous estrogen levels are high, phytoestrogens can act as antagonists on the ER due to their lower potency. Conversely, when natural estrogen levels are low, phytoestrogens can act as agonists on the ER [10]. Phytoestrogens can possess both endocrine-disrupting and potentially beneficial effects [11-13]. The role of phytoestrogens in prostatic diseases is a topic of widespread discussion (reviewed in [14-16]), but a recent meta-analysis [17] reported on the positive effects of daidzein and genistein on reductions of PCa risk.

While various techniques exist for detecting EDs in biological fluids like urine, plasma, or seminal plasma, there is currently a lack of analytical methods for directly assessing EDs in prostate tissue, despite the potential risks associated with EDs influencing prostate health. EDs, much like steroids, exist in both unconjugated and conjugated forms within the human body, primarily as sulfates and glucuronides. Unconjugated forms can be measured directly through straightforward methods involving precipitation or extraction steps, or through more time-intensive processes that include derivatization steps to enhance sensitivity. Conjugated forms can also be measured directly [18,19], but it is more common to first hydrolyze these analytes and then analyze them in their de-conjugated form.

This study addresses two aims. First, to develop and validate a method for the determination of estrogens and various groups of estrogen-like EDs (parabens, bisphenols, phytoestrogens, oxybenzone, nonylphenol) in prostate tissue in their unconjugated as well as conjugated forms. Second, to use this methodology to analyze 20 non-cancerous prostate tissues samples from patients that underwent robotic radical prostatectomy due to localized prostate carcinomas.

Materials and methods

Chemicals and reagents

The reference standards of estrone (E1), 17β-estradiol (E2) and estriol (E3) and deuterated standards of estrone (d_4E1) and estriol (d_2E3) were purchased from Steraloids (Newport, RI, USA). Standards of bisphenol A (BPA), bisphenol A glucuronide (BPAG), bisphenol A sulfate (BPAS) bisphenol S (BPS), bisphenol F (BPF), bisphenol AF (BPAF), bisphenol AP (BPAP), bisphenol P (BPP), bisphenol Z (BPZ), methylparaben (MP), ethylparaben (EP), propylparaben (PP), butylparaben (BP), benzylparaben (BenzylP), 4-*n*-nonylphenol (NP), daidzein, daidzein glucuronide, genistein, equol, oxybenzone and deuterated standards of BPA (d_{16}BPA), NP (d_4NP) and E2 (d_3E2) as well as ammonium fluoride trimethylchlorsilane (TMCS) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Deuterated standards of MP (d_4MP) and PP (d_4PP) were purchased from Chiron (Trondheim, Norway). Deuterated EP (d_4EP) and BP (d_4BP) were obtained from EQ Laboratories GmbH (Augsburg, Germany). Deuterated daidzein (d_4Daidzein) and genistein (d_4Genistein) were purchased from Cayman Chemical Company (Ann Harbor, MI, USA). Internal standards of BPZ (d_6BPZ), BPP (d_{16}BPP), BPAP (d_5BPAP) and BPAF (^{13}C12BPAF) were purchased from Toronto Research Chemicals Canada (Toronto, ON, Canada). Deuterated BPS (d_4BPS) was synthesized from tetrabrominated BPS via catalytic dehalogenation as reported previously [20]. Ethyl acetate (≥ 99.9 %) and n-hexane (≥ 99 %) were supplied by VWR International (Wayne, PA, USA). Methanol (≥ 99.9 %,) and water were from Honeywell Research Chemicals (Charlotte, North Carolina, USA). All solvents and reagents were of LCMS grade.

Study population and sample collection

The prostate samples were obtained from men attending the Department of Urology, Military University Hospital, Prague that underwent robot-assisted radical prostatectomy due to PCa. All procedures were performed with a da Vinci Xi Surgical System (Intuitive Surgical, Sunnyvale, CA, USA). The non-cancerous prostate tissue, as demonstrated by histopathological examination, was used in this study. Immediately after surgery the samples were frozen and stored in glass vials in -80 °C until analysis. Pooled tissue samples were used for validation experiments, and tissue samples from

20 patients were subsequently analyzed by the newly developed method.

The study adhered to the Declaration of Helsinki (2000) by the World Medical Association. The protocol received approval from the Ethics Committee of the Military University Hospital Prague under the reference number 108/15-26/2020. Informed written consent for the use of biological materials for research purposes was obtained from all subjects participating in the project.

Sample preparation

The method is based on a previously published method for determining EDs and estrogens in human plasma [21], with optimization for prostatic tissue preparations. A frozen prostate sample (approximately 20 mg) was wrapped in aluminum foil, placed in liquid nitrogen, and then pulverized with a hammer. The homogenized prostate tissue was transferred into a glass tube and weighed. Next, 1 ml of 0.9 % of saline was added, followed by liquid-liquid extraction by hexane-ethylacetate (v:v 3:2; 3 ml, 2 mins). The aqueous phase was frozen in dry ice, allowing the organic phase containing unconjugated steroids and EDs to be decanted into a clean glass tube. The organic phase was evaporated to dryness using a vacuum concentrator. Subsequently, the samples were reconstituted in 100 µL of 50 % methanol, transferred into an Eppendorf tube and underwent centrifugation (15000 g, 5 mins). The supernatant was moved to a glass vial with an insert and 30 µL was used for the LC-MS/MS analysis of unconjugated analytes.

To determine conjugated analytes, the aqueous phase with remaining conjugated steroids and EDs was precipitated by 2 ml of iced methanol. The samples were centrifuged (3000 rpm, 5 min) and the supernatant was transferred to a clean glass tube and evaporated to dryness. Chemical deconjugation by trimethylchlorsilane (TMCS) followed: 500 uL of 1 M TMCS was added to each sample and incubated 1 h in 55 °C. Then approximately 100 mg of sodium bicarbonate was added and again evaporated to dryness. The dry residues were finally reconstituted in 100ul of 50 % MeOH and processed the same way as unconjugated steroids.

Each batch consisted of two calibration sets, the samples and quality control (QC) samples; these included a doublet of the pooled matrix and a doublet of pooled matrix with the addition of 10pg of each analyte (except BPA, BPS and MP, where the addition was 40pg).

Liquid chromatography- tandem mass spectrometry (LC-MS/MS) conditions

Liquid chromatography was performed on an ExionLC AD liquid chromatography system (Sciex, Concord, Canada). A Kinetex Biphenyl column (100 mm × 3 mm, 2.6 µm; Phenomenex, Torrance, CA, USA) with corresponding Security Guard ULTRA cartridge system (UHPLC C18 for 3mm ID biphenyl column; Phenomenex, Torrance, CA, USA) was used for the separation. A water (A)- methanol (B) system was used with a post-column infusion of 6 mmol of ammonium fluoride in the water. A gradient elution started at a concentration of 50 % B during the initial 0-0.8 minutes, gradually ascending to 98 % B (0.8-6 min), followed by a further increase to 100 % B (6-9.4 min). Subsequently, there was a drop to 50 % B (9.4-9.6 min), after which the concentration was sustained at 50 % B from 9.6 to 12 min. The retention times for the analytes are indicated in Table 1.

A QTRAP 6500+ mass spectrometer (Sciex, Concord, Canada), equipped with an electrospray ionization (ESI) probe operating in negative ionization mode, was used for the detection of the analytes. The mass spectrometer conditions were as follows: ion spray voltage of -4500 V, temperature of 500 °C, curtain gas at 35.0 psi, ion source gas 1 at 40.0 psi, and ion source gas 2 at 50.0 psi. Multiple reaction monitoring (MRM) transitions are listed in Table 1.

Method validation

In order to be validated according to guidelines for bioanalytical method validation, the following parameters were examined: selectivity, specificity, sensitivity, accuracy, precision, recovery and stability.

The lower limit of quantification (LLOQ) was calculated as the 5x signal to noise ratio. Accuracy, precision and recovery were determined by a pentaplicate analysis of prostate tissue at 4 different concentrations: (I) pooled prostate tissue, (II) pooled prostate tissue with the addition of 10pg of all analytes except MP, BPA and BPS, which were spiked with 40 pg (into 20mg of tissue), (III) pooled prostate tissue with the addition of 50 pg of all analytes except MP, BPA, BPS with 200 pg (into 20 mg of tissue), (IV) pooled plasma with the addition of 120 pg of all analytes except MP, BPA, BPS with 480 pg (into 20mg of tissue). Within-run and between-run precision were expressed as the coefficient of variation and accuracy was calculated as follows:

$$Accuracy = \frac{c\,(A) - c\,(B)}{c\,(C)}\ x\ 100$$

where c (A), c (B) and c (C) represent the mean concentration of the analyte in the spiked sample, the mean concentration in the non-spiked sample and the spiked concentration, respectively.

Table 1. Retention times, LLOQs, precursor and fragment ions, and MS optimized conditions (declustering potential, DP; collision entrance potential, CEP; collision energy, CE; collision cell exit potential, CXP) for all analytes. Entrance potential (EP) was set to -10 V for all analytes. Values for the confirmation ion are given in the brackets.

Analyte	Retention time (min)	LLOQ (pg/mg tissue)	Precursor ion	Quantification ion	Confirmation ion	DP (V)	CE (V)	CXP (V)
E1	5.51	0.017	269.1	145.0	143.0	-100	-48 (-58)	-17 (-19)
d_4E1	5.49		273.0	147.0	145.1	-133	-52 (-72)	-13 (-17)
E2	4.57	0.027	271.0	183.2	145.2	-140	-52 (-54)	-17 (-13)
d_4E2	4.53		275.1	185.1	145.1	-105	-56 (-54)	-17 (-15)
E3	2.75	0.042	287.0	186.9	147.1	-5	-52 (-54)	-11 (-13)
d_2E3	2.74		289.1	173.0	147.1	-90	-48 (-52)	-19 (-21)
BPA	3.73	1.571	227.1	212.0	133.0	-30	-24 (-32)	-17 (-15)
d_{14}BPA	3.67		241.1	223.0	141.9	-45	-28 (-32)	-23 (-17)
BPS	1.73	2.000	248.9	108.0	91.9	-95	-34 (-40)	-5 (-11)
d_4BPS	1.72		252.9	110.0	94.0	-55	-36 (-42)	-51 (-11)
BPF	2.91	0.500	199.0	93.0	105.1	-45	-30 (-28)	-15 (-11)
d_{10}BPF	2.85		209.1	97.2	110.2	-105	-28 (-30)	-5 (-7)
BPAP	4.8	0.150	289.1	274.1	273.2	-45	-26 (-46)	-15 (-33)
d_5BPAP	4.78		294.1	279.2	199.8	-55	-30 (-36)	-31 (-23)
BPAF	3.77	2.857	335.0	265.0	197.0	-60	-30 (-50)	-17 (-23)
^{13}C12BPAF	3.76		347.0	277.1	188.2	-45	-32 (-58)	-29 (-33)
BPZ	5.03	0.150	267.2	173.0	223.1	-105	-36 (-44)	-21 (-19)
d_6BPZ	5.01		273.1	179.1	147.0	-90	-36 (-46)	-23 (-17)
BPP	5.9	0.450	345.2	330.2	133.0	-140	-34 (-42)	-29 (-13)
d_{16}BPP	5.86		361.2	343.0	325.2	-115	-36 (-52)	-55 (-45)
MP	2.14	1.212	151.0	92.0	136.0	-40	-26 (-20)	-13 (-15)
d_4MP	2.12		155.0	96.0	140.0	-35	-28 (-20)	-47 (-9)
EP	2.79	0.086	164.9	92.0	93.0	-40	-30 (-26)	-11 (-11)
d_4EP	2.77		169.0	96.0	141.1	-25	-30 (-20)	-11 (-9)
PP	3.48	0.162	179.0	92.0	136.0	-60	-28 (-22)	-13 (-15)
d_4PP	3.47		183.0	96.0	140.0	-45	-30 (-22)	-15 (-17)
BP	4.1	0.500	193.0	92.0	136.0	-60	-30 (-24)	-11 (-9)
d_4BP	4.11		197.0	96.0	141.0	-55	-30 (-22)	-47 (-19)
BenzylP	4.54	0.048	227.0	92.0	136.0	-45	-28 (-20)	-43 (-9)
NP	5.94	0.500	219.1	106.0	59.2	-80	-28 (-34)	-15 (-7)
d_4NP	5.93		223.2	110.0	141.0	-40	-30 (-12)	-15 (-23)
Oxybenzone	5.78	0.500	227.0	211.1	193.9	-60	-28 (-14)	-11 (-15)
d_3Oxybenzone	5.76		230.0	211.1	184.2	-40	-32 (-32)	-31 (-3)
Daidzein	2.9	0.385	253.0	207.8	132.0	-120	-40 (-52)	-23 (-15)
d_4Daidzein	2.89		257.0	211.9	227.1	-115	-46 (-38)	-23 (-19)
Genistein	3.46	0.500	268.9	133.0	132.0	-90	-38 (-48)	-17 (-17)
d_4Genistein	3.43		273.0	136.9	163.0	-85	-44 (-54)	-15 (-15)
Equol	3.39	0.250	241.1	121.0	135.1	-50	-20 (-22)	-13 (-21)

Autosampler stability was tested by analyzing the same set of 20 patients 24 hours after the first measurement, and the samples were compared with a freshly prepared calibration. The Wilcoxon test was used to evaluate the stability of the samples using MedCalc software version 22.009 (MedCalc software Ltd, Ostend, Belgium). Short-term temperature stability, long-term temperature stability, and freeze and thaw stability tests were not conducted, as the samples were and will be always processed immediately. The stock solution stability of EDs and steroids have been evaluated in previous studies [20,22]. In addition, the protocol was performed on dry ice to ensure constant cold conditions to prevent enzymatic activity in the tissue [23,24].

To evaluate the efficiency of the deconjugation process, we added a known quantity of commercially available sulfates (estrone sulfate – E1S, estradiol disulfate – E2-2S, estriol sulfate – E3S, bisphenol A sulfate – BPAS) and glucuronides (bisphenol A – BPAG, estrone glucuronide – E1G) to give a final amount of 100pg of unconjugated analyte. The experiment was performed in three sets of the 5 pooled prostate tissue samples and three sets of 5 pooled plasma matrix. The sulfates (E1S, E2-2S, E3S, BPAS) were added to the first set of 5 samples. E1G and BPAG were added to the second set of samples, and the third set of samples had no conjugates added.

Results

Method validation results

In the steroid and ED-free prostate tissue, there were no interferences at the retention times of the analytes, indicating good selectivity. The eight-point calibration curve was linear in the examined range (2-500 pg/tube for analytes except MP, BPA and BPS where 8-2000pg/tube was used), with coefficients of determination showing a fit of the calibration line at 0.98 and higher for all analytes. The method precision and accuracy parameters were in accordance with Food and Drug Administration [25] and European Medicines Agency [26] guidelines for bioanalytical method validation, as can be seen in Table 2. The LLOQ for each analyte is presented in Table 1.

Post-preparative (autosampler) stability tests were performed for analytes that were detected in at least 25 % of cases. No statistical difference was observed between freshly prepared samples and samples remaining 24 h in the autosampler. Accordingly, analytes that were not detected in freshly prepared samples were also not detected after 24h.

Table 2. Precision and accuracy for the individual analytes. The values that are below LLOQ, are marked as n.a (not applicable).

Analyte	Added (pg)	Precision (%)		Accuracy (%)
		intra-day	inter-day	
E1	0	n.a	n.a	
	10	10.1	5.9	95.4
	50	11.0	5.1	95.7
	120	9.2	8.4	98.0
E2	0	n.a	n.a	
	10	17.9	3.2	102.4
	50	6.7	3.8	110.4
	120	5.6	1.8	109.6
E3	0	n.a	n.a	
	10	17.0	17.9	88.3
	50	12.5	5.2	100.0
	120	8.3	2.7	96.7
BPA	0	n.a	n.a	
	40	17.9	5.2	107.9
	200	7.8	6.7	95.3
	480	6.5	5.1	92.1
BPS	0	n.a	n.a	
	40	13.5	13.1	95.4
	200	3.2	0.9	94.6
	480	3.2	1.0	96.5
BPF	0	n.a	n.a	
	10	10.2	4.1	91.3
	50	6.8	2.8	93.1
	120	5.3	1.8	92.1
BPAP	0	n.a	n.a	
	10	9.2	3.3	99.4
	50	3.5	2.5	100.6
	120	4.6	1.7	102.5
BPAF	0	n.a	n.a	
	10	15.8	6.9	95.0
	50	4.2	2.2	100.7
	120	3.8	1.7	101.4
BPZ	0	n.a	n.a	
	10	9.3	1.4	98.6
	50	6.0	0.7	102.7
	120	5.4	2.1	100.9
BPP	0	n.a	n.a	
	10	14.8	5.5	94.1
	50	7.0	2.2	97.0
	120	7.9	3.9	98.4
MP	0	n.a	n.a	
	40	15.5	14.8	104.8
	200	8.1	4.9	102.2
	480	5.4	1.6	99.9

Table 2. (continued)

EP	0	n.a	n.a	
	10	17.5	19.5	101.1
	50	6.9	3.9	98.3
	120	4.4	2.8	97.1
PP	0	n.a	n.a	
	10	10.0	0.9	104.8
	50	7.3	8.0	100.6
	120	4.5	3.9	102.2
BP	0	n.a	n.a	
	10	9.5	7.8	103.6
	50	2.6	0.8	105.7
	120	3.8	0.5	106.1
BenzylP	0	n.a	n.a	
	10	6.7	6.2	95.9
	50	10.5	1.7	102.6
	120	7.8	5.0	107.7
NP	0	n.a	n.a	
	10	11.4	9.2	93.8
	50	9.4	2.6	89.8
	120	6.3	5.9	93.3
Oxybenzone	0	n.a	n.a	
	10	22.4	7.0	107.9
	50	5.3	2.4	111.5
	120	4.7	5.9	104.5
Daidzein	0	n.a	n.a	
	10	12.5	6.1	95.5
	50	4.6	1.0	98.2
	120	4.7	2.4	99.0
Genistein	0	n.a	n.a	
	10	17.8	9.7	93.7
	50	10.1	3.7	98.9
	120	7.0	6.6	101.3
S-equol	0	n.a	n.a	
	10	7.6	4.4	97.6
	50	5.6	4.1	99.6
	120	6.8	4.2	101.0

The efficiency of the deconjugation process by TMCS was tested where standards of sulfates or glucuronides were commercially available. Overall, TMCS showed good deconjugation efficiency for sulfates and poor deconjugation efficiency for glucuronides as can be seen in Table 3. The results of deconjugation were similar in both examined matrices (pooled prostate tissue vs pooled plasma).

Table 3. Efficiency of deconjugation using TMCS, examined by adding a calculated amount of sulfates and glucuronides, respectively, to give a final amount of 100pg of unconjugated analyte. The values are expressed as deconjugation efficiency ± relative standard deviation (RSD)

Analyte	**Matrix**	
	prostate tissue	**pooled plasma**
E1S	87±3 %	89±4 %
E1G	20±18 %	1±8 %
E2-2S	107±5 %	97±7 %
E3S	75±8 %	77±16 %
BPAS	87±10 %	85±12 %
BPAG	-0.5±25 %	1.5± 12 %

Determination of EDs, phytoestrogens and estrogens from the non-cancerous prostate tissue of 20 patients

The newly validated method was used for the determination of EDs, phytoestrogens and estrogens in non-cancerous prostate tissues of 20 patients that underwent radical prostatectomy due to localized prostate carcinoma. Chromatogram of each analyte is given in Figure 1. The most frequently detected EDs were PP, MP, BPA, oxybenzone and EP (conjugated as well as unconjugated forms), as can be seen in Table 4. Of the estrogens, E1 was the most abundant steroid in unconjugated form, and conjugated E1 and E3 were detected in 100 % of cases. The concentrations of conjugated analytes were usually higher, with the exception of oxybenzone, EP and BPA. Also, daidzein and genistein were detected in their conjugated form in 2 cases (the same patients). Medians with 1st and 3rd quartiles are given in Table 4 for cases where the analyte was detected in more than 50 % of cases, as this gives more information about the concentration distribution. Where detected cases were lower than 50 %, the mean with standard deviation is shown.

Discussion

The method presented here was focused on the determination of endogenous as well as exogenous substances in the human prostate. Knowledge of the presence of these compounds can subsequently shed more light on prostate physiology and pathophysiology.

Numerous studies have proposed a potential role for estrogens in the initiation and advancement of PCa. (e.g. [27-31]) and the levels of unconjugated intraprostatic estrogens (mainly measured by radioimmunoassay) have been the subject of analysis of

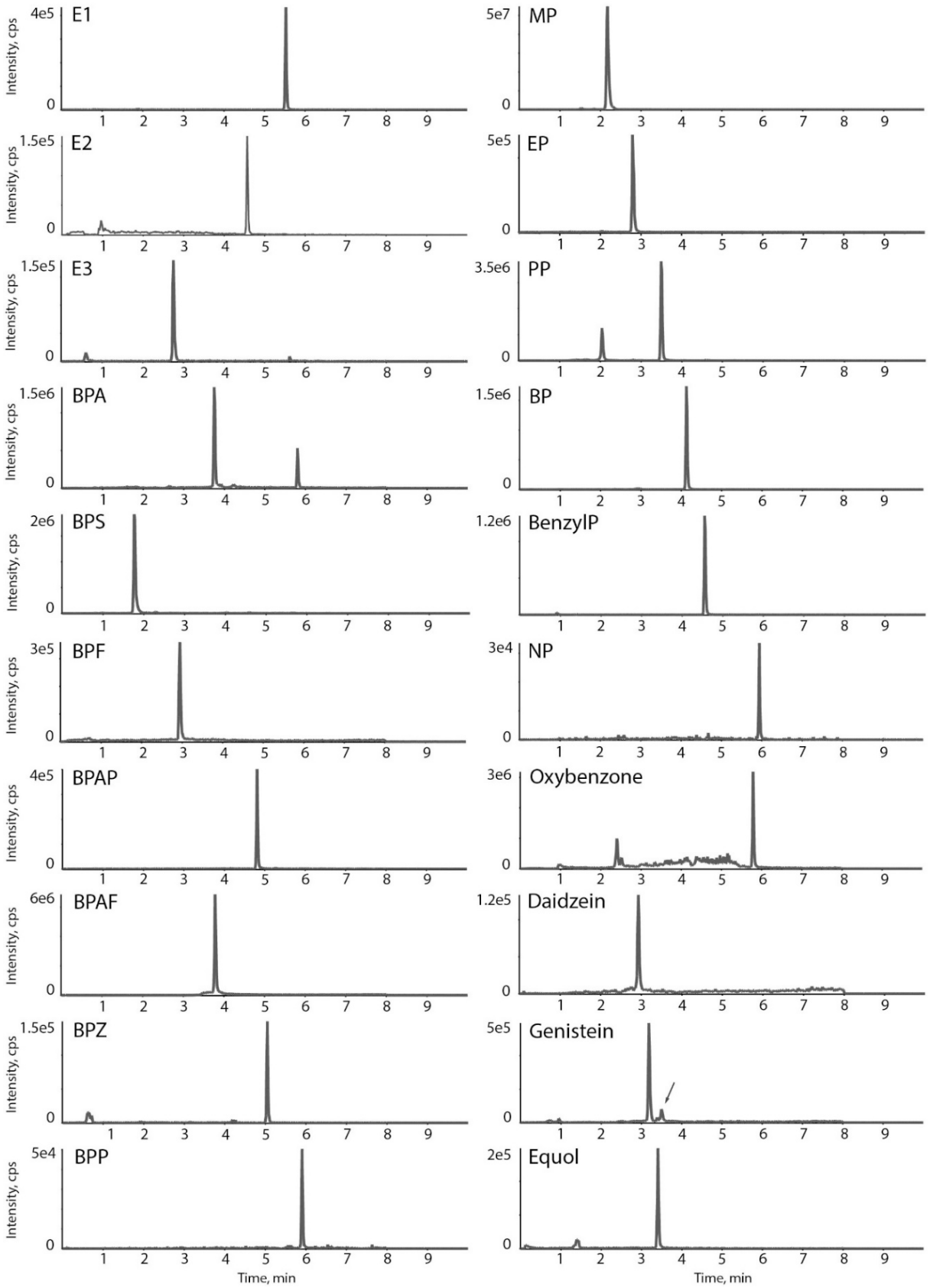

Fig. 1. Chromatograms of the 20 analytes included in the method in prostate tissue. In cases where the analyte was not detected, the chromatogram from the calibration curve is shown (16 pg/tube for BP, BenzylP, E2, BPAP, BPAF, BPP, BPZ, Equol, and 64 pg/tube for BPF and BPAF). Respective concentrations of analytes in prostatic tissues were as follows: c(E1)=2.43 pg/mg, c(E3)=3.19 pg/mg, c(MP)=49 pg/mg, c(EP)=0.568 pg/mg, c(PP) =3.66 pg/mg, c(BPA)=5.25 pg/mg, c(BPS)=2.66 pg/mg, c(oxybenzone)=12.30 pg/mg, c(daidzein)=1.18 pg/mg, c(genistein)=1.38 pg/mg.

Table 4. Counts and detection frequency, medians and interquartile ranges (IQR) for each analyte in unconjugated and conjugated form in a set of prostate tissue samples (n = 20). Median with IQR was replaced by mean ± standard deviation in cases where detection frequency was lower than 50 %. These values are marked with an asterisk.

Analyte	unconjugated			conjugated		
	cases detected	detection frequency (%)	Median (IQR) (pg/mg)	cases detected	detection frequency (%)	Median (IQR) (pg/mg)
E1	16	80	0.024 (0.017;0.025)	20	100	1.788 (1.082;2.474)
E2	1	5	0.027	1	5	0.351
E3	1	5	0.027	20	100	3.039 (2.424;3.828)
BPA	7	35	1.99±0.67*	12	60	1.675 (LLOQ;3.229)
BPS	0	0	0	2	10	2.06±0.18
BPF	0	0	0	0	0	0
BPAP	0	0	0	0	0	0
BPAF	0	0	0	0	0	0
BPZ	0	0	0	0	0	0
BPP	0	0	0	0	0	0
MP	9	45	2.46±3.36*	20	100	29.70 (23.00;42.21)
EP	5	25	0.37±1.15*	20	100	0.303 (0.199;0.525)
PP	20	100	0.439 (0.296;0.923)	20	100	1.478 (1.079;2.663)
BP	0	0	0	0	0	0
BenzylP	0	0	0	0	0	0
NP	0	0	0	0	0	0
oxybenzone	9	45	1.61±1.70*	9	45	1.86±2.87*
daidzein	0	0	0	2	10	0.43±0.18*
genistein	0	0	0	2	10	0.57±0.23*
S-equol	0	0	0	0	0	0

many studies over the years (reviewed in [32]). Concentrations of unconjugated estrogens in benign prostate tissue measured in our study were within the same concentration ranges as reported by other authors [33-35]. As in our study, levels of unconjugated E1 have been found to be higher than unconjugated E2 [33,36]. Levels of conjugated estrogens in prostate tissue were approximately 2 orders of magnitude higher than their unconjugated counterparts. Completing the picture, conjugated E3 was found to be the most abundant estrogen in prostate tissue in our study.

Findings from blood plasma/serum of healthy volunteers have found conjugated E1 levels one order of magnitude higher than unconjugated E1 [37,38]. In adult men, E1 sulfate is the most abundant circulating estrogen [31], and serum E1 sulfate has been suggested as a prognostic marker for PCa tumor aggressiveness, since mean E1 sulfate levels are significantly increased in patients with PSA >10ng/mL compared with patients with PSA < 10ng/mL [39]. Intraprostatic steroid concentrations have been reported to poorly correlate with their serum counterparts [33], so it would be interesting to investigate the relationship between intraprostatic E1 sulfate levels and tumor aggressiveness and prognosis in PCa patients.

To the best of our knowledge, no studies have analyzed EDs including phytoestrogens in the human prostate. Our study shows that EDs can spread to the human prostate from the blood, which is in accordance with the occurrence of EDs in other human organs such as adipose tissue [40-42], brain tissue [41,43] and liver [41]. Findings of associations between EDs and PCa from epidemiology studies are limited, though PCa patients were reported to have higher levels of urinary BPA than non-cancer patients, suggesting that BPA may serve as prognostic marker in PCa [44]. Given the ubiquity of EDs in the environment and human products, understanding their consequences on human health is crucial [45], and the ability to analyze intraprostatic EDs can be highly beneficial.

Oxybenzone is an endocrine disruptor with both antiandrogenic and (anti)estrogenic properties [46] that can be found not only in sunscreens to protect against UV radiation, but also in personal care products such as shampoos, creams and fragrances and in plastics, where it functions as a UV light absorber and stabilizer [47]. It is also ubiquitous in water, as waste water treatment plants are not able to effectively remove this substance [48]. Therefore, it is not surprising that detectable concentrations in urine were found in 96.8 % of US residents [49]. Oxybenzone levels in our study (ranging from 0 to 12.3 ng/g tissue) were similar to those reported by other authors in other human tissues such as breast [50] and adipose tissue [51].

Generally, two approaches for steroid and ED deconjugation are available. First, biological (enzymatic) deconjugation using enzymes from mammals, bacteria (from *Escherichia coli*) or molluscs (mainly from *Helix pomatia*). Second, chemical deconjugation using various reagents including trimethylchlorsilane (TMCS), hydrochloric acid (HCl) or sulfuric acid (H2SO4) can be used [52]. Both methods have some advantages and disadvantages, and it is important to know which conjugates can be measured by which methods. Regarding enzymatic deconjugation, enzymes have either only β-glucuronidase activity (enzyme from *E. coli*) or both β-glucuronidase and aryl sulfatase activity (*H. pomatia*). However, *H. pomatia* does not contain all the sulfatases needed for the deconjugation of sulfates; 3α-hydroxy-5β- and 3β-hydroxy-5-ene steroid sulfates are efficiently hydrolyzed, but 3α-hydroxy-5α-sulfates, C_{21} steroids sulfated at position 20 and C_{19} steroids sulfated at position 17 are not hydrolyzed [53]. Furthermore, *H. pomatia* also contains 3β-hydroxy-steroid dehydrogenase activity leading to conversions of some steroids, e.g., the transformation of androst-5-ene-3β,17β-diol to testosterone and the conversion of dehydro-epiandrosterone to androst-4-ene-3,17-dione, leading to inaccuracies in steroid measurements [54]. Because of this, chemical deconjugation by H_2SO_4 has been considered the method of choice for measuring sulfated androgens [55] and chemical deconjugation by HCl for estrogenic sulfates and glucuronides [56]. We assessed the efficiency of chemical deconjugation using TMCS with commercially available standards of conjugates. The results indicated that TMCS effectively deconjugates sulfates, while it poorly hydrolyzes glucuronides. This was clear when comparing the deconjugation efficiency between BPA glucuronide and BPA sulfate and E1G and E1S, respectively. However, these results have to be interpreted with caution as only limited number of sulfates and glucuronides was examined.

One of the strengths of this study lies in the measurement of deconjugation efficiency for individual analytes. This is of importance, considering that the deconjugation process varies across different analytes. However, many conjugated analytes do not have commercially available standards and therefore cannot be assessed in this way. Furthermore, estrogens and EDs were determined in a relatively small set of samples derived from patients suffering from Pca, so our results cannot be extrapolated to the general population. Nonetheless, this study lays the groundwork for future measurements of these analytes in larger cohorts.

Conclusion

Here we report a validated method for the measurement of unconjugated and conjugated estrogens and different classes of EDs in human prostate tissue. Using this method, we analyzed 20 non-cancerous prostate tissue samples from patients that underwent radical prostatectomy due to localized Pca. To the best of our knowledge, conjugated estriol as well as certain EDs such as parabens, bisphenols, phytoestrogens and oxybenzone were detected in human prostate tissue for the first time. These results give impetus to more detailed research into EDs and their role in prostate pathophysiology.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

This work was supported by Czech Health Research Council, project NU21J-01-00040 from the Czech Ministry of Health.

References

1. Liu S, He B, Li H. Bisphenol S promotes the progression of prostate cancer by regulating the expression of COL1A1 and COL1A2. Toxicology 2022;472:153178. https://doi.org/10.1016/j.tox.2022.153178

2. ÚZIS. Current epidemiological trends of neoplasms in the Czech Republic (in Czech). 2018 [accessed 4.9.2023]; https://www.uzis.cz/res/f/008352/novotvary2018.pdf
3. Culp MB, Soerjomataram I, Efstathiou JA, Bray F, Jemal A. Recent Global Patterns in Prostate Cancer Incidence and Mortality Rates. Eur Urol 2020;77:38-52. https://doi.org/10.1016/j.eururo.2019.08.005
4. Bergengren O, Pekala KR, Matsoukas K, Fainberg J, Mungovan SF, Bratt O, Bray F, Brawley O, Luckenbaugh AN, Mucci L, Morgan TM, Carlsson SV. 2022 Update on Prostate Cancer Epidemiology and Risk Factors-A Systematic Review. Eur Urol 2023;84:191-206. https://doi.org/10.1016/j.eururo.2023.04.021
5. Diamanti-Kandarakis E, Bourguignon JP, Giudice LC, Hauser R, Prins GS, Soto AM, Zoeller RT, Gore AC. Endocrine-disrupting chemicals: an Endocrine Society scientific statement. Endocr Rev 2009;30:293-342. https://doi.org/10.1210/er.2009-0002
6. Gore AC, Chappell VA, Fenton SE, Flaws JA, Nadal A, Prins GS, Toppari J, Zoeller RT. EDC-2: The Endocrine Society's Second Scientific Statement on Endocrine-Disrupting Chemicals. Endocrine Reviews 2015;36:E1-E150. https://doi.org/10.1210/er.2015-1010
7. Jambor T, Knížatová N, Lukáč N. Men's reproductive alterations caused by bisphenol A and its analogues: a review. Physiol Res 2021;70:S643-s656. https://doi.org/10.33549/physiolres.934742
8. Heudorf U, Mersch-Sundermann V, Angerer J. Phthalates: toxicology and exposure. Int J Hyg Environ Health 2007;210:623-634. https://doi.org/10.1016/j.ijheh.2007.07.011
9. Kolátorová L, Lapčík O, Stárka L. Phytoestrogens and the intestinal microbiome. Physiol Res 2018;67:S401-s408. https://doi.org/10.33549/physiolres.934022
10. Pilšáková L, Riečanský I, Jagla F. The physiological actions of isoflavone phytoestrogens. Physiol Res 2010;59:651-664. https://doi.org/10.33549/physiolres.931902
11. Domanska A, Orzechowski A, Litwiniuk A, Kalisz M, Bik W, Baranowska-Bik A. The Beneficial Role of Natural Endocrine Disruptors: Phytoestrogens in Alzheimer's Disease. Oxid Med Cell Longev 2021;2021. https://doi.org/10.1155/2021/3961445
12. Sridevi V, Naveen P, Karnam VS, Reddy PR, Arifullah M. Beneficiary and Adverse Effects of Phytoestrogens: A Potential Constituent of Plant-based Diet. Current Pharmaceutical Design 2021;27:802-815. https://doi.org/10.2174/1381612826999200917154747, https://doi.org/10.2174/18734286MTEw0MDMz4
13. Patisaul HB, Jefferson W. The pros and cons of phytoestrogens. Frontiers in Neuroendocrinology 2010;31:400-419. https://doi.org/10.1016/j.yfrne.2010.03.003
14. Goetzl M, Van Veldhuizen PJ, Thrasher JB. Effects of soy phytoestrogens on the prostate. Prostate Cancer Prostatic Dis 2007;10:216-223. https://doi.org/10.1038/sj.pcan.4500953
15. Ganry O. Phytoestrogens and prostate cancer risk. Prevent Med 2005;41:1-6. https://doi.org/10.1016/j.ypmed.2004.10.022
16. Morrissey O, Watson RWG. Phytoestrogens and prostate cancer. Current Drug Targets 2003;4:231-241. https://doi.org/10.2174/1389450033491154
17. Zhang Q, Feng H, Qluwakemi B, Wang J, Yao S, Cheng G, Xu H, Qiu H, Zhu L, Yuan M. Phytoestrogens and risk of prostate cancer: an updated meta-analysis of epidemiologic studies. Int J Food Sci Nutr 2017;68:28-42. https://doi.org/10.1080/09637486.2016.1216525
18. Jia Q, Hong MF, Pan ZX, Orndorff S. Quantification of urine 17-ketosteroid sulfates and glucuronides by high-performance liquid chromatography-ion trap mass spectroscopy. J Chromatogr B Biomed Sci Appl 2001;750:81-91. https://doi.org/10.1016/S0378-4347(00)00435-7
19. Labrie F, Bélanger A, Bélanger P, Bérubé R, Martel C, Cusan L, Gomez J, Candas B, Castiel I, Chaussade V, Deloche C, Leclaire J. Androgen glucuronides, instead of testosterone, as the new markers of androgenic activity in women. J Steroid Biochem Mol Biol 2006;99:182-188. https://doi.org/10.1016/j.jsbmb.2006.02.004
20. Kolatorova Sosvorova L, Chlupacova T, Vitku J, Vlk M, Heracek J, Starka L, Saman D, Simkova M, Hampl R. Determination of selected bisphenols, parabens and estrogens in human plasma using LC-MS/MS. Talanta 2017;174:21-28. https://doi.org/10.1016/j.talanta.2017.05.070
21. Vitku J, Horackova L, Kolatorova L, Duskova M, Skodova T, Simkova M. Derivatized versus non-derivatized LC-MS/MS techniques for the analysis of estrogens and estrogen-like endocrine disruptors in human plasma. Ecotoxicol Environ Saf 2023;260:115083. https://doi.org/10.1016/j.ecoenv.2023.115083

22. Vitku J, Chlupacova T, Sosvorova L, Hampl R, Hill M, Heracek J, Bicikova M, Starka L. Development and validation of LC-MS/MS method for quantification of bisphenol A and estrogens in human plasma and seminal fluid. TALANTA 2015;140:62-67. https://doi.org/10.1016/j.talanta.2015.03.013
23. McMahon MJ, Thomas GH. The metabolism of testosterone by human prostate in organ culture. J Endocrinol 1970;48:xx-xxi.
24. Kjoseland O, Tveter KJ, Attramadal A, Hansson V, Haugen HN, Mathisen W. Metabolism of testosterone in the human prostate and seminal vesicles. Scand J Urol Nephrol 1977;11:1-6. https://doi.org/10.3109/00365597709179684
25. Food and Drug Administration. Guidance for Industry, Bioanalytical method Validation. 2018 [accessed 4.9.2023]; http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm070107.pdf
26. European Medicines Agency. Guideline on bioanalytical method validation. 2011 [accessed 4.9.2023]; https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf
27. Kowalska K, Piastowska-Ciesielska AW. Oestrogens and oestrogen receptors in prostate cancer. Springerplus 2016;5. https://doi.org/10.1186/s40064-016-2185-6
28. Prins GS, Korach KS. The role of estrogens and estrogen receptors in normal prostate growth and disease. Steroids 2008;73:233-244. https://doi.org/10.1016/j.steroids.2007.10.013
29. Prezioso D, Denis LJ, Klocker H, Sciarra A, Reis M, Naber K, Lobel B, Pacik D, Griffiths K. Estrogens and aspects of prostate disease. International Journal of Urology 2007;14:1-16. https://doi.org/10.1111/j.1442-2042.2006.01476.x
30. Harkonen PL, Makela SI. Role of estrogens in development of prostate cancer. J Steroid Biochem Mol Biol 2004;92:297-305. https://doi.org/10.1016/j.jsbmb.2004.10.016
31. Giton F, Sirab N, Franck G, Gervais M, Schmidlin F, Ali T, Allory Y, de la Taille A, Vacherot F, Loric S, Fiet J. Evidence of estrone-sulfate uptake modification in young and middle-aged rat prostate. J Steroid Biochem Mol Biol 2015;152:89-100. https://doi.org/10.1016/j.jsbmb.2015.05.002
32. Šimková M, Heráček J, Drašar P, Hampl R. Determination of Intraprostatic and Intratesticular Androgens. Int J Mol Sci 2021;22. https://doi.org/10.3390/ijms22010466
33. Cook MB, Stanczyk FZ, Wood SN, Pfeiffer RM, Hafi M, Veneroso CC, Lynch B, Falk RT, Zhou CK, Niwa S, Emanuel E, Gao YT, Hemstreet GP, Zolfghari L, Carroll PR, Manyak MJ, Sesterhann IA, Levine PH, Hsing AW. Relationships between Circulating and Intraprostatic Sex Steroid Hormone Concentrations. Cancer Epidemiol Biomarkers Prev 2017;26:1660-1666. https://doi.org/10.1158/1055-9965.EPI-17-0215
34. Zhou CK, Stanczyk FZ, Hafi M, Veneroso CC, Lynch B, Falk RT, Niwa S, Emanuel E, Gao YT, Hemstreet GP, Zolfghari L, Carroll PR, Manyak MJ, Sesterhenn IA, Levine PH, Hsing AW, Cook MB. Circulating and intraprostatic sex steroid hormonal profiles in relation to male pattern baldness and chest hair density among men diagnosed with localized prostate cancers. Prostate 2017;77:1573-1582. https://doi.org/10.1002/pros.23433
35. Arai S, Miyashiro Y, Shibata Y, Kashiwagi B, Tomaru Y, Kobayashi M, Watanabe Y, Honma S, Suzuki K. New quantification method for estradiol in the prostatic tissues of benign prostatic hyperplasia using liquid chromatography-tandem mass spectrometry. Steroids 2010;75:13-19. https://doi.org/10.1016/j.steroids.2009.09.004
36. Neuzillet Y, Raynaud JP, Radulescu C, Fiet J, Giton F, Dreyfus JF, Ghoneim TP, Lebret T, Botto H. Sexual steroids in serum and prostatic tissue of human non-cancerous prostate (STERPROSER trial). Prostate 2017;77:1512-1519. https://doi.org/10.1002/pros.23429
37. Xu X, Roman JM, Issaq HJ, Keefer LK, Veenstra TD, Ziegler RG. Quantitative measurement of endogenous estrogens and estrogen metabolites in human serum by liquid chromatography-tandem mass spectrometry. Anal Chem 2007;79:7813-7821. https://doi.org/10.1021/ac070494j
38. Kafeenah H, Kuo CM, Chang TY, Jen HH, Yang JH, Shen YS, Wu CH, Chen SH. Label-free and de-conjugation-free workflow to simultaneously quantify trace amount of free/conjugated and protein-bound estrogen metabolites in human serum. Anal Chim Acta 2022;1232:340457. https://doi.org/10.1016/j.aca.2022.340457
39. Giton F, de la Taille A, Allory Y, Galons H, Vacherot F, Soyeux P, Abbou CC, Loric S, Cussenot O, Raynaud JP, Fiet J. Estrone sulfate (E1S), a prognosis marker for tumor aggressiveness in prostate cancer (PCa). J Steroid Biochem Mol Biol 2008;109:158-167. https://doi.org/10.1016/j.jsbmb.2007.10.005

40. Venisse N, Cambien G, Robin J, Rouillon S, Nadeau C, Charles T, Rabouan S, Migeot V, Dupuis A. Development and validation of an LC-MS/MS method for the simultaneous determination of bisphenol A and its chlorinated derivatives in adipose tissue. Talanta 2019;204:145-152. https://doi.org/10.1016/j.talanta.2019.05.103
41. Geens T, Neels H, Covaci A. Distribution of bisphenol-A, triclosan and n-nonylphenol in human adipose tissue, liver and brain. Chemosphere 2012;87:796-802. https://doi.org/10.1016/j.chemosphere.2012.01.002
42. Sousa S, Maia ML, Delerue-Matos C, Calhau C, Domingues VF. The role of adipose tissue analysis on Environmental Pollutants Biomonitoring in women: The European scenario. Sci Total Environ 2022;806:150922. https://doi.org/10.1016/j.scitotenv.2021.150922
43. van der Meer TP, Artacho-Cordón F, Swaab DF, Struik D, Makris KC, Wolffenbuttel BHR, Frederiksen H, van Vliet-Ostaptchouk JV. Distribution of non-persistent endocrine disruptors in two different regions of the human brain. Int J Environ Res Public Health 2017;14. https://doi.org/10.3390/ijerph14091059
44. Tarapore P, Ying J, Ouyang B, Burke B, Bracken B, Ho SM. Exposure to bisphenol A correlates with early-onset prostate cancer and promotes centrosome amplification and anchorage-independent growth in vitro. PLoS One 2014;9:e90332. https://doi.org/10.1371/journal.pone.0090332
45. Lacouture A, Lafront C, Peillex C, Pelletier M, Audet-Walsh É. Impacts of endocrine-disrupting chemicals on prostate function and cancer. Environ Res 2022;204:112085. https://doi.org/10.1016/j.envres.2021.112085
46. Kim S, Choi K. Occurrences, toxicities, and ecological risks of benzophenone-3, a common component of organic sunscreen products: a mini-review. Environ Int 2014;70:143-157. https://doi.org/10.1016/j.envint.2014.05.015, https://doi.org/10.5322/JESI.2014.23.1.143
47. DiNardo JC, Downs CA. Dermatological and environmental toxicological impact of the sunscreen ingredient oxybenzone/benzophenone-3. J Cosmet Dermatol 2018;17:15-19. https://doi.org/10.1111/jocd.12449
48. Schneider SL, Lim HW. Review of environmental effects of oxybenzone and other sunscreen active ingredients. J Am Acad Dermatol 2019;80:266-271. https://doi.org/10.1016/j.jaad.2018.06.033
49. Calafat AM, Wong LY, Ye X, Reidy JA, Needham LL. Concentrations of the sunscreen agent benzophenone-3 in residents of the United States: National Health and Nutrition Examination Survey 2003--2004. Environ Health Perspect 2008;116:893-897. https://doi.org/10.1289/ehp.11269
50. Barr L, Alamer M, Darbre PD. Measurement of concentrations of four chemical ultraviolet filters in human breast tissue at serial locations across the breast. J Appl Toxicol 2018;38:1112-1120. https://doi.org/10.1002/jat.3621
51. Artacho-Cordón F, Ríos-Arrabal S, León J, Frederiksen H, Sáenz JM, Martín-Olmedo P, Fernández MF, Olea N, Arrebola JP. Adipose tissue concentrations of non-persistent environmental phenols and local redox balance in adults from Southern Spain. Environ Int 2019;133:105118. https://doi.org/10.1016/j.envint.2019.105118
52. Gomes RL, Meredith W, Snape CE, Sephton MA. Analysis of conjugated steroid androgens: deconjugation, derivatisation and associated issues. J Pharm Biomed Anal 2009;49:1133-1140. https://doi.org/10.1016/j.jpba.2009.01.027
53. Shackleton CH. Profiling steroid hormones and urinary steroids. J Chromatogr 1986;379:91-156. https://doi.org/10.1016/S0378-4347(00)80683-0
54. Houghton E, Grainger L, Dumasia MC, Teale P. Application of gas chromatography/mass spectrometry to steroid analysis in equine sports: Problems with enzyme hydrolysis. Organic Mass Spectrometry 1992;27:1061-1070. https://doi.org/10.1002/oms.1210271016
55. Iannone M, Botrè F, Martinez-Brito D, Matteucci R, de la Torre X. Development and application of analytical procedures for the GC-MS/MS analysis of the sulfates metabolites of anabolic androgenic steroids: The pivotal role of chemical hydrolysis. J Chromatogr B Analyt Technol Biomed Life Sci 2020;1155:122280. https://doi.org/10.1016/j.jchromb.2020.122280
56. Liu ZH, Kanjo Y, Mizutani S. Deconjugation characteristics of natural estrogen conjugates by acid-catalyzed solvolysis and its application for wastewater samples. J Environ Monit 2010;12:1594-1600. https://doi.org/10.1039/c002952a