Chemical Research in Toxicology

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Article

Development of a Liquid Chromatography−Mass Spectrometry-Based In Vitro Assay to Assess Changes in Steroid Hormones Due to Exposure to Per- and Polyfluoroalkyl Substances

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Cite This: *Chem. Res. Toxicol.* 2022, 35, 1277−1288

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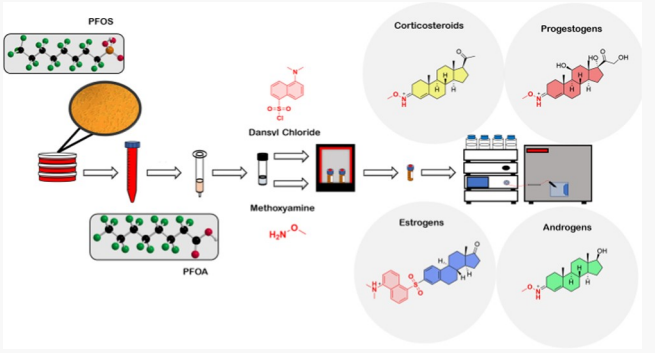
ABSTRACT: Per- and poly-fluorinated substances (PFASs) are organic pollutants that have been linked to numerous health effects, including diabetes, cancers, and dysregulation of the endocrine system. This study aims to develop a liquid chromatography with tandem mass spectrometry (LC-MS/MS) assay to measure changes in 17 hormones in H295R cell line (a steroid producing adrenocortical cells) upon exposure to PFASs. Due to the challenges in the analysis of steroid hormones using electrospray ionization MS, a chemical derivatization method was employed to achieve 0.07−2 μg/L detection limits in LC-MS/MS. Furthermore, a 10-fold concentration factor through solid-phase extraction (SPE) allows for consistent sub-parts per billion detections. Optimization of the derivatization conditions showed doubly-derivatized products in some hormone analytes, including progesterone, corticosterone, and cortisol, and gave improved ionization efficiency up to 20-fold higher signal than the singly-derivatized product. The use of SPE for sample cleanup to analyze hormones from cellular media using weak anion exchange sorbent yielded 80−100% recovery for the 17 targeted hormones. The method was validated by exposing H295R cells to two known endocrine disruptors, forskolin and prochloraz, which showed expected changes in hormones. An initial exposure of H295R cells with various PFAS standards and their mixtures at 1 μM showed significant increases in progestogens with some PFAS treatments, which include PFBS, PFHxA, PFOS, PFDA, and PFDS. In addition, modest changes in hormone levels were observed in cells treated with other sulfonated or carboxylated headgroup PFASs. This sensitive LC-MS/MS method for hormone analysis in H295R cells will allow for the investigations of the alterations in the hormone production caused by exposure to various environmental insults in cell-based assays and other *in vitro* models.



INTRODUCTION

Per- and poly-fluorinated alkyl substances (PFASs) are a class of halogenated chemicals used in a large pool of industrial applications and consumer products. Nearly 9000 PFASs exist varying in carbon chain-lengths, headgroups, degrees of fluorination, branching of the carbon chain backbone, and additional side chain functional groups, resulting in diverse chemical and physical properties.[1] Figure 1 provides examples of PFASs from different classes. PFASs are highly stable due to the combined smaller size and greater electronegativity of fluorine as compared to other halogens, resulting in stronger covalent bonds.[2] Applications of these compounds include but are not limited to uses in firefighting foams, lubricants, paint additives, textile surfactants, and metal plating.[3,4] Because of their widespread use, combined with the extreme stability of the carbon−fluorine bond, complex mixtures of PFASs have become ubiquitous environmental contaminants.

Increasing evidence of deleterious human health effects associated with PFAS exposure has been reported.[5] Consequently, the United States Environmental Protection Agency (USEPA) has rolled out lifetime health advisories for PFAS, including a suggested exposure level below 0.002 μg/kg/day for perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA), and a 0.07 μg/L concentration in drinking water to reduce the risk for noncancerous and cancerous health effects.[6] These compounds have low clearance rates in humans, with half-lives of up to 4 years for PFOS or PFOA that are present in the body, assuming no further intake.[7]

Studies have previously assessed the endocrine-disrupting effects of PFASs utilizing an adrenocarcinoma cell line, H295R.[8−12] One study based on PFOS and PFOA exposure of H295R cells to PFOS and PFOA at 10 and 100 μM for 24 h found that testosterone levels significantly decreased with 100

Received: April 13, 2022
Published: June 13, 2022

https://doi.org/10.1021/acs.chemrestox.2c00116
Chem. Res. Toxicol. 2022, 35, 1277−1288

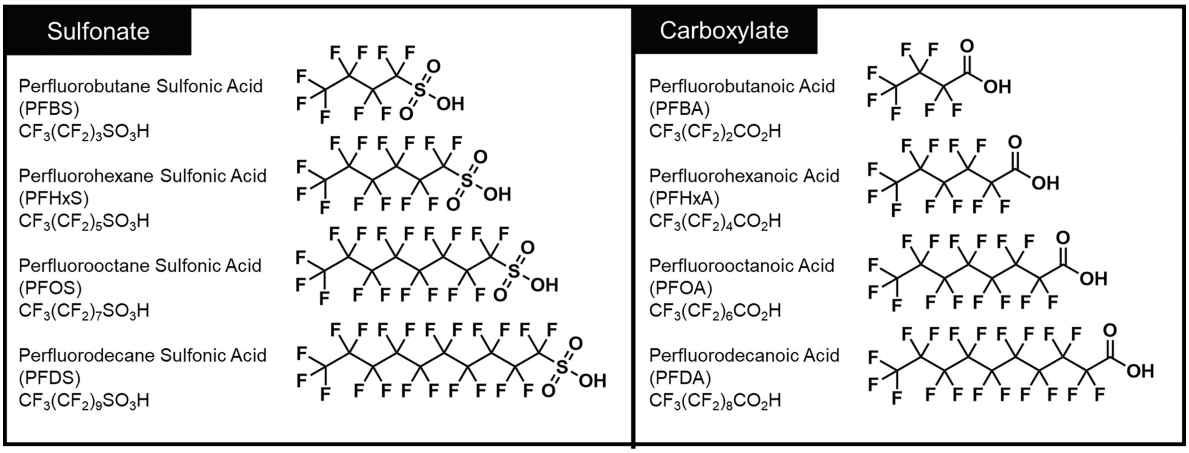


Figure 1. The per-and polyfluoroalkyl compounds (PFASs) evaluated in this study include perfluorobutane sulfonic acid (PFBS), perfluorobutanoic acid (PFBA), perfluorohexane sulfonic acid (PFHxS), perfluorohexanoic acid (PFHxA), perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorodecane sulfonic acid (PFDS), and perfluorodecanoic acid (PFDA).

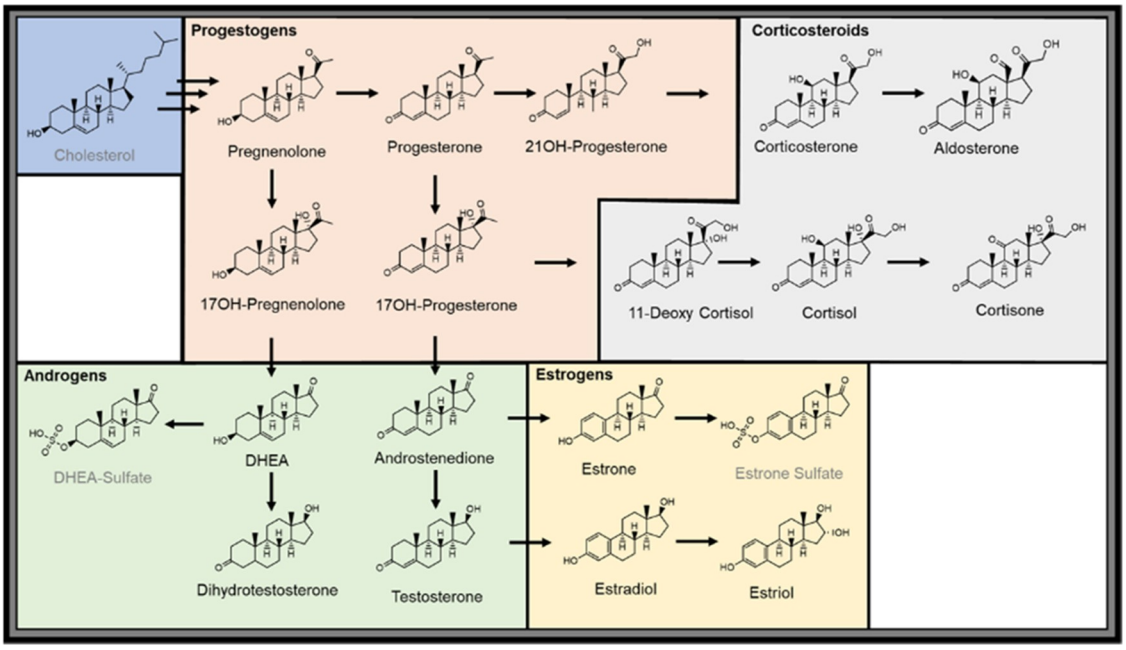


Figure 2. Scheme for the biosynthesis of 19 steroid hormones starting from cholesterol. Shown in black text are the hormones studied in the current work. Metabolites in gray text are not analyzed. This figure was created with permission using the information available in the online database KEGG (Kyoto Encyclopedia of Genes and Genomes).[12]

μM PFOS and PFOA, and levels of estradiol significantly increased with both PFOS and PFOA treatments.[9,10] A second study showed significant increases in estrone, estradiol, and progesterone with only 100 μM treatments after 24 h.[10] These studies utilized immunoassay kits specific for estrogen and testosterone and progesterone, which give high specificity for these hormones but fail to analyze the broader spectrum of other steroid hormones that could be affected by PFAS exposure. Immunoassay techniques also suffer from cross-reactivity and significant interferences from matrix effects. Another study that utilized liquid chromatography with tandem mass spectrometry (LC-MS/MS) under electrospray ionization with positive and negative mode switching showed that mixtures of various PFASs, polybrominated, and chlorinated persistent pollutants led to the increased expression of estrone but decreased expression of most progestogens and androgens.[11] These studies largely focused on legacy PFASs, such as PFOS and PFOA; however, there is still a need for the analysis of the effects of short- and long-chain PFASs and their mixtures, which are conditions that are more relevant in the environment. Past studies have focused on assessing the changes in a handful of hormones because of the analytical challenges associated with the simultaneous detection of these compounds at low concentrations.

The detection of the hormones can be accomplished in multiple ways, including the use of analyte-specific immunoassays,[13,14] derivatization followed by gas chromatography[15] with mass spectrometry, and liquid chromatography with tandem mass spectrometry (LC-MS/MS). LC-MS/MS is most commonly used for the analysis of steroid hormones in a variety of matrices and can achieve low limits of detection (i.e., low part per trillion levels).[16−19] However, steroid hormones

https://doi.org/10.1021/acs.chemrestox.2c00116
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do not have high ionization efficiencies under electrospray ionization (ESI) mode in LC-MS/MS, which makes their detection using ESI challenging.[16] Consequently, previous LC-MS/MS methods to study steroid hormones in H295R cells are limited by the poor ionization efficiency of pregnenolone or DHEA in positive ESI, or by the typically poorer ionization efficiency of negative mode.[20] To improve ionization efficiencies, method sensitivity, and the number of analytes included in this analysis, a derivatization strategy was used to add a readily ionizable functional group for increased ionization efficiency in the analysis of various hormones in H295R cells.[21]

The first goal of this study is to develop an assay that can measure 17 of the steroid hormones produced by H295R cells (Figure 2) to allow for a comprehensive evaluation of the endocrine-disrupting effects of PFASs. To this end, a two-part derivatization strategy, converting the ketone and phenol groups to readily ionizable amine groups, was utilized to overcome the poor ionization efficiencies of hormones in LC-MS/MS. The second goal of this study was to measure changes in hormone production by H295R cells upon exposure to endocrine disrupting chemicals, such as forskolin, prochloraz (Figure 3), and PFASs, for method validation. The forskolin

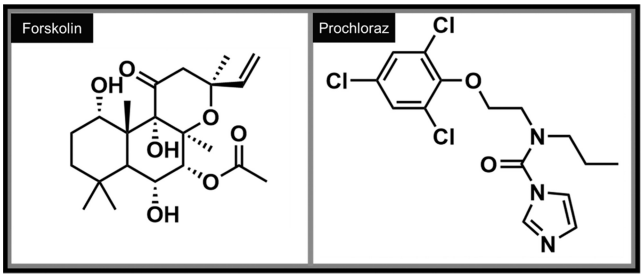

Figure 3. Structures of forskolin, an activator of hormone synthesis, and prochloraz, an inhibitor of hormone synthesis.

was used as the positive control for hormone accumulation because it is a known activator of adenylyl cyclase-producing cyclic adenosine monophosphate (cAMP), a signaling molecule, and a direct upstream regulator of hormone synthesis.[22] Prochloraz was used as a positive control for hormone depletion because it is a known inhibitor of multiple cytochrome 450 enzymes involved in steroidogenesis, including CYP11A1, CYP17A1, and CYP19A2.[23,24] Finally, the short-and long-chain legacy PFASs with sulfonate and carboxylate headgroups (Figure 1) were used as test compounds to determine the suitability of the developed analytical method to detect hormone alterations in H295R cells resulting from low-level exposure to PFAS mixtures.

MATERIALS AND METHODS

Materials. The following high-purity solid standards were purchased from Millipore Sigma (Burlington, MA): 17α-hydroxy progesterone (>95%), 21-hydroxy progesterone (>97%), Estrone (>99%), estriol (>97%), estrone-3-sulfate potassium salt (>98%), cortisone (>98%), corticosterone (>98%), and estrone-D4 and aldosterone (>95%). The following standard solutions were purchased from Millipore Sigma (Burlington, MA) at 1 mg/mL in methanol: testosterone, androstenedione, β-estradiol, dehydroepiandrosterone (DHEA), cortisol, dihydrotestosterone (DHT), 11-deoxycortisol, and dehydroepiandrosterone sulfate (DHEA-S). The following standard solutions were purchased from Millipore Sigma (Burlington, MA) at 100 μg/mL in methanol: 17-hydroxy pregnenolone, pregnenolone, testosterone-d3, 11-deoxycortisol-d5, cortisol-d5, 17-hydroxy progesterone-d8, pregnenolone-^{13}C2-d2, androstenedione-^{13}C3, and corticosterone-d4. Solid PFAS standards of perfluorobutanoic acid (PFBA), perfluorobutane sulfonic acid (PFBS), perfluorohexanoic acid (PFHxA), perfluorohexane sulfonic acid (PFHxS), perfluorooctanoic acid (PFOS), perfluorooctane sulfonic acid (PFOS), perfluorodecanoic acid (PFDA), and perfluorodecane sulfonic acid (PFDS) were purchased from Millipore Sigma (Burlington, MA). A ^{13}C-labeled PFAS mixture (MPFAC-24ES) was purchased from Wellington (Guelph, ON), which included MPFBA (^{13}C4), MPFBS (^{13}C3), MPFHxA (^{13}C5), MPFHxS (^{13}C3), MPFOA (^{13}C8), MPFOS (^{13}C8), and MPFDA (^{13}C6). Also purchased were methoxyamine hydrochloride, ammonium hydroxide (28%) acetone, LC-MS grade methanol, dimethyl sulfoxide (DMSO), and LC-MS grade acetonitrile. Solid sodium bicarbonate and formic acid (88%) were purchased from J.T. Baker (Radnor Township, PA). Sodium hydroxide was purchased from Fischer Scientific (Waltham, MA). Dansyl chloride was purchased from Fluka Chemical (Morris Plains, NJ). HPLC-grade methanol and ethyl acetate were purchased from J.T. Baker (Radnor Township, PA).

H295R adrenocarcinoma cells were purchased from the American Type Culture collection (ATCC) (Manassas, VA). Dulbecco's modified Eagle's media (DMEM)/Hams-F12 50:50, fetal bovine serum, trypsin and ITS$^+$ (insulin, transferrin and selenic acid) with 5% fetal bovine serum (FBS), 1% ITs$^+$ premix, and penicillin/streptomycin antibiotic cocktail were purchased from Corning (Corning, NY). Weak anion exchange (WAX) 6cc, 500mg, 30 μm cartridges for solid-phase extraction (SPE) were received from Waters (Milford, MA). An InertSustain Phenyl (3 μM 100 × 2.1 mm) HPLC column was received from GLS Sciences (Torrance, CA).

Separation for LC-MS/MS analysis was performed using an Agilent (Santa Clara, CA) 1200 series LC tower composed of a G13798 degasser, G1311A quaternary pump, G1367B high-performance autosampler, and G1330B autosampler thermostat. Mass analysis was done using a Thermo Scientific (Waltham, MA) TSQ Quantum Ultra triple quadrupole MS equipped with a heated electrospray ionization source and operated under positive mode ionization.

EXPERIMENTAL METHODS

Tissue culture. The H295R adrenocarcinoma cells were cultured in Dulbecco's modified Eagle's media (DMEM)/Hams-F12 50:50 with 5% fetal bovine serum (FBS), 1% ITs$^+$ premix, and 1% penicillin/streptomycin antibiotic cocktail. Cells were incubated at 37 °C under 5% CO_2 in a Thermo Scientific (Waltham, MA) Heracell incubator. Cells were grown to a confluency of 80−90% in 5−7 days with cellular media being replaced every 3 days. When the cells reached a confluency of 80−90%, the cells were passaged in a 1:3 ratio into 10 cm flasks for continued growth.

Cell Viability. Cell viability was assessed using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.[25] Treatments (24 h) included individual exposures of PFOS, PFOA, PFBS, PFBA, PFHxA, PFHxS, PFDA, PFDS, forskolin, and prochloraz, as well as mixtures of equal parts of PFOS and PFOA (mix A) and equal parts of PFBA, PFBS, PFHxA, PFHxS, PFOA, and PFOS (mix B). A vehicle control consisting of 1% (v/v) methanol (in final concentration) was added to the control wells (N = 5). After a 24 h incubation, the media from each treatment, control, and previously empty wells were aspirated and replaced with a 9% MTT solution. The absorbance was read with Bio-TEK (Winooski, VT) Synergy HT plate reader at 550 nm.

Positive and Negative Controls for Hormone Disruption. Forskolin and prochloraz were chosen as positive and negative controls, respectively, based on the Organization for Economic Co-operation and Development (OECD) test number 456.[1] Approximately 4.5 × 10^6 H295R cells were plated on sterile 10 cm plates in 10 mL of complete growth media (N = 3, for each condition). Cells were incubated at 37 °C under 5% CO_2 and were allowed to grow for 48 h to reach ~70 to 75% confluency. Stock concentrations for each treatment were prepared at a concentration 200 times higher than the

https://doi.org/10.1021/acs.chemrestox.2c00116
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Table 1. PFAS Surrogates Used for Quantification Based on Isotope Dilution MS, along with Their Corresponding Response Factor (*RF*)

compound	$[M-H]^-$	retention time (min)	labeled surrogate	$[M-H]^-$	retention time (min)	*RF*
PFBA	212.9	8.94	MPFBA	216.9	8.94	0.48
PFBS	298.9	10.21	MPFBS	301.9	10.22	0.06
PFHxA	312.9	9.93	MPFHxA	318	9.94	14.58
PFHxS	398.8	11.06	MPFHxS	401.9	11.07	1.10
PFOA	412.8	10.73	MPFOA	420.9	10.72	0.66
PFOS	498.8	12.09	MPFOS	506.9	120.8	1.20
PFDA	512.9	11.7	MPFDA	518.9	11.66	0.97
PFDS	598.9	13.56	MPFOS	506.9	120.8	0.73

intended concentration so that the final treatment solutions contained no more than 0.5% DMSO. Briefly, an addition of 50 μL to 10 mL provided the intended treatment concentrations of 1 and 10 μM for forskolin (hormone production inducer), and 0.1 and 1 μM for prochloraz (hormone production inhibitor). After a 24 h exposure, the cellular media were collected into sterile 15 mL Falcon tubes. The cellular media were then stored at −80 °C.

PFAS Treatments. Approximately 4.5 × 10^6 H295R cells were plated on sterile 10 cm plates in 10 mL of complete growth media (N = 3, for each condition). Cells were incubated at 37 °C under 5% CO_2 and allowed to grow for 48 h to reach 70−75% confluency. For this study, 10 treatment conditions were used. The first 8 treatments contained 1 μM individual solutions of either PFBA, PFBS, PFHxA, PFHxS, PFOA, PFOS, PFDA, or PFDS. The ninth treatment contained a mixture of 0.5 μM PFOS and 0.5 μM PFOA (mix A). The 10th treatment contained 0.16 μM of each PFBA, PFBS, PFHxA, PFHxS, PFOA, and PFOS to give a total PFAS concentration of 1 μM (mix B). After a 24 h incubation, the cellular media were collected into a sterile 15 mL Falcon tube and stored at −80 °C. The 10 cm plates were scraped, and the cell pellets were collected for each condition. Cell pellets were washed with PBS, and pellets were stored at −80 °C until further use.

PFAS Extraction from Cells. Frozen cell pellets were thawed on ice (10 min) and then resuspended with cold methanol containing 50 μ/L of PFHpA and PFHpS to act as surrogates. Cell pellets were vortexed (30 s) three times each and then sonicated using a sonicator probe (30 s). Cell lysates were centrifuged (2.4 g) for 10 min at 4 °C, and the supernatants were collected and then dried under a stream of N_2 gas. Cell lysates were resuspended to 1 mL of methanol with a 20 μg/L spike of a PFAS surrogate mixture. This includes seven isotopically labeled PFAS standards, including MPFBA, MPFBS, MPFHxA, MPFHxS, MPFOA, MPFOS, and MPFDA. Figure S1 shows the percent recovery for each PFAS, which was between 94 and 98% for the developed methanol extraction. PFAS quantitation in cell lysates was done by isotope dilution with a corrective back-calculation to account for the predicted recovery. Table 1 below provides the labeled PFAS surrogates used for isotope dilution. Equation 1 below shows the calculation done for isotope dilution. A retention factor (RF) was calculated for each PFAS quantitated. The RF was calculated as the ratio of native species' peak area to surrogate species at the same concentration.

$$\frac{[S]}{[X]} = \frac{I_s}{I_x} \times RF[S] \times I_x/I_s \times RF = [x] \quad (1)$$

X = unknown, S = surrogate, I = intensity, RF = response factor

Sample Cleanup Using Solid-Phase Extraction (SPE). For the analysis of hormones in the media, a method to clean up the samples was necessary to remove matrix components that will interfere with the LC-MS/MS analysis. Steroid hormones, such as estrogen and testosterone, were released into the media by H295R cells and therefore the media were selected for analysis.[26] Media were also analyzed for hormone levels based on the recommendation by the OECD 456 procedure.[1] The Waters Oasis weak anion exchange (WAX) SPE cartridges were used to capture both PFASs and steroid hormones on the hydrophobic−cationic hybrid stationary phase. WAX was chosen not only for its high percent recovery of hormones (Figure S2A) but it was also necessary to remove PFAS treatments to prevent concentration and potential instrument contamination. The media (10 mL) was diluted to 250 mL of Nanopure water in acid-washed amber glass jars and fortified with an internal standard mix consisting of testosterone-d3, 11-deoxycortisol-d5, cortisol-d5, 17-hydroxy progesterone-d8, pregnenolone-^{13}C2-d2, androstenedione-^{13}C3, corticosterone-d4, estrone-d4, and estradiol-d3, each with a concentration of 100 ppb in 1 mL. The SPE cartridges were conditioned with HPLC-grade methanol (5 mL) and Nanopure water (10 mL). SPE high-volume lines were used to load all samples onto the cartridges. Samples were passed through the SPE cartridges at 3 mL/min. After the samples were loaded, the cartridges were washed with Nanopure water (5 mL) and dried under vacuum for 1 h. The hormones were then selectively eluted using HPLC-grade methanol (8 mL), which was then dried under an N_2 stream. Samples were reconstituted in HPLC-grade methanol (1 mL).

Parallel Hormone Derivatization. A parallel derivatization strategy was used for the analysis of 17 hormones in each H295R test sample. Methoxyamine (MOA) was used to derivatize testosterone, dehydroepiandrosterone, dihydrotestosterone, pregnenolone, 17-hydroxy pregnenolone, progesterone, 17-hydroxy progesterone, 21-hydroxy progesterone, corticosterone, 11-deoxycortisol, cortisol, cortisone, aldosterone, and androstenedione.[21] Dansyl chloride (DC) was used to derivatize estrone, estradiol, and estriol.[21] The procedure for each derivatization step is as follows:

Methoxyamine Derivatization (MOA). A sample aliquot (200 μL) is dried down under an N_2 stream and resuspended with 100 mM of MOA in methanol (200 μL) at pH 4.0. The reaction vial is covered with aluminum foil and vortexed for 30 s, followed by incubation at 60 °C for 120 min. This reaction is then incubated at 4 °C on ice for 5 min to thermodynamically quench the reaction.

Dansyl chloride Derivatization (DC). A sample aliquot (200 μL) is dried down under an N_2 stream and resuspended with 100 μL of 100 mM sodium bicarbonate (pH 10.5) and 100 μL of 1 mg/mL DC in acetone. This reaction is capped with aluminum foil and vortexed for 30 s. This reaction is incubated at 60 °C for 30 min and then incubated at 4 °C on ice for 5 min.

Reaction Cleanup. After each reaction is complete, the two derivatized solutions are extracted using a liquid−liquid extraction. First, ethyl acetate (1 mL) is added to the MOA reaction vial, and then this solution is transferred completely into the DC reaction vial. The mixture of derivatized products is transferred to a 4 mL clear glass vial, vortexed for 30 s, and centrifuged at 0.7 (g) for 10 min at 25 °C. After centrifugation, the top ethyl acetate layer is transferred to a clean 1.5 mL amber glass vial and dried down under an N_2 stream. Then, another 1 mL of ethyl acetate is added to the lower aqueous layer for a second extraction. This mixture is vortexed and centrifuged again, and the top ethyl acetate layer is added to the dried sample in the 1.5 mL vial. This sample is dried again under an N_2 stream, and then resuspended with 200 μL of 1:1 acetonitrile: water for LC-MS/MS analysis.

LC-MS/MS Analysis of PFASs. Separation of PFASs was achieved using an Xbridge C18 analytical column equipped with an Agilent Eclipse Plus C18 delay column. A gradient mobile phase consisting of 5 mM ammonium acetate in water at pH 3.8 (mobile phase A) and

https://doi.org/10.1021/acs.chemrestox.2c00116
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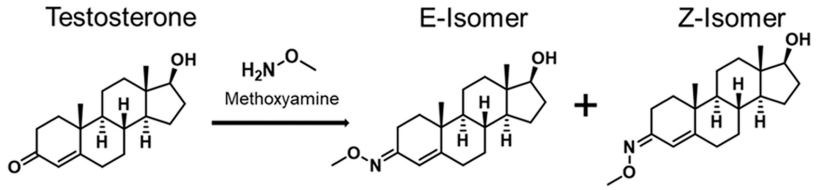

Figure 4. The nonspecific nature of the oxime reaction can generate *E* and *Z* geometric isomers. An example of this can be seen with the predicted *E* and *Z* isomer products of derivatized testosterone.

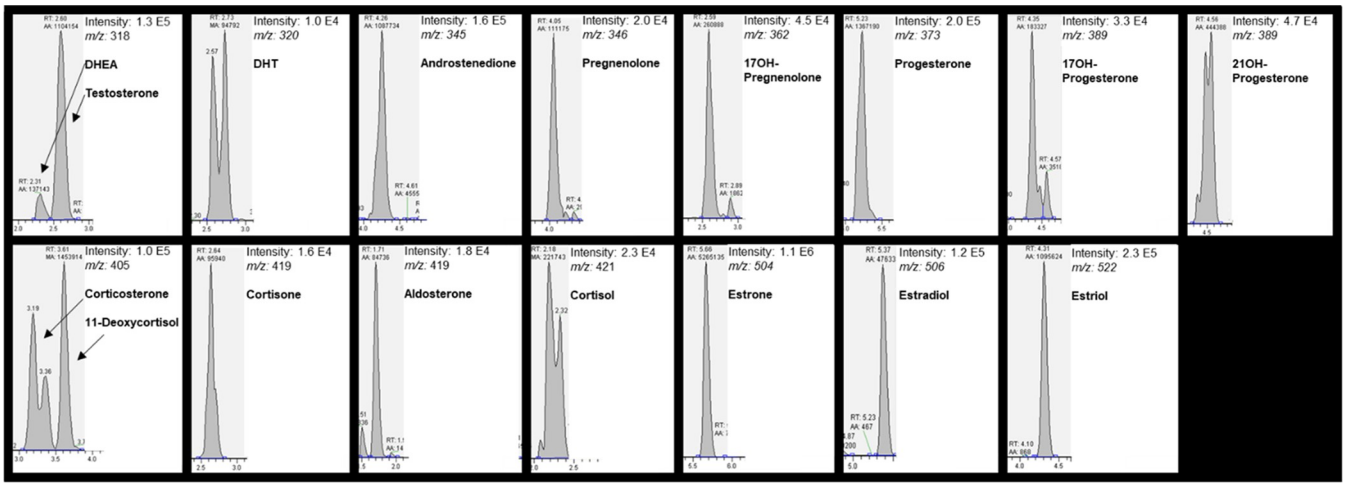

Figure 5. Extracted ion chromatograms (EICs) for the 17 hormones analyzed in this protocol. The EICs are shown for hormones spiked at 1 ppb in media. The spiked samples were extracted by solid-phase extraction (weak anion exchange) and concentrated 10× before derivatization, followed by LC/MS/MS analysis. DHEA/testosterone and 11-deoxycortisol/corticosterone are included in the same EIC because their precursor ions are the same, and their fragment ions are overlapping. These EICs were generated from known concentrations of standards and are representative of the hormones detected in H295R cell culture media. DHT, aldosterone, cortisol, and estriol were below the limits of detection.

acetonitrile (mobile phase B) was used at a flow rate of 200 μL/min. The gradient program starts at 5% mobile phase B, which is increased to 80% (0−5 min); this condition is held constant for 12 min. Then, the mobile phase is ramped to 90% B (12−17 min) and held constant for 10 min. Finally, the mobile phase is ramped to 95% B (27−28 min) and held constant for 4 min, followed by returning to the starting condition of 5% mobile phase B (32−45 min). The source conditions were: 300 °C source temperature, 300 °C vaporizer temperature, 35 (arb) (arbitrary unit) sheath gas, 30 (arb) auxiliary gas, −3000 V spray voltage, and 4 μAmp discharge current. The SRM settings used a 0.7 Q1 peak width of full width at half maximum (FWHM) and a 1-s cycle time. Table S1 provides all SRM transitions for each PFAS and internal standards used.[27]

LC/MS/MS Analysis of Hormones. An InertSustain Phenyl analytical column (100 × 2.1 mm, 3 μm) was used for the separation of DC and MOA-derivatized hormones. The mobile phase A was water with 1% formic acid at pH 2.8, and the mobile phase B was 20/80 (v/v) methanol/acetonitrile, with 1% formic acid. A 10 min LC method was developed for the separation of the 17 derivatized hormones. To improve detection, the separation of the *E*/*Z* isomers produced by the methoxyamine reaction was intentionally minimized to obtain a higher total area signal.[28] To improve the signal of each derivatized compound, the separated *E*/*Z* isomer peaks were fused into a single peak by utilizing a phenyl column. Initially, an InertSustain C18 analytical column (100 × 2.1 mm, 3 μm) was also tested, but this column resulted in a better separation of the *E*/*Z* isomers, giving lower peak intensity compared to the phenyl column. Figure 4 below shows examples of the predicted (*E*) and (Z) geometric isomer products. The *E*/*Z* products shown in Figure 4 likely form due to an SN2 reaction occurring on a planar ring with no functional groups contributing to steric hindrance. For some compounds, such as 17 and 21-hydroxy progesterone, the two peaks caused by diastereomers had some separation which could not be avoided (see Figure 5). However, even in these cases, the peaks were not fully resolved. For these compounds, both peaks were integrated during data analysis.

A 10 min gradient was used for the separation (flow was 500 μL/min) starting with 50% mobile phase B, which was held constant (0−0.5 min), followed by a ramp to 90% B (0.5−4 min). The mobile phase was held at 90% B (4−7.5 min) and then returned to the starting condition of 50% B (7.5−10 min). Ionization was achieved in the positive electrospray mode with a scan voltage of (+) 4250 V. Source conditions were as follows: capillary temperature at 200 °C and vaporizer temperature at 350 °C. The auxiliary gas and sheath gas pressures were set at 35 and 60 arb, respectively. A cycle time of 0.3 s was used, with a resolving power of 0.7 (FWHM) for Q1 and Q3. SRM was used for the analysis of all 17 derivatized hormones and labeled standards with MS parameters optimized by direct injection of each individual standard. Table 2 below shows a detailed report on the transitions, collision energies, tube lens values, limit of detection (LOD), and limits of quantifications (LOQ) for each analyte and deuterated standard. The LOD was determined from a 7-point calibration curve, ranging from 0.05−100 parts per billion (ppb) concentrations, and was calculated to be the signal corresponding to 3 times the standard deviation of the lowest concentration in the calibration curve, divided by the slope of the linear plot. The LOQ was determined as the concentration corresponding to a signal that is 10 times the standard deviation of the lowest concentration in the calibration curve, divided by the slope. Figure 5 illustrates extracted ion chromatograms of each hormone recovered from the cellular growth at 1 ppb and concentrated to approximately 10 ppb by SPE.

Statistical Analysis. Significance testing was performed using a two-way ANOVA comparing each treatment mean (N = 3) with a control (N = 3). Dunnett's test was used to correct for multiple comparison testing.

Software. Data acquisition and analysis were done using Thermo Xcalibur 2.2 SP1.48. Data workup and statistical analysis were

https://doi.org/10.1021/acs.chemrestox.2c00116
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Table 2. List of Derivatized Hormones, Including Their Molecular and Fragment Ions and Their Corresponding Limits of Detection (LOD) and Quantitation (LOQ)[a]

hormone	derivative	[M + H]+	tube lens	quant m/z	FvV	qual m/z	FvV	Rt min	LOD ppb	LOQ ppb	R^2	slope	Y-intercept
testosterone	MOA	318	136	138	25	126	26	2.7	0.13	0.46	0.9995	0.09	−0.0003
DHT	MOA	320	127	96	32	91	51	2.7	1.73	5.77	0.9820	0.01	0.0002
DHEA	MOA	318	74	253	17	157	18	2.3	0.34	1.12	0.9996	0.01	−0.0001
pregnenolone	MOA	346	87	100	28	301	15	4.0	0.96	3.19	0.9992	0.02	−0.0307
17OH pregnenolone	MOA	362	80	344	10	145	29	2.5	2.17	7.22	0.9993	0.04	−0.0570
androstenedione (DD)	MOA	345	104	260	39	283	24	4.2	0.27	0.90	0.9992	0.22	−0.2941
progesterone (DD)	MOA	373	118	286	30	126	43	5.1	0.29	0.97	0.9984	0.08	−0.0984
17OH progesterone (DD)	MOA	389	115	286	40	138	28	4.3	0.42	1.40	0.9976	0.11	−0.1815
21OH progesterone (DD)	MOA	389	115	126	26	138	30	4.5	0.23	0.75	0.9978	0.05	−0.1055
11-deoxycortisol (DD)	MOA	405	125	286	26	138	42	3.5	0.31	1.04	0.9982	0.06	−0.0457
corticosterone (DD)	MOA	405	115	343	26	152	37	3.5	0.23	0.75	0.9958	0.07	−0.1572
aldosterone (DD)	MOA	419	80	372	12	401	12	2.5	2.89	9.63	0.9970	0.06	−0.1717
cortisone (DD)	MOA	419	115	300	27	120	41	2.5	0.67	2.22	0.9985	0.10	−0.1649
cortisol (DD)	MOA	421	132	284	25	359	33	2.2	0.53	1.76	0.9976	0.83	−0.6832
estrone	DC	504	147	171	31	156	52	5.5	0.07	0.25	0.9982	0.07	−0.0983
estradiol	DC	506	135	171	36	156	57	5.3	0.92	3.08	0.9979	0.18	−0.1444
estriol	DC	522	134	171	32	156	34	4.3	0.09	0.26	0.9989	0.02	−0.0307
testosterone-D3	MOA	321	126	138.1	29	126.	29	2.7					
11-deoxycortisol-(DD)-D5	MOA	410	134	291.4	42	129.	27	3.5					
progesterone-(DD)-D9	MOA	382	115	293	45	142	31	5.1					
estrone-D4	DC	508	133	171	37	156	57	5.6					
androstenedione-C_{13}3	MOA	348	105	263.2	28	286.2	24	5.5					
pregnenolone-D2-C_{13}2	MOA	350	104	104.1	27	304.2	20	4.0					
estradiol-D3	DC	509	135	171.1	32	156.1	57	5.3					
corticosterone-D4	MOA	409	120	347.3	28	306.2	32	3.5					
cortisol-D4	MOA	425	116	363.3	28	288.2	30	2.2					
11-deoxycortisol-D5	MOA	410	134	291.2	27	129.1	42	3.5					

[a]DD: Double Derivatized Product; MOA: Methoxyamine; DC: Dansyl Chloride; FV: Fragmentation Voltage; Quant: Quantitative ion; Qual: Qualitative ion; and Rt: Retention Time.

performed using OriginPro 2020 (Academic) and GraphPad Prism (version 9.3.1).

RESULTS AND DISCUSSION

H295R Cells. Our goal was to treat H295R adrenocortical carcinoma cells under various PFAS exposure conditions to evaluate the endocrine-disrupting effects of PFASs. H295R cells were selected as a model test system because they are capable of producing the 17 steroid hormones shown in Figure 2, which are released into the cellular media.[26] The appropriateness of H295R cells as an endocrine-disruption model was tested in this study by treating them with a known inducer (forskolin)[22] and a known inhibitor (prochloraz), (Figure 3),[1,22,23] H295R cells were treated with 8 PFASs and two mixes representing environmental levels. Then, 10 mL of the collected media were analyzed for hormone levels relative to a control.

Solid-Phase Extraction of Hormones. Steroid hormones were extracted from the cellular media of PFAS-exposed H295R cells and were concentrated using Oasis™ WAX SPE cartridges prior to derivatization. The goals of this extraction were to reduce potential matrix interferences, concentrate the analytes, and remove PFASs that were spiked at high concentrations in the samples to prevent instrument contamination. The hybrid nature of the stationary phase in the WAX SPE cartridge, which consisted of polar, nonpolar, and charged functional groups, allowed for the retention of all 17 hormones and 8 PFASs. Steroid hormones were retained through hydrophobic interaction, while the charged PFASs were retained through electrostatic interactions with the stationary phase. Selection of elution solvent was done to only elute the analytes of interest but not PFASs. Methanol was chosen, as it was shown to elute the hormones, overcoming the hydrophobic interactions between the hormones and the stationary phase (Figure S2A). Methanol alone, however, did not elute the PFASs bound due to the strong electrostatic interaction between PFASs and WAX cartridge (Figure S2A, at most 20% recovery). It was necessary to add 5% ammonium hydroxide to show substantial recoveries for PFASs further verifying that PFASs did not elute with only methanol. Figure S2A provides the recoveries for each hormone using the WAX cartridges, which ranged from 80−100% when media was spiked with 200 parts per billion (ppb) hormone mix. The hormones with the lowest recoveries were DHT, estrone, cortisol, estradiol, and estriol (81−85%). Figure S2B shows 60−95% recoveries for 8 PFAS compounds with elution with methanol and methanol with 5% ammonium hydroxide.

Optimization of Derivatization Conditions. The mechanisms of the MOA and DC reactions are shown in Figure S3.[29] The Dansyl chloride reaction was optimized for total reaction time, which can be seen in Figure S4. Reaction

https://doi.org/10.1021/acs.chemrestox.2c00116
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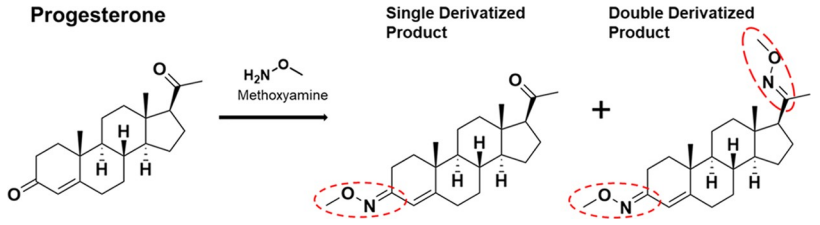

Figure 6. Progesterone contains two ketone groups, both of which can react with methoxyamine. The single derivatization (left) shows the most likely major single product, which is the oxime reacting with the sterically available ring. The double derivatization (left) shows the oxime reaction occurring on both the ring and less sterically available ketone in the alkyl group.

Table 3. List of Hormones and Their Respective Recovery and RSD after Extraction from 10 mL of Media (10 ppb) by Solid-Phase Extraction with Weak Anion Exchange Cartridges and Derivatized with Dansyl Chloride and Methoxyamine[a]

hormone	RSD (%)	recovery (%)	hormone	RSD (%)	recovery (%)
testosterone	5.2	88.9	corticosterone	2.6	91.9
DHEA	7.0	86.6	11-deoxycortisol	3.6	89.9
DHT	12.1	82.4	aldosterone	14.9	73.0
androstenedione	7.7	89.4	cortisone	1.5	74.8
pregnenolone	0.7	77.3	cortisol	6.5	86.9
17OH pregnenolone	6.6	81.5	estrone	4.7	70.6
progesterone	2.5	76.3	estradiol	5.2	71.9
17OH progesterone	0.6	86.0	estriol	6.2	74.5
21OH progesterone	2.6	85.2			

[a]Average percent recoveries are shown. Detailed information on the recoveries can be found in Figure S2A.

times of 10, 30, 60, and 120 min were tested, with 30 min showing the highest reaction efficiency, with no significant improvement with the 60 and 120 min reactions. Typically, 30 min was chosen for the reaction. Due to solubility issues, higher concentrations of DC were not considered. A pH of 10.5 was selected as the lowest pH (10.5) which would activate the phenol group of estrogens (pKa approximately 10.5)—while minimizing the risk of hydrolysis of the derivatives.[30] Multiple aspects of the MOA derivatization were optimized to improve the LOD of this method, including the optimum reaction time, reagent concentrations, and reaction pH. Many of the hormones have multiple ketone groups that provide multiple reactive sites for MOA derivatization. It was observed that at short reaction times and low reagent concentrations, there was a mixture of both singly- and doubly-derivatized products. Figure 6 shows an example of a single and double derivatized product with progesterone.

We first tested different derivatization times (30−180 min, Figure S5). It was observed that, with increased time, there was an increase in total product ion count for most species up to a 20-fold increase between 30 and 180 min. Considerable sample evaporation occurred after 180 min. Hence, 120 min was chosen. For hormone species that contained two reactive groups, such as progesterone and corticosterone, it was observed that the doubly-derivatized products were the dominant forms observed after a 120 min incubation. In addition, the total intensity of the doubly-derivatized products was also highest at the 120 min incubation. This higher intensity of the doubly-derivatized products relative to the singly-derivatized products may be due to the increased polarity that aids in source desolvation and ionization.[31] Due to the increases in signals associated with the m/z for the doubly-derivatized products, the latter was used for the analysis of all hormones that contain two ketones. It should be noted that cortisone contains three ketone groups, but only the doubly-derivatized product was observed.

We next considered an increased concentration of MOA to further drive the reaction toward the formation of the doubly-derivatized products. The MOA concentrations tested were 100, 200, 300, 400, and 500 mM, but concentrations higher than 100 mM MOA provided no significant increases in the derivatization products. Figure S6 summarizes the effect of MOA concentrations on the signal intensities of the derivatized products formed. Based on these, 100 mM MOA was used in the subsequent derivatizations of the hormones.

Finally, reaction pH is an important variable for this reaction. MOA is strengthened as a nucleophile in basic pH, while the reactivity of ketones increases under acidic conditions.[29] A study was performed to determine the optimal pH to maximize the products at both reactive sites. Figure S7 shows that MOA derivatization at pH 4 gave the highest peak intensities for each derivatized analyte. The acidic condition pH 3 and the more basic pH 5 both showed decreased peak intensities, suggesting a weaker nucleophile and a less reactive carbonyl. pH 4 was chosen for this reaction. Table 3 gives relative standard deviations of this methoxyamine reaction as well as Dansyl chloride in spiked media, showing reproducibility (N = 3) between 0.6 and 14.9%.

The method described in this section allows for a single LC-MS/MS run using positive ionization mode, with increased ionization efficiencies compared to the commonly used negative ionization mode for hormone analysis. We have achieved LODs between 0.07 and 2.89 ppb with extraction-based concentration factors, which further improved method sensitivity. We compared this method to recently published LC-MS/MS methods for hormone detections in complex matrixes. One study utilized online SPE and LC-MS/MS and obtained 0.1−5 ppb LODs in eggs.[19] A second study that relied on two separate ionization modes (ESI and APCI) using LC-MS/MS/MS to analyze hormones in human serum achieved 5−25 parts per trillion LODs in human serum.[18] However, none of these methods were able to account for all

https://doi.org/10.1021/acs.chemrestox.2c00116
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17 hormones analyzed in this study simultaneously. Finally, a recent LC-MS/MS method reported the analysis of the same 17 hormones in a cell-based assay with LODs between 0.02−7 ppb but required expensive automated extraction equipment for low volume analysis.[20] Overall, the method we developed here shows comparable or improved LODs to current literature on hormone analysis.

Validation of LC/MS/MS Method Using Known Endocrine Disruptors. We wanted to assess whether the developed derivatization and LC-MS/MS methods can quantify the changes in the hormone production of H295R cells. To this end, we exposed cell's known endocrine disruptors forskolin (upregulator) and prochloraz (down-regulator).

First, MTT-based viability assays[1] were done with forskolin and prochloraz to confirm that the concentrations to be used for the treatment are nonlethal to the cells. Figure S8 summarizes the viability results. Both forskolin and prochloraz do not induce toxicity at concentrations up to 10 μM. Based on these results and published guidelines,[1] 1 and 10 μM forskolin, and 0.1 and 1 μM prochloraz were used in H295R cells for a 24 h exposure. After 24 h, the growth media were collected, and hormones were extracted, derivatized, and analyzed, as described previously. Forskolin treatment showed increases in all targeted hormones, except for testosterone (which had no significant change) and cortisol and aldosterone (which were not detected) (Figure 7). It should be noted that while testosterone was not significantly increased, the immediate downstream product DHEA (Figure 2) showed a significant increase. Prochloraz treatment resulted in significant decreases in all targeted species, except for progesterone and pregnenolone, which instead increased. Cortisol and aldosterone were not detected, similar to the cells exposed to forskolin. Pregnenolone and progesterone increases can be attributed to the affected enzymes, CYP17A, and CYP19A that are directly upstream of hormone synthesis. Overall, forskolin and prochloraz treatments showed the expected changes in the hormone levels in cellular media, demonstrating the applicability of the derivatization and LC-/MS/MS methods in determining the endocrine-disrupting effects of chemicals in cell-based assays.

Exposure of H295R Cells to PFASs. MTT viability assays were carried out to determine the toxic effects of PFBA, PFBS, PFHxA, PFHxS, PFOA, PFOS, and two PFAS mixtures at the concentrations tested. Treatment concentration of 1 μM (total PFASs) was selected as the lowest test concentration, reflecting PFAS exposure through industrial wastes or spills in the environment.[32,33] Mixture A was composed of equal portions of PFOS and PFOA (0.5 μM each), the two most common PFASs found in the environment. Mixture B was composed of equal portions of PFBA, PFBS, PFHxA, PFHxS, PFOA, and PFOS (0.17 μM each), which are also typically found in serum and in short-chain PFASs that are known byproducts of longer-chain species.[34] Figure S9 shows the cell viability results from the treatments using 1, 3, 10, and 30 μM concentrations of PFASs. It was observed that PFAS treatments of up to 30 μM were not toxic to the cells, which is consistent with literature that suggests toxicity occurs at much higher PFAS concentrations (>100 μM).[10] Based on these results, 1 μM treatment for 24 h was chosen as the exposure condition to investigate the endocrine-disrupting effects of PFASs in the absence of any toxic response. The concentrations chosen in this study are also reflective of the low μM levels of PFOS and PFOA recently detected in human serum samples.[35,36]

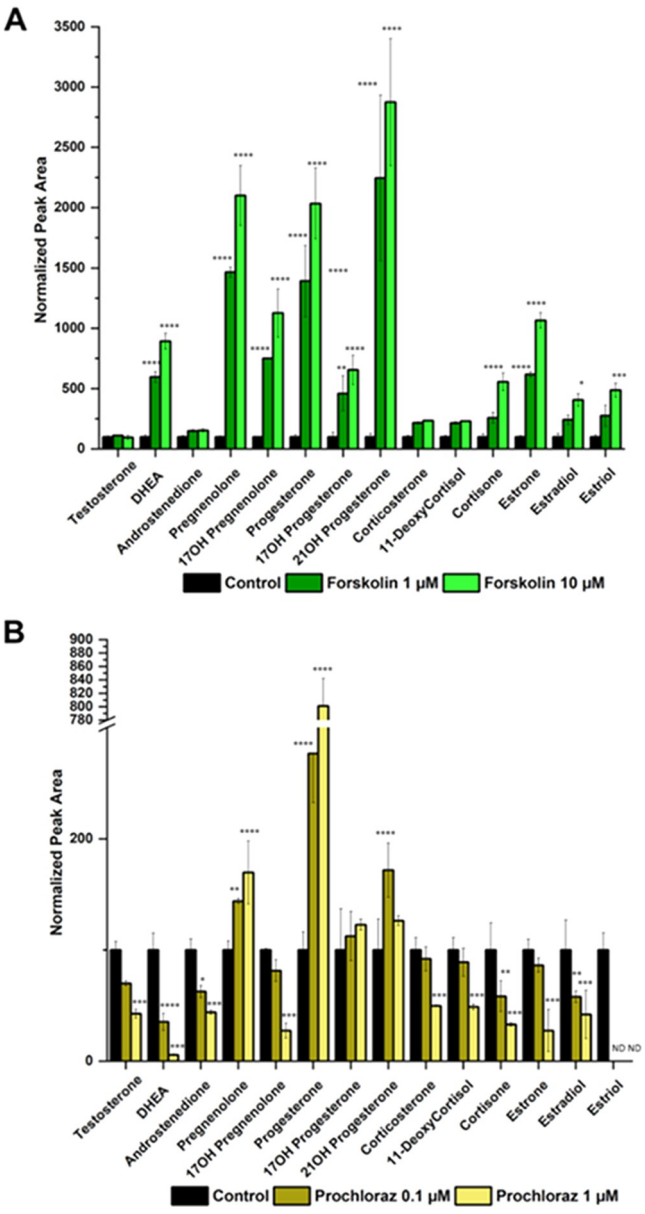

Figure 7. (A) Forskolin and (B) prochloraz affect hormone levels. (A) Significant increases in hormone levels can be seen with increased concentrations of forskolin (1 and 10 μM). (B) In contrast, prochloraz treatments showed mostly decreases in hormone levels except for pregnenolone and progesterone, which showed increases in total abundance (at 0.1 and 1 μM). Significance testing was done with a two-way ANOVA (* Adjusted P-value <0.05, ** Adjusted P-value <0.01, *** Adjusted P-value <0.001, and **** Adjusted P-value <0.0001).

Finally, to assess the effects of PFASs on steroid hormone production, H295R cells were treated with 1 μM of each PFBA, PFBS, PFHxA, PFHxS, PFOA, PFOS, PFDA, PFDS, Mix A, and Mix B for 24 h. Cells and growth media were collected separately to quantify the PFAS uptake by the cells and the hormone levels in the media. First, we analyzed the PFAS levels in cell pellets to determine the total PFASs associated with the cells and examined if the type of headgroup and the chain length of PFAS elicited different cellular responses. We also aimed to determine if any correlations

https://doi.org/10.1021/acs.chemrestox.2c00116
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occur between the changes in hormone levels and the type of PFAS the cells are exposed to. Table 4 shows the level of PFAS

Table 4. Total PFAS Concentrations Associated with H295R Cells Exposed to 1 μM of Individual PFAS, Mix A Containing PFOA and PFOS (Each at 0.5 μM), and Mix B Containing PFBA, PFBS, PFHxA, PFHxS, PFOA, and PFOS (Each at 0.17 μM)[a]

PFAS	PFAS individual (μg)	mix A	mix B
PFBA	ND		ND
PFBS	ND		ND
PFHxA	ND		ND
PFHxS	0.003 +/−0.0007		0.001 +/−0.002
PFOA	0.018 +/−0.005	0.009 +/−0.004	0.003 +/−0.002
PFOS	0.034 +/−0.006	0.025 +/−0.004	0.009 +/−0.001
PFDA	0.0203 +/−0.0003		
PFDS	0.95 +/−0.01		

[a]Values are given in μg/mg.

in each individual treatment and in Mix A and B. It was observed that with a 24 h exposure, the amount of PFASs associated with the cells correlated with chain length, with the eight- and ten-carbon-chain PFASs exhibiting higher cellular levels relative to the four and six carbon chain PFASs (Table 4). The apparent positive relationship between PFAS chain length and cell uptake may be attributed to the stronger interactions of the longer hydrophobic tails with the cell membrane.[37] Variation of uptake by headgroup was observed as well, where sulfonates have higher association than carboxylate given the same length of carbon chain. Similar trends were observed in the cells treated with mixes A and B. In Mix A and B, PFOS was higher than PFOA, and both were about half and a sixth of what was accumulated with the respective individual treatments (0.018 μg PFOA vs 0.009 μg PFOA in mix A vs 0.003 μg in mix B: Table 4). Mix B also showed longer-chain-length PFAS species having a higher association, and alternatively, smaller-chain-length PFAS not detected at all. These observations show that both chain length and headgroup play an important role in the cellular association of PFASs.

Next, the differences in the levels of hormones in the cell culture media produced by the H295R cells treated with 1 μM PFBA, PFBS, PFHxA, PFHxS, PFOA, PFOS, PFDA, PFDS, Mix A, and Mix B for 24 h were analyzed using ANOVA.[38] Figure 8 represents the relative levels of hormones for each treatment relative to the control cells (control), illustrated as a heat map (see Table S3 for hormone levels normalized to the control cells, $n = 3$). Based on previous literature and our total PFAS analysis in cell lysates, we anticipated some changes in total hormone expression after the 24 h treatment with a 1 μM test compound, especially in the cells that showed a higher cellular association with PFASs.[9−11] However, only a few of the hormone species showed significant changes (P-adjusted <0.05) in all treatments. First, DHT, cortisol, aldosterone, and estriol were not detected in any of the treated samples. Progesterone showed a significant increase of up to 2× higher than the control in PFDA-treated cells (P-adjusted <0.05). Pregnenolone shows an increase of up to 4× higher than the control in cells treated with PFHxA (P-adjusted <0.01). Lastly, 21-hydroxy-progesterone showed significant increases with PFBS (P-adjusted <0.05), PFOS (P-adjusted <0.01), PFDA (P-adjusted <0.001), and PFDS (P-adjusted <0.0001) treatments. All other changes observed as compared to the control were not significant (P-adjusted >0.05).

Analysis of the heat maps showed additional trends in hormone subgroups. Some androgens (testosterone, DHEA, and androstenedione) showed slight depletions compared to the control group. Progestogens (pregnenolone, 17OH pregnenolone, progesterone, 21OH progesterone, and 17OH progesterone) showed the highest degree of accumulation in this study. Specifically, progesterone and its two hydroxy derivatives showed accumulation when treated with longer-chain-length PFAS. Corticosteroids (corticosterone, 11-deoxycortisol, cortisone) and estrogens (estrone and estradiol) showed clear trends of depletion; however, these changes were not statistically significant (P-adjusted >0.05).

Interestingly, we observed increases in pregnenolone and progesterone upon treatments with PFHxA and PFDA, respectively. We earlier discussed a study, which showed increases in progesterone with high PFOA concentration (100 μM) and did not investigate the mechanisms that result in the modulation of progesterone levels.[10] However, other studies have shown decreases in pregnenolone and progesterone levels with PFOS exposure. One study showed that 1 μM treatments of PFOA in Theca and Granulosa cell lines (obtained from ovarian tissues) reduced progesterone, showing their role as endocrine disruptors even at low concentrations. However, similar to other studies, the mechanism of action was not discussed.[39] A second study showed *in vivo* effects of 5 mg/kg exposure of PFOA on mice and also found decreased total expression of progesterone and pregnenolone.[40] This study showed perturbed expression of *Cyp19a1*, the gene encodes for aromatase, an enzyme that transforms testosterone into estradiol. These results suggest that if aromatase activity is reduced or blocked by PFOA/PFOS, a decrease in estrogens would be observed and an increase in testosterone would occur. Based on these observations, in our current study, it is possible that progesterone and pregnenolone accumulate with PFAS treatment as a result of the inactivation of upstream enzymes involved in this pathway (Figure 2). Additional studies are needed to fully understand the mechanism of these changes caused by PFASs.

While our data shown detections for 13 of the 17 hormone our method can analyze for, it is important to discuss why DHT, aldosterone, cortisol, and estriol were not detected. A literature search of past experiments that quantified hormone abundances in H295R cell line gives an approximate idea of expected hormone levels, but various factors, including cell passage number or media composition, could affect total levels. Many hormones which we detect have been measured between 0.07 and 15 ppb. These include testosterone, DHEA, pregnenolone, progesterone, corticosterone, estrone, and estradiol.[41−43] Aldosterone has been reported at 0.06 ppb using radiolabeled analogue. This low abundance could explain why we did not detect this analyte.[42] Cortisol has been reported at 5 ppb, above our limit of detection, so it is possible that cortisol levels in our samples are lower than what has been reported.[43] We were unable to find existing data on DHT and estriol detections in other systems.

In the future, to further assess the endocrine-disrupting effects of PFASs, different conditions should be tested such as varying PFAS concentrations, prolonged exposures, and more intricate mixes. The mixes would be composed of different legacy and novel PFASs, including carboxylates, sulfonates, sulfonamides, fluorotelomers, and ethers. For example, while

https://doi.org/10.1021/acs.chemrestox.2c00116
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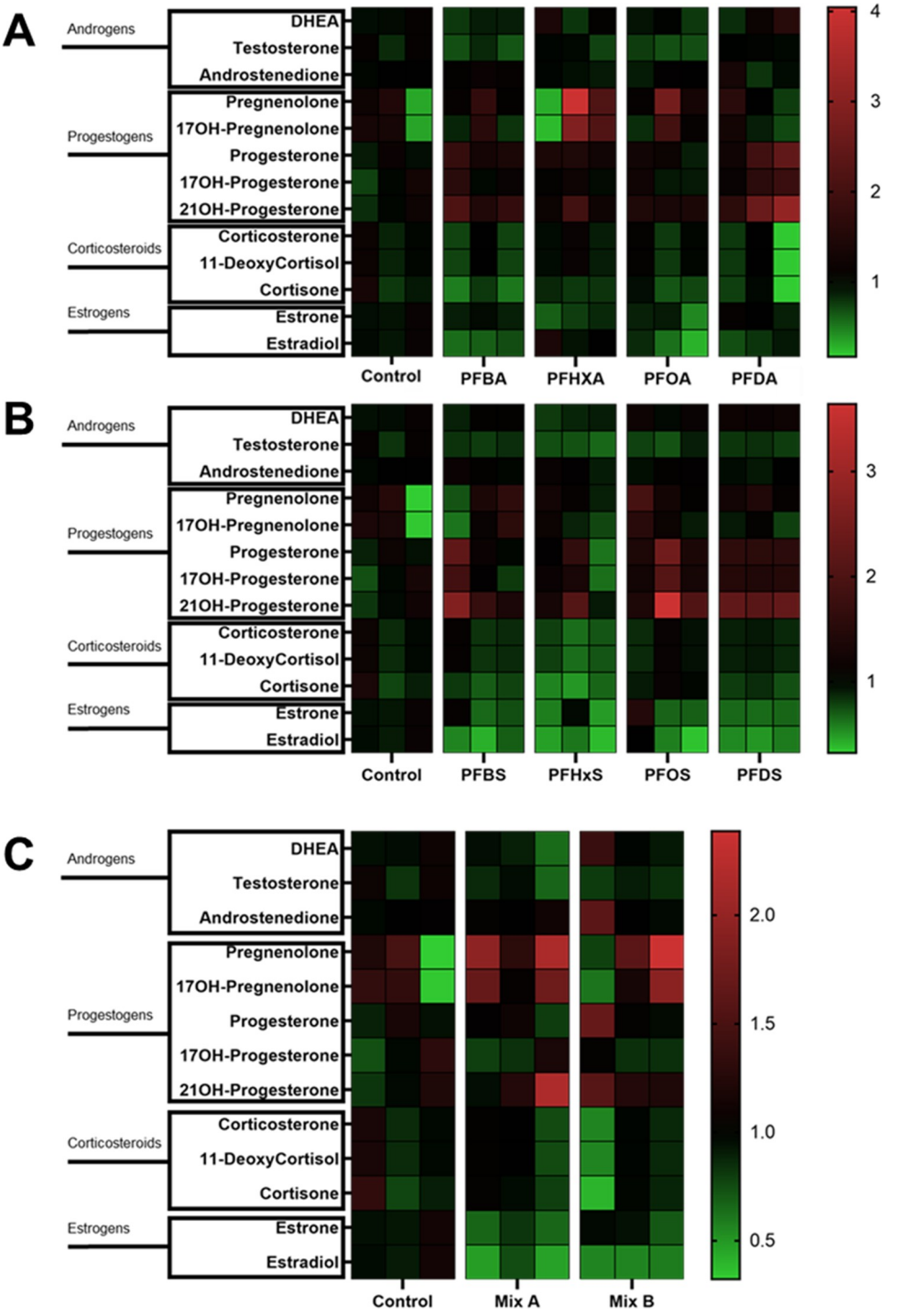

Figure 8. Heat maps produced in GraphPad Prism showing relative changes in hormones produced by H295R cells after 24 h treatment with (A) carboxylates; 1 μM of PFBA, PFHxA, PFOA, and PFDA; (B) sulfonates; 1 μM of PFBS, PFHxS, PFOS, and PFDA; (C) mixes; mix A and mix B. Heat maps are designated by hormone groups; androgens, progestogens, corticosteroids, and estrogens.

PFASs studied in this work are relevant due to their persistence in the environment, the results did not consider the effects of other PFAS structures such as the fluorotelomers, sulfonamides, and ether-containing PFASs. Evaluation of these compounds individually, as well as in mixtures, could further elucidate the endocrine-disrupting effects of PFASs at more environmentally relevant concentrations. The LC-MS/MS method we presented in this study will be a valuable tool in studying the endocrine-disrupting effects of PFASs and other contaminants found in the environment.

CONCLUSIONS

The LC-MS/MS method developed for the 17 hormones tested in this study has higher sensitivity relative to existing protocols because of the increased ionization efficiencies of the derivatized hormones under positive electrospray ionization mode. The LODs achieved for the MOA-derivatized hormones ranged from 0.13−2.89 ppb. For the DC-derivatized hormones, the LODs ranged from 0.07−0.92 ppb. Overall, the advantages of the method reported in this study are: (a)

https://doi.org/10.1021/acs.chemrestox.2c00116
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the detection of a broad range of hormones at low concentrations (i.e., ppt), with increased sensitivity upon a 10-fold concentration factor, and (b) the ability to discern biological changes in hormone levels in H295R cells. The induction of hormones was detected in H295R cells exposed to forskolin, while depletion in hormone levels was observed when cells were exposed to prochloraz. The results from this study provide evidence of the applicability of this sensitive LC-MS/MS method to detect subtle changes in hormones to assess endocrine disruption in cells. This rapid LC-MS/MS-based hormone assay can be used in the detection of a wide range of hormones and can be applied in the studies of suspected endocrine disruptors such as PFASs in a cell-based assay.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.2c00116.

MS/MS parameters for PFAS analysis; average detections of each hormone relative to PFAS treatment; extraction recovery for PFASs; solid-phase extraction recovery study for derivatized hormones; PFAS removal using solid-phase extraction; mechanism of hormone derivatization reactions; optimization of DC reaction time; optimization of MOA reaction time; effect of reagent concentration on MOA derivatization; effect of pH on MOA reaction efficiency; and MTT viability assay for prochloraz and forskolin, 8 PFAS compounds, and two mixes (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors gratefully acknowledge the support from the National Science Foundation (NSF) (CBET-2112201). Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the NSF.

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https://doi.org/10.1021/acs.chemrestox.2c00116
Chem. Res. Toxicol. 2022, 35, 1277−1288