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# **Analysis of steroids in urine by gas chromatography-capillary photoionization-tandem mass spectrometry**

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## **Abstract**

A new heated capillary photoionization (CPI) ion source design was developed to photoionize analytes inside a transfer capillary between a gas chromatograph (GC) and a mass spectrometer (MS). The CPI setup included a wide, oval-shaped vacuum-ultraviolet (VUV) transparent magnesium fluoride (MgF<sub>2</sub>) window to maximize photoionization efficiency and thus sensitivity. The source contained a nitrogen housing around the ionization chamber inlet to avoid undesirable hydrolysis and oxidation reactions with ambient air and to maximize the proportion of formed molecular radical cations of analytes. The feasibility of the ion source was studied by analyzing 18 endogenous steroids in urine as their trimethylsilyl (TMS) derivatives with gas chromatography-tandem mass spectrometry (GC-MS/MS). The method was validated and applied to human urine samples. To our best knowledge, this is the first time that a capillary photoionization ion source has been applied for quantitative analysis of biological samples. The GC-CPI-MS/MS method showed good chromatographic resolution (peak half-widths between 3.1 to 5.3 s), acceptable linearity (coefficient of determination between 0.981 to 0.996), and repeatability (relative standard deviation (RSD%) between 5 to 18%). Limits of detection (LOD) were between 2 to 100 pg mL<sup>-1</sup> and limits of quantitation (LOQ) were between 0.05 to 2 ng mL<sup>-1</sup>. In total, 15 steroids were quantified either as a free steroid or glucuronide conjugate from the urine of volunteers. The new CPI source design showed excellent sensitivity for analysis of steroids in complex biological samples.

## **Keywords**

Steroid; capillary photoionization; gas chromatography; tandem mass spectrometry; urine; dopant-assisted photoionization

## 1. Introduction

Steroids are hydrophobic compounds synthesized from cholesterol through enzymatic reactions via pregnenolone to other endogenous steroids [1]. Synthesis occurs in steroidogenic tissues (such as adrenal glands, gonads, and placenta) and in the central nervous system [1]. Steroids have multiple biological functions, particularly in the cell membrane and in cell signaling, inflammation, immune functions, salt and water homeostasis, pregnancy, and sexual characteristics [1, 2]. Steroids can be classified based on the number of carbons in their molecular structure (estrans [C<sub>18</sub>], androstanes [C<sub>19</sub>], pregnanes [C<sub>21</sub>]), or molecular function (glucocorticoids, mineralocorticoids, progestogens, androgens, and estrogens). Endogenous steroid concentrations in biological samples are important in clinical diagnostics of disorders and treatment of endocrine diseases [3-5]. Androgenic anabolic steroids (AAS) are widely used as doping agents in sports and even among non-competing amateurs to increase performance. As the use of AAS is prohibited by the World Anti-Doping Agency (WADA) [6] and steroids have important biological roles and potential in drug therapeutics, analytical methods for monitoring steroid levels are essential.

Urine, plasma, and serum have been primary samples in clinical and doping control analyses [4]. Urine is a non-invasive biofluid that is easy and painless to collect in large amounts. Steroids are excreted in urine (as free or in conjugated form) and thus makes it a suitable matrix for steroid analysis [5]. High sensitivity, accuracy, and selectivity are necessary as steroid concentrations in urine are usually low and variable (in the range of pg mL<sup>-1</sup> to ng mL<sup>-1</sup>) [7], steroids have isomeric structures, and the complex matrix may interfere with the analysis [4]. Steroids have been classically screened using immunoassays (IA), but these assays have been partly replaced by mass spectrometric methods due their superior specificity and sensitivity [4]. Traditional mass spectrometric steroid analyses have utilized gas chromatography-mass spectrometry (GC-MS) [4, 8]. Liquid chromatography-mass spectrometry is also widely used today [3, 4]. The three most important atmospheric pressure ionization (API) techniques, namely electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI) have all been applied in steroid analysis with LC-MS [9-11]. In ESI, the ionization efficiency of non-polar steroids is quite low and ESI can suffer from ion-suppression effects [12]. However, ionization efficiency can be improved with derivatization [13] and ESI has the advantage of permitting analysis of intact conjugates like glucuronides and sulfates [14, 15]. On the other hand, APPI has shown better sensitivity for non-polar compounds (such as steroids) [16, 17], has a wider

dynamic range [16], and has less matrix effects [17], although in APPI (and APCI) steroid conjugates are usually cleaved during the ionization process [18].

The popularity of GC-MS in steroid analysis relies on its resolving power (which exceeds that of LC), high peak capacity, and ability to separate steroid isomers [19]. GC-MS is also more robust, simple to use, easy to optimize, and uses a single mobile phase separation [19]. A drawback of GC-MS is the laborious sample pretreatment with need for chemical derivatization (usually with TMS for non-volatile compounds) and enzymatic hydrolysis prior to steroid analysis. Electron ionization (EI) is a commonly used ionization technique in GC-MS. EI typically results in extensive, characteristic fragmentation and the spectra can be searched against EI-MS spectral libraries. However, the fragmentation decreases sensitivity, selectivity, and may eliminate the formation of molecular ions, which are important for identification of analytes. It has been demonstrated, that the fragmentation in EI can be decreased by using ionization energy below the most commonly applied 70 eV, and still maintain the characteristic fragments needed for library matching [20, 21]. Even more effective approach to overcome the fragmentation is to connect the GC with MS using soft chemical ionization methods or API methods, such as APCI, APPI, ESI, and atmospheric pressure laser ionization (APLI) [19]. Use of e.g. triple quadrupole, quadrupole-time of flight, or ion trap MS enables the selective fragmentation of the molecular ion for reliable identification. The fragmentation of radical cations in MS/MS experiments follows the odd-electron fragmentation pattern and similar fragments are obtained as in EI-MS, enabling the usage of EI-spectral libraries [22]. When using API sources, both GC and LC can be connected to the same mass spectrometer equipped with an atmospheric pressure to vacuum ion optics. Expensive separate mass spectrometers for GC- and LC-MS analyses are therefore not needed.

GC-APCI-MS and GC-APPI-MS have shown to be potential methods for steroid analysis [22-25]. These methods provide good sensitivity, a gentle ionization process, and produce intense molecular ions or protonated molecules with minimal fragmentation. However, in conventional APCI and APPI, analytes are ionized in free space between the sprayer and MS inlet, and a part of the ions are lost before entering the vacuum of the mass spectrometer [26]. Consequently, ion transmission and sensitivity in conventional APCI or APPI is not maximal. Kersten *et al.* were the first to introduce photoionization inside a transfer capillary between the atmospheric pressure and vacuum with a system having a custom miniature VUV lamp inside the transfer capillary [27]. While the source showed good sensitivity for volatile compounds, it was not heated and was thus unsuitable for non-volatiles. Our group has also previously presented a

CPI method, which uses a direct interface for GC-MS and a photoionization method with high transmission efficiency [28]. Our CPI source is heated and VUV light enters the capillary through a VUV-transparent MgF<sub>2</sub> window. Recently, Mirabelli and Zenobi used a similar heated capillary photoionization source and interfaced it with solid-phase microextraction sampling [29]. In this study, we developed a new CPI source design with a wide opening under a MgF<sub>2</sub> window and an inert nitrogen atmosphere in the ionization chamber to increase sensitivity. Although the potential of capillary photoionization has been shown with standard solutions [27-29], thus far no studies have demonstrated the feasibility and validation of the method in quantitative analysis of complex biological or environmental samples. Here we studied the feasibility of a CPI ion source to analyze endogenous steroids in human urine with GC-CPI-MS/MS. A panel of 18 steroids representing various steroid structures and all steroid classes were chosen for the study, and their levels in free and glucuronide conjugate form were studied.

## 2. Materials and methods

### 2.1. Chemicals

The standard compounds 17 $\alpha$ -hydroxypregnenolone (17-OH-PREG,  $\geq 97\%$ ), 17 $\alpha$ -hydroxyprogesterone (17-OH-PROG,  $\geq 95\%$ ), 21-hydroxyprogesterone (21-OH-PROG,  $\geq 97\%$ ), aldosterone (A,  $\geq 95\%$ ), corticosterone (CORT,  $\geq 92\%$ ), cortisone (CS,  $\geq 98\%$ ), dehydroepiandrosterone (DHEA,  $\geq 99\%$ ), estrone (E1,  $\geq 99\%$ ),  $\beta$ -estradiol (E2,  $\geq 98\%$ ), estriol (E3,  $\geq 99\%$ ), etiocholanolone (ETIOL,  $\geq 98\%$ ), hydrocortisone (HC,  $\geq 98\%$ ), pregnenolone (PREG,  $\geq 98\%$ ), progesterone (PROG,  $\geq 99\%$ ), testosterone (T,  $\geq 98\%$ ), androsterone (ADT, 98.2%), and 17 $\alpha$ -methyltestosterone (Me-T, 99.5%) were from Sigma Aldrich (Steinheim, Germany). Androstenedione (AN,  $\geq 97\%$ ) was from Steraloids Inc. (Newport, RI, USA) and 11 $\alpha$ -hydroxyprogesterone (11-OH-PROG, 99.1%) from Santa Cruz Biotechnology (Dallas, TX, USA). Standard stock solutions (1 mg mL<sup>-1</sup> or 2 mg mL<sup>-1</sup>) of the steroids and their dilutions were prepared in methanol. The structures, trivial names, full names, abbreviations, steroid classes, molecular masses, monoisotopic masses, and molecular formulas of all analyzed steroids are presented in supplemental material (Table S1).

Methanol (LC-MS chromasolv®), heptane (HPLC-grade), chlorobenzene (HPLC-grade), diethyl ether (chromasolv® HPLC-grade), sodium dihydrogen phosphate ( $\geq 99.0\%$ ), sodium sulfate (99.0%),  $\beta$ -glucuronidase (*E. coli*, EC 3.2.1.31), N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, for GC-

derivatization  $\geq 98\%$ ), ammonium iodide (99.999 %) ( $\text{NH}_4\text{I}$ ), dithioerythritol (99.0 %) (DTE), potassium carbonate (99.7 %) ( $\text{K}_2\text{CO}_3$ ), and sodium hydrogen carbonate (99.5-100.5 %) ( $\text{NaHCO}_3$ ) were from Sigma Aldrich.

Artificial urine was made in-house and contained  $14 \text{ g L}^{-1}$  urea,  $1.7 \text{ g L}^{-1}$  diammonium hydrogen phosphate,  $700 \text{ mg L}^{-1}$  creatine,  $700 \text{ mg L}^{-1}$  glycine,  $700 \text{ mg L}^{-1}$  DL- $\alpha$ -alanine,  $700 \text{ mg L}^{-1}$  oxalic acid,  $350 \text{ mg L}^{-1}$  bovine serum albumin (all from Sigma Aldrich),  $350 \text{ mg L}^{-1}$  glucose (British Drug House Ltd., England), and  $250 \text{ mg L}^{-1}$  sodium chloride (Merck, Germany) in water purified with a Milli-Q system (Merck Millipore, Molsheim, France).

## 2.2. Samples and sample pretreatment

Artificial urine was used as a sample matrix in method development and validation. Samples (2.5 mL) were spiked with 50  $\mu\text{L}$  of steroid standard solution mixture (concentration levels 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000  $\text{ng mL}^{-1}$  as final concentration). Authentic urine samples were collected from male and female volunteers. Urine samples from four males (26, 34, 38, and 49 years old) and four females (26, 31, 34, and 40 years old) were used to verify the suitability of the method for the analysis of steroids in an authentic matrix. Each authentic sample was divided into two aliquots; one aliquot was used to determine free steroids and the other aliquot was used to analyze the sum concentrations of steroid glucuronide conjugates and free forms. The concentration of glucuronide conjugate was obtained by subtracting the concentration of free steroid from the sum concentration.

The glucuronide-conjugated steroids were hydrolyzed before extraction by adding 0.8 M sodium dihydrogen phosphate solution (1 mL, pH 7) and glucuronidase (50  $\mu\text{L}$ ) followed by incubation at  $50^\circ\text{C}$  for 1.5 hours. After enzymatic hydrolysis, steroids were extracted and derivatized similarly as the free steroid fraction of urine and artificial urine samples.

17 $\alpha$ -methyltestosterone was used as an internal standard with a final concentration of  $10 \text{ ng mL}^{-1}$ . The sample pH (2.5 mL) was adjusted to approximately pH 8 by dissolving  $\text{NaHCO}_3/\text{K}_2\text{CO}_3$  (125 mg, 2:1, w $^{-1}$ ) mixture in each sample. After pH adjustment, steroids were extracted with LLE to diethyl ether; diethyl ether (4 mL) and  $\text{Na}_2\text{SO}_4$  (1.5 g) were added to samples, followed by vortexing and centrifugation at  $2000 \times g$  for 10 min. Part of the organic phase (2.5 mL) was transferred into a new test tube and

evaporated under nitrogen flow. The derivatization reagent mixture MSTFA/NH<sub>4</sub>I/DTE (50  $\mu$ L, 1000:2:4, v w<sup>-1</sup>w<sup>-1</sup>) was added to the test tubes, tubes were vortexed, and mixtures were transferred to GC vials. The samples were incubated at 60°C for 15 min to derivatize all hydroxyl and keto groups of steroids with TMS. After derivatization, samples were ready for injection into GC.

## 2.3. Instrumentation

### 2.3.1. Gas chromatography

The analytes were separated with an HP 5892 II gas chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a CTC-A200S autosampler (CTC Analytics, Zwingen, Germany). Chromatographic separation was obtained by combining the following two analytical columns: a TR-5MS (15 m, i.d. 0.25 mm, film thickness 0.25  $\mu$ m) and a TR-50MS (15 m, i.d. 0.25 mm, film thickness 0.25  $\mu$ m). Both columns were obtained from Thermo Scientific (Waltham, MA, USA). A methyl-deactivated fused-silica retention gap (2 m, i.d. 0.25 mm) was installed in front of the first column and a deactivated fused-silica transfer capillary (1 m, i.d. 0.15 mm) after the second column; both were obtained from SGE Europe Ltd. (Milton Keynes, UK). All capillaries and columns were connected with pressfit connectors from BGB Analytik AG (Boeckten, Switzerland). The carrier gas was 99.996% pure helium (AGA, Espoo, Finland) with column pressure of 140 kPa. The GC temperature program started with a 1-min isothermal period at 190°C, after which the temperature was increased to 250°C at a rate of 10°C min<sup>-1</sup>, from 250°C to 260°C at a rate of 1°C min<sup>-1</sup> and from 260°C to final temperature of 330°C at a rate 7°C min<sup>-1</sup>. Temperature was kept at 330°C for 3 min to clean the columns. The total runtime was 30 min. Samples (3  $\mu$ L) were injected with 1-min splitless injection mode, and the temperature of the injector was 250 °C.

### 2.3.2. Gas chromatograph-ion source interface

Inside the GC oven, the GC transfer capillary was introduced through a stainless steel (SS) T-piece into a SS transfer tube (i. d. 0.5 mm). The SS transfer tube was heated (250°C) and served as a transfer line between the GC and MS. A few millimeters of this transfer tube and of the transfer capillary were introduced inside the CPI inlet capillary. A mixture of auxiliary gas nitrogen (flow rate 80 mL min<sup>-1</sup>) and dopant chlorobenzene (flow rate 3  $\mu$ L min<sup>-1</sup>) entered the T-piece through 1/16 in o.d. SS tubing. The dopant was pumped into the T-piece by a Pump II Elite syringe pump from Harvard Apparatus (Holliston, MA, USA) and vaporized and mixed with auxiliary gas before the T-piece. Inside the SS T-piece, the

mixture was directed into the SS transfer tubing and was transferred coaxially with the transfer capillary into the CPI capillary. The setup is presented in Fig. 1.

The intersection of the transfer tube and CPI capillary was open to ambient air. MS vacuum system pumps approximately 1 L min<sup>-1</sup> in to the MS, which means that in addition to GC eluent and dopant, also ambient air flows to the CPI ion source from the inlet. Nitrogen atmosphere was created around the CPI inlet capillary and the GC transfer tube interface by enclosing them in an aluminium housing, which was sealed and insulated with aluminium foil wrapped around the housing. Nitrogen was introduced into the housing through Teflon tubing with a flow rate of 4.5 L min<sup>-1</sup>. The housing was not completely gas tight to allow the vacuum system of the MS produce equal gas flow to that achieved without the nitrogen housing. However, the excess nitrogen prevented most of the ambient air from entering the ion source.

### *2.3.3. CPI ion source*

The CPI ion source contained a SS CPI capillary (i. d. 1.5 mm) connected to a flat SS part with an oval-shaped opening (length 17 mm, width 8 mm at the widest point, height 3 mm) which was covered with a 3-mm thick MgF<sub>2</sub> window from Thorlabs Sweden AB (Gothenburg, Sweden). A SS top plate with an 18-mm circular opening and graphite rings used as seals held the MgF<sub>2</sub> window in place. The inlet where the analytes entered the ion source was heated (250°C) by a resistance wire heater driven by a DC power supply (ISO-TECH IPS603, RS Components, Northants, UK). The main body of the CPI device was heated (250°C) with a 160 W cylindrical heater (diameter 6.5 mm, length 50 mm, Oy Meyer vastus Ab, Askola, Finland) which was controlled by a PID500 temperature controller (Tempatron, Essex, UK). The heater was embedded in a cylindrical aluminium block (diameter 35 mm, height 55 mm) which was attached to the bottom of the CPI device. The photoionization was performed with 10.0 and 10.6 eV photons from an rf krypton discharge vacuum UV lamp (PKR 106, Heraeus Noblelight Analytics Ltd., Cambridge, UK).

### *2.3.4. Mass spectrometry*

The mass spectrometer was an Agilent 6410 triple quadrupole (Agilent, Santa Clara, CA, USA). The CPI device was connected to the mass spectrometer glass capillary inlet. Data acquisition was performed with MassHunter Workstation Data Acquisition for Triple Quad software (version B.03.01) from Agilent Technologies. Data processing was performed with MassHunter Workstation Qualitative Analysis software (version B.06.00) and Quantitative Analysis software (version B.05.00) from Agilent Technologies. Precursor ions of the steroids (standard solution of 50 ng mL<sup>-1</sup>) were determined in full



scan mode with scan range  $m/z$  150 to 750 and a scan time of 300 ms. In the MRM experiments, the applied dwell time for each steroid was either 100 ms or 150 ms, depending on the time segment.

## 2.4. Validation

The developed method was validated following the validation instructions of The International Council for Harmonisation (ICH) and identification criteria of WADA [30, 31]. LOD, LOQ, linear range, linearity, repeatability, LLE recovery, and carryover were studied. In total, 21 different concentration levels: 0.001, 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000 ng mL<sup>-1</sup> spiked to artificial urine were tested for determination of linearity, linear range, LOD, and LOQ. A suitable linear range was selected based on the following criteria: maximum residual from the curve below 20% or below 30% in the low limit of quantitation level and quantifier/qualifier ion ratio in the range instructed by WADA [31]. Steroids were quantified with an internal standard method and identification was based on retention time and ion ratios of the selected product ions. For the calibration curves, 1/x weighting was applied. For ADT, ETIOL, and CS, the linear range was determined additionally with standard solutions (in artificial urine) which were diluted after extraction and derivatization. These calibration curves were applied to analyze high urine concentrations from samples that were also diluted after extraction and derivatization. LOD was determined based on the signal-to-noise (S/N) ratio using criteria  $S/N > 3$  with spiked standard compounds in artificial urine. As the noise was nonexistent for some of the compounds, a three-fold signal compared to reagent blank was also required. The LOQ criteria was  $S/N > 10$ , maximum deviation from the calibration curve 30%, and quantifier/qualifier ratio in the range accepted by WADA [31]. Repeatability and LLE recovery were determined at a concentration level of 10 ng mL<sup>-1</sup> from six replicates. Recovery was determined by comparing the responses of spiked extracted samples to standard solution spiked at the same concentration level. Carryover was studied by analyzing a blank sample (extracted artificial urine) after analysis of extracted 200 ng mL<sup>-1</sup> standard solution (in artificial urine) and by calculating the relative ratio of peak area of blank compared to the peak area of the standard solution.

## 3. Results and discussion

### 3.1. CPI ion source development

A new CPI source with a wide, low-depth ionization chamber under the wide MgF<sub>2</sub> window was designed to maximize ionization efficiency using a commercially available photoionization lamp. Since the

diameter of the window of the applied VUV lamp is 13 mm, the wide surface area of the opening provides high photon flux into the source, ensuring high ionization efficiency and thus sensitivity. The low depth and oval shape of the ion chamber were designed to minimize dead volume and dead angles. In fact, the volume of the ionization chamber (<0.4 mL) is negligible compared to the total gas flow rate through the chamber (approximately 1 L min<sup>-1</sup>). The ionization chamber was heated with a 160-W cylindrical heater embedded in a cylindrical aluminium block. PID temperature control of the heater and thermal mass of the aluminium block ensured stable and sufficiently high temperature to avoid adsorptions of the analytes. Together, these features ensured that the design of the ionization chamber did not cause peak tailing, broadening, or carryover as also shown experimentally (Table 1 and Fig. S1 in supplementary data).

In this design, a nitrogen housing was built around the CPI source inlet to provide an inert gas environment to the CPI source. Because ionization and the used MS require larger gas flow (approximately 1 L min<sup>-1</sup>) than provided by the GC and the dopant vaporization system, without the housing ambient air would flow into the CPI source and atmospheric changes could affect the photoionization process. When nitrogen housing was utilized, abundant analyte radical cations ([M]<sup>•+</sup>) were formed and formation of other ions was minimal. Without the housing, the intensities of other ions, such as [M+H]<sup>+</sup>, [M-TMS]<sup>•+</sup>, [M-TMSOH]<sup>•+</sup>, and [M+O<sub>2</sub>]<sup>•+</sup> were increased as presented and discussed in the supplementary material.

### 3.2. GC-MS/MS method development

The MS spectra of the TMS-derivatized steroids were measured to select the precursor ions for MS/MS analysis. The most intensive ion for all the steroids except for CS and HC was [M]<sup>•+</sup>, which was selected as the precursor ion. The protonated molecule [M+H]<sup>+</sup> was not observed for any of the TMS-derivatized steroids taking into account the isotopic distribution of the [M]<sup>•+</sup> ion (Fig. S2, Fig. S3 in supplementary data). The low ionization energy of TMS-derivatized steroids favors a charge-exchange reaction and formation of [M]<sup>•+</sup>. On the other hand, the relatively low proton affinity of the TMS-derivatized steroids does not favor a proton-transfer reaction. These results are consistent with previous studies [22, 28]. For CS- and HC-TMS, the most intense ion was [M-TMSOH]<sup>•+</sup>, which was selected as a precursor ion for these compounds. For almost all steroids, the [M-TMS]<sup>•+</sup> ion was also seen as a minor peak in the MS spectra (Fig. S4 in supplementary data). The MS/MS spectra of steroid radical cations showed similar

non-specific fragments corresponding to the loss of CH<sub>3</sub> [M-15]<sup>+</sup>, TMSOH [M-90]<sup>+</sup>, CH<sub>3</sub> + TMSOH [M-105]<sup>+</sup>, CH<sub>3</sub> + 2xTMSOH [M-195]<sup>+</sup>, and [TMS]<sup>+</sup> peak with *m/z* 73 (Fig. S2 and Fig. S3 in supplementary data). Specific fragments from the ring structure were also observed for all steroids. For the MRM method, two of the most intense sufficiently specific fragment ions were selected and collision energies for the transitions were optimized (Table S2 in supplementary data). However, for ADT, ETIOL, and internal standard, less abundant ions were selected for MRM. For ADT and ETIOL, selectivity was a more crucial parameter than sensitivity, since concentrations of these steroids are high in urine [7].

Two GC columns (TR-5MS and TR-50MS, see chapter 2.3.1.) connected in tandem were used to improve the separation of isobaric steroids and isotopes of co-eluting compounds as described in our previous study [22]. All steroids could be unambiguously detected and identified based on chromatographic separation and selective MRM transitions. Retention times of the steroids varied between 13.38 and 26.05 min (Table 1 and Table S2 in supplementary data); separation is shown in Fig. 2. Chromatographic separation with the method was good, and retention time RSDs below 1%. Peaks were symmetric and narrow with peak half widths in the range of 3.1 to 5.3 s (Table 1.). This indicates that the dead volume of the ion source was sufficiently small. Also, the ion source and transfer line materials and temperatures were adequate to avoid any cold traps, which could cause peak tailing and broadening. All the applied MRM transitions, MS parameters, and measured retention times are presented in Table S2 in supplementary data.

### 3.3. Method validation

The method was validated in terms of recovery of LLE, LOD, LOQ, linearity, repeatability, and carryover (Table 1). The separate MRM chromatograms for steroids in standard solution (5 ng mL<sup>-1</sup>), in the free steroid fraction of urine (male 38 years), in the glucuronide fraction of urine (male 38 years), and in a blank sample (extracted artificial urine) are shown in Fig. S1 in supplementary data. LODs were in the equal range of 0.002 to 0.02 ng mL<sup>-1</sup> for most of the steroids, with exception of A, ADT, ETIOL, and HC with slightly higher LODs (0.05-0.1 ng mL<sup>-1</sup>). The higher LODs for ADT and ETIOL can be explained by the selection of less abundant but more selective ions for quantitation. LODs reported in the literature for GC-EI-MS analysis of steroids from urine have ranged from 0.5 to 20 ng mL<sup>-1</sup> for AAS [32, 33], 0.08 to 100 ng mL<sup>-1</sup> for endogenous androgens [34, 35], 5 to 20 ng mL<sup>-1</sup> for corticosteroids [36,

37], 0.001 to 0.4 ng mL<sup>-1</sup> for estrogens [35, 38], and 0.3 to 2 ng mL<sup>-1</sup> for progestogens [35]. GC-EI-MS/MS LODs for AAS have ranged from 0.02 to 100 ng mL<sup>-1</sup> [39]. With LC-MS the LODs have ranged 0.1 to 10 ng mL<sup>-1</sup> for AAS [11] and 0.02 to 113 ng mL<sup>-1</sup> for corticosteroids [36, 37], and LOQs for estrogens ranged from 0.02 to 0.05 ng mL<sup>-1</sup>[38, 40]. LODs and LOQs of our GC-CPI-MS method were mainly lower than values reported previously in the literature [32-40], thus showing high sensitivity of the method. For estrogens our LODs and LOQs were slightly higher or in the same range as with GC-EI-MS reported previously [35, 38]. Considering that the measurements were performed with an over 10-year-old mass spectrometer, even higher sensitivity could be achieved if a modern mass spectrometer was used.

Repeatability of all steroids was reasonable with RSD values varying between 5 to 18% for six replicates at the level 10 ng mL<sup>-1</sup>. These RSD values were on the same range as reported previously for GC-EI-MS systems.[33-35] Linearity was acceptable with a coefficient of determination (R<sup>2</sup>) varying between 0.981 to 0.996. The linear range covered 2 to 4 orders of magnitude in the range 0.05 to 200 ng mL<sup>-1</sup>. Linear range coverage, linearity, and R<sup>2</sup> values showed similar analytical performance as obtained with GC-EI-MS systems [33-36]. LLE recoveries were in the range of 57.3 to 123.7%. Carryover was below 0.01% for all analytes, and only E2 showed some residual response in the blank sample above LOD but below LOQ.

### **3.4. Application to human urine samples**

The method was applied to analyze the urine of eight volunteers (4 females 26-40 years old and 4 males 26-49 years old) (Table 2 and Fig. S1 in supplementary data). Although sampling, normalization, circadian rhythm, menstrual cycle phase, and biological variation between individuals, and detection of different conjugates largely affect the measured urine concentrations of steroids, our data is in agreement with literature. As expected, concentrations of most of the steroid glucuronide conjugates were higher or at the same level compared to the respective free steroids, and only 11-OH-PROG and 17-OH-PROG showed levels below LOQ in all samples also as glucuronide conjugates (Table 2). The glucuronides of ADT and ETIOL are the main endogenous metabolites of testosterone and were excreted in urine at high concentrations (100-2000 ng mL<sup>-1</sup>) as also reported previously (median ADT 2260 ng mL<sup>-1</sup> and ETIOL 1850 ng mL<sup>-1</sup> in males, ADT 1470 ng mL<sup>-1</sup> and ETIOL 1480 ng mL<sup>-1</sup> in females)[7]. The detected concentrations of DHEA glucuronide were in the range of 10 to 40 ng mL<sup>-1</sup>; the mean value reported in

the literature in a study of over 3000 subjects was approximately 35 ng mL<sup>-1</sup>[7]. The corticosteroids CS and HC had the highest observed free-form concentrations (10-100 ng mL<sup>-1</sup>), being at the same level as their glucuronide conjugates. The average corticosteroid concentrations previously reported in the free form and glucuronide conjugate after hydrolysis with β-glucuronidase were 23.2 ng mL<sup>-1</sup> and 22.3 ng mL<sup>-1</sup> for hydrocortisone and 42 ng mL<sup>-1</sup> and 46 ng mL<sup>-1</sup> for cortisone, respectively [41]. The data showed clear differences in male and female samples regarding sex steroids; testosterone was higher in males (mean 16.9 ng mL<sup>-1</sup> in males, 1.8 ng mL<sup>-1</sup> in females) and estrogens were higher in females (>2 fold difference in the mean values), as also reported in the literature [7, 35, 40]. The detected mean estrogen glucuronide concentrations for females were 4.3 ng mL<sup>-1</sup> for E1, 1.4 ng mL<sup>-1</sup> for E2, and 3.9 ng mL<sup>-1</sup> for E3; these are consistent with the estrogen concentrations detected previously from premenopausal women (median 7 ng mL<sup>-1</sup> for E1, 2.5 ng mL<sup>-1</sup> for E2, and 7.8 ng mL<sup>-1</sup> for E3) [38]. Additionally, small concentrations of AN and CORT were detected in the free form and 17-OH-PREG, 21-OH-PREG, A, AN, CORT, and PREG as glucuronide conjugates.

Our results also agree with results obtained with open GC-APPI-MS-interface previously used in our laboratory with the same mass spectrometer [22]. However, the LODs were approximately one order of magnitude lower with the CPI as with the previous open APPI interface. With CPI we detected the free forms of DHEA and CORT (Fig. 3) and the glucuronides of 17-OH-PREG, CORT (Fig. 3), and A, which were not detected with the open APPI interface. These results show that CPI provides very high sensitivity and good quantitative performance for the analysis of steroids in urine samples.

#### 4. Conclusions

A new capillary photoionization (CPI) source design was developed and its quantitative performance evaluated in the analysis of selected steroids in urine. This is the first time that a CPI-type ion source has been applied in the quantitative analysis of a large analyte group from a real biological matrix. The developed GC-CPI-MS/MS method showed high chromatographic resolution, high sensitivity, specificity, and analytical performance to quantify steroids from human urine samples. Sensitivity of the system for analysis of steroids was better compared to GC-EI-system and it may be still improved with analyte specific optimization. This study shows that CPI source has a potential for analysis of non-polar compounds with high sensitivity from complex biological matrices and encourages to utilize CPI in more advanced GC applications, which might be challenging by EI-MS. We showed that the CPI ion source produces efficiently abundant steroid [M]<sup>+</sup> by charge-exchange reaction and formation of [M+H]<sup>+</sup> by

proton-transfer as well as fragmentation is minimal. This, and the possibility to combine GC with hyphenated high resolution API mass spectrometers via CPI introduces a possibility to use GC for non-targeted analysis to discover elemental compositions of unknowns. As there is some evidence in literature that the fragmentation of radical cations follows the same odd-electron fragmentation pathways as in EI, it might still be possible to use EI-spectral libraries for identification of MS/MS spectra. Furthermore, GC-EI-MS is limited to low carrier gas flow rates (1-2 mL min<sup>-1</sup>) due to the vacuum of ionization chamber. With CPI interface, carrier gas flow rates are not limited, which is expected to enable fast GC with wide or mega pore columns and large injection volumes.

### **Acknowledgment**

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### **Appendix A. Supplementary data**

The Supplementary data to this article is available at:

### **Notes**

Author Contributions: All authors have given approval to the final version of the manuscript.

The authors declare no competing financial interests.

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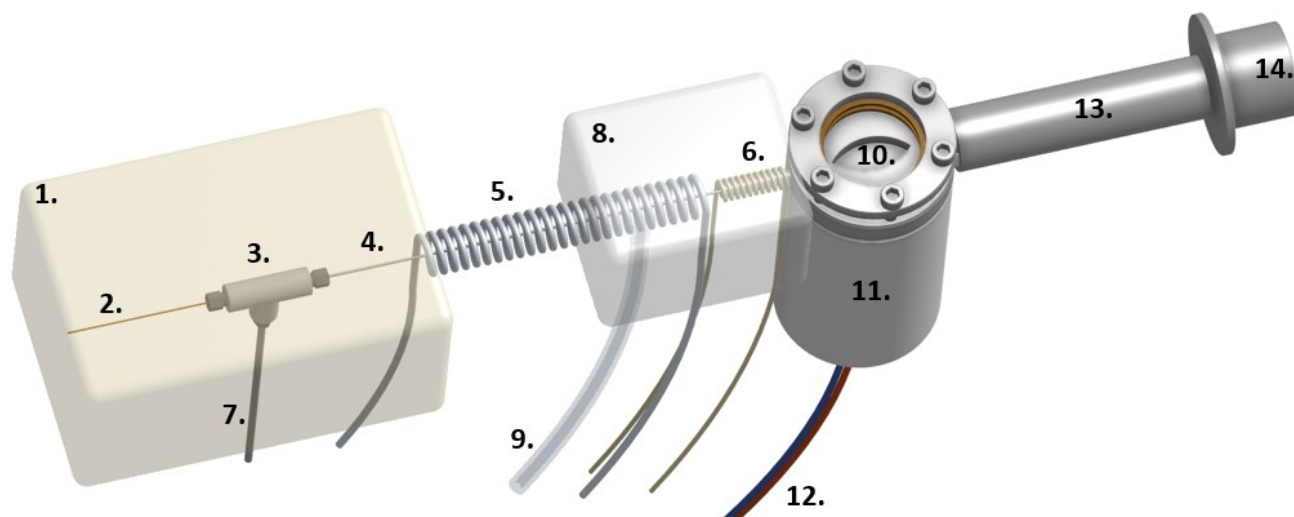
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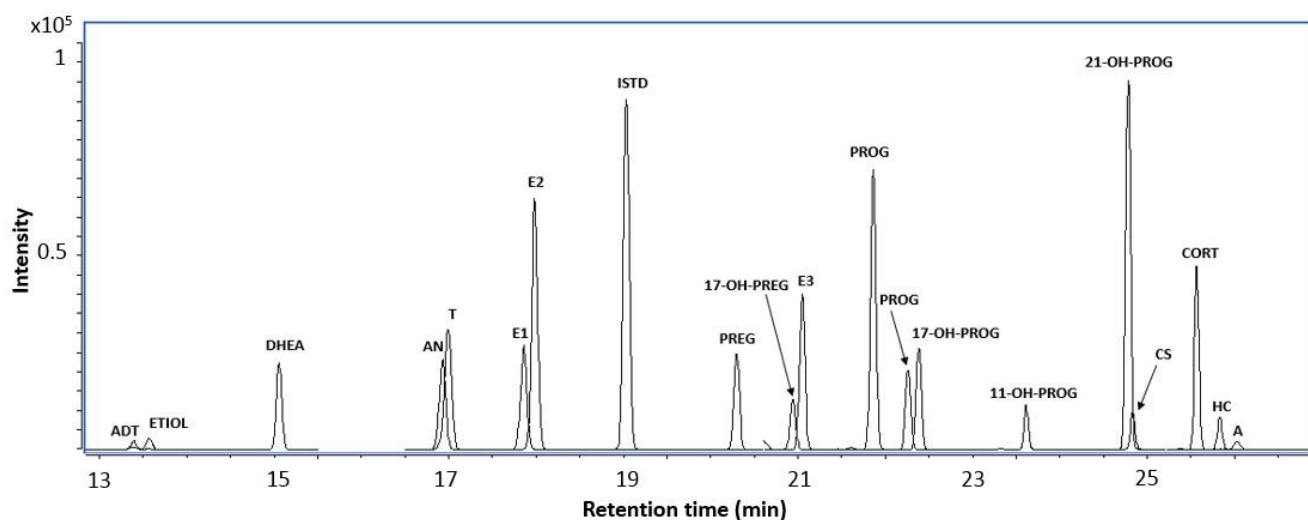


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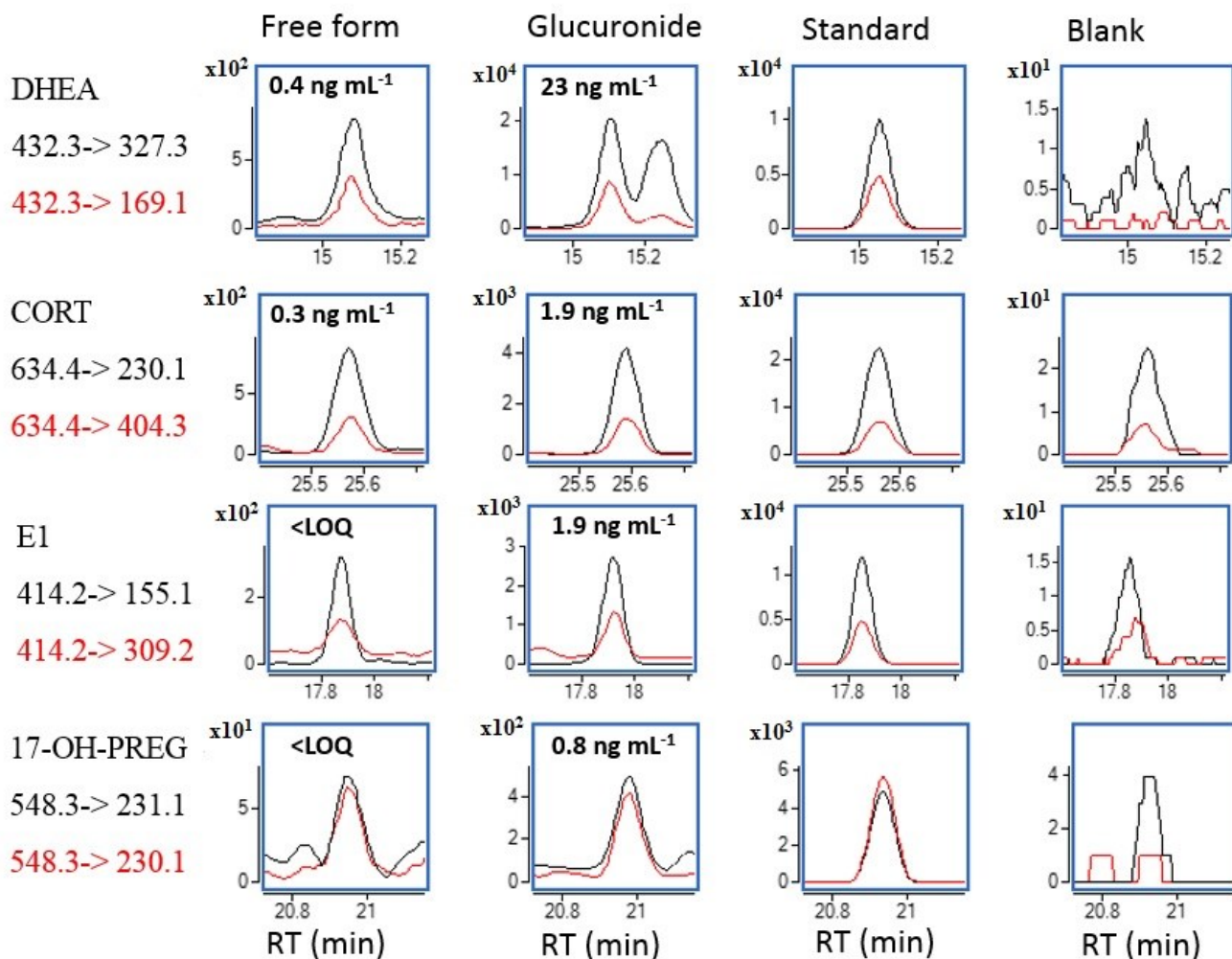
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**Fig. 1.** 3D-schematic of the GC-CPI ion source setup (available also as 3D-PDF). Parts marked in the figure; 1.GC oven, 2. GC transfer capillary, 3. SS T-piece, 4. SS transfer tube, 5. Heated transfer line, 6. Heated CPI inlet capillary, 7. Dopant and nitrogen gas line, 8. Nitrogen atmosphere in an aluminium housing, 9.Nitrogen flow through Teflon tubing, 10. Oval-shaped opening under MgF<sub>2</sub> window, 11. Heated aluminium block, 12. Temperature control of cylindrical heater 13. CPI capillary, 14. Inlet into mass spectrometer. Parts 5. and 8. were insulated with aluminium foil.



**Fig. 2.** Overlaid MRM chromatograms of quantifier ions of the studied steroids analyzed from 10 ng mL<sup>-1</sup> standard solution spiked in artificial urine.



**Fig. 3.** DHEA, CORT, E1, and 17-OH-PREG MRM chromatograms (quantifier as black and qualifier as red) in a male (38y) urine sample from free steroid and glucuronide fractions, standard compound (5 ng mL<sup>-1</sup>) spiked in artificial urine and blank sample. Note that different intensity scales are used for clear visualization

**Table 1.** Chromatographic peak parameters and validation results for steroids; chromatographic peak full width at half maximum (FWHM), symmetry factor, linear range, R<sup>2</sup> of calibration curve, LOD, LOQ, repeatability RSD% (n=6), LLE recovery %. \*From diluted standard solutions.

Steroid	RT (min)	RT RSD (%)	FWHM (s)	Symmetry factor	LOD (ng mL <sup>-1</sup> )	LOQ (ng mL <sup>-1</sup> )	Linear range (ng mL <sup>-1</sup> )	R <sup>2</sup>	Repeatability (n= 6)	Recovery (%)
11-OH-PROG	23.61	0.07	3.12	1.04	0.02	0.2	0.2-50	0.991	11.6	82.1
17-OH-PREG	20.93	0.07	4.50	1.01	0.01	0.1	0.1-100	0.988	12.9	122.1
17-OH-PROG	22.38	0.07	3.96	0.96	0.01	0.5	0.5-100	0.990	10.2	123.7
21-OH-PROG	24.77	0.05	3.84	1.12	0.002	0.2	0.2-100	0.995	5.0	62.9
A	26.02	0.04	5.28	1.25	0.1	2	2-200	0.992	12.6	70.3
ADT	13.38	0.20	4.62	1.18	0.1	2	2-100	0.988	8.5	70.2
ADT*							100-2000	0.994		
AN	16.92	0.14	5.10	0.91	0.02	0.5	0.5-50	0.992	7.5	79.4
CORT	25.56	0.04	3.42	0.92	0.01	0.2	0.2-20	0.993	11.1	83.4
CS	24.82	0.05	3.78	1.02	0.01	0.5	0.5-100	0.995	17.6	75.4
CS*							100-1000	0.997		
DHEA	15.05	0.13	3.78	1.00	0.01	0.05	0.05-100	0.996	5.3	75.7
E1	17.85	0.11	4.80	1.13	0.01	0.1	0.1-100	0.994	10.3	72.6
E2	17.98	0.11	4.74	0.93	0.01	0.1	0.1-100	0.995	10.3	73.6
E3	21.04	0.07	4.26	1.13	0.005	0.1	0.1-100	0.989	5.7	81.0
ETIOL	13.56	0.23	4.50	0.97	0.05	0.1	0.1-100	0.996	9.0	75.4
ETIOL*							100-2000	0.997		
HC	25.82	0.04	3.60	0.90	0.05	1	1-100	0.981	18.0	57.3
PREG	20.29	0.07	4.26	0.96	0.01	0.5	0.5-20	0.990	13.6	64.0
PROG	21.85	0.09	4.32	0.93	0.02	0.1	0.1-100	0.995	5.5	64.3
T	16.99	0.12	5.22	0.94	0.02	0.5	0.5-100	0.996	8.3	70.6

**Table 2.** Concentrations of analyzed steroids in urine of 8 volunteers either in the free or in the glucuronide-conjugated form. Concentrations of glucuronide conjugates were obtained by subtracting free steroid concentration from the concentration of total steroid after enzymatic hydrolysis. All values are given in ng mL<sup>-1</sup>. <LOQ corresponds to signal above LOD and below LOQ.

	11-OH-PROG	17-OH-PREG	17-OH-PROG	21-OH-PROG	A	ADT	AN	CORT	CS	DHEA	E1	E2	E3	ETIOL	HC	PREG	PROG	T
<b>Free fraction</b>																		
Male 26 y	-	-	<LOQ	-	-	-	0.9	-	18	0.3	<LOQ	-	-	<LOQ	2.7	<LOQ	-	<LOQ
Male 34 y	-	<LOQ	<LOQ	-	-	-	1.3	0.2	47	1.7	<LOQ	-	<LOQ	1.9	37	<LOQ	-	<LOQ
Male 38 y	-	<LOQ	<LOQ	-	-	-	1.1	0.3	66	0.4	<LOQ	-	<LOQ	4.9	38	<LOQ	-	<LOQ
Male 49 y	-	<LOQ	<LOQ	-	-	-	0.9	0.3	87	0.2	<LOQ	-	-	0.8	43	<LOQ	<LOQ	<LOQ
Female 26 y	-	<LOQ	<LOQ	-	-	-	0.7	<LOQ	41	0.7	<LOQ	-	<LOQ	1.3	22	<LOQ	-	<LOQ
Female 31 y	-	<LOQ	<LOQ	-	-	-	<LOQ	-	30	0.2	<LOQ	-	<LOQ	0.1	5.2	<LOQ	-	-
Female 34 y	-	<LOQ	<LOQ	-	-	-	0.6	0.2	58	0.2	<LOQ	-	<LOQ	0.8	16	<LOQ	0.1	<LOQ
Female 40 y	-	-	<LOQ	-	-	-	0.7	-	56	0.7	<LOQ	-	<LOQ	1.1	12	<LOQ	-	-
<b>Glucuronide fraction</b>																		
Male 26 y	-	0.3	<LOQ	<LOQ	<LOQ	270 <sup>b</sup>	0.2	<LOQ	7.8	11	0.7	0.2	0.8	150 <sup>b</sup>	3.0	0.6	-	6.5
Male 34 y	<LOQ	1.2	<LOQ	0.2	2.8	1000 <sup>b</sup>	0.5	1.1	48	34	1.7	0.8	3.2	1000 <sup>b</sup>	26	1.4 <sup>a</sup>	-	23
Male 38 y	-*	0.8	<LOQ	0.3	2.4	1400 <sup>b</sup>	0.7	1.6	81	23	1.9	0.6	1.6	1900 <sup>b</sup>	31	1.1 <sup>a</sup>	-	28
Male 49 y	<LOQ	0.8	<LOQ	0.2	4.2	720 <sup>b</sup>	0.3	1.7	90 <sup>b</sup>	21	1.1	0.3	1.3	570 <sup>b</sup>	33	0.7	-	10
Female 26 y	-	0.7	<LOQ	<LOQ	<LOQ	380 <sup>b</sup>	0.2	1.1	27 <sup>b</sup>	17	1.4	0.3	0.9	1200 <sup>b</sup>	13	0.8 <sup>a</sup>	-	1.3
Female 31 y	-	0.6	<LOQ	<LOQ	<LOQ	600 <sup>b</sup>	0.6	0.2	10	13	4.2	1.4	3.6	610 <sup>b</sup>	4.2	0.9 <sup>a</sup>	-	1.9
Female 34 y	<LOQ	0.2	<LOQ	<LOQ	2.1	470 <sup>b</sup>	0.2	1.0	42	21	3.0	0.9	3.5	1100 <sup>b</sup>	19	0.6 <sup>a</sup>	0.1	<LOQ
Female 40 y	<LOQ	0.7	<LOQ	<LOQ	3.6	1100 <sup>b</sup>	0.5	0.9	28	37	8.6	3.2	7.6	560 <sup>b</sup>	26	<LOQ	<LOQ	2.3

\*=interfering background noise above LOQ

a=interfering background in qualifier, quantifier/qualifier ratio above allowed limit

b=from diluted samples with diluted calibration curves

## Supplementary data

### Analysis of steroids in urine by gas chromatography-capillary photoionization-tandem mass spectrometry

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**Table S1.** Analyzed steroids with trivial names, full names, abbreviations, steroid classes, structures, molecular masses, monoisotopic masses, and molecular formulas.

**Table S2.** Steroid MRM transitions and optimized mass spectrometer parameters.

**Figure S1.** Extracted ion chromatograms of steroids from a male (38y) urine sample from free steroid and glucuronide fractions, standard compound (5 ng mL<sup>-1</sup>) spiked in artificial urine, and blank sample.

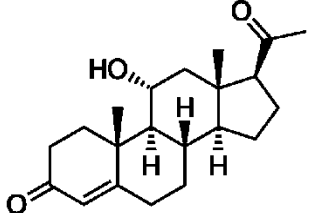
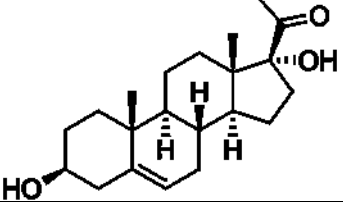
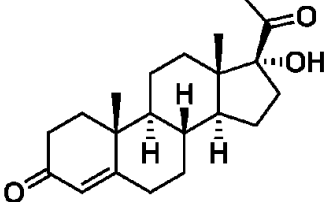
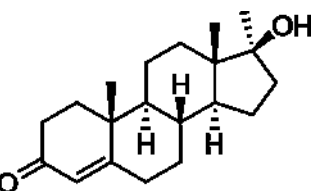
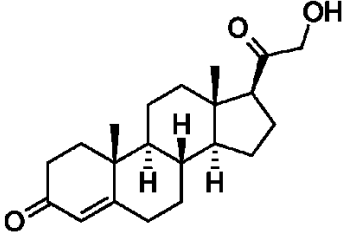
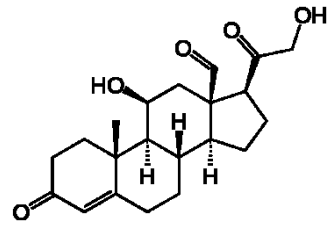
Detailed methods and results on the development of nitrogen housing

**Figure S2.** Background-subtracted mass spectra of ADT and DHEA measured from the same samples on the same day with and without nitrogen housing and product ion spectra of ADT and DHEA.

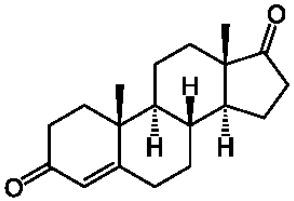
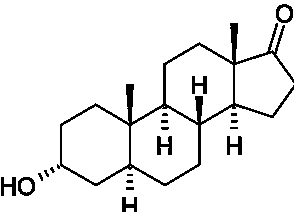
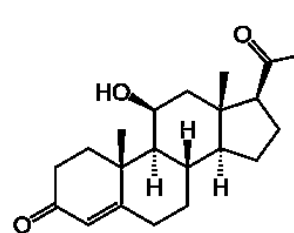
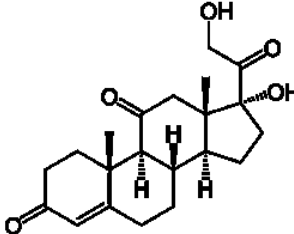
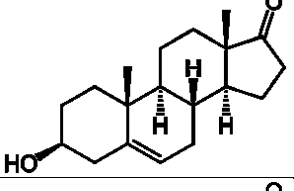
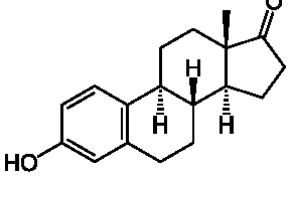
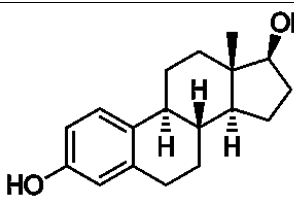
**Figure S3.** Background-subtracted MS-scan spectra of E2 with nitrogen housing (A and B) and without nitrogen housing (C and D).

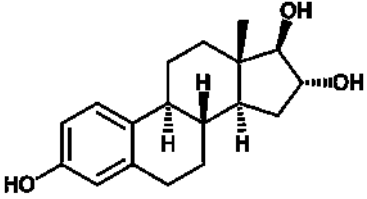
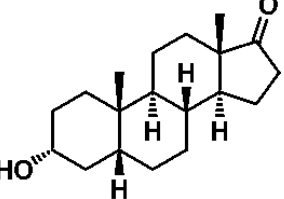
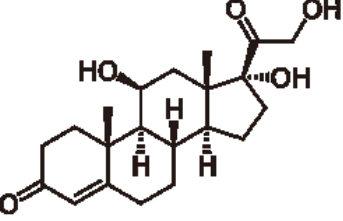
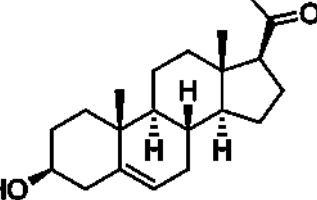
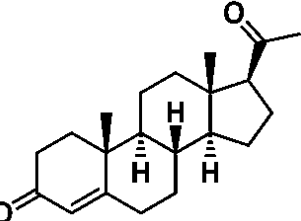
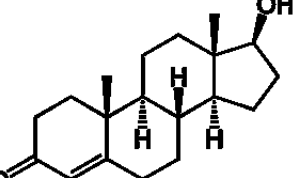
**Figure S4.** Mass spectra of TMS-derivatized steroid standard compounds.

**Table S1.** Analyzed steroids with trivial names, full names, abbreviations, steroid classes, structures, monoisotopic masses, and molecular formulas.

<b>Compound:</b> Trivial name Full name Abbreviation Steroid class	<b>Structure</b>	<b>Monoisotopic mass (g/mol)</b>  <b>Formula</b>
<b>11<math>\alpha</math>-Hydroxyprogesterone</b> 11 $\alpha$ -Hydroxy-4-pregnene-3,20-dione <b>11-OH-PROG</b> progestogen		330.22  $C_{21}H_{30}O_3$
<b>17<math>\alpha</math>-Hydroxypregnenolone</b> 3 $\beta$ ,17-dihydroxypregn-5-en-20-one <b>17<math>\alpha</math>-OH-PREG</b> progestogen		332.24  $C_{21}H_{32}O_3$
<b>17<math>\alpha</math>-Hydroxyprogesterone</b> 17 $\alpha$ -Hydroxy-4-pregnene-3,20-dione <b>17<math>\alpha</math>-OH-PROG</b> progestogen		330.22  $C_{21}H_{30}O_3$
<b>17<math>\alpha</math>-Methyltestosterone</b> 17 $\beta$ -Hydroxy-17-methylandro-4-en-3-one <b>Me-T (ISTD)</b> androgen		302.22  $C_{20}H_{30}O_2$
<b>21-Hydroxyprogesterone</b> 21-hydroxy-4-pregnene-3,20-dione <b>21-OH-PROG</b> glucocorticoid		330.22  $C_{21}H_{30}O_3$
<b>Aldosterone</b> 11 $\beta$ ,21-dihydroxy-3,20-dioxo-4-pregnen-18-al <b>A</b> mineralocorticoid		360.19  $C_{21}H_{28}O_5$

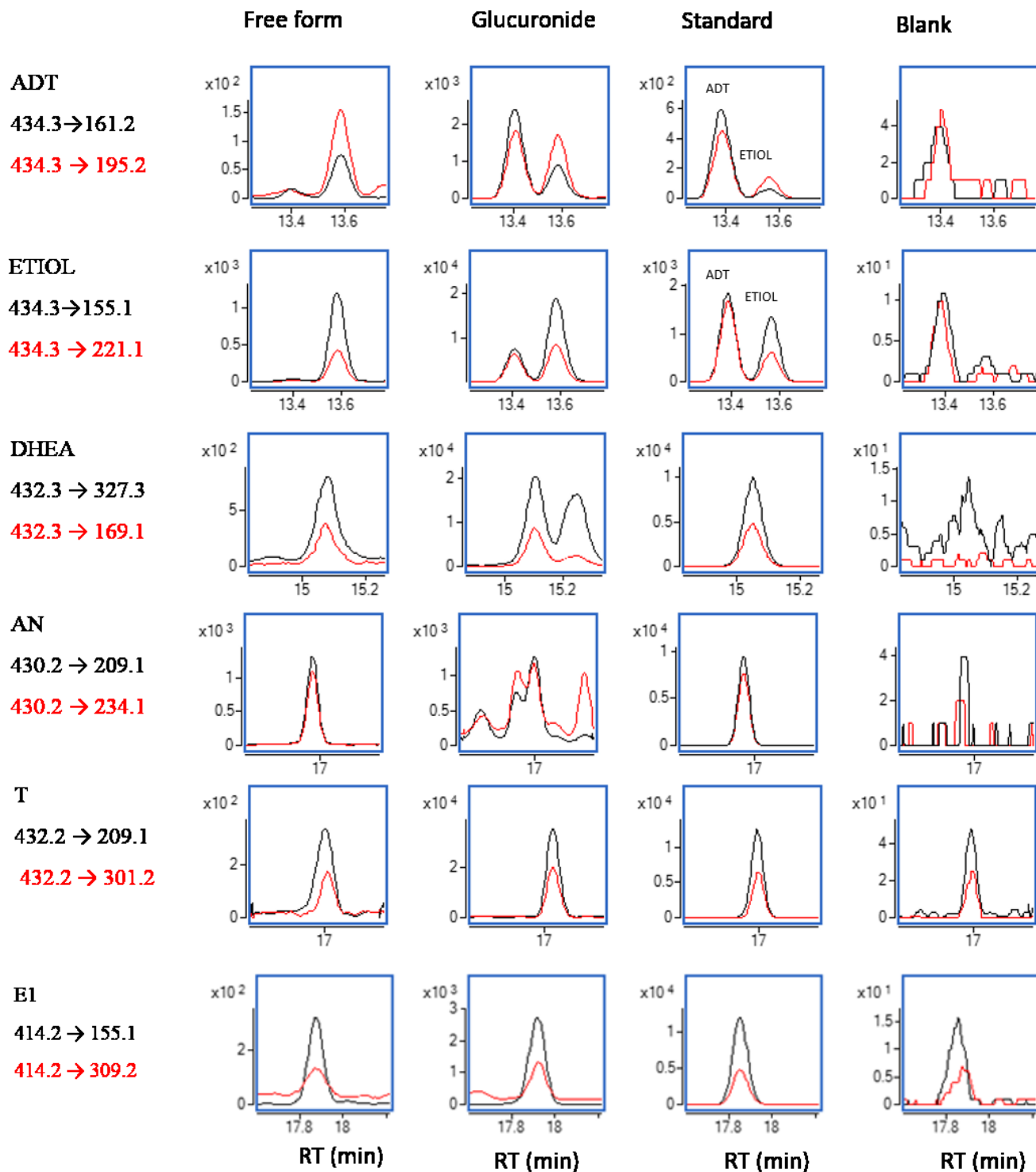


<p><b>Androstenedione</b> 4<math>\alpha</math>-androstene-3,17-dione AN androgen</p>		<p>286.19  C<sub>19</sub>H<sub>26</sub>O<sub>2</sub></p>
<p><b>Androsterone</b> 3<math>\alpha</math>-hydroxy-5<math>\alpha</math>-androstan-17-one ADT androgen</p>		<p>290.22  C<sub>19</sub>H<sub>30</sub>O<sub>2</sub></p>
<p><b>Corticosterone</b> 11<math>\beta</math>,21-dihydroxy-4-pregnene-3,20-dione CORT glucocorticoid</p>		<p>346.21  C<sub>21</sub>H<sub>30</sub>O<sub>4</sub></p>
<p><b>Cortisone</b> 17<math>\alpha</math>,21-dihydroxypregn-4-ene-3,11,20-trione CS glucocorticoid</p>		<p>360.19  C<sub>21</sub>H<sub>28</sub>O<sub>5</sub></p>
<p><b>Dehydroepiandrosterone</b> Androst-5-en-3<math>\beta</math>-ol-17-one DHEA androgen</p>		<p>288.21  C<sub>19</sub>H<sub>28</sub>O<sub>2</sub></p>
<p><b>Estrone</b> 3-hydroxyestra-1,3,5(10)-triene-17-one E1 estrogen</p>		<p>270.16  C<sub>18</sub>H<sub>22</sub>O<sub>2</sub></p>
<p><b><math>\beta</math>-estradiol</b> 3,17<math>\beta</math>-dihydroxy-1,3,5(10)-estratriene E2 estrogen</p>		<p>272.18  C<sub>18</sub>H<sub>24</sub>O<sub>2</sub></p>

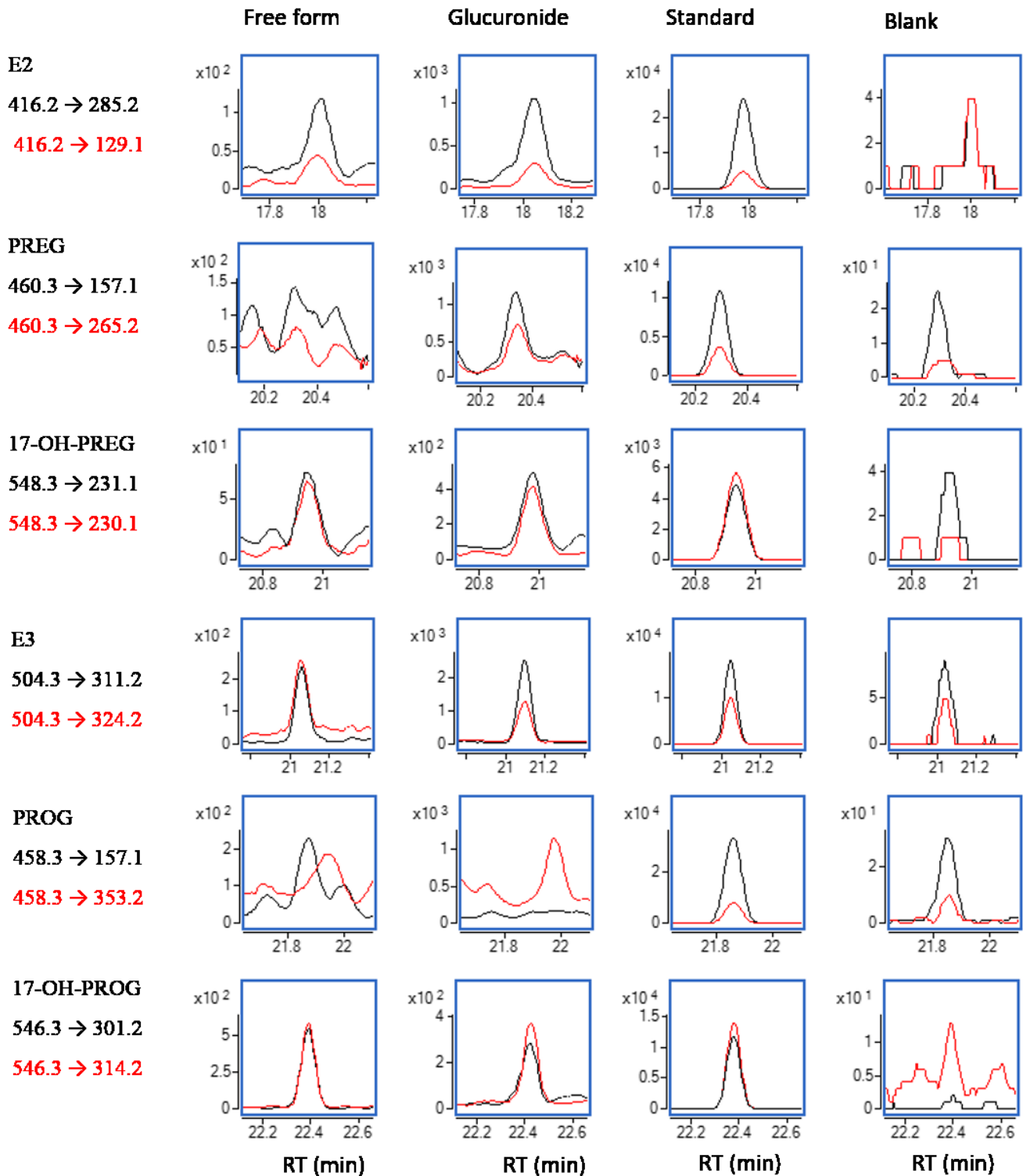
<p><b>Estriol</b> 3,17<math>\beta</math>-dihydroxy-1,3,5(10)-estratriene <b>E3</b> <b>estrogen</b></p>		<p>288.17 <b>C<sub>18</sub>H<sub>24</sub>O<sub>3</sub></b></p>
<p><b>Etiocholanolone</b> Etiocholan-3<math>\alpha</math>-ol-17-one <b>ETIOL</b> <b>androgen</b></p>		<p>290.22 <b>C<sub>19</sub>H<sub>30</sub>O<sub>2</sub></b></p>
<p><b>Hydrocortisone</b> 11<math>\beta</math>,17<math>\alpha</math>,21-trihydroxypregn-4-ene-3,20-dione <b>HC</b> <b>glucocorticoid</b></p>		<p>362.21 <b>C<sub>21</sub>H<sub>30</sub>O<sub>5</sub></b></p>
<p><b>Pregnenolone</b> 3<math>\beta</math>-hydroxypregn-5-en-20-one <b>PREG</b> <b>progestogen</b></p>		<p>316.24 <b>C<sub>21</sub>H<sub>32</sub>O<sub>2</sub></b></p>
<p><b>Progesterone</b> 4-pregnene-3,20-dione <b>PROG</b> <b>progestogen</b></p>		<p>314.22 <b>C<sub>21</sub>H<sub>30</sub>O<sub>2</sub></b></p>
<p><b>Testosterone</b> 17<math>\beta</math>-hydroxy-4-androsten-3-one <b>T</b> <b>androgen</b></p>		<p>288.21 <b>C<sub>19</sub>H<sub>28</sub>O<sub>2</sub></b></p>

**Table S2.** Steroid MRM transitions and optimized mass spectrometer parameters.

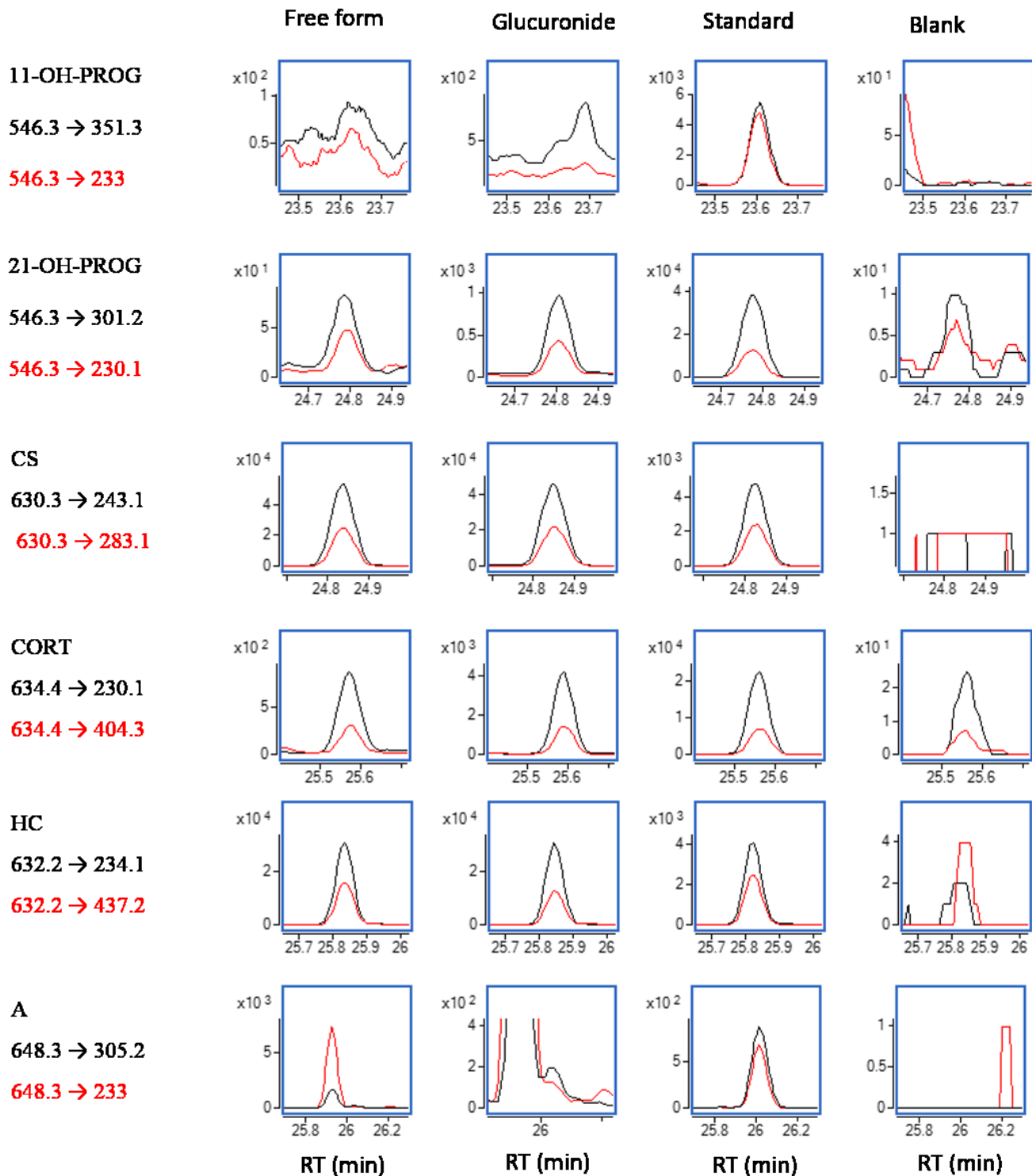
compound	time segment	RT (min)	precursor ion type	attached TMS-groups in M	precursor ion (m/z)	fragmentor voltage (V)	quantifier ion (m/z)	quantifier ion CE (V)	qualifier ion (m/z)	qualifier ion CE (V)
ADT	1	13.38	M <sup>+</sup>	2 TMS	434.3	180	161.2	20	195.2	20
ETIOL		13.56	M <sup>+</sup>	2 TMS	434.3	170	155.1	25	221.1	40
DHEA	2	15.05	M <sup>+</sup>	2 TMS	432.3	170	327.3	20	169.1	25
AN	3	16.92	M <sup>+</sup>	2 TMS	430.2	190	209.1	30	234.1	30
T		16.99	M <sup>+</sup>	2 TMS	432.2	180	209.1	30	301.2	35
E1	4	17.85	M <sup>+</sup>	2 TMS	414.2	180	155.1	25	309.2	25
E2		17.98	M <sup>+</sup>	2 TMS	416.2	180	285.2	25	129.1	25
Me-T (ISTD)	5	19.04	M <sup>+</sup>	2 TMS	446.3	180	301.2	30	314.3	25
PREG		20.29	M <sup>+</sup>	2 TMS	460.3	160	157.1	30	265.2	30
17-OH-PREG	6	20.93	M <sup>+</sup>	3 TMS	548.3	180	231.1	30	230.1	35
E3		21.04	M <sup>+</sup>	3 TMS	504.3	180	311.2	20	324.2	20
PROG	7	21.85	M <sup>+</sup>	2 TMS	458.3	170	157.1	30	353.2	30
17-OH-PROG		22.38	M <sup>+</sup>	3 TMS	546.3	170	301.2	35	314.2	30
11-OH-PROG	8	23.61	M <sup>+</sup>	3 TMS	546.3	170	351.3	30	233	35
21-OH-PROG	9	24.77	M <sup>+</sup>	3 TMS	546.3	170	301.2	35	230.1	30
CS		24.82	[M-TMSOH] <sup>+</sup>	5 TMS	630.3	190	243.1	40	283.1	40
CORT	10	25.56	M <sup>+</sup>	4 TMS	634.4	170	230.1	35	404.3	25
HC		25.82	[M-TMSOH] <sup>+</sup>	5 TMS	632.2	180	234.1	40	437.2	40
A		26.02	M <sup>+</sup>	4 TMS	648.3	190	305.2	30	233	30



**Figure S1.** Extracted ion chromatograms of steroids from a male (38y) urine sample from free steroid and glucuronide fractions, standard compound ( $5 \text{ ng mL}^{-1}$ ) spiked in artificial urine and blank sample (note that different intensity scales are used for clear visualization). Quantifier transition and chromatogram is marked with black and qualifier with red color. Figure continues.



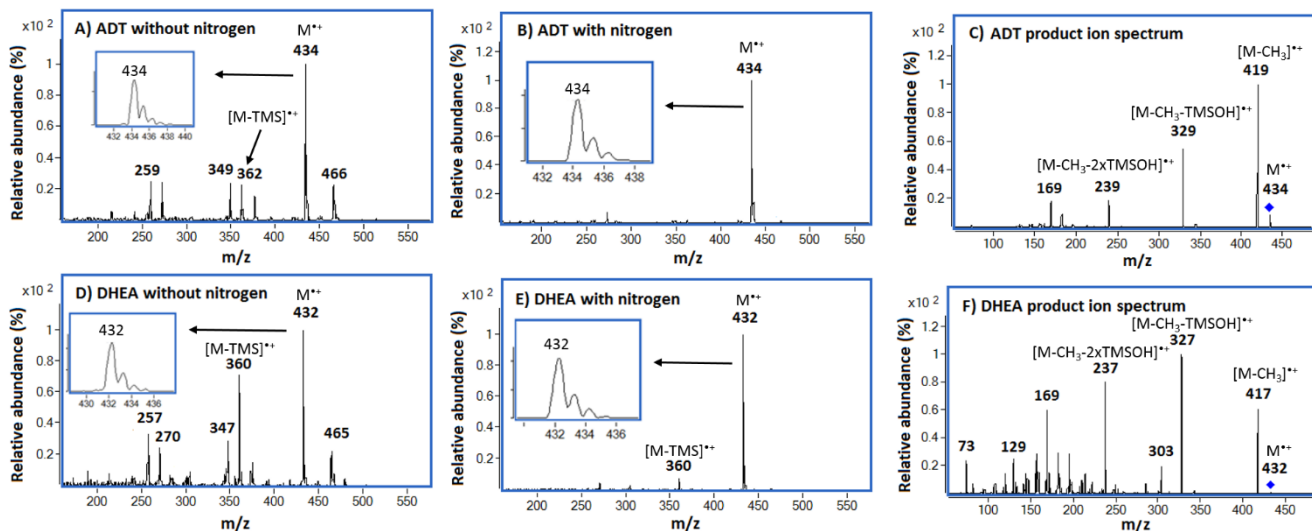
**Figure S1.** Extracted ion chromatograms of steroids from a male (38y) urine sample from free steroid and glucuronide fractions, standard compound ( $5 \text{ ng mL}^{-1}$ ) spiked in artificial urine and blank sample (note that different intensity scales are used for clear visualization). Quantifier transition and chromatogram is marked with black and qualifier with red color. Figure continues.



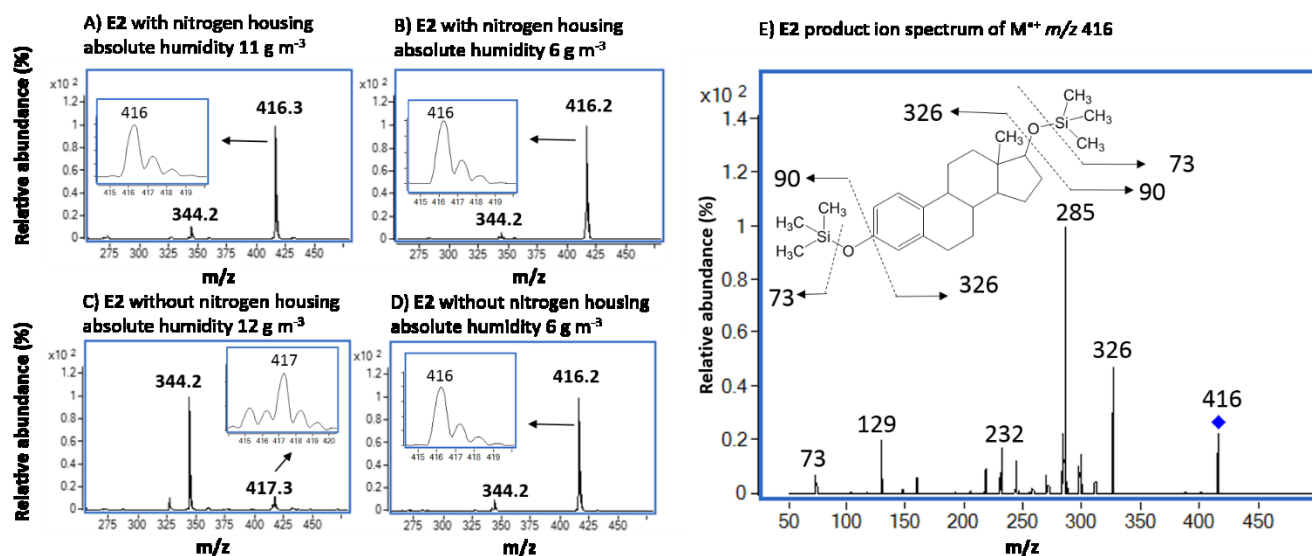
**Figure S1.** Extracted ion chromatograms of steroids from a male (38y) urine sample from free steroid and glucuronide fractions, standard compound (5 ng mL<sup>-1</sup>) spiked in artificial urine and blank sample (note that different intensity scales are used for clear visualization). Quantifier transition and chromatogram is marked with black and qualifier with red color.

## Detailed methods and results on the development of nitrogen housing

As gas flow towards MS was higher than the gas flow provided by GC and dopant system, ambient air could flow into the CPI, possibly affecting to photoionization process, which was mainly prevented by building up a nitrogen atmosphere around the ion chamber. The effect of the nitrogen atmosphere was studied by measuring the spectra of the TMS derivatives of steroids with and without the nitrogen housing. Results for E2, ADT, and DHEA are shown as examples in Fig. S2 and Fig. S3. An abundant radical cation ( $[M]^{+\bullet}$ ) was formed with nitrogen housing and formation of other ions was minimal. Without the housing, the intensities of  $[M+H]^+$ ,  $[M-TMS]^{+\bullet}$ ,  $[M-TMSOH]^{+\bullet}$ , and  $[M+O_2]^{+\bullet}$  were increased, possibly due to reactions with molecules of ambient air such as oxygen and water. The formation of  $[M+H]^+$  is most likely due to atmospheric water, which, in photoionization, produces protonated water cluster reagent ions that favor proton transfer reactions [32].  $[M-TMS]^{+\bullet}$  was not observed in the product ion spectra of the steroids (Fig. S2, Fig. S3 in supplementary data) and thus it is unlikely that it is formed by in-source fragmentation of the radical cation. The reason for observing  $[M-TMS]^{+\bullet}$  may be that atmospheric water promotes gas-phase hydrolysis of the TMS derivatives, and the hydrolysis product  $[M-TMS]$  is then ionized by a charge exchange reaction in the CPI source. The reaction may also be photocatalyzed, and the OTMS group can be substituted with photon-generated hydroxyl radical prior to ionization. To demonstrate the effect of the changing atmospheric environment to the photoionization process, spectra were also measured on two separate days in a laboratory kept at approximately 21°C without a dehumidifier and with a two-fold difference in absolute humidity between the measurement days (Fig. S3). Without nitrogen housing, the humidity clearly correlated with the formation of  $[M+H]^+$  and  $[M-TMS]^{+\bullet}$  ions (Fig. S3) although other fluctuations in the laboratory air contents may also contribute to the obtained result. With nitrogen housing, the spectra showed no significant changes related to ambient conditions or absolute humidity. This demonstrates that in GC-APPI-MS analysis, the variations in ambient air composition may have a significant effect on the photoionization process and these variations can be eliminated by using nitrogen housing as shown in Fig. S3 or by using a fully closed CPI ion source.

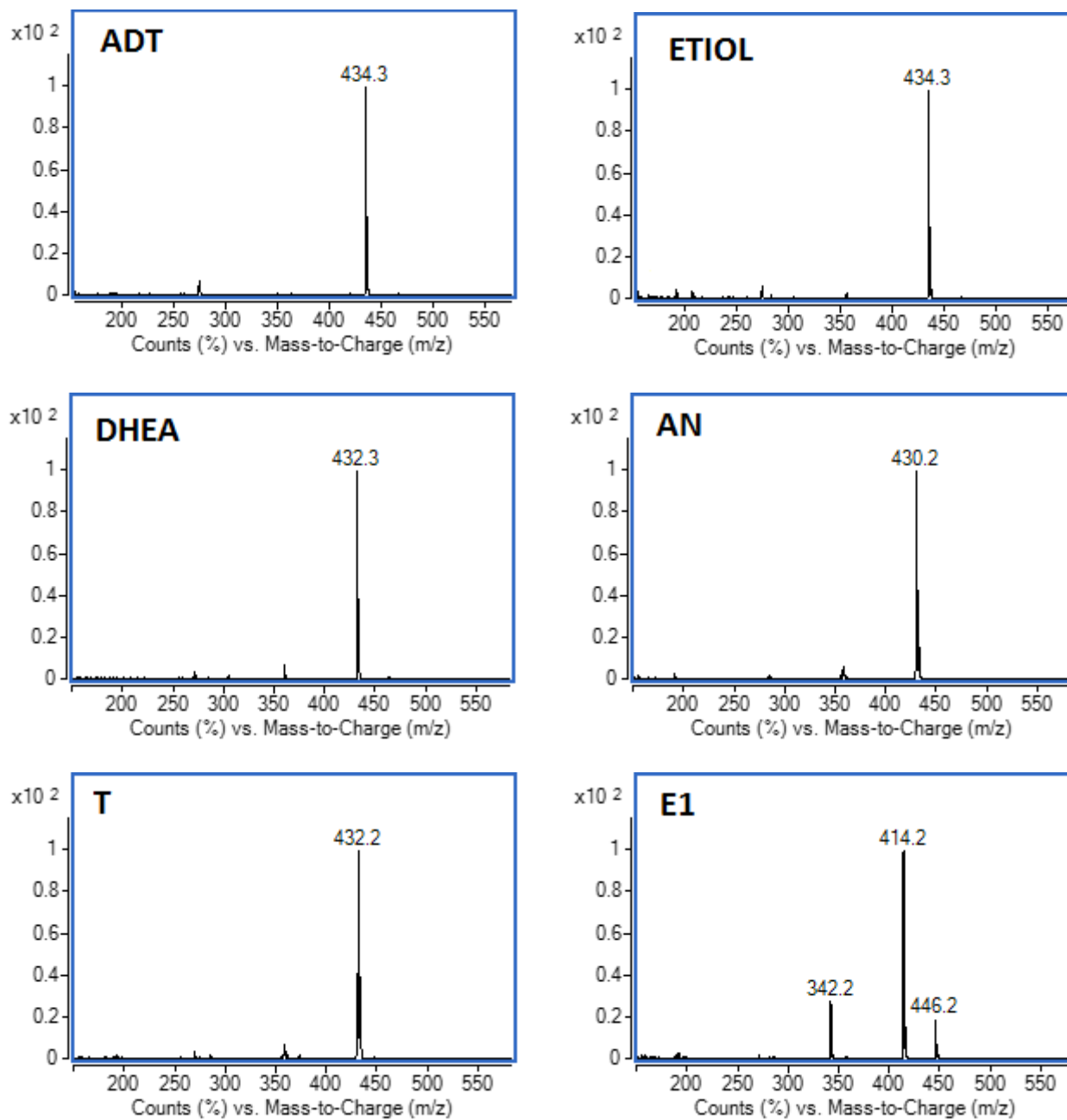


**Figure S2.** Background-subtracted mass spectra of ADT (A,B) and DHEA (D,E) measured from the same samples on the same day with (B,E) and without (A,D) nitrogen housing. Product ion spectra of ADT (collision energy 20V) and DHEA (collision energy 25V) radical cations (C and F).

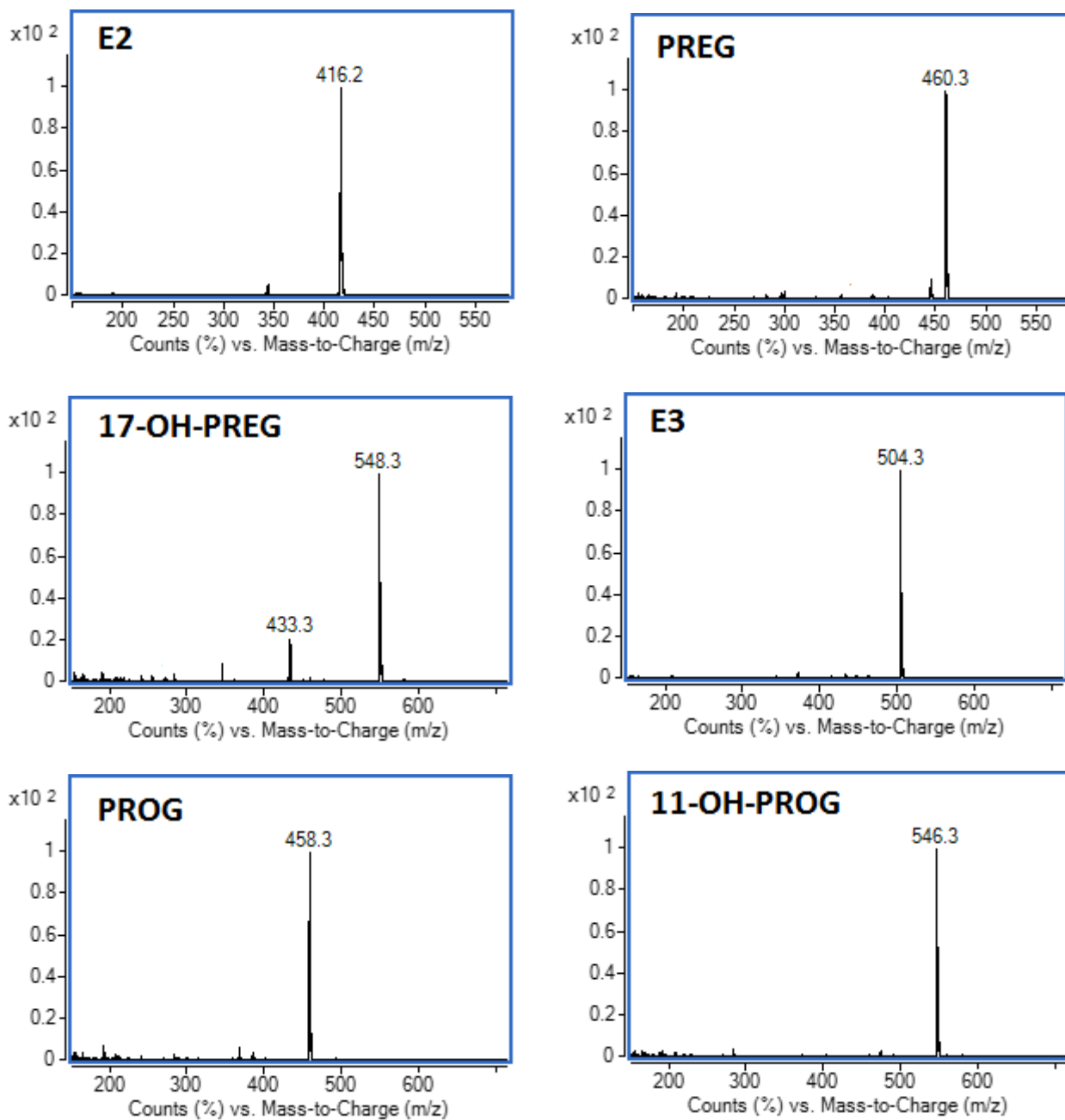


**Fig. S3.** Background-subtracted MS-scan spectra of E2 with nitrogen housing (A and B) and without nitrogen housing (C and D). The absolute humidity of the measurement days were 6  $\text{g m}^{-3}$ \* (B, D); 12  $\text{g m}^{-3}$ \* (C); and 11  $\text{g m}^{-3}$ \* (A). Figure 2E shows the product ion spectrum of E2 radical cation ( $[M]^{+\bullet}$ ) at  $m/z$  416) with collision energy 20V and scan range 50-500. \*Absolute humidity was calculated based on the average observations (temperature, pressure, relative humidity) on the measurement day provided by the Finnish Meteorological institute.

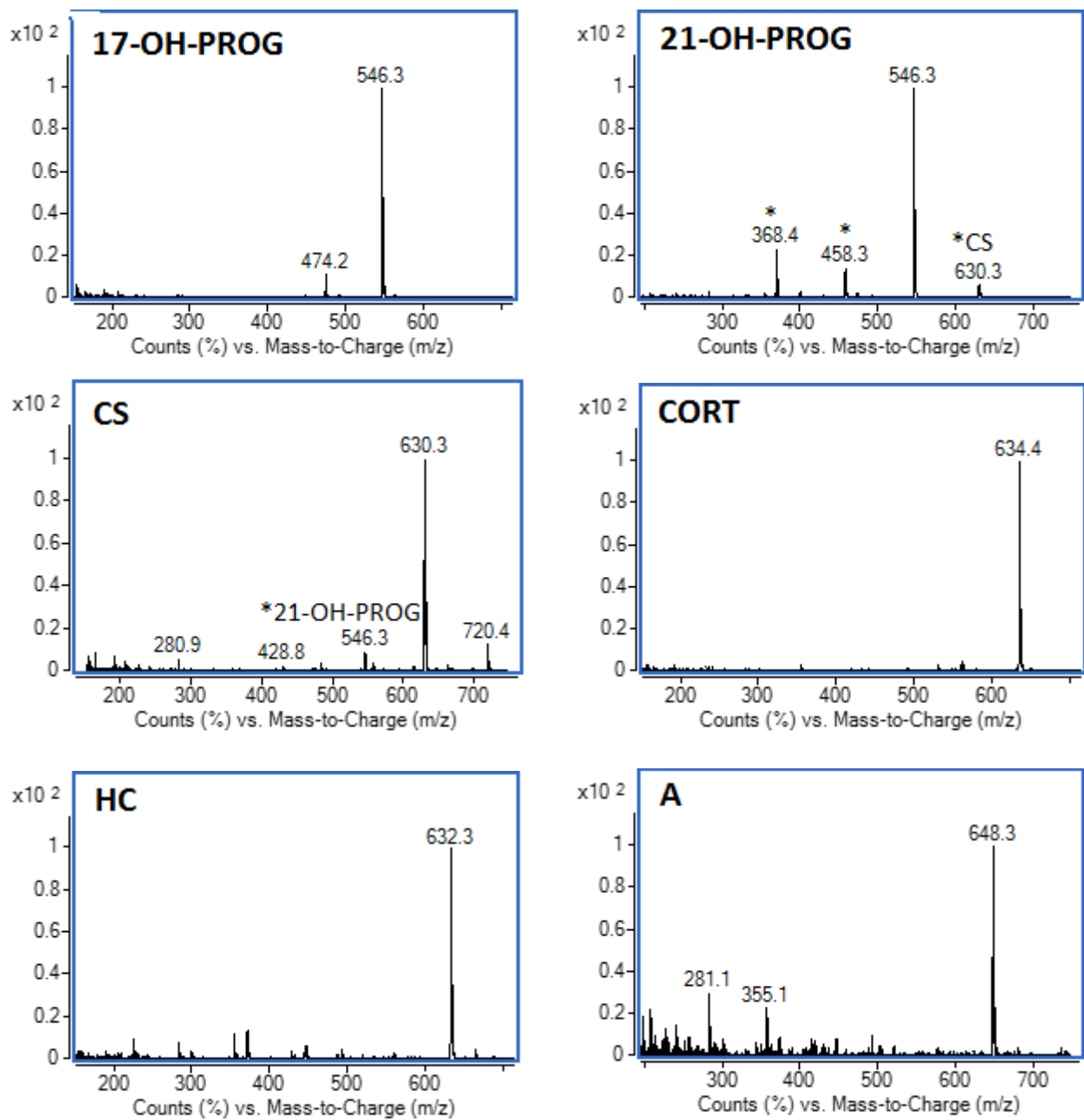




**Figure S4.** Mass spectra of TMS-derivatized steroid standard compounds. Peaks from co-eluting compounds marked with \*. Figure continues.



**Figure S4.** Mass spectra of TMS-derivatized steroid standard compounds. Peaks from co-eluting compounds marked with \*. Figure continues.



**Figure S4.** Mass spectra of TMS-derivatized steroid standard compounds. Peaks from co-eluting compounds marked with \*.