Quantification of Steroid Hormones in Plasma Using a Surrogate Calibrant Approach and UHPLC-ESI-QTOF-MS/MS with SWATH-Acquisition Combined with Untargeted Profiling

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Keywords: Steroidomics, Lipidomics, SWATH, Data-independent acquisition, Estradiol, Testosterone, Clinical Analysis

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ABSTRACT

In spite of demonstrated lack of accuracy and consistency, quantification of steroid hormones is still most commonly executed via immunoassays. Mass spectrometric methods with triple quadrupole instruments are well established and, because of their proven robustness and sensitivity, best suited for targeted analysis. However, recent studies have shown that high-resolution mass spectrometers, like quadrupole time-of-flight instruments (QTOF), show comparable performance in terms of quantification and can generate additional sample information via untargeted profiling workflows. We demonstrate that adequate accuracy and selectivity for estradiol and testosterone can be achieved with a QTOF by data-independent acquisition with sequential window acquisition of all theoretical fragment-ion mass spectra (SWATH). Besides potential combination of targeted quantification and untargeted profiling, SWATH offers advantages with respect to sensitivity because the reduced total number of MS/MS experiments could be used to increase accumulation time without increasing cycle time. By applying a surrogate calibrant method leading to successful validation, a reliable method for absolute steroid quantification and high potential for steroid profiling has been developed. Linear calibration was achieved in the range from 10 - 1,000 pg mL^{-1} for $^{13}C_3$-estradiol and from 20 - 15,000 pg mL^{-1} for $^{13}C_3$-testosterone. Results for inter-day precision ($^{13}C_3$-estradiol: 4.5 - 10.2 %; $^{13}C_3$-testosterone: 5.1 - 7.8 %) and inter-day accuracy ($^{13}C_3$-estradiol: 94.6 - 112.8 %; $^{13}C_3$-testosterone: 98.2 – 107.7 %) were found to be well acceptable. Eventually, the method has been utilized to measure clinical samples of a study in which male volunteers obtained transdermal estradiol patches and sex hormone levels were quantified in plasma.

1. INTRODUCTION

17β-estradiol (E) and 17β-testosterone (T), the main steroid sex hormones in women and men, play crucial roles in human physiology and are frequently monitored analytes in routine diagnostics and clinical studies [1]. Despite the well-known disadvantages, like impact of matrix effects and cross-reactivities [2], the majority of steroid analytics is still performed via immunoassays. Numerous studies have already shown inconsistency between assay results, especially in critical patient groups with low steroid levels [3-9]. Accurate results, however, are mandatory for effective therapy and study interpretation. Consequently, the demand for reliable techniques, in particular liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), is emerging in clinical analysis and clinical studies [10].

Another challenge of steroid quantification in plasma is the absence of true blank matrix for calibration and assessment of assay selectivity. To overcome this problem, various alternative methods are described [11]. In order to obtain an authentic analytical environment, a surrogate calibrant approach [12] was selected for this method. Herein, calibration is done via an analyte-related substance, preferably a stable-isotope-labeled analogue (SIL), which is spiked into the true matrix. After initial matching of SIL response to target analyte response and verification of parallelism [13], the surrogate calibration is used for sample quantification.

The goal of this study was to develop and validate a sensitive LC-MS/MS method for the quantification of E and T in human plasma to verify and complement results previously gathered by a competitive chemiluminescent enzyme immunoassay. A large number of quantitative assays using LC hyphenated to triple-quadrupole (QqQ) instruments were already published for these steroid hormones [10, 14-26]. To reach low concentration levels of target analytes in various matrices, pre-column derivatization is often carried out, using

e.g. Girard-P [27, 28], dansyl chloride [27], aminoxypropyl trimethylammonium bromide [29] (Amplifex Keto) for ketolic steroids such as T, and dansyl chloride [27] or 1,2-dimethylimidazole-5-sulfonyl chloride [21] for phenolic steroids such as E. Due to robustness, high sensitivity and wide linear range, LC-ESI-QqQ is the method of first choice for targeted quantitative analysis of steroid hormones.

Recently, however, quantification by LC coupled to high-resolution (HR) MS raised some interest due to good performance [30-32]. Usually, quantitative data with such HR-MS instruments (quadrupole/time-of-flight or quadrupole/orbitrap) are acquired in MRM^{HR} (also called parallel reaction monitoring, PRM) or data-dependent acquisition (DDA) [33, 34]. In former acquisition mode, after a full scan MS experiment (survey scan) MS/MS experiments are programmed for the selected targets whereby precursor selection occurs by a quadrupole mass analyzer with unit mass followed by fragmentation and analysis of the product ions in the HR-mass analyzer. Highly selective MS/MS chromatograms can be extracted for the programmed targets (i.e. EICs for fragment ions of the selected precursors), while untargeted profiling is still possible at the MS level [35, 36]. In DDA, subsequent to the full scan MS experiment, a series of MS/MS experiments, in which the most intensive precursor ions detected in the survey scan are fragmented, is carried out. Thus, MS/MS data are not collected comprehensively across the entire chromatogram and all study samples. The consequence is that quantitative analysis can be only performed with the precursor ion from the MS experiment. This restriction can be overcome by untargeted profiling with data-independent acquisition (DIA). In DIA, MS/MS fragmentation occurs without dependence on information from the survey scan. All precursors of the entire m/z range co-isolated by the quadrupole are co-fragmented simultaneously (termed MS^E, all ion fragmentation) [37]. This yields complex composite spectra, which is the reason why this acquisition mode has not

become very popular. However, precursor selection can also be performed in a stepped manner with sequential, intermediate-sized Q1 windows (e.g. 20-50 Da), thus covering the entire m/z range of interest. This acquisition mode has been developed for proteomics [38] but has been recently tested for small molecules as well, including metabolomics and lipidomics [39-45]. Better performance than with DDA has been documented for this DIA called SWATH (sequential window acquisition of all theoretical fragment-ion mass spectra) due to better analyte (metabolite) coverage, better reproducibility, and less complex composite spectra [43]. Moreover, comprehensive MS/MS data are available and can be used for quantitative analysis. The application of a QTOF with SWATH acquisition for quantitative purposes has recently shown promising results [41, 46]. Here, we wanted to utilize the advantageous properties SWATH offers in terms of sensitivity, especially when surrogate calibration is used. In contrary to previous works, fully optimized SWATH experiments for generating specific and sensitive MS/MS fragment ion signals for quantification of target analytes without derivatization was established.

Concluding, we demonstrate the performance of UHPLC-ESI-QTOF-MS/MS analysis by DIA with SWATH for the simultaneous targeted quantitative analysis of E and T in human plasma samples from a clinical study in which male subjects were treated with transdermal E patches. Extension of the method to a combined targeted/untargeted profiling method is illustrated as well. Furthermore, reliable quantification based on peak areas of extracted MS/MS chromatograms of characteristic fragment ions in SWATH experiments is demonstrated.

2. EXPERIMENTAL SECTION

2.1 Materials. T, 17β-testosterone-2,3,4-$^{13}C_3$ ($^{13}C_3$T, 100 µg mL^{-1} in methanol), E, 17β-estradiol-2,3,4-$^{13}C_3$ ($^{13}C_3$E), 17α-estradiol (epiestradiol, epiE) and phosphoric acid (85 %, w/v, ACS grade) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). 17β-testosterone-2,2,4,6,6-$^{2}H_5$ (d_5T, 106.7 µg mL^{-1} in methanol) was purchased from IsoSciences (King of Prussia, PA, USA). 17β-estradiol-2,4,16,16,17-$^{2}H_5$ (d_5E, 100 µg mL^{-1} in acetonitrile) and 17α-testosterone (epiT; 1.0 mg mL^{-1} in acetonitrile) were purchased from Cerilliant (Round Rock, TX, USA). Details on standard solutions, (surrogate-) calibrants and quality controls can be found in supplementary data (Appendix A.). Cortisone and cortisol were purchased from Cayman Chemical (Ann Arbor, MI, USA). Type I purity water was obtained from a Purelab Ultra purification system (ELGA LabWater, Celle, Germany). Immunoassay measurements of study samples were done with an Immulite 2000 system (Siemens Diagnostics, Erlangen, Germany) using complying E and T kits for total quantification.

2.2 Immunoassay. In this fully automated, competitive chemiluminescent enzyme immunoassay the solid phase consist of beads coated with rabbit polyclonal antibodies specific for the respective target analyte. After introduction of the sample (T: 20 µL; E: 25 µL) and alkaline-phosphate conjugated with E or T, respectively, the target analytes compete with the analyte-enzyme complexes for the limited binding sites during an incubation period of 60 minutes. After washing to remove excess material and reagents, a chemiluminescent substrate (adamantly dioxetane phosphate ester) is added. Hydrolization of the substrate by alkaline phosphatase yields unstable anions, which, as a result of decomposition, generate constant emission of photons. Accordingly, light intensity is inversely proportional to target analyte concentration in the sample. Lyophilized serum quality controls (MassCheck Steroid Panel 2, tri-level) were purchased from Chromsystems (Graefelfing, Germany). Subjects

providing blood samples gave written informed consent to the study that conformed to the Declaration of Helsinki as revised in 2008 and was approved by the local Ethics Committee on Research Involving Humans.

2.2 Sample preparation. 500 µL of EDTA plasma were diluted with 500 µL of 5 % H_3PO_4 (w/v) that contained 1.0 ng mL^{-1} of d_5E and 0.4 ng mL^{-1} of d_5T as internal standards (IS). After vortexing, the sample was loaded onto a dry Oasis PRiME HLB SPE cartridge (1 cc / 30 mg, Waters, Milford, MA, USA). Samples were processed applying negative pressure with a Vacmaster 20 manifold (Biotage, Uppsala, Sweden). After the first loading step, the cartridges were washed with 1 mL of 50 % MeOH in H_2O (v/v). Analytes were then eluted with 2 × 500 µL MeOH and the eluate was dried using a Savant ISS110 SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA, USA). After reconstitution in 100 µL MeOH, samples were centrifuged for 5 min at 15,000 × g and 4 °C with a 5415R microcentrifuge (Eppendorf, Hamburg, Germany). The supernatant was transferred into a vial, which was crimped and stored at 4 °C in the autosampler. Samples were analyzed as soon as possible after preparation.

2.3 LC-method. The chromatographic system consisted of a 1290 Infinity UHPLC system (Agilent Technologies, Waldbronn, Germany) and a PAL HTC-xt autosampler (CTC Analytics, Zwingen, Switzerland). Separation was performed on a Kinetex C18 column (50 mm × 2.1 mm, 2.6 µm, 100 Å pore size) with a KrudKatcher Ultra in-line filter (Phenomenex, Aschaffenburg, Germany) for column protection. Mobile phase A consisted of H_2O + 0.1 % formic acid (v/v) and mobile phase B of MeCN + 0.1 % formic acid (v/v). The flow rate was 0.3 mL min^{-1} with a constant oven temperature of 30 °C. Injection volume was set to 10 µL. The following gradient was applied: 5 - 30 % B from 0.0 – 0.5 min, 30 – 45 %

169 B from 0.5 – 3.2 min, 45 – 95 % B from 3.2 – 3.5 min, holding 95 % B from 3.5 – 4.0 min,
170 95 – 5 % B from 4.0 – 4.2 min, equilibration with 5 % B from 4.2 – 5.0 min.
171 **2.4 MS-method.** Mass spectrometric detection was performed on a TripleTOF 5600+ mass
172 spectrometer with a DuoSpray source (Sciex, Concord, Ontario, Canada). Optimized ion
173 source parameters were as follows: curtain gas (N_2) 35 psi; nebulizer gas (N_2) 50 psi; heater
174 gas (N_2) 80 psi, ion source voltage floating 4,000 V, source temperature 600 °C. Samples
175 were measured in positive electrospray ionization (ESI) mode, running one TOF-MS
176 experiment in the mass range of *m/z* 30 – 1,000 (survey scan; resolution ≥ 30,000, FWHM @
177 829.5393 Da) and four SWATH-MS/MS experiments (resolution ≥ 15,000, FWHM
178 @ 397.2122 Da) per cycle (method 1, see Table 1). Accumulation time (t_{Acc}) was set to the
179 following values: TOF-MS scan: 20 ms; SWATH of T/$^{13}C_3$T: 50 ms; SWATH of d_5T: 50 ms;
180 SWATH of E/$^{13}C_3$E: 300 ms; SWATH of d_5E: 100 ms. Total cycle time (t_{Cyc}) was delimited
181 to 570 ms to attain at least ten data points per peak in regard to average peak widths of about
182 6 s. Enhanced product ion mode was enabled. For SWATH experiments of d_5-internal
183 standards, enhancement was set to the monoisotopic mass of the used fragment, respectively.
184 For SWATH experiments that covered two compounds, target analytes and surrogate
185 calibrants, the enhancement mass was set to the calculated mean mass of both corresponding
186 fragments. Mass calibration was done via infusion of sodium acetate (0.1 mg mL^{-1} in
187 MeCN:H_2O, 1:1, v/v) every 25th injection. The whole analytical system was controlled by
188 the Analyst 1.7 TF software (Sciex).
189 **2.5 Data analysis and quantification.** Calibration curves were constructed using weighted
190 least-square linear regression (weighting factor: 1/x) of six different calibrant levels by
191 plotting peak area ratios of $^{13}C_3$E/d_5E and $^{13}C_3$T/d_5T against respective surrogate calibrant
192 concentrations. The resulting equations were used to determine target analyte concentrations

in real samples via E/d_5E and T/d_5T ratios, respectively. Two QCs, QC_{low} ($^{13}C_3$T: 60 pg mL^{-1}; $^{13}C_3$E: 30 pg mL^{-1}) and QC_{high} ($^{13}C_3$T: 12,000 pg mL^{-1}; $^{13}C_3$E: 800 pg mL^{-1}) were embedded after every 20th sample in the sequence to verify stable method performance. To control for accuracy and linearity of calibration, five determinations of the calibration were equally distributed across the whole sequence. Quantification was based on fragment ions (Table 1). Fragment peak areas were extracted using a ± 10 mDa mass window in the associated SWATH experiments. Automated integration with the MultiQuant 3.0 software (Sciex) was done using a MQIII algorithm, Gaussian smoothing (width: 2 data points), noise percentage of 90 %, baseline substraction window of 0.1 min and a peak splitting factor of 2. Excel 2007 (Microsoft, Redmond, WA, USA), SPSS Statistics 23 (IBM, Armonk, NY, USA) and Origin 2017 (OriginLab, Northampton, MA, USA) were used for further data evaluation.

3. RESULTS AND DISCUSSIONS

3.1 Sample preparation. E and T are bound to plasma proteins like SHBG (sex hormone-binding globulin) [47]. Their release by organic solvents used for protein precipitation would demand an evaporation step prior to SPE which is needed for E/T enrichment. Hence, 5 % H_3PO_4 was selected for protein precipitation [48-51] because the resultant supernatant could be directly loaded onto the Oasis PRiME HLB material, which does not require pre-conditioning and equilibration prior to the loading step. 50 % MeOH in H_2O (v/v) was selected as optimum washing eluent and complete analyte elution with good recoveries of E, $^{13}C_3$E, T and $^{13}C_3$T could be achieved with 2 × 500 µL MeOH. By drying and reconstitution in 100 µL MeOH, a total sample pre-concentration factor of 5 was achieved to reach sufficient levels of sensitivity (for details see Appendix A.).

3.2 LC-MS method. A fast UHPLC method with gradient elution (5 min including re-equilibration) was developed using a core-shell C18 column (Kinetex® C18, 2.6 µm). Faster elution by higher flow rates was not considered because the detection sensitivity significantly dropped at flow rates higher than 0.3 mL min^{-1} [52]. Close to baseline separation of E and T was achieved (R_S = 0.98) (Fig. A.3C) and in spite of a fast gradient sufficient assay specificity was ensured by selective mass spectrometric detection.

The low concentrations of E expected in male plasma samples required dedicated optimization of MS parameters to reach maximal sensitivity for E. For assessment of most sensitive conditions, ionization efficiencies of analytes were tested with APCI and ESI in positive and negative mode. Best sensitivity for E was achieved in negative APCI mode, but ionization of T was inacceptable in negative APCI and negative ESI. Accordingly, analysis in positive mode was mandatory since polarity switching in ms time scale is not possible for the TripleTOF 5600+. Whereas the $[M+H]^+$-precursor ion could be detected for T, E only showed an in-source fragmentation product $[M-H_2O+H]^+$, which was selected as the precursor. For acquisition, data-independent acquisition mode using SWATH, a sequential window-based MS/MS acquisition methodology with intermediate Q1 precursor window sizes, was executed. It allows flexible adjustment and thus optimization of MS parameters for each SWATH window separately and leads to a comprehensive set of MS/MS data in the selected Q1 precursor windows. Since SWATH acquisition used parameters, which secured ≥ 10 spectra available across each peak, enough data points were available to enable generation of MS/MS chromatograms, i.e. EICs of fragments, with some advantages as described below (see also Fig. A.4). Activation of the enhanced product ion mode showed > 3 times increase in signal intensities. This feature optimizes the ion pulsing process for a specific fragment and improves the duty cycle [53]. However, only a narrow m/z-region around the targeted

fragment is enhanced by this process and ions outside this region are lost for detection and excluded. Because of this effect, precursor ions of analytes were not observed in the SWATH-MS/MS experiments in the present case (Fig. A.5).

3.3 Comparison of SWATH and MRM^{HR} sensitivity by their instrumental LODs. Instead of individual product ion MS/MS experiments with unit mass Q1 precursor selection (MRM^{HR}) for each analyte, SWATH-MS/MS experiments were created (Table 1). By selection of appropriate window sizes (4 Da for E, T and their corresponding $^{13}C_3$ analogues; 5 Da for the deuterated internal standards), fragments of target analytes and corresponding surrogate calibrants could be detected in the same SWATH window. Because of fragmentation interferences, separate SWATH windows had to be created for d_5-analogues. Optimized window sizes assured assay specificity for the fragment ions used for quantification. Sensitivity, on the other hand, generally increases with increasing accumulation time t_{Acc} (see Fig. A.6 and Fig. A.7).

SWATH acquisition allowed to reduce the total number of MS/MS experiments and allowed to distribute the maximally available t_{Acc} between fewer experiments. This enabled to increase t_{Acc} for each analyte as compared to MRM^{HR}. In order to compare the sensitivity of MRM^{HR} and SWATH, the instrumental limits of detection (LODs) were determined for three different methods: The SWATH method with the parameters described in section 2. and Table 1, an MRM^{HR} method with t_{Acc} equal to the SWATH experiment (i.e. 300 ms for E and 50 ms for T) (MRM_{eq}) and an MRM method with half the t_{Acc} (i.e. 150 ms for E and 25 ms for T) ($MRM_{1/2}$). The $MRM_{1/2}$ method was designed since it represents the most realistic equivalent to the SWATH method as only half the t_{Acc} is available due to the double number of experiments if each of the analyte and $^{13}C_3$-calibrant is acquired by separate product ion MS/MS experiments. Besides t_{Acc}, all other mass spectrometric parameters (see subchapter

2.4 and Table 1) were kept identical for each method to ensure best achievable comparability. Furthermore, all methods were run with enabled "enhanced product ion mode", had identical cycle times t_{Cyc} and a uniform t_{Acc} (20 ms) for the mandatory TOF-MS experiment. To assess instrumental LODs in the low concentration range, an 8-point calibration of both target analytes in MeOH was analyzed in triplicate. Instrumental LODs were lowest for the SWATH method (5.8 and 8.1 pg mL^{-1} for E and T, respectively; about factor 2-3 lower than with MRM^{HR} even at equal t_{Acc}; see also Table A.7).

3.4 Assay specificity. While SWATH was shown to increase sensitivity, specificity is lost owing to the broader Q1 isolation window. Validation therefore ultimately requires verification of sufficient assay specificity. First of all, possible interferences deriving from SILs have to be ruled out. The attempt to cover target analyte and corresponding $^{13}C_3$- and d_5-analogues in one single 8 Da-wide SWATH window, respectively, failed since interferences were observed both for E and T. Investigation showed that fragmentation of d_5-standards caused significant interference due to overlapping isotope patterns of d_5-fragments and $^{13}C_3$-fragments. Accordingly, a separate 5 Da SWATH window was created for analysis of each d_5-standard. Further optimization showed that two additional SWATH experiments of 4 Da width are adequate to cover corresponding pairs of target analytes and surrogate $^{13}C_3$-calibrants, respectively. Fragmentations in these windows were free of interference and showed sufficient specificity (see Fig. A.11 – A.16). In untargeted SWATH methods, windows are usually overlapping by 1 Da. In our targeted approach a gap of at least 1.5 Da had to remain between the SWATH windows to avoid interferences. This is owed to the fact that the Q1 is not capable of doing an exact cutout of m/z ranges. Also ions with an m/z slightly (~ 1 Da) below or above SWATH window limits will pass through the Q1, which can lead to unwanted interference. Cross-validation via commercial quality controls (see 3.6) has

finally been utilized to verify assay specificity. Also, epiT and epiE, epimers of T and E with identical fragmentation, were analyzed and showed chromatographic baseline separation (epiT to T, Δt_R: 0.42 min; epiE to E, Δt_R: 0.27 min) (see Fig. A.10). Assay specificity (i.e. lack of interferences) of $^{13}C_3$- and d_5-standards was determined by analyzing six different blank plasma samples. No interfering peaks in a retention time window of ± 0.1 min of the respective analyte were detected.

3.5 Calibration and limits of quantification. With optimized conditions, both E and T could be detected with high sensitivity. Unfortunately, for T the signal leveled off at concentrations above 1,000 pg mL^{-1} due to detector saturation. De-optimization, by raising DP from 120 to 200 V, led to a shift of the linearity range which then covered the relevant concentration range between 20 pg mL^{-1} (instrumental LLOQ) to the upper limit of quantification (ULOQ) of 15,000 pg mL^{-1} (see also Fig. A.8).

Due to absence of blank matrix for matrix-matched calibration, a surrogate calibrant approach was adopted. To ensure accuracy of quantification via $^{13}C_3$-surrogate calibrants, parallelism of the calibration curves between surrogate calibrants and the corresponding standard addition curve of the target analyte has to be verified [13]. In the present case, the maximum difference of the slopes of T and $^{13}C_3$T during three inter-day measurements was 3.7 % (slope of $^{13}C_3$T divided by slope of T) and 3.2 % for E and $^{13}C_3$E (slope of $^{13}C_3$E divided by slope of E) (see Fig. A.18). Therefore, $^{13}C_3$T and $^{13}C_3$E have been found to be adequate surrogate calibrants for quantitative analysis of T and E in human plasma.

LLOQs in real samples were determined adopting the criteria set forth by the FDA guideline for bioanalytical method validation (analyte response at least 5 times the response of the blank response, precision of 20 % and accuracy of 80 - 120 %). Thus, 10 pg mL^{-1} for E and

311 $^{13}C_3E$, and 20 pg mL^{-1} for T and $^{13}C_3T$ were set as LLOQs in real samples (Fig. 1). During
312 validation these values were shown to meet the acceptance criteria for LLOQs.
313 **3.6 Method validation.** Method validation was performed on the basis of the FDA guideline
314 on bioanalytical method validation with minor modifications (e.g. 5 replicates over 3
315 independent days instead of one replicate over 5 independent days for assessment of inter-day
316 accuracy and precision). The detailed results can be found in Appendix A. Matrix effect
317 (ME), extraction recovery (RE) and process efficiency (PE) were evaluated according to
318 Matuszewski et. al [54] with $^{13}C_3$-labelled analogues of analytes, which are expected to suffer
319 equally from ME as the coeluted target analytes. The results are shown in Table 2. E shows
320 an average ME (ion suppression) of 81.3 % and T a more significant average ME of 60.4 %,
321 which made its correction by internal standards (here d_5-analogues) mandatory. Average
322 recoveries for E and T were 88.0 and 84.4 %, respectively.
323 Intra-assay and inter-day precisions and accuracies were determined in plasma using the
324 surrogate calibrants. Four QCs were used to validate precision and accuracy: QC_{LLOQ}, QC_{3x}
325 $_{LLOQ}$, QC_{Mid}, QC_{ULOQ}. These QCs were measured on three days in quintuplicate (n = 5)
326 (Table 3). Precisions were < 10 % in the entire range and accuracies between 95 and 115 %
327 recoveries clearly confirm that assay specificity of the current SWATH methodology is
328 adequate. Adequate method performance was further confirmed by cross-validation with
329 commercial QCs (lyophilized true plasma matrix controls with certified E and T
330 concentrations). Results are shown in Table A.13. Precisions matched those of above
331 validation study and bias remained within acceptable limits (6 - 15 %). Adequate analyte
332 stability during sample storage, freeze-thaw cycles, autosampler stability and short-term
333 stability at ambient temperature was verified as well (see Table A.11).

3.7 Intra-assay cross-validation with alternative quantifiers. With the employed DIA using SWATH, comprehensive high-resolution MS/MS data are available across the steroid hormone peaks in all samples. Thus, it becomes possible to select post-acquisition the most appropriate ion from a peak group, viz. precursor ion from either TOF-MS or MS/MS experiments, or any fragment ion from MS/MS experiments, to generate the EIC chromatograms for quantification. This opens up the possibility to use the most intensive ion as quantifier ion, provided it has sufficient specificity, and any of the other ion traces as qualifier ions, similar to QqQ-based quantification assays but with high mass resolution readout and no need of pre-acquisition decision on the selected ion transitions. It enables another level of validation via controlling the results by additional fragment or precursor ion EICs or ion ratios [55]. In other words, in order to control if the chosen fragment for quantification is selective, other fragments or precursors of the same analyte can be used for quantification and both sets of results can be compared. For example, for T a linear calibration from 500 to 15,000 pg mL^{-1} could be achieved for the precursor from the MS experiment. Also, a second fragment with m/z 97.0648 (MS/MS fragment 2), with comparable sensitivity to the original quantifier fragment of m/z 109.0648, yielded a linear calibration function for the entire range. All patient sample concentrations were also calculated for these alternative signals. Using MS/MS fragment 2 as alternative quantifier the results were in good agreement to the original results (scatter plot linear regression: $y = 1.000$ (± 0.003) $x + 158.2$ (± 16.7), $R^2 = 0.99684$) (Fig. A.21A). Using the precursor ion trace of the TOF-MS experiment, the agreement was still acceptable yet significantly worse (scatter plot linear regression: $y = 0.9541$ (± 0.006) $x - 126.0$ (± 31.6), $R^2 = 0.9875$) (Fig. A.21B) indicating the potential problem in terms of specificity of single stage MS data.

Consequently, also these results consolidate adequate assay specificity and method performance.

3.8 Combined targeted/untargeted profiling (towards steroidomics). Contrary to classical targeted assays with triple quadrupole instruments the current method provides simultaneously lipid profiles in an untargeted manner. Additional information can be derived from TOF-MS experiments (survey scans) or SWATH-MS/MS experiments. In many cases, signals in TOF-MS lack of specificity or show insufficient sensitivity (see Fig. 4). Comprehensive data of superior quality can be acquired by additional MS/MS experiments. To demonstrate the potential of SWATH for steroidomic analysis, an exemplary MS-method with six extra SWATH experiments was created to cover the relevant mass range of interest (Table A.14; method 2). The four previously optimized SWATH windows for E and T quantification remained unaltered, so that the capability of combined untargeted profiling and targeted quantification of E and T can be documented. A mass range from m/z 250 - 370 was additionally covered by SWATH MS/MS experiments, which mostly comprises unconjugated steroids. To use this narrower range for MS/MS experiments allows to design smaller precursor selection windows which is favorable for assay specificity in steroid analysis. The peak spotting plot in Fig. 2 and Fig. A.22 reveals a total of 1,613 molecular features in the TOF-MS survey scan.

For verification of the utility of this expanded steroidomics profiling method, the trilevel commercial controls were analyzed. These commercial QCs specify concentrations for a variety of other steroids, besides E and T, dehydroepiandrosterone, dehydroepiandrosterone-sulfate, androstenedione, hydroxyprogesterone, dihydrotestosterone (DHT) and progesterone which could be identified by matching precursor mass, isotope pattern and MS/MS fragmentation (see Fig. 3). Furthermore, verification of identity was achieved by controlling

for linearity of the obtained three-point calibration of the trilevel controls (see Table A.15). Cortisol, cortisone, epiE and epiT could also be specifically identified in samples by comparison with available standards (see Fig. 3). Other steroids only annotated by exact mass and coherent fragmentation were aldosterone, corticosterone, deoxycortisol, deoxycorticosterone, estrone and pregnenolone. Furthermore, several acylcarnitines could be identified via spectral matching to the LipidBlast [56] database, concluding that also other non-steroidal, lipophilic compounds are captured by sample preparation.

The currently employed combined targeted/untargeted profiling by data-independent acquisition with SWATH provides other benefits. Availability of comprehensive MS/MS data within the dedicated m/z range across the chromatogram and all samples allows to extract both MS chromatograms of precursors but also MS/MS chromatograms of fragments which is not possible with common data-dependent acquisition. This enables uncompromised retrospective data processing post-acquisition. Quantitative analysis can be either performed on precursors or fragments, which ever gives better assay specificity and/or higher sensitivity. This is documented in Fig. 4 exemplarily for non-targeted dihydrotestosterone (DHT). DHT is a bioactive metabolite of T formed by the enzyme 5α-reductase and is the biologically most active form of T. In a targeted assay with a triple quadrupole and SRM acquisition for E and T, no information on DHT could be obtained. In the combined targeted/untargeted screening approach, presented in Table A.14, DHT is detected in the different samples as well. This allows deriving information, at least for relative quantification (e.g. for differential steroidomics between sample groups). However, the signal is very poor in the TOF-MS chromatogram of the precursor (S/N (PeakView estimate) = 2.3) due to many interferences and a high noise level (Fig. 4A). Although the signal is reduced in the MS/MS chromatogram of the precursor (Fig. 4B), the S/N ratio was significantly improved due to a lower noise

405 level. Upon extraction of the MS/MS chromatogram from the precursor with m/z 255.2113
406 the S/N ratio increased by a factor of about 4 because the majority of interferences were
407 eliminated and the noise level further reduced (Fig. 4C). Data processing on this signal is
408 certainly advantageous for retrospective relative quantification of samples. The fact that in
409 DIA with SWATH all signals are acquired and comprehensive MS as well as MS/MS data
410 are available, provides researchers the flexibility to use the optimal MS or MS/MS signal for
411 data processing. If taken into account that MS parameters were not optimized for the
412 untargeted SWATH experiments, even higher sensitivity might be possible. Also, ion ratios
413 can be further processed for confirmation underpinning the advantage of DIA [55].
414 **3.9 Clinical Study and comparison with immunoassay results.** In a clinical study, the
415 effect of E and insulin on food intake in men was investigated. Here, two groups of healthy
416 young men (each n = 16) received transdermal E (100 µg/24h) or placebo via transdermal
417 patches for three days. According to a 2 × 2 design, the experiment comprised two individual
418 sessions in each subject with intranasal insulin (160 IU) and, respectively, placebo
419 administration. In each session, plasma samples were collected at five different time points,
420 totaling 320 samples. These samples were measured by method 1 (Table 1) and also
421 quantified by a competitive chemiluminescent enzyme immunoassay (IA, Immulite 2000).
422 Whereas E levels of 22.2 % of samples were below the LLOQ of the immunoassay (20 pg
423 mL^{-1}), only one sample (0.3 %) could not be quantified by mass spectrometry (LLOQ: 10 pg
424 mL^{-1}). IA results were compared to mass spectrometric (MS) results by correlation scatter
425 plots (Fig. 5) and Bland-Altman plots (Fig. 6). At first sight, the scatter plot for E presumes
426 acceptable agreement between methods. However, the Pearson correlation coefficient r of
427 0.8913 expresses the high variability in the lower region between 40 - 100 pg mL^{-1}. This gets
428 more clearly visible in the Bland-Altman plot, where we can see that differences increase

with lower E levels and reach over ± 60 %. The scatter plot for T shows disagreement, especially in the upper region above 5,000 pg mL^{-1}. A further look into the Bland-Altman plot shows that there is strong variability over the whole range. Although 2s limits are narrower than for E, differences of over ± 50 % can be observed, which is inacceptable for clinical measurements. The reason for the partially strong disagreement could be the known disadvantages of immunoassays, as they are prone to cross reactivity, general sample condition like lipemia or hemolysis [57] or other interferences. Several groups already investigated agreement between different methodologies for steroid quantification and found large discrepancy exceeding clinical acceptance limits [3-9]. Vesper et. al [8] reported the high variabilities of estradiol assays in general and found mean bias of up to 22.5 % for MS methods compared to up to 235 % for immunoassays. Wang et. al [9] found that the Immulite 2000 is likely to systematically underestimate T concentration and showed discrepancy of over 60 % compared to LC-MS/MS, which correlates well to our observations. Overall, variability of quantitative results was found to be substantially smaller for MS methods than for immunoassays [58]. Consequently, institutions like the Joint Committee for Traceability in Laboratory Medicine (JCTLM) only accept MS assays as reference methods [59] and the National Institute of Standards and Technology (NIST) is working on establishing reliable LC-MS/MS methods for steroid quantification [23].

To control for trueness of the mass spectrometric method, commercial QCs with known concentration levels were purchased and quantified (see Table A.13). By reaching the clinical acceptance limit of 85 - 115 % accuracy and < 15 % precision, the MS method was proven to yield reasonable results.

Moreover, processing of survey scan data revealed additional information on study participants. For instance, a 3.2-fold increase of hydroxyprogesterone (d_5T-normalized) in

placebo patch groups compared to E patch groups could be displayed (Fig. 7). Application of transdermal E therefore seems to interact in hydroxyprogesterone metabolism. To support this hypothesis, we retrospectively analyzed hydroxyprogesterone/d_5T response ratios in QC samples (QC_{low} and QC_{high}; n = 36), which were run equally distributed across the entire sample sequence and were derived from an identical plasma pool. Precision, calculated as relative standard deviation, was 23.6 %. Moreover, hydroxprogesterone ratios in commercial QCs (n = 9 per level) showed following precision: Level I (0.30 ng mL^{-1}): 29.7 %; Level II (1.54 ng mL^{-1}): 16.6 %; Level III (8.96 ng mL^{-1}): 8.3 %. These values are well below the biological variance observed in the study samples and below the common acceptance limit for assay precision of 30% RSD for biomarker studies (usually applied as criteria in untargeted profiling methods). Other examples of significantly regulated steroids were found as well (Fig. A.23, Fig A.24). In general, it is shown that such an assay combined favorably hypothesis-driven targeted quantification and untargeted profiling which allowed to generate new hypotheses without extra measurements, without additional samples, and without additional human/animal experiments. Consequently, such a combined targeted/untargeted assay can be regarded in line with the 3R-principle for human and animal studies (3R principle means to avoid animal experiments altogether (**R**eplacement), to limit the number of animals (**R**eduction) and their suffering (**R**efinement) in tests to an absolute minimum), because it collects more information per sample.

4. CONCLUSIONS

The DIA technique SWATH, primarily designed for untargeted analysis of peptides in proteomics, was shown capable of accurate and reliable quantification via HR-MS/MS data. While controlling for specificity, advantageous analysis in terms of analyte coverage and

sensitivity compared to regular MRM^{HR} was demonstrated. This way simultaneous low-level quantification of E and T was achieved without derivatization nor polarity switching. Especially for endogenous compounds that require alternative quantification via surrogate calibrants, SWATH enables beneficial experiment design by reduction of the total number of MS and MS/MS experiments favorable for keeping cycle times short. Owing to the feature of combined targeted/untargeted analysis, valuable secondary information is recorded and accessible post-acquisition. High quality untargeted MS/MS data, e.g. for steroid profiling, can be collected by optional, user-modulated SWATH experiments. Validation according to international guidelines (with some minor modifications) and accurate quantification of certified, commercial quality controls underline the value of this acquisition technique. By exploiting the potential of SWATH for sensitive and quantitative steroid analysis, most likely in conjunction with extended chromatography, the avenue towards steroidomics has been paved herein.

ACKNOWLEDGMENTS

We acknowledge the financial support by the “Struktur- und Innovationsfonds Baden-Württemberg (SI-BW)”, the German Science Funds (DFG no. INST 37/821-1 FUGG) and by grants from the German Federal Ministry of Education and Research (BMBF) to the German Center for Diabetes Research (DZD e.V.; 01GI0925).

AUTHOR INFORMATION

Author Contributions. M.H. conceived and designed the clinical study. M.L. and B.D. conceived and designed analytical method. B.D. performed the experiments and

measurements, and analyzed the data. The manuscript was written through contributions of all authors.

Notes. The authors declare no competing financial interest.

Appendix A. Supplementary data

TABLES AND FIGURES

Table 1. Overview of method-parameters for targeted analysis of E and T (method 1).[a]

Analyte	Ion Species	Precursor [m/z]	Q1 window [m/z]	Fragment [m/z]	DP [V]	CE [V]	t_R [min]
E	$[M\text{-}H_2O+H]^+$	255.1743	254.5 - 258.5	159.0804	90	25	3.076 ± 0.011
$^{13}C_3$E	$[M\text{-}H_2O+H]^+$	258.1844		162.0905			3.076 ± 0.012
d_5-E	$[M\text{-}H_2O+H]^+$	260.2057	260.0 - 265.0	161.0930	90	25	3.044 ± 0.012
T	$[M+H]^+$	289.2162	288.5 - 292.5	109.0648	200	33	3.159 ± 0.009
$^{13}C_3$T	$[M+H]^+$	292.2263		112.0749			3.158 ± 0.009
d_5T	$[M+H]^+$	294.2476	294.0 - 299.0	113.0899	120	33	3.136 ± 0.010

[a]Declustering potential (DP), collision energy (CE), retention time (t_R). SWATH (Q1) windows covered both, target analyte and corresponding surrogate calibrant. Their specific fragments have a mass difference of 3 Da and do not show interferences. To create a SWATH-MS/MS experiment, also a TOF-MS experiment has to be performed in each cycle. This precedented TOF-MS run (30 - 1,000 Da) had a t_{Acc} of 20 ms, CE of 10 V and a DP of 100 V.

Table 2. Matrix effect, extraction recovery, process efficiency.[b]

Analyte		ME [%]	RE [%]	PE [%]
$^{13}C_3E$	QC_{LLOQ}	82.2 ± 12.7	94.2 ± 11.1	77.4 ± 11.5
	$QC_{3x\ LLOQ}$	79.6 ± 11.8	93.3 ± 9.7	74.3 ± 14.1
	QC_{Mid}	83.3 ± 5.7	79.4 ± 9.2	66.1 ± 8.4
	QC_{ULOQ}	80.2 ± 6.7	85.2 ± 3.9	68.4 ± 3.7
$^{13}C_3T$	QC_{LLOQ}	55.2 ± 13.3	86.9 ± 12.2	48.0 ± 6.5
	$QC_{3x\ LLOQ}$	58.7 ± 14.5	89.2 ± 10.9	52.4 ± 9.8
	QC_{Mid}	63.2 ± 4.5	78.7 ± 5.8	49.8 ± 6.8
	QC_{ULOQ}	64.4 ± 7.1	82.6 ± 5.5	53.2 ± 4.8

[b]Single determinations of 5 different lots were used to create QCs in neat standard solution, post-extraction spiked plasma and pre-extraction spiked plasma. Error was calculated by addition of relative errors of mean values. T shows a relatively ineffective PE. Since the LLOQ of 20 pg mL^{-1} is below normal reference levels in patients, a PE of around 50 % can be accepted. Concentrations, see Table 3 footnote c.

519 **Table 3.** Validation results of precision and accuracy.[c]

Analyte		QC_{LLOQ}		$QC_{3x\ LLOQ}$		QC_{Mid}		QC_{ULOQ}	
		Accuracy [%]	Precision [%]	Accuracy [%]	Precision [%]	Accuracy [%]	Precision [%]	Accuracy [%]	Precision [%]
$^{13}C_3E$	Intra-day (n = 5)	114.7	3.9	99.2	2.5	96.9	4.4	93.9	8.5
	Inter-day (n = 15)	112.8	5.5	99.2	5.0	94.6	4.5	96.9	10.2
$^{13}C_3T$	Intra-day (n = 5)	111.7	4.7	102.8	2.7	108.2	4.5	95.7	6.6
	Inter-day (n = 15)	107.7	7.8	102.1	6.5	104.1	7.6	98.2	5.1

520 [c]Concentrations were as following: QC_{LLOQ}, 10 pg mL^{-1} for $^{13}C_3E$ and 20 pg mL^{-1} for $^{13}C_3T$;

521 $QC_{3x\ LLOQ}$, 30 pg mL^{-1} for $^{13}C_3E$ and 60 pg mL^{-1} for $^{13}C_3T$; QC_{Mid}, 250 pg mL^{-1} for $^{13}C_3E$ and

522 2,500 pg mL^{-1} for $^{13}C_3T$; QC_{ULOQ} 1,000 pg mL^{-1} for $^{13}C_3E$ and 15,0000 pg mL^{-1} for $^{13}C_3T$.

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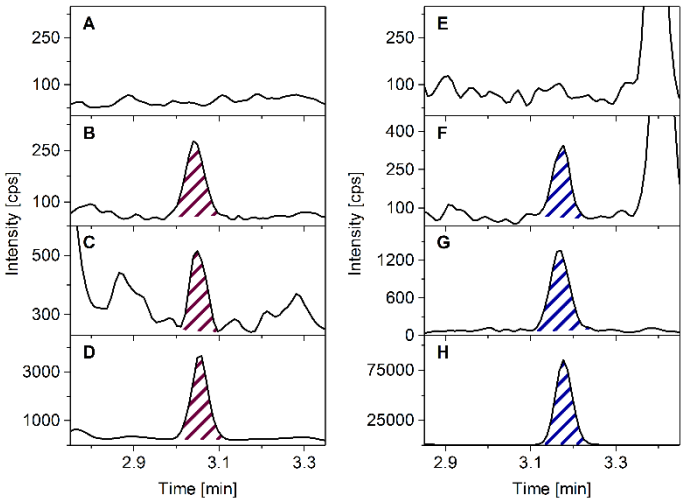


524

525 **Fig. 1.** Chromatograms (product ion EIC) in true plasma matrix. A: $^{13}C_3$E in blank matrix; B:

526 $^{13}C_3$E spiked at LLOQ (10.0 pg mL^{-1}); C: E in real sample at LLOQ (10.7 pg mL^{-1}); D: E in real

527 sample (242 pg mL^{-1}); E: $^{13}C_3$T in blank matrix; F: $^{13}C_3$T spiked at LLOQ (20.0 pg mL^{-1}); G: T

528 in commercial control Level I (201 pg mL^{-1}, lowest concentration of all samples); H: T in real

529 sample (7,507 pg mL^{-1}).

530

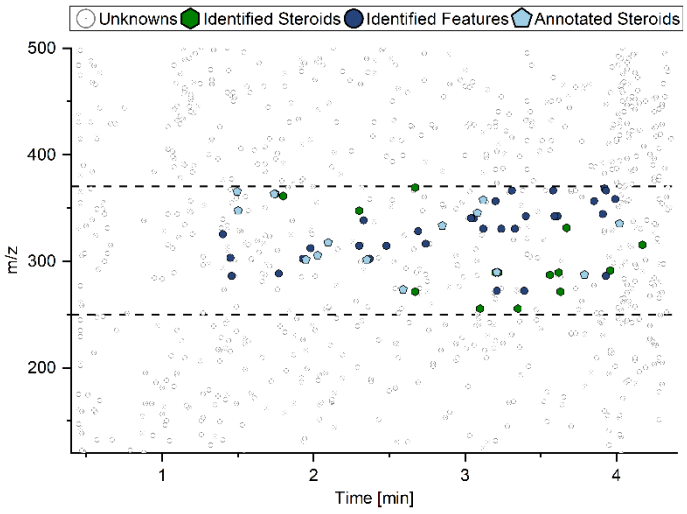


Fig. 2. Aligned peak spotting in 9 repeated measurements of a pooled plasma QC sample (m/z range from 120 to 500 is shown, for extended overview see Fig. A.22) applying method 2 (see Table A.14). 1,613 molecular features with a peak intensity over 2,000 cps were found in the survey scan after blank subtraction, de-isotoping and de-adducting. Dashed lines represent the mass range covered comprehensively by SWATH MS/MS experiments. Identified steroids were verified by injection of authentic standards and matching of t_R and mass spectra. Identified features showed matching precursor m/z and high level agreement of mass spectra (LipidBlast [56], MassBank [60]) identified by MS-DIAL [61] software. Annotated steroids were found by matching m/z of precursors from steroids covered in the LipidMaps [62] database.

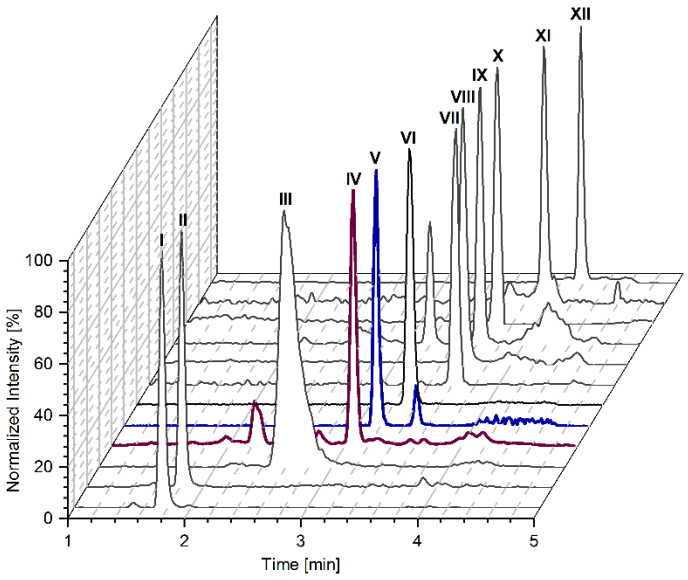
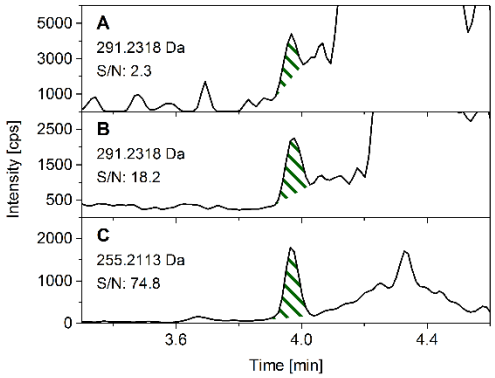


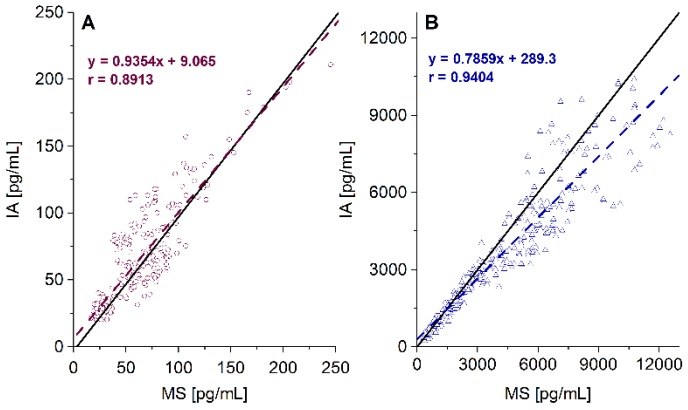
Fig. 3. Overlay of normalized chromatograms of identified steroids (targets IV and V; non-targeted steroids I-III and VI-XII) in commercial control. I: Cortisol (fragment, m/z 327.1955 ± 0.02); II: Cortisone (fragment, m/z 343.1904 ± 0.02); III: Dehydroepiandrosterone-Sulfate (fragment, m/z 213.1638 ± 0.02); IV: Estradiol (fragment, m/z 159.0804 ± 0.02); V: Testosterone (fragment, m/z 109.0648 ± 0.02); VI: epiE (fragment, m/z 159.0804 ± 0.02); VII: epiT (fragment, m/z 109.0648 ± 0.02); VIII: Androstenedione (fragment, m/z 97.0648 ± 0.02); IX: Dehydroepiandrosterone (precursor, m/z 271.2062 ± 0.02); X: Hydroxyprogesterone (fragment, m/z 97.0648 ± 0.02); XI: Dihydrotestosterone (fragment, m/z 255.2113 ± 0.02); XII: Progesterone (fragment, m/z 97.0648 ± 0.02). Method 2 (see Table A.14).



553

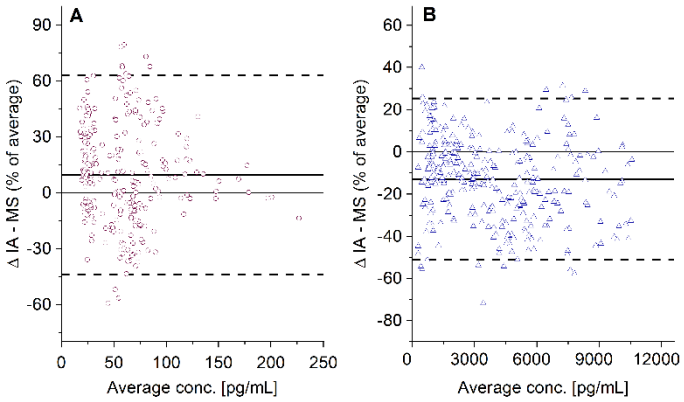
554 **Fig. 4.** Comparison of signal quality for non-targeted dihydrotestosterone. Signals were obtained
555 from commercial QC Lvl. III (1,050 pg ml^{-1}). A: TOF-MS of precursor ion; B: SWATH-MS/MS
556 of precursor ion; C: SWATH-MS/MS of fragment ion. Method 2 (see Table A.14). (S/N values
557 are estimates calculated with PeakView).

558

559 

560 **Fig. 5.** Scatter plots for comparison of results from immunoassay (IA) and mass spectrometry
561 (MS). Plot (A) shows results for E and plot (B) for T. Solid lines resemble the optimum line of
562 parity. Dashed lines are results of linear regression analysis of results obtained with the two
563 methods.

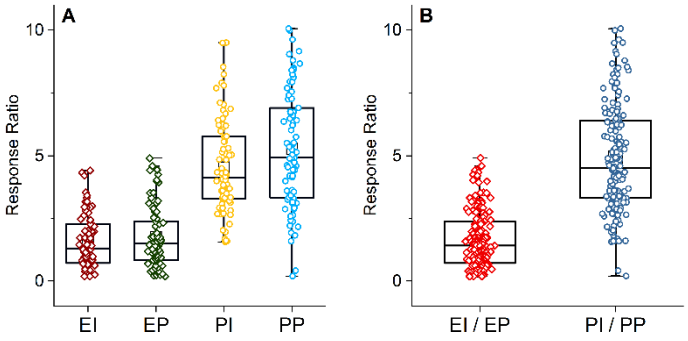
564



565

566 **Fig. 6.** Bland-Altman plots for comparison of results from immunoassay (IA) and mass
567 spectrometry (MS). Plot (a) for E with mean difference (9.7 %, solid line) and 2s limits (95 %
568 limits of agreement; +2s = 63.1 % , -2s = - 43.7 %, dashed lines). Plot (b) for T with mean
569 difference (-13.0 %, solid line) and 2s limits (+2s = 25.3 % , -2s = - 51.2 %, dashed lines).

570



571

572 **Fig. 7.** Relative quantification of hydroxyprogesterone. EI: E patch & insulin treatment; EP: E
573 patch & placebo treatment; PI: placebo patch & insulin treatment; PP: placebo patch and placebo
574 treatment. Boxplots for each of the four groups (A) and for grouped E patch and grouped placebo
575 patch samples (B). For B, a 3.2-fold increase (median values) in hydroxyprogesterone was found
576 in placebo patch groups (U-test, p-value: 3.3×10^{-47}). Signals were obtained from TOF-MS scan
577 (precursor signals).

578

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