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**How to cite**

OLESTI, Eulalia et al. Steroid profile analysis by LC-HRMS in human seminal fluid. In: Journal of Chromatography. B, 2020, vol. 1136, p. 121929. doi: 10.1016/j.jchromb.2019.121929

This publication URL: https://archive-ouverte.unige.ch/unige:133089
Publication DOI: 10.1016/j.jchromb.2019.121929

# Steroid profile analysis by LC-HRMS in human seminal fluid

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## Abstract

Steroids are essential hormones that play a crucial role in homeostasis of many biological processes including sexual development, spermatogenesis, sperm physiology and fertility. Although steroids have been largely studied in many biological matrices (such as urine and plasma), there is very limited information of the steroid content and their study as potential indicators of the quality of the seminal fluid. In this study, a LC-HRMS strategy has been developed in order to obtain the extended steroid profile of human seminal fluid. A comparison between supported liquid extraction (SLE) and solid liquid extraction (SPE) was carried out and the chosen SPE method was further optimized to evidence the largest possible number of compounds. Steroids were automatically annotated by using DynaStI, a publicly available retention time prediction tool developed in our lab, to match the experimental data (i.e. accurate mass and $t_R$). Altogether, these resources allowed us to develop a post-targeted approach able to consistently detect 41 steroids in seminal fluid (with half of them being androgens). Such steroid pattern was found stable across different extraction times and injection days. In addition to accurate mass and retention time, the identity of 70% of the steroids contained in such steroid profile was confirmed by comparing their fragmentation patterns in real samples to those of pure commercial standards. Finally, the workflow was applied to compare and distinguish the steroid profile in seminal fluid from healthy volunteers (n=7, with one of them being a vasectomized subject). In all, the developed steroidomics strategy allows to reliably monitor an extended panel of 41 steroids in human seminal fluid.

## Keywords

Steroid profile, seminal fluid, LC-HRMS

## Highlights

- A workflow for the relative quantification of 41 steroids in seminal fluid was developed.
- The steroids' identification was achieved by using a post-targeted approach.
- The steroid profile in seminal fluid was evaluated in seven healthy subjects.

## 1. Introduction

Steroids are synthesized in several tissues (e.g. adrenal glands, gonads, brain, skin) and since they play a critical role in several biological processes, they can be involved in a great diversity of pathologies [1,2]. In men, androgens play a crucial role in mediating reproductive functions including spermatogenesis, sperm maturation and the functionality of the sexual accessory organs [3,4]. Indeed, the increased number of male infertility cases[5] has pointed out the important role of steroid homeostasis in sex-related organs, and alterations on the steroid profile have been associated with increased risks to develop prostate cancer and higher levels of sperm abnormalities[6–8].

In targeted steroid analysis, pre-defined sets of steroids have been quantified [1] in different human samples (such as urine [9,10], plasma or serum [11,12], hair [13], saliva[14], etc.) and using different analytical strategies, ranging from gas chromatography-mass spectrometry (GC-MS) [15] to liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) [9,16,17]. The use of untargeted approaches for steroid analysis is mainly exploratory and remains limited to some of the most common matrices, such as urine and plasma, either by GC-MS [17–19] or LC-MS/MS [20–23]. However, untargeted steroidomic analysis for alternative matrices (such as seminal fluid) still remains unexplored despite its large potential.

The absolute quantitation of a restricted number of steroids in human seminal fluid has been already shown to be a useful tool to evaluate seminal fluid quality and for the evaluation and monitoring of fertility treatments. As an example, higher levels of estrogens with lower levels of androgens (such as testosterone) have been associated to impaired spermatogenesis production [24] and the testosterone/estradiol ratio has been used as a good indicator of the normal spermatogenesis function [3,24].

However, the steroid content of the seminal fluid has been only poorly explored so far and there is very limited information of the steroid composition of the seminal fluid and its potential role as biomarkers of male infertility or other conditions [23]. The steroid mapping in seminal fluid has the potential to provide relevant information to diagnose and monitor the evolution of different diseases, infer xenobiotics' toxicological effects, and to find out the origin of some unexplained cases of the low seminal quality [25,26]. Indeed, the characterization of the steroid profile in seminal fluid can provide a closer and more accurate picture of the testis function and physiology, which could be extremely relevant in fertility related pathologies [3,7]. Furthermore, the methodologies currently used to analyze

the steroid profile in other matrices (such as blood), cannot be directly used as diagnostic biomarkers for the testis' or the prostate's statuses [27], since contribution from other organs acts as a confounding factor in blood plasma.

The aim of this manuscript is to describe a complete workflow for mapping an extended steroid profile of seminal plasma by identifying the largest possible number of steroids using an straightforward analytical approach, what is a prerequisite for a broad adoption in routine clinical laboratories. To do so, an untargeted acquisition strategy was chosen since it enables a large coverage of the steroid profile with potential capacity for biomarker discovery and allowing the re-exploration of the acquired data.

## 2. Materials and methods

### 2.1. Chemicals and reagents

The available standards of endogenous steroids and androstenedione-$d_7$ (used as isotopically-labelled internal standard (IS)) were obtained from Sigma-Aldrich (Buchs, Switzerland) and Steraloids (Newport, RI, USA) with a purity >98% for all standards. Phosphoric acid ($H_3PO_4$), zinc sulphate ($ZnSO_4$) and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich. Formic acid (FA) was obtained from Biosolve (Valkenswaard, The Netherlands) and acetonitrile (MeCN), water ($H_2O$), methanol (MeOH) and dichloromethane (DCM) from Fisher Scientific (Loughborough, UK).

### 2.2. Standards and solutions

Stock solutions of steroid standards were prepared at 1 mg/mL in methanol (100%) containing 0.1% FA. Then, according to each analyte ionization response, working solutions ranging from 0.1 µg/mL to 2 µg/mL were prepared in $H_2O$/MeOH (95:5). The internal standard solution contained androstenedione-$d_7$ at 50 ng/mL in MeOH.

### 2.3. Biological samples

Seminal samples were obtained from healthy volunteers from the University of Geneva after informed consent and anonymization (n=9). From these samples, all of them were used for the method development and optimization and also as surrogated QC. For the proof of concept, n=6 samples from this set were used and an additional sample from a vasectomized volunteer was included. All samples were incubated at 37ºC during 20-45 min to allow liquefaction and then centrifuged 10 min at 700 × g to separate the seminal fluid from the spermatozoa [26]. Afterwards, seminal fluid was aliquoted and stored at -80ºC until steroid extraction.

### 2.4. Liquid chromatography and mass spectrometry conditions

LC separations were performed on an Acquity H-Class UPLC System (Waters, Milford, USA) coupled to an Orbitrap Q-Exactive Focus mass spectrometer (Thermo Fischer, Waltham, USA), equipped with an ESI source (HESI II probe) operated in positive ionization mode. The steroids' separation was achieved on a Kinetex C18 column (2.1 mm × 150 mm, 1.7 µm, from Phenomenex, Torrance, CA) with a flow rate of 0.3 mL/min and a column temperature of 30 ºC. Mobile phase A was water and mobile phase B was acetonitrile, both containing FA at 0.1% (v/v). A generic linear gradient program was used for the separation of the steroids with a total run of 25 min. The percentage of B solvent (acetonitrile) changed as follows: 0 min, 2 %; 14 min, 98%; 17 min, 98%; 17.1 min, 2%; 25 min, 2%. The ESI source was set with a heater temperature of 425 ºC and the sheath gas and auxiliary gas pressures were set to 50 and 15 arbitrary units, respectively. The ion spray voltage was set to 3 kV, with a capillary temperature of 300 ºC and the S-Lens RF level was 55%. The detection of steroids was achieved in Full Scan mode using a mass resolution of 70,000 with a mass range from 200 to 600 m/z. A window of acquisition from 120 to 1000 m/z was used, for an acquisition target (AGC) of $3\times10^6$ ions with a maximum filling time of the C-Trap (IT Fill Time) of 250 ms. All extracted chromatograms were obtained using an m/z tolerance of 5 ppm. For the steroid identity confirmation, a parallel reaction monitoring (PRM) method was developed. An isolation window of 0.4 m/z was set and the maximum number of precursor ions to be multiplexed in a scan event (MSX count) was from 2 to 8, depending on the proximity of the analytes in terms of retention times. The chromatographic and general MS conditions of the PRM were the same as the ones used in the full scan analysis. For each steroid, the most intense parent ion was chosen at the corresponding retention time of the chromatographic peak. Mass calibration (< 3ppm) was performed once a week using the Pierce® Velos ESI Positive Ion Calibration standard mixture (Thermo Fisher Scientific) containing *n*-butylamine, caffeine, MRFA (peptide of Met-Arg-Ala acetate salt) and Ultramark 1621. The selected precursors ions then undergo in a collision-induced fragmentation (10 to 30 eV) in order to conduct the MS/MS analysis. Xcalibur 4.1 (Thermo Fischer, Waltham, USA) was used for the instrument control and data acquisition.

### 2.5. Sample preparation and extraction

Two different extractions procedures were compared to find the most adapted one for steroidomic profiling in seminal fluid: SLE on ISOLUTE SLE+ 400 Extraction plates from Biotage, Uppsala, Sweden; and SPE with HLB µElution plates (Waters, Milford, MA, USA). In both cases, 200 µL of the samples and 10 µL of the IS were diluted with 200 (SLE) or 500 µL (SPE) of aqueous 4% $H_3PO_4$ before loading into the extraction plates. For the SLE extraction, the samples were extracted by gravity with 0.6 mL of DCM. Then, a positive pressure of 3 psi was applied for 30 s to complete the elution of the organic solvent. In the case of SPE, a washing step was performed with 400 µL of $H_2O$:MeOH (95:5) prior to eluting the steroids with 50 µL of $H_2O$:MeCN (10:90). The eluates obtained after SLE or SPE

were evaporated to dryness using a SpeedVac (Thermo Fischer Scientific, Waltham, MA, USA) and reconstituted in 100 µL of $H_2O$:MeOH (50:50). Finally, 10 µL were injected for LC-HRMS analysis.

### 2.6. Method optimization

Different experimental parameters were evaluated to enhance extraction efficiencies: protein precipitation (assayed with aqueous $ZnSO_4$ 10% or TCA 10%), washing conditions ($H_2O$:MeOH 60:40 or 95:5), SPE elution solvent ($H_2O$:MeOH, 10:90 and 50:50) and elution volumes for SLE (0.3, 0.6 and 0.9 mL of DCM were assayed). The reconstitution solvent was also compared between two conditions ($H_2O$:ACN, 90:10 and $H_2O$:MeOH, 50:50). The method optimization was performed by using a pool of seminal fluid samples obtained from healthy subjects (n=9).

### 2.7. Evaluation of within/between day variability

Once the optimized conditions for identifying the major number of steroids in the seminal fluid matrix were selected, a within/between-day variability comparison was performed over three days. During this time period, the steroids from the seminal fluid pool (nine volunteers) were repeatedly extracted each day and injected in different analytical batches into the LC-HRMS system to evaluate the repeatability and fidelity of the steroid profile obtained with the developed methodology [28]. The repeatability was expressed as the square root of the within-day variation (calculated as the average of the variances of each day (j=3)) divided by the average of the mean of the ratio (analyte area/internal standard area). The fidelity interval was estimated as the square root of the between-day variation (which was calculated as the variance of the 3 daily averages minus the within-day variance divided by the number of extracted samples (n=6 aliquots from the pool)) divided by the average of the mean ratios (analyte area/internal standard area)[28].

### 2.8. Data treatment

Xcalibur (version 4.1; Thermo Fischer, Waltham, USA) and Skyline MS 4.2 (MacCoss Lab Software) [29] were used for data analysis. The preliminary annotation of the steroids' peaks was performed by using their accurate masses and retention times, obtained either from authentic standards or with the support of DynaStI, an open tool providing retention times for steroids separated under reverse-phase conditions. This tool is freely accessible on the website: http://dynasti.vital-it.ch [30–32]. The DynaStI database contains RP-retention information from 188 steroids, either measured from commercial reference standards (92) or predicted *in silico* (96). The steroids confirmatory identification was achieved by comparing the fragmentation patterns obtained in biological samples to those from commercial standards analysed in PRM mode.

Multivariate analysis was conducted on SIMCA 15 (Umetrics Sartorium Stedim, Umeå, Sweden) and graphs were plotted using OriginPro 2018 (Origin Lab, Massachusetts, USA).

### 2.9. Method applicability

As a proof of concept of the presented methodology, the newly developed workflow was applied to seminal fluid samples from healthy subjects (n=7). One of the subjects was vasectomized and the sample was collected about one year after the surgery (id 7). Additionally, we analysed a pool of the extracted volunteers (post-extraction pool of the id 1 to 6, all the non-vasectomized individuals) and we extracted different surrogated QC (pre-extraction QC from different healthy volunteers) in order to monitor the stability of the steroid profile in the analytical batch. All samples were processed in duplicates and the steroid profile was evaluated for all samples in the same randomized analytical batch.

## 3. Results and Discussion

### 3.1. Method optimization

The main analytical challenges of the steroid profiling in semen are i) the poor ionization of some steroids [33], ii) the low concentration compared to blood plasma for some molecules (such as testosterone, androstenedione or cortisol [3]), iii) the structural similarities between steroids [1], and iv) the high viscosity of the seminal fluid (around three times more viscous than blood plasma) [34,35]. For steroids not showing a suitable ionization behaviour, a derivatization step can enhance its detectability [33]. With regard to the other challenges mentioned above, different strategies were assayed to perform the steroids analysis in seminal fluid. Firstly the spermatozoa were discarded form the sample by centrifugation and only the seminal plasma was collected. Secondly, pre-concentration, cleaning and extraction steps (SLE and SPE) were optimized for the maximum metabolite coverage of steroids as described below. Finally, the use of reference standards, an in-house database [30,31] and the PRM acquisition mode, made possible the reliable annotation and identification of most of the steroids. An example chromatogram of a set of steroids identified in a real seminal plasma sample is shown in **Figure 1**.

For the pre-concentration and cleaning steps, a comparison between SLE and SPE extraction cartridges was performed to find the best conditions to detect and identify the extended steroid profile in human seminal fluid. Different protein precipitation solutions ($ZnSO_4$ 10% w/v or TCA 10%) were considered as a pre-treatment step before SPE and SLE, but no significant increment of the number of found steroids, nor the intensity of their peaks was observed ( **Figure S1**). For SLE, different volumes of elution (0.3, 0.6 and 0.9 mL of DCM) and reconstitution solvents (aqueous acetonitrile or methanol mixtures) were evaluated. For SLE, 0.6 mL of DCM for the elution step and reconstitution in 50 µL of methanol:water (1:1) yielded the best results, in accordance with similar procedures published in other

matrices such as serum or urine [9,11,36]. However, the optimized SPE extraction allowed the extraction of a larger panel of molecules compared to SLE (see **Figure 3**). While SLE allowed to monitor 22 steroids, SPE extracted 46 steroids from the seminal fluid samples, providing a broader picture of the steroid profile. A panel of 18 common steroids including testosterone, cortisol or androstenedione were unambiguously identified with both extraction methods. The limited performance of SLE with regard to SPE for steroid profiling in seminal fluid can be explained by considering the viscosity of the matrix. Indeed, to facilitate sample handling, a dilution step with water is needed prior to sample loading on either SPE or SLE cartridges. While SPE can cope with more diluted samples by just passing a larger volume through the cartridge to eventually yield similar analyte recoveries, in SLE the amount of sample that can be loaded is limited by the surface of the sorbent, thus compromising the sensitivity of the technique if previous sample dilution is required. For subsequent SPE optimization, different washing solutions (with different water percentages aqueous methanol) and different elution solvents (methanol or acetonitrile) were also assayed for the steroidomic mapping. In all, we observed that the SPE extraction, washed with water:methanol (95:5), eluted with water:acetonitrile (10:90) and reconstituted with water:methanol (50:50) yielded the largest coverage of steroids in human seminal fluid.

### 3.2. Reproducibility of the steroid profile

One of the main difficulties of the steroid profile determination is the analytical variability between different analyses, which compromises the robustness of the steroidomic profile [37]. Therefore, in order to evaluate the whitin/between-day variability of the post-targeted analyses, the stability of the profile was evaluated during different extraction and injection days (3 different days (j=3) with n=6 samples from the common pool). From the 46 steroids found with the SPE approach, 41 were consistently found over the different injection days (3 extraction days and in the SPE-SLE comparison experiment) with similar peak intensities (see **Figure 3**). Peak area correction using androstenedione-$d_7$ as an internal standard allowed to reduce variability coming from the sample preparation and instrumental analysis.

Regarding the within-day variation and the between-day variation (**Table 1**), 27 out of the 41 steroids identified in the seminal fluid presented a repeatability and intermediate fidelity ≤ to 30%. These results showed a good reproducibility of the developed methodology and an acceptable accuracy of the profile, even in the absence of any normalization procedure and by only using a single internal standard (androstenedione-$d_7$). Most steroids yielding poor repeatability and fidelity (≥ to 30%) came from annotations without MS/MS confirmation (see Section 3.3). Such subset of compounds presented a low peak intensity, what could explain the higher variations of the areas among the three different days presented in **Table 1**. Furthermore, the use of specific internal standards for each analyte would have contributed to obtain a lower within and between-day variation in a targeted strategy.

### 3.3. Steroid profile confirmation

By combining the use of an open steroid database (https://dynasti.vital-it.ch) [30–32] with empirical data (i.e. exact mass, MS/MS and $t_R$) a post-targeted annotation of the detected steroids in seminal fluid was achieved. This strategy reduced the size of the dataset by filtering the analytes of interest (steroids) from the untargeted acquisition data set [1]. In all, 41 steroid were considered at level 1 of annotation [1,38] (matching in exact mass and $t_R$ with standards analysed in identical conditions) (see **table 1**). From these 41 potential steroids, the identification of around 70% of them (28 steroids) was additionally confirmed by comparing the fragmentation pattern of each steroid in seminal plasma samples to those obtained from pure standards (see **Figure 2**). The combination of retention time, accurate mass and fragmentation pattern provided the highest level of confidence for peak identification [20]. The un-confirmed profile (30% left, about 12 compounds) were found to be consistent among the different working days and did not correspond to any identified compound.

### 3.4. Classification of the steroids profile in semen

The evaluation of the stable steroid profile containing 41 compounds in total (with similar peak intensities during three different extraction days), revealed that about half of the steroids (44%) found in the seminal fluid were androgens (see **Table 1**). This finding is in accordance with the study performed by Kwan TK et al in 1992 were many androgens (such as testosterone or 5α-dihydrotestosterone) were also found in seminal fluid [39]. Furthermore, the described essential role of intra-testicular androgens in the regulation of spermatogenesis and the development of functional male reproductive organs also supports our findings [40]. Then, about one fifth of the detected steroids (20%) were corticosteroids (glucocorticoids and mineralocorticoids) [17] previously described to influence the testosterone biosynthesis in animal models through their receptors in the Leydig cells [3,41]; and about 10% of the steroid profile are oestrogens, which have been also found in the masculine gonads (such as estradiol and estriol) and have a key role in fertility [1,2,42,43]. The remaining steroids were progestogens (12.2%), which also play a key role in the male fertility [7,44,45], and some cholic acid derivatives (12.2%).

### 3.5. Proof of concept

To demonstrate the applicability of the presented methodology, the steroid profile in human seminal fluid from different volunteers with no known health issues (n=7) was independently assessed. Due to limited sample availability, a set of surrogated QCs was generated from the pool of seminal fluid samples (n=9, already used for the method development and optimization). Six aliquots of this pool were separately submitted to sample preparation and LC-MS analysis steps. This set allowed to evaluate the variability derived from the whole analytical process. Unsupervised multivariate analysis (Principal Component Analysis, PCA) (see **Figure 4**), showed that all quality controls (QCs) were tightly clustered although not completely centred, as expected from surrogate QCs. Then, the analysis of the different

individuals (IDs 1a/b to 7a/b, see **Figure 4**) demonstrated the low variation of the obtained steroid profile in duplicate extractions of the same samples performed simultaneously. The steroid profile of these subjects (id1 to id 7) shared characteristic features among their patterns and the developed methodology allowed to highlight the differences found between individual samples and a reference average profile (see **Figure 3**). Interestingly, one of the healthy volunteers (id 7) was vasectomized one year before the sample collection and no-apparent differences were found in the steroid profile of this subject in comparison with the rest of the volunteers. Further studies between non-vasectomized and vasectomized individuals (with samples collected at different times after the surgery) would be required in order to find further alterations in the steroid profile and evaluate their potential differences on the steroid fingerprint. Upon inspection of the PCA loadings plot (**Figure 4b**), it can be observed that cortisol and testosterone exhibit opposite behaviours on the first principal component (PC1), which accounts for the main source of variability within the set of variables. This result is in agreement with the well-known anticorrelation between stress (cortisol) and procreation (testosterone) steroid levels, a natural evolutionary output that promotes reproduction and childbearing in safe environments while hindering it under stress situations [46,47]. Data from the present analyses show how individuals with higher testosterone levels (negative PC1 loadings) tend to have lower cortisol levels than the ones with lower testosterone concentrations (positive PC1 loadings) and vice-versa. However, in order to further confirm this exploratory result in human seminal plasma, a larger population would be required. The evaluation of this small cohort demonstrates the successful combination obtained after an untargeted acquisition mode with a post-targeted data treatment strategy. Even though different improvements could be made to enrich and complement the presented study (such as adding non-fertile patients or develop an absolute quantification of the steroid profile), the developed strategy has permitted to map the steroidome in human seminal fluid. Research involving a larger number of participants (e.g. available Swiss cohorts [26,27]) will be carried out in order to evaluate the usefulness of steroids as potential indicators of quality of the seminal fluid by applying the developed methodology.

### 3.6. Strengths and limitations

An optimization of the extraction and pre-concentration steps was conducted and a post-targeted metabolomics approach applied to establish a steroid profile by using LC-HRMS. An exploratory, untargeted strategy was applied for the discovery of steroids in the human seminal fluid. Key steroids such as cortisol, testosterone or 17b-estradiol were detected in seminal fluid along with XXXXX steroids found for the first time in human seminal plasma to the best of our knowledge. The method was validated to assess its reproducibility, fidelity precision. MS/MS confirmation was performed on AMRT-annotated steroids. The method was eventually applied to study the steroid profile of healthy subjects, illustrating its versatility and potential role in fundamental research and/or clinical applications. Since the goal of the developed methodology is the characterization of large cohorts through steroid fingerprinting, only one LC-MS mode was considered.

## 4. Conclusions

The identification of steroids present in human seminal fluid has remained almost unexplored to this day. However, a better assessment of steroid profile in human seminal fluid may provide opportunities to better understand the links between human semen quality and fertility. In this study, SPE conditions were optimized to obtain the largest number of steroids from human seminal fluid. We have developed a workflow for the mapping of an extended steroid profile comprising 41 steroids consistently found in all the seminal fluid samples along different extraction days. The annotation of the steroid profile was achieved thanks to an untargeted acquisition mode with a post-targeted identification strategy using an in-house developed, publicly available, retention time database and prediction tool. The identity 28 of steroids was confirmed by their MS/MS fragmentation pattern. The majority of the steroids were androgens. The developed methodology highlighted the differences found between different subjects and a reference steroid profile. The analysis of the steroid profile in healthy subjects has proved the versatility of the methodology and its potential use for studying male related infertility.

## 5. Acknowledgments

The authors would like to acknowledge the Swiss Centre for Applied Human Toxicology (SCAHT, Switzerland) and the Swiss National Science Foundation (grant 31003A_166658) for funding this work.

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# Figures and Tables

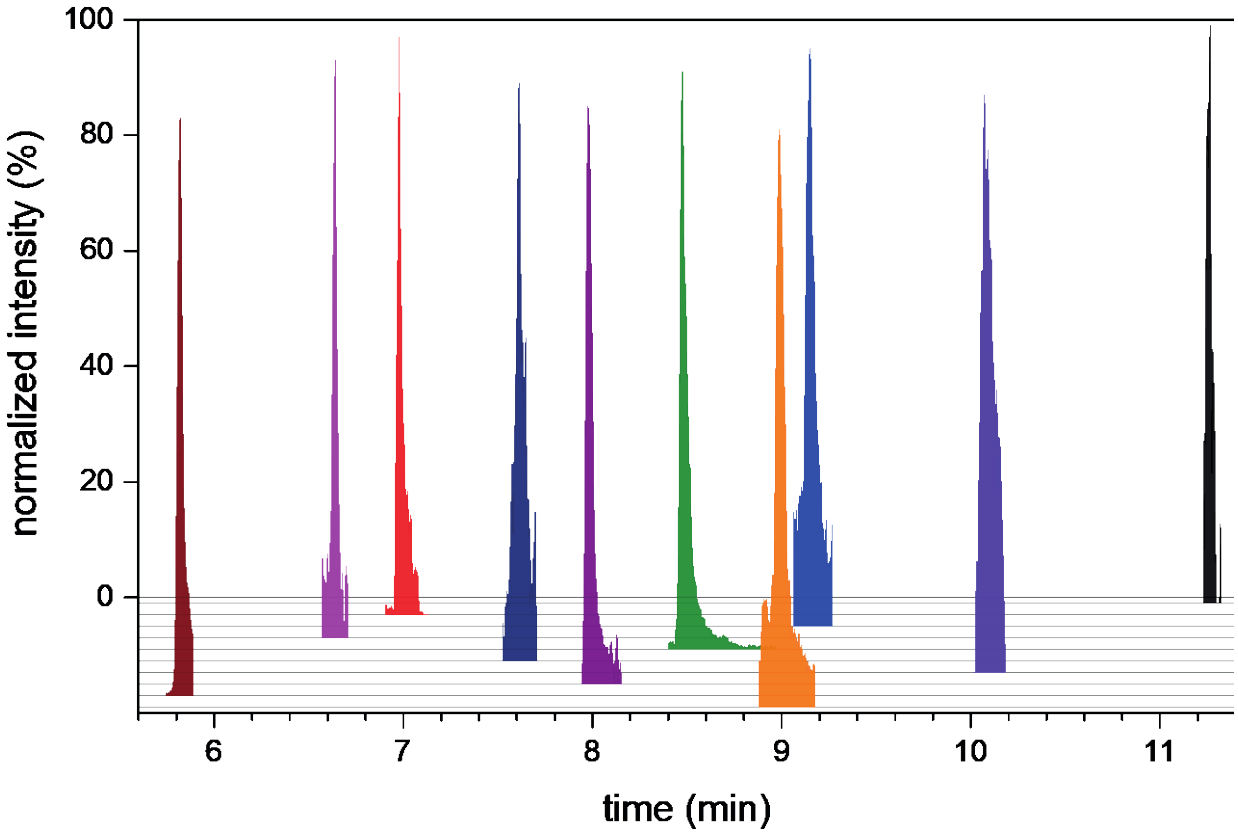


**Figure 1**. Chromatogram of a set of 10 representative steroids found in a real sample of human seminal fluid. From left to right, peaks correspond to: 2-Hydroxyestriol, 16a,17b-estriol, cortisol, 11-dehydrocorticosterone, glycocholic acid, 11-ketoetiocholanolone, cholic acid, testosterone, 5a/b-dihydrotestosterone, and pregnenolone. The peak heights were plotted as the normalized intensity (100%).

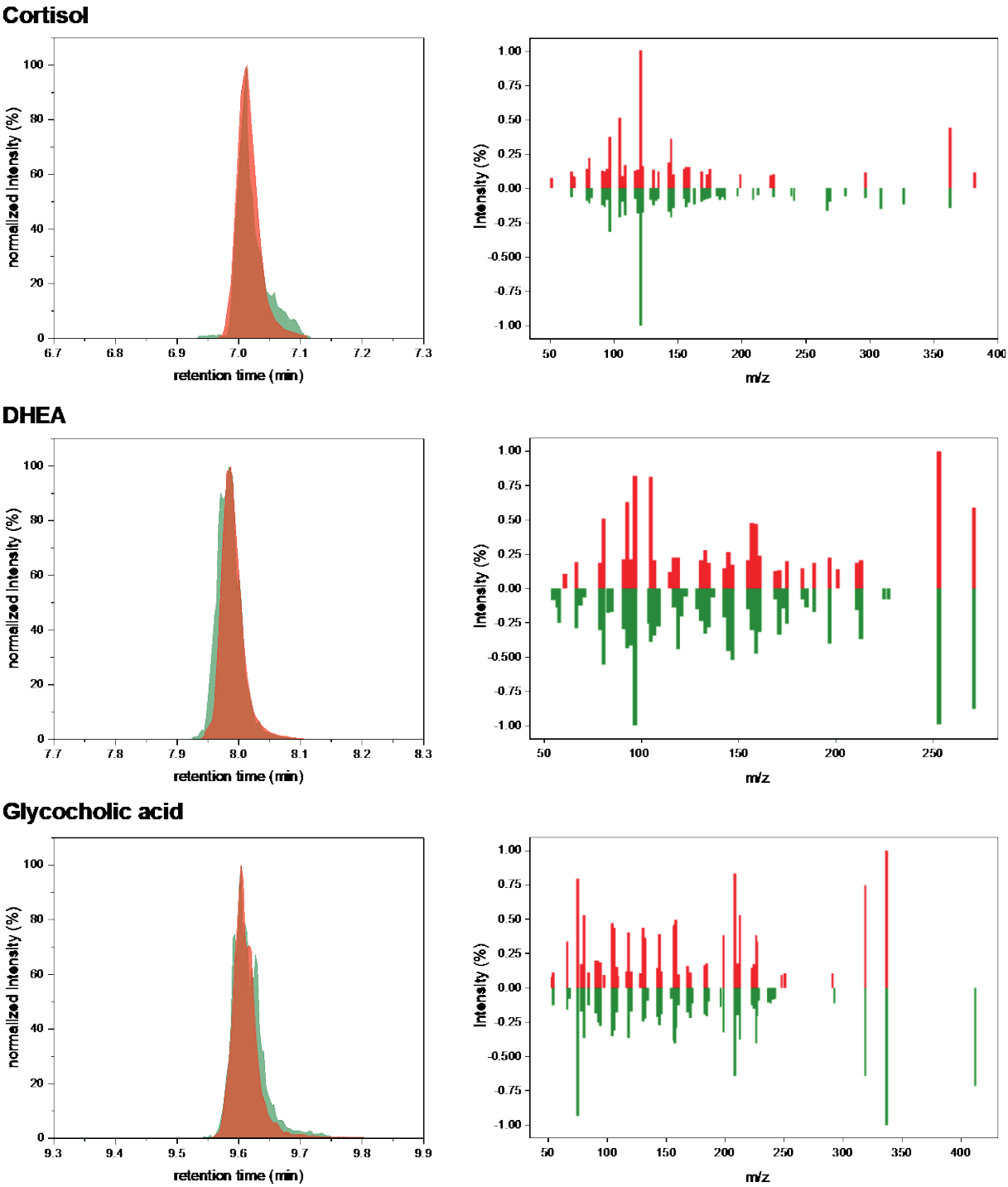


**Figure 2**. Comparison of the experimental (green) and reference (red) chromatograms and mass spectra.

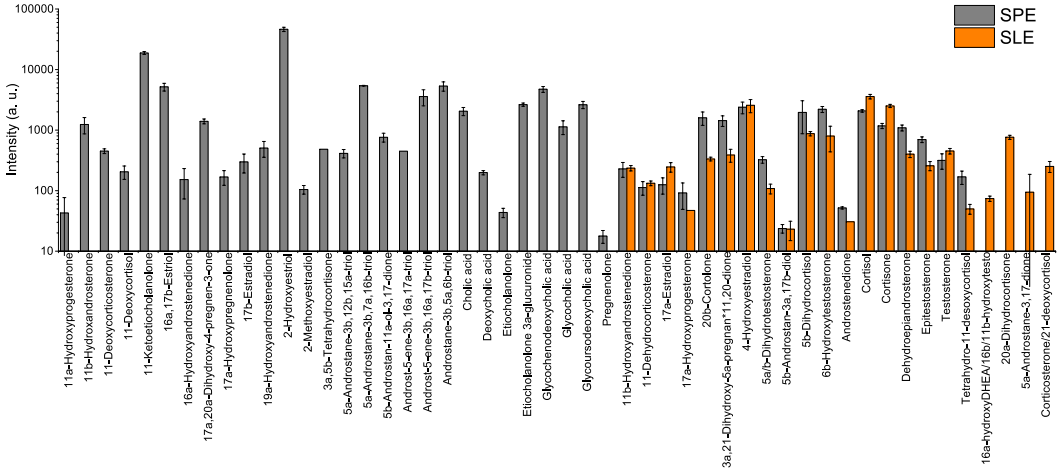


**Figure 3**. Steroids observed in seminal fluid (j=3) after the using the optimized conditions for SPE extraction (in grey) and SLE extraction (in orange). The mean of the intensity of the peaks is plotted in a logarithmic scale with the error bars showing the standard deviation.

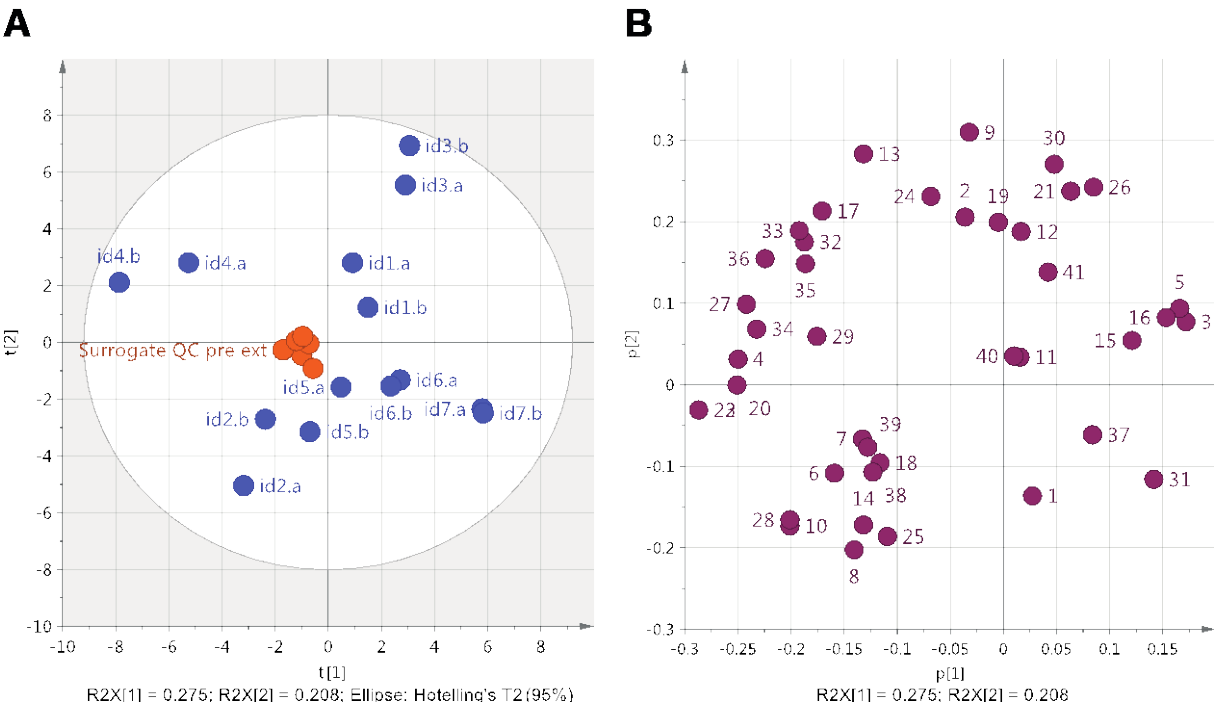


**Figure 4.** Multivariate analysis of the steroid profile in healthy volunteers (seven individuals; id 7 was a vasectomized subject). **A)** Principal Component Analysis (PCA) of the steroid profile from volunteers (n=7, in blue colour) and the surrogate quality controls pre-extraction (orange colour, n=9). **B)** Scatter plot of the steroid contribution in the PCA. The numbers correspond to the following steroids: 1) 2-methoxyestradiol, 2) 2-hydroxyestriol, 3) glycocholic acid, 4) 11-ketoetiocholanolone, 5) glycochenodeoxycholic acid, 6) glycoursodeoxycholic acid, 7) 16a,17b-estriol, 8) androstane-3b,5a,6b-triol, 9) 5a-androstane-3b,7a,16b-triol, 10) 4-hydroxyestradiol, 11) 11b-hydroxyandrosterone, 12) 3a,21-dihydroxy-5a-pregnan-11,20-dione, 13) androst-5-ene-3b,16a,17b-triol, 14) cholic acid, 15) cortisol, 16) cortisone, 17) 6b-hydroxytestosterone, 18) androst-5-ene-3b,16a,17a-triol, 19) 20b-cortolone, 20) 11-deoxycortisol, 21) 5b-dihydrocortisol, 22) DHEA, 23) epitestosterone, 24) 19-hydroxyandrostenedione, 25) 11a-hydroxyprogesterone, 26) 11b-hydroxyandrostenedione, 27) testosterone, 28) 16a-hydroxyandrostenedione, 29) DHT/5b-dihydrotestosterone, 30) 3a,5b-tetrahydrocortisone, 31) deoxycholic acid, 32) androstenedione, 33) 17a-hydroxypregnenolone, 34) etiocholanolone, 35) 17a-hydroxyprogesterone, 36) pregnenolone, 37) 17a,20a-dihydroxy-4-pregnen-3-one, 38) 11-dehydrocorticosterone, 39) 5b-androstan-3a,17b-diol, 40) etiocholanolone 3a-glucuronide and 41) 17b-estradiol.

| Steroids compounds | MS/MS confirmation | Chemical formula | Classification | Species | Rt | Repeatability | Fid. Int |
|---|---|---|---|---|---|---|---|
| 11a-Hydroxyprogesterone | yes | $C_{21}H_{30}O_3$ | Progestogen | $[M+H]^+$ | 8.52 | 22% | 34% |
| 11b-Hydroxyandrosterone | no | $C_{19}H_{30}O_3$ | Androgen | [M+H]+; [M-2H2O+H]+ | 8.42 | 29% | 41% |
| 11b-Hydroxyandrostenedione | yes | $C_{19}H_{26}O_3$ | Androgen | $[M+H]^+$; $[M-H_2O+H]^+$ | 8.05 | 21% | 22% |
| 11-Dehydrocorticosterone | yes | $C_{21}H_{28}O_4$ | Glucocorticoid | $[M+H]^+$ | 7.64 | 74% | 88% |
| 11-Deoxycortisol | yes | $C_{21}H_{30}O_4$ | Glucocorticoid | $[M+H]^+$ | 8.09 | 55% | 70% |
| 11-Ketoetiocholanolone | yes | $C_{19}H_{28}O_3$ | Androgen and Glucocorticoid | $[M+H]^+$; $[M-H_2O+H]^+$ | 8.50 | 14% | 15% |
| 16a,17b-Estriol | yes | $C_{18}H_{24}O_3$ | Estrogen | $[M+H]^+$ | 6.65 | 22% | 24% |
| 16a-Hydroxyandrostenedione | no | $C_{19}H_{26}O_3$ | Androgen | $[M+H]^+$; $[M-H_2O+H]^+$ | 7.69 | 22% | 22% |
| 17a,20a-Dihydroxy-4-pregnen-3-one | no | $C_{21}H_{32}O_3$ | Progestogen | $[M+H]^+$ | 8.72 | 14% | 16% |
| 17a-Hydroxypregnenolone | yes | $C_{21}H_{32}O_3$ | Progestogen | $[M-H_2O+H]^+$ | 9.40 | 20% | 20% |
| 17a-Hydroxyprogesterone | yes | $C_{21}H_{30}O_3$ | Progestogen | $[M+H]^+$ | 9.64 | 27% | 71% |
| 17b-Estradiol | no | $C_{18}H_{24}O_2$ | Estrogen | $[M-H_2O+H]^+$ | 8.94 | 8% | 8% |
| 19-Hydroxyandrostenedione | yes | $C_{19}H_{26}O_3$ | Androgen | $[M+H]^+$ | 7.14 | 32% | 34% |
| 20b-Cortolone | no | $C_{21}H_{34}O_5$ | Glucocorticoid / Mineralocorticoid | [M+H]+; [M-H2O+H]+ | 7.11 | 23% | 53% |
| 2-Hydroxyestriol | yes | $C_{18}H_{24}O_4$ | Estrogen | $[M+H]^+$ | 5.82 | 11% | 11% |
| 2-Methoxyestradiol | no | $C_{19}H_{26}O_3$ | Estrogen | $[M+H]^+$; $[M-H_2O+H]^+$ | 9.27 | 13% | 13% |
| 3a,21-Dihydroxy-5a-pregnan*11,20-dione | yes | $C_{21}H_{32}O_4$ | Glucocorticoid / Mineralocorticoid | $[M+H]^+$ | 8.20 | 37% | 87% |
| 3a,5b-Tetrahydrocortisone | yes | $C_{21}H_{32}O_5$ | Glucocorticoid | $[M+H]^+$ | 7.62 | 33% | 52% |
| 4-Hydroxyestradiol | no | $C_{18}H_{24}O_3$ | Estrogen | $[M+H]^+$; $[M-H_2O+H]^+$ | 7.96 | 17% | 18% |
| 5a-Androstane-3b,7a,16b-triol | yes | $C_{19}H_{32}O_3$ | Androgen | $[M-2H_2O+H]^+$ ; $[M-H_2O+H]^+$ | 6.85 | 12% | 12% |
| 5b-Androstan-3a,17b-diol | no | $C_{19}H_{32}O_2$ | Androgen | $[M-H_2O+H]^+$ | 9.61 | 27% | 27% |
| 5b-Dihydrocortisol | no | $C_{21}H_{32}O_5$ | Glucocorticoid | $[M+H]^+$; $[M-H_2O+H]^+$ | 7.76 | 30% | 71% |
| 6b-Hydroxytestosterone | yes | $C_{19}H_{28}O_3$ | Androgen | $[M+H]^+$; $[M-H_2O+H]^+$ | 6.88 | 12% | 12% |
| Androst-5-ene-3b,16a,17a-triol | yes | $C_{19}H_{30}O_3$ | Androgen | $[M-2H_2O+H]^+$ | 7.80 | 28% | 57% |
| Androst-5-ene-3b,16a,17b-triol | no | $C_{19}H_{30}O_3$ | Androgen | $[M-2H_2O+H]^+$ ; $[M-H_2O+H]^+$ | 6.64 | 19% | 20% |
| Androstane-3b,5a,6b-triol | yes | $C_{19}H_{32}O_3$ | Androgen | $[M-2H_2O+H]^+$ | 9.52 | 8% | 23% |
| Androstenedione | yes | $C_{19}H_{26}O_2$ | Androgen | $[M+H]^+$ | 9.57 | 20% | 22% |
| Cholic acid | yes | $C_{24}H_{40}O_5$ | Bile Acid | $[M+NH_4]^+$ | 8.93 | 32% | 45% |
| Cortisol | yes | $C_{21}H_{30}O_5$ | Glucocorticoid | $[M+H]^+$ | 7.01 | 14% | 16% |
| Cortisone | yes | $C_{21}H_{28}O_5$ | Glucocorticoid | $[M+H]^+$ | 7.10 | 17% | 25% |
| Deoxycholic acid | no | $C_{24}H_{40}O_4$ | Bile Acid | $[M-2H_2O+H]^+$ | 10.63 | 20% | 24% |
| DHEA | yes | $C_{19}H_{28}O_2$ | Androgen | $[M-H_2O+H]^+$ | 9.60 | 27% | 30% |
| 5a/b-Dihydrotestosterone | yes | $C_{19}H_{30}O_2$ | Androgen | $[M+H]^+$; $[M-H_2O+H]^+$ | 10.12 | 24% | 26% |
| Epitestosterone | no | $C_{19}H_{28}O_2$ | Androgen | $[M+H]^+$ | 9.62 | 15% | 25% |
| Etiocholanolone | no | $C_{19}H_{30}O_2$ | Androgen | [M-H20]+ | 10.33 | 37% | 41% |
| Etiocholanolone-3a-glucuronide | yes | $C_{25}H_{38}O_8$ | Androgen | $[M+NH_4]^+$ | 8.13 | 20% | 25% |
| Glycochenodeoxycholic acid | yes | $C_{26}H_{43}NO_5$ | Bile Acid | $[M+H]^+$ | 9.12 | 15% | 17% |
| Glycocholic acid | yes | $C_{26}H_{43}NO_6$ | Bile Acid | $[M+H]^+$ | 7.98 | 15% | 26% |
| Glycoursodeoxycholic acid | yes | $C_{26}H_{43}NO_5$ | Bile Acid | $[M+H]^+$; $[M-H_2O+H]^+$ | 8.11 | 14% | 28% |
| Pregnenolone | yes | $C_{21}H_{32}O_2$ | Progestogen | [M-H20]+ | 11.25 | 19% | 21% |
| Testosterone | yes | $C_{19}H_{28}O_2$ | Androgen | $[M+H]^+$ | 9.15 | 29% | 29% |

**Table 1**. Summary of the principal characteristics of the steroids stable profile found in seminal fluid, with LC-HRMS parameters of the steroids (MS/MS confirmation, chemical formula, MS species and Retention time (Rt)), Steroids biochemistry classification and repeatability and fidelity interval calculations.

# Supplementary Material

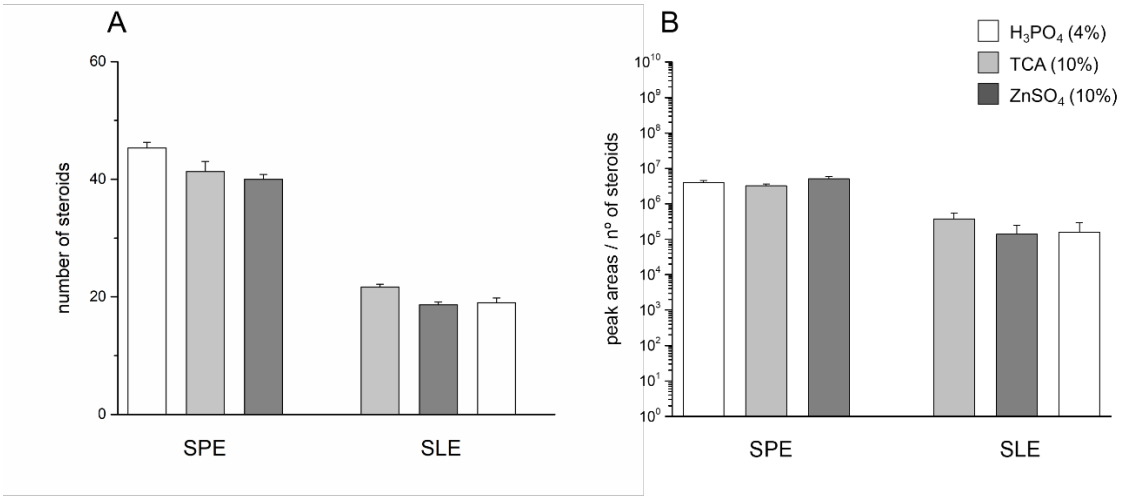


**Figure S1**. Bar graphs of the mean (and standard deviation) of the A) number of steroids and B) peak areas/number of steroids after the steroid extraction with SPE or SLE. Bars are colored according to the protein precipitation procedure: in white, $H_3PO_4$ (aq. 4%), in light grey TCA (aq. 10%) and in dark grey $ZnSO_4$ (aq. 10%).