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Review

Mass spectrometry: Future opportunities for profiling and imaging steroids and steroid metabolites

Ruth Andrew^{1,2} and Natalie Z. M. Homer²**Abstract**

Steroid hormone profiling has historically underpinned advances in endocrine investigation and research, crucially dependent on selective and sensitive hormone assays. Mining the “steriodome” by mass spectrometry (MS) provides greater specificity than immunoassays. Building on a 50 year legacy, gas and liquid chromatography-MS continue to evolve (e.g. sequential derivatisation, mobile phase modifiers). Exciting new technology (e.g. imaging, ion mobility, supercritical fluid), sample preparation (microextraction, molecular imprinted polymers) are advancing the field. Automated analysis of wider profiles of steroids is within reach, in smaller and more varied sample types, including molecular mapping of tissue sections. These new analytical dimensions require rigorous validation and advanced statistics. This review highlights that MS continues to open new windows of biochemical understanding in endocrinology.

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Steroid, Estrogen, Mass spectrometry, Chromatography, Imaging, Extraction.

Abbreviations

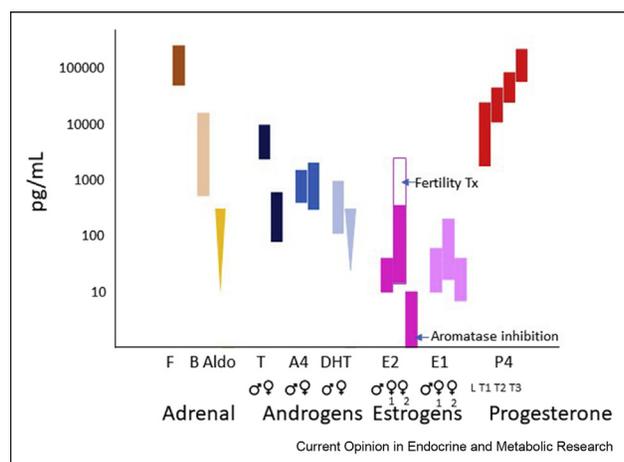
CCS, collision cross-section; DESI, desorption electrospray ionisation; GC, Gas Chromatography; HILIC, hydrophilic interaction liquid chromatography; HPLC, high performance liquid chromatography; IMS, ion mobility separation; LC, Liquid chromatography; LESA, liquid extraction surface analysis; MALDI, Matrix assisted laser desorption ionisation; MIP, Molecular Imprinted polymers; MS, Mass spectrometry; SLE, supported liquid extraction; SPE, solid phase extraction; UHPLC, ultra-HPLC.

Introduction

During the last century, endocrinologists have become increasingly reliant on sensitive and specific measurements of hormones to understand the pivotal roles of steroids in health and disease, dissecting intricate regulation of homeostatic processes (reproduction, stress responses, metabolism to name a few). Immunoassays opened the first doors to steroid detection in the 1960s, underpinning many key discoveries and are still widely used today, appreciated for their rapidity and ease of automation and use. However when levels of steroids are low, specificity of immunoassays has been called into question [1]. Cross-reactivity with similar compounds and over-estimation can be particularly problematic with sex steroids, notably estrogens and 5 α -dihydrotestosterone, creating conflicting data in subjects of certain age groups, between genders and following treatments. No matter how careful one is, there may be unknowns in the biological matrix that cross react with the antibody leading to inaccurate measurements. Moreover, immunoassays for steroids are usually restricted to measuring single analytes rather than multi-steroid profiles, although multiplexing has been achieved in other settings, e.g. cytokine bead arrays. Although immunoassays continue to evolve (e.g. immunocomplexes [2], surface enhanced Raman scattering [3] and lateral flow [4] assays), these concerns prompted the Endocrine Society to publish a position statement in 2013 [5], and to assemble the “Sex Steroid Assays Reporting Task Force” recommending that clinical steroid biochemistry should be supported by mass spectrometry (MS) and guiding current publishing standards [6].

MS is an analytical technique that measures charged molecules or ions in the gaseous state. Samples are ionised, separated in a mass analyser according to their mass-to-charge ratio (m/z) and ions quantified by relative abundances. Coupled to separation techniques such as gas (GC) or liquid chromatography (LC), MS is regarded as the gold standard for steroid hormone analysis, based on its precision and accuracy, but the aspiration of global application to comprehensively profile steroids across wide dynamic ranges (Figure 1) has been hard to achieve [7]. Successful implementation of MS to steroid bioanalysis has required technological advances of all stages of analytical workflows, including sample preparation, separation, ionisation and

Figure 1



Steroids circulate in concentrations, which span across 5 orders of magnitude and in an even wider ranges in disease or in children. The graph shows concentrations in plasma/serum in adults, in some cases separated by males and females and stages of the menstrual cycle and pregnancy. F = cortisol; B = corticosterone; Aldo = aldosterone; P4 = progesterone; E1 = estrone; E2 = estradiol; T = testosterone; A4 = androstenedione; DHT = dihydrotestosterone; 1 = pre-menopausal; 2 = post-menopausal; L = Luteal phase; T1 = trimester 1; T2 = Trimester 2; T3 = Trimester 3; Tx = treatment. Triangular columns depict steroids where lower amounts are less clear use to limits of assay sensitivity.

mass analysis (Figure 2). MS has become more accessible and automated meaning it can be employed in wider settings, but specialist scientists are still essential to drive innovation. Technology advances have allowed application to smaller - “microsampling” - and more varied sample types [8,9]. We can now study spatial intracrinology rather than only levels in fluids, through MS imaging. Here we highlight the advances over the last few years which continue to unravel endocrine, paracrine and intracrine complexity and place MS at the forefront in delivering systems endocrinology in the post-genomic era.

Ionisation for detection

At the heart of any MS analysis is the intrinsic need to ionise molecules of interest, which for steroids presents the first challenge. Steroids must either gain (often for glucocorticoids, progestogens and androgens) or lose charges (often for estrogens and aldosterone) but these lipophilic molecules have a low propensity to ionise. This problem is exacerbated in the presence of competing biomatrix molecules, so-called “matrix effects” which can present as ion suppression or ion attenuation. Overcoming these phenomena – essentially battling invisible, unknown confounders – is key to minimising analytical variability and has required decades of innovation (Figure 2) with still more required, especially when trying to down-size, analyse

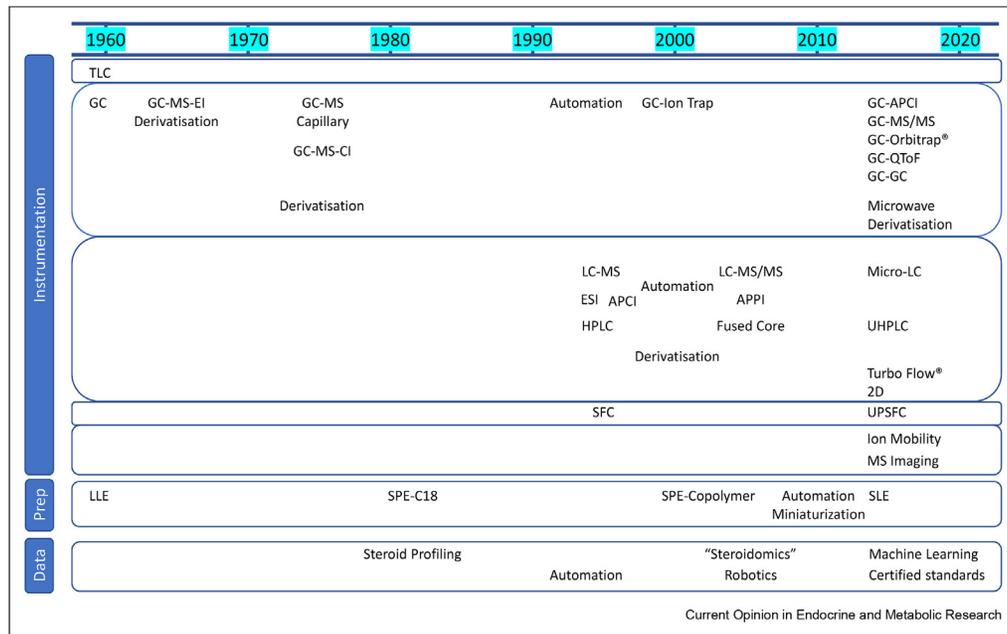
tissue and extend clinical quality standards into the preclinical arena.

The art of separation

Separation of steroids in advance of MS analysis is common, hailing from pioneering work by Shackleton et al. in the 1970s [10], profiling steroids using GC to characterise in-born errors of metabolism, and this approach has been adopted world-wide. This initial profile of 17 steroids has led the way for detailed screening in clinical biochemistry and research labs, with iterations benefitting from instrument advances e.g. single quadrupole mass analysers transitioning to tandem MS for enhanced specificity [11,12]. Ion trap technology has been implemented but thus far proved less popular [13]. The high resolving power of GC column technology has enabled increasing numbers of analytes (e.g. 52 by Wang et al. [14]) to be recovered from a wider range of matrices. Recently a drive to understand the holistic steroidome [15] has sparked interest in approaches that exploit sequential derivatisation [12] of different chemical groups characterising several steroid classes within one assay, and thus enabling chemical sub-types of steroids to be screened concomitantly. Innovative use of sub-fractionation combining electron impact and atmospheric pressure chemical ionisation in conjunction with GC have added breadth [16]. Derivatisation has also been used effectively to encourage extraction from tissues, demonstrated with breast adipose [17]. These approaches offer new opportunities in pathway mapping, while being highly beneficial in reducing sample volume [12]. GC has only recently been interfaced with high resolution MS which will bring new opportunities and GC/GC is an exciting field to watch [18].

While GC yields excellent separation and remains a mainstay, applications using LC-MS/MS emerged once electrospray ionisation was more widely adopted, paving the way to the practical realisation of the position statement [5] in clinical biochemistry; LC often offers higher throughput by faster preparation workflows and reduced analysis time. Testosterone and cortisol, being abundant, were relatively swiftly switched to MS analysis in clinical biochemistry and research settings, but estrogens, particularly in lower ranges (Figure 1), presented challenges, for example assessing effectiveness of aromatase inhibitors in women recovering from breast cancer, where residual hormone may promote tumour regrowth [19]. Although the resolving power of LC is not as good as GC, ongoing improvements in LC technology are enabling multi-steroid profiling, albeit some steroids having poorer sensitivity and isomer separation remains challenging [20,21]. A further benefit of LC over GC is the ability to include conjugates in the analyte profile [22]. Moreover steroids are now an integral part of metabolomics screens [23] e.g. cancer

Figure 2



50 Decades of Advances in Steroid Mass Spectrometry. 2D = 2 dimensional; APCI—atmospheric pressure chemical ionisation; APPI = atmospheric photoionisation; CI = chemical ionisation; EI = electro impact; ESI = electrospray; GC = gas chromatography; HPLC = high performance liquid chromatography; LC = liquid chromatography; LLE = liquid–liquid extraction; MS = mass spectrometry; MS/MS = tandem MS; Prep = sample preparation; qToF = quadrupole-time of flight; SFC = supercritical fluid; SLE = supported liquid extraction; SPE = solid phase extraction; UHPLC = ultra-HPLC; UPSFC = ultra performance SFC.

biomarkers. Over time column performance has improved, with alternative chemistries (e.g. biphenyl [24]) and smaller particle sizes offering greater surface area for interactions. As the field progressed from high performance liquid chromatography (HPLC) to ultra-HPLC (UHPLC), MS sensitivity has benefitted from associated reduced flow of mobile phase. Further miniaturisation through micro-LC [25], is now possible with associated economic, environmental and sustainability benefits. Like with GC, 2 dimensional automated separation is starting to gain traction [26].

Although sensitivity improves with instrument advances, derivatisation combined with LC to improve ionisation remains beneficial for ultimate sensitivity [22,27,28]. Derivatives append to specific chemical groups, such as ketones or hydroxyls, and there is now increased interest in combining measurements of derivatised and underderivatised steroids (e.g. estrogens and glucocorticoids respectively [29]), or derivatised steroids with conjugates [22], or parallel [30] derivatisation of two distinct chemical groups — all these approaches widen analytical profiles. Judicious use of mobile phase additives is gaining popularity to enhance sensitivity including stable lithium adducts [31] avoiding dehydration, and ammonium fluoride [32] which allows positive-negative switching and hence combined

analysis of “negative”- estrogens and aldosterone - and “positive”- steroids - glucocorticoid progestogens and androgens. Summed Multiple Reaction Monitoring [7,32] is more commonplace, whereby multiple specific transitions are consolidated to increase sensitivity.

What lies beneath

MS workflows look for “known knowns” and, despite the excellent qualities of LC and GC, analytical specialists are always acutely aware of what might be hidden from view. Numerous steroidal isomers and closely related molecules within the biomatrix can co-elute and share m/z values and thus the field must continue to explore orthogonal separation to peel away the layers of chemical complexity. Capillary electrophoresis drew early interest as an alternative separation approach [33,34] but this has not yet reached common use. In contrast however, ultra-performance supercritical fluid chromatography (UP-SFC) has found a renaissance with revised instrument design enabling safety and throughput [35]. Whether this approach will find a leading edge remains untested, but is a chromatographic space to watch [36,37], with great potential to improve isomer resolution through orthogonal molecular interactions and also enhanced sensitivity through more facile desolvation. By way of example, De Kock et al. separated 19 steroids within 5 min using this approach [36].

The shape of things to come

Ion mobility separation (IMS) is the exciting new kid on the block, separating ions through collision cross section (CCS) and bringing a yet further dimension to separation. Ion mobility was developed to separate larger molecules and, although differences in CCS of steroids are much smaller, they appear tractable to the technique [38], particularly with the resolving power of IMS already improving e.g. with cyclic IMS. Ion mobility can be used in conjunction with chromatography to filter out unknown isobaric interferences and increasing confidence in data. In the absence of chromatography, ion mobility allows shotgun approaches and improves specificity in imaging workflows. Again derivatisation has a place, to enhance CCS differences (e.g. estrogen isomers [39]), while increasing ion intensity in the fight against matrix effects.

Fishing in the biological pool

Sole reliance on chromatographic separation for sample clean-up can be a mistake that will come back to bite the analyst and indeed simple methods reliant on protein precipitation can suffer badly from matrix effects. Extraction of steroids from biological matrices is highly beneficial prior to introduction into the instrument. In recent years sample preparation has moved away from manual liquid–liquid extraction, firstly to supported liquid extraction (SLE) [40], and increasingly towards automated and miniaturised solid phase extraction [32]. A bewildering array of high-quality support matrices are available, with reverse and ion-exchange chromatography being most popular, and new technologies constantly evolving e.g. cyclodextrin based polymers [41]. These technologies have advanced with increasingly robustness, less reliance on wetting and offering facile removal of phospholipids, the most common source of matrix effects. In the last few years we see the field teetering on bespoke “analyte matched SPE” using Molecular Imprinted Polymers (MIPs [42–45] which act as “chemical antibodies” through mirroring the SPE matrix to the shape of the analyte, with multiplexing possible. This approach may allow stereospecific extraction, an important conceptual interface with biology through the many examples of steroid isomer pairs in vivo with differing bioactivity (e.g. 17 α and β isomers of testosterone and estradiol and the many hydroxy metabolites of estrogens [28]). Many novel approaches to extraction are coming to the fore through the environmental field, including microwave extraction [46,47], metal organic frameworks [48], magnetic ionic liquid extraction [49,50], and pressurized liquid extraction [51].

Scanning the depths

Additional analytical specificity can be achieved through high-resolution MS, where mass is determined with

greater accuracy. Empirical formulae may be assigned and more granular measurement of m/z achieved to distinguish ions. High resolution instrumentation has become more accessible during the last 10 years, but there remains much debate as to its benefits as an alternative to tandem MS for quantitation. Despite the apparent advantages in selectivity, doubts remain regarding the dynamic range and the robustness of quantitation. Opportunities in this space are still to be fully exploited, with only a few applications as yet coming through [18,26,52,53].

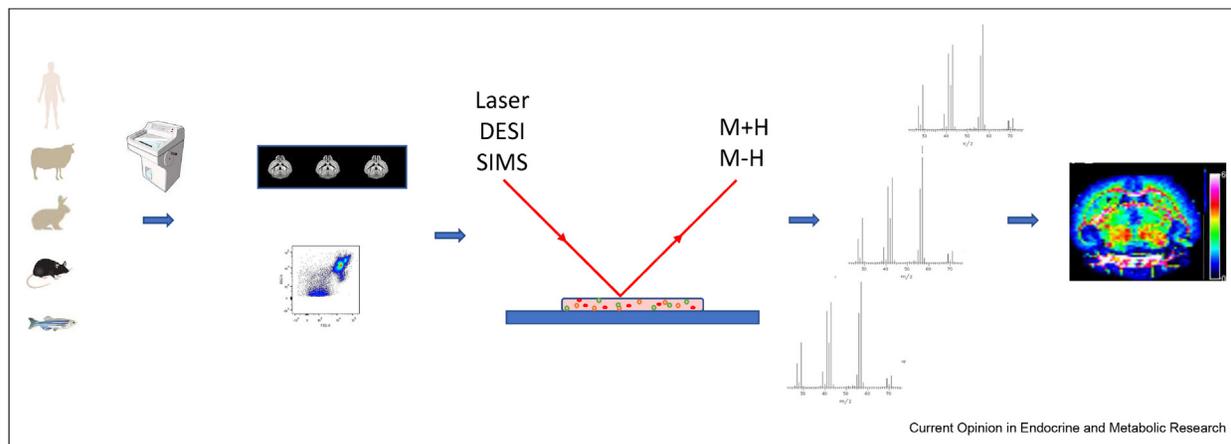
The spatial frontier

Sampling without chromatography through tissue imaging of steroids was first achieved by Cobice et al. [54] for glucocorticoids. The approach of MS imaging is an exciting application of matrix assisted laser desorption ionisation (MALDI) and desorption electrospray ionisation (DESI) technology, allowing a sampling device to raster across tissue surface harvesting the ions of interest and then reconstruction of regional maps (Figure 3). Steroids, with their low ionizability, require derivatisation in this setting, matched to steroid class. Basic MS imaging does not permit separation but innovations such as ion mobility and chromatography coupled to liquid extraction surface analysis (LESA) technology [55] offer routes to overcoming isobaric interferences that otherwise would limit data value e.g. DHEA interfering with testosterone due to being the same mass. This may also be overcome by fragmentation/MS3 in conjunction with imaging [56,57]. The approach is semi-quantitative and requires careful multi-dimensional data analysis and normalisation, and standardisation is challenging. Mass cytometry is also reaching clinical application [58] but will require step-changes in sensitivity to allow visualisation of steroids at a cellular level. New matrices are coming to the fore better suited to ionize steroids, such as poly(3,4-ethylenedioxythiophene)/graphene [59].

Data complexity, validation and community standards

Lastly it is important to acknowledge that with increasing data complexity comes the need for different approaches to data handling and a greater focus on community standards. A number of (inter)national QC schemes operate now allowing both clinical and research labs to align, benefitting from bespoke reference ranges [60] where stable-isotope dilution is the gold-standard for quantitation with the added advantage of chromatographic peak tracking. Samples are enriched with an isotopologue of the analyte to correct for variation introduced through matrix effects, sample processing and instrumentation. Stable-isotope labelled internal standards are increasingly available commercially for clinically relevant steroids but for bespoke research assays may need to be synthesized in-house, a highly challenging and skilled task, which requires high purity

Figure 3



MS Imaging to Visualise Intracrinology. Mass Spectrometry imaging offers the ability to localise steroid distribution across tissue sections. Ionisation can be achieved through means such as matrix assisted laser desorption ionisation (MALDI) and desorption electrospray ionisation (DESI) from tissue or cells and images reconstructed into heatmaps which can be aligned with histology.

as an end point. ^{13}C and deuterium are the most popular isotopes, where the internal standard selected must carry sufficient labels to avoid interference with naturally occurring isotopologues, for steroids, enrichment with a minimum of 3 heavy isotopes. Analysts must ensure the lack of contribution of measurable unlabelled analyte in the internal standard, or make quantitative adjustment during validation if present. Although less common these days, in the absence of stable-isotope labelled standards, analysts may correct to positional isomers or molecules from the same chemical class but the inferiority of this approach is evident in poorer precision and accuracy [28]. The position of the enriched isotopes also matters so that ideally the precursor and product ions both carry the labels. ^{13}C standards are preferred for derivatisation approaches where deuterio-hydrogen exchange may occur in the presence of chemical catalysts and labels lost during sample processing and even in-source. This has been a focus of new derivatisation approaches in recent years [28,61].

Validation criteria for analytical methods have become better described by the Food and Drugs (FDA) [62], European Medical Agencies (EMA) [63] and the bio-analytical community in the last decade for non-endogenous compounds, where stringent requirements are in place for assay linearity, precision and accuracy (e.g. 15% relative standard deviation (RSD) in replicates except at the lower limit of quantitation, where 20% RSD is acceptable). However these benchmarks still remain controversial for endogenous biomarkers, mainly due to the lack of analyte-free matrix. Selecting an appropriate surrogate matrix is an important step in validation and in the case of steroids, charcoal-stripped plasma or serum can be used but detectable levels,

sometimes remain, even with double stripping, and must be assessed before validation. Draft guidelines from The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use [64] suggest that 25% RSD for endogenous biomarkers in method validation is acceptable, as long as this can be scientifically justified [65]. The FDA recognise that some characteristics of validation may not apply but have not as yet changed their guidelines to address this.

The use of certified standard solutions and certified reference materials is expected in regulatory studies and is fast becoming a prerequisite for publication to raise quality and agreement in the field; some Quality Control schemes make patient samples available to allow comparison between clinical labs, but still only for a limited number of most clinically relevant steroids. In general quantitation of steroids should continue aim to match to FDA and EMA [62,63] guidance and there are some excellent tutorial articles available to guide [66,67].

Plug-in methods by manufacturers are making the field more robust and increasing its accessibility, and best practice for sampling handling and storage is being addressed formally [68]. However to fully mine the rich multi-profile datasets, engagement with new methods of data handling are needed, for example isotope deconvolution analysis to improve calibration [69]. Harvesting the concept of the “steroid metabolomic profile” [70] requires multi-variate statistical analysis and machine learning has recently been explored to exciting effect in diagnostics of adrenal cancer [15].

Conclusions

Steroidomics, harvesting 2D or 3D multi-hormone profiles generated by mass spectrometry, has blossomed over the last few years, building on a rich history of innovation. Over the last few years researchers have taken a more holistic look at endocrine health and disease, in conjunction with other strands of the 'omics field. This field of analytical chemistry continues to benefit from technology advances in chromatography and mass spectrometry, and is poised to harvest current opportunities presented at the interface of chemistry and systems biology.

Conflict of interest statement

Nothing declared.

Priority papers

No 5: Position statement from the Endocrine Society marking the need for mass spectrometry to improve the quality of steroid analysis. This statement drove the need for researchers to work with analytical chemists to apply MS techniques to their best advantage. Over recent years this has meant that researchers have continued to explore the most recent instrument and chemical advances in the field to push down limits of detection, minimise sample volume, improve quality of analysis and explore alternative matrices.

No 15: This paper demonstrates how machine learning can be applied to GCMS data to devise fingerprints of health and disease. The researcher successfully identified profiles of biomarkers of adrenal carcinoma.

No 18: Gas Chromatography coupled with MS has been the mainstay of steroid analysis and the technology remained relatively static until recently where we now see it being interfaced with high-resolution MS and also converted to 2 dimensional chromatography. This paper shows an approach that combines GC/GC with high resolution MS and demonstrates the benefits that can be gained from the extra levels of specificity. It opens doors to untargeted screening and demonstrates the value of accurate MS in identifying unknowns.

No 37: Supercritical Fluid chromatography (SFC) has been recognised as a potential separating technique for several decades but has recently become more accessible through instrument advances. This recent paper makes detailed comparisons between SFC and gas chromatography, showing the strength of the technique in multi-hormone profiling.

No 38: Hernandez- Mesa et al. developed a database of collision cross sections of steroidal molecules demonstrating this parameter is a useful orthogonal approach to

achieve separation and developing a publicly available database to enable the field. Ion mobility is poised to be used both in conjunction with chromatographic and imaging workflows to enhance specificity and enable shotgun approaches.

No 54: Cobice et al. were the first group to achieve tissue mapping of steroids using MS imaging. They applied derivatisation approaches to the tissue surface to enhance the signal of keto-steroids and developed a novel approach to map steroids by MALDI sampling. This approach has been developed further to other keto-steroids and oxysterols.

References

1. Stanczyk FZ, Xu X, Slussc PM, Brinton LA, McGlynn KA: **Do metabolites account for higher serum steroid hormone levels measured by RIA compared to mass spectrometry?** *Clin Chim Acta* 2018, **484**:223–225.
2. Leivo J, Laura Kivimäki L, Juntunen E, Pettersson K, Lamminmäki U: **Development of anti-immunocomplex specific antibodies and non-competitive time-resolved fluorescence immunoassay for the detection of estradiol.** *Anal Bioanal Chem* 2019, **411**:5633–5639.
3. Wang R, Chon H, Lee S, Cheng Z, Hong SH, Yoon YH, Choo J: **Highly sensitive detection of hormone estradiol E2 using surface-enhanced Raman scattering based immunoassays for the clinical diagnosis of precocious puberty.** *ACS Appl Mater Interfaces* 2016, **8**:10665–10672.
4. Oh H-K, Kim JW, Kim JM, Kim MG: **High sensitive and broad-range detection of cortisol in human saliva using a trap lateral flow immunoassay (trapLFI) sensor.** *Analyst* 2018, **143**: 3883.
5. Handelsman DJ, Wartofsky L: **Requirement for mass spectrometry sex steroid assays in the Journal of Clinical Endocrinology and Metabolism.** *J Clin Endocrinol Metabol* 2013, **98**: 3971–3973.
6. C.a.L.S. Institute: *Mass spectrometry for androgen and estrogen measurements in serum.* CLSI Guideline C57. Wayne, PA, USA: CLSI; 2015.
7. Smy L, Straseski JA: **Measuring estrogens in women, men, and children: recent advances 2012–2017.** *Clin Biochem* 2018, **62**:11–23.
8. Spooner N, Anderson KD, Siple J, Wickremsinhe ER, Xu Y, Lee M: **Microsampling: considerations for its use in pharmaceutical drug discovery and development.** *Bioanalysis* 2019, **11**.
9. Gomez-Gomez A, Miranda J, Feixas G, Arranz Betegon A, Crispi F, Gratacós E, Pozo OJ: **Determination of the steroid profile in alternative matrices by liquid chromatography tandem mass spectrometry.** *J Steroid Biochem Mol Biol* 2020, **197**:105520.
10. Shackleton CHL, Honour JW: **Simultaneous estimation of urinary steroids by semi-automated gas chromatography. Investigation of neonatal infants and children with abnormal steroid synthesis.** *Clin Chim Acta* 1976, **69**:267–283.
11. Homer NZM, Rutter A, Kothiya S, Walker BR, Andrew R: **Gas chromatography tandem mass spectrometry offers advantages for urinary steroid analysis.** *Anal Biochem* 2017, **538**: 34–37.
12. Ankarberg-Lindgren C, Dahlgrena J, Andersson MX: **High-sensitivity quantification of serum androstenedione, testosterone, dihydrotestosterone, estrone and estradiol by gas chromatography–tandem mass spectrometry with sex- and puberty-specific reference intervals.** *J Steroid Biochem Mol Biol* 2018, **183**:116–124.

13. Gaudl M, Kratzsch J, Bae JY, Kiess W, Thiery J, Ceglarek U: **Liquid chromatography quadrupole linear ion trap mass spectrometry for quantitative steroid hormone analysis in plasma, urine, saliva and hair.** *J Chromatogr A* 2016, **1464**: 64–71.
14. Wang R, Hartmann MF, Tiosano D, Wudy SA: **Characterizing the steroidal milieu in amniotic fluid of mid-gestation: a GC-MS study.** *J Steroid Biochem Mol Biol* 2019, **193**:105412.
15. Chortis V, Bancos I, Nijman T, Gilligan LC, Taylor AE, Ronchi CL, O'Reilly MW, Schreiner J, Asia M, Riestler A, Perotti P, Libé R, Quinkler M, Canu L, Paiva I, Bugalho MJ, Kastelan D, Denny MC, Sherlock M, Ambroziak U, Vassiliadi D, Bertherat J, Beuschlein F, Fassnacht M, Deeks JJ, Biehl M, Arit W: **Urine steroid metabolomics as a novel tool for detection of recurrent adrenocortical carcinoma.** *J Clin Endocrinol Metab* 2020, **105**:e307–e318.
16. Hennig K, Antignac JP, Bichon E, Morvan ML, Miran Mu, Delalogue S, Feunteun J, Le Bizec B: **Steroid hormone profiling in human breast adipose tissue using semi-automated purification and highly sensitive determination of estrogens by GC-APCI-MS/MS.** *Anal Bioanal Chem* 2018, **410**:259–275.
17. Moon J-Y, McNamara K-M, Lee J-J, Chung BC, Sasano H, Choi MH: **Improved detectability of sex steroids from frozen sections of breast cancer tissue using GC-triple quadrupole-MS.** *J Steroid Biochem Mol Biol* 2018, **178**:185–192.
18. Bileck A, Verouti SN, Escher G, Vogt B, Groessl M: **A comprehensive urinary steroid analysis strategy using two-dimensional gas chromatography - time of flight mass spectrometry.** *Analyst* 2018, **143**:4484–4494.
19. Kallak TK, Baumgart J, Evers AS, Poromaa IS, Moby L, Kask K, Norjavaara E, Kushnir MM, Bergquist J, Nilsson K: **Higher than expected estradiol levels in aromatase inhibitor-treated, postmenopausal breast cancer patients.** *Climacteric* 2012, **15**: 473–480.
20. Wang Z, Wang H, Peng Y, Chen F, Zhao L, Li X, Qin J, Li Q, Wang B, Pan B, Guo W: **A liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based assay to profile 20 plasma steroids in endocrine disorders.** *Clin Chem Lab Med* 2020, <https://doi.org/10.1515/cclm-2019-0869>.
21. Son HH, Yun WS, Cho H-S: **Development and validation of an LC-MS/MS method for profiling 39 urinary steroids (estrogens, androgens, corticoids, and progestins).** *Biomed Chromatogr* 2020, **34**, e4723.
22. van der Berg C, Venter G, van der Westhuizen FH, Erasmus E: **Development and validation of LC-ESI-MS/MS methods for quantification of 27 free and conjugated estrogen-related metabolites.** *Anal Biochem* 2020, **590**:113531.
23. Sun C, Gao M, Wang F, Yun Y, Sun Q, Guo R, Yan C, Sun X, Li Y: **Serum metabolomic profiling in patients with Alzheimer Disease and amnesic mild cognitive impairment by GC/MS.** *Biomed Chromatogr* 2020 Sep, **34**(9), e4875, <https://doi.org/10.1002/bmc.487>.
24. Lindner JM, Vogeser M, Grimm SH: **Biphenyl based stationary phases for improved selectivity in complex steroid assays.** *J Pharmaceut Biomed Anal* 2017, **142**:66–73.
25. Márta Z, Bobály B, Fekete J, Magda B, Imre T, Mészáros KV, Bálint M, Szabó PT: **Simultaneous determination of thirteen different steroid hormones using micro UHPLC-MS/MS with on-line SPE system.** *J Pharmaceut Biomed Anal* 2018, **150**: 258–267.
26. Blokland MH, Zoontjes PW, Van Ginkel LA, Van De Schans MGM, Sterk SS, Bovee TFH: **Multiclass screening in urine by comprehensive two-dimensional liquid chromatography time of flight mass spectrometry for residues of sulphonamides, beta-agonists and steroids.** *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 2018, **35**: 1703–1715.
27. Liu Q, Chi Q, Fan R-T, Tian HD, Wang X: **Quantitative-profiling method of serum steroid hormones by hydroxylamine-derivatization HPLC-MS.** *Nat Products Bioprospect* 2019, **9**: 201–208.
28. Denver N, Khan S, Stasinopoulos I, Church C, Homer NZM, MacLean MR, Andrew R: **Novel derivatization strategy for analysis of estrogens and their metabolites in human plasma by liquid chromatography tandem mass spectrometry.** *Anal Chim Acta* 2019, **1054**:84–94.
29. S. Laforest, M. Pelletier, K. Ennou-Idrissi, N. Denver, B. Poirier, S. Nguyen, F. Durocher, N.Z.M. Homer, C. Diorio, A. Tchernof, R. Andrew, Simultaneous quantification of estrogens and glucocorticoids in human adipose tissue by liquid-chromatography-tandem mass spectrometry, *J Steroid Metab Mol Biol*, **95**, 105496, 1-9.
30. Qin Q, Feng D, Hu C, Wang B, Chang M, Liu X, Yin P, Shi X, Xu G: **Parallel derivatization strategy coupled with liquid chromatography-mass spectrometry for broad coverage of steroid hormones.** *J Chromatogr A* 2020, **1614**:460709.
31. Wang Q, Shimizu K, Maehata W, Pan Y, Sakurai K, Hikida T, Fukada Y, Takao T: **Lithium ion adduction enables UPLC-MS/MS-based analysis of multi-class, 3-hydroxyl group-containing keto-steroids.** *J Lipid Res* 2020, **61**: 570–579.
32. Li XS, Li S, Kelleman G: **Simultaneous determination of three estrogens in human saliva without derivatization or liquid-liquid extraction for routine testing via miniaturized solid phase extraction with LC-MS/MS detection.** *Talanta* 2018, **178**: 464–472.
33. Britz-McKibbin P, Ichihashi T, Tsubota K, Chen DDY, TerabE S: **Complementary on-line preconcentration strategies for steroids by capillary electrophoresis.** *J Chromatogr A* 2003, **1013**: 65–76.
34. Görög S, Gazdag M, Kemenes-Bakos P: **Analysis of steroids part 50. Derivatization of ketosteroids for their separation and determination by capillary electrophoresis.** *J Pharm Biomed Anal* 1996, **14**:1115–1124.
35. Storbeck K-H, Gilligan L, Jenkinson C, Baranowski ES, Quanson JL, Arit W, Taylor AE: **The utility of ultra-high performance supercritical fluid chromatography-tandem mass spectrometry (UHPSFC-MS/MS) for clinically relevant steroid analysis.** *J Chromatogr B* 2018, **1085**:36–41.
36. de Kock N, Acharya SR, Kumari SJ, Ubhayasekera A, Bergquist J: **A novel targeted analysis of peripheral steroids by ultra-performance supercritical fluid chromatography hyphenated to tandem mass spectrometry.** *Sci Rep* 2018, **8**: 16993.
37. Teubel J, Wüst B, Schipke CG, Peters O, Parr MK: **Methods in endogenous steroid profiling – a comparison of gas chromatography mass spectrometry (GC-MS) with supercritical fluid chromatography tandem mass spectrometry (SFC-MS/MS).** *J Chromatogr A* 2018, **1554**:101–116.
38. Hernández-Mesa M, Le Bizec B, Monteau F: **A.M. García-Campaná, D.-P. G., Collision cross section (CCS) database: an additional measure to characterize steroids.** *Anal Chem* 2018, **90**:4616–4625.
39. Ahonen L, Fasciotti M, Gennäs GB, Kotiaho T, Daroda RJ, Eberlin M, Kostianen R: **Separation of steroid isomers by ion mobility mass spectrometry.** *J Chromatogr A* 2013, **1310**: 133–137.
40. Owen LJ, Keevil BG: **Supported liquid extraction as an alternative to solid phase extraction for LC-MS/MS aldosterone analysis.** *Ann Clin Biochem* 2013, **50**:489–491.
41. Manaf NA, Saad B, Mohamed MH, Wilson LD, Latiff AA: **Cyclodextrin based polymer sorbents for micro-solid phase extraction followed by liquid chromatography tandem mass spectrometry in determination of endogenous steroids.** *J Chromatogr A* 2018, **1543**:23–33.
42. Tang J, Wang j, Yuan L, Xiao Y, Wang W, Yang Z: **Trace analysis of estrogens in milk samples by molecularly imprinted solid phase extraction with genistein as a dummy template molecule and high performance liquid chromatography-tandem mass spectrometry.** *Steroids* 2019, **145**:23–31.

43. Guć M, Schroeder G: **Molecularly imprinted polymers and magnetic molecularly imprinted polymers for selective determination of estrogens in water by ESIMS/FAPA-MS.** *Biomolecules* 2020, **10**:672.
44. Czarny K, Szczukocki D, Krawczyk B, Juszcak R, Skrzypek S, Gadzała-Kopciuch R: **Molecularly Imprinted Polymer film grafted from porous silica for efficient enrichment of steroid hormones in water samples.** *J Separ Sci* 2019, **42**:2858–2866.
45. Kellens E, Bové H, Vandenryt T, Lambrichts J, Dekens J, Drijkoningen S, D'Haen J, De Ceuninck W, Thoelen R, Junkers T, Haenen K, Ethirajan A: **Micro-patterned molecularly imprinted polymer structures on functionalized diamond-coated substrates for testosterone detection.** *Biosens Bioelectron* 2018, **118**:58–65.
46. Ran J, Liang X, Du H, Sun J: **Optimization of DHEA extraction from sweet potato pomace by ultrasonic-microwave synergistic employing response surface methodology.** *J AOAC Int* 2019, **102**:680–682.
47. Dévier MH, Labadie P, Togola A, Budzinski H: **Simple methodology coupling microwave-assisted extraction to SPE/GC/MS for the analysis of natural steroids in biological tissues: application to the monitoring of endogenous steroids in marine mussels *Mytilus Sp.*** *Anal Chim Acta* 2010, **657**:28–35.
48. Gao G, Li S, Lia S, Wang Y, Zhao P, Zhang X, Hou X: **A combination of computational–experimental study on metal-organic frameworks MIL-53(Al) as sorbent for simultaneous determination of estrogens and glucocorticoids in water and urine samples by dispersive micro-solid-phase extraction coupled to UPLC-MS/MS.** *Talanta* 2018, **180**:358–367.
49. Moreira F, Santana ER, Spinelli A: **Ionic liquid-supported magnetite nanoparticles as electrode modifier materials for estrogens sensing.** *Sci Rep* 2020, **10**:1955.
50. Feng X, Xu X, Liu Z, Xu S, Zhang L: **Novel functionalized magnetic ionic liquid green separation technology coupled with high performance liquid chromatography: a rapid approach for determination of estrogens in milk and cosmetics.** *Talanta* 2020, **209**:120542.
51. Wang M, Wang Y, Peng B, Zhao N, Zhao J, Xiong Z, Zhao L: **Multi-class determination of steroid hormones and antibiotics in fatty hotpot ingredients by pressurized liquid extraction and liquid chromatography–tandem mass spectrometry.** *J Pharmaceut Biomed Anal* 2019, **171**:193–203.
52. Honda L, Becerra-Herrera M, Richter P: **Liquid chromatography–time-of-flight high-resolution mass spectrometry study and determination of the dansylated products of estrogens and their hydroxylated metabolites in water and wastewater.** *Anal Bioanal Chem* 2018, **410**:7909–7919.
53. Olesti E, Garcia A, Rahban R, Rossier MF, Boccard JF, Nef S, González-Ruiz V, Rudaz S: **Steroid profile analysis by LC-HRMS in human seminal fluid.** *J Chromatogr B* 2020, **1136**.
54. Cobice DF, MacKay CL, Goodwin R, McBride A, Langridge-Smith P, Webster SP, Walker BR, Andrew R: **MS imaging for dissecting steroid intracrinology within target tissues.** *Anal Chem* 2013, **85**:11576–11584.
55. Yutuc E, Anglini R, Baumert M, Mast N, Pikuleva I, Newton J, Clench MR, Skibinski DOF, Howell OW, Wang Y, Griffiths WJ: **Localization of sterols and oxysterols in mouse brain reveals distinct spatial cholesterol metabolism.** *Proc Natl Acad Sci* 2020, **117**:5749–5760.
56. Takeo E, Sugiura Y, Uemura T, Nishimoto K, Yasuda M, Sugiyama E, Ohtsuki S, Higashi T, Nishikawa T, Suematsu M, Fukusaki E, Shimma S: **Tandem mass spectrometry imaging reveals distinct accumulation patterns of steroid structural isomers in human adrenal glands.** *Anal Chem* 2019, **91**:8918–8925.
57. Smith KW, Thompson PD, Rodriguez EP, Mackay CL, Cobice DF: **Effects of vitamin D as a regulator of androgen intracrinology in LNCAP prostate cancer cells.** *Biochem Biophys Res Commun* 2019, **519**:579–584.
58. Ravkov EV, Charlton CM, Barker AP, Hill H, Peterson LK, Slev P, Tebo A, Voelkerding KV, Wittwer CT, Heikal N, Delgado JC, Lázár-Molnár E, Kumánovics A: **Evaluation of mass cytometry in the clinical laboratory.** *Cytometry B Clin Cytom* 2019, **96**:266–274.
59. Huang S, Ye N, Chen G, Ou R, Huang Y, Zhu F, Shena J, Ouyang G: **A robust and homogeneous porous poly(3,4-ethylenedioxythiophene)/graphene thin film for high-efficiency laser desorption/ionization analysis of estrogens in biological samples.** *Talanta* 2019, **195**:290–297.
60. Ackermann D, Groessl M, Pruijm M, Ponte B, Escher G, d'Uscio CH, Guessous I, Ehret G, Pechère-Bertschi A, Martin P-Y, Burnier M, Dick B, Vogt B, Bochud M, Rousson V, Dhayatl NA: **Reference intervals for the urinary steroid metabolome: the impact of sex, age, day and night time on human adult steroidogenesis.** *Plos One* 2019, **14**, e0214549.
61. Faqehi AMM, Cobice DF, Naredo G, Mak TCS, Upreti R, Gibb FW, Beckett GJ, Walker BR, Homer NZM, Andrew R: **Derivatization of estrogens enhances specificity and sensitivity of analysis of human plasma and serum by liquid chromatography tandem mass spectrometry.** *Talanta* 2016, **151**:148–156.
62. C f v Medicine. *Guidance for industry: Bioanalytical method validation in: C.f.D.E.a. Research*; 2018. www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf; 2018.
63. *EMA guideline for bioanalytical method validation.* European Medicines Association; 2011. EMEA/CHMP/EWP/192217/2009.
64. C f H M Product. *ICH guideline M10 on bioanalytical method validation.* European Medicines Association; 2019. EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2.
65. Yu C, Bashaw ED: **Regulatory perspective of biomarker bioanalysis during drug development.** *Bioanalysis* 2019, **11**:607–610.
66. Krueve A, Rebane R, Kipper K, Oldekop M-L, Evard H, Herodes K, Ravió P, Leito I: **Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part I.** *Anal Chim Acta* 2015, **870**:29–44.
67. Krueve A, Rebane R, Kipper K, Oldekop M-L, Evard H, Herodes K, Ravió P, Leito I: **Tutorial review on validation of liquid chromatography–mass spectrometry methods: Part II.** *Anal Chim Acta* 2015, **870**:8–28.
68. Handelsman DJ, Desai R, Seibel MJ, Le Couteur DG, Cumming RG: **Circulating sex steroid measurements of men by mass spectrometry are highly reproducible after prolonged frozen storage.** *J Steroid Biochem Mol Biol* 2020, **197**:105528.
69. Pitarch-Motellón J, Fabregat-Cabello N, Le Goff C, Roig-Navarro AF, Sancho-Llopis JV, Cavalier E: **Comparison of isotope pattern deconvolution and calibration curve quantification methods for the determination of estrone and 17β-estradiol in human serum.** *J Pharmaceut Biomed Anal* 2019, **171**:164–170.
70. Gawlik AW, Shmoish M, Hartmann MF, Wudy SA, Hochberg Z: **Steroid metabolomic signature of insulin resistance in childhood obesity.** *Diabetes Care* 2020, **43**:405–410.