Method Development for Mass Spectrometric Detection of Neurosteroids in Cerebrospinal Fluid as Potential Biomarkers of Cognitive Disorders

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Research of the present study was conducted from 2013 till 2020 under supervision of Prof. Dr. Maria Kristina Parr at the Institute of Pharmacy of the Freie Universität Berlin.

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2nd Reviewer: Prof. Dr. Francesco Botrè

Date of defense: February 10th, 2021

Independence Declaration

I hereby affirm that I have completed the presented cumulative dissertation independently and without unauthorized assistance. No aids other than these listed in the text were used in the writing of the dissertation. A doctoral procedure has never been completed at any other university or applied to another department.

Juliane Teubel

To raise new questions, new possibilities, to regard old problems from a new angle, requires creative imagination and marks real advance in science.

- Albert Einstein -

Acknowledgements

None of us got to where we are alone.
Whether the assistance we received was obvious or subtle, acknowledging someone's help is a big part of understanding the importance of saying thank you.

- Harvey Mackay -

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1 Introduction and Aim of the Project

Profiling of steroid hormones that were synthesized by the human body plays an important role in various fields of endocrinology. A special group of endogenous steroids are neurosteroids [1], that are synthesized in the central (CNS) or peripheral (PNS) nervous system [2-4], which is not only a source, but also a target for NS. Research in this field has been increasingly intensified within the last years, as they are discussed as potential biomarkers in various cognitive disorders, such as depression or dementia [5-7].

The only accessible matrix in human living beings which most likely reflects the situation in brain is cerebrospinal fluid (CSF). It therefore has become a remarkably interesting matrix for neurosteroid analysis. Nevertheless, the knowledge on effects of neurosteroids and their metabolism is limited and only few data on NS levels in CSF are available. Many reasons for this are conceivable, such as limited available amounts of CSF for analysis, ultra-trace-levels of steroids that require extremely sensitive analytical methods, and difficult achievement of selectivity for structurally highly similar endogenous steroids in multi analyte methods. Further, the few available studies report high neurosteroid level deviations. For example, in controls reported concentrations of dehydroepiandrosterone range from not detectable to 840 pg/ml [8, 9], or rather high concentrated hydrocortisone levels range from 0.2 – 19 ng/ml in alive controls [10, 11] up to 125 ng/ml in post-mortem CSF [12]. This might be due to high individual variability of study participants but may also be a sign for truly challenging analysis.

Steroid profiling in various body fluids is predominantly performed with gas chromatography coupled to (tandem) mass spectrometry (GC-MS(/MS)), (ultra) high performance liquid chromatography coupled to (tandem) mass spectrometry ((U)HPLC-MS(/MS)), or multiple immunoassays (IA). Each method offers different advantages and disadvantages [13-16].

Immunoassays are performed with a specific antibody for the target analyte which is detected by radioactive labelling (RIA) or other labelling agents, such as enzyme-linked immunosorbent assay (ELISA) or different luminescence assays (LIA, chemical CLIA, electrochemical ECLIA). However, a major drawback is the hazard of cross-reactivities between structurally related endogenous steroids, and the limited number of simultaneously detectable analytes.

Since decades, GC-MS(/MS) analysis of derivatized steroids is the method of choice for multi analyte screenings covering up to 30-70 steroids [17-20]. For enhancement of volatility and stability and generation of characteristic electron ionization (EI) spectra, numerous well established derivatization procedures for either oxo groups, hydroxy groups, or both are available [21, 22], e.g. trimethylsilyl ethers (TMS) or methoximes (MO) or mixed derivatives (MO-TMS). However, even with the elaborate, commonly used two-step derivatization procedures, some steroids yield multiple isomeric derivatives.

HPLC-MS(/MS) was increasingly favoured for steroid profiling during the last years catching up with GC-MS, but still remaining a complementary technique [17, 19, 20, 23]. Advantageously, it does not necessarily require a derivatization of the analytes, although

derivatization can be used for an improvement in sensitivity. Further, it enables direct analysis of conjugated steroids without prior cleavage, as necessary in GC-MS(/MS). However, even with UHPLC separation for some isomers can still not be achieved, as resolution is rather limited.

Currently, supercritical fluid chromatography (SFC) returns to analytical science as sustainable and green analytical method, since a lot of effort has been made in optimization of instrumentation. It has shown to be very powerful in separation efficiency [24, 25], and it is orthogonal to reversed phase chromatography in most cases [26]. This chromatographic behaviour presents new and promising opportunities for application in steroid profiling, which was first reported for exogenous steroids in the context of anti-doping [27] analysis, but was recently also started to be investigated for endogenous steroids [28-30].

Summarized, it can be stated that IA, HPLC, SFC and GC are feasible in ascending numbers of analytes that are intended to be detected.

This project aims to develop a comprehensive and sensitive method for profiling of an as large as possible set of steroids in one human CSF aliquot. The selection of steroids for method development and evaluation was based on comprehensive literature research of neurosteroids already determined in CSF and was expanded with steroids covering major peripheral pathways of biosynthesis and metabolism. This resulted in a set of 51 steroids, including (pro-)gestagens, androgens, corticoids, estrogens and steroid sulfates. Their structural formulas, nomenclature and abbreviations are displayed in section 10.1. The selection further includes numerous configurational isomers, e.g. androsterone and epiandrosterone, as well as constitutional isomers with possible similar mass spectrometric properties, e.g. 5α-dihydrotestosterone and androsterone that therefore inevitably require chromatographic separation for quantification.

The two most-promising methods for endogenous steroid profiling, GC-MS and SFC-MS(/MS), were intensively investigated within this work. First, the gold standard GC-MS with different derivatization procedures was explored, and the formation of multiple derivatives described and confirmed. Overall, none of the derivatization procedures provided acceptable results for further method development. With its unique selectivity, SFC-MS/MS was able to overcome this issue and to distinguish between all assorted steroids with fast separations on different columns. Altogether, SFC-MS/MS has demonstrated its high potential in endogenous steroid profiling and its superiority to GC-MS within the requirements of this project.

2 Theoretical Background

2.1 Neurosteroids

Pioneering studies of Baulieu et al. [31] coined the term “neurosteroid” as special group of endogenous steroids already in the beginning of the 1980s. According to this, neurosteroids are steroids that that are synthesized in the central (CNS) or peripheral (PNS) nervous system. Later, in the 1990s, the concept of neurosteroids was expanded by Paul and Purdy [32] to “neuroactive steroids” (NAS). This term also includes steroids that were synthetized in the periphery but were also capable of modifying neural activities. Synthetic steroids that affect nervous system were also included since then [33]. In order to improve readability, in the following only the term neurosteroid for both will be used.

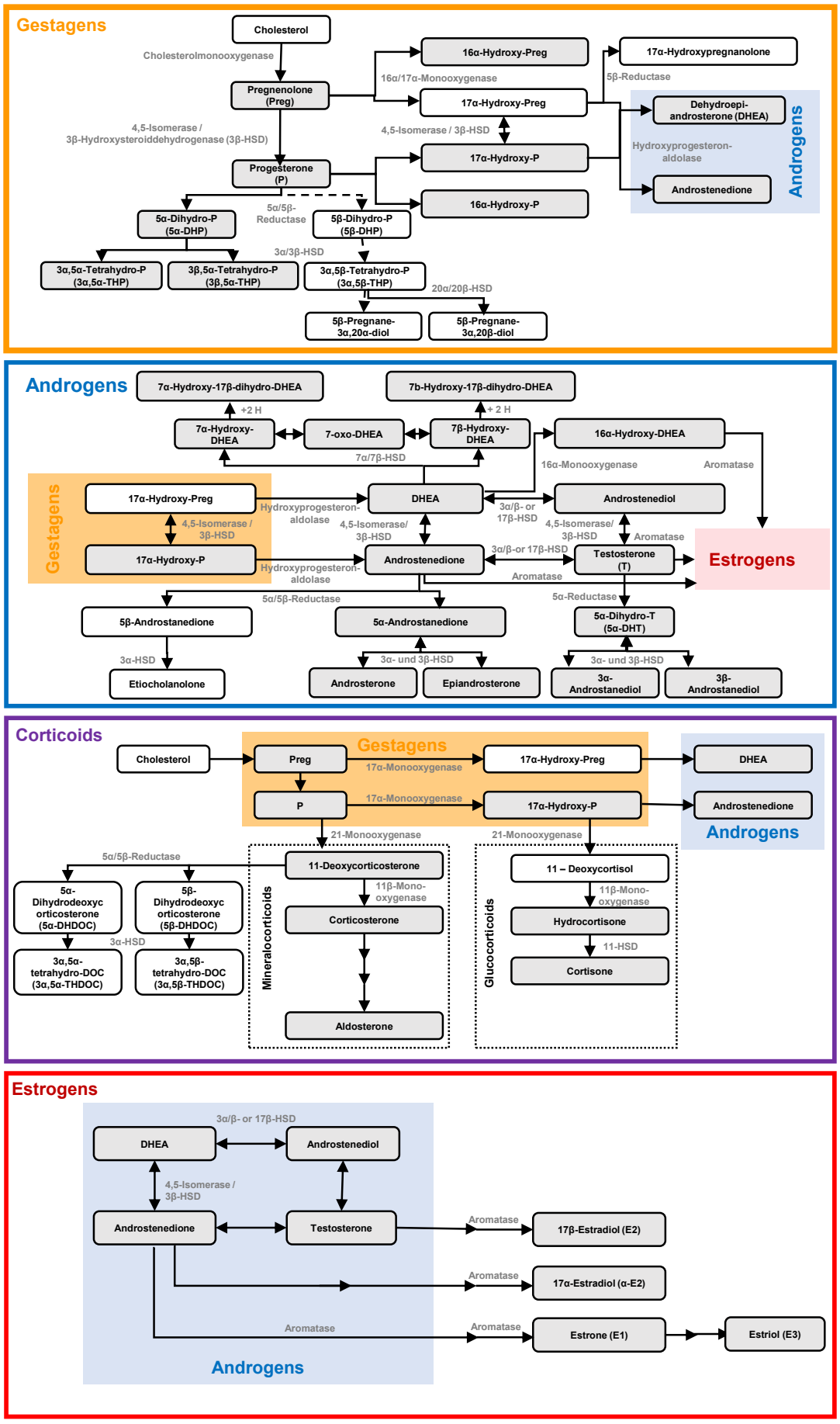
Their *de novo* synthesis from cholesterol and metabolism in the brain was subsequently validated by several studies [4, 34-37]. Both are likely to be sex dimorphic during developmental states and whole life span as indicated by results from animal and model experiments [33]. Figure 1 displays an overview on main metabolism pathways in periphery that were also found to take place in the brain. NS that were already under investigation in human cerebrospinal fluid are highlighted in gray fields. Generally, only few data on NS levels in human CSF are available. Reported levels of NS from different steroid classes are reviewed and discussed in detail in Manuscript No. 1.

Initial animal studies by Baulieu et al. [38] hinted independent NS regulation from peripheral fluid metabolism, whereas more recent investigations in animals demonstrated its dependence [33, 39]. However, these studies did not reveal a simple direct correlation between NS levels of peripheral origin and different brain regions or other nerval tissues [33, 39, 40]. These interactions require NS passage through blood-brain-barrier (BBB) and/or blood-CSF-barrier (BCB) and interchange between different brain regions. Further, local nervous tissue steroidogenesis may contribute to differences observed within the different compartments. Knowledge on mechanisms and extents of those very complex processes is poor not only in animal experiments, but even more so in human beings and requires further clarification [33, 40-43].

In the periphery, effects of endogenous steroids are mediated by interaction with their intracellular receptors through classical receptor-dependent regulation of gene transcription. As the much more rapid interactions of NS cannot be mediated by those pathways, alternative pathways were intensively investigated. This revealed effect mediation via various non classical pathways through binding to different membrane receptors (e.g. NMDA and GABA) and regulation of non-genomic signalling cascades [44-47].

Physiological effects of NS are manifold, including influence on brain development, rapid modulation of neuronal excitability, brain plasticity, and behaviour and may further be sex dimorphic. Alterations in NS pattern seem to be involved in numerous diseases, such as

cognitive diseases (e.g. dementia), neurological diseases (e.g. multiple sclerosis, status epilepticus, Parkinson, idiopathic pain), psychological diseases (e.g. schizophrenia, depression) and behavioural shifts (e.g. aggression, addiction). For example, dehydroepiandrosterone (DHEA) and pregnenolone (Preg) and their sulfates were some of the first investigated NS, both are mainly associated with cognition, learning and memory and seem therefore to be involved e.g. in dementia diseases. As effects of NS are too multifarious for short discussion, several extensive reviews on mechanisms of actions in humans and animals are available elsewhere for the interested reader [3, 6, 7, 45, 48-63].



black: biosynthesis and metabolism, grey: enzymes

Figure 1: Overview of major metabolism pathways of (neuro-)steroids, adapted from Manuscript No. 1

2.2 Cerebrospinal Fluid (CSF)

2.2.1 Physiological CSF

Cerebrospinal fluid (CSF) of healthy individuals is a clear and colourless body fluid that is present around the brain. The total CSF of human adults' volume is approx.120 – 200 ml, from which around 25% are in the ventricular system and the rest is distributed in the subarachnoid spaces. Being in direct contact with the extracellular fluid, CSF is the most likely fluid to reflect brain metabolism, although this might be restricted to several brain areas [41, 64, 65].

CSF is separated from the vascular system by blood-CSF-barrier (BCB), whereas the brain parenchyma is separated from vascular system by blood-brain-barrier (BBB). Their morphology and transport properties differ, and therefore also the type and amount of substances that undergo influx or efflux, although both have similar functions [41, 43, 66].

Main physiological functions of CSF are the neutralization of brain gravity and its protection from mechanical harms, as it physically acts like a water cushion for the brain. Despite this, it maintains CNS homeostasis and removes metabolic waste products from brain.

CSF production mainly takes place in the choroid plexus of the brain ventricles. It is mainly, but not exclusively, filtrated out of blood. The daily production rate is about 500 – 700 ml per day, which corresponds to complete exchange for approximately four times per day. Absorption into venous blood mainly takes place via the arachnoid villi in the skull space but also spinal absorption and lymphatic pathways are still under investigation [67].

CSF composition is still part of investigation in several studies [68-72]. However, CSF consists predominantly out of water. Nevertheless, the composition is manifold with more than 450 small molecule metabolites according to CSF metabolome database [73], even if the amount of nearly all components can be considered as low. This refers for example to low protein content, which is approx. 0,5% of serum content in varying composition, already including possible steroid binding globulins. Cellular content is also very low, mainly containing white blood cells. Further components of CSF included in small proportions are amino acids, neurotransmitters, hormones and neuropeptides, enzymes and enzyme inhibitors, immune and inflammatory mediators, sugars and carbohydrates, trace metals and vitamins. Components, which are rather high concentrated, are electrolytes and lipids. The electrolyte amount is similar to blood and differs only slightly in composition. The content of various lipids ranges around 10 - 20 µg/ml. Thereof, up to 5 µg/ml are accounted to the steroid precursor cholesterol which is high in comparison to expected NS levels in nanomolar and picomolar range. Other lipidic substances in CSF are fatty acids, triglycerides, glycerophospholipids, sphingolipids, lipoproteins, and prostaglandins [68, 74].

CSF withdrawal requires strong clinical indication for ethical reasons. The most common type of CSF withdrawal is single lumbar puncture (LP), where usally 10-15 ml of CSF are withdrawn. As this material is usually aliquoted for different clinical analytical examinations, one can expect a volume of around 2 ml for a single examination as planned in this project.

2.2.2 Artificial CSF (aCSF)

Validation of methods for endogenous substances in ultra-trace-levels is challenging and necessitates an analyte free surrogate matrix, which should be as similar as possible to real matrix for achievement of best results in matrix effect investigations. Therefore, an artificial cerebrospinal fluid (aCSF) was developed for method validation purposes.

Although CSF component content is rather low, due to its diversity possible interfering components must be considered as they can heavily interfere in MS detection and potentially lead to ion suppression or ion enhancement [75-78].

Depending on later sample preparation, two major groups seem likely to cause matrix effects. Whereas the high electrolyte content in the hydrophilic phase may cause problems, the lipidic components will be simultaneously extracted with neurosteroids in commonly used lipophilic extraction procedures. As already mentioned, especially the content of cholesterol is remarkably high in comparison to NS levels and might be added to surrogate matrix to likely reflect real conditions. As protein content in CSF is generally low, protein precipitation as often performed within other body fluids analysis such as blood, is thus considered controversial or even useless.

Within this project, Tanja Naumann [79] developed a model experiment in her bachelor thesis for investigation of the previous considerations. The results and the final aCSF composition and preparation are reported in 3.1.1.

2.3 (Neuro-)Steroid Analysis with GC-MS

2.3.1 Introduction

Since decades GC-MS(/MS) analysis is considered as the “gold standard” in steroid profiling and was therefore initially chosen to achieve the project aim.

Considering instrumentation, GC-MS offers effective separation with high sensitivity, whereas GC-MS/MS is predominately used in targeted ultra-trace analysis because of lower background noise and also for profound structure elucidation, as the second MS enables further fragmentation of the analyte. Despite of this, untargeted analysis and structure elucidation are commonly performed with high resolution mass spectrometry (HRMS). GC-MS with EI ionization well complies with the requirements within this project for initial investigation of the selected steroids and was therefore performed applying separation methods based on standard procedures for steroid profiling reported in literature [18, 80-82]. The configuration of the instrumentation of this project is schematically displayed in Figure 2.

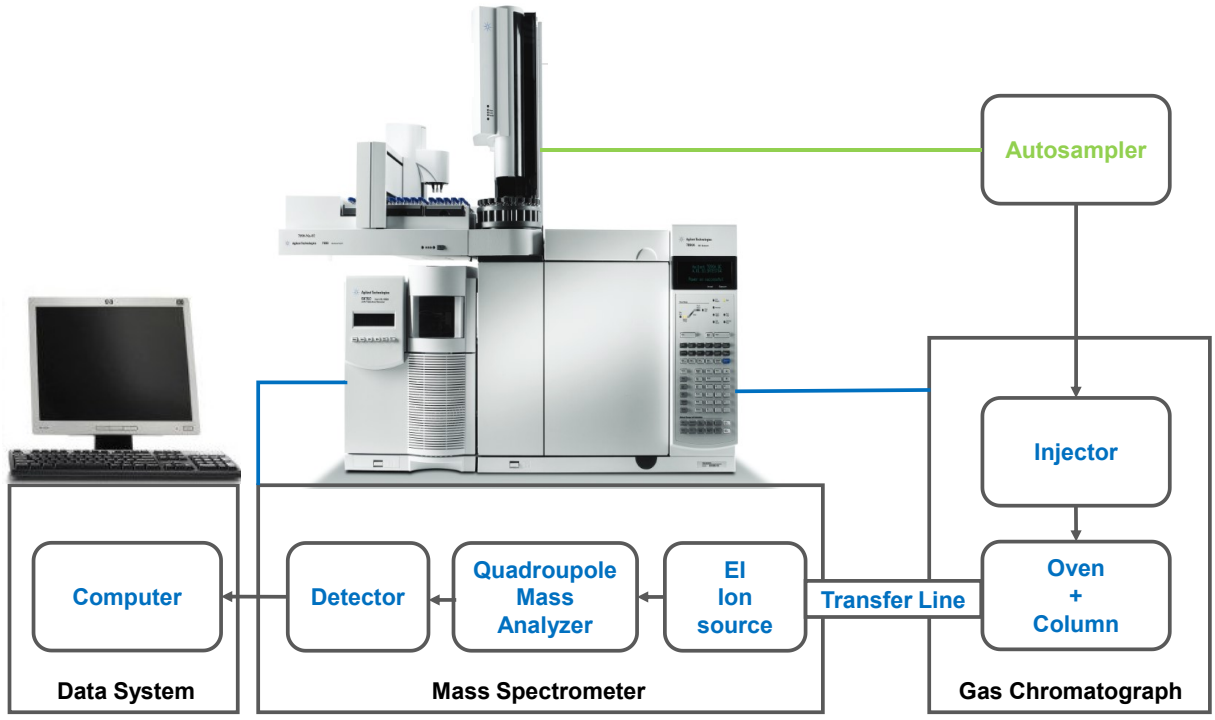


Figure 2: Schematic configuration of the GC-EI-MS system used within this project, consisting of an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass selective detector; images with courtesy of Agilent Technologies

GC-MS(/MS) of steroids is predominantly performed with derivatization of analytes prior to injection, due to their chemical properties. Depending on the ionization type that is used, e.g. electron ionization (EI) or chemical ionization (CI), different derivatization reactions may be required. Main reasons for derivatization are improved chromatographic properties, increasing substance volatility and enhanced thermal stability, improved signal intensity and lower detection limits. For optimum results derivatizations reactions should be quantitative.

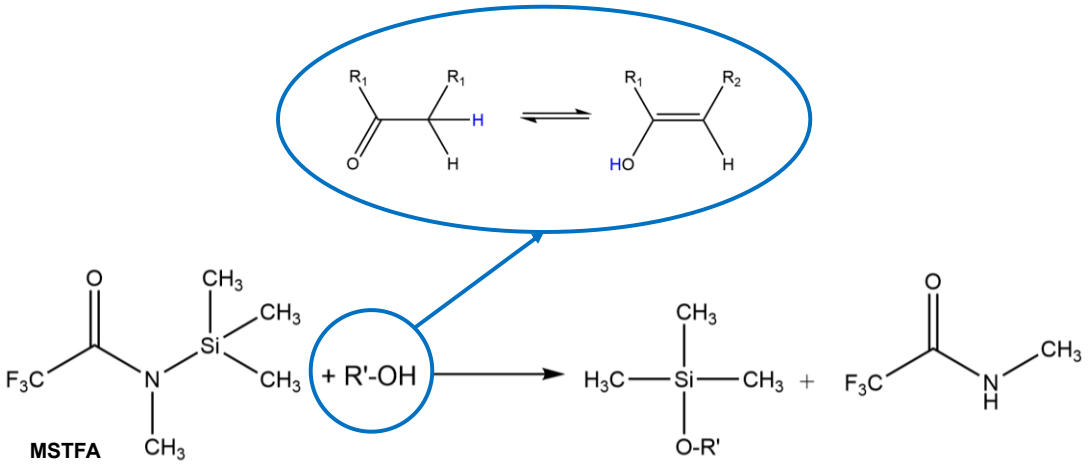
Within here, the panel of selected steroids impedes the selection of a suitable derivatization procedure, due to polyfunctionality. There are steroids with oxo-groups (at position 3,11,17,20), aliphatic hydroxy-groups (at position 3,7,11,16,17,20), phenolic hydroxy-groups (at position 3) or steroids with both, oxo-and hydroxy-groups. Type, number and position of functional groups for each individual steroid are outlined in Table 3. The selection further includes 10 steroid sulfates that cannot be easily derivatized directly for GC-EI-MS. During the last decades they were cleaved prior to derivatization and subsequently separated analogical to non-conjugated steroids. However, recent research by Polet et. al. [83]. reported promising direct GC-MS analysis of non-hydrolyzed sulfated steroids by CI and low-energy-electron ionization (LE).

Numerous derivatization procedures for steroids covering for either oxo groups, hydroxy groups or both are well established [21, 22, 84-87]. Derivatization as trimethylsilyl ethers (TMS) or methoximes (MO) or mixed derivatives (MO-TMS) are predominantly reported. They are

easy to prepare and produce highly volatile and stable derivatives with characteristic EI spectra. Therefore, different procedures for generation of these derivatives were investigated (Manuscript No. 2). However, there are many further opportunities for steroid derivatization covering a wide spectrum of derivatization reactions and agents for different ionization techniques that can be read up elsewhere by the interested reader. [21, 22].

2.3.2 Silylation

This derivatization is based on replacement of the active proton in hydroxy groups by the trimethylsilyl group by nucleophilic attack of the heteroatom to silica as shown in Figure 3. One must be aware, that due to the type of hydroxy group (primary, secondary, tertiary, phenolic) and steric orientation their rate of silylation markedly differs, which may lead to incomplete derivatization.



Si-(CH3)3 = TMS = Trimethylsilyl-Group // R' = -alkyl, -alkenyl // MSTFA = N-Methyl-N-(trimethylsilyl)trifluoroacetamide

Figure 3: General mechanism of silyl ether formation with MSTFA and keto-enol-tautomerization

Derivatization of oxo-groups requires prior enolization by keto-enol-tautomerization (chemical equilibrium between two isomeric forms of an aldehyde or ketone) that can be reinforced in presence of several silylation agents with acidic or basic catalysts [21]. This is simultaneously the major drawback of this technique as this may led to formation of isomeric enol-derivatives that may separate depending on chromatographic conditions. For example, in steroids this occurs frequently in position C3 or C20 as exemplified in Figure 4.

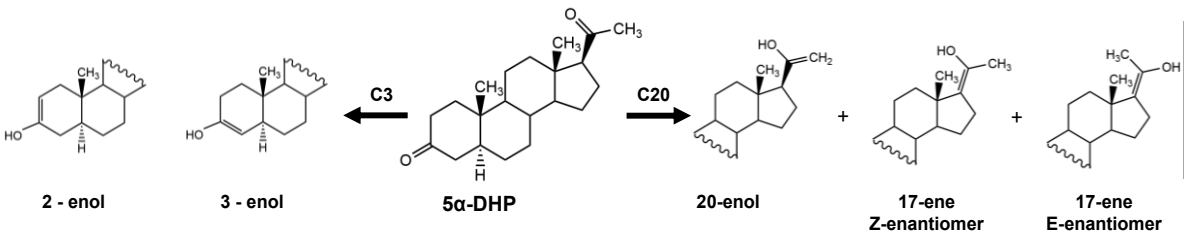


Figure 4: Enolization at position C2 and C3 and C20 exemplified for 5α-dihydroprogesterone

This can be avoided by introduction of a protection group such as by oximation as described in 2.3.3 prior to silylation procedure.

2.3.3 Oximes and Mixed Derivatives

Basically, the reaction of aldehydes or ketones with primary amines leads to formation of imines. If the aldehydes or ketones contain the grouping C=N−OH as a functional group, oxidized imines, so called oximes, were formed. If hydroxylamine is used as reagent, the slightly acidic hydroxy group can be further derivatized which is commonly performed as silylation reaction. However, to avoid this second step, utilization of O-alkyl hydroxylamine is in favor, which results in formation of O-alkyl-oximes. A frequently used agent is O-methyl hydroxylamine (Figure 5), forming methoximes (MO). For steroids containing oxo-groups only, oximation can be performed as alternative to silylation. Nevertheless, steroids with only hydroxy groups cannot be derivatized with this procedure. If steroids with both functional groups need to be analyzed, the most common practice is the oximation of the oxo group as protection step for prevention of enolization reaction prior to silylation of the remaining hydroxy groups. Those derivatives were called MO-TMS and herein MO-TMSIM due to the derivatization with MSTFA with basic catalyst imidazole as performed within this work.
One major drawback of this technique is the formation of syn (Z)- and anti (E)-isomers (Figure 5) that may separate in high resolution conditions.

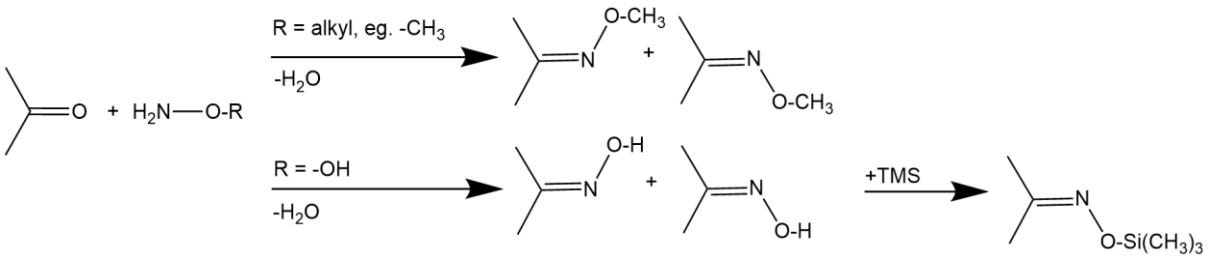


Figure 5: Oxime formation and possible further silylation reaction with formation of syn- and anti-isomers

2.4 Analytical Supercritical Fluid Chromatography (SFC)

2.4.1 Introduction

SFC had many different names in the past, like “critical state chromatography”, high-pressure gas chromatography above critical temperatures”, “dense gas chromatography”, “subcritical fluid chromatography” or “high performance liquid chromatography with enhanced fluidity” [88-91]. All of them tried to describe the exceptional physiochemical properties of a supercritical fluid: a highly compressible fluid which acts like a solvent in SFC.
The supercritical region is a transient region above the critical point (approx. 31 °C / 80 bar) [92], where no distinct liquid or gaseous phase exists. Figure 6 shows the phase diagram of carbon dioxide (CO_2). Small changes in pressure and temperature will not result in a phase

transition but evoke large changes of the density of the fluid and therefore retention behaviour of the analytes [88, 90, 93].

In dependency of different parameters during performance, the actual physical state of such a fluid may switch between the two domains of supercritical state (a fluid with higher than critical pressure and temperature) or subcritical state (a liquid with close to critical pressure, but little lower than critical temperature) [88, 90, 93, 94] (Figure 6). Although they describe different physical states both essentially have similar chromatographic properties [88, 95]. However, the term supercritical fluid has become established over time regardless of the actual state of the fluid used [88].

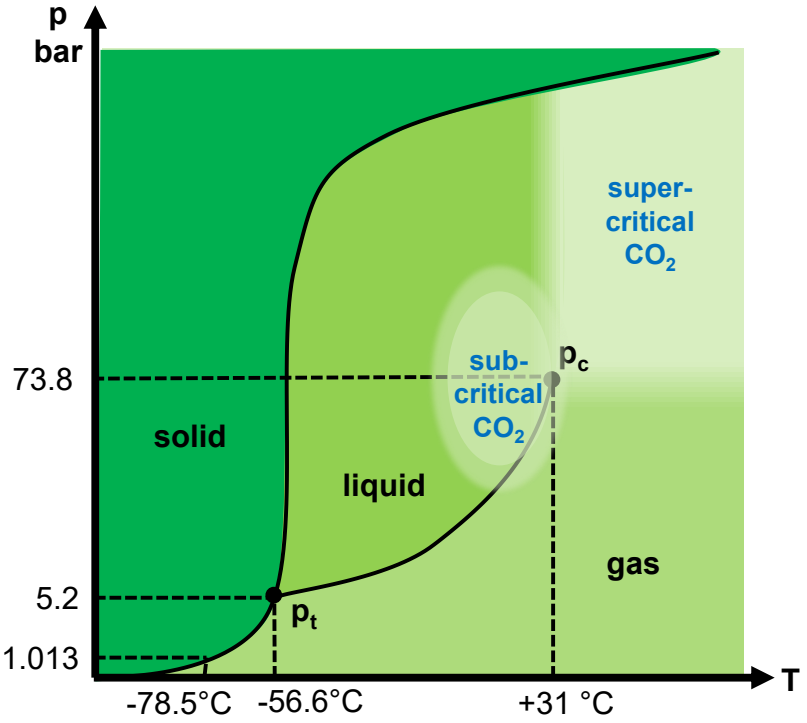


Figure 6: Phase diagram of carbon dioxide

As was already obvious when trying to name it, the physicochemical properties of SFC are unique [90, 96]. With a view to practice this further becomes obvious by comparison with LC and GC conditions as shown in Figure 7. First, density corresponds to solvation power: the higher the density the higher the solvation. Due to the density of the supercritical fluid between LC and GC conditions, SFC results in higher solvation power than GC and more similar to LC conditions. Secondly, the diffusion coefficient corresponds to mass transport: the higher the diffusion coefficient, the higher the mass transport. With a diffusion coefficient between LC and GC conditions, SFC enables higher mass transport than in LC. Importantly, there is no condition, where a supercritical fluid can have both the solvation power of a liquid and the high diffusion of a gas at the same time. Nevertheless, depending on the applied temperature and

pressure, the physicochemical properties can be shifted towards more LC or GC like conditions [97].

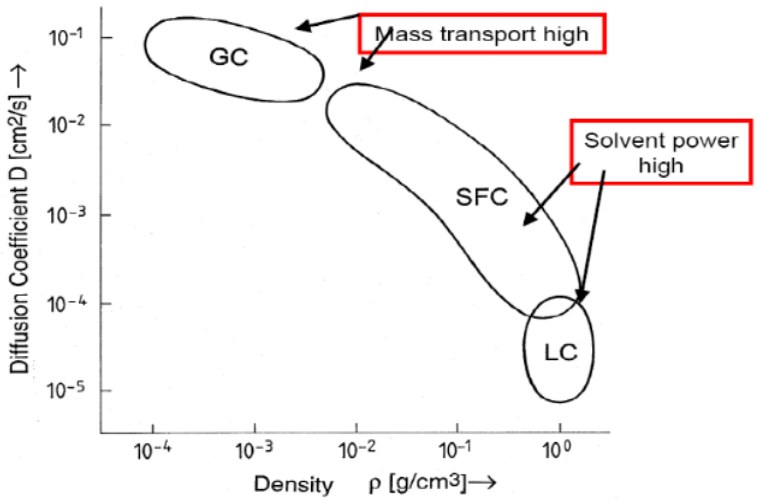


Figure 7: Comparison of physicochemical properties of GC, SFC and LC [98]

Summarized, SFC is a powerful separation technique, where supercritical fluids are used as mobile phase with an instrumentation that is almost identical to that used in HPLC.

2.4.2 History

During the last years SFC celebrates a comeback und returns to analytical science as a renewed and promising green method for the future. However, it has a long history with incidental discoveries and misunderstandings that have been partially cleared up over the decades and turned out to be meaningful on what we know today as SFC [88, 89, 94, 97, 99]. Whereas the first gas chromatographs were described in the 1950s, it was only little later in 1957, when Jim Lovelock first proposed chromatography with gas above its critical point as mobile phase [89]. In 1962 the first publication with chromatographic conditions we can call SFC was published by Ernst Klesper [100], who pioneer-like demonstrated the separation of metal porphyrins with dense chlorofluorocarbons as mobile phase. Until the end of the 1970s further work on SFC was tendentially supressed by fundamental misunderstandings [89] of physiochemical properties of supercritical fluids by Giddings [101] and results of Miles Novotny [102] and L.B. Rodgers [103], who indicated that SFC seemed to be unlikely to produce results as good as HPLC after they observed serious problems with density gradients along the length of the column. At least, Sie and Rijnders where the first to introduce the term “supercritical fluid chromatography” in 1967 [88]. During this time modifications of GC conditions and instrumentation were predominantly in focus of science and SFC nearly vanished. However, scientists of a research group of Hewlett-Packard (HP, now Agilent Technologies) published ongoing research on dense gas chromatography at the end of the 1970s which lead to further customer demand and the introduction of the first commercially available packed-column SFC

in the early 1980s by HP followed by Jasco [89]. About the same time, a capillary version of SFC was introduced by Miles Novotny and Milton Lee and was seen as the future of SFC, due to the lack of commercially available packed-column SFC-instrumentation [88, 94]. However, for both systems many physiochemical properties remained uncovered and it took some further years of research to unravel misconceptions and fundamentally change the perception of SFC. These results ended up in a renaissance of SFC in the 1990s and the introduction of the 2nd commercially available SFC by HP and the predominance of packed-column SFC over capillary SFC. However, there was still no large establishment on the market. This changed in 2009 with the introduction of Aurora SFC (later Agilent Technologies), that made modern SFC more similar to HPLC than GC by possible conversion of an HPLC system to SFC, followed by introduction of Waters Corporation UPC^2 - system in 2012 and other supplier's SFC instruments (e.g. Shimadzu) that were subsequently introduced in the market [89]. Since then, SFC was and still is likely to be used to a much greater extent due to its unique selectivity properties and wide application areas and the growing demand for sustainable chemistry.

2.4.3 Mobile Phase and Control Variables

In SFC, the mobile phase principally consists of a supercritical fluid. Supercritical fluids that were investigated in the past include for example carbon dioxide (CO_2), ammonia (NH_3), nitrous oxide (NO_2), sulfur hexafluoride (SF_6) and xenon (Xe) [92]. Today, SFC is almost exclusively performed using carbon dioxide CO_2 as supercritical fluid, as most practicable option considering issues like easily accessible critical point and miscibility with more polar solvents, low costs, environmental compatibility, and safety concerns [24, 92]. In the following carbon dioxide is referred to as supercritical fluid, or respectively supercritical carbon dioxide, $scCO_2$.

$ScCO_2$ is a highly nonpolar solvent with a polarity like n-hexane or heptane [104, 105]. Alteration of chromatographic behavior can be achieved by addition of more polar organic cosolvent(s) to $scCO_2$, also termed as modifier [104, 106, 107]. The effects of modifier addition are complex, including change of mobile phase density, increase of solute solubility, adsorption on stationary phase surface, or change of mobile phase polarity [107-109]. The elution strength mainly follows well established Snyder`s P scale or Hildebrandt solvents scale for silica as stationary phase like in normal phase HPLC [88]. Snyder further developed a solvent selectivity classification scheme (Figure 8). It is based on the assumption that Van der Waals forces were universal and plots the solvents according to their ability to interact with proton donors, acceptors or as dipole, ending up in eight solvent families, where members of each group have a similar selectivity [88, 106]. Consequently, it is worthwhile to try a modifier of a different solvent family instead of one from the same if separation fails with the chosen modifier. Frequently used modifiers are various alcohols, but also addition of ethyl acetate, acetonitrile, or other organic solvents or even water may be helpful to overcome separation issues [107].

Methanol is the by far most widely used modifier due to complete miscibility with $scCO_2$, easy availability, and low UV cut-off in case of SFC hyphenation with UV detection. Water has a limited solubility of approximately 1% in $scCO_2$ but can be added up to 10% in $scCO_2$/methanol (V/V) mixtures [88] and may be beneficial, especially for chiral separations [110].

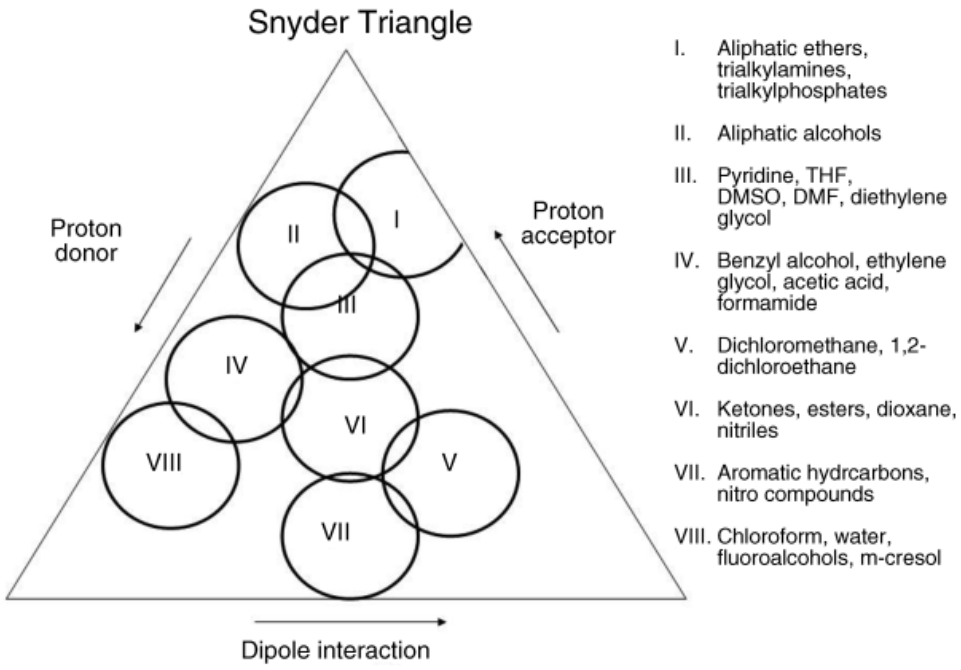


Figure 8: Snyder's solvent triangle [111]

Further improvement of chromatographic behaviour may be achieved by addition of a small amount of so-called additives (acids, bases, salts) dissolved in the modifier [112, 113]. This might be necessary for highly polar analytes and amphoteric compounds with too intense interactions with the stationary phase that elute with poor peak shapes or even fail to elute. Additives affect the interaction environment and may result in modification and stabilization of mobile phase pH-value, suppression of solute ionization, cover active sites of the stationary phase or form neutral pairs with the analyte [88, 112-115]. As a rule of thumb, addition of acids improves peak shapes of acids and addition of bases improves peak shapes of bases. However, it might be the other way round due to ion pairing and amphoteric analytes might require multiple ions for good results [88]. Frequently used additives are acids (e.g. formic acid, acetic acid), bases (e.g. diethylamine, trimethylamine), salts (e.g. ammonium formate, ammonium acetate) or their respective mixtures with concentration ranges between 0.1 and 1% in the modifier [112, 113]. To improve their solubility in the modifier and further improve chromatographic quality, a small portion of water is usually added [112, 113].

Further impact on separation performance can be exerted by change of different parameters with SFC instrumentation that were evaluated by screening experiments during method

development. Generally, separation performance depends on density of the mobile phase. An increase in density increases the solvent strength, and therefore leads to decreasing retention [105]. However, the more modifier is added to the highly compressible, low viscosity $scCO_2$, the less impact can be achieved by change of the parameters [105]. The following parameters are considered to influence the density of the mobile phase in descending order [88]:

- modifier concentration
- column temperature
- backpressure
- flow rate

As many physicochemical properties of supercritical fluids and their mixtures still remain unclear, one has to be aware that the following part describes generalized expected retention behavior. However, many unusual and unexpected retention behaviors were reported in the past [116-118].

Considering modifier concentration, typically ranges between 5 and 50% were applied [88, 93, 107]. However, that high amounts of cosolvents result in no longer supercritical conditions in its pure sense. They result in subcritical (or even liquid) conditions. Predominantly, analytical SFC is performed with composition of the mobile phase programmed from low to high polarity by gradient programming from low to high modifier. Adjustment of modifier concentration is the most effective way to impact retention and selectivity. As mentioned, increasing density results in higher solvation strength, and therefore decreasing retention [105]. Doubling modifier concentration, approximately halves retention time. Especially small amounts of modifier added can have large effects on retention as outlined in 2.4.4. Such large retention shifts with small concentration shifts complicate reproducibility. Therefore, as a rule of thumb, concentrations of modifier below 5% should be avoided [88, 118].

Changes of temperature have less effect on retention than modifier concentration but can result in significant shifts in selectivity with change of elution order and peak reversals. This can be explained by affection of mobile phase density as the mobile phase becomes more or less compressible by changing temperature [104, 119]. Normally employed temperatures range between 25 °C and 50 °C, but also unconventional temperatures from -5 °C up to 80 °C were explored and may be beneficial [119]. A decrease or increase of 10 degree already indicates if selectivity improves [88].

Pressure is a major control parameter with pure $scCO_2$ [104]. The higher the modifier concentration becomes, the lower effects were observed on density and retention and therefore selectivity [120]. However, especially with low modifier concentrations it may be worthful to examine an achievement of subtle changes, as changes of pressure also directly influence mobile phase density [121, 122].

Changes in flow rate have only little impact on selectivity and retention as by changing the flow rate only the pressure inside the column varies which only slightly influences the density as the backpressure regulator still maintains the overall pressure in the system. However, as long as the chromatographic behaviour meets the requirements of the envisaged separation, increasement of the flow rate may be supportive to shorten run times [95].

2.4.4 Stationary Phases

Many stationary phases that were used in packed column SFC were originally designed for HPLC. However, during the last years development for specific SFC stationary phases heavily increased [24, 123].

Generally, for polar analytes polar stationary phases (e.g. bare silica, cyano, amino, diol, 2-ethylpyridine) were used and for low polarity analytes reversed phase columns (e.g. C18, C8). Lesellier et al. [124, 125] intensively studied stationary phase properties in SFC over the last years and developed a classification system that visualizes differences in column selectivity in form of a spider diagram. Usually several different similar stationary phases can be used for the same purpose as the methods are generally optimized by changing of mobile phase conditions, for example all the classic polar phases that fairly cluster together according to this classification system. Analogous to the solvent selection according to Snyders triangle, it is more worthful to switch to a column from another group instead of investigating another one from the same group if separation of analytes was not achieved.

Alteration of stationary phase characteristics may occur by absorption of $scCO_2$, modifier, and additives. It was already in the 1990s when Parcher et al. [108] demonstrated that the first small additions of modifier methanol result in extensive adsorption on a stationary phase by production of monolayers, whereas at higher concentrations only small amounts were absorbed. Similarly, monolayers of $scCO_2$ can also be adsorbed [118, 126]. More recently, Fairchild et al. [127] proposed silyl ether formation as major contributor to retention and selectivity. An also well-known phenomenon is strong adsorption of additives to polar stationary phase even at concentrations below 1 % [113, 114] which may change column characteristics temporarily or permanently. If, and to which extent different adsorption phenomena affect column characteristics certainly need to be further clarified in the future.

Considering the column dimensions, older applications predominantly used 4.6-mm id and 250mm-length columns packed with 5 µm particles. However, the use of sub-2 µm particles in 3-mm id and 100-mm length columns has become the state of the art due to shorter runtimes with same separation efficiency, when operated at optimum velocity [124, 128, 129].

2.4.5 Sample Dissolution Solvents

Peak distortions caused by sample diluent solvent are a well-known phenomenon, also called “strong solvent effect” [130]. According to HPLC good laboratory practice, samples are

recommended to be diluted in the same solvent as the mobile phase. However, this is not possible with supercritical fluids and sometimes not with the sample itself. Basically, any compound soluble in methanol or less polar solvent is analyzable with SFC, whereas highly polar compounds are rather poor candidates [131]. Recent studies indicate possible distortions between the sample, the solvent and the mobile phase [130, 132]. Desfontaine et al. [132] further reported distortions due to competitive adsorption of the analyte and the diluent to the stationary phase. Generalized, samples in SFC should be dissolved in a solvent close to (or even better weaker than) the chosen mobile phase with as small as possible sample injection volume. Therefore, sample dissolution solvent screening necessarily needs to be part of the method development as a function of the analyte, the mobile phase and the selected stationary phase.

2.4.6 Advantages of SFC

SFC has physiochemical properties that make it a unique separation technique. Resulting advantages can be further clarified with the help of the van Deemter (Knox) equation that describes the kinetic performance of a column:

$$H = A^{0.33} + \frac{B\ D_{1,2}}{\mu} + \frac{C\ d_p^2\ \mu}{D_{1,2}}$$

H = theoretical plates
$A^{0.33}$ = Eddy diffusion
B = axial diffusion
C = radial diffusion
$D_{1,2}$ = solute binary diffusion coefficient in the mobile phase
μ = mobile phase linear velocity
d_p^2 = particle diameter

- Term B (contribution of axial diffusion): higher diffusion coefficient results in higher optimum linear velocity
- Term C (contribution of radial diffusion and mass transport): higher diffusion coefficient results in less efficiency at higher flow rates
- Term B and C further contain the ratio of the solute binary diffusion coefficient in the mobile phase $D_{1,2}$ to the linear velocity of the mobile phase μ

The major advantages of SFC can be derived from this:

- Faster analysis times: because of low intramolecular interaction of $scCO_2$ diffusion coefficients of molecular analytes in pure $scCO_2$ are approx. 10 – 15 times higher than in aqueous solutions and remain high in compressed $scCO_2$. Addition of modifier significantly decreases diffusion coefficients, but they remain higher than in HPLC. Over all, SFC with 5-50% modifier is 3-5 times faster than HPLC, at the same particle size and the same efficiency [88, 128].
- Lower pressure drops: because of low intramolecular interaction of $scCO_2$ molecules the viscosity of the supercritical fluid is low, even with addition of modifier. Low viscosity of the supercritical fluid results in smaller pressure drops along the column. Pressure drop in SFC is approx. 3-5 times smaller than in HPLC at 3-5 times of the speed, even with sub 2 µm-columns [88, 128].
- High efficiency: because of low pressure values in SFC multiple columns can be coupled for enhancement of number of count of theoretical plates [88, 123, 129].

Further significant advantages of SFC include:

- Selectivity: orthogonal retention behavior compared to reversed phase, normal phase, and HILIC HPLC and GC provides chromatographic alternatives [26, 106, 133].
- Low operating costs: worldwide distribution infrastructure for beverage grade CO_2 and high throughput lowers operating costs [24, 88].
- Green technology: less use of hazardous solvents and use of from other industries recycled CO_2 reduces environmental pollution and liquid waste [24, 134].

2.4.7 Applications and Steroid Analysis with SFC

SFC largely covers the same application space as HPLC as displayed in Figure 9. It has proven to be very powerful in separation of enantiomers and isomers and analytics focused on these applications for decades [105]. However, during the last years SFC was also explored for many other applications [24, 135] as illustrated in Figure 9. A lot of extensive references are available elsewhere for the interested reader, as for example analysis of pharmaceuticals [134, 136-138], natural products [139], food [140], bioanalysis [105, 141], metabolomics [142], forensics [143], anti-doping [144-146] or lipid profiling [147, 148].

Until know only few publications that considered exogenous or endogenous steroids [27-29, 145, 149-151] or steroid sulphates [30] demonstrated the suitability of SFC for this class of compounds. Considering this project, SFC opens the unique opportunity to separate steroids under normal phase conditions instead of reversed phase mode in HPLC or as widely applied with GC. The promising results are presented in Manuscript No. 2.

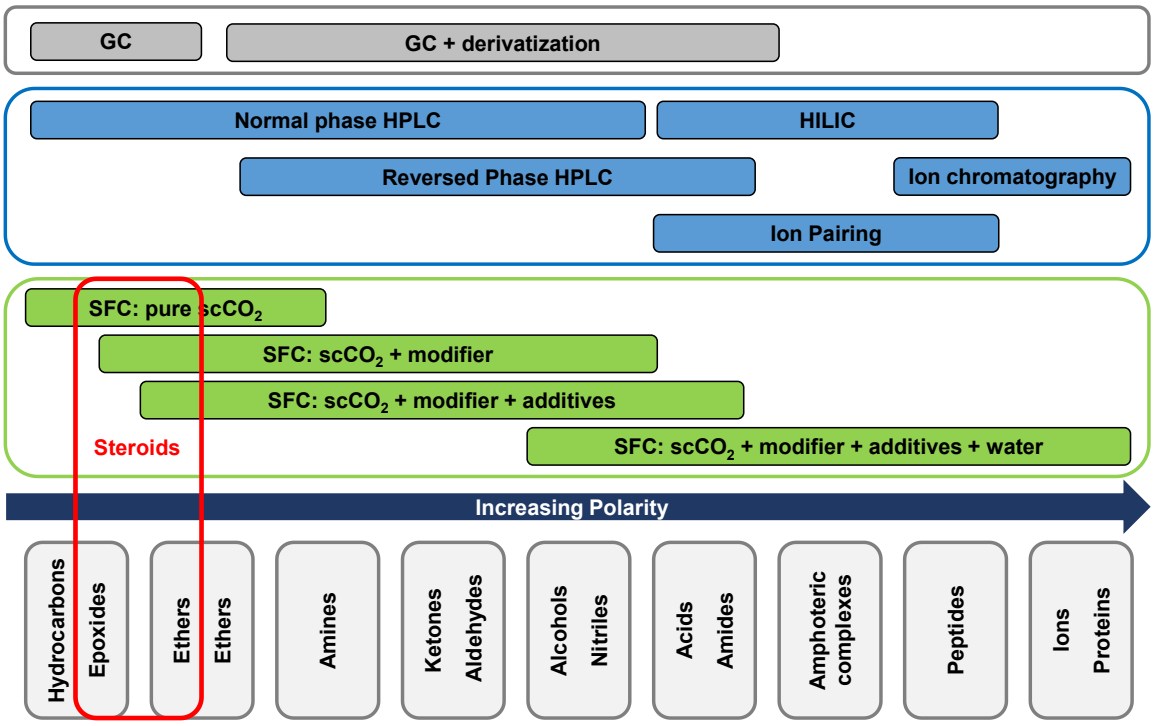


Figure 9: Potential analytes for SFC and comparison of various SFC conditions to those used in HPLC and GC, steroids as application are highlighted in red; adapted from [152, 153] and completed with notes specific to this section

2.4.8 SFC Instrumentation and Hyphenation with Mass Spectrometry

A comprehensive work on SFC instrumentation for analytical use was published and compared with (U)HPLC instrumentation by Terry Berger in 2015 [154]. Basically, hardware in SFC instrumentation is similar to (U)HPLC-instrumentation and many compartments like solvent degasser, modifier pump, thermostatic oven/column compartment and tubing can be used for both with some minor modifications due to the usage of $scCO_2$ as the main component of the mobile phase. Because of the high compressibility and low viscosity of supercritical fluids, especially the CO_2-pump, and the autosampler require the most significant modifications.

Considering the pump, most SFC instruments use binary pumps, one for the modifier and one for $scCO_2$ to control composition and volumetric flowrate of the mobile phase. In many labs CO_2 is commonly supplied by steel cylinders at approximately 55 bars at 20°C. However, the pressure is temperature dependant. Changes of temperature influence the equilibrium of the high-density liquid CO_2 phase on lower density gaseous headspace. It may potentially cause cavitation in the pump by fluid vaporization in case of increasing temperature. The higher the temperature become, the lower the density of the fluid become, and the more compression (higher compression ratio) must be applied by the subsequent pump system. To overcome this, the pump heads are usually chilled to 4-5°C and the fluid is also pre-chilled. Further, the use of a booster pump highly improves flow and pressure fluctuations, by pre-compressing the CO_2 to a pressure few bars below the column head pressure in front of the metering pump,

which then only meters the flow. However, coping compressibility issues with supercritical fluids is much more complicated and cannot be overcome with chilling and usage of a booster pump, only. Many further modifications of hardware (e.g. valves, pistons, tubing) are necessary and can be read up elsewhere by the interested reader [94, 97, 154-156].
In conventionally used modern (U)HPLC autosamplers, the compressed mobile phase in needle, tuning and syringe would be exposed to atmospheric pressure and therefore decomposed when the sample is loaded, which must be avoided in SFC. Therefore, a fixed-loop approach, where a previously filled loop is switched into the pressurized $scCO_2$ stream, is state-of-the-art technique in many commercially available SFC instruments. This technique enables high precision injection of full loops, but with partial loop fillings, precision lowers. Further, the required loop overfills lead to waste of sample. New approaches are therefore under development, such as injection of flexible sample volumes, which are pressurized in a loop before injection into the $scCO_2$ stream [157].
Finally, to ensure that the supercritical fluid remains a single dense phase throughout the whole chromatograph a back-pressure regulator at the system outlet is necessarily required.

Interfacing of SFC can be realized with different potential interfaces commonly known from HPLC [88] but necessarily requires careful design due to high pressure that needs to be maintained after the analytical column. Considering MS/(MS)-interfacing, several interface designs to find suitable solutions were configured over the years and intensively discussed [158-162]. Only recently, splitless hyphenation which allows complete transfer of the sample resulting in improved sensitivity but also higher solvent loads was made available [158, 160].
Electrospray ionization (ESI) is predominantly used, but also atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI) were applied [135, 160, 163-165]. Schematic configurations of ESI, APCI and APPI ion source design and their applicability are illustrated in Manuscript No. 3. Compared with LC instrumentation SFC may require a post-column make-up pump to ensure complete transfer of the sample to the ion source as the mobile phase supercritical fluid will have vaporized before, especially if SFC is performed with low modifier concentrations [166]. Moreover, this is one of the unique features of SFC which presents the possibility to achieve optimal support of ionization utilizing a make-up solvent independent from mobile phase composition [160]. Main results of make-up optimization experiments are presented in Manuscript No. 3 and supported by further results presented in section 3.3.2. The schematic configuration of the instrumentation in this project is presented in Figure 10.

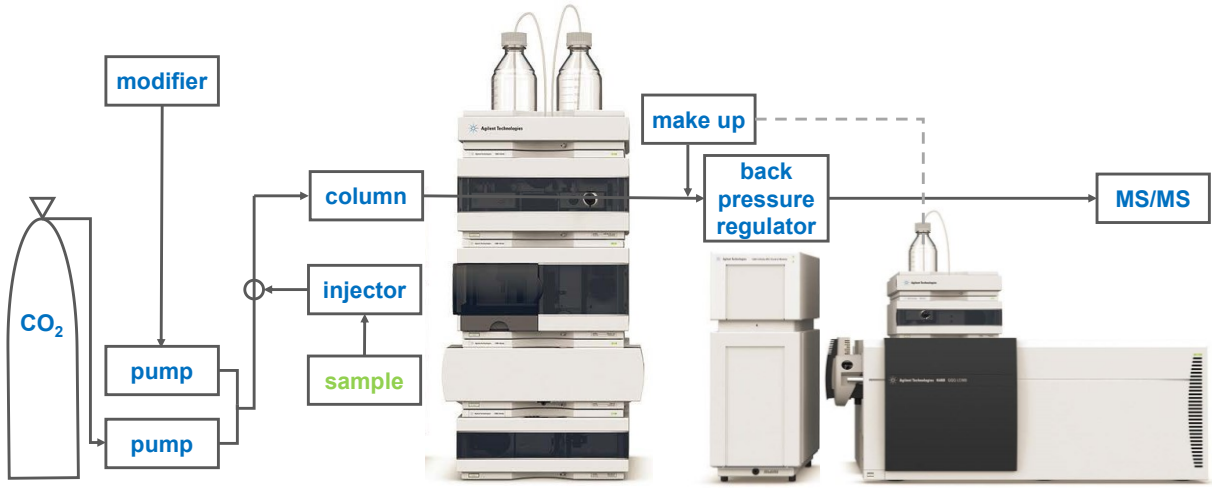


Figure 10: Schematic configuration of the SFC-ESI-MS/MS instrumentation in this project, consisting of an Agilent 1260 Infinity 1 SFC system splitlessly coupled to an Agilent 6495 triple quadrupole mass spectrometer; images with courtesy of Agilent Technologies

2.4.9 SFC-MS(/MS) Method Development

Method development of a new SFC-MS(/MS) method requires careful investigation of chromatographic and mass spectrometric parameters, ideally followed by robustness testing. Considering chromatographic parameters, comprehensive tutorials on SFC method development were by published by Terry Berger [88], Ashraf-Khorassani et al. [131] and Lucy Nováková et al. [104] for instance. Basically, chromatographic method development includes the previously discussed steps (section 2.4.3-2.4.5):

- stationary phase screening
- modifier screening
- adjustment of modifier concentration, column temperature, backpressure, and flow rate of the modifier
- sample dissolution solvent screening

Despite of this, method development must also include investigation of the mass spectrometric parameters as previously outlined in section 2.4.8 and Manuscript No. 3, such as:

- selection of ion source
- make-up composition and flow-rate screening
- ion source parameter optimization

Beyond, much effort is put into achieving analysis results with an appropriate quality not only for pharmaceuticals but also for bioanalysis which are both strongly regulated by various international and national guidelines, such as ICH, USP, FDA, or GTFCh. To meet the continuously tightening requirements on validation, robustness testing becomes increasingly more important [167]. Initially, robustness testing was performed by the end of method validation to indicate factors that could affect results of interlaboratory studies.

As this may result in re-development and re-validation, associated with cost increase and loss of time, nowadays robustness testing is recommended to performed already at the end of method development or at the beginning of validation procedure [167-169], as is the case within this project.

ICH and USP define robustness as follows: “The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage” [167]. A measure for robustness is the range in which the result is independent of changing a parameter [170].

Only a few examples of SFC robustness studies were published during the last years [168] although the high potential to develop robust SFC methods was only recently demonstrated by method transfer between 19 participating analytical laboratories, on 4 continents in 9 countries [171]. Nevertheless, the few available references impede implementation of an experimental set-up for robustness testing. According to USP/ICH robustness testing is usually performed by either an one-variable-at-a-time (OVAT) procedure or an experimental design procedure, which were discussed in detail by Dejagher et al. [167]. In short, the OVAT procedure varies and evaluates the levels of a given factor while keeping the other factors at nominal level, with the nominal level is referred to as standard method parameters. This method is a simple method, as long as not too many factors have to be considered. However, this univariate method cannot take factor interactions into account, which would require more extensive robustness testing. With the more comprehensive experimental design procedure, the effect of a given factor is calculated at several level combinations of the other factors.

Another strategy for robust method optimization is the in pharmaceutical development well established Quality by Design (QbD) concept [169], “a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding based on sound science and quality risk management” [168]. A key component of this concept is the design space (DS), which is defined as the space of chromatographic conditions that will ensure the quality of the separation within the chromatographic method [168]. If this robust optimization strategy is successfully performed, a conventional robustness testing as outlined before is no longer required according to USP and therefore speeds up the analytical life cycle. This concept is discussed in more detail by Dispas et al. [168, 172] for the interested reader. With this strategy, successful method transfer between columns with different lengths [173] or between different instrumentations (Waters and Agilent) could be demonstrated [172].

Concluding, the workflow that was used for method development within this project covering these stages is presented in section 3.3.1. Results of chromatographic development were discussed in Manuscript No. 2, whereas results of mass spectrometric investigations were mainly presented in Manuscript No. 3, supplemented by section 3.3.2. Robustness testing is presented in section 3.3.3.

3 Supplementary Results

3.1 Cerebrospinal fluid

3.1.1 Composition and Preparation of Artificial Cerebrospinal Fluid (aCSF)

An artificial cerebrospinal fluid (aCSF) was developed as analyte free surrogate matrix for method validation purposes. As described in 2.2.2, cholesterol might be necessarily added to aCSF to reflect real CSF conditions most likely. For this reason, Tanja Naumann [79] developed a model experiment in her bachelor thesis within this project. First, the chromatographic behavior of cholesterol was examined and compared to that of testosterone. For this purpose, thin-layer chromatography (TLC) was carried out under SFC-like conditions instead of SFC-MS/MS, since underivatized cholesterol is barely detectable using electrospray ionization. The preliminary test confirmed that cholesterol coeluted with steroids, so that matrix effects cannot be ruled out. To investigate possible interferences, in a worst-case scenario increasing amounts of cholesterol were added to a high concentrated solution with all 51 steroids, which led to steroid-to-cholesterol ratios from zero cholesterol to 1:1000, which is comparable to possible physiological conditions. As no matrix effect was observed, it seemed not necessary to add cholesterol to aCSF.

Therefore, it was decided to only prepare an electrolyte solution which reflects physiological conditions as aCSF. The composition is shown in Table 1. For this purpose, two solutions, A and B (Table 2) are initially produced. Solution A (electrolytes) is cloudy whereas solution B (buffer solution pH 7.4) is clear. Both are freshly mixed in a 1:1 ratio if needed. The solution then shows only a slight cloudiness. The pH of the aCSF mixture is around 7.8 due to the high proportion of hydrogen carbonate in solution A. This pH is slightly above the physiological pH of CSF. However, especially after storage of CSF samples loss of CO_2 may lead to an observable shift from the physiological pH 7.3-7.4 to slightly alkaline areas [174, 175] which justifies the aCSF composition.

Table 1: Comparison of the electrolyte contents of physiological CSF [68] and prepared aCSF

Ion	concentration in CSF [mEq*l^{-1}]	concentration in CSF [mmol*l^{-1}]	concentration in a CSF [mmol*l^{-1}]
Na^+	141	141	141
Ka^+	2.9	2.9	2.9
Ca^{2+}	2.3 – 3.2	1.15 – 1.6	1.5
Mg^{2+}	2.0 – 2.5	1 – 1.25	1
Cl^-	120	120	124.7
PO^{43-}	1.2 – 2.0	1.2 – 2.0	1.5
HCO_3^-	21.5 – 25.8	21.5 – 25.8	21.5

Table 2: Preparation of solution A and B. The following substances are dissolved in 500 ml of high-purity, sterile-filtered water each

Substance in solution A	**Weight [m = g]**	**Molar mass [M = g*mol^{-1}]**	**Concentration [mmol*0,5 l^{-1}]**
Sodium chloride (NaCl)	6.826	58.44	116.8
Potassium chloride (KCl)	0.216	74.55	2.9
Calcium chloride ($CaCl_{2)}$	0.166	110.98	1.5
Magnesium chloride ($MgCl_2$)*	0.095	95.21	1
Sodium bicarbonate ($NaHCO_3$)	1.806	84.01	21.5

* weight as magnesium chloride hexahydrate ($MgCl_2$*$6H_2O$) (M=203.3 g•mol^{-1}; m = 0.2033g)

Substances in Solution B	**Weight [m = g]**	**Molar mass [M = g*mol^{-1}]**	**Concentration [mmol*0,5 l^{-1}]**
Disodium hydrogen phosphate (Na_2HPO_4)	0.176	141.96	1.2
Sodium dihydrogen phosphate (NaH_2PO_4)*	0.036	119.98	0.3

* weight as sodium dihydrogen phosphate monohydrate (NaH_2PO_4*H_2O) (M=137.99 g•mol^{-1}; m = 0.0414g)

3.2 (Neuro-)steroid Analysis with GC-MS

Manuscript No. 2 comprehensively evaluates the results of the GC-EI-MS experiments conducted within this project and provides further theoretical background on the investigated derivatization procedures. Summarized, there was no derivatization technique that provided acceptable results to meet the requirements of the project for further GC-MS method development. Therefore, supercritical fluid chromatography coupled to mass spectrometry was alternatively explored.

Within the performed derivatization experiments, optimum results were obtained by either MSTFA with addition of trimethyliodosilane (TMIS) or methoximes (MO) or mixed derivatives of MO and MSTFA with the addition of alkaline catalyst imidazole (TMSIM) referred to as MO-TMSIM. It was not possible to obtain more than 65% of the derivatives with only one peak as desired. A detailed overview of the outcome of the different derivatization procedures is provided in Table 3. Structural formulas of all steroids (underivatized) are available in section 10.1. Due to the polyfunctionality of the selected steroids many of them yield multiple isomeric derivatives by these different procedures which becomes particularly noticeable within the high-resolution chromatographic conditions required within this project. As mentioned, Manuscript No. 2 already presents the main results. Therefore, the following section provides supporting data, only.

Table 3: Investigated steroids, overview on their functional groups and number of derivatives obtained

Analyte and Abbreviation	IUPAC	number of oxo-groups (C-Position)	number of hydroxy-groups (C-Position)	number of double bonds (C-Position or configuration)	No. of derivatives				
					MSTFA	TMIS	TMSIM	MO	MO - TMSIM
(Pro-)gestagens									
Pregnenolone (Preg)	3β-Hydroxypregn-5-en-20-one	1 (20)	1 (3β)	1 (5)	1	3	1	1	1
16α-Hydroxypregnenolone (16α-OH-Preg)	3β,16α-Dihydroxypregn-5-en-20-one	1 (20)	2 (3β, 16α)	1 (5)	1	1	1	1	1
17α-Hydroxypregnenolone (17α-OH-Preg)	3β,17α-Dihydroxypregn-5-en-20-one	1 (20)	2 (3β, 17α)	1 (5)	5	2	1	1	1
Progesterone (P)	Pregn-4-ene-3,20-dione	2 (3, 20)	-	1 (4)	2	3	1	1	2
17α-Hydroxyprogesterone (17α-OH-P)	17α-Hydroxypregn-4-en-3,20-dione	2 (3, 20)	1 (17α)	1 (4)	4	1	3	1	1
16α-Hydroxyprogesterone (16α-OH-P)	16α-Hydroxypregn-4-en-3,20-dione	2 (3, 20)	1 (16α)	1 (4)	3	1	3	3	2
5α-Dihydroprogesterone (5α-DHP)	5α-Pregnan-3,20-dione	2 (3, 20)	-	- (5α)	2	3	2	2	2
5β-Dihydroprogesterone (5β-DHP)	5β-Pregnan-3,20-dione	2 (3, 20)	-	- (5β)	2	3	2	1	1
3α-5α-Tetrahydroprogesterone (3α-5α-THP)	3α-Hydroxy-5α-pregnan-20-one	1 (20)	1 (3α)	- (5α)	1	3	1	1	1
3β-5α-Tetrahydroprogesterone (3β-5α-THP)	3β-Hydroxy-5α-pregnan-20-one	1 (20)	1 (3β)	- (5α)	1	3	1	1	1
3α-5β-Tetrahydroprogesterone (3α-5β-THP)	3α-Hydroxy-5β-pregnan-20-one	1 (20)	1 (3α)	- (5β)	1	2	1	1	1
17α-Hydroxypregnanolone	3α,17α-Dihydroxy-5β-pregnan-20-one	1 (20)	2 (3α, 17α)	- (5β)	6	1	1	1	1
5β-Pregnan-3α, 20α-diol	5β-Pregnan-3α,20α-diol	-	2 (3α, 20α)	- (5β)	1	1	1	1	1
5β-Pregnan-3α, 20β-diol	5β-Pregnan-3α,20β-diol	-	2 (3α, 20β)	- (5β)	1	1	1	1	1
Androgens									
Testosterone	17β-Hydroxyandrost-4-en-3-one	1 (3)	1 (17β)	1 (4)	2	1	2	2	2
5α-Dihydrotestosterone (5α-DHT)	17β-Hydroxy-5α-androstan-3-one	1 (3)	1 (17β)	- (5β)	2	1	3	2	2
Androstenediol	Androst-5-ene-3β,17β-diol	-	2 (3β, 17β)	1 (5)	1	1	1	1	1
Dehydropeiandrosterone (DHEA)	3β-Hydroxyandrost-5-en-17-one	1 (17)	1 (3β)	1 (5)	1	1	2	1	1
16α-Hydroxy-Dehydroepiandrosterone (16α-Hydroxy-DHEA)	3β,16α-Dihydroxyandrost-5-en-17-one	1 (17)	2 (3β, 16α)	1 (5)	1	1	1	1	2
7α-Hydroxy-Dehydroepiandrosterone (16α-Hydroxy-DHEA)	3β,7α-Dihydroxyandrost-5-en-17-one	1 (17)	2 (3β, 7α)	1 (5)	1	2	1	1	1
7β-Hydroxy-Dehydroepiandrosterone (16α-Hydroxy-DHEA)	3β,7β-Dihydroxyandrost-5-en-17-one	1 (17)	2 (3β, 7β)	1 (5)	1	1	1	1	2

Table 3 continued

Analyte and Abbreviation	IUPAC	number of oxo-groups (C-Position)	number of hydroxy-groups (C-Position)	number of double bonds (C-Position or configuration)	No. of derivatives				
					MSTFA	TMIS	TMSIM	MO	MO - TMSIM
7α-Hydroxy-17β-Dihydro-Dehydroepiandrosterone (7α-Hydroxy-17β-Dihydro-DHEA)	Androst-5-ene-3β,7α,17β-triol	-	3 (3β, 7α, 17β)	1 (5)	1	3	1	1	1
7β-Hydroxy-17β-Dihydro-Dehydroepiandrosterone (7β-Hydroxy-17β-Dihydro-DHEA)	Androst-5-ene-3β,7β,17β-triol	-	3 (3β, 7β, 17β)	1 (5)	1	1	1	1	1
Androstenedione	Androst-4-ene-3,17-dione	2 (3, 17)	-	1 (5)	3	1	1	2	2
5α-Androstanedione	5α-Androstan-3,17-dione	2 (3, 17)	-	1 (4)	2	1	3	2	2
5β-Androstanedione	5β-Androstan-3,17-dione	2 (3, 17)	-	- (5α)	3	2	3	1	1
Androsterone	3α-Hydroxy-5α-androstan-17-one	2 (3, 17)	1 (3α)	- (5β)	1	1	1	1	1
Epiandrosterone	3β-Hydroxy-5α-androstan-17-one	1 (17)	1 (3β)	- (5α)	1	1	1	1	1
Etiocholanolone	3α-Hydroxy-5β-androstan-17-one	1 (17)	1 (3α)	- (5α)	1	1	2	1	1
Corticoids									
11-Desoxycortisol	17α,21-Dihydroxypregn-4-ene-3,20-dione	2 (3, 20)	2 (17α, 21)	1 (4)	3	2	4	1	2
Hydrocortisone (Cortisol)	11β,17α,21-Trihydroxypregn-4-ene-3,20-dione	2 (3, 20)	3 (11β, 17α, 21)	1 (4)	2	2	1	1	2
Cortisone	17α,21-Dihydroxypregn-4-ene-3,11,20-trione	3 (3, 11, 20)	2 (17α, 21)	1 (4)	3	2	2	3	2
11-Desoxycorticosterone	21-Hydroxypregn-4-ene-3,20-dione	2 (3, 20)	1 (21)	1 (4)	4	1	2	3	1
Corticosterone	11β,21-Dihydroxypregn-4-en-3,20-dione	2 (3, 20)	2 (11β, 21)	1 (4)	3	1	1	2	2
5a-Dihydrodeoxycorticosterone (5α-DHDOC)	21-Hydroxy-5a-pregnan-3,20-dione	2 (3, 20)	1 (21)	- (5α)	5	1	3	5	2
5b-Dihydrodeoxycorticosterone (5β-DHDOC)	21-Hydroxy-5b-pregnan-3,20-dione	2 (3, 20)	1 (21)	- (5β)	6	1	2	1	1
3α,5β-Tetrahydrodeoxycorticosterone (3α,5β-THDOC)	3a,21-Dihydroxy-5b-pregnan-20-one	1 (20)	2 (3α, 21)	- (5β)	2	1	2	3	1
3α,5α-Tetrahydrodeoxycorticosterone (3α,5α-THDOC)	3a,21-Dihydroxy-5a-pregnan-20-one	1 (20)	2 (3α, 21)	- (5α)	2	1	2	5	1
Estrogens									
17α-Estradiol	Estra-1,3,5(10)-trien-3,17α-diol	-	2 (3 phenolic, 17α)	aromatic (1, 3, 5)	1	1	1	1	1
17β-Estradiol	Estra-1,3,5(10)-trien-3,17β-diol	-	2 (3 phenolic, 17β)	aromatic (1, 3, 5)	1	1	1	1	1
Estrone	3-Hydroxyestra-1,3,5(10)-triene-17-one	1 (17)	1 (3 phenolic)	aromatic (1, 3, 5)	1	1	1	2	1
Total with one peak only					**20**	**27**	**24**	**28**	**27**

3.2.1 Silylation

The trimethylsilyl (TMS) group is the most popular group utilized for silylation. Therefore, a variety of trimethylsilylating agents with different properties has been developed. One of the most important silylating agents, N-methyl-N-(trimethylsilyl)-trifluoracetamide (MSTFA), was already introduced by M. Donike [86] in the 1960s. It is volatile and can be also used as injection solvent, which is incredibly useful within sample preparation procedure. Addition of different catalyst results in derivatization of different groups of polyfunctional steroids [84]. Shortly summarized, as described in Manuscript No. 2 the selection of the silylating agents that were investigated [21, 22, 176] based on the following assumptions:

- MSTFA only: derivatization of hydroxyl groups only, with no derivatization of sterically hindered hydroxy groups,
- MSTFA with alkaline catalyst imidazole (TMSIM): derivatization of hydroxy groups only, particularly promoted for sterically hindered hydroxy groups without stimulating formation of enol-ethers,
- MSTFA with addition of trimethyliodosilane (TMIS): promoted for quantitative derivatization of hydroxy groups and also oxo groups by enhancement of silylation power of MSTFA by catalyzing enolization (except of position 11 due to steric hindered access by the methyl groups at C18 and C19).

Considering the results presented in Table 3, it can be stated that the previous hypotheses cannot be fully confirmed.

First, the assumptions indicate that MSTFA only and TMSIM do only derivatize hydroxy groups. This cannot be confirmed as also steroids without hydroxy groups such as the (pro-)gestagens, progesterone (P) and 5α- and 5β-dihydroprogesterone (5α- and 5β-DHP) with oxo-groups in C3 and C20, and androgens, androstenedione, and 5α- and 5β-androstanedione with oxo groups in C3 and C17 (Figure 11) were derivatized, which can only be result of prior enolization of oxo groups. For example, Figure 12 displays the outcome for progesterone with only oxo-groups in position C3 and C20. Formation of TMS-ether(s) is possible as 2-/or 3-enol, 17-enol Z-/or E-enantiomer, or 21-enol. Derivatized with MSTFA only, P shows peaks for underivatized analyte as expected, but also two mono-TMS-derivatives. These might be one mono-TMS-ether of C3 and C20 each, or TMS derivatives formed of isomeric enols of either C3 or C20 position. 5α- and 5β-DHP, androstenedione and 5α- and 5β-androstanedione also resulted in non-derivatized analyte or mono-derivative(s) with MSTFA only. Formation of TMS-ethers for the androgens is possible as 2-/or 3-enol, or 16-enol. Derivatization of P (Figure 12) and androstenedione with TMSIM resulted in no underivatized analyte but a single mono-derivative. This initially may support the assumption that formation of enol-ethers is at least fewer promoted by derivatization with MSTFA and alkaline catalyst imidazole. However, regarding TMSIM outcome of 5α- and 5β-DHP as well

as 5α- and 5β-androstanedione this cannot be confirmed either. There, TMSIM derivatization resulted in two (5α- and 5β-DHP) up to three (5β-androstanedione) noticeable mono-TMS peaks or even two mono- and one bis-TMS-derivative (5α-androstanedione). However, also here it is not clear if enolization occurs at C3 and/or C20 for (pro-)gestagens. Over all, both derivatization procedures unfortunately resulted in mixtures of underivatized, mono-, bis-, tris-, etc. derivatives for many of the selected steroids (Table 3).

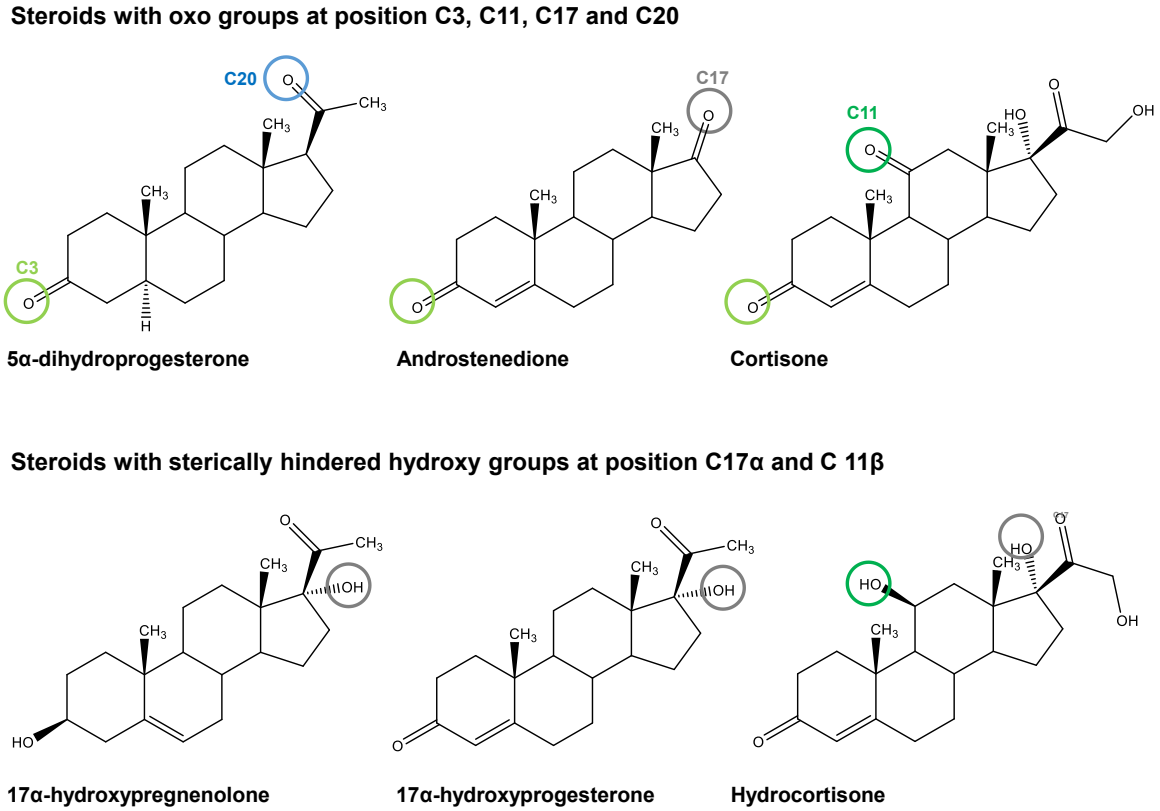


Figure 11: Structural formula of examples of steroids with oxo groups in position C3, C11, C17 and C20 and steroids with sterically hindered hydroxy groups in position C11β, C17α

Secondly, TMSIM was highlighted to particularly derivatize sterically hindered hydroxy groups. Within the selected steroid panel this refers several steroids (Table 3) with tertiary hydroxy groups in 17α-position and the hydroxy group at position C11ß, which is sterically hindered by the methyl groups at C18 and C19. Overall, derivatization outcomes in this study do not allow for confirmation of this hypothesis without further structure elucidation experiments. For example, all (pro-)gestagenes with tertiary hydroxy groups, namely 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 17α-hydroxypregnanolone (Figure 11) resulted in bis-TMS-derivatives. For 17α-hydroxypregnenolone and 17α-hydroxypregnanolone these might be bis-TMS ethers of hydroxy groups in position C 3 and 17, but also enols of Position C20 as outlined before. At least for 17α-hydroxyprogesterone formation of a bis-TMS derivative is only possible with formation enol-formation at position C3 and/or C20. Corticoids with a tertiary hydroxy-group in 17α-position and without an additionally hydroxy group at C11 are 11-desoxycortisol

and cortisone. Both predominantly resulted in incomplete derivatized tris-derivatives. Two of the assorted steroids (Table 3) with a hydroxy group in C11β position resulted in their quantitative derivatized pentakis-TMS-derivative (hydrocortisone) or tetrakis-TMS-derivative (corticosterone). Nevertheless, hydrocortisone predominantly resulted in a tetrakis-TMS-derivative. This might be due to incomplete derivatization of the sterically hindered tertiary hydroxy group C17α or the hydroxy group at C11β, or due to incomplete derivatization of oxo groups at C3 or C 20. As silylation power of TMSIM is rather low and therefore more suitable for hydroxy groups, the latter assumption seems to be more relevant. This assumption can also be underlined with the observation, that all the steroids with oxo groups only (P, 5α- and 5β-DHP, androstenedione and 5α- and 5β-androstanedione) resulted in mono-derivatives and therefore incomplete derivatization as mentioned before.

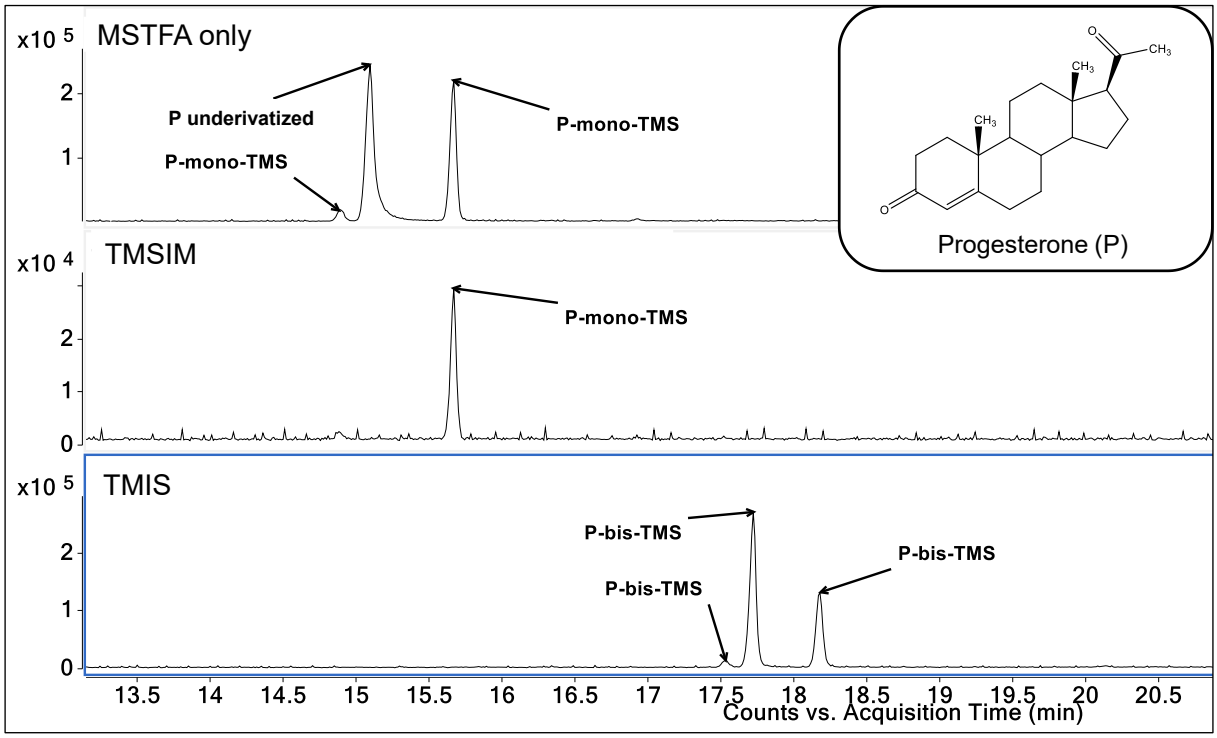


Figure 12: Chromatograms of progesterone derivatized with MSTFA only, TMSIM and TMIS

Thirdly, TMIS is assumed to quantitatively derivatize all hydroxy and oxo groups. This can be confirmed for all the analytes with oxo groups only, P, 5α- and 5β-DHP, androstenedione and 5α- and 5β-androstanedione. For example, derivatization of P with TMIS resulted in detection of three (out of six possible) bis-TMS-derivatives, confirming quantitative derivatization of oxo-groups but also showing enolization isomers as shown in Figure 12. 5α- and 5β-DHP, androstenedione and 5α- and 5β-androstanedione also show quantitative derivatization to bis-TMS-derivatives, with three peaks for the (pro-)gestagens and mainly one peak for androgens. This implicates that enolization at C20 may occur more abundantly than in C3 which both groups had in common. Quantitative derivatization for all steroids with (partly tertiary) hydroxy

groups only can also be confirmed, as steroids with two aliphatic hydroxy groups (5β-pregnane-3α,20α-diol, 5β-pregnan-3α,20β-diol, androstenediol) ore a phenolic and aliphatic hydroxy group (17α-estradiol, 17β-estradiol) resulted in a single TMS-bis derivative each and steroids with three hydroxy groups (7α-hydroxy-17β-dihydro-DHEA, 7β-hydroxy-17β-dihydro-DHEA) in a single tris-TMS-derivative each as expected. Further, all other steroids (Table 4) with oxo and hydroxy groups resulted in quantitatively derivatized TMS-ethers. This also includes steroids with a oxo group in C11 (cortisone) or hydroxy group in position C11β (hydrocortisone, corticosterone), which are described to be more difficult to access for TMS-ether formation due to steric hindrance by the methyl groups at C18 and C19 [84]. Over all, all investigated steroids (Table 3) were quantitively derivatized, but in many cases this procedure was accompanied by enolization resulting in multiple derivatives.
To sum up, for detailed outcomes of all derivatization variations further investigations would be necessary to unravel the particular positions of derivatization and the position and extent of enolizations. However, those experiments were not part of this work anymore, as no derivatization technique provided acceptable results to meet the requirements of the project for further GC-MS method development and investigations.

Considering mass spectra, ionization with EI resulted in characteristic and reproducible fragmentation patterns that are helpful in structure elucidation und substance identification with many reference EI spectra are available in spectra libraries [177].
There are some general types of fragmentation of TMS derivates that give high abundances and where continuously observable within the experiments and are shown in Figure 13:

- loss of a methyl group $[M-CH_3]^+$, $[M-15]^+$
- loss of trimethylsilanol (TMSOH) $[M\text{-}TMSOH]^{\bullet+}$, $[M-90]^{\bullet+}$, especially observed in polyhydroxy steroids or vicinal TMS-groups
- concerted or sequential loss of a methyl and trimethylsilanol group $[M-TMSOH-CH_3]^+$, $[M-105]^+$
- ion at m/z 147 $[(CH_3)_3SiOSi(CH_3)_2]^+$ “TMS-O-DMS” due to migration of TMS group residues in steroids with more than one TMS-group, particularly in vicinal structures
- ion at m/z 129, $[C_3H_4OTMS]^+$ or $[M\text{-}C_3H_4OTMS]^+$, $[M-129]^+$ by A-ring or D-Ring fragmentation, particularly abundant in steroids with 3-hydroxy-5-ene-groups or steroids with TMS-derivatized 3- or 17-hydroxy functions
- ions at m/z 73 $[(CH_3)_3)Si]^+$ and m/z 75 $[(CH_3)_2)SiOH]^+$ for the TMS ethers themselves

Despite of this, various further fragmentations, such as ring losses and fragmentations, side chain cleavage or different group migrations or rearrangements are known and reviewed literature with very detailed information on fragmentation mechanisms which are available elsewhere [16, 21, 178-182].

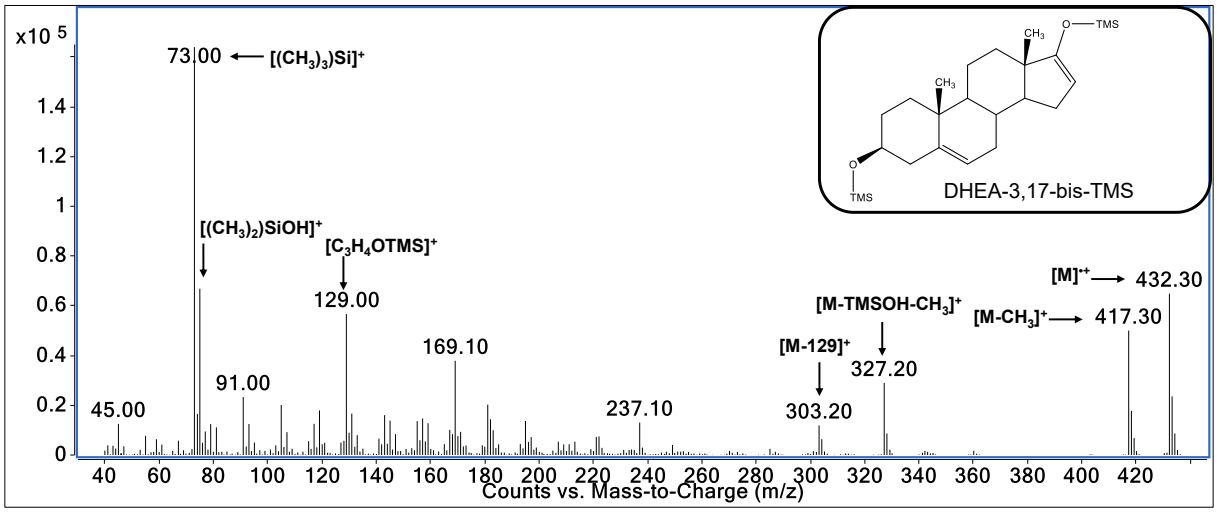


Figure 13:Typical mass spectrum of TMS derivatized steroids, exemplified by DHEA-3,17-bis-TMS

3.2.2 Oximes and Mixed Derivatives

As already mentioned, one major drawback of this technique is the formation of syn (Z) – and anti (E)-isomers that may separate in high resolution conditions. However, although steric hindrance in steroids' backbone strongly influences the share, this is still observable within the assorted steroid setting as exemplary shown in Figure 14.

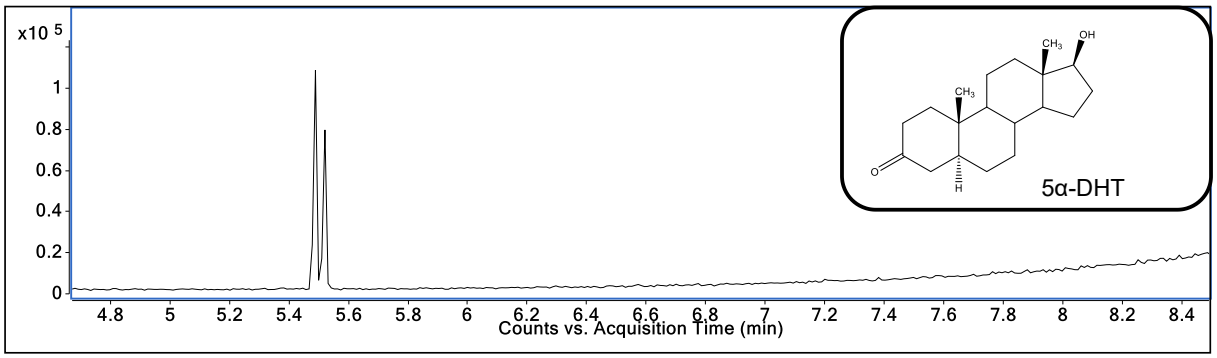


Figure 14: Chromatogram of 5α-dihydrotestosterone (5α-DHT) syn- and anti-isomers

Like silyl ethers, MO and mixed MO-TMSIM derivatives also show characteristic fragmentation patterns. Within the selected steroid panel, the following fragmentations of MO were therefore observable with high abundance as exemplified for DHEA in Figure 16:

- loss of a methyl group $[M-CH_3]^+$, $[M-15]^+$
- loss of water $[M-H_2O]^+$, $[M-18]^+$
- loss of the whole MO-group $[M\text{-}OCH_3]^+$, $[M-31]^+$

A comprehensive study on fragmentations of oximes of different steroids that were also included within this study with oxo groups at C3, C17 and C20 was published by Griffiths et al. [183] (Figure 15). Although steroids were ionized with electrospray and fragmentation was

induced by collision-induced dissociation (CID) within this study, many of these fragmentation patterns of ring dissociation and side chain cleavages also occur in EI.

In conclusion, mass spectra of MO-TMSIM show characteristic fragments of both oximation and silylation derivatization reaction as described before. An example is displayed in Figure 16.

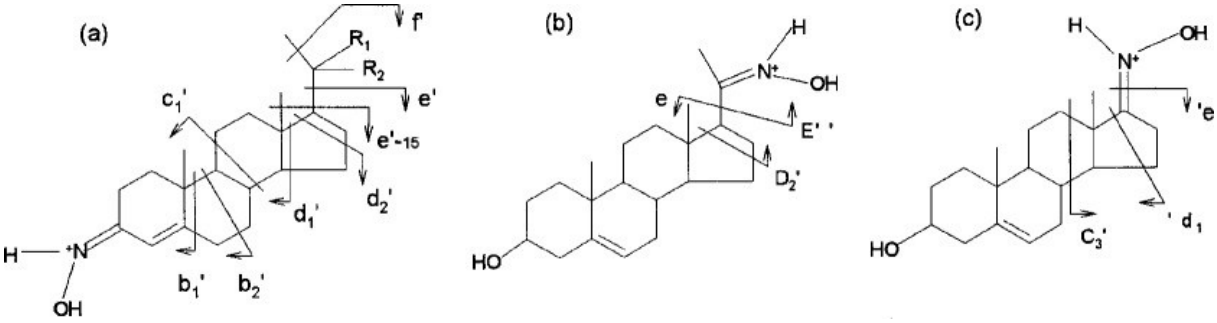


Figure 15: LC-ESI-MS/MS fragmentation of protonated steroid oximes. (a) 3-oxo-4-ene-steroid oximes, for 3,20-bisoximes R1=R2=NOH, for 20-hydroxy steroids R1=H, R2=OH; (b) 20-oxosteroid oximes; (c) 17-oxosteroid oximes according to [183]

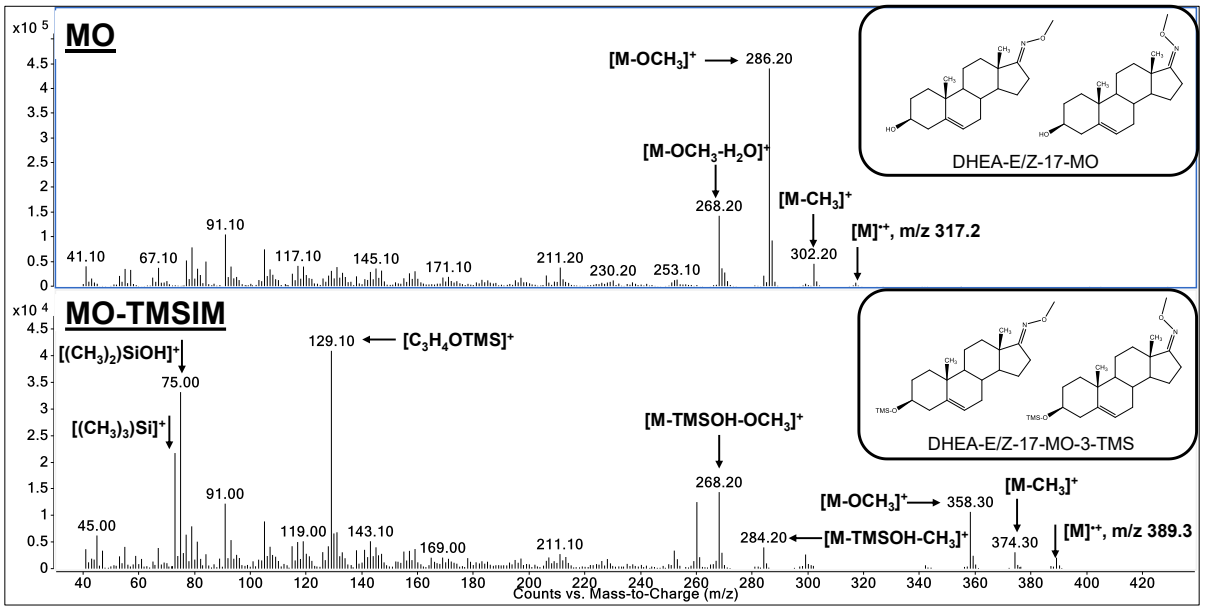


Figure 16: Typical mass spectrum of MO and MO-TMS derivatized steroids, exemplified by DHEA-E/Z-17-MO (upper) and DHEA-E/Z-17-MO-3-TMS (lower)

3.3 Analytical Supercritical Fluid Chromatography (SFC)

3.3.1 Development of a New SFC-ESI-MS/MS Method within this Project

Figure 17 presents the workflow that was used for method development within this project which was planned as described in section 2.4.9.

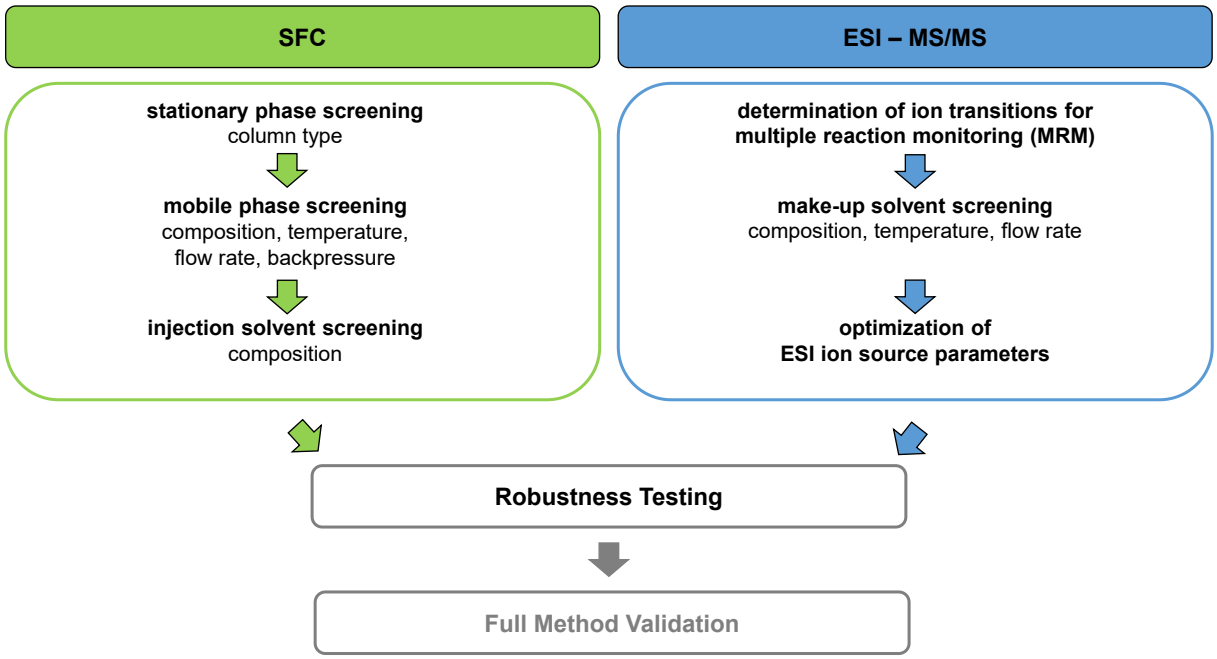


Figure 17: Workflow for method development of the SFC-ESI-MS/MS method

The method development aimed to establish a comprehensive method for the determination of 51 selected steroids and ended up in three SFC-ESI-MS/MS methods with excellent separation for the difficult task of endogenous steroid profiling. A short overview is presented in Table 4, whereas the detailed information are outlined in Manuscript No. 2.

.

Table 4: Overview of developed SFC-MS/MS methods

	Steroids I	**Steroids II**	**Steroid sulfates**
No. of Analytes	32 steroids	9 steroids	10 steroid sulfates
Column	2 serially coupled Agilent Zorbax RxSil (3.0x100 mm, 1.8µm)	Waters Torus Diol (3.0x150 mm, 1.7µm)	Waters BEH 2-Ethylpyridin (2-EP) (3.0 x 100 mm, 1.7µm)
Modifier	isopropanol (gradient mode)	methanol (gradient mode)	$MeOH/H_2O$ (95/5 v/v) and 40 mM NH_4FA (gradient mode)
Make-up	$MeOH/H_2O$ (95/2.5 v/v) and 1 mM NH_4F	$MeOH/H_2O/FA$ (95/2.5/0.1 v/v/v) and 1 mM NH_4F	$MeOH/H_2O/FA$ (95/2.5/0.1 v/v/v) and 5 mM NH_4FA
Make-up flow rate	0.150 $mL*min^{-1}$	0.150 $mL*min^{-1}$	0.150 $mL*min^{-1}$

3.3.2 Optimization of Mass Spectrometric Conditions within this Project

As first stage of method development prior to SFC optimization process suitable ion transitions were evaluated with Agilent Optimizer Software by direct injection using an Agilent HPLC Infinity II UHPLC system coupled to an Agilent 6495 triple quadrupole mass spectrometer with ESI ion source. All steroids were ionized in ESI+ or ESI- mode and at least two reasonable diagnostic transitions (one qualifier and one or more quantifiers) were selected for each steroid for multiple reaction monitoring. As known to be common in steroid analysis not only the quasi molecular ions $[M-H]^-$ and $[M+H]^+$ but also fragments of water losses $[M+H-H_2O]^+$ or $[M+H-2H_2O]^+$ with higher intensity were selected as precursors in ESI+ mode.

As mentioned, SFC-MS(/MS) offers the unique possibility to support ionization utilizing a make-up solvent different to modifier solvent used in chromatography. Based on common ESI knowledge, analysis was initially performed with MeOH/water/FA (97.4/2.5/0.1 v/v/v) and NH_4FA (5mM) as make up with a flow rate of 0.250 $mLmin^{-1}$ which was sufficient during chromatographic method development.

However, ionization efficiency of steroids is found rather low except for those containing an α,β-unsaturated oxo function (e.g. testosterone, progesterone) and worthful to be further optimized.

Following the chromatographic development, a systematic investigation of make-up composition and flow rate, and steroid ionization in connection to SFC with the different ionization techniques ESI, APCI and APPI was carried out, followed by ion source parameter optimization which was assisted by Agilent Mass Hunter Source Optimizer. Manuscript No. 3 reports the evaluation of this data in detail.

Overall, for the majority of analytes ESI was superior to the other techniques and therefore the by far best compromise to cover all assorted steroids for this project. Figure 18 displays the increase of peak areas after ion source parameter optimization compared to initially applied ion source parameters during method development of exemplary steroids of final method steroids I. Basically, method steroids I included 32 steroids (Table-S 1), separated on two serially coupled Agilent Zorbax RxSil (3.0x100 mm, 1.8 µm) with modifier isopropanol in gradient mode, with a make-up composition consisting of $MeOH/H_2O$ (95/2.5 v/v) and 1 mM NH_4F as additive, with a make-up flow rate of 0.150 $mL•min^{-1}$ (Table 4, page 33). Ion source parameters that were part of optimization procedure included sheath gas temperature and flow, drying gas temperature and flow, nebulizer pressure, capillary voltage, nozzle voltage, high pressure funnel / low pressure funnel voltage. Compared with ion source parameters previous to optimization, the obtained data show a slight increase for 3-oxo-4-ene steroids (testosterone, progesterone, androstenedione) which already showed highly abundant ESI+ quasi molecular ions $[M+H]^+$ before. Medium to very high increase was achieved for hydroxylated or phenolic steroids with fragments of water losses $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$ or ESI- quasi molecular ions $[M-H]^-$ which had shown very low (e.g. androstenediol, 5α-DHT) or

medium abundances before. Especially the latter ones massively benefitted from optimization procedure and underline the high value of ionization optimization.

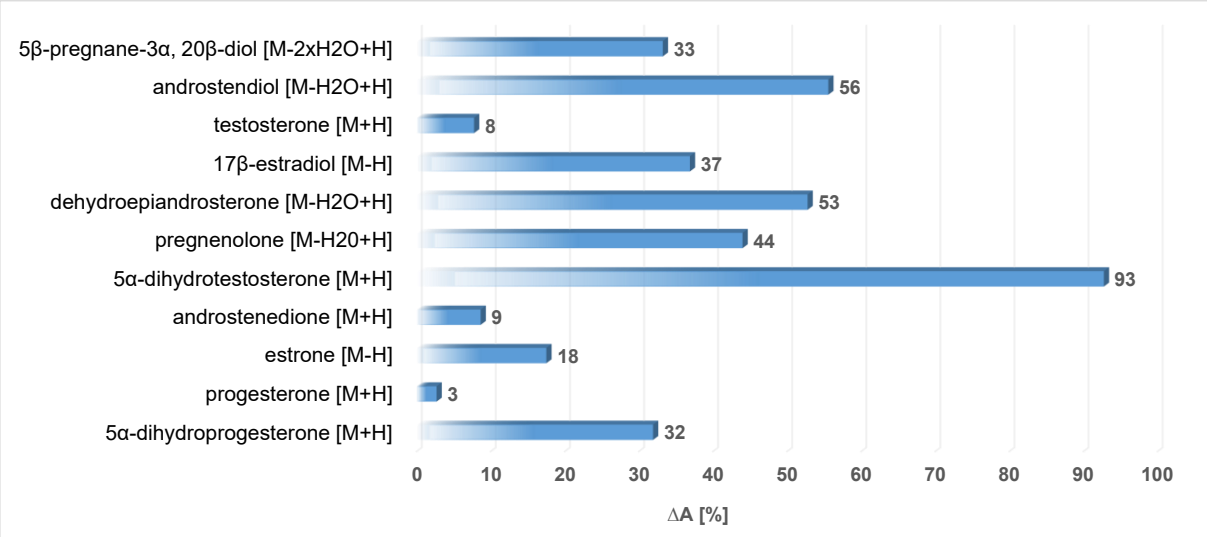


Figure 18: Increase of peak area ΔA [%] after ion source parameter optimization compared to initially applied ion source parameters during method development of exemplary steroids of final method steroids I (method details available at Table-S 3)

3.3.3 Robustness of the Developed Methods

Within this project, the OVAT strategy was applied for initial evaluation of robustness, as at this stage in-house laboratory performance in research and development (R&D) environment is intended. However, in case of transfer to routine use in other laboratories, further investigations may be required.

Robustness testing of the three developed SFC methods was conducted by Tanja Naumann within her bachelor thesis [79].

As described in 2.4.8 SFC instrumentation allows the adjustment of the method parameters modifier concentration, flow rate, backpressure, and column temperature. Since modifier concentration (programmed as gradient mode) no longer changes within the finalized methods, robustness testing was performed with the remaining parameters flow rate, backpressure and column temperature to which a variation of +/-10% was applied.

The assumption of constant modifier concentration mainly bases on the use of pure solvent isopropanol and methanol for methods steroids I and II. However, modifier of method steroid sulfates consists of MeOH/H_2O (95/5 v/v) and 40 mM NH_4FA additive added (Table 4, page 33). Fluctuations in the mixture were not part of the investigations, as preparation of modifier mixture is assumed to be accurately performed by appropriately trained lab personnel. Further, the pumping system may cause fluctuations in gradient composition and pressure control. As the instrumentation underlies comprehensive periodical maintenance, combined with continuous monitoring of pump system pressure within running methods, the instrumentation is assumed to constantly perform and was not further investigated within robustness testing.

The chromatographic robustness was evaluated by means of the relative standard deviation (RSD) of the retention times (RT) for all steroids (contained in the respective method Steroids I, II and steroid sulfates (Table-S 1). The retention times were compared with the mean RT obtained by measurements at the original backpressure, temperature, and flow rate for each method to identify the most relevant chromatographic parameters for robustness considerations. The obtained results from this evaluation are displayed in Figure 19.

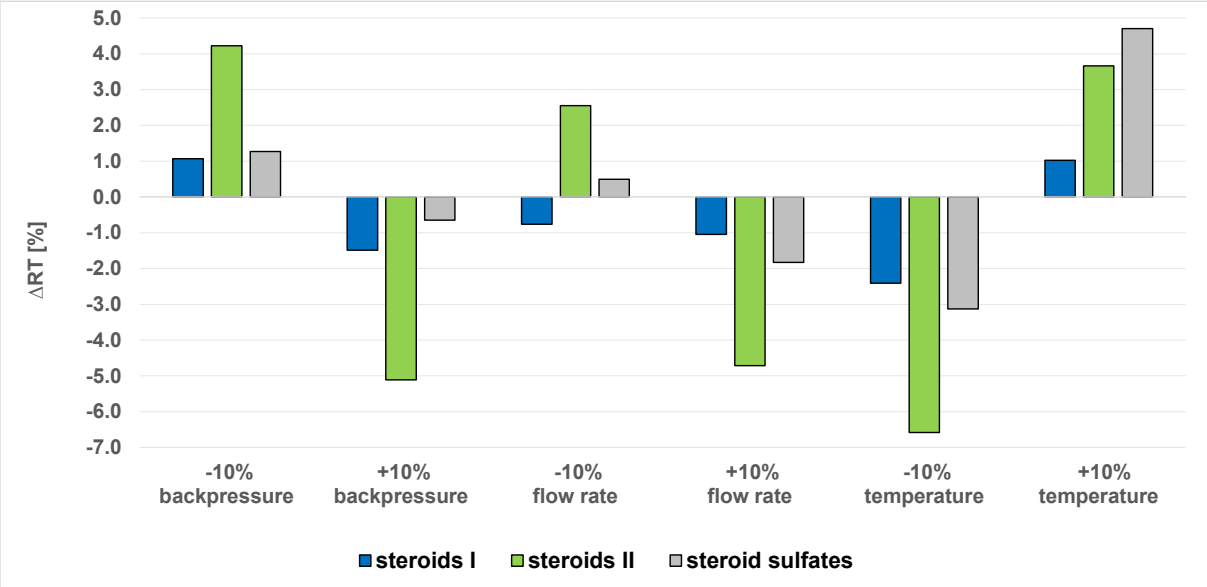


Figure 19: Percentage shift ΔRT [%] in the retention time of the changed method parameters backpressure, flow rate and temperature compared to the original method parameters for all steroids contained in the respective method Steroids I, II and steroid sulfates (Table-S 1), (n=4)

Within the chosen range for robustness testing the following conclusions on retention time shifts can be generalized for all three methods: lowering of backpressure and flow rate but elevation of temperature lead to increasing retention times and vice versa. On top of that, the influence caused by changes in flow rate is smaller than by back pressure and temperature. This is explainable by immediate impact of backpressure and temperature on the mobile phase density whereas the flow rate only indirectly influences the density as outlined in 2.4.3.

A closer look to the dataset further reveals decrease in devations in RT values of the individual measurements to the mean RT of the original parameters with increasing RT and run time of the method (data and illustrations available in [79]). More detailed, in all methods RT from early eluting analytes are more affected by changes in method parameters than later eluting analytes, as displayed in Figure 20. This is also well in accordance with earlier considerations that the initial addition of small amounts of modifier to $scCO_2$ in the beginning of the gradient programm (in normal phase mode) highly impacts supercritical fluid physicochemical properties and stationary phase behaviour, whereas this efect lowers with increasing amount of modifier (2.4.3 and 2.4.4).

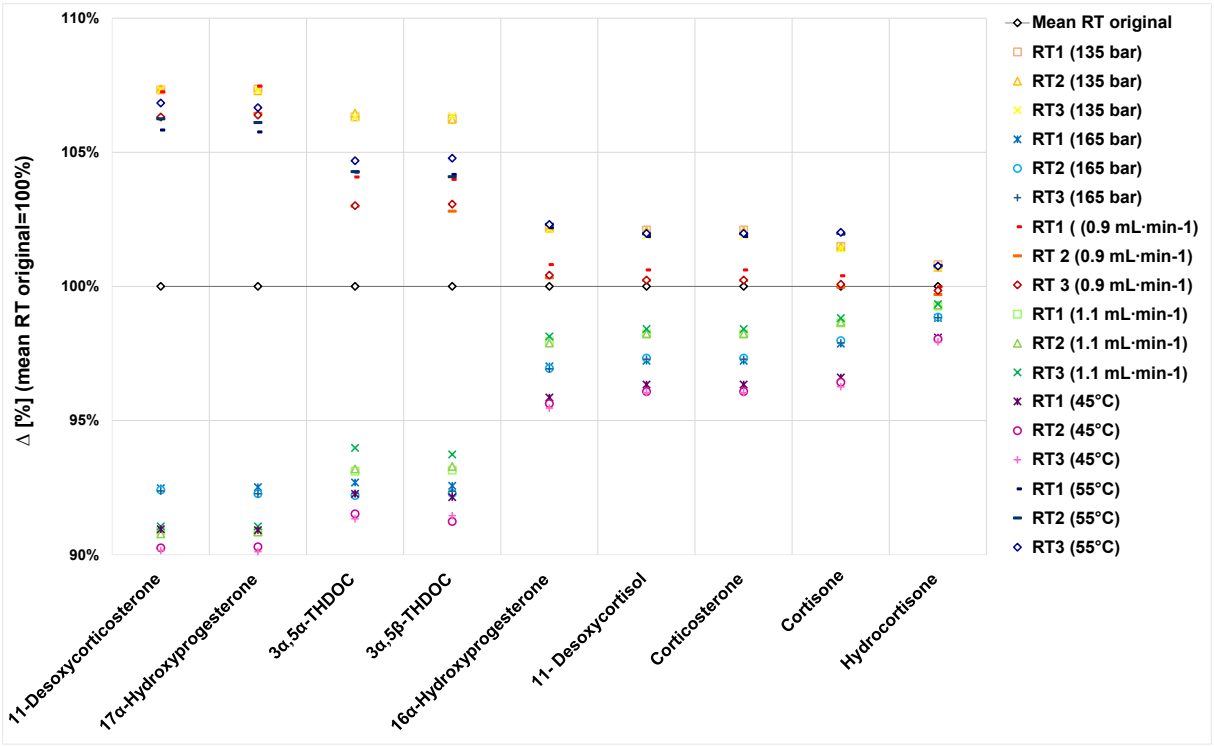


Figure 20: Deviation of retention times of individual measurements from mean retention time recorded with original chromatographic parameters for all steroids included in method steroids II (Table 4,Table-S 1), reprinted from ref. [79]

In summary, the most critical parameter for all three methods is variation of temperature, particularly lowering of the temperature. Method steroids II (Table 4, page 33) turned out to be a little more sensitive to parameter changes than the other ones. However, all three developed methods were proven to be robust within the selected range of method parameters.

4 Project Highlights, Conclusions and Future Perspectives

Neurosteroids are a special and fascinating group of steroids, on which research has been increasingly intensified within the last years. However, until today still only few data are available on reference levels of NS in human CSF, which is the only accessible matrix in living human beings that most likely reflects the situation in the brain and has therefore become a noteworthy interesting specimen for analysis. To the authors knowledge Manuscript No. 1 was the first publication that systematically reviews the published literature of the last twenty years with focus on determined NS levels in human CSF for all major steroid classes, including (pro-)gestagens, androgens, corticoids, estrogens, and steroid conjugates. Within these 20 years, only eighty-one relevant publications were available under this focus. The reported levels of all NS show very high variations, not only in patients, but also in the control groups. This may be a result of challenging analysis or high individual variability of the participants steroidome. Clinical study design and choice of participants must be undertaken very carefully due to different endogenous and exogenous factors that may alter NS levels. Beyond that, CSF is a very rare specimen, which is hardly available from larger collectives. As a result, there is a huge lack of satisfactory baseline data. Without this, so far obtained data should be interpreted with caution and seem certainly not to be suitable for transfer to routine clinical utilization yet.

Another main reason for the lack of data is the truly demanding analysis of NS in human CSF for different reasons, such as ultra-trace levels of NS, low amounts of available CSF and high structural similarities of endogenous NS. Therefore, a comprehensive method, highly selective and sensitive for a large range of concentrations for different NS in one aliquot is desired.

The development of such a suitable analytical method that meets the previously explained expectations is presented in Manuscript No. 2.

Initially, for method development and evaluation, a large set of 51 endogenous steroids (Table-S 1) was selected, based on literature research of NS already determined in CSF and expanded with steroids covering major pathways of biosynthesis and metabolism (Manuscript No. 1). The selection includes several configurational isomers that inevitably require chromatographic separation for quantification.

As described in Manuscript No. 1, although CSF has supposedly only few components, they are manifold and should be considered as possible interfering factors in analytical method development. Therefore, within this project the appropriate surrogate matrix aCSF was developed as described in 3.3.1 for method development and challenging validation of endogenous substances that necessitates analyte free matrix.

Steroid profiling is predominantly performed with gas chromatography (GC) coupled to (tandem) mass spectrometry (MS(/MS) as the method of choice, but also multiple immunoassays or high performance liquid chromatography (HPLC)-MS/MS were utilized – each method offering different advantages and disadvantages, which were intensively

discussed in Manuscript No. 1. An alternative chromatographic approach is SFC, as it has shown to be very powerful in separation efficiency, which was barely explored for endogenous steroids until now (2.4.7).

Comparing those well-established methods, as a first step, the gold standard GC–MS(/MS) seemed most suitable and was therefore further investigated. However, as comprehensively outlined in Manuscript No. 2 and section 3.2 this method did not meet the expectations for the intended analytical task. In short, sulfate quantification requires cleavage prior to derivatization, which is subject to natural fluctuations. At least 35% of the steroids formed more than one derivative, no matter which derivatization procedure was used. Further, coelution of side and main peaks of different endogenous steroids with same *m/z* occurred. Taken together, this hampers proper quantification.

Therefore, SFC-MS/MS was investigated as orthogonal and promising technique for this project. This included careful investigation of chromatographic and mass spectrometric parameters and final robustness testing. Initially, the chromatographic method development intended to determine all 51 steroids in a single run. However, as described in Manuscript No. 2, also SFC has shown limitations. With view on quantification, chromatographic behavior and time expenses, it was more meaningful to set up three methods with different column selectivity. All three SFC-MS/MS methods offer excellent separation with the time expenses of the three methods together nearly equal to one run in GC–MS.

Method development further required careful investigation of mass spectrometric parameters, as ionization efficiency of steroids is found rather low and worthful to be further optimized. Within this project, SFC was splitlessly hyphenated to MS-instrumentation (2.4.8). Manuscript No. 3 and section 3.3.2 evaluate the hyphenation of SFC with MS by APCI, APPI, and ESI exemplified by steroids. As SFC-MS(/MS) offers the unique possibility to support ionization utilizing a make-up solvent different to modifier solvent for chromatography, a systematic investigation of make-up composition and flow rate and its influence on steroid ionization in connection to SFC with the different ionization techniques was carried out, followed by ion source parameter optimization. Within these investigations, ESI was superior to the other techniques for the majority of analytes and therefore the by far best compromise to cover all assorted steroids for this project. Interestingly, each of the three SFC - methods requires a different make-up solvent and different ion source parameters for optimum results.

Finally, all three methods were proven to be robust within the selected range of method parameters as described in section 3.3.3.

Taken together, SFC-MS/MS has shown its potential as alternative to GC-MS in steroid profiling. To the authors knowledge this was the first time that such a high number of endogenous steroids was demonstrated to be analyzed with SFC. Hyphenation with MS/MS and ESI ionization was proven to achieve optimum results within this multi-analyte design. The developed method further scores with facilitated sample preparation and reduced time

expenses compared to GC, as no time-consuming derivatization step and/or sulfate cleavage is required. Moreover, as sulfate cleavage is not required, different sulfates of the same steroid, e.g. 3- and 17-estradiol sulfate and 3,17-estradiol-disulfate that may initiate different physiological effects can be distinguished. The instrumentation used in this project (2.4.8) is equipped with two column compartments, containing three column ovens, which enables high-throughput analysis of the samples as all methods can be easily operated in one sequence. Transfer to other systems with less column ovens is still possible by the quickly done exchange of different columns and due to short equilibration times in SFC. Finally, this method helps to advance environmentally sustainable chemical research and development for the future.

Within this project, the development of a robust SFC-ESI-MS/MS method for determination of NS has been completed. In a subsequent project, validation for matrix CSF is ongoing with the herein developed aCSF as surrogate matrix. In the future, the SFC-ESI-MS/MS method will offer the opportunity to quantify a large set of NS within the rare specimen human CSF and to distinguish between several isomers that may have different physiological effects. NS, that were not determined so far or included in previous investigations, may be additionally detected. As mentioned, until now the determined NS show very high variations, already in the control groups. By providing a reliable tool for challenging NS analysis, variations due to analytical issues can be eliminated. The method may contribute to determination of satisfactory baseline data and clarification of individual variability of the human brain steroidome. However, this also requires careful study design with appropriate collective sizes. Prospectively, expansion of this method to other body fluids like blood seems also very promising and may serve for various purposes, such as correlation of NS in CSF with blood levels which seems not sufficient for meaningful correlation yet, but is highly desirable for possible simplification of diagnosis in disease states in the future.

5 Publications

5.1 Manuscript No. 1

“Determination of Neurosteroids in Human Cerebrospinal Fluid in the 21st Century: a Review”

Juliane Teubel, Maria Kristina Parr
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Determination of steroid hormones synthesized by the human body plays an important role in various fields of endocrinology. Neurosteroids (NS) are steroids that are synthesized in the central (CNS) or peripheral nervous system (PNS), which is not only a source but also a target for neurosteroids. They are discussed as possible biomarkers in various cognitive disorders and research interest in this topic raises continuously. Nevertheless, knowledge on functions and metabolism is still limited, although the concept of neurosteroids was already introduced in the 1980s.

Until today, the analysis of neurosteroids is truly challenging. The only accessible matrix for investigations of brain metabolism in living human beings is cerebrospinal fluid (CSF), which therefore becomes a very interesting specimen for analysis. However, neurosteroid concentrations are expected to be very low and the available amount of cerebrospinal fluid is limited. Further, high structural similarities of endogenous neurosteroids challenges analysis. Therefore, comprehensive methods, highly selective and sensitive for a large range of concentrations for different steroids in one aliquot are required and under continuous development.

Although research has been increasingly intensified, still only few data are available on reference levels of neurosteroids in human cerebrospinal fluid. In this review, published literature of the last twenty years, as a period with relatively contemporary analytical methods, was systematically investigated. Considerations on human cerebrospinal fluid, different analytical approaches, and available data on levels of in analogy to periphery

conceivable occurring neurosteroids, including (pro-) gestagens, androgens, corticoids, estrogens, and steroid conjugates, and their interpretation are intensively discussed.

5.2 Manuscript No. 2

"Methods in Endogenous Steroid Profiling – a Comparison of Gas Chromatography Mass Spectrometry (GC–MS) with Supercritical Fluid Chromatography Tandem Mass Spectrometry (SFC-MS/MS)"

Juliane Teubel, Bernhard Wüst, Carola G. Schipke, Oliver Peters, Maria Kristina Parr
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In various fields of endocrinology, the determination of steroid hormones synthesised by the human body plays an important role. Research on central neurosteroids has been intensified within the last years, as they are discussed as biomarkers for various cognitive disorders. Their concentrations in cerebrospinal fluid (CSF) are considered to be regulated independently from peripheral fluids. For that reason, the challenging matrix CSF becomes a very interesting specimen for analysis. Concentrations are expected to be very low and available amount of CSF is limited. Thus, a comprehensive method for very sensitive quantification of a set of analytes as large as possible in one analytical aliquot is desired.
However, high structural similarities of the selected panel of 51 steroids and steroid sulfates, including numerous isomers, challenges achievement of chromatographic selectivity.
Since decades the analysis of endogenous steroids in various body fluids is mainly performed by gas chromatography (GC) coupled to (tandem) mass spectrometry (MS(/MS)). Due to the structure of the steroids of interest, derivatisation is performed to meet the analytical requirements for GC–MS(/MS).Most of the laboratories use a two-step derivatisation in multi-analyte assays that was already published in the 1980s. However, for some steroids this elaborate procedure yields multiple isomeric derivatives. Thus, some laboratories utilize (ultra) high performance liquid chromatography ((U)HPLC)–MS/MS as alternative but, even UHPLC is not able to separate some of the isomeric pairs. Supercritical fluid chromatography (SFC) as an orthogonal separation technique to GC and (U)HPLC may help to overcome these issues.
Within this project the two most promising methods for endogenous steroid profiling were investigated and compared: the "gold standard" GC–MS and the orthogonal separation technique SFC-MS/MS. Different derivatisation procedures for gas chromatographic detection were explored and the formation of multiple derivatives described and confirmed. Taken together, none of the investigated derivatisation procedures provided acceptable results for further method development to meet the requirements of this project. SFC with its unique selectivity was able to overcome these issues and to distinguish all selected steroids, including

(pro-)gestagens, androgens, corticoids, estrogens, and steroid sulfates with appropriate selectivity. Valued especially in the separation of enantiomeric analytes, SFC has shown its potential as alternative to GC. The successful separation of 51 steroids and steroid sulfates on different columns is presented to demonstrate the potential of SFC in endogenous steroid profiling.

5.3 Manuscript No. 3

"Splitless hyphenation of SFC with MS by APCI, APPI, and ESI exemplified by steroids as model compounds"

Maria Kristina Parr, Bernhard Wüst, Juliane Teubel, Jan Felix Joseph

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A systematic evaluation of splitless hyphenation of supercritical fluid chromatography (SFC) with mass spectrometry (MS) was performed using different techniques for ambient pressure ionization. Interfaces commonly known from HPLC-MS/MS, i.e. electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photo ionization (APPI), were tested for their suitability in SFC-MS/MS. A triple quadrupole MS was used for data evaluation in a targeted multi-analyte design using endogenous steroids as model compounds. Individual optimization of the ionization parameters was performed in multi-dimensional design for best support of ionization in all three techniques. A post-column make-up was used to avoid analyte precipitation in the transfer capillary but also to support ionization independently from mobile phase composition. Buffer choice and concentration as well as temperature were found crucial in ESI and APCI. Best results for the multi-analyte method were obtained in both techniques using ammonium fluoride as make-up buffer. Instead of buffer solutions different organic solvents were used as dopants in APPI to support ionization. The mobile phase constituent isopropanol was already found to support ionization in APPI, however, for many analytes the addition of toluene resulted in superior results in terms of intensity. Comparing the optimized methods in terms of limit of detection (LOD), limit of quantification (LOQ), and sensitivity (slope of calibration curve) ESI was the best choice for the multiple analyte design. Only a few analytes resulted in a different optimum ionization, if focused on separately. In terms of linear dynamic range, APCI and APPI proved superior to ESI, where calibration over the whole range of concentrations (from LOD up to 5000 pg $*$ μL^{-1}) required quadratic regression.

6 Summary

Neurosteroids (NS) are a special of group of endogenous steroids, which are synthesized in the central (CNS) or peripheral nervous system (PNS), which is not only a source but also a target for NS. As they are discussed as potential biomarkers for various cognitive disorders, research interest in this topic remarkably increases. In this context CSF becomes an interesting specimen for NS analysis, as it is the only accessible matrix which most likely reflects brain metabolism in living human beings.

Analysis of NS is truly challenging for different reasons, such as low amounts of available matrix, ultra-trace-levels of expected steroids, and high structural similarity of endogenous steroids. Therefore, the aim of this work was the development of a comprehensive and sensitive method for profiling of an as large as possible set of NS in one aliquot of human CSF. For method development and evaluation, a set of fifty-one steroids, including (pro-)gestagens, androgens, corticoids, estrogens and steroid sulfates was selected, based on comprehensive literature research of neurosteroids already determined in human CSF and expanded with steroids covering major peripheral pathways of biosynthesis and metabolism.

Profiling of endogenous steroids is well-established with gas chromatography (tandem) mass spectrometry (GC-MS(/MS)), (ultra) high performance liquid chromatography (tandem) mass spectrometry ((U)HPLC-MS(/MS)), or immunoassays (IA) – each method offering different advantages or disadvantages. As IA, HPLC and GC based methods were reported for ascending number of analytes that were detected, GC-MS was initially selected within this project regarding the selected large set of analytes.

Analysis with GC-MS requires derivatization of analytes, therefore several well-established derivatization procedures were examined. Overall, none of the derivatization procedures provided acceptable results for further method development and analyte quantification, due to formation of multiple derivatives of some steroids and occurrence of coelution of side and main peaks of different endogenous steroids with same *m/z*.

As alternative approach supercritical fluid chromatography (tandem) mass spectrometry (SFC-MS(/MS)) presents new and promising application opportunities and currently experiences a comeback to analytical science was therefore investigated for steroid profiling. Full method development included investigation of chromatographic and mass spectrometric parameters, followed by robustness testing. One of the unique features of SFC instrumentation is the opportunity to use a different make-up solution for ionization than mobile phase. Considering the rather poor ionization capacity of steroids, the different ion sources ESI, APCI, and APPI were tested for their suitability in combination with different make-ups.

Finally, with its unique selectivity SFC-MS/MS was able and to overcome the derivatization issues of GC-MS and to distinguish between all selected steroids. Method development resulted in three optimized methods on different columns with ESI ionization that offer excellent separation in reasonable time and can be easily operated in one sequence as high-throughput

approach. SFC-MS/MS further scores with facilitated sample preparation and reduced time expenses compared to GC-MS, as no time-consuming derivatization step and/or sulfate cleavage is required. In addition, the development of SFC-based methods also contributes to environmentally sustainable chemical research for the future.

Altogether, considering the requirements of this project, the high potential of SFC-MS/MS in endogenous steroid profiling and its superiority towards GC-MS was demonstrated. In a subsequent project, the method will be validated with the CSF matrix. Prospectively, expansion of this method to other body fluids like blood seems also very promising. In the future, the SFC-ESI-MS/MS method will offer the opportunity to quantify a large set of NS within the rare specimen human CSF and may contribute to clarification of knowledge on metabolism and functions of NS.

7 Zusammenfassung

Neurosteroide (NS) sind eine spezielle Gruppe endogener Steroide, die im zentralen (ZNS) oder peripheren Nervensystem (PNS) synthetisiert werden, welches nicht nur eine Quelle, sondern auch ein Target für NS ist. Das Forschungsinteresse an diesem Thema nimmt bemerkenswert zu, da NS als potenzielle Biomarker für verschiedene kognitive Störungen diskutiert werden. In diesem Zusammenhang wird Cerebrospinalflüssigkeit (CSF) zu einem interessanten Material für die NS-Analyse, da sie die einzige zugängliche Matrix ist, welche am wahrscheinlichsten den Hirnstoffwechsel des lebenden Menschen widerspiegelt.

Die Analyse von NS ist aus verschiedenen Gründen anspruchsvoll, wie z.B. durch geringe Mengen an verfügbarer Matrix, sehr niedrige Konzentrationen der erwarteten endogenen Steroide und deren hoher struktureller Ähnlichkeit. Ziel dieser Arbeit war es, eine umfassende und empfindliche Methode zur Detektion einer möglichst großen Menge von NS in einem Aliquot menschlicher CSF zu entwickeln.

Für die Methodenentwicklung und -evaluierung wurden einundfünfzig Steroide, einschließlich (Pro-)Gestagenen, Androgenen, Kortikoiden, Östrogenen und Steroidsulfaten ausgewählt. Die Auswahl erfolgt basierend auf einer umfassenden Literaturrecherche zu bereits in CSF bestimmter NS und wurde um Steroide, welche wichtige Biosynthese- und Stoffwechselwege in der Peripherie abdecken, erweitert.

Das Screening endogener Steroide mittels Gaschromatographie-(Tandem)-Massenspektrometrie (GC-MS(/MS)), (Ultra-) Hochleistungs-Flüssigkeitschromatographie-(Tandem)-Massenspektrometrie ((U)HPLC-MS(/MS)) oder Immunoassays (IA) ist gut etabliert, wobei jede Methode unterschiedliche Vor- oder Nachteile bietet. Weil IA-, HPLC- und GC-basierte Methoden für eine aufsteigende Anzahl von zu detektierenden Analyten geeignet sind, wurde im Rahmen dieses Projekts zunächst GC-MS im Hinblick auf die ausgewählte große Anzahl von Analyten ausgewählt.

Die Analyse mittels GC-MS erfordert eine Derivatisierung der Analyten. Daher wurden zahlreiche gut etablierte Derivatisierungsverfahren untersucht. Auf Grund der Bildung multipler Derivate einiger Steroide und des Auftretens von Koelution von Neben- und Hauptpeaks verschiedener endogener Steroide mit demselben *m/z*, lieferte insgesamt betrachtet keines der Derivatisierungsverfahren akzeptable Ergebnisse für die weitere Methodenentwicklung und Quantifizierung der Analyten.

Als alternativer Ansatz erfährt die überkritische Flüssigkeitschromatographie-(Tandem)-Massenspektrometrie (SFC-MS(/MS)) derzeit ein Comeback in der analytischen Wissenschaft und bietet neue und vielversprechende Anwendungsmöglichkeiten und wurde daher für die Entwicklung einer Steroid-Screening-Methode eingehend untersucht. Die vollständige Methodenentwicklung umfasste die Untersuchung chromatographischer und massenspektrometrischer Parameter, gefolgt von Robustheitstests. Eines der einzigartigen Merkmale der SFC-Instrumentierung ist die Möglichkeit, eine von der mobilen Phase

abweichende Make-up-Lösung für die Ionisierung zu verwenden. In Anbetracht der eher geringen Ionisierungseffizienz von Steroiden wurden die verschiedenen Ionenquellen ESI, APCI und APPI auf ihre Eignung in Kombination mit verschiedenen Make-up-Lösungen getestet.

Die SFC-MS/MS war in der Lage die Derivatisierungsprobleme der GC-MS zu überwinden und mit ihrer einzigartigen Selektivität zwischen allen ausgewählten Steroiden zu unterscheiden. Die Methodenentwicklung führte zu drei optimierten Methoden auf verschiedenen Säulen mit ESI-Ionisation, die eine exzellente Trennung in angemessener Zeit bieten und als Hochdurchsatz-Ansatz leicht in einer Sequenz betrieben werden können. Die SFC-MS/MS punktet darüber hinaus mit einer vereinfachten Probenvorbereitung und einem geringeren Zeitaufwand im Vergleich zur GC-MS, da kein zeitaufwändige Derivatisierungsschritt und/oder Sulfatspaltung erforderlich ist. Darüber hinaus trägt die Entwicklung von SFC-basierten Methoden auch zu einer ökologisch nachhaltigen chemischen Forschung für die Zukunft bei.

Insgesamt konnte unter Berücksichtigung der Anforderungen dieses Projektes das hohe Potential der SFC-MS/MS bei der Analyse endogener Steroide und ihre Überlegenheit gegenüber der GC-MS nachgewiesen werden. In einem sich dieser Arbeit anschließenden Projekt erfolgt die Validierung der Methode mit der Matrix CSF. Perspektivisch erscheint auch die Ausweitung dieser Methode auf andere Körperflüssigkeiten wie Blut sehr vielversprechend. In der Zukunft wird die SFC-ESI-MS/MS-Methode die Möglichkeit bieten, eine große Menge von NS innerhalb des menschlichen CSF zu bestimmen, und könnte somit zur weiteren Aufklärung des Wissens über Metabolismus und Funktionen von NS beitragen.

8 Declaration of Own Contribution

In the following, the author's own contributions to the individual publications, which are the basis for this cumulative work, are presented in detail:

Manuscript No.1:

- Comprehensive literature research on neurosteroid determinations in human cerebrospinal fluid and neurosteroid analysis
- Systematic evaluation of revealed data
- Manuscript preparation in cooperation with the co-authors

Manuscript No. 2:

- Conception and execution of GC-MS experiments
- Conception and execution of full method development in SFC-MS/MS experiments
- Evaluation of the corresponding data in cooperation with the co-authors
- Manuscript preparation in cooperation with the co-authors

Manuscript No 3.:

- Conception and implementation of SFC-MS/MS ESI ion source optimization experiments
- Evaluation of the corresponding data in cooperation with the co-authors
- Manuscript preparation in cooperation with the co-authors

Manuscript No.	Conception	Data collection	Data evaluation	Manuscript preparation
1	100%	100%	100%	90%
2	100%	90%	90%	90%
3	33 %	33 %	33 %	33 %

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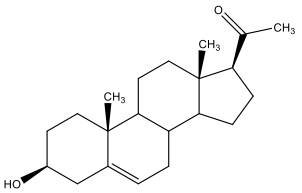
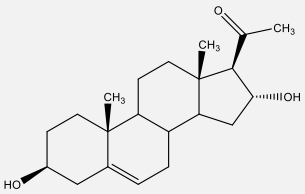
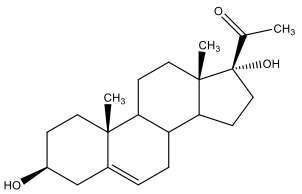
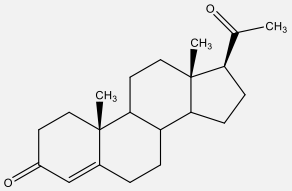
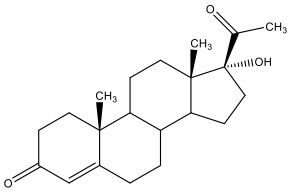
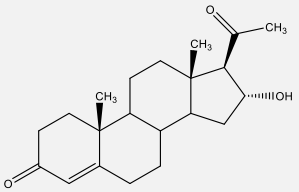
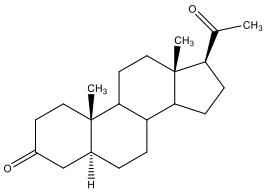
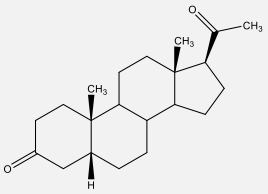
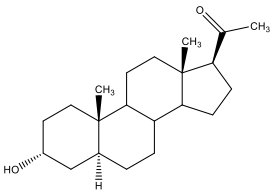
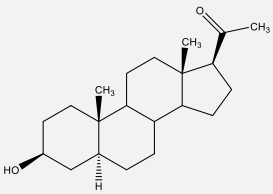
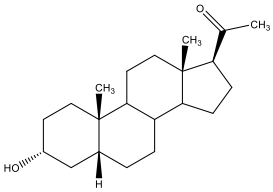
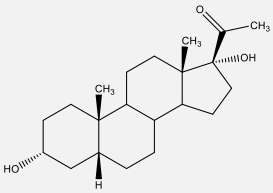
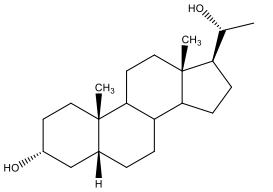
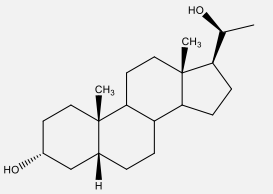
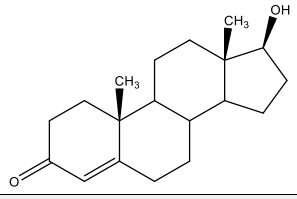
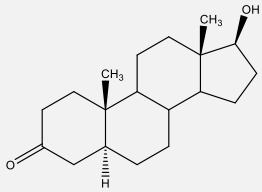
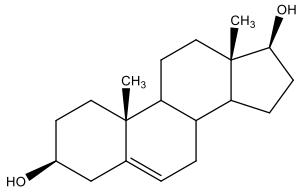
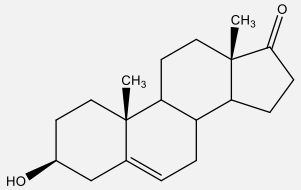
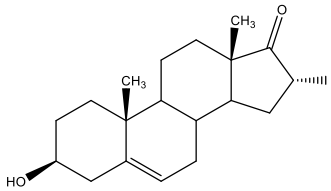
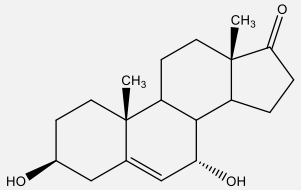
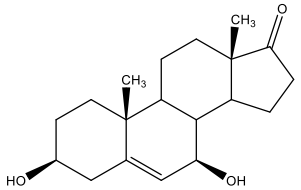
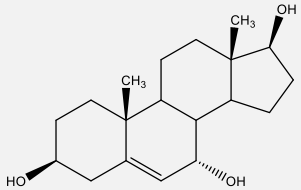
10 Annex

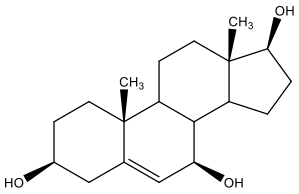
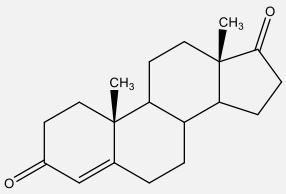
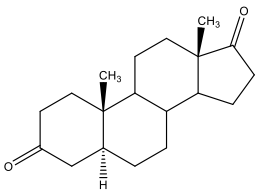
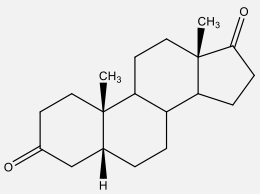
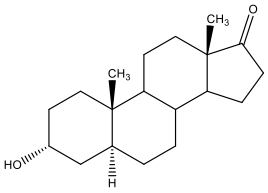
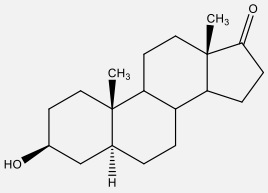
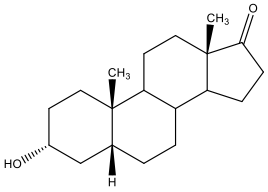
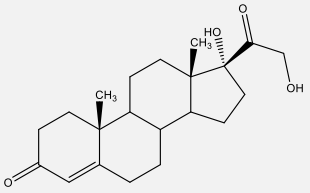
10.1 Structural Formula, Nomenclature, Synonyms and Abbreviations of investigated Steroids and Assignment to final SFC-Method

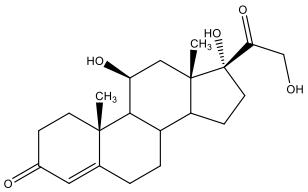
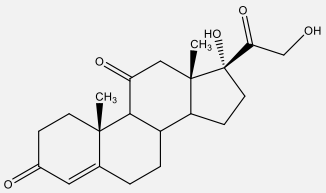
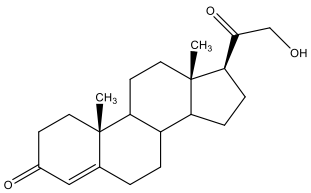
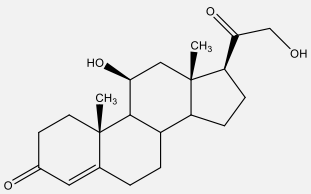
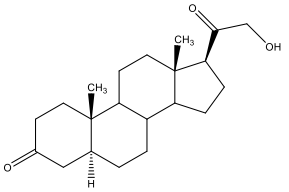
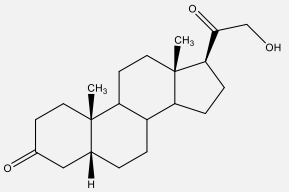
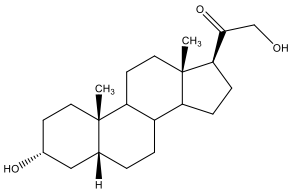
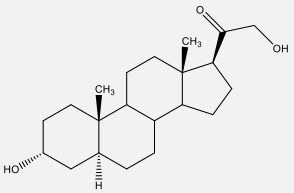
Table-S 1: Structural formula, nomenclature, synonyms and abbreviations of investigated steroids and assignment to final SFC-Method

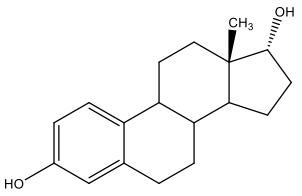
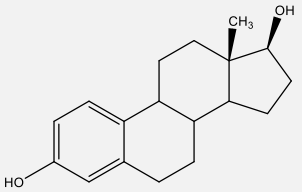
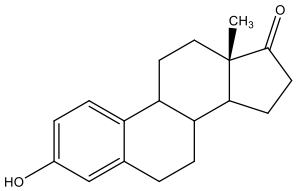
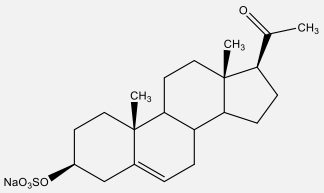
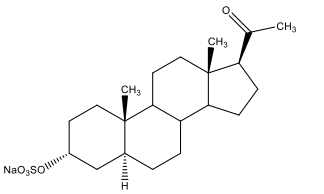
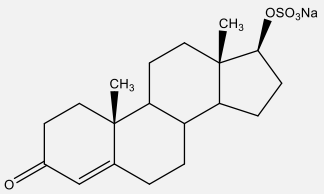
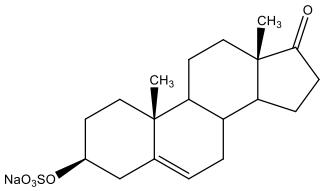
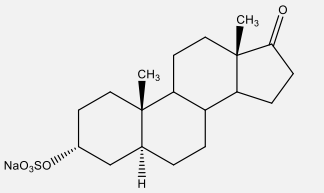
No.	Analyte and Abbreviation IUPAC Nomenclature *Synonyms*	Structural Formula	SFC Method
	(Pro-)gestagens		
1	**Pregnenolone (Preg)** 3β-Hydroxypregn-5-en-20-one		Steroids I
2	**16α-Hydroxypregnenolone (16α-OH-Preg)** 3β,16α-Dihydroxypregn-5-en-20-one		Steroids I
3	**17α-Hydroxypregnenolone (17α-OH-Preg)** 3β,17α-Dihydroxypregn-5-en-20-one		Steroids I
4	**Progesterone (P)** Pregn-4-ene-3,20-dione		Steroids I
5	**17α-Hydroxyprogesterone (17α-OH-Prog)** 17α-Hydroxypregn-4-ene-3,20-dione		Steroids II
6	**16α-Hydroxyprogesterone (16α-OH-Prog)** 16α-Hydroxypregn-4-ene-3,20-dione		Steroids II

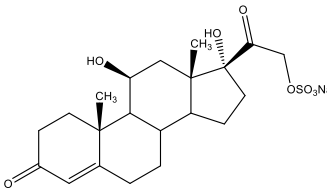
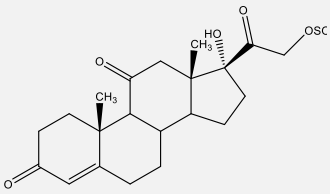
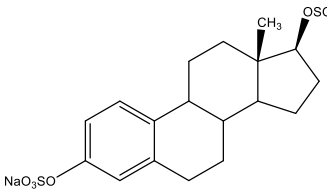
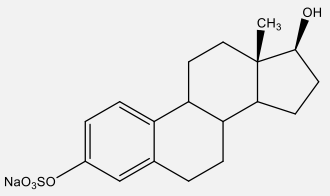
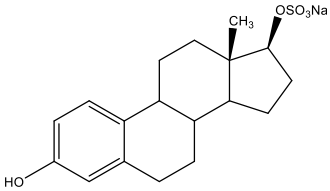
No.	Analyte and Abbreviation IUPAC Nomenclature *Synonyms*	Structural Formula	SFC Method
7	**5α-Dihydroprogesterone (5α-DHP)** 5α-Pregnane-3,20-dione *Allopregnandione*		Steroids I
8	**5β-Dihydroprogesterone (5β-DHP)** 5β-Pregnane-3,20-dione *Pregnandione*		Steroids I
9	**3α,5α-Tetrahydroprogesterone (3α,5α-THP)** 3α-Hydroxy-5α-pregnan-20-one *Allopregnanolone*		Steroids I
10	**3β,5α-Tetrahydroprogesterone (3β,5α-THP)** 3β-Hydroxy-5α-pregnan-20-one *Epiallopregnanolone*		Steroids I
11	**3α,5β-Tetrahydroprogesterone (3α,5β-THP)** 3α-Hydroxy-5β-pregnan-20-one *Pregnanolone*		Steroids I
12	**17α-Hydroxypregnanolone** 3α,17α-Dihydroxy-5β-pregnan-20-one		Steroids I
13	**5β-Pregnane-3α,20α-diol** 5β-Pregnane-3α,20α-diol *Pregnandiol*		Steroids I
14	**5β-Pregnane-3α,20β-diol** 5β-Pregnane-3α,20β-diol		Steroids I

No.	Analyte and Abbreviation IUPAC Nomenclature *Synonyms*	Structural Formula	SFC Method
	Androgens		
15	**Testosterone (T)** 17β-Hydroxyandrost-4-en-3-one		Steroids I
16	**5α-Dihydrotestosterone (5α-DHT)** 17β-Hydroxy-5α-androstan-3-one *5α-Dihydrotestosterone*		Steroids I
17	**Androstenediol** Androst-5-ene-3β,17β-diol		Steroids I
18	**Dehydroepiandrosterone (DHEA)** 3β-Hydroxyandrost-5-en-17-one		Steroids I
19	**16α-Hydroxydehydroepiandrosterone (16α-Hydroxy-DHEA)** 3β,16α-Dihydroxyandrost-5-en-17-one		Steroids I
20	**7α-Hydroxydehydroepiandrosterone (7α-Hydroxy-DHEA)** 3β,7α-Dihydroxyandrost-5-en-17-one		Steroids I
21	**7β-Hydroxydehydroepiandrosterone (7β-Hydroxy-DHEA)** 3β,7β-Dihydroxyandrost-5-en-17-one		Steroids I
22	**7α-Hydroxy-17β-dihydro-dehydroepiandrosterone (7α-Hydroxy-17β-dihydro-DHEA)** Androst-5-ene-3β,7α,17β-triol *7α-Hydroxyandrostenediol*		Steroids I

No.	Analyte and Abbreviation IUPAC Nomenclature *Synonyms*	Structural Formula	SFC Method
23	**7β-Hydroxy-17β-dihydro-dehydroepiandrosterone (7β-Hydroxy-17β-dihydro-DHEA)** Androst-5-ene-3β,7β,17β-triol *7β-Hydroxyandrostenediol*		Steroids I
24	**Androstenedione** Androst-4-ene-3,17-dione		Steroids I
25	**5α-Androstanedione** 5α-Androstane-3,17-dione		Steroids I
26	**5β-Androstanedione** 5β-Androstane-3,17-dione		Steroids I
27	**Androsterone** 3α-Hydroxy-5α-androstan-17-one		Steroids I
28	**Epiandrosterone** 3β-Hydroxy-5α-androstan-17-one		Steroids I
29	**Etiocholanolone** 3α-Hydroxy-5β-androstan-17-one *5β-Androsterone*		Steroids I
	Corticoids		
30	**11-Deoxycortisol** 17α,21-Dihydroxypregn-4-ene-3,20-dione *Cortexolone*		Steroids II

No.	Analyte and Abbreviation IUPAC Nomenclature *Synonyms*	Structural Formula	SFC Method
31	**Hydrocortisone** 11β,17α,21-Trihydroxypregn-4-ene-3,20-dione *Cortisol*		Steroids II
32	**Cortisone** 17α,21-Dihydroxypregn-4-ene-3,11,20-trione		Steroids II
33	**11-Deoxycorticosterone** 21-Hydroxypregn-4-ene-3,20-dione *Cortexon*		Steroids II
34	**Corticosterone** 11β,21-Dihydroxypregn-4-ene-3,20-dione		Steroids II
35	**5α-Dihydrodeoxycorticosterone (5α-DHDOC)** 21-Hydroxy-5a-pregnane-3,20-dione *5α-Dihydrodeoxycorticosterone*		Steroids I
36	**5β-Dihydrodeoxycorticosterone (5β-DHDOC)** 21-Hydroxy-5b-pregnane-3,20-dione *5β-Dihydrodeoxycorticosterone*		Steroids I
37	**3α,5β-Tetrahydrodeoxycorticosterone (3α,5β-THDOC)** 3α,21-Dihydroxy-5β-pregnan-20-one *3α,5β-Tetrahydrodeoxycorticosterone*		Steroids II
38	**3α,5α-Tetrahydrodeoxycorticosterone (3α,5α-THDOC)** 3α,21-Dihydroxy-5α-pregnan-20-one *3α,5α-Tetrahydrodeoxycorticosterone*		Steroids II

No.	Analyte and Abbreviation IUPAC Nomenclature *Synonyms*	Structural Formula	SFC Method
	Estrogens		
39	**17α-Estradiol (α-E2)** Estra-1,3,5(10)-triene-3,17α-diol		Steroids I
40	**17β-Estradiol (E2)** Estra-1,3,5(10)-triene-3,17β-diol		Steroids I
41	**Estrone (E1)** 3-Hydroxyestra-1,3,5(10)-trien-17-one		Steroids I
	Steroid Sulfates		
42	**Pregnenolone-3-sulfate (Preg-3-S)** Sodium 20-oxopregn-5-en-3β-yl-sulfate		Steroid Sulfates
43	**3α,5α-Tetrahydroprogesterone-3-sulfate (3α,5α-THP-3-S)** Sodium 20-oxo-5α-pregnan-3α-yl-sulfate		Steroid Sulfates
44	**Testosteron-17-sulfate (T-17-S)** Sodium 3-oxoandrost-4-en-17β-yl-sulfate		Steroid Sulfates
45	**Dehydroepiandrosterone-3-sulfate (DHEA-3-S)** Sodium 17-oxoandrost-5-en-3β-yl-sulfate		Steroid Sulfates
46	**Androsterone-3-sulfate** Sodium 17-oxo-5α-androstan-3α-yl-sulfate		Steroid Sulfates

No.	Analyte and Abbreviation IUPAC Nomenclature *Synonyms*	Structural Formula	SFC Method
47	**Hydrocortisone-21-sulfate** Sodium 11β,17α-dihydroxy-3,20-dioxo-pregn-4-en-21-yl-sulfate		Steroid Sulfates
48	**Cortisone-21-sulfate** Sodium 17α-hydroxy-3,11,20-trioxo-pregn-4-en-21-yl-sulfate		Steroid Sulfates
49	**17β-Estradiol-3,17-disulfate** Disodium Estra-1,3,5(10)-trien-3,17β-yl-disulfate		Steroid Sulfates
50	**17β-Estradiol-3-sulfate** Sodium 17ß-Hydroxy-Estra-1,3,5(10)-trien-3-yl-sulfate		Steroid Sulfates
51	**17β-Estradiol-17-sulfate** Sodium 3-Hydroxy-Estra-1,3,5(10)-trien-17ß-yl-sulfate		Steroid Sulfates

10.2 Materials and Methods

10.2.1 Instrumentation and Methods

Table-S 2: GC-MS instrumentation and methods

GC-MS instrumentation	Agilent 7890A gas chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 5975C mass selective detector (MSD)
Method: TMS derivatives	*Steroids:* • 41 steroids • steroids No. 1-41; assignment according to Table-S 1 *GC parameters:* • column: Agilent HP1-Ultra (17 m × 200 µm × 0.11 µm) • carrier gas: helium, 1 mL*min^{-1}, constant flow • oven temperature program: 183°C, 3°C*min^{-1} to 232°C, 40°C*min^{-1} to 310°C (2 min hold) • injection volume: 2 µL *MS parameters:* • ionization: 70 eV, EI, full scan mode
Method: MO derivatives	*Steroids:* • 41 steroids • steroids No. 1-41; assignment according to Table-S 1 *GC parameters:* • column: Agilent DB-5HT (30 m × 250 µm × 0.1 µm) • carrier gas: helium, 1 mL*min^{-1}, constant flow • oven temperature program: 120°C for 0.5 min, 50°C*min^{-1} to 200°C, then 25°C*min^{-1} to 390°C (5 min hold) • injection volume: 2 µL *MS parameters:* • ionization: 70 eV, EI, full scan mode

Table-S 3: SFC-MS/MS instrumentation and methods

SFC-MS/MS Instrumentation	Agilent 1260 Infinity I analytical SFC system splitlessly coupled to an Agilent 6495 triple quadrupole mass spectrometer using a low dispersion nozzle in the SFC module, make-up solvent was added prior to the ion source (after the backpressure regulator) using an Agilent 1260 isocratic HPLC pump
Method: Steroids I	*Steroids:* • 32 steroids • steroids No. 1-4, 7-29, 35, 36, 39-41; assignment according to Table-S 1 *SFC parameters:* • column: two serially coupled Agilent Zorbax Rx-Sil Rapid Resolution HT (3.0 × 100 mm, 1.8 µm) columns • mobile phase: modifier PrOH (mobile phase B) in $scCO_2$ (mobile phase A) in gradient mode (A:B): 98:2 during 0–1 min, linear gradient to 95:5 at 6 min, 90:10 at 10 min, 87:13 at 12 min, 55:45 at 17 min and 55:45 during17–21 min (re-equilibration) • back pressure: 150 bar • mobile phase flow rate: 1 $mL*min^{-1}$ • column temperature 50°C • injection volume 5 µL using ACN as sample solvent • make-up solvent: MeOH:H_2O (97.5:2.5, v/v) with 1 mM NH_4F • make up flow rate: 0.150 $mL*min^{-1}$ *MS parameters:* • sheath gas temperature: 390°C • sheath gas flow: 12 $*min^{-1}$ • dry gas temperature: 165°C • drying gas flow:19 $L*min^{-1}$ • nebulizer: 25 psi • capillary: ES+: 2500 V/ES-: 2500 V • nozzle ES+: 500 V/ES-: 2000 V • HPRF/LPRF: ES+: 110/80 V/ES-:190/160 V
Method: Steroids I	*Steroids:* • 9 steroids • steroids No. 5, 6, 30-34, 37, 38; assignment according to Table-S 1

	SFC parameters: • column: Waters Acquity UPC^2 Torus Diol (3.0 × 150 mm, 1.7 µm) • mobile phase: modifier MeOH (mobile phase B) in $scCO_2$ (mobile phase A) in gradient mode (A:B): 93:7 during 0–3.5 min, and linear gradient to 70:30 at 10 min • back pressure: 150 bar • mobile phase flow rate: 1 mL*min^{-1} • column temperature 50°C • injection volume 5 µL using ACN as sample solvent • make-up solvent: MeOH:H_2O (97.5:2.5, v/v) with 1 mM NH_4F and 0.1% FA • make-up flow rate: 0.150 mL*min^{-1} *MS parameters:* • sheath gas temperature: 390°C • sheath gas flow: 12 L*min^{-1} • dry gas temperature: 150°C • drying gas flow:17 L*min^{-1} • nebulizer: 35 psi • capillary: ES+: 3000 V/ES-: 3000 V • nozzle ES+: 500 V/ES-: 1300 V • HPRF/LPRF: ES+:110/120 V/ES-:90/160 V
Method: Steroid Sulfates	*Steroids:* • 10 steroid sulfates • steroids No. 42-51; assignment according to Table-S 1 *SFC parameters:* • column: Waters Acquity UPC^2 BEH 2-ethyl-pyridine (2-EP) (3.0 x 100 mm, 1.7 µm) column • mobile phase: modifier MeOH:H_2O (97.5:5, v/v) with additive 40 mM NH_4FA (mobile phase B) in $scCO_2$ (mobile phase A) in gradient mode (A:B): 87.5:12.5 at 0 min, and linear gradient to 82:18 at 6.5 min, 82:18 during 6.5–11 min, 81:19 at 11.9 min, and 60:40 during 11.91–14 min. (re-equilibration) • back pressure: 150 bar • mobile phase flow rate: 1.7 mL*min^{-1} • column temperature: 50°C • injection volume: 5 µL using ACN as sample solvent

	• make-up solvent: MeOH:H_2O (97.5:2.5, v/v) with 5 mM NH_4FA and 0.1% FA • make-up flow rate: 0.150 mL*min^{-1} *MS parameters:* • sheath gas temperature: 390°C • sheath gas flow: 12 L*min^{-1} • dry gas temperature: 180°C • drying gas flow:11 L*min^{-1} • nebulizer: 10 psi • capillary: ES+: 4500 V/ES-: 4500 V • nozzle: ES+: 500 V/ES-: 0 V • HPRF/LPRF: ES+:170/160 V/ES-:210/140 V

Table-S 4: Miscellaneous instruments

LC instrumentation for ion transition determination prior to SFC method development	Agilent 1290 Infinity II UHPLC system coupled to an Agilent 6495 triple quadrupole mass spectrometer with Agilent Optimizer software
Sample concentrator	Techne, Bibby Scientific, Stone, UK
Fresh ultrapure water system	LaboStar 2-DI/UV system (SG Wasseraufbereitung und Regeneration GmbH, Barsbüttel, Germany) equipped with LC-Pak Polisher and a 0.22 µm membrane point-of-use cartridge (Millipak)

Additionally, the usual laboratory glass ware has been utilized together with further conventionally laboratory equipment (e.g. centrifuge, pipettes).

10.2.2 Chemicals, Reagents, and Materials

Table-S 5: Chemicals, reagents, and materials

Item	Manufacturer
all reference steroids (Table-S 1)	Steraloids (Newport, USA) or Sigma–Aldrich (Taufkirchen, Germany)
Acetonitrile (ACN)	VWR Chemicals (Darmstadt, Germany
Ammonium fluoride (NH4F)	Sigma-Aldrich (Taufkirchen, Germany)
Ammonium formate (NH_4FA)	Sigma-Aldrich (Taufkirchen, Germany)
Ammonium iodide (NH_4I)	Sigma-Aldrich (Taufkirchen, Germany)
Calcium chloride	VWR Chemicals (Radnor, USA)
Disodium hydrogen phosphate	VWR Chemicals (Radnor, USA)
Ethanthiol	Sigma-Aldrich (Taufkirchen, Germany)
Ethylacetate	Sigma-Aldrich (Taufkirchen, Germany)
Formic Acid (FA)	Sigma-Aldrich (Taufkirchen, Germany)
Hexane	VWR Chemicals (Darmstadt, Germany)
Imidazole	Merck (Darmstadt, Germany)
Magnesium chloride hexahydrate	VWR Chemicals (Radnor, USA)
Methanol (MeOH)	VWR Chemicals (Darmstadt, Germany)
Methoxyamine hydrochloride	Sigma-Aldrich (Taufkirchen, Germany)
N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA)	KarlBucher (Waldstetten, Germany)
Potassium chloride	VWR Chemicals (Radnor, USA)
Propan-2-ol, isopropanol	VWR Chemicals (Darmstadt, Germany)
Pyridine, water free	VWR Chemicals (Darmstadt, Germany)
Sodium bicarbonate	VWR Chemicals (Radnor, USA)
Sodium chloride	VWR Chemicals (Radnor, USA)
Sodium dihydrogen phosphate monohydrate	VWR Chemicals (Radnor, USA)
t-Butyl-methyl ether (TBME)	Applichem GmbH (Darmstadt, Germany)
Water, fresh ultrapure	obtained from LaboStar 2-DI/UV system (Table-S 4)

Depending on the respective filed of application, all chemicals and reagents were from analytical, LC-MS or GC-MS grade.

10.3 Abbreviations

ACN	acetonitrile
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photo ionization
CO_2	carbon dioxide
CNS	central nerval system
CSF	cerebrospinal fluid
DS	design space
EI	electron ionization
ESI	electrospray ionization
ESI-	negative electrospray ionization
ESI+	positive electrospray ionization
FA	formic acid
FDA	Department of Health and Human Services Food and Drug Administration
GC	gas chromatography
GTFCh	Society of Toxicological and Forensic Chemistry
H_2O	water
HPLC	high performance liquid chromatography
HPRF	high pressure funnel
IA	immunoassay
ICH	International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
LPRF	low pressure funnel
MeOH	methanol
MO	methoxime
MS	mass spectrometry
MS(/MS)	tandem mass spectrometry
MSTFA	N-Methyl-N-(trimethylsilyl)trifluoracetamide
NH_4F	ammonium fluoride
NH_4FA	ammonium formate
NH_4I	ammonium iodide
NAS	neuroactive steroid
NS	neurosteroid(s)
OVAT	one-variable-at-a-time
PNS	peripheral nerval system
QbD	Quality by Design concept
RT	retention time
$scCO_2$	supercritical carbon dioxide
SFC	supercritical fluid chromatography
TMIS	trimethyliodsilane
TMS	trimethylsilyl
TMSIM	trimethylsilyl catalyzed with imidazol
UHPLC	ulltra-high performance liquid chromatography
USP	united states pharmacopoeia

10.4 List of Figures

10.5 List of Tables

Relevant presentations and posters in international conferences

Oral presentations

SFC - MS/MS - A New Way of Steroid Profiling

J. Teubel

King's College London (KCL) – Freie Universität Berlin (FUB) Joint Workshop Initiative, Berlin (2019)

Supercritical Fluid Chromatography: a Powerful Tool for Steroid Profiling

J. Teubel

24th International Symposium on Electro- and Liquid Phase- Separation Techniques (ITP), Gdansk (2017)

Method Development for Determination of Neurosteroids in Cerebrospinal Fluid as Potential Biomarkers of Post-Operative Cognitive Dysfunction using GC-MS within the Focus Area DynAge

J. Teubel, O. Peters, C. Schipke, B.Bert, M. Parr

Deutsche Pharmazeutische Gesellschaft, Tag der Pharmazie, Berlin (2014)

Poster Presentations

Sensitivity Improvement for Steroid Sulfates in ESI-Mass Spectrometry by Ion Source Optimization

J. Teubel, T. Naumann, J.F. Joseph, B. Wüst, M.K. Parr
Deutsche Pharmazeutische Gesellschaft, Tag der Pharmazie, Berlin (2018)

Potential of Supercritical Fluid Chromatography (SFC) in Steroid Profiling

J. Teubel, B. Wüst, M.K. Parr

SFC 2016, Green Chemistry Group, Vienna (2016)

Method development for mass spectrometric detection of neurosteroids in cerebrospinal fluid as potential biomarkers of post-operative cognitive dysfunction

J. Teubel, B. Wüst, C. Schipke, O. Peters, M.K. Parr

Deutsche Pharmazeutische Gesellschaft, Tag der Pharmazie, Berlin (2016)

Mass spectrometric detection of neurosteroids in cerebrospinal fluid as potential biomarkers of post-operative cognitive dysfunction

J. Teubel, B. Wüst, C. Schipke, O. Peters, M.K. Parr

3rd Congress on Steroid Research, Chicago (2015)