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Titel der Masterarbeit / Title of the Master's Thesis

„Development of an alternative method to detect the exogenous origin of testosterone and its metabolites via GC/C/IRMS"

verfasst von / submitted by

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I. Abstract

The use of anabolic androgenic steroids in sports is prohibited, as it would give the athlete an illicit advantage over clean competitors. Nonetheless there are always frauds in professional sports who disregard the anti-doping rules set by the International Olympic Committee and the World Anti-Doping Agency. The detection of those illegal substances is a challenge for the analytical laboratories, that changes continuously.

Especially the abuse of endogenous anabolic androgenic steroids like Testosterone is difficult to determine. Since those substances are produced by the body on its own, their origin has to be confirmed. The only way to distinguish between endogenously produced and exogenous administered Testosterone is the comparison of the carbon isotope ratio of Testosterone and its metabolites, determined by isotope ratio mass spectrometry. A downside of this method is its susceptibility to errors caused by impurities. As steroids are mainly excreted as conjugates combined with a complex urine matrix the sample preparation has to be extensive and thorough.

The current method employed by the anti-doping laboratory in Seibersdorf relies on the enzymatic hydrolysis of steroid glucuronides followed by a chromatographic separation of the free steroids. The collected chromatographic fractions are then analysed by gas chromatography coupled to combustion isotope ratio mass spectrometry.

During this proof-of-concept thesis an alternative method for the sample preparation was developed. The conjugated steroids are first separated by high performance liquid chromatography and fractions of selected glucuronides are collected. Afterwards the glucuronides are deconjugated by acidic hydrolysis. Finally the carbon isotope ratio is determined by isotope ratio mass spectrometry as in the established method.

The developed method delivers results that are comparable those from the routine analysis. Measurements of positive and negative control samples fulfil the criteria set by the world anti-doping agency, therefore the method is fit for purpose.

II. Zusammenfassung

Die Einnahme von anabolen androgenen Steroiden ist im Sport generell nicht erlaubt, es würde dem Athleten einen verbotenen Vorteil gegenüber sauberen Konkurrenten verschaffen. Dennoch kommt es immer wieder zu Betrugsskandalen von Sportlern, die die Anti-Doping Regelungen des Internationalen Olympischen Komitees und der Welt Anti-Doping Agentur missachten. Die Detektion von solchen illegalen Substanzen stellt die entsprechenden Labors immer wieder vor sich ständig ändernde Herausforderungen.

Besonders der Missbrauch von endogenen anabolen androgenen Steroiden, wie zum Beispiel Testosteron, ist schwer bestimmbar. Diese Stoffe werden auch körpereigen gebildet, daher muss zusätzlich die Herkunft bestimmt werden. Die einzige Möglichkeit exogen zugeführtes Testosteron vom endogenen unterscheiden zu können, ist der Vergleich der Verhältnisse stabiler Kohlenstoffisotope von Testosteron und seinen Metaboliten, diese Analyse erfolgt mittels Isotopenverhältnis-Massenspektrometrie. Ein Nachteil dieser Methode ist die Anfälligkeit auf Fehler durch Verunreinigungen. Im Urin, der verwendeten Probenmatrix für Dopingtests, liegen die Steroide als Konjugate vor, zusätzlich noch viele weitere Matrixsubstanzen. Daher muss die Probe vor einer endgültigen Analyse einer umfangreichen und gründlichen Probenvorbereitung unterzogen werden.

Die derzeit im Anti-Doping Labor Seibersdorf angewandte Methode setzt die enzymatische Hydrolyse zur De-konjugation von Steroid-Glucuroniden ein. Anschließend werden die freien Steroide über eine präparative Hochleistungsflüssigchromatographie in einzelne Fraktionen aufgeteilt. Diese werden danach über Chromatographie gekoppelt mit Verbrennungs-Isotopenverhältnis-Massenspektrometrie auf das Kohlenstoffisotopenverhältnis analysiert.

Im Rahmen dieser Machbarkeitsstudie wurde eine alternative Methode für die Probenvorbereitung von Urinproben entwickelt. Dabei werden die Steroid-Konjugate zunächst über präparative Hochleistungsflüssigchromatographie aufgetrennt und ausgewählte Glucuronide in Fraktionen gesammelt. Anschließend erfolgt die Dekonjugation durch saure Hydrolyse der Glucuronide. Letztendlich wird das Kohlenstoffisotopenverhältnis der freien Steroide wie in der Routine mittels Isotopenverhältnis-Massenspektrometrie bestimmt.

Die entwickelte Methode liefert Ergebnisse die vergleichbar sind mit jenen der Routine-Methode. Analyseergebnisse von positiven und negativen Qualitätskontrollproben erfüllen alle entsprechenden Kriterien der Welt Anti-Doping Agentur, daher ist die Methode prinzipiell für den Zweck geeignet.

III. Table of contents:

IV. List of abbreviations

AAS:	anabolic androgenic steroids
EAAS:	endogenous anabolic androgenic steroids
GC/C/IRMS:	gas chromatography coupled to combustion isotope ratio mass spectrometry
HPLC:	high performance liquid chromatography
P450:	Cytochrom P450
HSD:	hydroxysteroid dehydrogenase
DHEA:	Dehydroepiandrosterone (3β-Hydroxyandrost-5-en-17-one)
16EN:	16-Androstenol (5α-Androst-16en-3α-ol)
T:	Testosterone (17β-Hydroxyandrost-4-en-3-one)
PD:	Pregnanediol (5β-pregnane-3α,20α-diol)
11keto:	11-keto-etiocholanolone (3α-Hydroxy-5β-androstane-11,17-dione)
Epi:	Epitestosterone (17α -Hydroxyandrost-4-en-3-one)
DHT:	Dihydrotestosterone (Androstane-17β-ol-3-one)
5αAdiol:	5α-Androstane-3α,17β-diol
5βAdiol:	5β-Androstane-3α,17β-diol
UGT:	Uridine-diphospho-glucuronosyltransferase
AAF:	adverse analytical finding, positive sample
T/E:	Testosterone to Epitestosterone ratio calculated from urinary glucuronides
CIR:	Carbon isotope ratio
$\delta^{13}C$:	delta-value, ratio of $^{13}C/^{12}C$ in ‰
VPDB:	Vienna PeeDee Belemnite
RuBisCO:	Ribulose-bisphosphate-carboxylase/-oxygenase
CAM:	Crassulacean acid metabolism
TC:	target compound
ERC:	endogenous reference compound
WADA:	World Anti-Doping Agency
$\Delta\delta^{13}C$:	difference in $\delta^{13}C$-values between the ERC and TC
SPE:	Solid Phase Extraction
TMS:	Trimethylsilyl-
EI:	Electron Ionisation
CI:	Chemical Ionisation
GC/MS:	Gas chromatography coupled to mass spectrometry
GC/MS^2:	Gas Chromatography coupled to two dimensional mass spectrometry
MSTFA:	N-Methyl-N-(trimethylsilyl)trifluoracetamide
TMSI:	Trimethylsilyl-iodide
QC:	quality control sample, either positive (QCP) or negative (QCN)
OHE:	11β-Hydroxyetiocholanolone
OHA:	11β-Hydroxyandrosterone

V. Acknowledgements

There is an old saying that goes: “You are what you eat.”

That is especially true for the carbon isotope ratios, as they are a direct result of one’s diet. But in a much broader sense, the saying can also mean that certain influences can have an impact on the outcome. Regarding my studies and personal life there are several people who affected me so I want to thank them for the positive influence they had.

First of all I want to thank my family for the support and my colleagues from work for help during the everyday routine, especially my colleague Christian Östreicher for introducing me to the world of IRMS and my boss Günter Gmeiner for giving me the opportunity of this project. I am also grateful to Caroline Emery for questioning some aspects and giving me new ideas and finally to Gunda Köllensperger for supervision of the master thesis.

Then there are a few people who deserve to be mentioned extra as they had a bigger influence on my personal life. I am lucky to have them as my friends and want to thank them from the bottom of my heart.

Katharina Zanitzer, who is always there for me and tries to help me with personal development, while keeping both of us smiling. Evelyn Fülöp, who is an excellent conversation partner during long extended coffee breaks. Stefan Solé, who not only appreciates my bad jokes but makes me laugh with his own jokes. Alexander Kastner, who is able to look at most things in life with a wink, while emphasizing on good aspects. Cornelia Schmutz, who is never afraid of being honest in a constructive way. Lukas Kapitany, who is always open for spontaneous events. Stefan Hauer, who supports and enhances all the good and bad ideas.

1. Introduction

1.1. Aim of this study

The goal of the described study is the attempt to design an alternative method for the $^{12}C/^{13}C$-ratio determination of endogenous anabolic androgenic steroids (EAAS) via gas chromatography, coupled to combustion stable isotope ratio mass spectrometry (GC/C/IRMS). The main points of development are the purification and hydrolysis of steroid conjugates, opposed to the existing method. In the established procedure the phase II metabolites of steroids in urine are purified through solid phase extraction and afterwards glucuronide conjugates are enzymatically hydrolyzed. The resulting free steroids are cleaned up and isolated by high performance liquid chromatography (HPLC) into separate fractions. These clean fractions can be analyzed for the steroids isotopic ratio by GC/C/IRMS.

A different approach applied in this thesis is the HPLC clean-up of steroid glucuronides in contrast to purification of free steroids. The objective of the experimental design is to have a different separation of steroids and possible improvements of chromatographic fractionation.

The conjugates purified by this method can be hydrolyzed to obtain the free steroids which are needed for GC/C/IRMS analysis. Another discussed issue is deconjugation by acidic hydrolysis opposed to the enzyme catalyzed reaction of the standard method. Since enzyme mixes contain not only the pure enzyme, but remains of the producing organism, additional contaminations are imminent and have to be removed. Pure acid is supposed to be much cleaner and also cheaper in comparison to any enzyme mix. A desired outcome is an inexpensive alternative hydrolysis with fewer contaminations. The chemical hydrolysis should also be able to deconjugate steroid sulfates, which are left out in the current method.

1.2. Steroid Structure

All steroids have the same structural cyclopentanophenanthrene core consisting of four connected cyclic hydrocarbons. The first three (A, B and C) are cyclohexanes, while the last (D) has a cyclopentane structure.

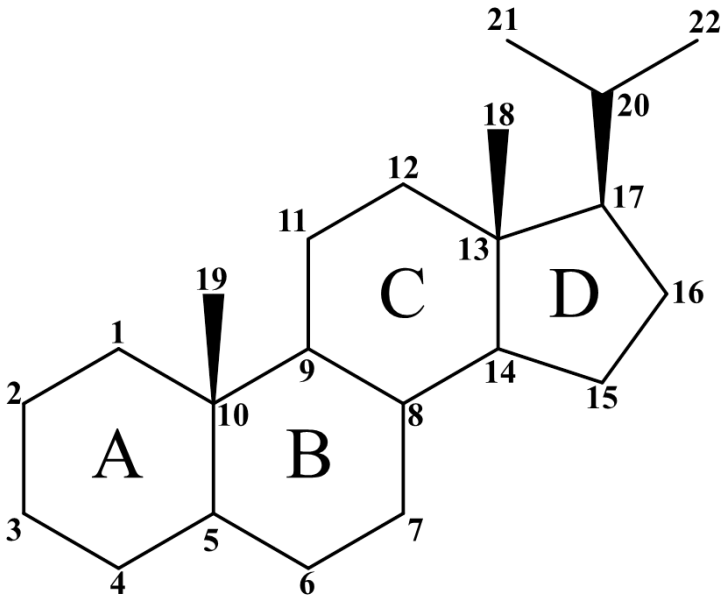


Fig. 1: *structure of a steroid with numeration of the carbon atoms*

Depending on the number of carbon atoms the steroid belongs to a different class, for example Estranes (C_{18}), Androstanes (C_{19}) and Pregnanes (C_{21}). Since the real structure of a steroid is not planar as illustrated on paper, it is important to depict the relative configuration. Therefore substituents bound above the plane are connected with a solid wedged line (β-bond) and those below the plane are connected with a dashed line (α-bond). A result of these variations is the three-dimensional structure of steroids and effects of the position of the substituents, especially at C-5.

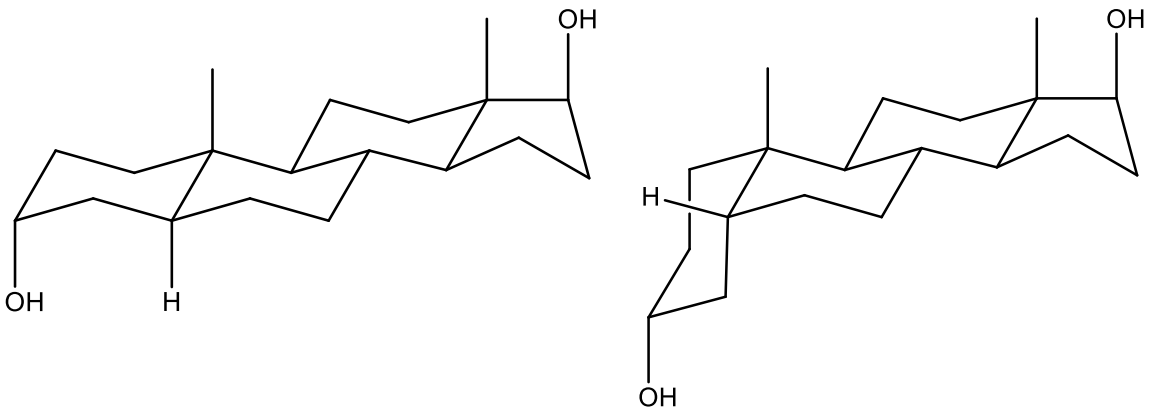


Fig. 2: *comparison of the three-dimensional structure of the two Androstane-3α,17β-diol isomers. Left:5αAdiol; Right: 5βAdiol*

In 5α-Steroids the cyclohexane rings are oriented similar to each other in the chair-confirmation whereas the A-Ring is rotated in 5β-Steroids. This difference is of importance for the chemical, physiological and chromatographic properties of steroids[1].

1.3. Steroid biosynthesis

The complex biosynthesis of steroids starts with the formation of cholesterol, involving 30 steps that will not be fully discussed within the scope of this thesis. Basically the human body is able to fully synthesize cholesterol from acetate with squalen as an intermediate product, most of it being produced in the liver or intestines[2].

The key enzymes for the steroid genesis are either from the cytochrome P450 family (P450) or hydroxysteroid-dehydrogenases (HSD). There are several reactions mediated by cytochrome P450, while HSDs generally convert secondary alcohols to ketones. The HSD reactions are reversible by principle but on the other hand the hydroxylations and bond cleavages by cytochrome P450 are irreversible.

In the beginning cholesterol is converted to Pregnenolone via consecutive hydroxylation followed by side chain cleavage by P450scc, an enzyme that only steriodogenic cells can express[3]. This is the rate limiting step of the steroid biosynthesis, as the expression of P450scc determines the amount of steroids produced. Pregnenolone is a potential substrate for two enzymes in the steroid biosynthesis. The first one, 3βHSD has an additional functionality than the ability of converting secondary alcohols to ketones, an isomerase activity. In the case of Pregnenolone, the double bond is shifted to the A-ring of the steroid, via a 3-keto intermediate forming Progesterone[4]. The other enzyme, P450c17 shows 17α-hydroxylase as well as 17,20-lyase activity and preferably catalyses the reaction of Pregnenolone to 17α-Hydroxypregnenolone and in the second step to Dehydroepiandrosterone (DHEA)[5]. A side reaction is the formation of Androsta-5,16-dien-3β-ol from Pregnenolone[6], after several steps this compound is metabolized to Androst-16en-3α-ol (16EN). In the case of Progesterone as a substrate 17α-Hydroxyprogesterone and from that Androst-4ene-3,17-dione are formed via P450c17 catalysis. Finally another HSD (17βHSD) converts Androst-4ene-3,17-dione to 17β-Hydroxyandrost-4-en-3-one (Testosterone)[7]. But testosterone can also be produced via two HSD catalysed reactions of DHEA.

Pregnanediol (PD) is produced over several steps starting from Progesterone starting with 20α-reduction and 3α-hydrogenation by 3αHSD. Corticosteroids are produced by the hydroxylation through P450c21 and P450c11, for example 17α-hydroxyprogesterone is converted to cortisol[8] and further to 11Keto-etiocholanolone (11keto).

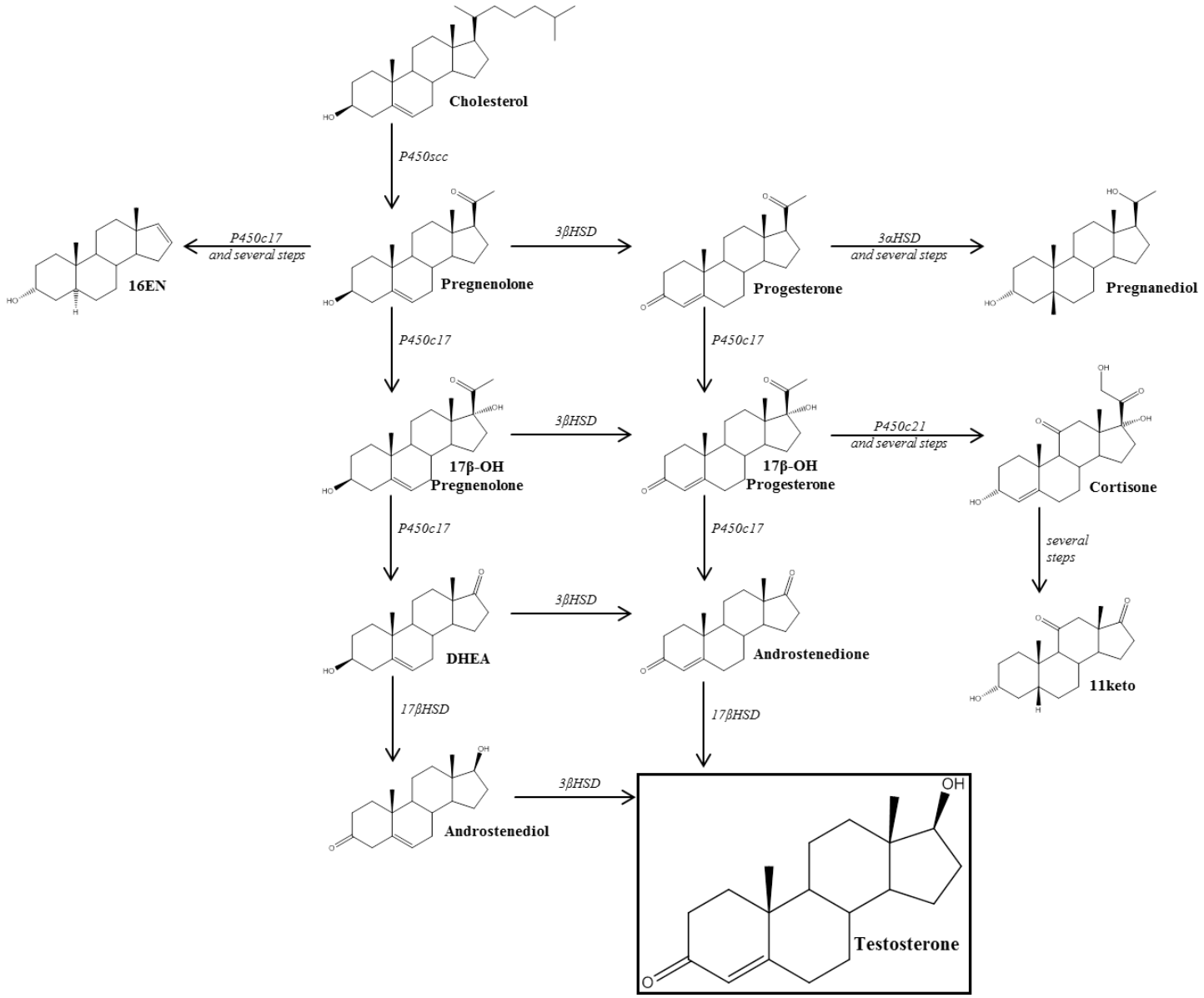


Fig. 3: *Androgen biosynthesis from cholesterol to DHEA and Testosterone, with additional branching to 16EN, PD and 11keto, compiled from literature*[9]

The steroids 16EN, PD and 11-keto have no androgenic or anabolic activity, but can be used as endogenous references independent of further androgen metabolism. This is especially important for the detection of Testosterone misuse, as discussed later on. Epitestosterone (Epi), the 17α-isomer of Testosterone is formed similar to Testosterone starting from Androstenedione or DHEA[10].

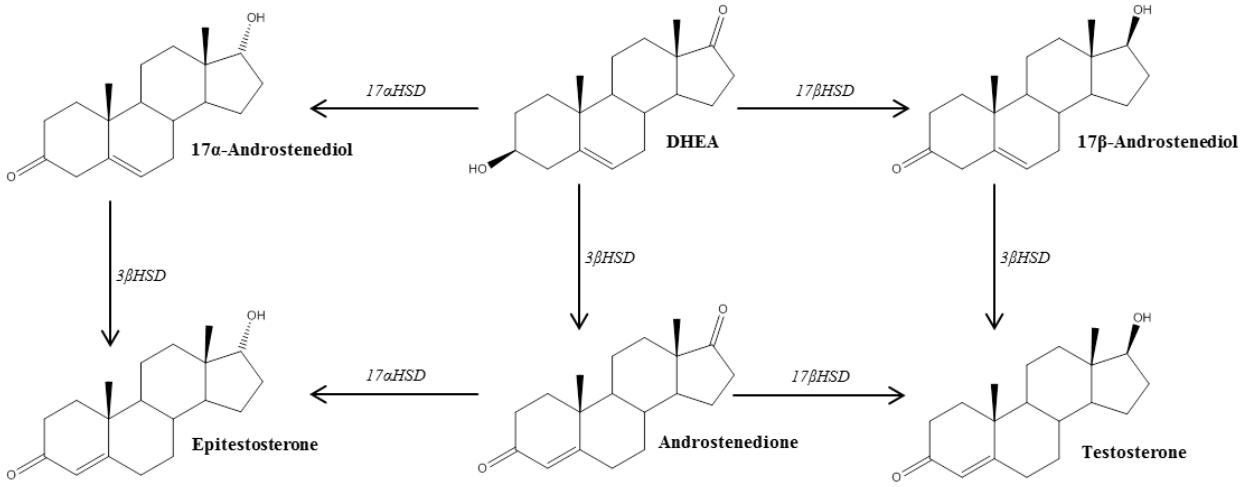


Fig. 4: *schematic metabolic pathway for the production of Testosterone and Epitestosterone. The reactions are directed towards T and Epi even though they are reversible.*

1.4. Testosterone Metabolism

Phase I metabolism of steroids takes place mainly in the liver with the primary goal of deactivation. Starting from Testosterone or Androstenedione, the first step is the reduction of the double bond in the steroidal A-ring. This reaction can be done by either 5α- or 5β-reductase, leading to 5α- or 5β-reduced steroids[11]. This step is irreversible, and in case of 5b-reduction a true inactivation of the compound. 5α-reduction principally enhances the androgenic activity of testosterone, but only in other tissue than the liver. The reduced steroids are then further metabolized by 3α-HSD to form their 3α-hydroxylated counterparts. Basically the reactions, except the reduction, are reversible, but the equilibrium is shifted to the main metabolites Androsterone (Andro) and Etiocholanolone (Etio). Hence the concentrations of 5α-Androstane-3α,17β-diol (5αAdiol) and its 5β-counterpart (5βAdiol) are lower.

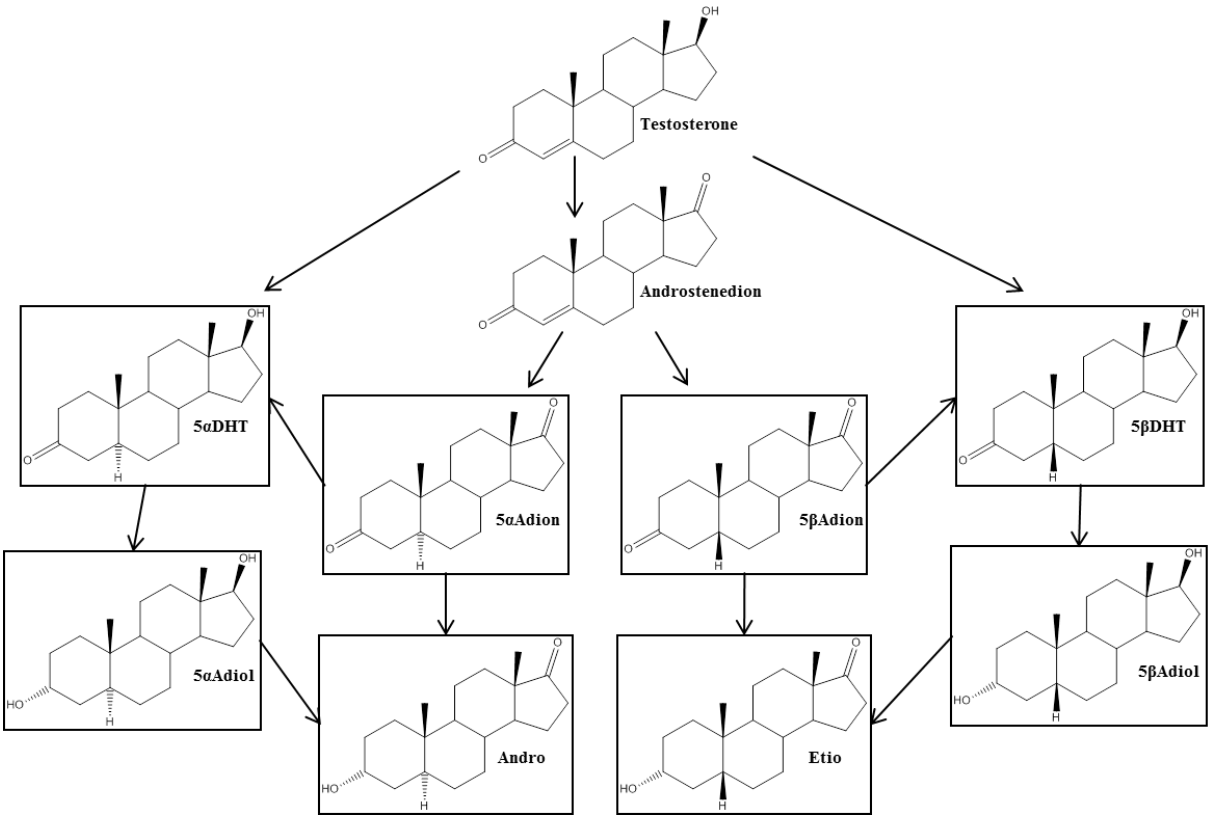


Fig. 5*: metabolism of Testosterone adapted from literature*[12]*, even though HSD catalysed reactions are reversible they are directed towards the main metabolites Etio and Andro in this scheme. The enzymes that catalyse the reactions are named in the text above.*

During phase II metabolism steroids become more water-soluble through conjugation to help the urinary excretion. The main metabolites of steroids are glucuronides and to a lesser extent sulpho-conjugated moieties. Since the focus of this thesis lies on the analysis of steroid glucuronides, only their formation and not of sulphates will be covered in the next paragraph. Glucuronidation not only makes the steroids more polar but also completely removes the bioactivity. The functional group is taken from Uridine-diphosphate glucuronic acid, and transferred onto free hydroxyl groups of steroids resulting in β-D-glucuronides of the steroids.

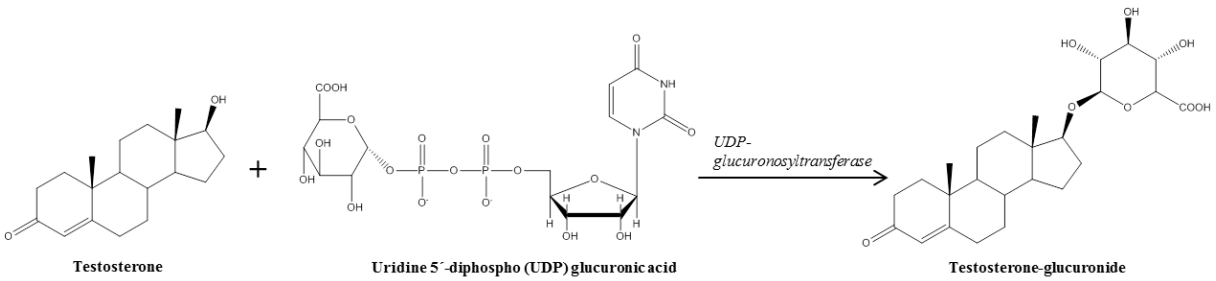


Fig. 6*: schematic of the glucuronidation reaction, in this example the formation of Testosterone-glucuronide*

The enzyme class responsible for the biotransformation of steroids are the uridine diphospho-glucuronosyltransferases (UGTs). They are divided in two families with subfamilies, based on

sequential identities. The primary site of expression is the liver, where most of the steroid metabolism takes place[13].

The polymorphism of the enzyme *UGT2B17* is of special significance, since it has testosterone as a substrate and plays a role in elevation the T/E ratio after exogenous testosterone application. Individuals with no copy of the gene, the *del/del* genotype, usually have T/E ratios below 1 and rarely reach the threshold of 4 after testosterone administration. The other genotypes *ins/ins* and *ins/del* show higher ratios which rise above the threshold after testosterone application. Therefore the T/E ratio alone is not applicable for the detection of administered exogenous testosterone as it can lead to false negatives for *del/del* genotypes of *UGT2B17*. This is important as it was shown that the del/del type is more prevalent in Asian than in Caucasian ethnicities[14,15].

1.5. Physiological effects of anabolic androgenic steroids

Since the structural elucidation and isolation of testosterone by Ruzicka in the 1930s[16] the use of steroids was on the rise. At first they were used to support the recovery of the athletes, later on the anabolic effects were more important. As a result anabolic androgenic steroids have been responsible for the most positive doping cases (adverse analytical findings, AAF) after the respective laboratories were able to detect these in the 1950s[17]. One of the milestones was the introduction of the Testosterone to Epitestosterone ratio (T/E) by Donike to aid in the detection of the application of endogenous steroids[18].

There are several performance enhancing effects of AAS, but discussing all of them would go beyond the scope of this thesis[19]. Androgens promote skeletal muscle growth and thus athletic performance that is reliant on strength in the long run. The androgen receptor positively affects the protein synthesis[20] and reuse of amino acids[21], two factors which increases the muscle growth. Additionally the growth hormone secretion is stimulated, which further increases the build-up of muscle mass[22]. Another positive characteristic of androgens is their erythropoietic effect that results in an increased haemoglobin production[23]. Finally that leads to better blood circulation, nutrient supply for the muscle and improved oxygen transport.

Androgens not only physiologically affect the body, but also have psychological influences on the body. Higher androgen concentrations can lead to better cognitive functions, paired with improved motoric movements, as well as a higher motivation for competition and the will to take risks[24]. Downsides in this regard are increased aggressive behaviour[25] and sometimes even manic disorders[26]. However those traits can even be beneficial in athletic competition.

There is a relation of the steroids structure to its anabolic potency, based on the binding affinity to the androgen receptor. In the case of endogenous steroids the position of keto- and hydroxyl-groups has to be considered. The 3-keto group of testosterone favors binding, reducing it to an alcohol reduces the

binding affinity no matter which isomer. On the other hand, the conformation of the 17-hydroxyl group is very important for the potency. Here the 17β-isomer is androgenic active, while a 17α-hydroxyl group shows barely affinity to the androgen receptor. That is clearly visible in the comparison of testosterone and its inactive isomer epitestosterone, regarding their androgenic potency. Another factor important for the activity is the spatial arrangement of the steroid backbone. A fully saturated even structure shows the best binding properties. This means that the straight 5α-steroids are favoured for binding to the androgen receptor compared to the slightly curved 5β-isomers. A double bond also brings distortion into the steroid system. Hence 5α-DHT is a stronger androgen than Testosterone[27]. From those observations it is clear, that the final metabolites of testosterone are not as potent as their precursor.

1.6. Detection of Misuse of endogenous anabolic androgenic steroids

As the body produces steroids on its own it is not trivial to distinguish between endogenous and exogenous origin. Nevertheless, there are several indications of unauthorized application of endogenous anabolic steroids. The first indication utilized was the ratio of Testosterone to its inactive isomer Epitestosterone, but it was later found out that this rule of thumb was too general and led to false positive tests as well as the already discussed downsides of false negatives. Therefore the so called athlete steroidal passport was introduced, which is the longitudinal steroid profile with a subject based approach. This steroid profile contains the concentrations of Testosterone, Epitestosterone, its metabolites Andro, Etio, 5αAdiol, 5βAdiol and the precursor DHEA as markers for doping. Even though the concentrations of these substances can vary, the overall ratios should not change over time. Using a Bayesian network and integrating the individual factors sex, age, ethnicity and UGT genotype, lower and upper limits are calculated for every steroid ratio[28]. Once a urine sample of an athlete is out of bounds regarding the steroid profile, it has to be confirmed with an additional analysis and the origin of EAAS determined. If that sample turns out to be negative the athlete's personal limits are adjusted.

In steroid analysis there are several confounding factors that can distort the athlete's steroid profile and thus have to be analyzed as well. Diuretics for example lead to a higher urine volume and thus dilution of the steroids, even though the androgens are not affected, the concentration might be below the detection limit. Another factor is the consumption of alcohol, which can result in elevated T/E-ratios. This is because ethanol inhibits the conversion of testosterone to androsterone during the phase I metabolism[29]. To monitor the athlete's alcohol consumption the urinary excretion of the metabolite ethylglucuronide is measured. A class of substances that have an influence on the steroid metabolism are 5α-reductase inhibitors like Finasteride, they reduce the production of the 5α-metabolites 5α-DHT, 5αAdiol and Andro.

If the sample is neither clean nor stored properly and contaminated with bacteria, an influence on the steroid profile can be seen as well. Steroidal glucuronides are deconjugated by bacterial activity leading to elevated concentrations of the unconjugated moieties. Through the bacterial enzyme 3-hydroxysteroid-dehydrogenase the hydroxyl-groups of androsterone and etiocholanolone can be transformed to keto-functions resulting in the formation of 5α-androstanedione and 5β-androstanedione[30].

If one of those confounding factors is present in the sample its steroid profile has to be considered with special precautions or even regarded as invalid.

1.7. Differences in stable carbon isotope ratios

The only way to detect the true origin of EAAS is the determination of the Carbon isotope ratio (CIR). To understand the principle behind the method and the reasoning for the criteria of positive samples, natural distribution and circulation of stable carbon isotopes has to be considered.

The Element Carbon with the atomic number 6 and therefore 6 protons has two stable isotopes. The most common one is the isotope with 6 neutrons, ^{12}C with 98.93% natural abundance; it has an atomic mass of exactly 12, since the atomic mass unit is defined as 1/12 of the mass of ^{12}C. The second stable and natural occurring isotope is ^{13}C with 1.07%. Other than the two stable isotopes ^{14}C can also be found in nature, being produced through cosmic radiation. It has a half-life of 5715 years and is used in radio carbon dating of organic material. The second unstable isotope with a practical application is ^{11}C, but it has to be synthesized in a cyclotron. It has a half-life of 20 minutes and is used for positron-emission tomography.

A tool to compare the isotopic composition of natural compounds is the ratio of the stable isotopes. As carbon only has two stable isotopes the strategy is quite obvious. From the ratio of 13C/12C the so called delta values (δ13C) are calculated.

$$\delta^{13}C = \left(\frac{\left(\frac{^{13}C}{^{12}C}\right)_{sample}}{\left(\frac{^{13}C}{^{12}C}\right)_{standard}} - 1 \right) * 1000‰$$

Eq. 1*: equation to calculate the $\delta^{13}C$-values of a whole sample or substance. VPDB is used as a virtual reference standard, as described in the following paragraph*

Therefore a homogenous and stable reference material is needed as foundation for comparison. Since the origin of the method lie in geology the measurement scale is based on minerals. For a long time a marine carbonate from the rostrum of an extinct mollusk (*Belemnitella Americana*) found in the Peedee Formation in America, was used with the abbreviation PDB. This fossil had an unusually high fraction of ^{13}C compared to other minerals. After the original source of this reference material was exhausted, a new one of virtual nature was defined by the International Atomic Energy Agency, the so-called Vienna Peedee Belemnite (VPDB). Today all results of carbon isotope ratios are annotated versus VPDB; a negative value means less ^{13}C than the primary reference material, which denotes the zero point of the scale. As the usage of a non-existing

reference material is not practical, calibration materials have to be used. For example NBS 19, a piece of marble with a $\delta^{13}C$-value of +1.95‰ was used to define the VPDB-scale[31–33]. But for routine analysis it makes more sense to use certified reference materials that were calibrated against a calibration material.

The overall natural abundance of carbon isotopes is not changing, but organic compounds can have a range of different delta-values, depending on their origin. Since the isotopes have small differences in physical properties, isotopic discrimination can occur in various physical and chemical processes. Humans and other animals commonly incorporate carbon through dietary intake; the isotopic composition of their metabolites is dependent on their nutrition. Only plants contribute to the fixation of carbon from the atmosphere and they have different mechanisms, resulting in distinct isotopic ratios. There are three different pathways for the CO_2 uptake in plants, each resulting in different Carbon isotope ratios.

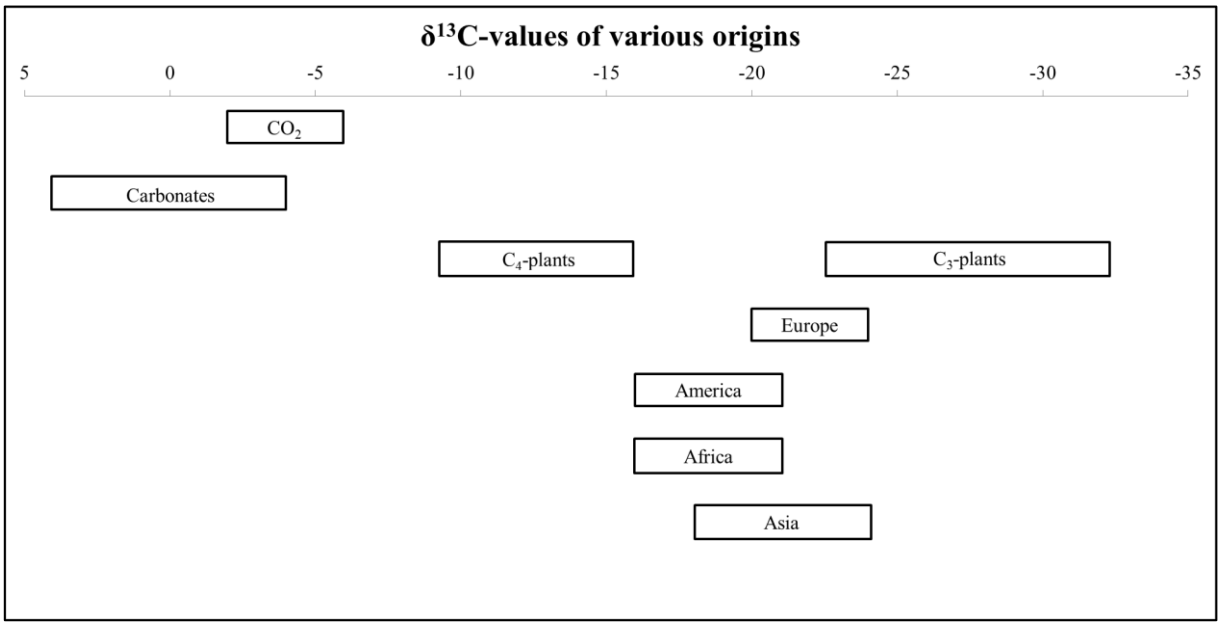


Fig. 7*: δ13C-values of various origins, compiled from literature*[34–36]*. The δ13C-values from the different continents are from urinary steroids of doping control tests.*

Plants fixate CO_2 via the carboxylation of ribulose bisphosphate by the enzyme Ribulose-bisphosphate-carboxylase/-oxygenase (RuBisCO) in the Calvin-Cycle. Depending on the type of plant there can be additional steps in the Carbon uptake.

This is the case for C_4-plants as they utilize a pre fixation step of dissolved CO_2 in a separate location from the final carboxylation. The first Carbon fixation in the mesophyll is the formation of oxaloacetate catalyzed by phosphoenolpyruvate carboxylase. Afterwards the oxaloacetate is transported to bundle sheath cells where the reaction is reversed and CO_2 is set free to be finally incorporated by the RuBisCO carboxylation.

Contrary to that in C_3 plants the whole fixation takes place in the mesophyll and only consists of the RuBisCO carboxylation reaction. The third group of plants based on the mechanism of CO_2 absorption utilizes the Crassulacean Acid Metabolism (CAM) which is a mixture of C_3 and C_4 pathways depending on environmental conditions like temperature, humidity and daytime. The RuBisCO catalyzed carboxylation is isotopically discriminating, whereas the diffusion of CO_2 into the plant shows minimal isotopic fractionation. Depending on the photosynthesis pathway the subsequent metabolic products of the plant show different delta-values. C_3 plants show more depleted ^{13}C contents compared to air, since the carboxylation of ribulose bisphosphate is the only and therefore limiting step in carbon uptake. On the other hand in C_4 plants the diffusion of CO_2 is more critical and limiting all further reactions resulting in less ^{13}C depletion than in C_3 plants. CAM plants show isotopic similarities to C_3 plants during daytime, whereas the Carbon fixation process during nighttime resembles their C_4 counterparts. Examples for dietary relevant C_3 plants are rice and soy, for C_4 plants maize and sugar cane and pineapple as a CAM plant[37,38].

Within the human metabolism little to no isotopic discrimination takes place, so substances of the same classes should have comparable delta-values. As a result a large difference of the delta-values indicates manipulation through intake of synthetic substances. To calculate the so called delta delta-values a point of reference is needed. For the analysis of EAAS it should be another endogenous steroid that has neither androgenic nor anabolic function and is not influenced by the metabolism of the target substances.

$$\Delta\delta^{13}C = \delta^{13}C_{ERC} - \delta^{13}C_{TC}$$

Eq. 2: *equation to calculate the differences of $\delta^{13}C$-values between ERC and TC*

PD is chosen as the primary endogenous reference compound (ERC), 16EN, 11β-Hydroxyandrosterone (11OHA) or 11keto are acceptable as well[39].

1.8. Criteria for an adverse analytical finding

Due to measurement uncertainties and natural variations the delta delta values can have a range. Results out of this range imply intake of synthetic EAAS and therefore lead to a positive testing result. From reference population data and excretion studies the world anti-doping agency (WADA) has set thresholds for the $\Delta\delta^{13}C$-values of each relevant substance.

As the metabolites are depleted regarding their ^{13}C percentage compared to their origin, they should have more negative delta values further down the metabolism. A minor isotopic fractionation takes place during formation of Etio from Testosterone, as the Enzyme 5β-reductase appears to discriminate against heavy isotopologues[36].

That is clearly visible in the relation of the $\delta^{13}C$ -values of the final metabolites, where Etio is usually 1‰ more negative than Andro. Considering this, the $\Delta\delta^{13}C$ threshold for Etio is set higher than for Andro.

Table 1*: criteria for an AAF for EAAS according to the WADA technical document, adapted from*[39]*. If only all criteria from any row are met, the sample result is regarded as an adverse analytical finding.*

positive criteria	$\Delta\delta^{13}C_{ERC\text{-}TC}$				
	Testosterone	**Epi**	**Andro**	**Etio**	**Androstandiol**
i	> 3‰				> 3‰ both Adiols
ii					> 3‰ both Adiols
iii		> 4‰			
iv			> 3‰		
				> 4‰	
v			2-3‰		> 3‰ either Adiol
				3-4‰	> 3‰ either Adiol
vi					$\Delta\delta^{13}C_{ERC\text{-}5\alpha Adiol}$ > 4‰ and 5αAdiol ≤ -27‰

To confirm a positive sample the whole analysis has to be stable and unambiguous even with measurement uncertainties, therefore additional precautions have to be made. As the compounds of relevance have to be pure for the final stable isotope analysis, the sample preparation starting from crude urine has to be quite extensive, thorough and involves several cleaning steps. The techniques that play a major role to purify the sample in this thesis will be described in the following paragraphs.

1.9. Solid Phase Extraction

A simple method to reduce sample volume and unwanted matrix interferences is solid phase extraction (SPE). In principle it works similar to chromatographic separation, as the analyte molecules are retained on a solid phase and can be eluted later on. With the right conditions impurities are washed out and target molecules stay trapped on the stationary phase. There are different types of materials as in column chromatography, depending on the analytes characteristics. Most particles are based on a silica backbone, modified with functional groups to provide various binding mechanisms. In this thesis a C18 column is used for SPE. The silica particles are modified with octadecyl-chains to create a non-polar stationary phase. The partitioning mechanism is based on hydrophobicity and should separate the water soluble polar urinary compounds from the desired substances. The steroid conjugates on the other hand are supposed to adsorb onto the stationary phase.

In general a solid phase extraction procedure consists of four steps: conditioning of the sorption material, addition of the sample, washing, and as last step the elution of target molecules. The first step is to wet the surface of silica particles to ensure that molecules can access the functional groups of the sorption material and bind to them. It is usually performed by rinsing with a strong solvent and then washing with the same solvent the sample is dissolved in. For urine the last conditioning step before sample addition is done with water. After conditioning of the solid phase

material the sorption material should not run dry as this decreases the ability to bind molecules. As the next step the sample is added and washed carefully. Goal of the washing step is to retain the target molecules on the stationary phase but to remove matrix interferences. Before elution with the right solvent the cartridge can be dried to remove excess washing solution. Elution mixtures should be chosen to barely dissolve the desired molecules from the solid phase and retain impurities. An important aspect on solvent selection is pH-Value. Molecules like glucuronides show different retention characteristics whether they are protonated, deprotonated or have no charge. To keep the pH of a sample close to constant buffers can be used.

The adjustment of the flow rate in solid phase extraction is necessary because otherwise the extraction process would be inefficient and take too long. The process can be sped up by either applying vacuum on one side or pressure on the other side of the cartridge. A downside of too high flow rates is missing time for equilibrium between the analytes and the solid phase, which results in bad sorption and therefore inferior retention. Some steps are more prone to mistakes and have to be performed more thoroughly than others. Conditioning can be done fast and with a high flow rate. All operations afterwards should be run with continuous dropwise flow rates and not too rapid. In case of clogging due to samples with lots of solid matrix interferences the process should be accelerated.

1.10. Hydrolysis

Since Steroids are undergoing conjugation during the phase II metabolism, they have to be hydrolyzed to analyze their respective free form. This step is mandatory for analysis by gas chromatography, as the steroid conjugates are neither thermally stable nor volatile. There are two practical approaches to the deconjugation reaction, either biological (enzymatic) or chemical.

In theory enzymes have the advantage of catalyzing only one specific reaction with an expected outcome, but the enzyme can contain additional activities for other reactions. β-glucuronidase is the enzyme of choice for the hydrolysis of steroid glucuronides and can be produced from different organisms. The two sources are bacteria like E.Coli and molluscs like Helix pomatia. The enzyme from Helix pomatia shows has sulfatase activity as well, so these conjugates can be cleaved as well. Downsides are other enzymatic activities that lead to unwanted side reactions, possible degradation of steroids and formation of artefacts. The β-glucuronidase from E.Coli on the other hand is highly specific for the cleavage of β-glucuronides only and is therefore more widely used[40]. Chemical hydrolysis is more universal, as both glucuronide and sulfate moieties can be cleaved off[41]. Downsides are possible unwanted side reactions and the chance of steroid degradation.

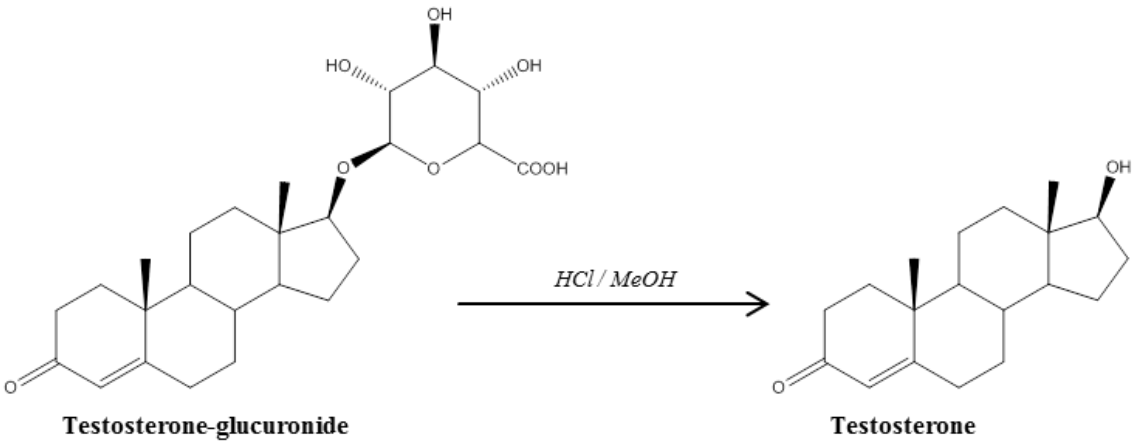


Fig. 8*: Acidic hydrolysis of testosterone-glucuronide with hydrochloric acid, the formed free glucuronic acid is intentionally left out of the picture*

To harmonize the procedures of all anti-doping laboratories for the hydrolysis of steroid conjugates WADA permits the use of β-Glucuronidase from E.Coli only[42]. Since in equine Sports there is no comparable authority like WADA every lab can autonomously decide about their hydrolysis procedure, so chemical hydrolysis is an alternative[43].

1.11. High Performance Liquid Chromatography

Chromatography is a technique to separate molecules in solution based on their different properties. The separation is generally based on the distribution of the substances between the solid stationary phase and the liquid mobile phase. The stationary phase has to be carefully selected to meet the analytical requirements, as is a higher impact on the chromatography than the other parameters. There are several types of columns with their own mechanism of separation; the most common ones are briefly described in the following paragraph.

Normal phases have the simplest composition; the stationary phase particles are highly polar silica, the mobile phase is in contrary to that non-aqueous and non-polar. The sample separation process is based on polar features as dipol-dipol interactions between the stationary phase and the substances. In this technique water should be avoided, therefore it is not suited for many life-science applications. To accommodate samples that are in aqueous solutions the stationary phase has to be modified. One approach to that is the modification of the silica surface with straight alkyl chains, hence completely changing the polarity and creating so-called reversed phases. Reversed phases are non-polar and retention mechanism is based on hydrophobic interactions and can be used for water soluble organic molecules. It is primary used for aqueous samples as the mobile phase consists of a mixture of water and an organic solvent.

To separate highly polar and charged molecules a different type of phase is needed. The surface of the stationary phases in these ion exchange columns are charged so that molecules of the opposite charge stick to them. The elution process is initiated by addition of stronger ions than the analyte molecules.

Further properties of the stationary phase that can have an influence on the efficiency are particle geometry and size as well as column dimensions. In general columns with smaller particles perform better and have higher resolving power, but are accompanied with much higher backpressure. The particle structure can range from fully porous to particles with an impermeable core and an outer shell where the separation takes place. The advantage of these core-shell particles is that they have a higher resolution, as the mass transfer during chromatography is reduced resulting in less diffusion. To further improve the partitioning process the length of the column can be increased, as more stationary phase provides more separation. A downside is the subsequent rise in analysis time with longer columns, so a compromise has to be made.

The column used in this thesis has a C18-modified reversed stationary phase with core-shell particles. Free silanol groups of the particle that are not already modified with the C18-alkyl chain are end capped with trimethylsilyl groups to prevent unwanted interactions with those. The application of fraction collection brings the limitation of a minimum flow rate as the automated collector cannot handle to small volumes. Therefore the particle size cannot be too small, as the pressure would rise above the limits of the capillaries. For the same reason a shorter column than usual preparative HPLC columns was chosen. After unpublished in-house tests a Phenomenex Kinetex C18 with 4.6mm inner diameter, 150mm length, 2.6µm particle and 100Å pore size was chosen.

Modern HPLC instruments consist of an autosampler, a pumping system, column compartment and some kind of detector. For the use of a mobile phase consisting of two solvents the HPLC has to have at least two pumps and a gradient mixer to correctly prepare the wanted composition of the mobile phase. The samples are stored in the autosampler at a defined temperature to prevent degradation or unwanted evaporation of the solvent. During the injection a specific volume is transferred with a syringe in the sample loop which is then flushed onto the column. With this technique the column flow is not interrupted and sample transfer is kept consistent. All tubings should be as short as possible to reduce unnecessary flow paths and possible void volumes. Since the viscosity of the solvent is temperature dependent, the column has to be kept inside the column oven at controlled constant temperature. The last part after the column is the detector to monitor the eluting substances. Mass spectrometry is often used not only to quantify the analyte but also give structural information about molecules of interest in the sample. A big downside is the destructive nature of the ionization process; therefore it cannot be used for preparative applications. A different detection option is optical spectroscopy, either with UV- or visible light. These methods are not as universal as a mass spectrometer, some substances are not possible to detect and unwanted signals from contaminations can overlap everything else. But in this case a UV-detector is well suited for this task, where the UV absorption is measured in a flow cell right before the automated fraction collector.

There are two approaches to the elution process, either isocratic or with a mobile phase gradient. Isocratic means that the composition of the mobile phase stays the same during the whole

chromatography. This works for simple samples with a small number of analytes, as the solvent conditions are optimized for only one of the components, the others usually show peak broadening. For more complex samples with several different substances a changing mobile phase gradient helps in the elution process. The gradient starts with an isocratic part whereby all analytes are just retained on the column and barely travel through the stationary phase; this part of the gradient is to sharpen the sample band and eventually the resulting peaks. Afterwards the percentage of the stronger solvent is increased continuously to release the analyte molecules into the mobile phase and finally elute them from the column. The slope of the gradient has a big influence on the peakwidth and resolution. Faster gradients with a larger change in eluent composition result in sharper peaks, as the eluting power of the mobile phase has a larger increase in the same amount of time than on a slow gradient. A downside of quick eluting methods is the possible lack of separation between the peaks. This is no problem if the detection method can easily distinguish between different substances in potential double peaks like mass spectrometry. However for preparative HPLC a good baseline separation is crucial to collect only the wanted substances with adequate purity.

Especially for IRMS the collection of the whole peak is vital, as isotopic fractionation happens during chromatography. As heavier isotopologues elute faster than their lighter counterparts, the end of the peak is more depleted than the beginning.

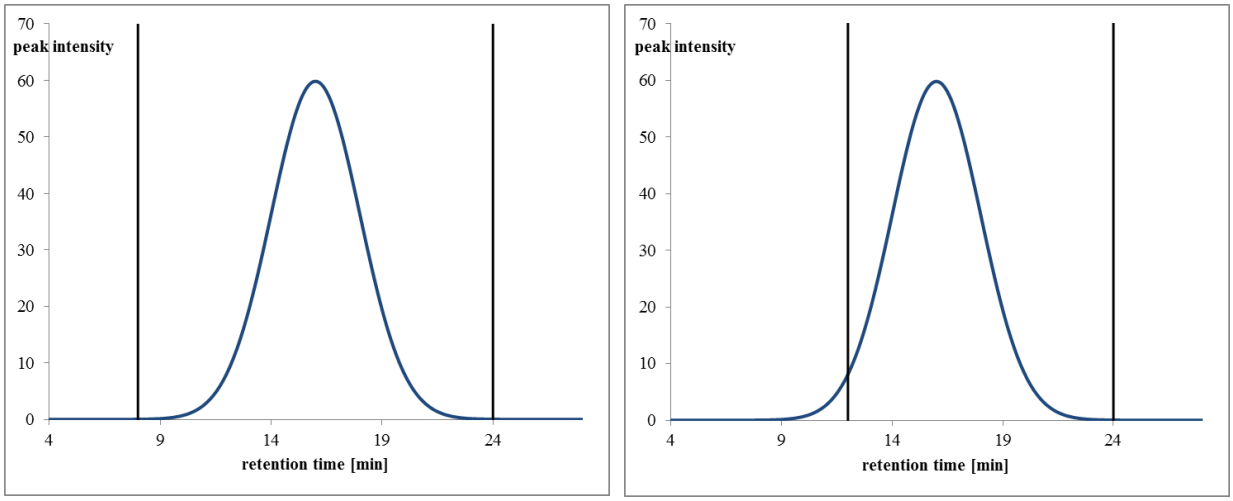


Fig. 9*: schematic representation of an ideal HPLC peak, the vertical lines representing the fraction collection time windows. On the left side fraction collection and the resulting $\delta^{13}C$-value is correct. On the right side collection starts too late, resulting in a too negative $\delta^{13}C$-value.*

An option to ensure the correct fraction collection is to put a standard mixture or control sample with known delta values at the end of each HPLC run. If the results from the IRMS analysis match the theoretical delta values, the fraction collection was adequate[44].

1.12. Gas Chromatography

In gas chromatography substances are separated by their volatility and interactions with the surface of the stationary phase. Analytes have to be thermally stable and volatile in the chosen temperature range.

A gas chromatograph consists of the injector, the column within the oven and a detector. Inside the injector, also called inlet, lays the liner, a deactivated glass tube with a distinct volume for gas. The liner is closed off through a septum on one side, directly connected to the column on the other side and can be heated independent of the column oven. The sample is injected with a syringe through the septum into the liner and evaporated. Purpose of a liner is to vaporize the sample and solvent uniform and at once. Afterwards the now gaseous sample has to be transferred efficient onto the column without any interactions with the liner wall. Uneven sample transfer results in undesirable peak shapes like tailing or even peak splitting. Liner dimensions are a limitation to sample volume, as the sample expands through vaporization. If the liner is too small, parts of the sample can be lost through the septum purge flow, which results in poor reproducibility. Too large liners lead to additional dead volume that increases the sample transfer time and eventually causes peak tailing. To improve peak shape liners can be packed with glass wool. Surface area of the liquid sample increases, as it deposits on the wool, which leads to more uniform vaporization.
In general there are different techniques to inject the sample, used depending on the properties of the analyte molecules and their concentration. Split and splitless injection can be operated with the same instruments.
For split injection a part of the carrier gas flow is distributed to the split vent, and blown out of the system, therefore the sample is also partially lost. The split ratio determines how much of the sample is loaded on the column. As the carrier flow is much higher than the actual column flow the sample is transferred rapidly on the column. A disadvantage is discrimination of analytes with different boiling points. Less volatile components do not have enough time to evaporate completely and are diluted in another way compared to substances with lower boiling points. Hence the sample composition on the column is different than prior to the injection. Since the technique is simple, robust and possible contaminants are also flushed out, it is widely used for qualitative analysis. However through the uneven dilution of the analytes it is not always suitable for quantitative analysis and cannot be used for trace analysis.

In splitless mode the split vent is kept closed and the whole sample is transferred to the column. Sample loading takes much longer, because it has to be done with the column flow of the carrier gas. To prevent peak broadening caused by these long transfer times the sample has to be re-concentrated on the column. This can be achieved by cold trapping and utilizing the solvent effect. Directly after the inlet the column has a lower temperature, therefore the sample is affected by a negative temperature gradient. High-boiling components have a reduced mobility and are compressed into tight bands which start to move only after the column oven heats up during the temperature program. Despite the name, the split vent is closed only during the injection process and is kept open otherwise with a higher carrier flow to compensate the split. The splitless time is an important method parameter that can heavily affect the chromatography and has to be optimized before. Modern GCs have an additional feature for faster sample transfer onto the column in splitless mode, called splitless with

surge. During the surge time a higher pressure is applied in the inlet to reduce the sample transfer time and eventually sharpen the peaks.

A special injection mode to prevent discrimination is utilizing programmable temperature vaporization (PTV). It is a universal technique but requires an inlet capable of controlling the temperature and retaining the liquid sample until evaporation. The sample can be injected either in a cool or hot liner and it can be operated in split or splitless mode. If the sample is introduced to a cool liner that is gradually heated the lower boiling solvent evaporates first. By opening the split vent in the start of the temperature gradient the solvent can be removed efficiently. This technique allows much larger sample volumes as the solvent is not a problem anymore. Another possibility to inject the sample is directly on the column. This technique is especially suited for high boiling substances which would not be transferred completely in split or splitless mode. The instrumentation setup requires a syringe needle that fits exactly into the column and deposits the liquid on the first centimeters of the column. Thus the samples contained impurities are also transferred on the column, which makes this method more susceptible to contaminations than other injection techniques.

Then the substances are flushed through the column with the carrier, the mobile phase. This gas has to be inert towards the analyte molecules and should not react with the stationary phase. The three most commonly used gases are Hydrogen, Helium and Nitrogen. The main gas properties for gas chromatography are the viscosity and diffusivity. Gas viscosity increases with temperature, which results in lower average linear velocity. In constant pressure mode the inlet and column start temperature is set to a fixed value to keep the pressure stable. As the column temperature changes through the temperature program of the oven the flow rate and therefore linear velocity of the carrier in the column varies with a consistent pressure. On the other hand, if the gas chromatography is operated in constant flow, the injector pressure changes over the run time to keep the carrier gas flow constant. Hydrogen has the lowest viscosity therefore chromatographic runs with hydrogen are faster and require lower pressure for higher flow rates. Diffusivity describes how well solute molecules are able to diffuse in the gas phase. Diffusion in the column can be described by its direction, radial towards the column and longitudinal alongside the column. More radial diffusion of the analytes leads to more possible contacts with the column coating per time and increasing partitioning. Longitudinal diffusion is unwanted as it results in no additional interaction with the coating and causes peak broadening. Lesser carrier velocity leads to more time for contacts with the coating and thereby better separation, but also to wider peaks due to longitudinal diffusion. Hydrogen and Helium have similar diffusivities, but since Hydrogen is easily combustible Helium is preferred. Nitrogen is as inert as Helium; however its diffusivity is about a quarter of Helium therefore chromatography with Nitrogen as a carrier has to be performed at lower velocity and takes accordingly longer.

There are two types of GC columns, packed and capillary columns. The packing of packed columns consist of small inert particles coated in the actual stationary phase, similar to LC columns. Nowadays

capillary columns made of polyimide are more common, as the separation is more efficient and faster than on packed columns. The walls of the capillary are coated with the stationary phase. Depending on the composition of this polymer the polarity can change from non-polar to highly polar[45].

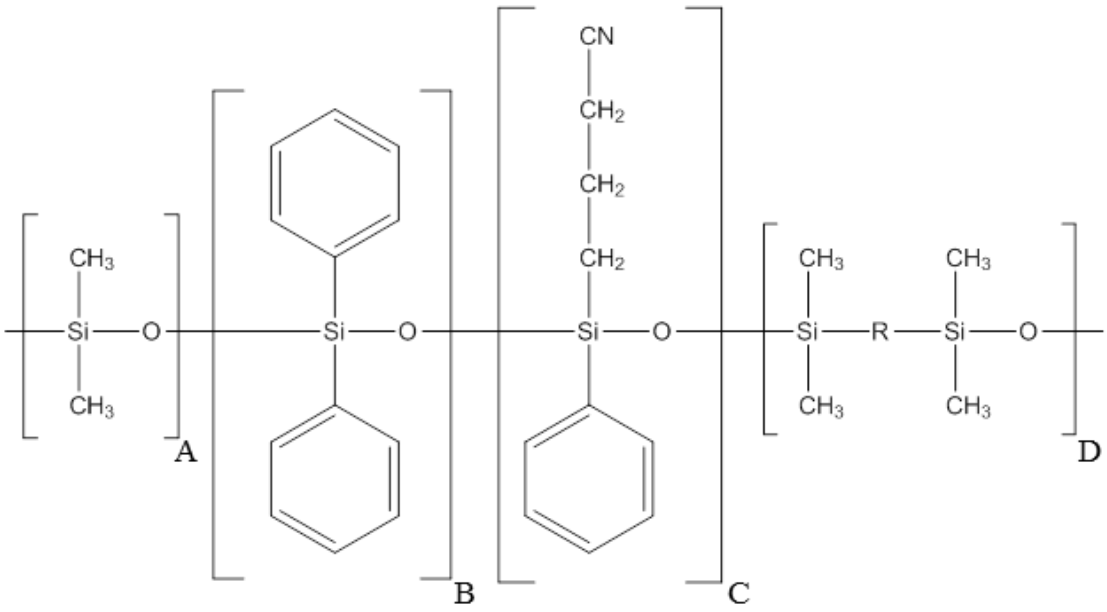


Fig. 10*: generic composition of a polysiloxane coating for a stationary phase in capillary gas chromatography. The monomers in brackets are denoted with letters as placeholders for their percentage, depending on the type of stationary phase. A: dimethyl-, B: phenyl-, C: cyanopropylphenyl-, D: a proprietory phase from Restek*

The general structure of such polymer is shown in **Fehler! Verweisquelle konnte nicht gefunden werden.**. The least polar stationary phase only consists of dimethyl-polysiloxane, the polarity increases with the addition of more phenyl groups. For even higher polarity the phenyl groups are exchanged with cyanopropylphenyl groups. The last optional section shown in **Fehler! Verweisquelle onnte nicht gefunden werden.** is introduced to increase the stability of the polymer and reduce column bleed. For example the column used in this thesis has an unknown portion of it incorporated. Since it is a property of the manufacturer no further information is given.

For the separation of steroids there are a few potential choices, depending on the sample preparation. In the laboratory's initial testing procedure steroids are analyzed as trimethylsilyl (TMS) derivatives, therefore a non-polar column with 100% dimethyl polysiloxane is used. Acetylated steroids are best separated on a (50% phenyl)-dimethylpolysiloxane column, since they are more polar than their TMS-analogs[46]. As free steroids are in between the two beforehand mentioned derivatives regarding their polarity, there are more column options. The established method in-house can either utilize only a (50% phenyl)-dimethylpolysiloxane column or a combination together with a (5% phenyl)-dimethylpolysiloxane precolumn for additional separation mechanisms. Other groups make use of just (5% phenyl)-dimethylpolysiloxane for the analysis of free steroids[47]. A type of polar stationary phase not previously mentioned is polyethylene glycol, but it is not usable for the separation of steroids.

Every chromatographic system needs a detector afterwards to assess the samples composition. A simple way to visualize the eluting substances is the measurement of the gas stream thermal conductivity compared to the pure carrier gas. These thermal conductivity detectors (TCD) lack sensitivity but are nondestructive; therefore another detector can be used afterwards. One of the most common detectors is the flame ionization detector (FID), where the stream of gas is introduced into a hydrogen flame. The sample is thereby incinerated and gets thermally ionized. Through the capture of the resulting electrons a voltage corresponding to the concentration of the substance can be measured. A universal detection method for GC is mass spectrometry where the molecules are ionized and analyzed according to their mass to charge ratio (m/z). With this instrumentation not only quantitative but qualitative information can be ascertained as well.

1.13. Gas chromatography coupled to mass spectrometry (GC/MS)

As the analyte molecules are already in the gas phase, a suitable ionization technique has to be used. The two main options for producing ions after GC are Electron Ionization (EI) and Chemical Ionization (CI). Both techniques base on the ionization through a beam of electrons, in EI the sample is directly ionized, while in CI a reaction gas ionized by the electron beam helps in the formation of secondary ions from target molecules. The electrons are produced by a heated filament and accelerated with an adjustable potential towards a positive electrode. Depending on the resulting wavelength of the individual electrons in the beam they have distinct energies. As these wavelengths are in the range of bond lengths, the energy can easily be transferred to the molecules in the gas phase, leading to excitation and eventually to emission of electrons from the molecule, resulting in the ionization[48]. With increasing electron energy these processes occur more often, resulting in higher ionization rates. If the electron energy is too high, the resulting wavelength is much shorter than bond lengths and therefore passing through the molecule without excitation. Around 10eV is usually enough to ionize all organic compounds, additional energy leads to fragmentation. Most molecules show the maximum number of produced ions at electron energies of 70eV, thus this value has been chosen as standard condition for EI. As this is more than enough energy to form ions, most molecules are heavily fragmented and the initial molecular ion is barely existent. Lower energy settings in the ion source lead to less fragmentation, but also to decreasing signal intensity.

One of the strongest points of EI is the fact, that most spectra were produced under these standard conditions for a long time resulting in databases, the so-called spectral libraries. With enough experience and knowledge, structural information of a molecule can be figured out from its fragmentation pattern. Additional comparison with spectral libraries helps with the elucidation of unknown compounds.

CI is a softer ionization technique leading to less fragmentation, but depending on the chosen reaction gas, the type of resulting ions can be influenced. Modern instruments are equipped with a

multifunctional ion source that can work in EI as well as in CI. The complementary information from the two techniques can be combined altogether to give a better understanding of unknown molecules.

After the ion source uncharged particles have to be filtered out and ions are separated according to their m/z. As the pressure decreases further inside the mass spectrometer, the ions are sucked in and accelerated by electronic potential differences. They are guided through the ion optics by lenses and redirected to get rid of uncharged particles, since they are not affected by electrical fields and continue on a straight path. The ion beam is focused further and led to the mass analyser, where the distinction between the different ions is taking place. There are several types of mass analysers, depending on the analytical aspect as well as the type of ionization utilized. The two types used in this thesis are quadrupole and magnetic sector field analysers, so only those are described in the following paragraphs.

A quadrupole basically consists of four electrically charged metal rods. By connecting the two respective opposing rods and applying current, an electrical field is generated. The two connected rods have the same polarities and in addition an alternate current with a phase shift of 180° is applied between the two pairs. As a result positive charged ions fly towards the rod with the negative direct current. At the same time the alternate current superimposes on this rod with a positive charge and repels the cation. After the current alternates the ion is attracted again and travels on this plane in a sinus curve like path. Since there are not only two but four rods the real path of the ions is better described by motions of spinning tops through the quadrupole. By changing the voltage of the direct current and the amplitude of the alternate current different ions are stabilized. If only the alternate current is applied, all ions pass the analyser in this radio frequency only mode and no selection takes place, resulting in a resolution of zero. The quadrupole is a scanning analysator, which means it can go through the optima for many ions in a short time with highest possible resolution. This is achieved by a ramped change of the currents, with the limitation of only being able to analyse ions with only one m/z-ratio at high resolution. But this also means that it cannot analyse two single charged ions with a mass difference at the same time. If two ions are to be monitored at the same time, the loss of quantity is around 50%, as the quadrupole can only analyse one at a time, making it unsuitable for reliable quantitative information on isotopic compositions[48].

The quadrupole is an uncomplicated and cheap mass analyser for routine analysis, although it lacks in resolution for structural elucidation. The magnetic sector field is simpler by principle, but has different limitations. An electromagnet is employed to create a magnetic field for the separation of ions. There the ion beam is guided between the two magnetic poles and is deflected perpendicular to the magnetic field as well as perpendicular to the direction of the ions. The radius on which the ions travel is dependent on the mass to charge ratio and the kinetic energy of the charged particles. But if all ions are accelerated with the same voltage in the ion source, then the kinetic energy of single charged ions with the same mass should be identical. Lighter particles are more affected by the magnetic field and

therefore travel on smaller radii. The electromagnet only reaches a certain angle and afterwards the ions travel again in a straight line to the detector. Since the adjustment of the magnet takes a long time and is accompanied by errors due to hysteresis, the magnet settings are fixed. This setup is optimal for the analysis of isotope ratios, as a small range of masses is always available for simultaneous detection being channelled in distinct ion beams[32].

Therefore multiple detectors are positioned to capture the ions from different m/z ratios. Those have to be robust and deliver signals that are directly related to the amount of ions that arrive at the detector, characteristics that Faraday Cups accomplish. Those are metal cups, that are grounded and every time an ion hits the surface a current flows through the grounding resistor. This results in a true measurement of ion abundance, independent of the charge, mass or energy of the ion. To prevent errors the cups are coated with carbon to inhibit the emission of secondary electrons. Depending on the application three to five such Faraday Cups are used as detectors after a magnetic sector field to measure the isotope ratios. They are set up so that the three in the middle have a mass difference of 1 and in the case of carbon they can analyse the masses 44, 45 and 46. To take small deviations of kinetic energy in the source into account the second and fourth cup are wider than the third in the middle. The biggest downside of Faraday cups is the slow response time, which proves to be problematic for measurement of unstable ion currents as in scanning mass analysers.

Thus another detector type has to be used for quadrupole mass spectrometers, usually an electron multiplier. There the incoming ions impact on a highly charged electrode, the conversion dynode, and eject secondary charged particles. The conversion dynode has to have the opposite charge than the detected ion. The secondary particles, mostly electrons, are then amplified by striking following dynodes with decreasing potential until this cascade ends at the ground level where it is converted to an electric signal. Electron multipliers have a higher signal amplification rate than Faraday Cups, but the conversion of the original ion to the secondary electrons is dependent on the nature of the ions. Heavier ions have a lower velocity and result in lesser electrons produced[48].

1.14. GC/MS analysis of endogenous steroids

Limiting factors of GC is the missing thermal stability and volatility of some substances. Even if the substances are stable, some structural elements within the molecule are difficult to work with. Functional groups containing oxygen often result in problems like peak tailing or can even react with the coating of the column. To accommodate these problems the sample preparation has to be adapted and a derivatisation step introduced. In the case of steroids their hydroxyl- and keto-groups often lead to less desirable results. These functionalities can be converted to TMS-derivatives, which have much better properties regarding gas chromatography[49]. A common reagent for that is N-Methyl-N-(trimethylsilyl)trifluoracetamide (MSTFA), that in combination with trimethylsilyl-iodide (TMSI) is a potent silylation reagent[50].

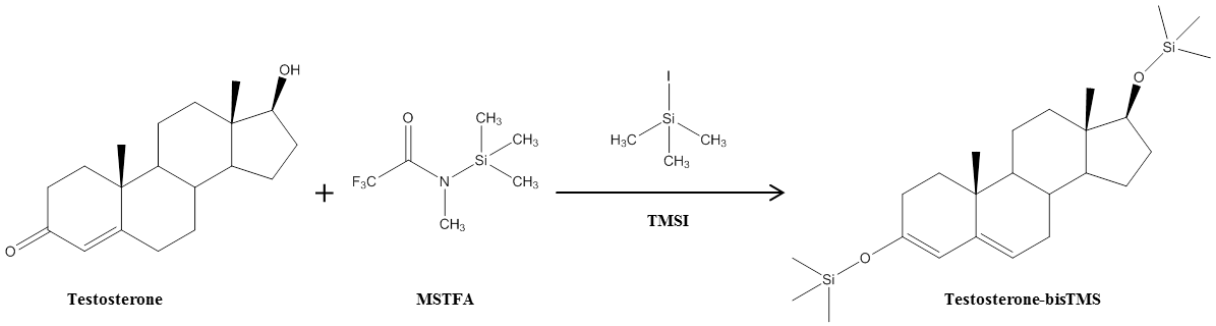


Fig. 11*: Derivatisation of Testosterone with MSTFA/TMSI*

The sample extract has to be absolute water free for this reaction to work properly and especially for quantitative analysis the yield of the reaction has to be monitored in some way. For the derivatisation of endogenous steroids the ratio of mono to Androsterone-bisTMS is used as an indicator, as it has one hydroxyl- and one keto-group that should be derivatised[51]. If the reaction is incomplete most of the steroids are only converted to mono-derivatives and the reaction has to be repeated for acceptable results.

Although IRMS is not a quantitative analysis it is important to know the steroid concentrations beforehand. With known quantities of the substances it is possible to define a specific amount that is injected into the system. This is needed to have the resulting signals within the linear range of the instrument and the analytical method. Therefore every sample has to undergo the semi quantitative in-house determination of endogenous steroids prior to IRMS confirmation. An aliquot of the urine is enzymatically hydrolysed at a certain pH-value, after an extraction step the free steroids are derivatised by MSTFA/TMSI and finally analysed on GC-MS^2. This initial testing procedure is done for almost every sample with additional substances, hence the chromatographic separation leads to less desirable results. The usage of a triple quadrupole mass analyser simplifies the allocation of the steroids to their respective peaks and fragment ratios can lead to guaranteed assignments.

1.15. GC/C/IRMS

IRMS is a technique to determine the ratio of stable isotopes in substances. In hyphenation with GC it is mostly used for Carbon, Nitrogen, Oxygen and Hydrogen. Before the mass spectrometric analysis the substances have to be converted in an easy to detectable molecule with optimally only one occurrence of the element of interest. In the case of Carbon, all organic compounds are oxidized to Carbon dioxide in a combustion interface. To accommodate the oxidation step and a continuous flow of gas into the mass spectrometer combined with the optional analysis of a reference gas a GC/C/IRMS is built different from a normal GC/MS. The main points of difference are the combustion interface, the control of the gas flows, the open split port and even the type of mass spectrometer.

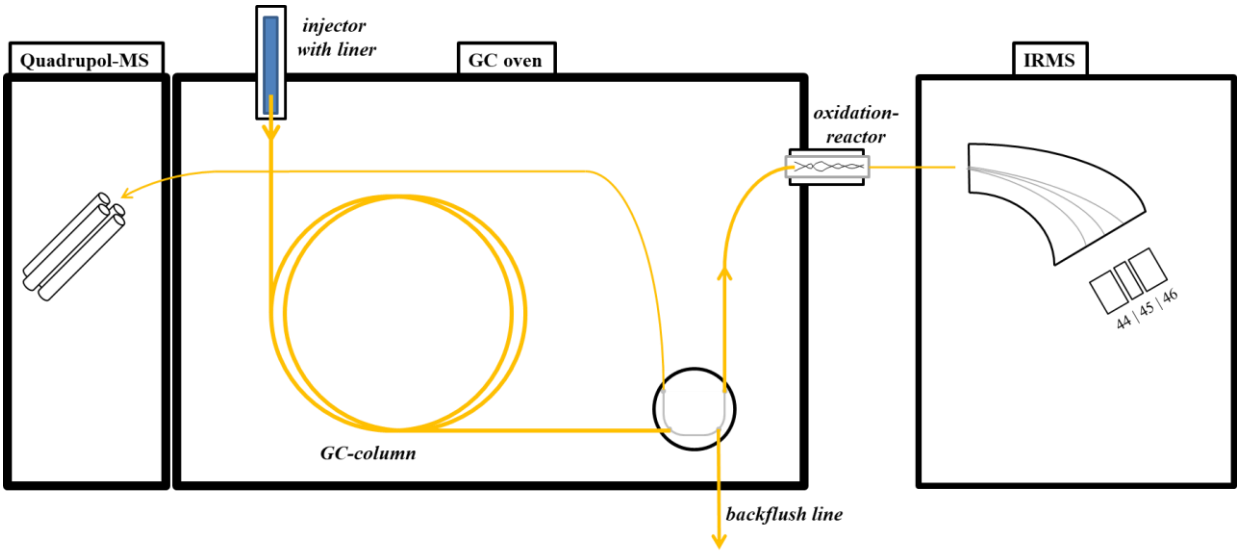


Fig. 12: *simplified schematic of a GC/C/IRMS instrument hyphenated with an additional quadrupole mass spectrometer as used in this thesis. The direction of the flows is dependent on the operation mode, except of the GC column.*

The first part after the end of the GC-column is a connection to a three or four way splitter inside the oven. One way each goes into the backflush, the combustion reactor and the last optional one into the quadrupol mass spectrometer. If the instrument is put in backflush mode an additional helium flow from the mass spectrometer side of the instrument is employed through the vent and the backflush valve is opened. As a result the column flow is directed out of the system, sparing the reactor and the mass spectrometer afterwards. This is crucial for flushing out the solvent from the injection. Another application for an open backflush valve is the conditioning of the reactor. As this is done by flushing the reactor with pure oxygen the column must be protected. Therefore the excess oxygen is guided out of the system through the backflush valve. But for the analysis of substances it has to be closed so that the column flow is guided through the combustion interface to the mass spectrometer.

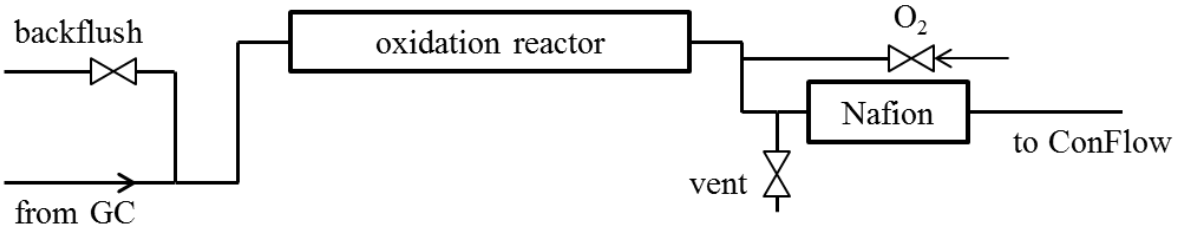


Fig. 13: *Schematic of the gas pathways inside a GC/C/IRMS*

The oxidation takes place in the reactor of the combustion interface, an alumina tube filled with metal wires. For carbon IRMS the normal reactor has one nickel and copper wire coated with their oxides and an additional platinum wire. It is operated typically at 950°C oxidizing all organic matter to CO_2 and H_2O with the oxides being reduced back to their respective metals. The three metal wires have different properties. CuO is stable at temperatures around 600°C and oxidizes efficiently without additional oxygen, but degrades at higher temperatures releasing oxygen. NiO shows even at temperatures over 1000°C no oxygen bleed and is stable for a much longer time than CuO. But if no

free oxygen is present at high temperatures, it tends to pyrolyze organic compounds rather than oxidizing them. Therefore the mixture of CuO and NiO is crucial, as both oxides contribute to the oxidation reaction. As CuO degrades it provides the O_2 for NiO to oxidize the sample properly. Pt only acts as a catalyst for the oxidation reaction[52]. Another type of reactor mostly used for the simultaneous determination of nitrogen and carbon isotope ratios only consists of NiO inside a Ni-tube[53].

All reactors need to be regenerated from time to time, as the oxygen either is consumed by the organic material or bleeds out. The re-oxidation of the metal wires is done by flushing the reactor with oxygen at working conditions. For a brand new reactor the oxidation of the material and conditioning is initially done at 600°C and the temperature is increased to working temperature. There are different approaches to take care of the re-oxidation. One of them, utilized in the used methods, flushes the reactor with oxygen for a short time before every sample, the so-called seed oxidation. The other option is to stop with the chromatography every set number of samples and have an oxidation time of a few hours. The number of samples that can be run at once is higher with seed oxidation, but before the sequence the reactor needs to be conditioned with oxygen for several hours or even overnight. After the oxidation reactor the stream of gas should only consist of the carrier He and the oxidation products CO_2 and H_2O. Water in the ion source can lead to problems, as it is able to protonate CO_2 producing HCO_2^+. These ions mimic heavy CO_2 resulting in false values for the masses 45 and 46.

Therefore the gas stream has to be dried after the oxidation reactor. In the standard instrument configuration the gas flow goes through a Nafion membrane after the reactor. The membrane is only permeable for water, which gets carried away by a reverse flow of dry helium on the outside. Additionally a water freezing trap can be employed using liquid nitrogen to remove water[54]. An unpublished method is the usage of immobilized phosphorous pentoxide in a small glass tube. The gas stream flows through the tube and water is absorbed by the desiccant However these small water traps do not last very long and have to be prepared once per week. This method is used in-house on one instrument. The preparation is simple but takes practice, so the use of commercial Nafion membrane is preferred.

The dried sample stream is transferred to a manifold where all gas flows are controlled, the so-called ConFlow. To measure accurate isotope ratios the mass spectrometer needs a low stable flow of gas into the ion source and the possibility of switching to a reference gas. To accommodate high flows of an elemental analyser the sample flow needs to be reduced in an open split. This is not so much the case for GC/C/IRMS hyphenation, but the same principle is used in the reference open split. The open split is a volume at atmospheric pressure, where the transfer capillary to the mass spectrometer, the sample or reference gas line and an additional helium capillary for dilution are connected.

In the sample open split a minimum flow of helium is always applied to prevent air from coming in. This would dilute the incoming sample gas from the GC too much, so the MS capillary is designed to

be moveable and can slide inside the sample capillary for complete. With this loose connection the mass spectrometer is not overloaded and excess gas is flushed out with the constant helium flow to purge the volume. In the reference open split the capillary leading to the mass spectrometer is not directly linked to the reference gas capillary. The vacuum from the mass spectrometer constantly draws gas from the reference open split, excess is guided out of the system. By applying additional flows of helium into the open split the initial reference gas flow is diluted.

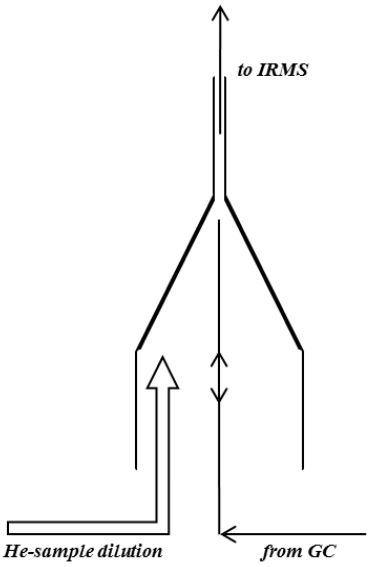


Fig. 14*: schematic of the open split in the ConFlow of an IRMS instrument*

The IRMS mass spectrometer utilizes electron ionisation, a magnetic sector analyser and multiple faraday cups as detectors. They have been described in a previous chapter. The mass to charge ratios measured in the three middle detector cups are 44, 45 and 46, the most common isotope combinations of CO_2, which are listed in **Table 2**.

Table 2*: the three ions measured and their possible CO_2 isotopic compositions, ranked by natural occurance.*

m/z	Isotopic composition
44	$^{12}C^{16}O^{16}O^{+}$
45	$^{13}C^{16}O^{16}O^{+}$, $^{12}C^{17}O^{16}O^{+}$
46	$^{12}C^{18}O^{16}O^{+}$, $^{13}C^{17}O^{16}O^{+}$, $^{12}C^{17}O^{17}O^{+}$

As one can deduct from the table, stable oxygen isotopes also have an influence on carbon dioxide isotopologues. To avoid overestimation of the ^{13}C proportion the amount of ^{17}O has to be subtracted from the 45m/z signal. The exact oxygen isotope ratios of the used gas are not known, but the share of ^{17}O is calculated based on the assumed relationship of $^{18}O/^{17}O$. Furthermore the abundance of ^{18}O is deduced by comparison of 46m/z and 44m/z, as most of the contribution to the 46m/z signal should come from ^{18}O[55].

Since VPDB is only a virtual primary reference material and therefore not usable for steroid analysis, the instrument has to be calibrated with suitable certified reference materials. To factor in the chromatographic separation in front of the IRMS, these calibrating standards should be similar to the substances of interest. As a result common standard mixtures of alkanes cannot be used, since these substances show very different properties than steroids. Therefore different certified steroid standards from Cornell University with the codes CU/USADA are utilized[56]. These standards are measured before and after every run to check the stability of the instrument. If needed the $\delta^{13}C$-value of the reference gas can be adjusted to match the reference materials. The $\delta^{13}C$-value of the reference gas is supposed to be stable, so usually this adjustment only needs to be made when the instrument configuration or the gas cylinder is changed. Every analysis starts and ends with short impulses of the reference gas to which a $\delta^{13}C$-value has been assigned before. These signals are within the backflush

time and do not interfere with the chromatographic separation. One of those, but not the first or last, is used as a reference point of this specific chromatographic analysis for the $\delta^{13}C$ determination.

The integration of chromatographic peaks in GC-C-IRMS is also not trivial, as there are many possible mistakes. Since the isotopes have different physical properties, these small but perceivable distinctions are passed to the molecules built from them. This is noticeable in the composition of the chromatographic peaks, as heavier molecules show less interactions with the stationary phase and elute faster, resulting in an earlier peak start of 45 m/z[57].

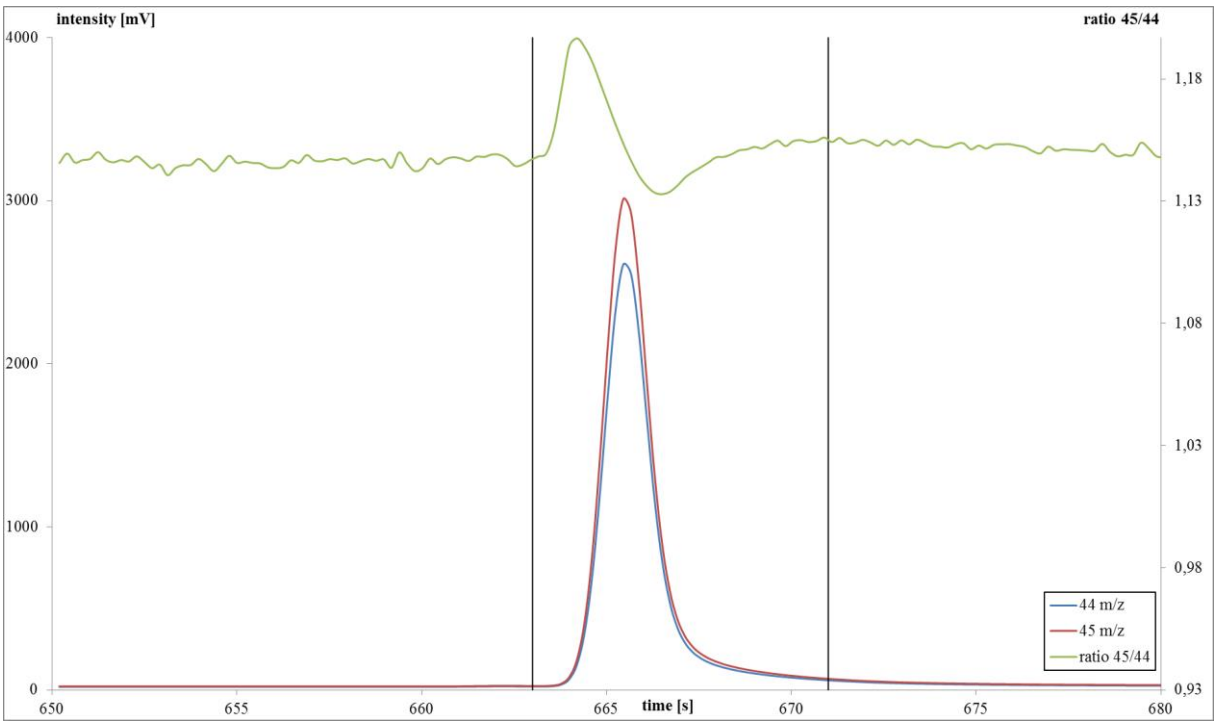


Fig. 15*: presentation of a GC/C/IRMS peak, the masses 44 and 45 are depicted together with their ratio. The intensity of the peaks is shown on the left main vertical axis, while the ratio is shown on the secondary axis. The vertical lines represent the optimal borders for peak integration.*

For data evaluation it is important, that the whole peak is integrated correctly and these differences in retention time are accounted. This can be done by either looking at both data traces for 44 and 45 m/z or observing 44 m/z and the ratio of 45/44. In the beginning of the elution process the ratio rises, then changes to lower values than the background and finally swings back to the ratio it had before. Peak integration must start before the rise of the ratio and has to end after the ratio returned to the value before the peak, otherwise the resulting $\delta^{13}C$ values are incorrect.

The 45/44 ratio can also be used to assess a peak for coelution and possible contaminations. These are visible if the ratio has an asymmetric swing and or additional extrema. Peak evaluation is especially important, if the GC is not additionally coupled to a scanning mass spectrometer. Even if the identity and purity of the substance within the peak can be assured simultaneously on a different detector, it is faster and more convenient to observe the ratio of the CO_2 isotopologues. A downside of this method is that different substances with the exact same chromatographic behavior cannot be distinguished.

Special emphasis lies on peak background settings, since they serve as a base for integration. The chosen background should be stable and show a constant signal, or the d^{13}C-value of the peak is false. By default only the trace of 44 m/z is shown, so it is important to monitor the 45/44 ratio as well to see if there is no change in the background. A background richer in ^{13}C leads to peaks with more negative ^{13}C-values. Therefore the wrong integration setting can result in false positive samples, if the background for the target compounds was chosen incorrectly. If a sample fraction is clearly contaminated and or no acceptable separation can be achieved it is in the judgment of the certifying scientist to reject the results. In some dirty samples peaks of some substances show broad shoulders or even appear as double peaks. Even though the integration over the whole thing would result in a reasonable δ^{13}C-value, it has to be viewed cautiously.

One approach to get better chromatographic results with fewer problems regarding the integration is the derivatisation. This enhances the thermal stability, decreases the boiling point and increases the structural differences between some steroid isomers. The combustion interface between the GC and the mass spectrometer limits the options of derivatisation. Usually steroids are converted to TMS derivatives for GC by silylation with MSTFA/TMSI, but the introduction of other elements than carbon, hydrogen and oxygen can affect the combustion reactor. In the case of silicon from the silylation agent siliconoxide is formed and tends to coat the metal wires or even clogs the reactor tube, making it unusable. Another unwanted side reaction is the formation of copper-silicon compounds[58]. Therefore another derivatisation technique is needed which is quantitative, selective for hydroxyl groups and contains no additional elements.

Thus acetylation of free hydroxyl groups is a common derivatisation for steroids in IRMS. The reaction is simple, fast and results in quantitative yields, all characteristics that are essential. Finally it fulfils an important criterion for the analysis of stable isotope ratios, no isotopic discrimination during the derivatisation reaction[59].

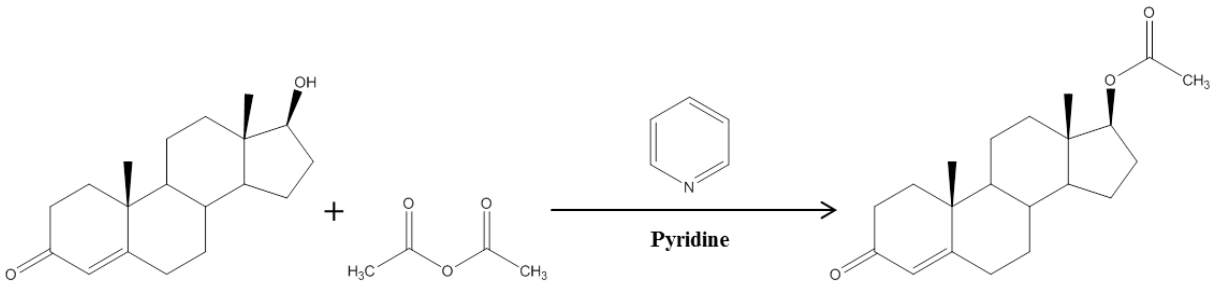


Fig. 16*: Acetylation reaction of Testosterone with Acetic anhydride resulting in Testosterone acetate*

However with the attachment of the Acetyl-group two additional carbon atoms are introduced to the molecule per hydroxyl group. Therefore the exact isotopic ratio of the derivatization reagent has to be known and considered in the final calculation of the delta values.

$$\delta^{13}C_{St} = \left(\delta^{13}C_{dSt} * n_{dSt} - \delta^{13}C_{d} * n_{d}\right)/n_{St}$$

Eq. 3*: equation to calculate the final $\delta^{13}C$ value of the original steroid (St) based on the analysis of a derivatised steroid (dSt) where n is the number of carbon atoms. The derivatising compound is denoted with d.*

The δ^{13}C-value of the acetic anhydride can be determined by acetylation of certified reference materials with known δ^{13}C-values. This is done by comparing theoretical value of the free and the measured value of the acetylated steroid and the difference per carbon atom in the substances. Although derivatisation can positively affect the steroids chromatographic behaviour it is an additional step in the sample preparation that is accompanied by possible analyte loss and impurities. The two methods compared in this thesis do not make use of this technique to keep the already complex sample preparation simpler.

As mentioned before the biggest downside of Carbon-IRMS is the missing information about the substance since all organic matter is transformed to CO_2. A comparison with pure standards allows identification only by retention time, which is not satisfactory. To elucidate the peaks a second analysis has to be performed without the oxidation step prior to the mass spectrometer. This can be done either with a second injection on a different mass spectrometer or on a modern instrument that is coupled to an additional mass analyzer. These types of machines have a junction in the oven after the GC-column next to the backflush line. The gas flow is split to the oxidation reactor and a small amount to the second mass spectrometer.

2. Materials & Methods

All steroids and steroid conjugates were purchased from Steraloids Inc., the source of the other materials in given in brackets.

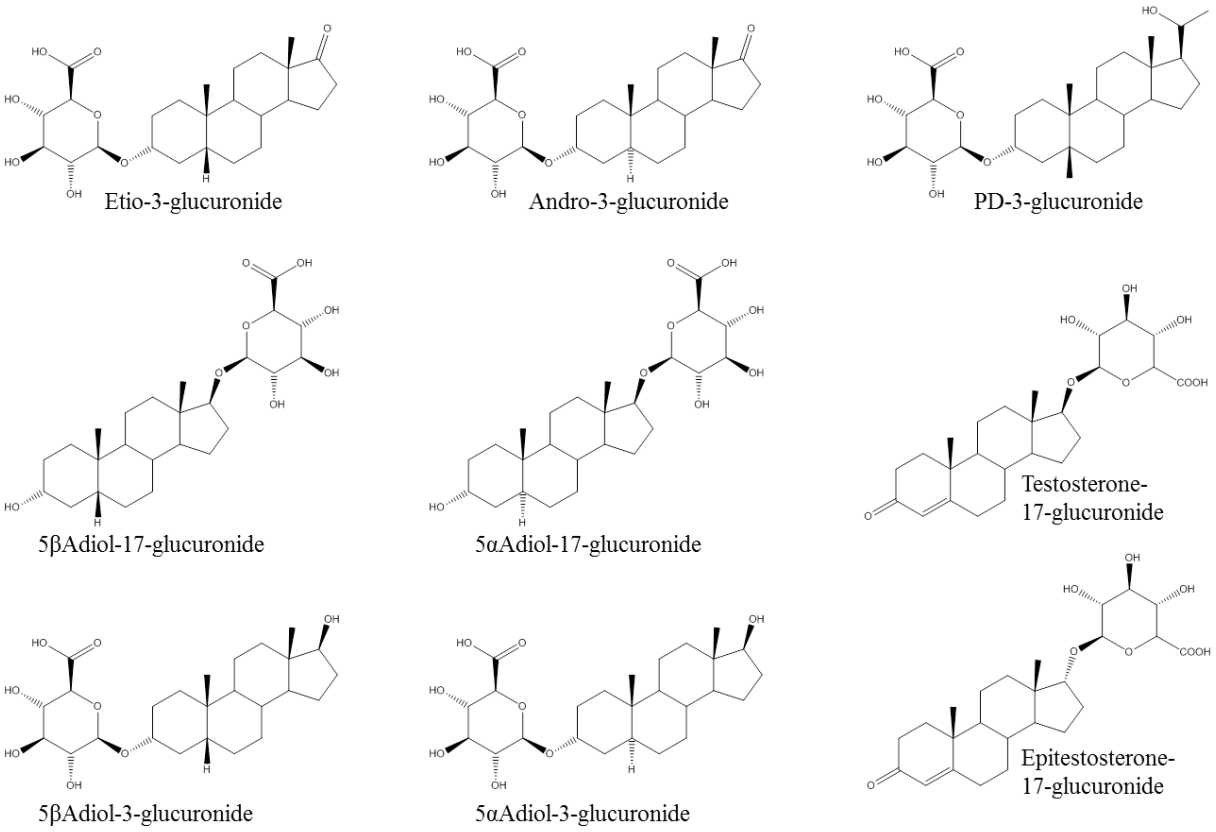


Fig. 17*: structural formulas of the steroid conjugates that were used in this thesis.*

2.1. Samples

Positive (QCP) and negative (QCN) quality control sample are chosen for the tests. Those are measured in routine analysis with every batch, so the steroid profiles as well as the corresponding $\delta^{13}C$-values are known. QCN originates from a colleague (male, 20 years) with no prior exposure to any steroids or related substances. QCP is a mixture of different urine samples from an androstenedione excretion study, done by a coworker (male, 51 years). Additionally a sample of the author's urine (male, 27 years) is used as a sample with known steroid profile but no information about the $\delta^{13}C$-values. This is

Table 3*: Sample concentrations of the relevant steroids, determined by the initial testing procedure used in-house for the analysis of endogenous steroids.*

Substance	QCP (10mL)	QCN (12mL)	TL (15mL)
	Concentration in ng/mL		
Testosterone	69	63	79
Epi	22	30	39
5βAdiol	423	165	623
5αAdiol	131	85	75
Etio	7401	2068	3984
Andro	4044	3115	2970
PD	237	399	587

to simulate an unknown negative sample. A batch of five QCPs, five QCNs and six aliquots of the author's urine were submitted to the final sample preparation procedure.

2.2. Solid Phase Extraction:

The first step is an adaption of the SPE procedure of the routine method on C18 cartridges (Biotage, ISOLUTE C18, 500mg/6mL). Conditioning of cartridges is achieved by consecutive washing steps with MTBE (Honeywell, CHROMASOLV), MeOH (VWR, HiPerSolv CHROMANORM) and ultra-pure water (purified by Milli-Pore device to 18.2 MΩ.cm and <2ppb TOC). After loading the sample the cartridge is rinsed three times with methanol in water (5%) and dried under vacuum. The sample is eluted with MeOH and a mixture of MeOH and MTBE (1:2). After removing the organic solvents the extracts are vacuum dried over phosphorus pentoxide for 30 minutes.

2.3. Separation and Purification by HPLC:

A chromatography system with an automated fraction collector is used (UltiMate 3000 with AFC3000 from Thermo Fisher), with Chromeleon (Version 7.2 SR4) as software. The column (Phenomenex Kinetex C18, 2.6µm, 100Å, 4.6x150mm) with a precolumn (Phenomenex Security Guard C18, 5µm) is installed in the column department at 30°C. Water (solvent A) (VWR, HiPerSolv CHROMANORM) and acetonitrile (solvent B) (VWR, HiPerSolv CHROMANORM) with each 0.1% formic acid (Sigma-Aldrich, 98-100% puriss.) are used as solvents for the chromatography. Before every run, the solvent lines are purged and afterwards the column flow is increased to the final flow of 0.7mL/min. The solvent composition is set to the starting conditions of the gradient and kept until a stable backpressure is reached. The autosampler is kept at 15°C, an injection volume of 43µL is used and UV-absorption at 190nm and 245nm is monitored during the analysis. The first injection of a sequence is always a methanol blank; afterwards multiple standard mixtures are injected to adapt the fraction collection time windows.

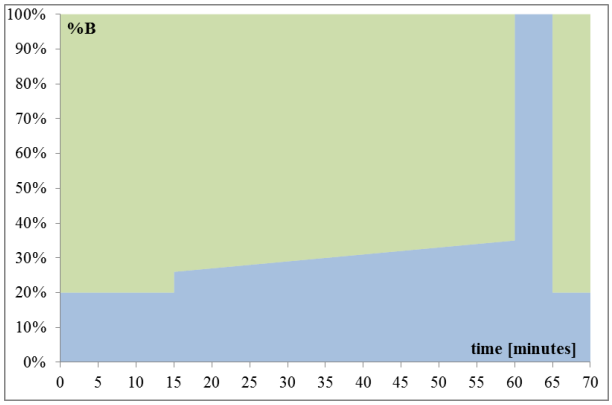


Fig. 18*: presentation of the used gradient for the HPLC separation of steroid glucuronides. The gradient starts at 20%B, after 15 minutes the slope starts at 26%B and ends at 60 minutes and 35%B, afterwards a washing step is utilized.*

After the last pre-sample standard the automated fraction collector is set back to starting point and labelled glass tubes are put in the right position. Samples are diluted with 45µL of a mixture of water, acetonitrile and methanol (75:20:5, all HiPerSolv CHROMANORM). After the last sample a post-sample standard is analyzed to monitor the stability of the retention times. The fractions in the glass tubes are checked for correct sample collection, as the same fractions should have identical volumes of

liquid. The solvent is removed in a vacuum centrifuge at 45°C for at least 90 minutes or until completely dry before the next step.

Table 4*: composition of the standard mixture used to set the collection time. These times have inter-sequential differences and the given times are just examples from one run.*

Substance	Concentration [µg/mL]	Retention time [min]	Collection Time [min]	Fraction
Testosterone-17-glucuronide	60	27.2	26.5-28.0	1
5βAdiol-3-glucuronide	100	35.8	35.1-39.0	2
5αAdiol-3-glucuronide	100	36.5		
Epitestosterone-glucuronide	100	37.1		
5αAdiol-17-glucuronide	60	37.1		
5βAdiol-17-glucuronide	100	38.2		
Androsterone-3-glucuronide	60	44.3	43.2-47.2	3
Etiocholanolone-3-glucuronide	60	46.3		
Pregnanediol-3-glucuronide	100	57.3	55.8-58.3	4

2.4. Acidic hydrolysis of steroid glucuronides:

The dry extract is purged with Argon for at least 30 seconds. Under a constant flow of Argon 500µL MTBE (Honeywell, CHROMASOLV) and HCl (Sigma Aldrich, 1.25M in methanol, for GC derivatization) are added and tubes are sealed. They are stored in a water bath at 45°C overnight for hydrolysis. Afterwards the liquid gets removed by evaporation in a vacuum centrifuge at 45°C. For liquid-liquid extraction and neutralization 20% carbonate buffer (pH 8.6) and of a mixture of equal parts n-pentan (Merck, UniSolv) and MTBE is added. After shaking for 10 minutes and centrifuging the organic layer containing the free steroids is taken off into a separate tube. After evaporation of the solvent the dry extract is transferred from the tube with MTBE into smaller autosampler vials for GC/C/IRMS analysis.

2.5. GC/C/IRMS:

Table 5*: summary of the used dilutions of the final fractions for GC/C/IRMS analysis.*

	QCP	QCN	TL
Fraction 1	30	30	30
Fraction 2	100	100	200
Fraction 3	850	750	900
Fraction 4	30	45	75

Before the analysis the dry extract is diluted with Androstanol (17ng/µL in MTBE) as a retention time standard, according to the approximate concentrations of the corresponding steroids. The goal is to get a dilution that produces signal intensities in the linear range of the analysis method. The instruments used from Thermo Fisher Scientific are a Trace GC 1300, with a TriPlus RSH Autosampler and an ISQ3000. The IRMS is a Delta V Advantage Plus with the GC IsoLink II and a ConFlow IV. To set up the GC Chromeleon (Version 7.2.8) is used and for IRMS IsoDat (Version 3.0).

A column with intermediate polarity (Restek, Rxi-17-Sil-MS, 30m, 0.25mm ID, 0.25µm film thickness) is used for separation and a deactivated Borosilicate liner (Restek, Topaz single taper 2.0mm ID) is used in splitless mode with surge. Samples and standards are stored in the autosampler at 15°C. The syringe is washed three times with iso-propanol (Merck, LiChrosolv) and iso-octane (Merck, SupraSolv) before and after every injection. For every sample and standard a fixed volume of 1.2µL is injected.

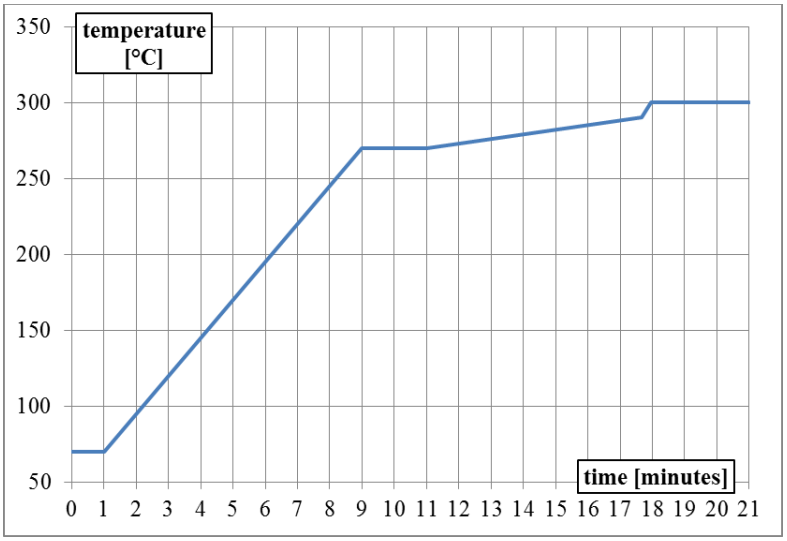


Fig. 19*: visualization of the used temperature gradient for GC with the heat rates, target values and hold times in the table below.*

Table 6*: temperature gradient used in GC/C/IRMS*

Heat rate [°C/min]	Target value [°C]	Hold time [min]
0	70	1
25	270	2
5	290	0
35	300	3

The inlet is kept at 275°C and a constant column flow is set to 1.6mL/min. After a splitless time of 1.5min a split flow of 99mL/min is employed, this results in a split ratio of 62. During the splitless time the purge flow of 5mL/min is stopped and a surge pressure of 270kPa is applied to the inlet for better sample transfer to the column. The same temperature gradient that is used for routine analysis is also employed in this method. Since MTBE with a boiling point of 55°C is chosen to dilute the samples the solvent effect cannot be utilized at a starting temperature of 70°C. The final temperature of 300°C is used to bake out the column and get rid of higher boiling impurities.

The oxidation reactor is kept at 950°C and is flushed for 0.3 minutes with O_2 prior to every measurement for seed oxidation. Before the acquisition starts the magnet finds the peak center, by automatically focusing on the reference gas CO_2. The first 540 seconds of the analysis are in backflush mode with five 20 second long pulses from the reference gas. The fourth pulse is used to set the $\delta^{13}C$ value for the whole measurement. After the peak of the last compound of interest the backflush valve is opened again at 1140 seconds. Afterwards two final reference gas pulses are employed to compare the stability before and after the sample elution.

The single quadrupole is set to start scanning at 8.5 minutes until the end of the GC run time in a range of 40-350m/z with a scan time of 0.2 seconds. The ion source is operated in electron ionization mode at 250°C and the MS transfer line is heated to the same temperature.

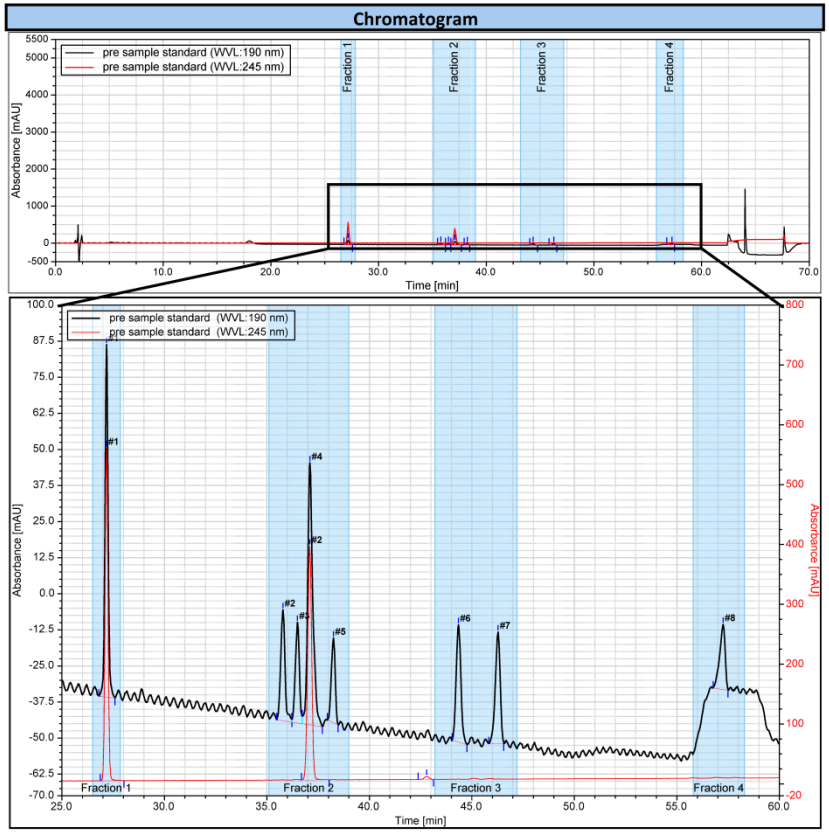
3. Results

Within this proof of concept study an alternative sample preparation method for the detection of EAAS with exogenous origin in urine by GC/C/IRMS was developed.

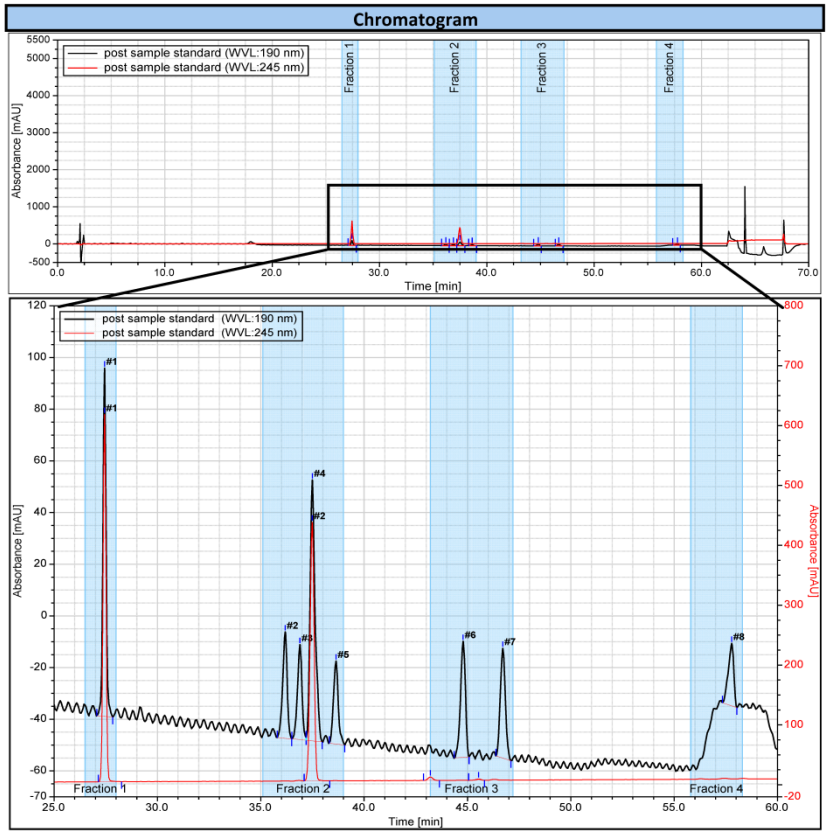
The employed liquid chromatography is efficient enough to separate steroid glucuronides for fraction collection with one or more substances per fraction. Although the clear separation is only visible in a mix of pure standards with no added matrix, it also applies to urine samples. The correct fraction collection of samples is confirmed by the following GC/C/IRMS analysis.

The comparison of standards before and after samples during the sequence gives the stability of retention times. All substances are within the fraction collection windows before and after the injection of 16 samples.

After chromatographic separation and chemical hydrolysis the fractions are individually analysed on GC/C/IRMS resulting in $\delta^{13}C$-values for every detected substance. Peak identity is ensured by analysis on a hyphenated single quadrupol mass spectrometer.



Peak#		Substance		Retention Time [min]		Fraction#
WVL:190 nm	WVL:245 nm	WVL:190 nm	WVL:245 nm	WVL:190 nm	WVL:245 nm	
1	1	Testosterone-Gluc	Testosterone-Gluc	27.18	27.18	1
2	n.a.	5ß-Androstanediol-3Gluc	n.a.	35.79	n.a.	2
3	n.a.	5a-Androstanediol-3Gluc	n.a.	36.49	n.a.	2
n.a.	2	Epitestosterone-Gluc	Epitestosterone-Gluc	n.a.	37.09	2
4	n.a.	5a-Androstanediol-17Gluc	n.a.	37.10	n.a.	2
5	n.a.	5ß-Androstanediol-17Gluc	n.a.	38.24	n.a.	2
6	n.a.	Etiocholanolone-Gluc	n.a.	44.34	n.a.	3
7	n.a.	Androsterone-Gluc	n.a.	46.28	n.a.	3
8	n.a.	Pregnanediol-Gluc	n.a.	57.26	n.a.	4



Peak#		Substance		Retention Time [min]		Fraction#
WVL:190 nm	WVL:245 nm	WVL:190 nm	WVL:245 nm	WVL:190 nm	WVL:245 nm	
1	1	Testosterone-Gluc	Testosterone-Gluc	27.44	27.44	1
2	n.a.	5ß-Androstanediol-3Gluc	n.a.	36.18	n.a.	2
3	n.a.	5a-Androstanediol-3Gluc	n.a.	36.89	n.a.	2
n.a.	2	Epitestosterone-Gluc	Epitestosterone-Gluc	n.a.	37.49	2
4	n.a.	5a-Androstanediol-17Gluc	n.a.	37.49	n.a.	2
5	n.a.	5ß-Androstanediol-17Gluc	n.a.	38.64	n.a.	2
6	n.a.	Etiocholanolone-Gluc	n.a.	44.79	n.a.	3
7	n.a.	Androsterone-Gluc	n.a.	46.71	n.a.	3
8	n.a.	Pregnanediol-Gluc	n.a.	57.78	n.a.	4

Fig. 20: *comparison of the chromatographic separation of a standard mixture before (shown left) and after (shown right) the injection of urine samples. The absorbance at 190 (shown in black) and 245nm (shown in red) is plotted over time. The table below contains the detected substances and their retention times and their respective fractions of the automated fraction collection.*

Table 7*: Presentation of the IRMS results. For each sample and substance the $d^{13}C$-value is depicted with the respective peak intensity of the mass 44 if detectable. The table's columns represent the substances grouped according to their HPLC fractions. The samples are grouped in QCP, QCN and TL, named after the donor of the additional negative sample. The first line of each sample block shows the urinary concentration of the respective steroid. Samples marked with as asterisk were analysed without the internal standard Androstanol. QCP5 already showed no signals during HPLC, therefore the contents must have been lost before. Not detected peaks are denoted with the abbreviation n.d.*

	Fraction 1	**Fraction 2**			**Fraction 3**		**Fraction 4**
	Testosterone	**5bAdiol**	**5aAdiol**	**Epi**	**Etio**	**Andro**	**PD**
QCP	69 ng/mL	423 ng/mL	131 ng/mL	22 ng/mL	7401 ng/mL	4044 ng/mL	237 ng/mL
1*	-28,8 *(200mV)*	-28,5 *(1300mV)*	-27,6 *(400mV)*	n.d.	-28,3 *(4800mV)*	-25,7 *(2800mV)*	-22,1 *(800mV)*
2	-26,6 *(100mV)*	-30,0 *(1600mV)*	-27,6 *(500mV)*	n.d.	-28,8 *(4700mV)*	-28,0 *(2500mV)*	-22,7 *(600mV)*
3	-29,8 *(100mV)*	-29,4 *(1000mV)*	-27,7 *(300mV)*	n.d.	-29,1 *(4700mV)*	-28,2 *(2500mV)*	-22,4 *(500mV)*
4	-28,8 *(300mV)*	-29,5 *(1800mV)*	-27,1 *(700mV)*	n.d.	-29,1 *(5100mV)*	-28,2 *(2700mV)*	-22,4 *(600mV)*
5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
QCN	63 ng/mL	165 ng/mL	85 ng/mL	30 ng/mL	2068 ng/mL	3115 ng/mL	399 ng/mL
1*	-21,9 (500mV)	-22,0 *(600mV)*	-23,0 *(600mV)*	n.d.	-21,9 *(2400mV)*	-21,7 *(3400mV)*	-20,9 *(1600mv)*
2	-22,0 (500mV)	-23,8 *(700mV)*	-23,2 *(700mV)*	n.d.	-22,2 *(2000mV)*	-21,3 *(3000mV)*	-21,6 *(1300mV)*
3	-21,8 (500mV)	-23,5 *(600mV)*	-21,5 *(500mV)*	n.d.	-22,5 *(2100mV)*	-22,0 *(3100mV)*	-22,1 *(2200mV)*
4	-22,5 (400mV)	-23,0 *(700mV)*	-23,0 *(600mV)*	n.d.	-22,1 *(2100mV)*	-21,8 *(3100mV)*	-21,5 *(1900mV)*
5	-21,1 (400mV)	-23,0 *(500mV)*	-22,1 *(500mV)*	n.d.	-22,0 *(1900mV)*	-21,9 *(2800mV)*	-21,5 *(1600mV)*
TL	79 ng/mL	623 ng/mL	75 ng/mL	39 ng/mL	3984 ng/mL	2970 ng/mL	587 ng/mL
1*	-23,2 *(700mV)*	-22,3 *(3000mV)*	-23,1 *(1200mV)*	n.d.	-21,8 *(5000mV)*	-22,4 *(3000mV)*	-22,2 *(2300mV)*
2	-23,2 *(1100mV)*	-22,8 *(3100mV)*	-23,2 *(1200mV)*	n.d.	-22,8 *(5400mV)*	-22,6 *(3800mV)*	-22,1 *(3500mV)*
3	-23,0 *(1000mV)*	-22,5 *(2800mV)*	-22,8 *(1100mV)*	n.d.	-22,2 *(5200mV)*	-22,2 *(3200mV)*	-21,6 *(2200mV)*
4	-22,4 *(900mV)*	-22,4 *(3200mV)*	-23,3 *(1000mV)*	n.d.	-22,3 *(5400mV)*	-22,3 *(4600mV)*	-22,1 *(3200mV)*
5	-22,6 *(700mV)*	-22,6 *(3600mV)*	-23,2 *(1300mV)*	n.d.	-22,3 *(6000mV)*	-22,5 *(3500mV)*	-21,7 *(2500mV)*
6	-22,2 *(100mV)*	-22,6 *(3100mV)*	-23,9 *(1200mV)*	n.d.	-22,3 *(5800mV)*	-22,6 *(3200mV)*	-21,8 *(3200mV)*

From those results of the $\delta^{13}C$-values average values with their standard deviations can be calculated. To get an overview of the validity of the method the results are compared to those of the already employed routine analysis. Since Epi is not detectable in any sample it is excluded from further results and discussion.

Table 8: *Summary of the $\delta^{13}C$-values of the QCN and QCP, with a comparison of the routine method and the new alternative method. The $\delta^{13}C$-values are displayed with its standard deviation.*

QCN			**QCP**	
$\delta^{13}C \pm s_d$	$\delta^{13}C \pm s_d$		$\delta^{13}C \pm s_d$	$\delta^{13}C \pm s_d$
routine (n=17)	*new (n=5)*	Substance	*routine (n=17)*	*new (n=4)*
-21.70 ±0.14	-21.86 ±0.45	Testosterone	-28.37 ±0.37	-29.13 ±0.47
-21.83 ±0.93	-23.06 ±0.61	5βAdiol	-28.93 ±0.90	-29.35 ±0.54
-21.43 ±0.32	-22.96 ±0.47	5αAdiol	-27.83 ±0.31	-27.50 ±0.23
-21.48 ±0.10	-22.16 ±0.21	Etio	-28.28 ±0.28	-28.88 ±0.38
-22.23 ±0.39	-21.74 ±0.24	Andro	-29.03 ±0.38	-28.10 ±0.10
-20.78 ±0.50	-21.52 ±0.38	PD	-22.43 ±0.64	-22.43 ±0.18

As shown in **Table 8**, the QCP and QCN prepared with the developed sample preparation are comparable to the routine method. The QCP is positive according to the technical Document for IRMS from WADA and the QCN and the unknown test sample are negative. The $\delta^{13}C$-values of the QCs are within the two standard deviation range of the routine analysis, which proves that the method is fit for purpose. Those results are summarized for better visualization into a graph with the WADA rules concerning positive samples.

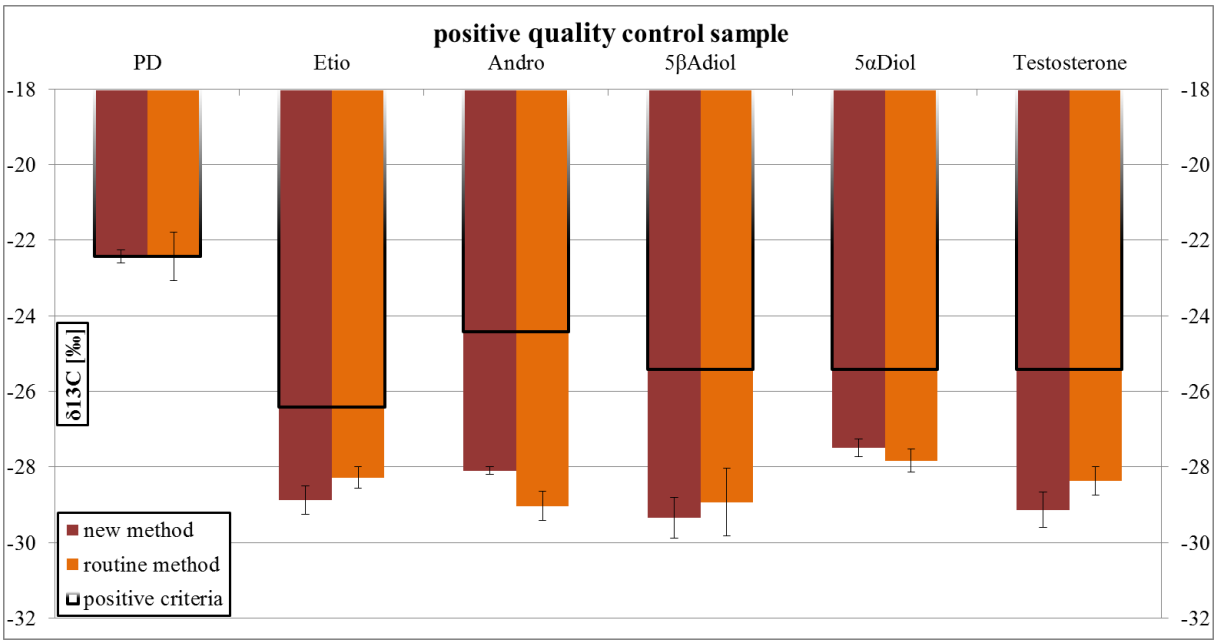


Fig. 21: *compilation of the results of the QCP. The $\delta^{13}C$-values of the new and routine method are shown with their respective standard deviations. The WADA criteria for a positive sample are depicted, based on PD of the new method as ERC.*

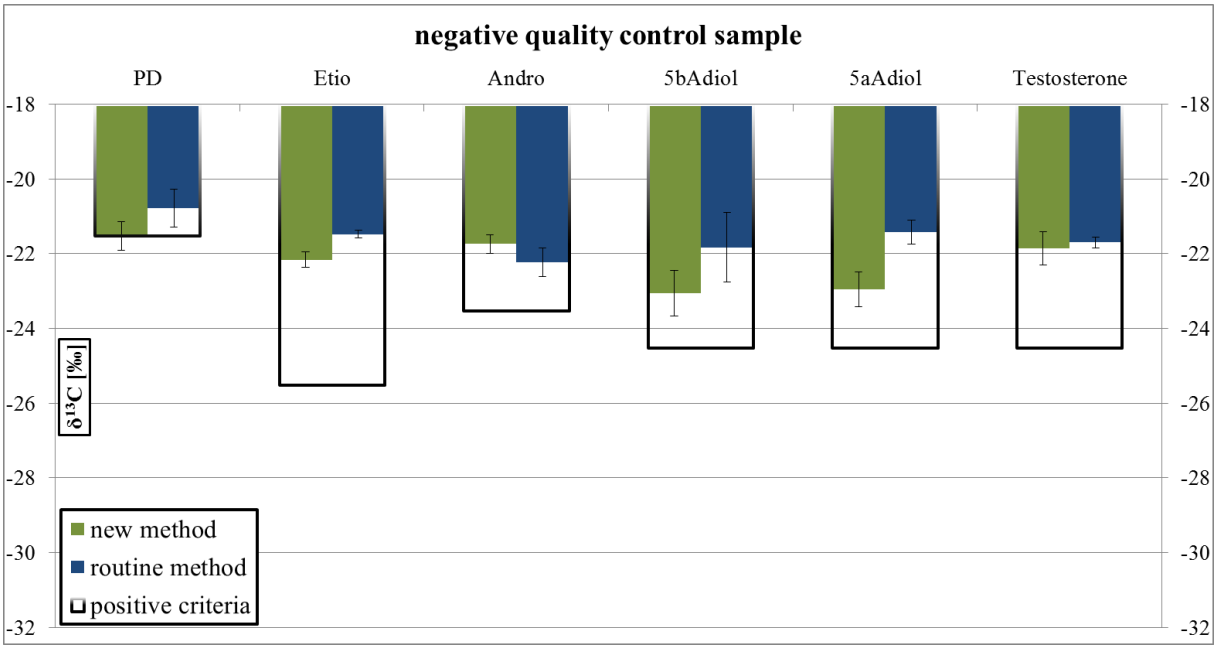


Fig. 22: *compilation of the results of the QCN. The $\delta^{13}C$-values of the new and routine method are shown with their respective standard deviations. The WADA criteria for a positive sample are depicted, based on PD of the new method as ERC.*

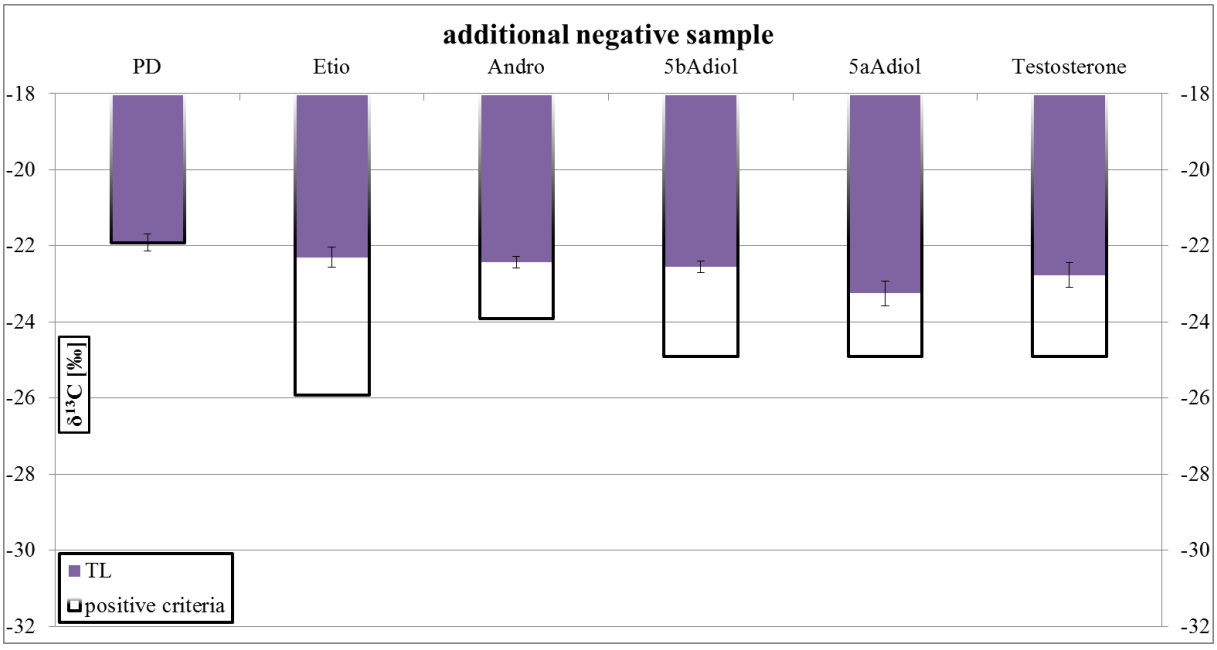


Fig. 23: *compilation of the results of the additional negative sample. The $\delta^{13}C$-values of the new and routine method are shown with their respective standard deviations. The WADA criteria for a positive sample are depicted, based on PD as ERC.*

4. Discussion

As the method consists of several important points, all of them have to be viewed separately to discuss the individual problems and potential improvements.

4.1. Solid Phase Extraction

A tested approach to reduce matrix interferences was usage of a second SPE to further separate urinary contents from the extract. The first SPE was planned to be utilized for reduction of the large sample volume. Afterwards a second SPE with a higher selectivity and stronger retention is chosen to further purify the extract. Therefore a HLB (Waters, 60mg) was used. This step was thought to be needed to protect the HPLC column, as the sample prior to HPLC is really dirty. However it made no significant difference in sample purity or matrix signals.

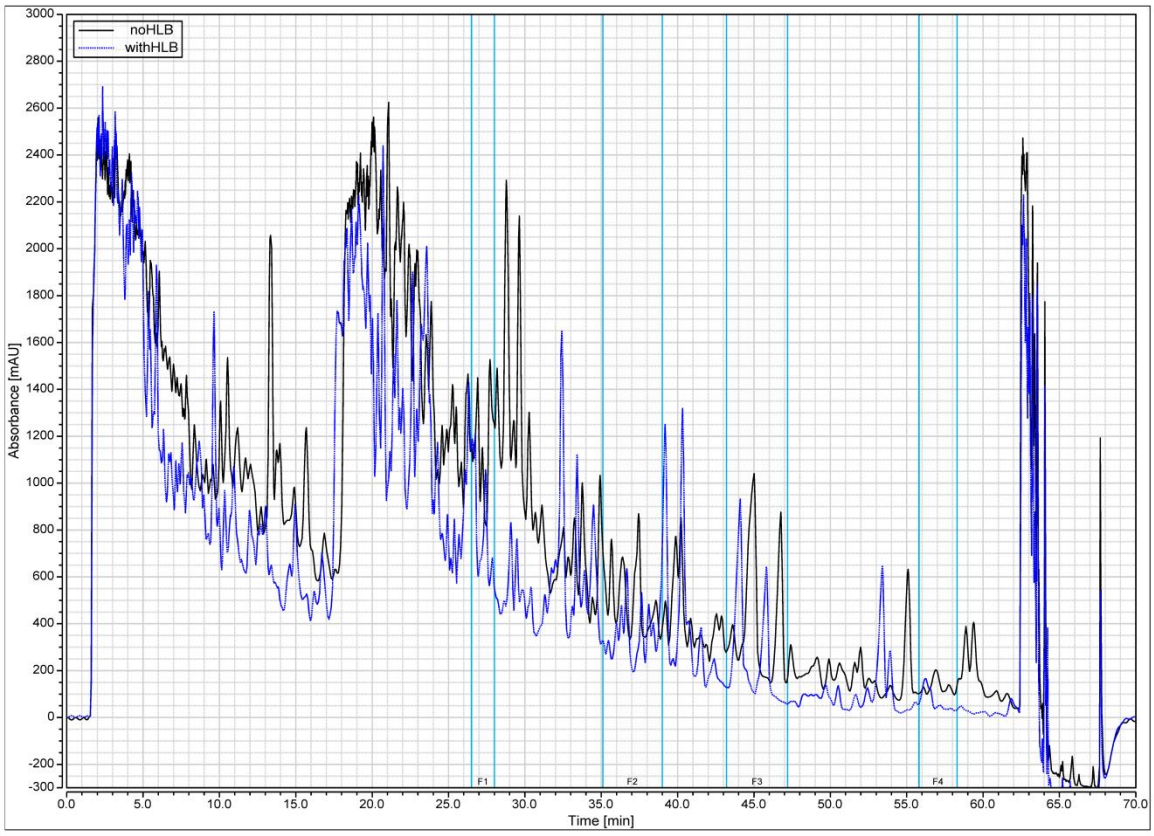


Fig. 24*: Chromatograms of the two compared sample extraction versions with the absorbance over time. The double extraction with a C18 first and a HLB cartridge afterwards is depicted in blue. The single extraction with only a C18 cartridge is shown in black. In both chromatograms 190nm is chosen as the wavelength for detection. The collected fractions are represented by the vertical blue lines. They are corresponding to the final version of the extraction and would be slightly different for the other extraction method, as the retention times and therefore the chosen fraction collection times may vary.*

The second extraction implicated potential loss of sample with no decrease in interfering matrix compounds. The additional time investment was estimated to be too much to be competitive with the routine method. Finally the idea of a double SPE was discarded and the method was carried out only with C18-material, similar to the routine sample preparation.

An idea that has not been tested yet is the usage of a completely different separation mechanism for SPE. As the steroid glucuronides are slightly acidic anion exchange should be able to separate them from other urinary compounds.

4.2. HPLC

The column-type was chosen based on published experiments[60] and an in-house testing with a LC-MS method for steroid conjugates. The goal of this method is to inject the entirety of the concentrated sample and collect well separated substances. To achieve the wanted peak capacity and separation, a wider column diameter than the usual LC-MS columns was selected. The combination of HPLC and an automated fraction collector leads to a few restrictions. With too low flows the volume of a fraction is too small to be collected properly. Higher flows lead to a higher backpressure, which the system is not able to handle. As a compromise a flowrate of 0.7mL/min was chosen, as it results in a pressure the tube fittings are capable of withstanding. Since there is only a UV detector and no mass spectrometer, the peaks have to be much purer than with conventional LC-MS, where impurities can be filtered out easily. In supplementary to the standard detection wavelength of 190nm a second channel specific for the conjugated double bonds of Testosterone and Epi at 245nm is added. Without acid in the eluents, no peaks for steroid conjugates are observed since glucuronides have to be protonated to show acceptable retention behaviour. This is achieved by adding 0.1% formic acid. The addition of formic acid to the eluents brings elevated background noise levels at 190nm, but is a necessity. It was shown[61] that the separation of steroid isomers is more efficient at lower temperatures. Therefore the temperature of the column oven was lowered compared to the routine method to 30°C. But no tests concerning the temperature dependence of the chromatographic separation have been conducted.

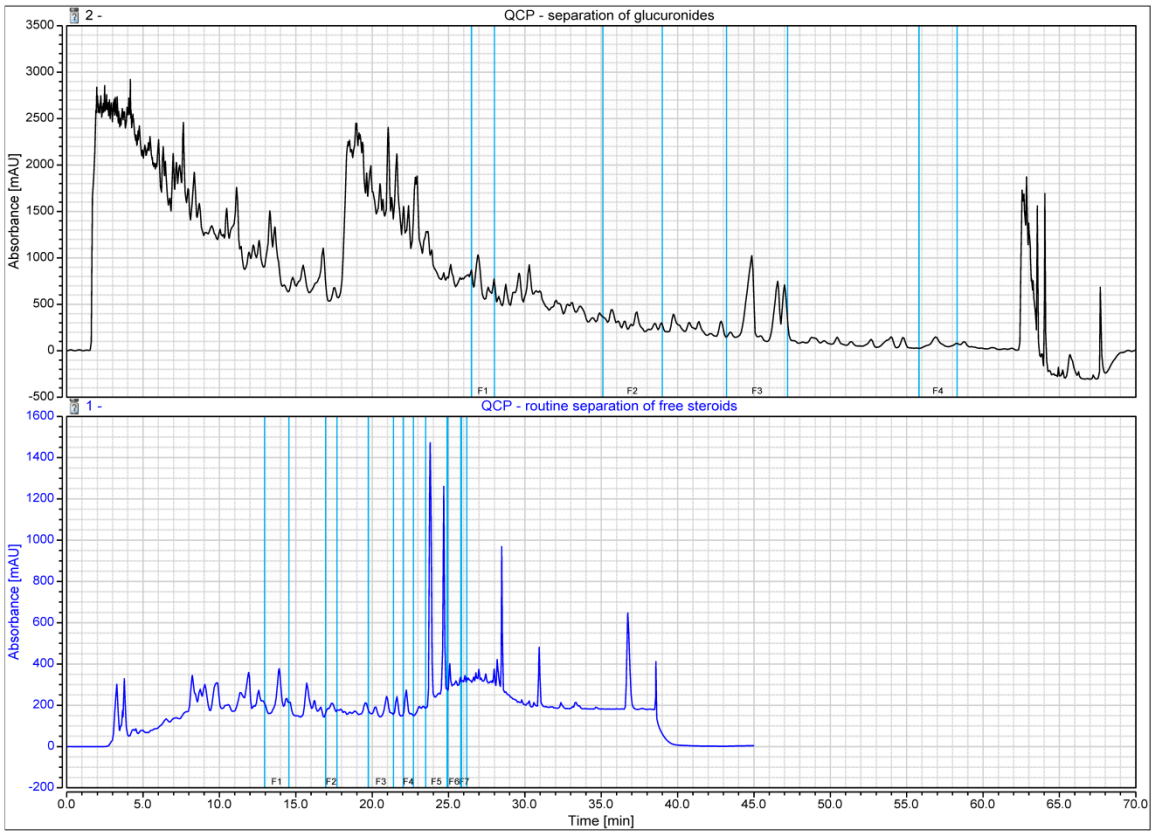


Fig. 25: *comparison of the HPLC separation of free and conjugated steroids. On top in black is the developed method and below the routine method in blue. In both chromatograms the absorbance at 190nm wavelength is shown over time. In addition the fractions that were collected in the specific sample are depicted by the vertical blue lines. The routine HPLC separation is done on a combination of two consecutive columns (XBridge Shield C18 & ACE C18, both with 5µm particles, 100Å, and 150x4.6mm) with a comparable gradient, but in only 45 instead of 70 minutes and a flow rate of 1mL/min.*

It is clearly visible that a large part of the matrix interferences are hydrophilic substances. In the newly developed method they are not as well removed in comparison to the routine method. In the standard method the steroid glucuronides are hydrolysed and then the free steroids are extracted with MTBE and n-pentane, therefore leaving hydrophilic substances behind in the aqueous phase. The gradient for the separation of the glucuronides tries to exclude most interference by employing a longer step with low content of organic solvent. That works well enough to get signals for the substances of the first two fractions. The later fractions are usually no problem, as there are not so many matrix components with a similar retention behaviour. And a factor that is much more important is the concentration difference. The glucuronides of the two main metabolites, Etio and Andro, which have high concentrations in urine, are in the third fraction. Therefore the contaminants in this fraction are diluted much more than the contaminants from the first fraction. PD-glucuronide is also not a problem, as the concentrations are sufficiently high enough, especially in women. Separation of the Androstanediol-glucuronides is a challenging aspect, as there are two hydroxyl-groups per molecule and therefore two potential sites for glucuronidation. In the preliminary tests only the 17β-glucuronide was used and so the fractions contained only the end of the peak of 5αAdiol-3-glucoronide, resulting in completely

wrong δ^{13}C-values in the IRMS measurements. With the used column it is impossible to isolate the Androstane-diols into separate fractions. This is not too much of a problem for the used QCP and QCN, since the concentrations of the two substances are in a comparable range. But in real samples the concentrations between those can have a more than tenfold difference. An idea to address this problem is a second HPLC after the hydrolysis for certain fractions. The same stationary phase that is utilized in the routine method for the separation of free steroids can be employed. This should work well, as the Androstanediols are well separated. An additional benefit of this approach would be an extract that should have a higher purity, than the Androstanediol fractions from the routine method. This is important, since especially the 5αAdiol fraction is often heavily polluted with matrix interferences. There are two downsides; the major one would be the loss of analytes during the second HPLC. Furthermore is consecutive HPLC a large time investment of approximately half a day.

Usually a retention time standard is added to samples to check for the stability of chromatography. In the standard method Estradioldiacetat is used as it elutes really stable during the final highly organic part of the gradient. A big difference regarding the solvents is the addition of formic acid, which is not needed in the routine method. Estradioldiacetat has the downside of not being stable under acidic conditions, as it dissociates. As alternatives Trenbolone, Trenbolonacetat and Methyltestosterone were evaluated. Methyltestosterone was impractical, since it coelutes with PD on HPLC and GC with the used methods. Trenbolonacetat has the same issues as Estradiolacetat and dissociates in the used solvents. Trenbolone on the other hand elutes between the first and second fraction and is therefore not collected. However this only works well in the standard mixture with no interfering matrix. Between the first fractions the background signal is really high and the peak for Trenbolone is overshadowed. So the final method uses only the standard mixtures before and after the sample to indicate the chromatographic stability.

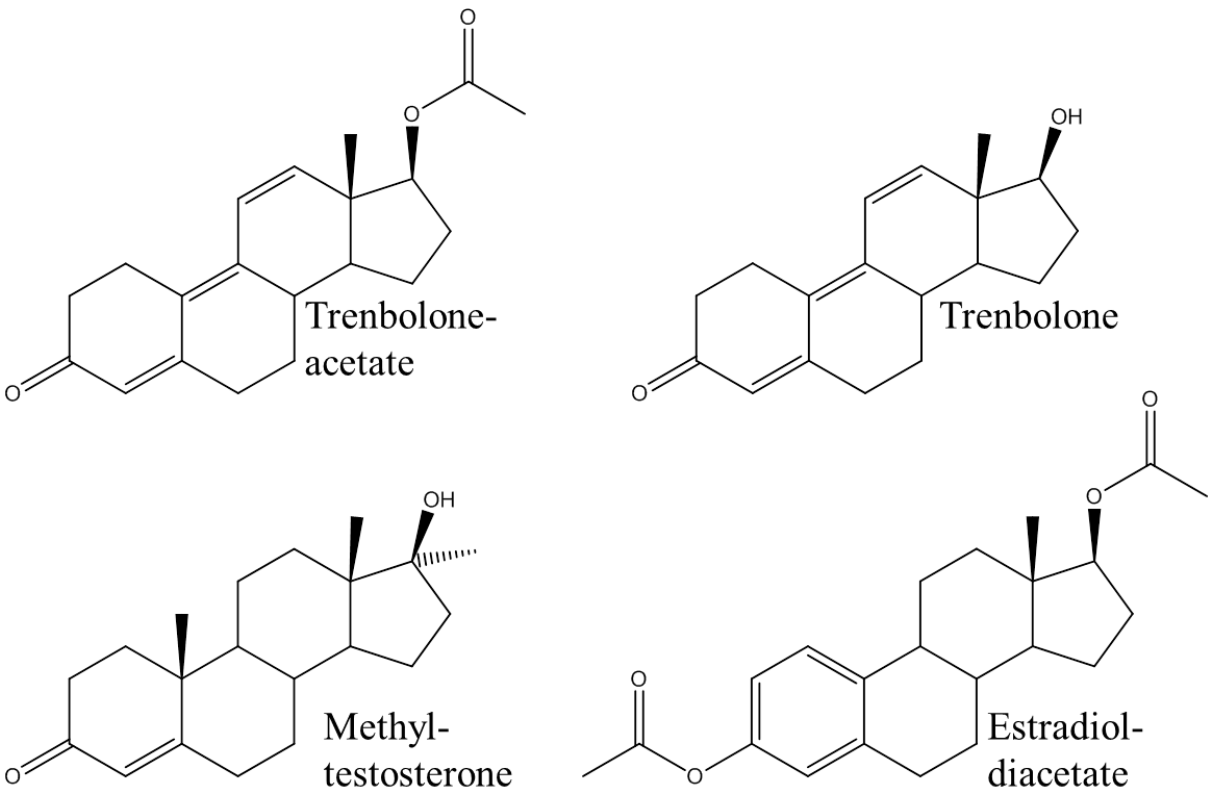


Fig. 26: *structural formulas of four potential retention time standards for the HPLC separation of steroid-glucuronides*

A different approach is to use a substance that is already in urine as a retention time standard. With this method there is no need of adding anything to the sample and therefore removing a step that includes potential contaminations. The substance has to be relatively non-polar and elute late during the gradient. This is to ensure the visibility on the chromatogram, as the background signal decreases with increasing amount of organic solvent in the mobile phase. Since the SPE beforehand is supposed to purify steroid conjugates, those substances should be considered as well. But not only the wide range of concentrations in urine samples is a problem, but also that all substances monitored in this thesis elute in the more aqueous part of the gradient. PD, the only collected substance that elutes late, shows low UV-absorption and is therefore not suited as a retention time standard. Other sample preparation protocols for free steroids[62] use therefore 16EN, as it is always present in urine. 16EN elutes during the 100% organic part of the gradient and has the advantage that it can be used as an ERC. So the idea is to try out 16EN-glucuronide to check the stability of the retention times and collect it as well for the final IRMS analysis. A big problem is the limited availability of steroid conjugate standards, at the moment no 16EN-glucuronide standard can be bought from merchants that sold the other steroid conjugates.

4.3. Hydrolysis

To check the efficiency of the steroid hydrolysis the free steroids were converted to TMS-derivatives with MSTFA/TMSI and measured with the laboratory's standard method for steroid analysis on GC/MS^2.

The first experiment was based on a publication by Tang[63], who states that a short time at elevated temperature is enough time to fully hydrolyse steroid glucuronides with hydrochloric acid. The test was conducted with 50ng testosterone-glucuronide and 500µL 1.25M HCl in methanol. After 10 minutes at 60°C the mixture was dried, the free steroids extracted with MTBE and derivatised. Unfortunately no free steroids were found in the GC/MS^2 analysis. Therefore subsequent experiments were done with a longer hydrolysis time. A suggestion was to add a non-polar solvent into the mixture[64]. The idea behind that is to help with better dissolution of free steroids in organic solvents compared to their conjugated moieties. Two organic solvents were evaluated, Ethylacetate and MTBE. Another test was carried out, adding 500µL Ethylacetate and 500µL 1.25M HCl in Methanol to 50ng of Testosterone-glucuronide. The mixture was closed, and stored for 24h at room temperature. After derivatization and analysis it was clear that this approach was working, since clear signals for TMS-testosterone could be observed. The approach of analysing TMS-derivatives is fast and gives semi quantitative results, as it is done in selected reaction monitoring. A downside is that it yields no information about potential side products of the reaction. Therefore the experiment was repeated without derivatization. The free steroids were then analysed in a full scan mode on a single quadrupol GC/MS with the same column that is used in GC/C/IRMS. However, the analysis showed not only signals for Testosterone, but Testosterone-acetate as well. This is a result of the addition of Ethylacetate to the reaction mixture and the transesterification from glucuronides to acetates. This is accompanied by a loss of analyte with an unknown effect on the δ^{13}C-value. In addition to this problem subsequent measurement on the same instruments showed peak tailing and loss of signal intensity. A potential reason for the impairment of the column is the acidic activation of the stationary phase. Although the HCl is supposedly completely removed by evaporation the free glucuronic acid still remains in the sample extract and might have ruined the GC-column.

After this epiphany MTBE was chosen as an unreactive nonpolar solvent. The free glucuronic acid was removed by neutralization with carbonate buffer followed by organic liquid extraction of the free steroids with MTBE.

Concerning the reaction yield and optimal parameters a set of three mixtures was incubated at the respective temperature. The first solution contained only the internal standard for the initial testing procedure of endogenous steroids, the second solution consisted of the free steroids with internal standard and the third solution included the glucuronidated versions and the internal standard. The mixture of free steroids contained 200ng of each steroid, and the mixture of the conjugated steroids had the same equivalent concentration of free steroids. To each of these standard mixtures the acid and organic solvent was added and kept at the chosen temperature. Each hour an aliquot was taken out and neutralized with Carbonate buffer. Out of this solution the free steroids were extracted with MTBE, dried, converted to TMS-derivatives and analyzed on GC/MS^2. Afterwards the ratios of the free steroids and their respective internal standards are compared over reaction time. First the peak areas

are corrected with the internal standards to include small deviations. The corrected peak areas of the steroids from the glucuronide mixture are divided by the corrected peak areas from the mixture of steroids without hydrochloric acid. Every experiment was just conducted once, so no statistical evaluation is possible, hence the result of this hydrolysis kinetics study is just for estimation of reaction parameters.

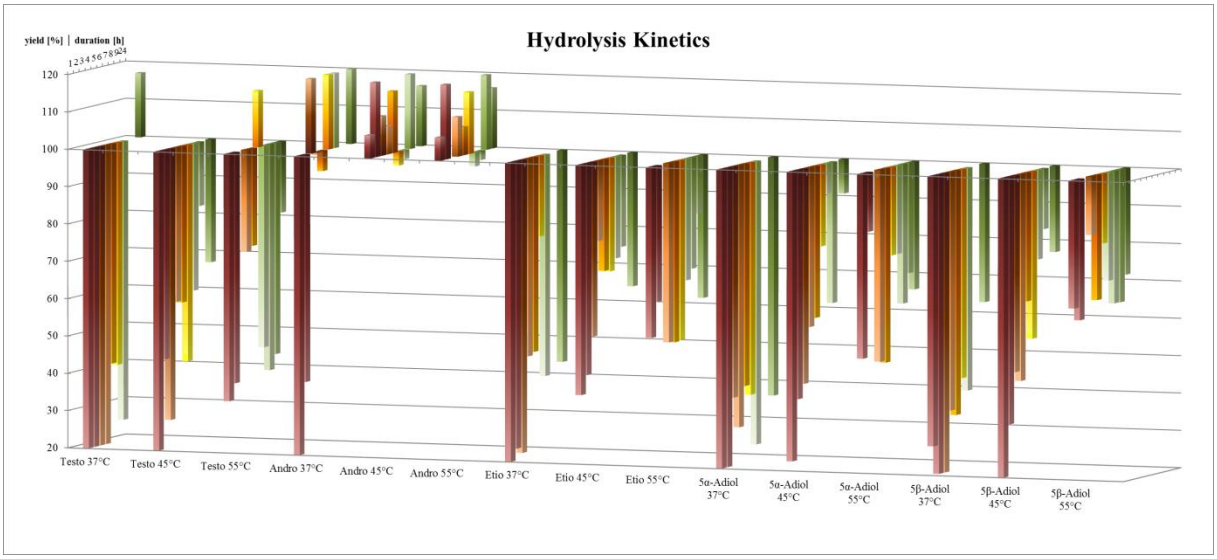


Fig. 27: *Presentation of the results from the hydrolysis kinetics experiment. The hydrolysis yields are compared for the individual steroids over time at certain temperatures. The steroids are abbreviated for better visualization. The yield is given in percent on the vertical axis, the depth of the graph shows the hydrolysis time in hours. An additional horizontal plane cuts the graph at 100%. Values below 20% and above 140% are cut off.*

The values for PD were left out, as the initial testing procedure that was used for semi-quantitative analysis is not optimised for PD, thus the results are unusable.

Interestingly the yield of Andro was unusually high, as its glucuronide has bad efficiency for enzymatic hydrolysis[51]. Unfortunately a possible reason for the high amounts of hydrolysed Andro is an unwanted side reaction, as found out later. From the tests it is clear that the acidic hydrolysis takes longer than a few hours at the chosen temperatures. As expected the hydrolysis of steroid glucuronides appears to be faster at higher temperatures. But the difference between the temperatures is decreasing with longer hydrolysis duration. To reduce the steroids heat exposure over a longer period of time a lower temperature is preferred, even though there is no indication for degradation. Finally reaction parameters of 45°C and hydrolysis overnight are chosen, to keep the temperature low and the reaction time as short as reasonable. All further experiments are conducted under those conditions. However the first sample that was hydrolysed after HPLC separation showed odd results. Especially the second fraction containing both the Androstanediols showed additional peaks for Andro and Etio. The fractions were collected properly and even that would not explain the presence of Andro, as it elutes not in the beginning of the next fraction. The only explanation for that is the oxidation of the secondary hydroxyl-groups to ketones. In that case the 5αAdiol is converted to Andro and its 5β counterpart to Etio. Since this happens in pure standards as well as in urine samples it appears that

oxygen from air acts as an oxidant. To prevent the formation of these undesired products the air in the reaction vial was displaced by Argon. Working under complete inert conditions was not possible, because of limitations in glassware. Thus the vials with the dry extracts were purged with Argon for at least 30 seconds before adding acid, solvent and closing under a constant gas stream. First tests regarding the yield of hydrolysis and optimal temperature and time parameters were carried out without the inert gas atmosphere. Therefore they are excluded from the results and only listed in this discussion of the thesis. But further investigations should follow, and subsequent tests should include more analytes. One of the predicted benefits of this hydrolysis method was the possible cleavage of sulfo-conjugated steroids. This was not yet tested and leads to potential for future research.

4.4. IRMS

As already shown in the results, the δ^{13}C-values of the QCs prepared with the alternative method are comparable to those from the routine method. Not only do they meet the WADA criteria[39] for a positive or negative sample, their average is also within the limits of two standard deviations. But another factor that has to be taken into consideration is the apparent recovery of the analytes. Since the instrument method and data processing is the same as for routine analysis a relation can be made between the QCs from the newly developed and the standard method. QCs that were analysed two weeks before the experiments of this thesis were chosen as basis of comparison. The signal intensities of 44 m/z give a short overview of the differences in sample loss. With the same concentration the signal intensities should be comparable, or even higher if less final volume is used. Concerning the signal intensities the only disparity between the two methods is the fact, that in the routine method 5α- and 5βAdiol are in separate fractions and therefore the final volume can be adjusted differently.

Table 9*: comparison of the (rounded) signal intensities of the mass 44 and the final AS volumes for the routine method and the developed alternative method in the example of one QCP and QCN each.*

Substance	QCP new method		QCP routine		QCN new method		QCN routine	
	volume [µL]	Int.(44) [mV]	volume [µL]	Int.(44) [mV]	volume [µL]	Int.(44) [mV]	AS vol [µL]	Int.(44) [mV]
T	30	300	35	400	30	500	35	500
5βAdiol	100	1800	60	2800	100	600	35	2300
5αAdiol	100	700	35	1100	100	500	35	1000
Etio	850	5100	900	4200	750	2100	750	1700
Andro	850	2700	900	2200	750	3100	750	2300
PD	30	600	35	1200	45	2200	45	2200

It appears that no general predication about the apparent recovery can be made from the signal intensities alone, as it depends not only on the substance but on the sample as well. PD results in similar signals in QCN, but the QCP only shows half the intensity compared to the routine method. Testosterone signals are slightly worse in the alternative method. The two main metabolites Andro and Etio show comparable signals in QCP and even higher ones in QCN. Both the Androstanediols are in QCN for both methods alike and in QCP the alternative method gives higher signals.

Apparently there are some contents within the QCP that have a negative influence on the recovery of PD-glucuronide with the newly developed method. But to be sure, further tests should be conducted with also with other samples. A potential for analyte loss is bacterial degradation[30]. If hygienic conditions during sample collection are suboptimal and the sample is not frozen properly bacteria can grow inside the urine bottle. These microorganisms can hydrolyse the glucuronides, cleaving and digesting the glucuronic acid. It was shown, that it has no influence on the δ^{13}C-value of the steroids[65]. Nonetheless, the bacterial degradation increases the amount of free steroids in urine, which are not collected during the HPLC, therefore resulting in a loss. In case of the samples used during this thesis it should have no influence, since the QCP and QCN do not undergo multiple thawing and refreezing processes as routine samples, and the negative test sample was fresh morning urine.

Concerning the δ^{13}C-values of the substances the only surprising outcome is the smaller difference between Andro and Etio than expected. Usually they differ by more than 1‰, with Etio being more negative. However in the final results of the new method they are within a range of 0.5‰ of each other. Still, the results are within the 2 standard deviation borders of the QCs.

In the IRMS-analysis there are not only the peaks of the monitored substances visible, but others as well. Unfortunately, the comparison of δ^{13}C-values is not too useful, as they might have been only partially collected during HPLC fractionation.

4.5. GC/MS

The advantage of the hyphenation from the GC/C/IRMS and the single quadrupole mass spectrometer is the additional structural information of the eluting substances. In theory the mass spectra can be compared to those of databases, as the electron ionization is usually standardized. Sadly in practice this is not as easily applicable. In this instrument setting the lenses of the ion source are tuned to have the maximum resolution and signal intensity between 200-350m/z, the mass range for the parent ions of androgens and their most significant fragments. Even though the standardized 70eV electron energy is used, the acquired mass spectra differ from the libraries a little bit. Furthermore the available libraries only contain information about the most common steroids like Testosterone. As a consequence it is not possible to fully identify substances without the usage of standards. But with the strategy of comparing the spectra of peaks to the spectra of standards with the same retention time the identity is assured. For unknown peaks only an estimate about the approximate chemical formula can be made. In addition the pattern of the mass spectra can be compared and similarities between unknown peaks and known substances can be assumed. The data analysis of the mass spectra of known and unknown peaks was done in Xcalibur (Version 4.1).

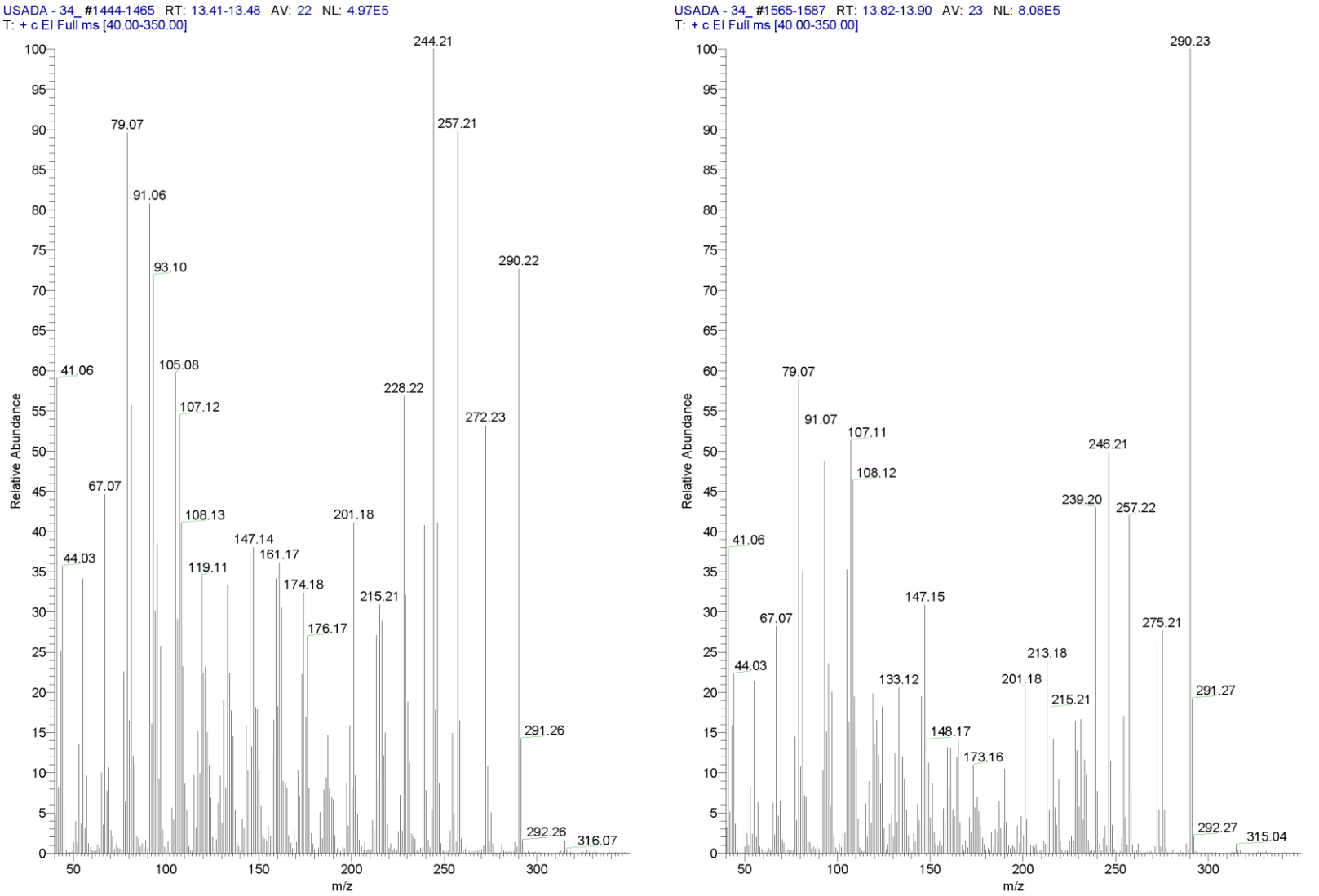


Fig. 28: *mass spectra of the two isomers Etio (left) and Andro (right). The retention times of Etio (13.35min) and Andro (13.86min) are aligned to the internal standard Androstanol with a retention time of 10.82 minutes. Mass spectra are in the range of 40-350 m/z and are normalized to the base peak.*

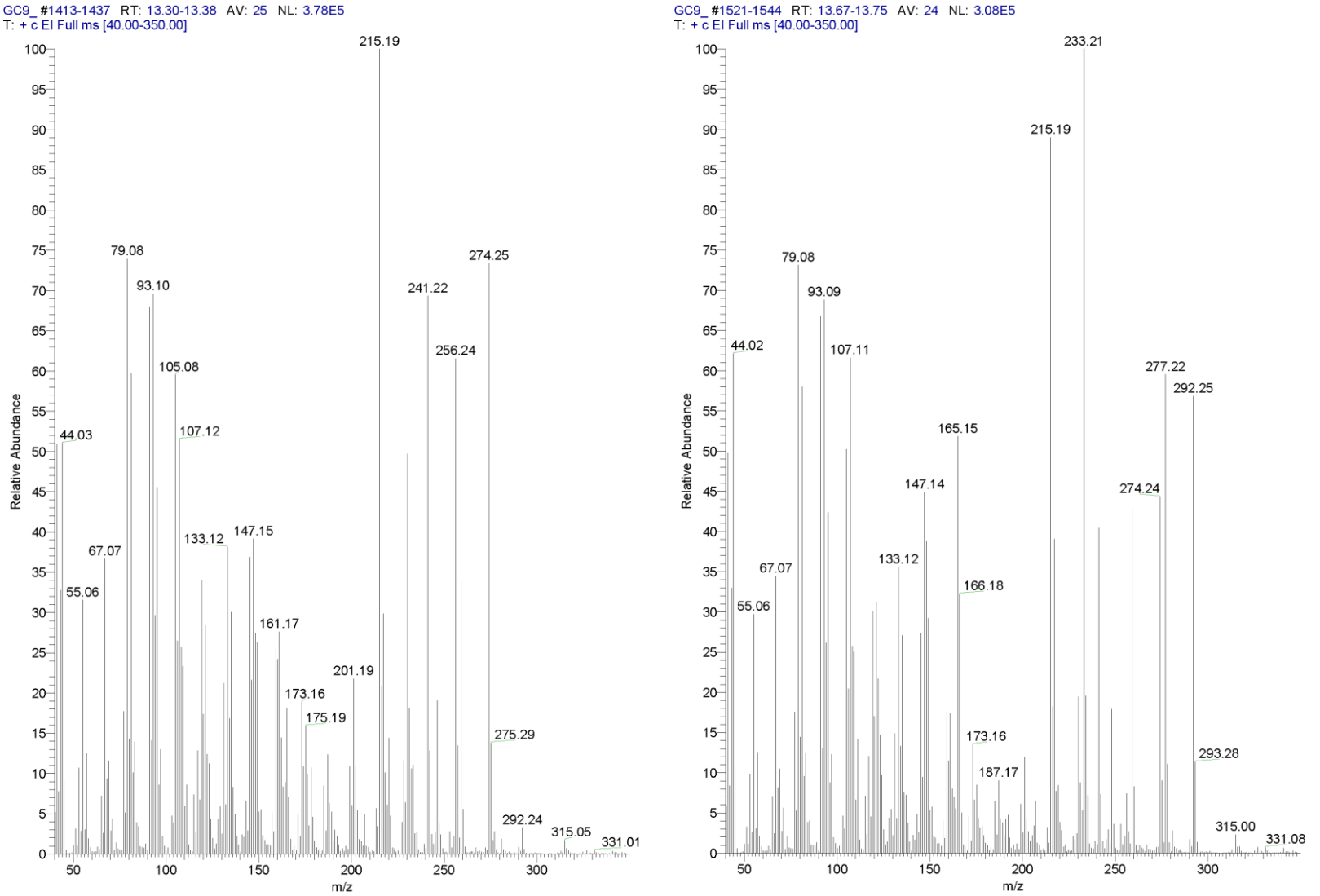


Fig. 29: *mass spectra of the two isomers 5βAdiol (left) and 5αAdiol (right). The retention times of 5βAdiol (13.34min) and 5αAdiol (13.70min) are aligned to the internal standard Androstanol with a retention time of 10.82 minutes. Mass spectra are in the range of 40-350 m/z and are normalized to the base peak.*

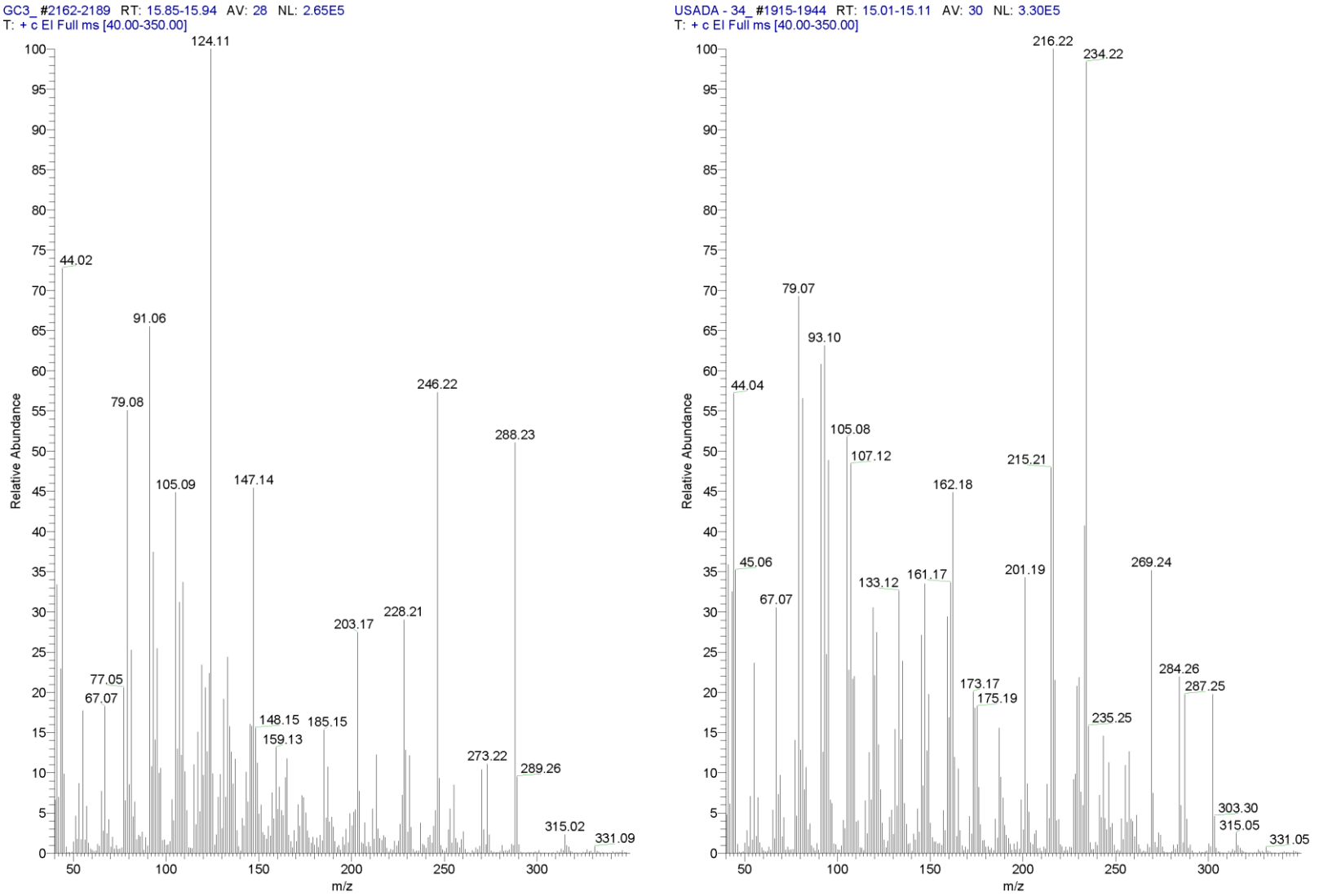


Fig. 30: *mass spectra of the monitored substances Testosterone (left) and PD (right). The retention times of Testosterone (15.90min) and PD (15.05min) are aligned to the internal standard Androstanol with a retention time of 10.82 minutes. Mass spectra are in the range of 40-350 m/z and are normalized to the base peak.*

The fragmentation pattern of steroids is complex, but the small differences in structure have a large influence on the processes during ionization. With the used settings of the ion source the molecular peak is visible for most steroids. It has the m/z value of the corresponding molar mass of the intact molecule. Usually a loss of water (-18m/z) and a loss of a methyl-group (-15m/z) can be seen as well. Those two fragments are common for steroids, and not specific to the more characteristic features of the molecule.

The conjugated double bond in the A-ring of Testosterone can be rearranged to a cyclobutane-ring (-42m/z) resulting in a fragment with 246m/z. However the partial loss of the D-ring (-44m/z) is specific for 17-keto steroids as Andro and Etio and also leads to a fragment with 246m/z. But the ratio of the formation of these fragments is different and can therefore be used to distinguish the substances. In steroids with a 17-hydroxyl group an extended elimination of the D-ring can occur (-59m/z). That is especially good visible in 5αAdiol, as the fragment resulting from it (233m/z) is the base peak of the spectrum[66,67].

But those are not all fragmentation pattern, listing and considering all of them would go beyond the scope of this thesis.

From the mass spectra it is clearly visible, that the two pairs of isomers are not only distinguishable by retention time, but through comparison of the fragment ratios as well. To evaluate the purity of the collected fractions one representative example of them is taken and the peaks are compared to those of the pure standards.

In the following discussions about the purity of the fractions and the identity of peaks the developed method is compared to the old routine method. Therefore a negative quality control sample from a sequence approximately two weeks prior to the measurements of the new method is taken into account. Right after that, the column was changed, so the retention times are shifted, and have to be adjusted according to the retention time standard androstanol. The reason for the column change is the deterioration of the peakshape, especially PD is affected.

The last fractions that contain PD and the pair of Etio and Andro are easy to interpret, as they include almost no visible contaminations or other substances. Since those two fractions are highly diluted to accommodate their high amount in urine the absence of other peaks is expected. But for samples with lower concentrations than the ones used there is the possibility of emerging peaks from the background.

4.5.1. Pregnanediol

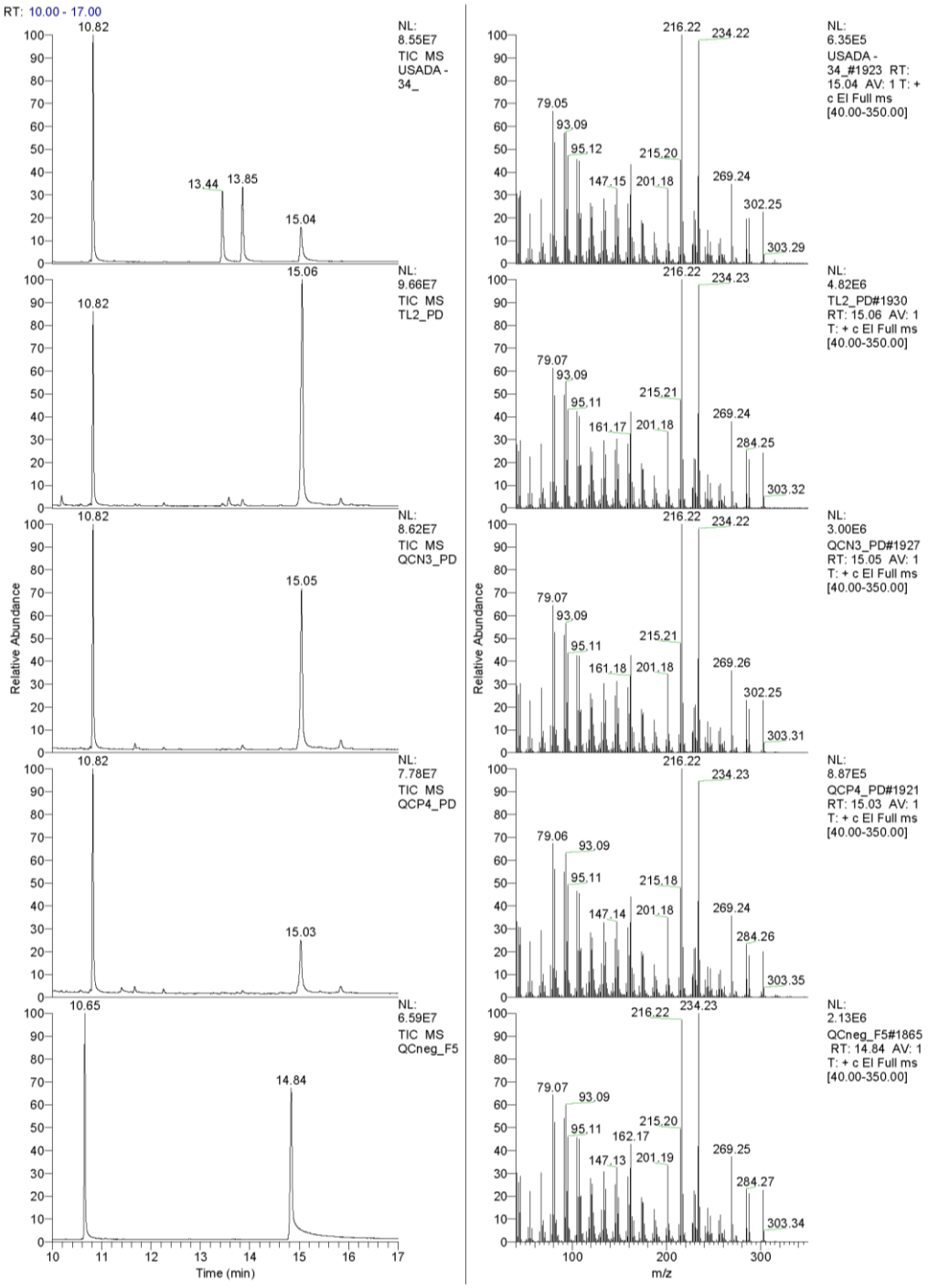


Fig. 31: *Comparison of the fractions containing pregnanediol. On the left side, the chromatograms are shown and right next to it the corresponding mass spectra of the pregnanediol-peak. From top to bottom are shown a reference standard containing androstanol, etiocholanolone, androsterone and pregnanediol, a negative sample, negative quality control and positive quality control from the alternative method and finally the negative control sample from the routine analysis. The chromatograms show the total ion current normalized to the highest peak over time from 10 to 17 minutes. The peaks are compared to the internal standard androstanol at 10.82 minutes regarding their retention time. The last chromatogram is from a different sequence, the retention times are therefore uniformly shifted by 0.17 minutes. The mass spectra are derived from the peak apex at 15.04 minutes or 14.84 in the last chromatogram and are normalized to the base peak.*

As shown in **Fig. 31**, the peak in the last fraction is pure and only contains PD. The chromatograms are comparable to the routine method as well. In this case the routine analysis shows a worse chromatogram with peak tailing.

4.5.2. Etiocholanolone & Androsterone

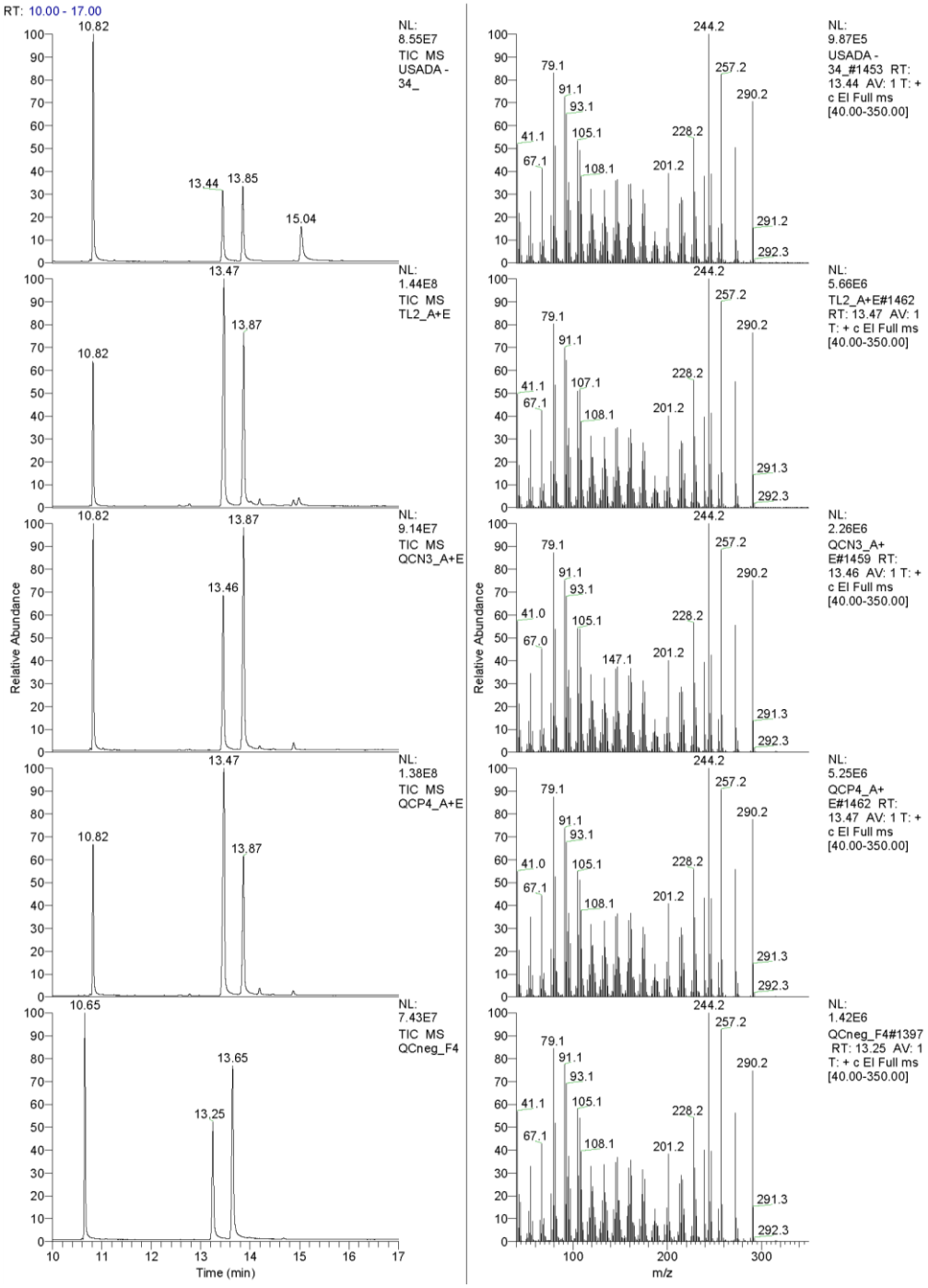


Fig. 32: *Comparison of the fractions containing etiocholanolone. On the left side, the chromatograms are shown and right next to it the corresponding mass spectra of the etiocholanolone-peak. From top to bottom are shown a reference standard containing androstanol, etiocholanolone, androsterone and pregnanediol, a negative sample, negative quality control and positive quality control from the alternative method and finally the negative control sample from the routine analysis. The chromatograms show the total ion current normalized to the highest peak over time from 10 to 17 minutes. The peaks are compared to the internal standard androstanol at 10.82 minutes regarding their retention time. The last chromatogram is from a different sequence, the retention times are therefore uniformly shifted by 0.17 minutes. The mass spectra are derived from the peak apex at 13.36 minutes or 13.25 in the last chromatogram and normalized to the base peak.*

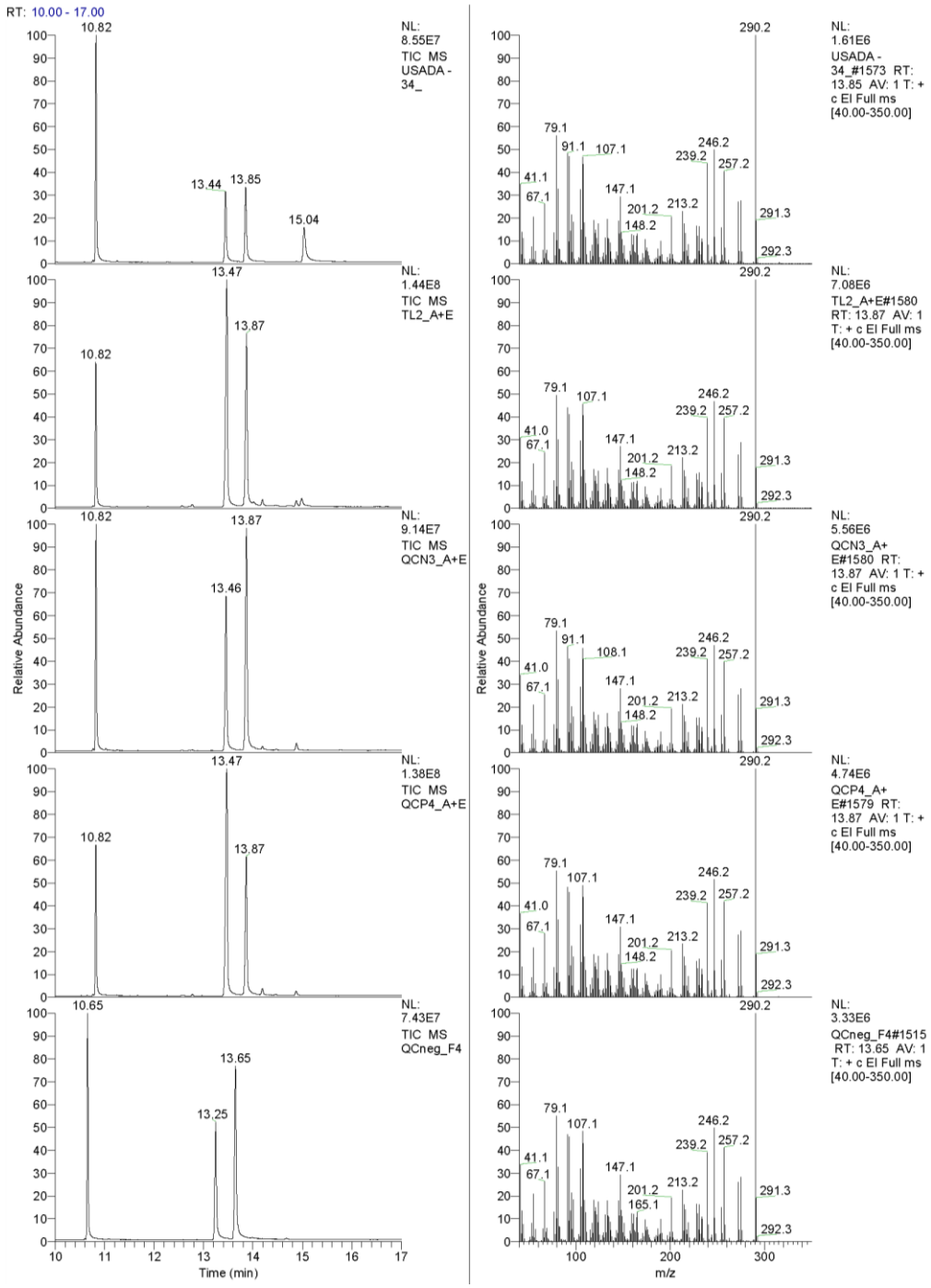


Fig. 33: *Comparison of the fractions containing androsterone. On the left side, the chromatograms are shown and right next to it the corresponding mass spectra of the androsterone-peak. From top to bottom are shown a reference standard containing androstanol, etiocholanolone, androsterone and pregnanediol, a negative sample, negative quality control and positive quality control from the alternative method and finally the negative control sample from the routine analysis. The chromatograms show the total ion current normalized to the highest peak over time from 10 to 17 minutes. The peaks are compared to the internal standard androstanol at 10.82 minutes regarding their retention time. The last chromatogram is from a different sequence, the retention times are therefore uniformly shifted by 0.17 minutes. The mass spectra are derived from the peak apex at 13.86 minutes or 13.65 in the last chromatogram and normalized to the base peak.*

As shown in **Fig. 32** and **Fig. 33**, the peaks in the fraction containing Etio and Andro are pure and only contain the respective substances. The chromatograms are comparable to the routine method as well.

4.5.3. Androstandiols

The fraction collection via the employed separation of glucuronides leads to a mixed fraction containing both Androstandiols. On the other hand the routine method is able to fully separate both substances and divide them into different fractions.

The 5α-isomer is collected in an individual tube, while 5βAdiol is combined with Pregnanetriol, Epi and DHEA. The final dilution in this fraction is adjusted according to the concentration of 5βAdiol. Therefore the other substances are not detectable if they are not present in high amounts. Since the routine method is not validated for the substances in this fraction except 5βAdiol, the others only give bonus information about the sample. Pregnanetriol can be used as an additional ERC as it should have the same δ^{13}C-values as PD. DHEA can also be used as an anabolic steroid[68] and if applied gives positive δ^{13}C-values, but is not part of the technical document for IRMS of the world anti-doping agency. The same can be applied for Epi, but this substance is rarely even detectable in the routine analysis.

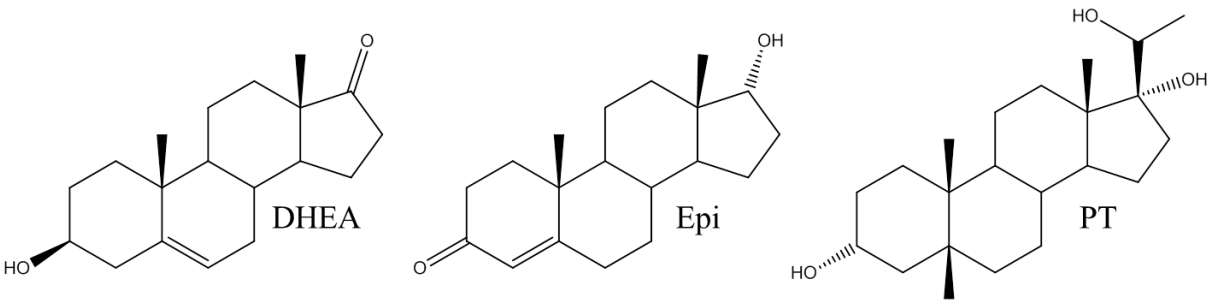


Fig. 34: *structural formulas of the substances Pregnanetriol, Epi and DHEA. Those are additionally monitored together with 5βAdiol in the routine method.*

With the routine method Epi and DHEA are below the linear range of the IRMS in both the QCs because of low concentrations. So from a solely chromatographic point of view there is little difference between the positive and negative QC. Therefore only one of them prepared with the standard method is discussed in the scope of this thesis.

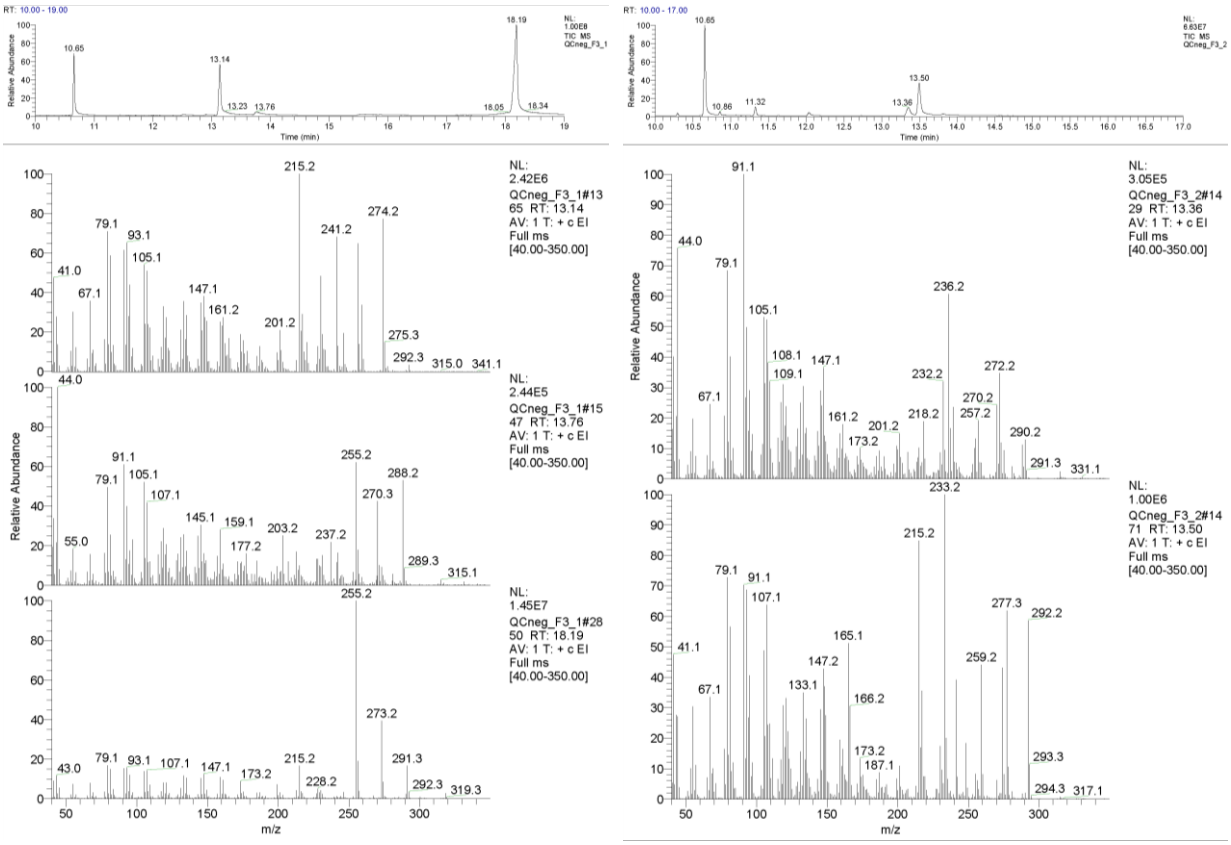


Fig. 35: *Display of the fractions containing Androstandiol isomers from the routine method. On the left side is the fraction containing 5βAdiol and on the right side the fraction containing 5αAdiol. The chromatograms show the total ion current normalized to the highest peak over a time range from 10 to 19 minutes and 10 to 17 minutes. The internal standard androstanol at 10.65 minutes is left out of the discussion. The mass spectra of the noteworthy peaks are depicted below the chromatograms. On the left side those are the mass spectra from the apex of the peaks at 13.14, 13.76 and 18.19 minutes. On the right side the mass spectra for the peaks at 13.36 and 13.50 minutes are shown. All mass spectra are in the range of 40-350 m/z and normalized to the base peak.*

Fig. 35 shows the fraction containing 5βAdiol and the aforementioned additional not validated steroids on the left side. The first peak at 13.14 minutes is the compound of interest, 5βAdiol, as it fits the retention times and the mass spectra, when compared to standards. The smaller peak at 13.76 most likely consists of DHEA. But since this substance is not monitored on a regular basis, there was no standard for it in the sequence, so not a matching chromatogram and mass spectrum. The large peak at 18.19 minutes is Pregnanetriol, which is present in high concentrations in the used QCN. Pregnanetriol tends to have bad peaks if the GC column is not in optimal shape. The three hydroxyl groups within the molecule lead to bad chromatographic properties, as it has the most possibilities of unwanted side reactions with the stationary phase.

In **Fig. 35** the fraction containing 5αAdiol is shown on the right side. The peak is detected at 13.50 minutes, retention time and mass spectrum fit the standard. Right before the peak for 5αAdiol, an unknown compound elutes at 13.36 minutes. A molecular peak at 290 m/z can be estimated, that corresponds to a steroidal sum formula of $C_{19}H_{30}O_2$ like Andro and Etio. But retention times and fragmentation pattern are different. Therefore no conclusion about the structure of this unknown substance can be made at the moment.

As already mentioned the alternative sample preparation leads to a mixed androstandiol fraction with several unknown peaks in addition to the compounds of interest.

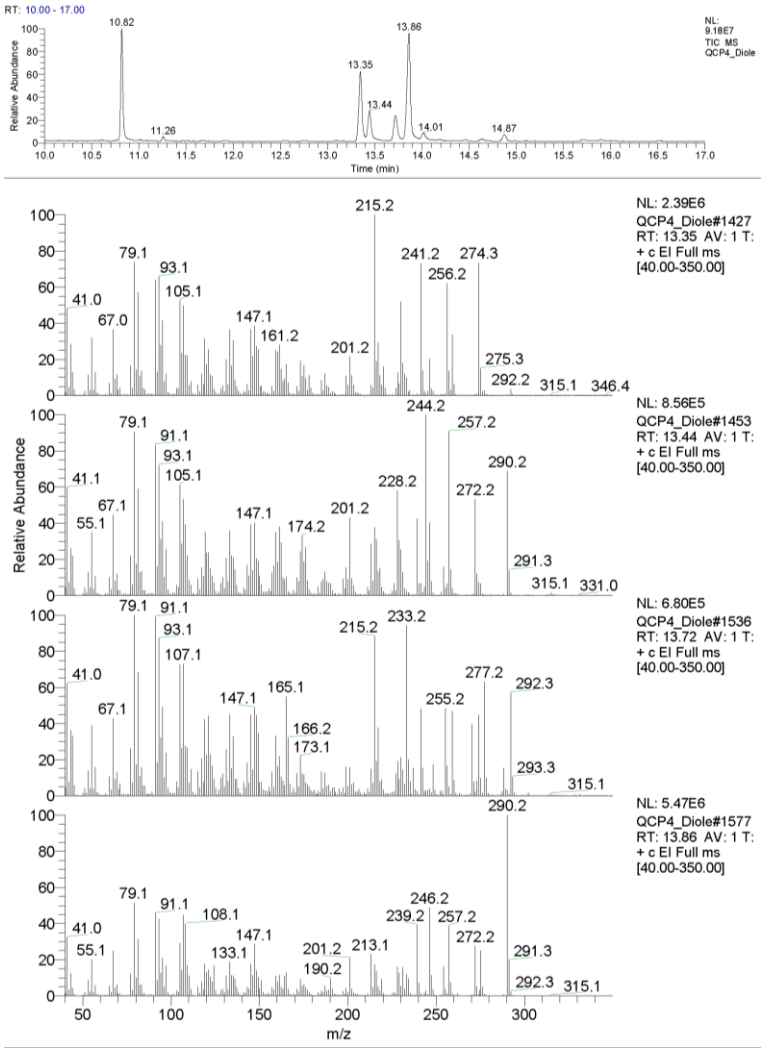


Fig. 36: *chromatogram of the fraction containing both Androstandiol isomers from the positive control sample and the mass spectra of noteworthy peaks. The chromatogram on top shows the total ion current normalized to the highest peak over a time range from 10 to 17 minutes. The mass spectra are derived from the peaks at 13.35, 13.44, 13.72 and 13.86 minutes. All mass spectra are in the range of 40-350 m/z and normalized to the base peak. The internal standard androstanol at 10.82 minutes is intentionally left out of the discussion.*

There are four noteworthy peaks present in the positive control sample. The first one at 13.35 minutes being the monitored 5βAdiol with matching retention time and mass spectrum. The second peak at 13.44 minutes shows an apparent molecular peak at 290 m/z, which likely corresponds to steroidal sum formula of $C_{19}H_{30}O_2$. Interestingly the fragmentation pattern matches neither the unknown substance in the 5αAdiol fraction from the routine method nor any monitored steroid. The third peak at 13.72 is the other substance of interest, 5αAdiol and the retention time as well as the mass spectrum fits the standard. The last unknown peak has the same fragmentation pattern and retention time as Andro. Andro-glucuronide elutes during the HPLC much later, therefore the origin has to be from somewhere else. This leads to the assumption, that purging with argon is not sufficient enough to prevent the oxidation of 5αAdiol to Andro. However the $\delta^{13}C$-values of the two peaks do not match, and differ by several ‰. Hence this Andro-peak has to originate from another source.

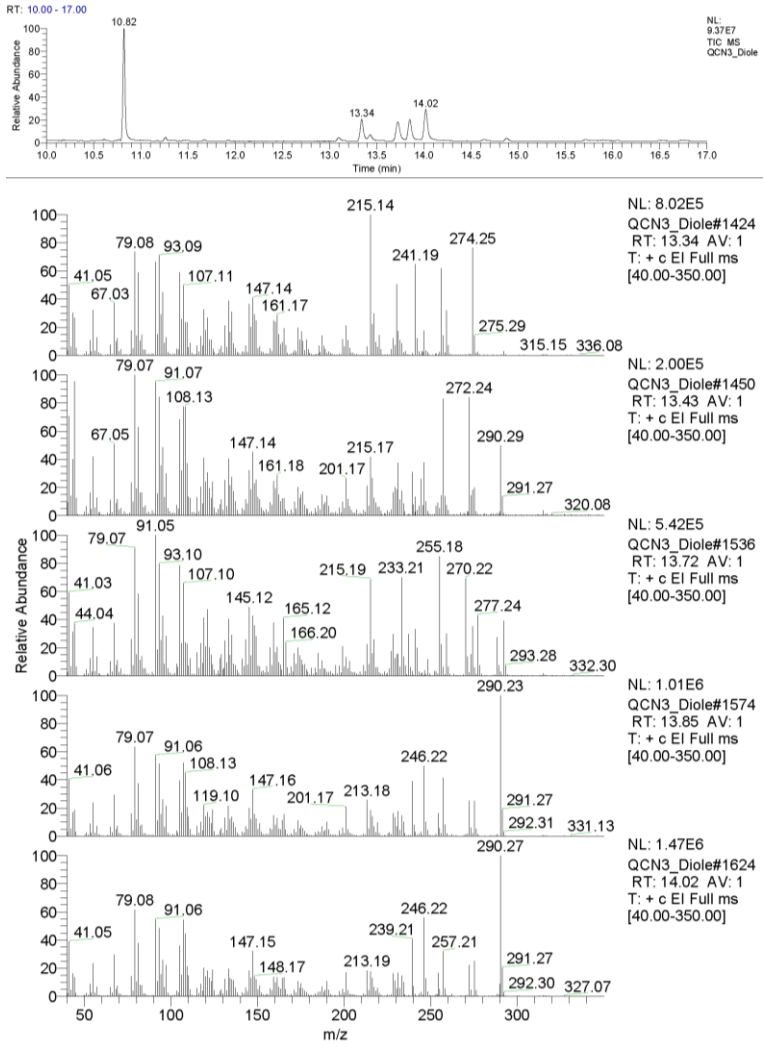


Fig. 37: *chromatogram of the fraction containing both Androstandiol isomers from the negative control sample and the mass spectra of noteworthy peaks. The chromatogram on top shows the total ion current normalized to the highest peak over a time range from 10 to 17 minutes. The mass spectra are derived from the peaks at 13.34, 13.43, 13.72, 13.85 and 14.02 minutes. All mass spectra are in the range of 40-350 m/z and normalized to the base peak. The internal standard androstanol at 10.82 minutes is intentionally left out of the discussion.*

The negative control sample shows similar peaks to the positive control sample. The two substances of interest are clearly visible with matching retention times. 5βAdiol elutes at 13.34 minutes and shows the right mass spectra, 5αAdiol elutes at 13.72 minutes. However the peak containing 5αAdiol shows additional fragments for 288, 270 and 255m/z, which could come from a contamination from an unknown steroid. Interestingly, the first unknown compound at 13.44 minutes shows a different fragmentation pattern than the one in the positive control sample. Either it is a different compound, as it matches no other substance, or the peak is contaminated by one or more other substances. This is a possibility, since the peak intensity is quite low and not fully baseline separated from the peak before.

The peak at 13.85 is comparable to the one in the positive control sample. It also has the same retention time and fragmentation pattern as Andro. In addition to the mismatching $d^{13}C$-values the ratio of 5αAdiol to alleged Andro is completely different than in the positive control sample. That

strengthens the hypothesis, that the origin of this substance is not the oxidation of 5αAdiol, but some other substance with diverging concentrations.

However, an additional peak emerges from the background at 14.02 minutes. This peak was just faintly visible in the negative control sample. Again, the fragmentation pattern is similar to Andro, but with small deviations. As the retention time is also far off from Andro, it must be a different substance, but with a similar structure.

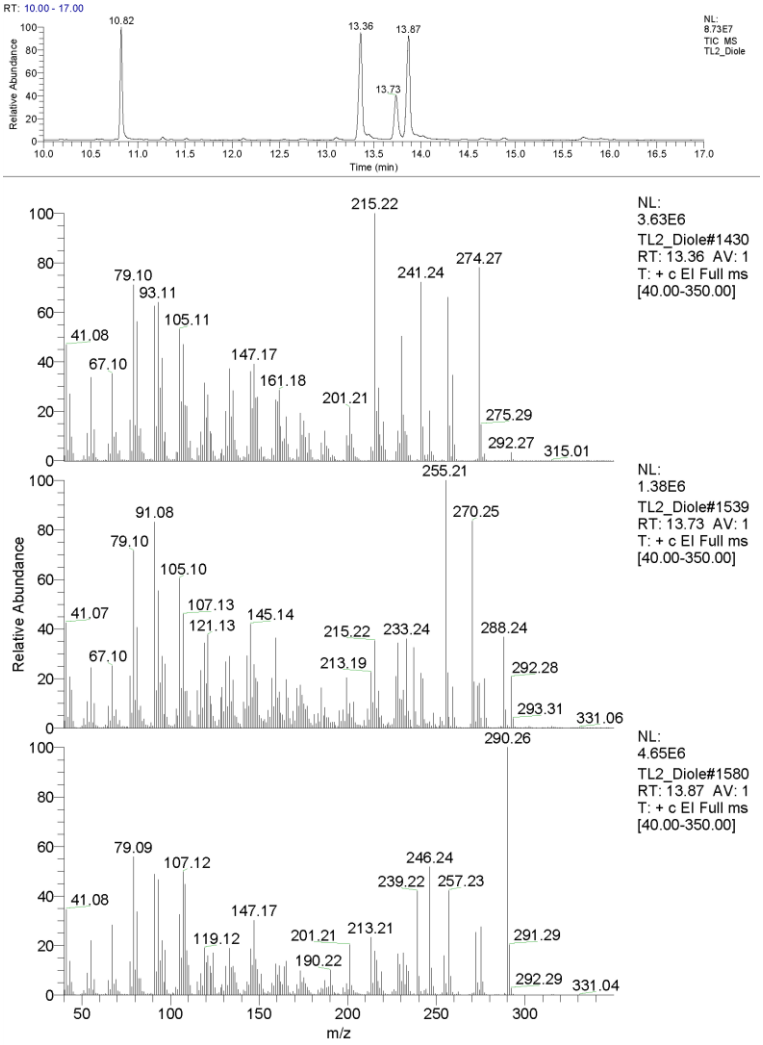


Fig. 38: *chromatogram of the fraction containing both Androstandiol isomers from the additional negative sample and the mass spectra of noteworthy peaks. The chromatogram on top shows the total ion current normalized to the highest peak over a time range from 10 to 17 minutes. The mass spectra are derived from the peaks at 13.36, 13.73 and 13.87 minutes. All mass spectra are in the range of 40-350 m/z and normalized to the base peak. The internal standard androstanol at 10.82 minutes is intentionally left out of the discussion.*

The other negative sample shows fewer peaks than the others in the Androstandiol fraction. At 13.36 minutes the first substance of interest, 5βAdiol is distinctly visible with matching retention time and fragment ions. 5αAdiol is also clearly detectable at 13.73 minutes with the right retention time. However the peak containing 5αAdiol shows additional fragments for 288, 270 and 255m/z, which could come from a contamination from an unknown steroid. The peak at 13.87 minutes shows the same retention time and fragment ions as the corresponding ones in the positive and negative control

sample. However the ratio of peak intensity compared to 5αAdiol is different again. The last peak with the Andro-like fragmentation pattern is not present. These observations can also be seen as proof, that those compounds are not an oxidation product but sample specific substances with variable concentration.

4.5.4. Testosterone

In the routine method the fraction containing testosterone is usually always contaminated with an unknown substance with similar retention behavior as testosterone. This compound elutes about 20 seconds before testosterone, an efficient separation if the peaks have no tailing. Interestingly the peak intensity ratio is relatively stable for negative samples but gets inverted for positive samples. That ratio is used as a first unofficial indicator in the routine method, if the testosterone peak is higher than the peak of the unknown substance the sample is usually positive. This observation can be seen in the comparison of the positive and negative quality control from the routine method as shown in **Fig. 39**.

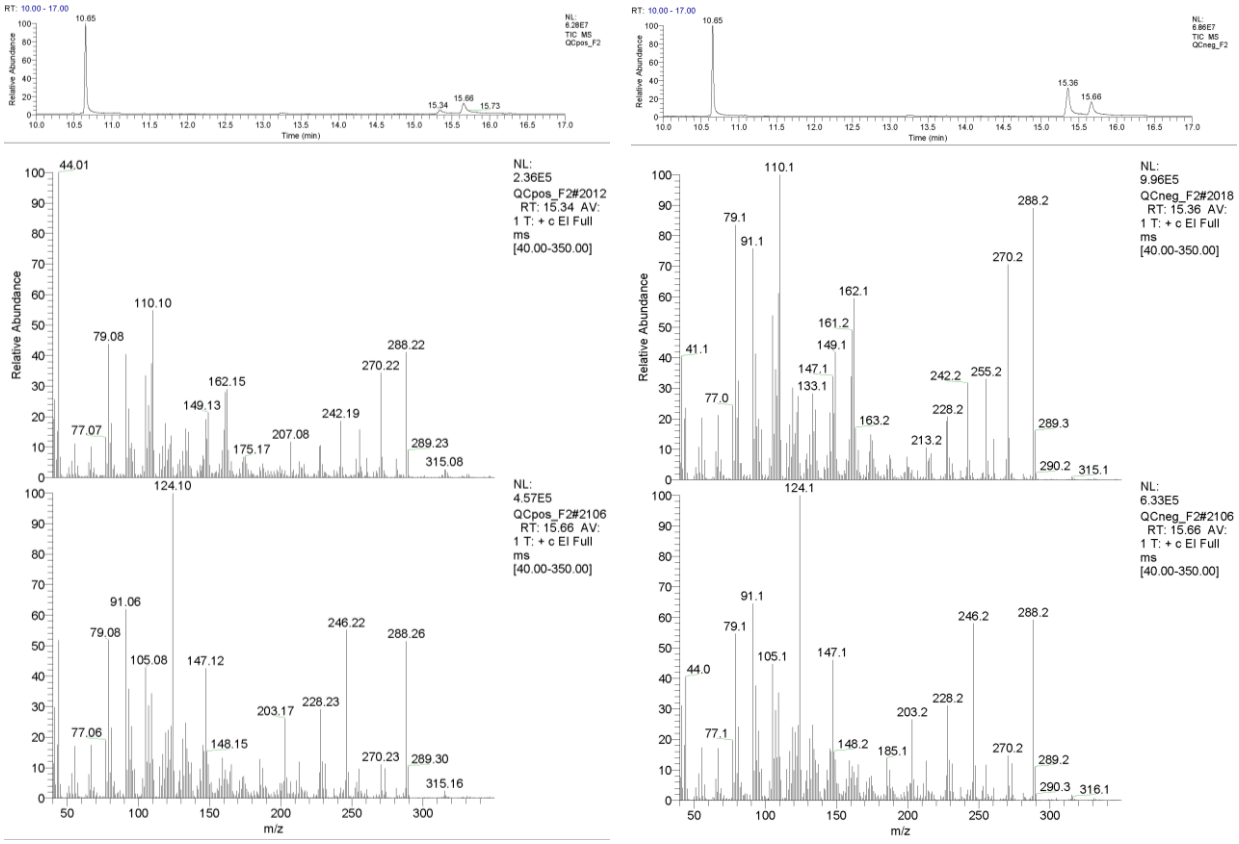


Fig. 39: *presentation of the testosterone fraction from the routine analysis. On the left side the QCP is shown and the QCN right next to it. The chromatograms on top are shown as total ion currents normalized to the highest peak over a time range from 10 to 17 minutes. The mass spectra below the chromatograms are derived from the peaks at 15.34 and 15.66 minutes for the positive control sample and at 15.36 and 15.66 minutes for the negative control sample. All mass spectra are in the range of 40-350 m/z and normalized to the base peak. The internal standard androstanol at 10.65 minutes is intentionally left out of the discussion.*

The unknown compound in the testosterone fraction from the standard sample preparation results in large signals for ions with the masses 288, 270 and 255. The fragmentation pattern is similar to the unknown substance, that contaminates 5aAdiol in the new alternative method, but the retention times

do not match. With testosterone having a nominal molecular mass of 288 g/mol, it is likely that the unknown substance is a steroid with the same sum formula ($C_{19}H_{28}O_2$). The peak at 270m/z is a result of water loss and the peak at 255m/z has the same mass difference (33m/z) to the molecular peak as a fragment peak in the 5βAdiol standard. Other substances that show the same loss of 33m/z are Andro and DHEA. This could imply structural similarities, but no clear answer has been found yet about this compound.

With the HPLC-separation of the steroid glucuronides this unknown steroid is completely removed from the testosterone fraction. But a downside is the inclusion of other substances, one of them having only less than ten seconds difference in retention time from testosterone. Since the completeness of fraction collection for those substances is not ensured, the δ^{13}C-values are not considered during this discussion.

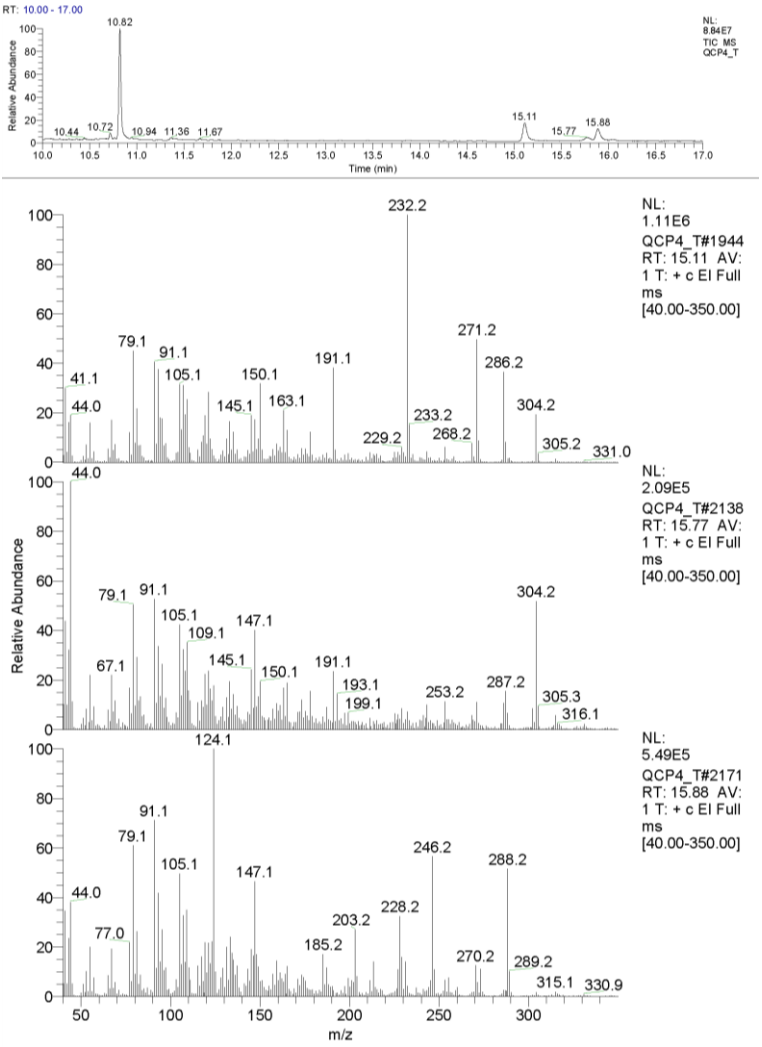


Fig. 40: *Testosterone fraction of the QCP from the alternative sample preparation. Below the chromatogram the mass spectra of noteworthy peaks with the retention times 15.11, 15.77 and 15.88 minutes are shown. All mass spectra are in the range of 40-350 m/z and normalized to the base peak. The internal standard androstanol at 10.82 minutes is intentionally left out of the discussion.*

The first two peaks at 15.11 and 15.77 minutes in the QCP show an apparent molecular ion of 304 m/z. This is a mass difference of 16 to testosterone, so a possible oxidation or hydroxylation product. The third peak at 15.88 minutes is clearly testosterone, the retention time and the mass spectrum fits to the standard.

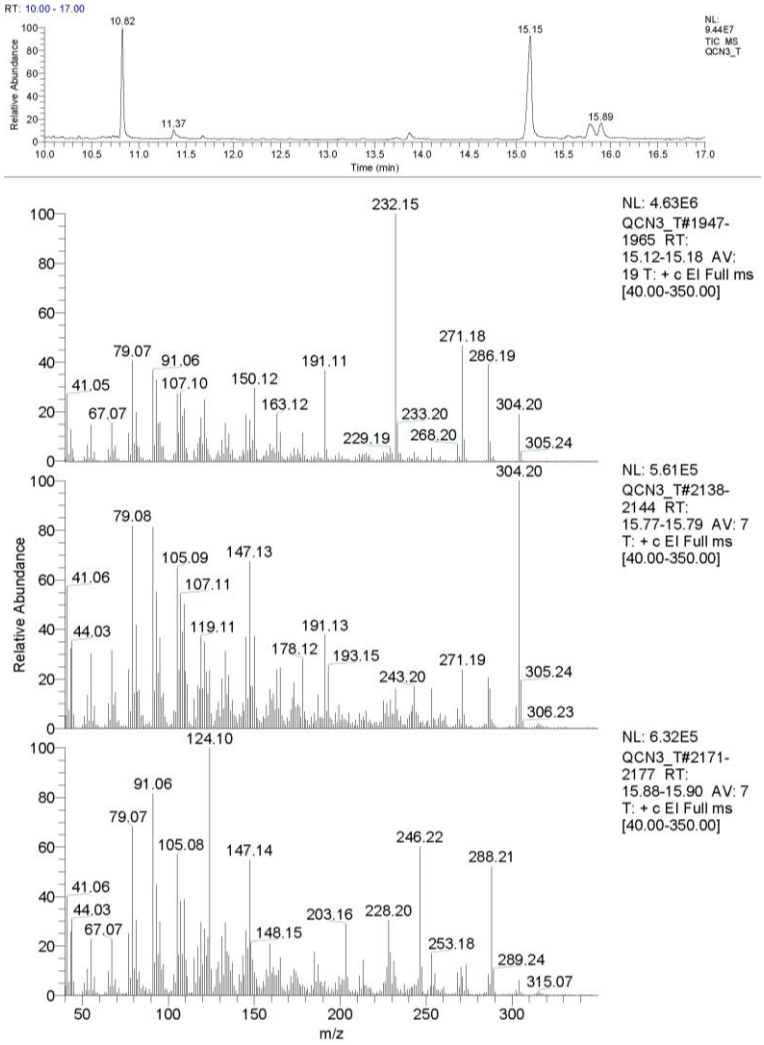


Fig. 41: *Testosterone fraction of the QCN from the alternative sample preparation. Below the chromatogram the mass spectra of noteworthy peaks with the retention times 15.15, 15.78 and 15.88 minutes are shown. All mass spectra are in the range of 40-350 m/z and normalized to the base peak. The internal standard androstanol at 10.82 minutes is intentionally left out of the discussion.*

Again, the third peak at 15.89 consists of Testosterone, with fitting retention time and mass spectrum. Interestingly the first two peaks at 15.15 and 15.78 minutes are higher in the QCN than in the QCP. This leads to the presumption, that it is not an oxidation product, but a metabolite that has different concentrations between the samples. Since the positive and negative QC have approximately the same concentrations of Testosterone and the first fraction is treated identically an oxidation product should be visible at the same rate. There is a possibility that the peak consists of an endogenous hydroxyl- or oxo-steroid. To test this hypothesis, a mixture of three substances with these structural features was also analyzed, shown in **Fig. 43**. Those three substances are independent of the testosterone metabolism and can also be used as ERCs[39].

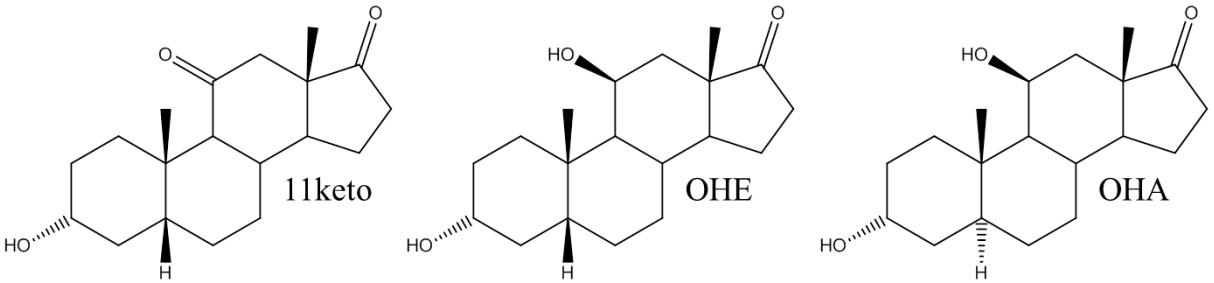


Fig. 42: *structural formulas of the three alternative ERCs 11keto, 11β-Hydroxyetiocholanolone (OHE) and 11β-Hydroxyandrosterone (OHA).*

After the comparison of the peak in the QCs shortly after 15.10 minutes with the peak of 11keto it is visible, that the fragment pattern is similar and the retention times only differ by less than 0.1 minutes. Unfortunately the 11keto concentrations in the samples are unknown. Otherwise the ratio of concentrations between the samples should correspond to the ratio of signal intensities.

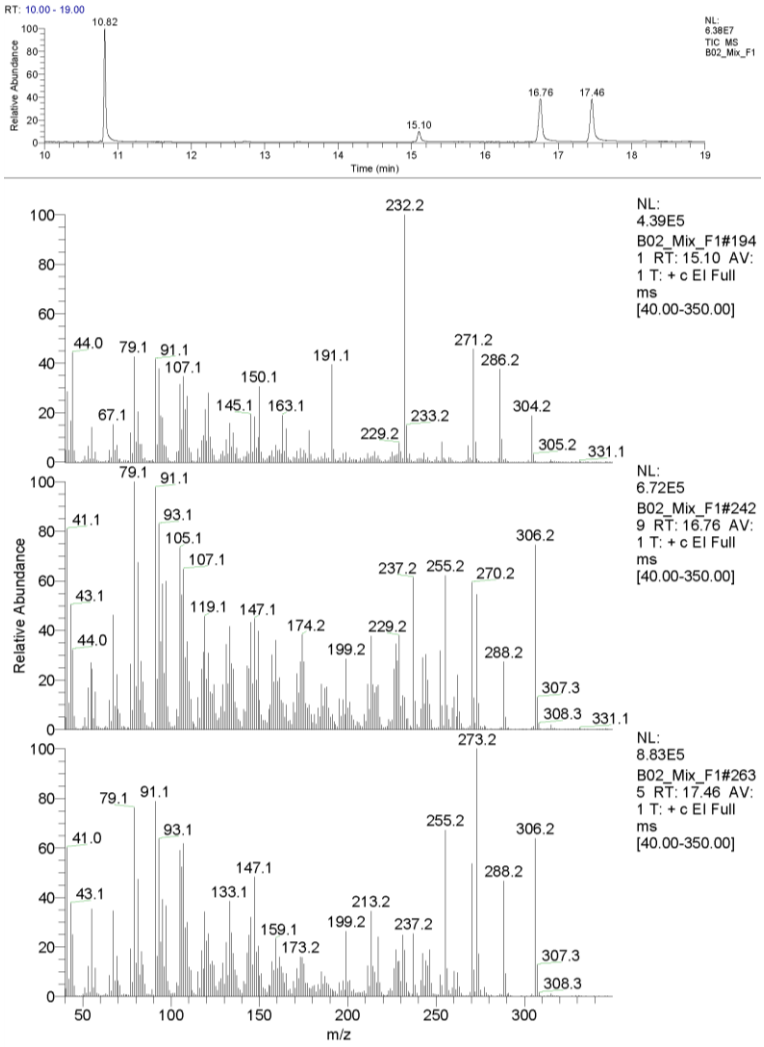


Fig. 43: *presentation of a reference standard containing the following substances, with their retention times in minutes in parenthesis: androstanol (10.82), 11keto (15.10), OHE (16.76) and OHA (17.46). The chromatogram is the total ion current normalized to the highest peak over the time range from 10 to 19 minutes. All mass spectra are in the range of 40-350 m/z and are normalized to the base peak. The internal standard androstanol at 10.82 minutes is intentionally left out of the discussion.*

However the δ^{13}C-values are completely off with values more negative than -30‰. They should be in the same range as PD, the primary ERC. This leads to the assumption, that the window of HPLC-fraction collection is not optimized for 11keto-glucuronide.

Furthermore there is no 11keto-glucuronide standard available, so the optimization of the HPLC-fraction collection times is not plannable at the moment.

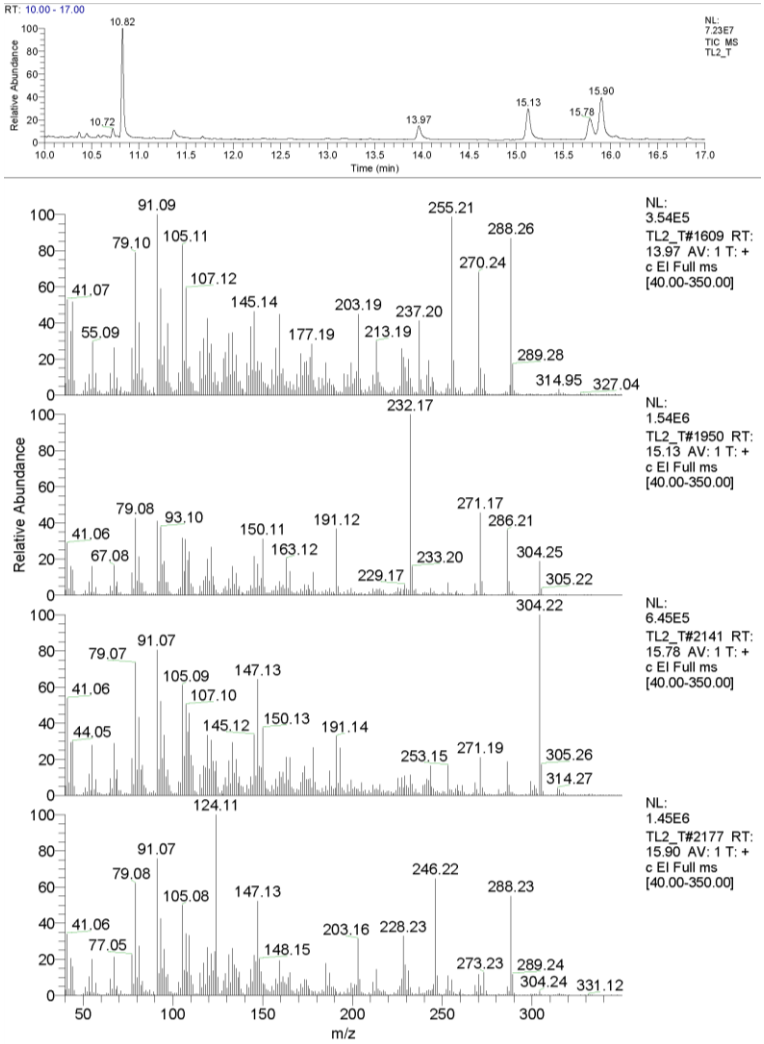


Fig. 44: *Testosterone fraction of the additional negative sample from the alternative sample preparation. Below the chromatogram the mass spectra of noteworthy peaks with the retention times 13.97, 15.15, 15.78 and 15.88 minutes are shown. All mass spectra are in the range of 40-350 m/z and normalized to the base peak. The internal standard androstanol at 10.82 minutes is intentionally left out of the discussion.*

In the additional negative sample there is an extra peak at 13.97 minutes visible as well as the testosterone peak at 15.90 minutes, the presumed 11keto peak at 15.13 and the unknown oxo- or hydroxyl-steroid at 15.78 minutes. This peak is not detected in other samples, and shows similar fragments as the unknown compound in the Testosterone fraction of the routine method, but with a completely different retention time. Again, no further predictions about this substance can be made other than a potential sum formula comparable to testosterone ($C_{19}H_{28}O_2$) and structural similarities to 5βAdiol.

5. Conclusion & Outlook

During this proof-of-concept study a working and promising method for alternative sample preparation to detect the origin of endogenous steroids via IRMS was developed. However at its current state it cannot substitute the routine method or provide any additional information. There are several issues that have to be addressed, if the method shall be implemented in the doping control routine.

Considering the time investment, the hydrolysis has to be reviewed again. Perhaps it can be sped up to compete with the enzymatic option.

The final chromatograms are crowded with peaks compared to the routine method, so an additional clean-up is needed. This can be accomplished by a second preparative chromatography of the free steroids after hydrolysis. The already established routine method can be used as a base for further development.

The apparent recovery is comparable to the standard method or worse, so the manual steps during the SPE and the hydrolysis have to be optimized as well to prevent further losses. The samples that were used for the development have higher concentrations of steroids than most routine samples, which helps the detection. They are also considered as clean, as there are little interferences from the matrix. Also there are no other exogenous or even prohibited substances present. The tested athlete could potentially take a lot of supplements or other medication, which might influence the first chromatographic separation of the glucuronides. Also urine samples with the steroids excreted as unconjugated moieties or with heavy microbial contamination cannot be analyzed. More substances need to be included, 11keto as a second and 16EN as a third ERC, since WADA regulations require at least two ERCs, if PD is compromised.

One point that was one of the potential big benefits of the separation of steroid conjugates and the acidic hydrolysis has not been addressed yet. The implementation of steroids sulfates should be possible and might bring new insights, therefore further tests should be conducted.

6. References

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