University of South Bohemia in České Budějovice

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Derivatization study of selected steroid compounds

Bachelor Thesis

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Abstract

For the purpose of this study, three steroid hormones, namely 1,4-androstadiene-3,17-dione (ADD), 1,4-androstadiene-3-one-17β-ol (boldenone) and 17β-estradiol were selected for derivatization and analysis. The investigation of the enolisation-silylation reaction of the three analytes was in the focus of this bachelor work. Several different experiments were conducted in order to examine the influence of a variety of derivatization agents, the reaction conditions and the stability of the derivatives. Gas chromatography coupled to mass spectrometry was used as the method for analysis and quantitation.

I hereby declare that I have worked on this bachelor thesis independently and used only the sources listed in the bibliography.

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Abbreviations

AAS	anabolic androgenic steroids
ADD	1,4-androstadiene-3,17-dione
BSTFA	*N,O*-bis-(trimethylsilyl)-trifluoroacetamide
DMDCS	dimethyldichlorosilane
EDC	endocrine disrupting chemical
GC	gas chromatography
GC-MS	gas chromatography – mass spectrometry
GPSCs	growth-promoting steroids and steroid-like compounds
ISTD	internal standard
LC-MS	liquid chromatography – mass spectrometry
LOEL	lowest observable effect level
m/z	mass-to-charge ratio
MSTFA	*N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide
MTBSTFA	*N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide
R.S.D.	relative standard deviation
RT	retention time
SPE	solid phase extraction
STW	sewage treatment works
TMIS	trimethyliodosilane
TMS	trimethylsilyl
U.S. EPA	United States Environmental Protection Agency
WWTP	waste water treatment plants

1. Aims of bachelor thesis

For the purpose of this study, three different steroid hormones were examined. A proper analysis of these steroids, however, is rather difficult and requires derivatization prior to analytical measurements. The compounds chosen contained polar keto and hydroxyl groups. Hydroxyl groups can easily be derivatized by silylation. On the other hand, keto groups have to be transformed into their enol form before silylation is possible.

Due to the lack of literature findings concerning the optimal conditions of the derivatization procedure, the improvement of the derivatization method, namely an enolisation-silylation reaction, was the main objective of this bachelor thesis.

The experiments were divided into three parts. First of all, different derivatization agents were tested for their efficiency in combination with various catalysts. Subsequently, studies on the reaction conditions were conducted by varying temperature and duration of the procedure. In addition, the stability of the obtained derivatives during storage was evaluated.

Gas chromatography coupled to mass spectrometry was chosen as the method for analysing and quantifying the derivatives of the individual experimental series. Finally, a statistical evaluation was performed.

2. Introduction

Steroids compromise a variety of chemical compounds and thus feature several functions and fields for commercial applications. This group of chemicals include natural substances such as hormones, but also a number of artificial compounds often produced for their anabolic properties.

Due to various forms of usage especially in human and veterinary medicine, steroid hormones can find their way into the environment. Since they exhibit endocrine-disruptive properties and a tendency for bio-accumulation, they can be regarded as environmental hazards. Understanding the detection and analysis of steroids is therefore crucial to consequently being able to prevent their effects on nature and wildlife.

2.1. Steroids in the environment

Steroid hormones are naturally occurring chemicals and are endogenous to many species. Humans and animals produce steroid hormones in their bodies. Those hormones are subsequently released into the environment via excretion.

Studies have revealed that human males excrete about 10 mg of androgens (mainly testosterone and androstenedione) per day, whereas approximately 5 µg/day of each of the estrogens estrone and estradiol can be found in the excrements of females. During pregnancy these values can be significantly higher, reaching 1000 fold amounts [1,2]. The levels of steroids found in the urine or faeces of humans can also be increased due to the intake of hormone-containing pharmaceuticals such as the contraceptive pill for women or cortisone-containing drugs which are used for pain-relief, as anti-inflammatory agents or immunosuppressives.

Not only humans release endogenous steroids into the environment via excretion, but also animals. Considerable amounts of these hormones are produced by domestic livestock. Animal manure, a mixture of animal faeces, urine and bedding, is considered to be a major source of natural steroids in the environment [1]. In tables 1 and 2 the results of studies about the yearly amount of steroid hormones released into the environment by farm animals in the European Union and the USA are presented [1,3].

Table 1: Estimated steroid excretion of farm animals in the European Union in the year 2000

Species	Amount [million]	Estrogens [tons]	Androgens [tons]	Gestagens [tons]
Cattle	82	26	4.6	185
Pigs	122	3.0	1.0	79
Sheep	112	1.3		58
Chickens	1002	2.8	1.6	
Total	**1318**	**33**	**7.1**	**322**

Table 2: Estimated steroid excretion of farm animals in the United States in the year 2000

Species	Amount [million]	Estrogens [tons]	Androgens [tons]	Gestagens [tons]
Cattle	98	45	1.9	253
Pigs	59	0.83	0.35	22
Sheep	7.7	0.092		3.9
Chickens	1816	2.7	2.1	
Total	**1981**	**49**	**4.4**	**279**

Animals do not only excrete endogenously produced hormones. When kept for agricultural purposes, they are often treated with veterinary drugs which might contain steroid substances as well. Excess steroids then add up to naturally produced compounds found in the animal faeces. Furthermore, the feeding of growth-promoting steroids and steroid like

compounds (GPSCs) is a common method to increase animal growth rates or muscle mass in the U.S. beef cattle industry [4,5]. As illustrated in table 2, cattle contribute about 90% of all estrogens and gestagens excreted by farm animals in the United States and more than 40% of the excreted androgens [3].

The excrements of animals are often used in the form of manure and are applied to fields for the purpose of natural fertilization. The steroids present in the manure are spread alongside. Distribution of the hormonal compounds takes place in soil or through the hydrological cycle. The possible pathways of GPSCs into the environment are shown in figure 1 [4]. The same principle is, of course, also applicable for all naturally produced hormones.

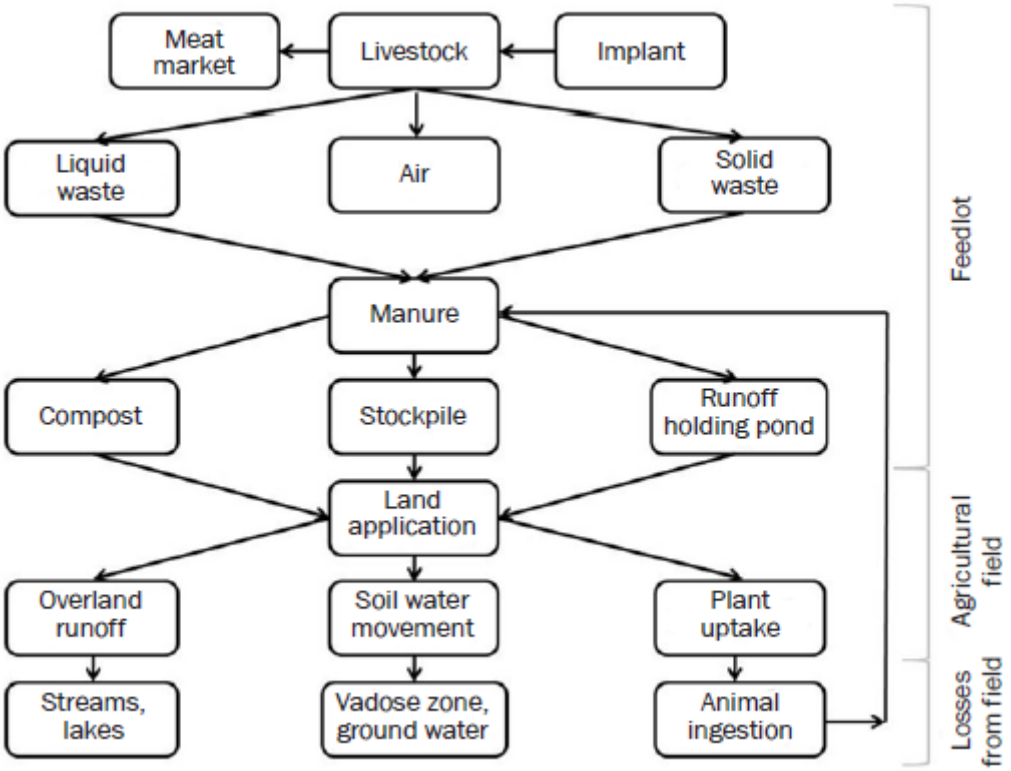


Figure 1: Environmental pathways of steroid compounds excreted by farm animals (source: [4])

As mentioned above, there are several possibilities for the steroids to be distributed in the environment. Steroid compounds are generally considered as lipids and thus they are poorly soluble in water. This low water solubility leads to their enrichment in organic matter like soil. When present in an aqueous environment, steroid compounds may leave the aqueous phase through adsorption to sediment or sludge. Mineralization in biosolids or soils is also a possibility [1]. Moreover, considerable hormone amounts can be detected in sewage waters and run-offs from fields which have previously been fertilized with animal manure [6]. From these sources the hormones can further be transported into surface waters or streams.

Steroids excreted by human beings usually can be prevented from entering the environment as they are concentrated in the sewage system and degraded in waste water treatment plants (WWTP) [1]. Due to their stability, however, a small part of the hormones might pass through the sewage treatment and might be released to surface waters alongside the treated water [7].

Due to their presence and persistence in the environment, steroid hormones, like estrogens, can be considered as environmental pollutants [8]. The estrogens estrone and 17β-estradiol can be found in concentrations of tens of nanograms per litre in effluents of sewage treatment works (STW). These values are above the lowest observable effect level (LOEL) of 10 ng/L for organisms like fish and plants [9,1]. Additionally, it has been found out that even these small concentrations of steroids present in the STW effluents can affect aquatic animals [10].

Based on their chemical and structural properties, steroid hormones can function as endocrine disruptors and as such have an impact on the reproduction of animal species [11]. The effects of such endocrine disrupting chemicals (EDCs) are particularly distinct for aquatic life forms and include reproductive abnormalities, such as reduced gonadal growth, delayed sexual maturity or depressed sex steroids in fish. Exposure to EDCs can be observed to cause reduced fertility or intersexuality in fish [12]. Estrogens, like estrone or 17β-estradiol, or the synthetic compound 17α-ethinylestradiol, the active ingredient used in contraceptive pills, are suspected to lead to feminization of male fish [13,14]

EDCs like steroid hormones do not only affect wildlife, but can also have an impact on humans. Studies suggest that the exposure of endocrine disruptors has an influence on the sex ratio of newborns in disfavour of males, meaning that an unusually small proportion of male children is born [15]. The impacts, however, might not always be self-evident. Also long-term effects such as mutations causing metabolic dysfunctions, infertility and possibly cancer might occur [16,17].

2.2. Analysis of steroids in previous studies

Due to the fact that steroid compounds are accumulated in the environment and can have severe impacts on wildlife, it is important to improve the knowledge about their distribution and effects in order to be able to develop preventive methods and solutions for the environmental issues. Several studies on the analysis of steroids by LC-MS and GC-MS have already been conducted. Regarding GC-MS, many of these studies have focused on silylation or enolisation-silylation reactions for derivatization prior to quantitative analysis – a method which has also been the core theme of this bachelor thesis.

An extensive study has been implemented by the U.S. Geological Survey and devoted to the determination of 20 different steroid hormones as well as related compounds in filtered and unfiltered water. A new method for analysis could be developed. Water samples were taken from groundwater or surface waters, from WWTP effluents or from agricultural runoffs. After sampling, the analytes of interest were isolated via solid-phase extraction (SPE) followed by silylation of active functional groups and final analysis by gas chromatography with tandem mass spectrometry. Derivatization by silylation was performed with an MSTFA reagent, similarly to the method applied for the experimental work conducted in the course of this bachelor thesis. The performance of the method was tested by analysis of spiked replicate samples featuring steroid concentrations of 10 and 100 ng/L. Recovery rates between 60 and 120 % were desired and obtained. Moreover, the persistence of analytes in reagent water before derivatization was tested. Stabilities of 8 days when the analytes were stored refrigerated at +4°C or of 56 days when stored frozen at -15°C were suggested [18].

The optimization of the silylation reaction of estrogen compounds has been the goal of another study by Shareef et al. in 2006. GC-MS was again the method of choice for analysis and quantitation. MSTFA, BSTFA and the third reagent *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA) were tested for derivatization. MTBSTFA, however, turned out to be not suitable for the derivatization of the tested estrogen 17α-ethinylestradiol as it promotes the steroid's breakdown to estrone. Degradation also occurred when using MSTFA and BSTFA reagents in pyridine solvent at temperatures above 75°C. When using dimethyl formamide as solvents, optimum reaction was observed for temperatures between 75 and 80°C. 30 minutes seemed to be a sufficient time period for complete derivatization [19]. Other experiments about derivatization reaction conditions have revealed that silylation can be performed under mild conditions at temperatures between 50 and 100°C for 15 to 30 minutes. The derivatization of hindered functional groups requires stronger conditions and the use of catalysts [20].

3. Studied compounds

The group of compounds studied for this bachelor thesis are classified as so-called steroid compounds.

Steroids, in general, are organic compounds exhibiting a polycyclic hydrocarbon skeleton and are described as lipids according to their properties. The basic framework consists of 17 carbon atoms arranged in a certain configuration of four rings – three six-membered rings and one five-membered ring. The four rings are usually labelled with letters A to D as depicted in figure 2. The three cyclohexane rings (A, B and C) form the skeleton of a perhydro-derivative of phenanthrene. The D-ring has a cyclopentane structure. In addition to their core structure, steroids feature a variety of functional groups in different positions.

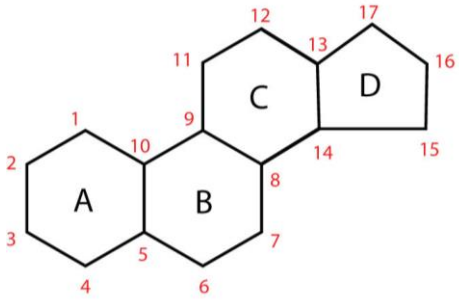


Figure 2: General framework of steroid compounds
(source: *http://catalog.flatworldknowledge.com/bookhub/reader/2547?e=gob-ch17_s04*)

Many steroids act as hormones in plants and animals including humans and are responsible for a variety of biochemical functions. Widely known representatives of this group of substances include bile acids, the dietary chemical cholesterol, but also vitamins (e.g. Vitamin D3 – also called ‘cholecalciferol’, a steroid molecule with one open ring) and sexual hormones such as estrogen or testosterone.

For the experiments three different steroid hormones featuring hydroxyl and keto groups in positions 3 and 17 were chosen.

3.1. ADD

ADD is the abbreviation for 1,4-androstadiene-3,17-dione which is also known under the name of ‘boldione’. It is known as a prohormone or precursor of the anabolic compound boldenone. The precursor ADD can be converted to the metabolically active form of boldenone via enzymatic reactions in the human or animal body. For this reason, the intake of prohormones has become a popular option for sportspeople to achieve the doping effect of anabolic drugs [21- 23].

As depicted in figure 3, ADD possesses a keto group in each of the positions 3 and 17.

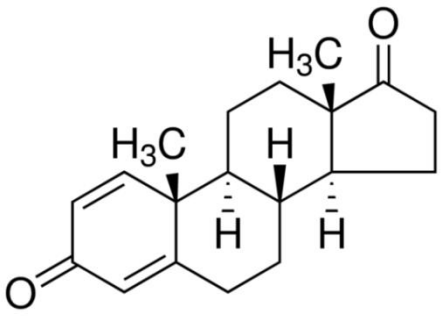


Figure 3: Structure of ADD (source: *http://www.sigmaaldrich.com*)

3.2. Boldenone

Boldenone (or 1,4-androstadiene-3-one-17β-ol) is classified as an anabolic androgenic steroid (AAS). These AAS compounds are derivatives of the male endogenous hormone testosterone and are synthetically produced and modified to enhance their anabolic effects and simultaneously lower their androgenic activity. These anabolic effects include protein synthesis, muscle growth and synthesis of red blood cells (erythrocytes). The compound boldenone was initially developed for veterinary purposes, but has also found illegal usage as a growth promoter in cattle. Moreover, boldenone is used as a doping agent for racehorses as well as human athletes [21-26].

In comparison to its precursor ADD, boldenone features a hydroxyl group instead of a keto group in position 17. The rest of their structures is identical (compare figures 3 and 4).

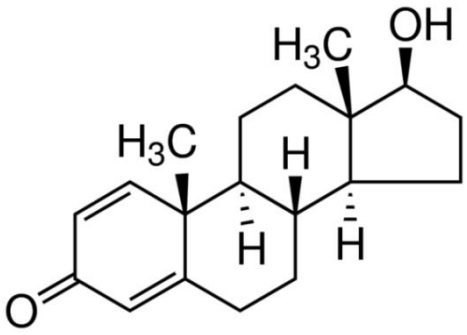


Figure 4: Structure of boldenone (source: *http://www.sigmaaldrich.com*)

3.3. 17β-Estradiol

17β-estradiol (often simply called ‘estradiol’) is described as the primary biologically active estrogen [27]. Estrogens are the primary female sexual hormones and are synthesized in all vertebrates and in some insects [28]. They control the female reproductive system as well as the development of female secondary sex characteristics.

Estradiol is frequently used as a component of oral contraceptives [29] and has shown to have anti-inflammatory effects on the nervous system and the ability to reduce neuropathic pain [30, 31].

17β-estradiol was chosen for this study as it exhibits two hydroxyl groups located at the positions 3 and 17 (figure 5).

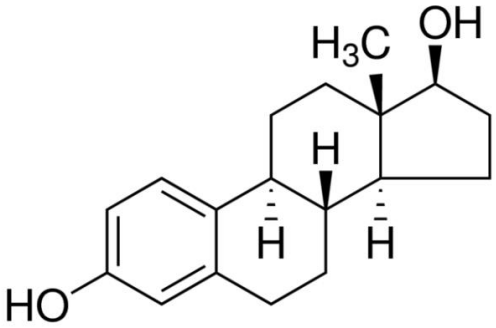


Figure 5: Structure of 17β-estradiol (source: *http://www.sigmaaldrich.com*)

4. Theory of experimental methods

4.1. Derivatization

Derivatization is a method frequently used in chemistry. It allows the quantitative transformation of chemical compounds into related species called ‘derivatives’. The general structure of the initial compound is thereby maintained. Usually the functional groups are the target of transformation.

Special derivatization reagents are used for the procedure. They react with the initial compound and form the product derivative. There is a variety of commercially available derivatization agents that feature different characteristics based on different requirements. Typical reagents are methylation agents which cause the introduction of methyl groups to the studied compound. Another common derivatization technique is silylation, a method leading to the formation of silyl derivatives.

The general purpose of derivatization is to adjust a compound to certain requirements. Chemical substances might be stabilized via derivatization and their chemical properties might be changed, which can facilitate analysis or make them more suitable for particular processes.

4.2. Enolisation-silylation

The steroid compounds studied in these experiments feature hydroxyl and keto groups. Analysis was performed by GC-MS which is the method of choice for studying these chemicals due to its high resolution and a relatively fast analysis speed [32]. Unfortunately, steroids are rather unsuitable for the applied GC-MS method due to their weak volatility, thermal instability and their polar functional groups. Derivatization prior to GC-MS analysis is therefore necessary to avoid adsorption in the column and to enhance their sensitivity for analytical detection [33].

For this study, silylation was chosen as the method of derivatization. This technique is commonly applied for the derivatization of organic compounds containing functional groups with active hydrogen atoms such as hydroxyl (-OH), carboxylic (-COOH) or amino ($-NH_2$) groups. The resulting silyl derivatives are less polar and more stable than the original compounds [20]. Moreover, their volatility is increased, which facilitates separation by gas chromatography. In addition, the silyl derivatives feature a higher molecular weight as well as a larger mass-to-charge-ratio (m/z) of their characteristic mass spectrometric ions, which makes identification and quantitation less vulnerable to interferences [18].
In the case of this experiment, silylation reagents were used which caused the generation of trimethylsilyl (TMS) derivatives. A general reaction equation for this process can be written as:

$$R\text{-}OH + Me_3Si\text{-}X \rightarrow R\text{-}O\text{-}SiMe_3 + H\text{-}X$$

Silylation can be easily applied to any compound featuring an active hydrogen atom in their functional group, which is also true for the selected analytes containing hydroxyl groups (boldenone and 17β-estradiol). However, some of the analytes (ADD and boldenone) also exhibit keto functional groups which cannot simply be derivatized by silylation as they lack free active hydrogen atoms. The method of enolisation-silylation, on the other hand, allows the silylation of keto groups in a special way and was therefore utilized in the experiments conducted for this study. In a first step, the keto groups of the steroids are converted into their enol tautomers. In the course of this enolisation, the hydrogen atom attached to the α-carbon is migrated to the oxygen of the keto group. In the process a new carbon-carbon

double bond is formed and a hydroxyl group is generated. The mechanism of the keto-enol equilibrium is depicted in figure 6.

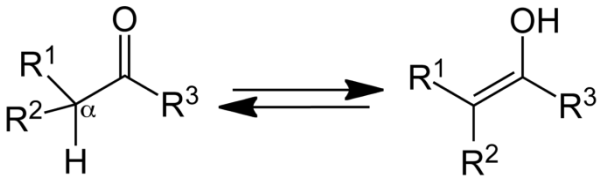


Figure 6: Keto-enol tautomerism (source: *Wikipedia*)

The newly formed enol compound features a hydroxyl group which can be derivatized by silylation resulting in an enol-silyl derivative product. The enolisation-silylation reaction is a beneficial and profitable method as it directs the keto-enol equilibrium towards the enol side and simultaneously forms the respective enol-silyl derivative [33].
Enolisation-silylation can be achieved by trimethylsilylation agents like MSTFA in combination with some catalysts (e.g. trimethyliodosilane (TMIS) or ammonium iodide) [33].
In figure 7, the derivatization by enolisation-silylation of the steroid androsterone, a compound featuring both a hydroxyl and a keto group, is illustrated [34].

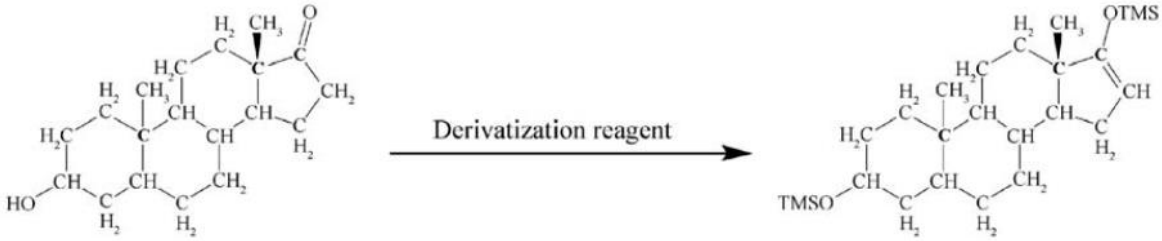


Figure 7: Enolisation-silylation reaction of androsterone (source: [35])

5. Experimental procedure

5.1. Chemicals and reagents

The three analytes ADD, boldenone and 17β-estradiol were prepared in the form of mixed stock solutions in toluene. For the GC-MS analysis, calibration standards containing all three analytes at five different concentration levels were prepared. Toluene again served as the solvent. In addition to the external calibration, an internal standard (ISTD), namely cholesterol (structure see figure 8), was used. It was added to both the samples and the calibration standards.

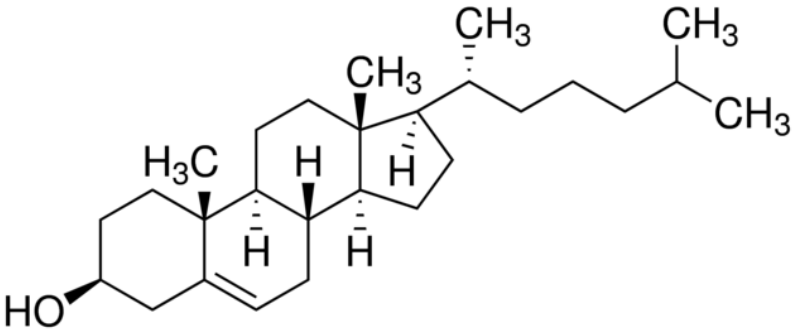


Figure 8: Structure of cholesterol (source: *http://www.sigmaaldrich.com*)

For the silanization of the glassware the silanizing agent Sylon-CT™, a solution of 5% of DMDCS (dimethyldichlorosilane) in toluene, was used. Moreover, pure toluene and methanol were required.

For the derivatization reaction several derivatization agents were tested. For that purpose, the reagents MSTFA (*N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide) and BSTFA (*N,O*-bis-(trimethylsilyl)-trifluoroacetamide) in combination with a variety of catalysts were used. Figures 9 and 10 depict the basic structures of MSTFA and BSTFA. In table 3 all derivatization agents as well as the activating compounds present in some of the solutions are listed.

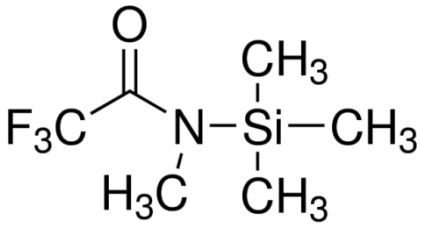


Figure 9: Structure of MSTFA (source: *http://www.sigmaaldrich.com*)

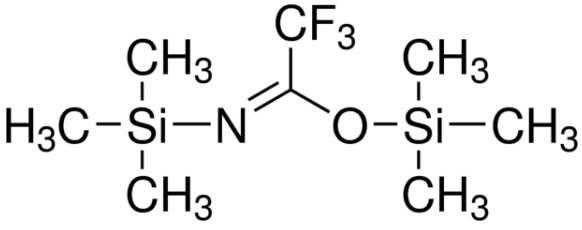


Figure 10: Structure of BSTFA (source: *http://www.sigmaaldrich.com*)

Table 3: Derivatization agents

Reagent	Activated with
MSTFA	
MSTFA I	ethanethiol and ammonium iodide
MSTFA II	2-(trimethylsilyl)ethanethiol
MSTFA III	imidazole
BSTFA	

The inactivated derivatization agents MSTFA and BSTFA were used in the form of a 1:1-mixtures with pyridine. Moreover, an experiment was conducted where the reagent MSTFA II, an already activated solution, was mixed with the additional catalyst pyridine. For the described experiments, an anhydrous form of pyridine was utilized.

For re-dissolving the analytes prior to GC-MS measurement, *n*-hexane was chosen as the appropriate solvent.

5.2. Silanization of glassware

Steroid compounds class among the lipids and their core structure is built up by an apolar hydrocarbon framework. This apolar character allows the compounds to adsorb to different surfaces such as plastic or glass and makes them vulnerable to loss of sample due to adhesion to laboratory equipment during experimental procedure.
For this reason, the interior surfaces of the vials used for reaction had to be deactivated prior to usage. Deactivation was achieved by silanization. Sylon-CT^{TM} was used as silanization reagent.
In the first part of the silanization procedure, the glass surface of the small volume vial inserts required for the experiments was coated by rinsing with the reagent for at least one minute. The coating step was repeated two more times. The excess reagent was reused during each coating step by transferring the solution from one glass vial to the next. Subsequently, the vial inserts were rinsed two times with toluene and a third time with methanol. The deactivated surface was then dried under a gentle stream of nitrogen.

5.3. Sample preparation

For the purpose of the following experiments, sample solutions containing all three steroid analytes were required.
First of all, initial stock solutions with a concentration of 1 mg/mL were prepared for each analyte. For that, respective amounts of the individual solid steroids were weighed out and dissolved in toluene in the case of ADD and boldenone or in methanol in the case of 17β-estradiol. In addition, a stock solution for the internal standard cholesterol in toluene was prepared featuring a concentration of 1 mg/mL as well.
For the actual experiments, a mixed solution containing all three analytes at a concentration of 10 µg/mL was used. This working solution was prepared by dilution of the above mentioned stock solutions with toluene and included the internal standard cholesterol at a concentration of 30 µg/mL.

5.4. Derivatization reactions

The selected compounds all featured hydroxyl and keto groups in positions 3 and 17 of their core steroid skeleton. Derivatization by silylation had to be achieved for both of these functional groups leading to the generation of bis-trimethylsilyl (TMS) derivatives.
The experiments conducted for this study were split into three different setups with individual goals. First of all, different derivatization agents were tested. The most promising reagent determined in this experiment was then used for further investigations on the reaction conditions and the derivatives' stability. The division of the experiment enabled a broad and comprehensive examination of the aspects and efficiency of the enolisation-silylation derivatization reaction.

For all experiments, the mixed sample solution containing 10 µg/mL of each analyte and 30 µg/mL of ISTD in toluene was used. Three samples were prepared and measured for each setup or condition to guarantee statistical significance and to facilitate a statistical analysis.

5.4.1. Experiment No. 1 – Derivatization agents

The first part of the study focused on the testing of different derivatization agents and aimed to determine one reagent which is best suitable for all three steroid analytes.

For the derivatization, 70 µL of sample solution were transferred to a GC-MS vial containing a silanized small-volume insert. The solvent was evaporated to dryness under a stream of

nitrogen at a temperature of 25 to 30°C. After evaporation 50 µL of one of the derivatization agents respectively were added. When pyridine was used as a catalyst, an additional amount of 50 µL of this compound was added to the vial to achieve a 1:1-ratio of reagent and pyridine. The vials were then placed into a heating block and the mixtures were heated at 65°C for 60 minutes. Afterwards, the vials were removed from the heating block and allowed to cool to room temperature. Subsequently, evaporation under a stream of nitrogen was performed to remove excess amounts of the derivatization reagents. The analyte residues were then redissolved with 70 µL of *n*-hexane.

To following table summarizes the reagents which were used for this experiment.

Table 4: Reagents studied for derivatization reaction

No.	Reagent	Addition of	Ratio
1	MSTFA	pyridine	1:1
2	MSTFA I		
3	MSTFA II		
4	MSTFA III		
5	BSTFA	pyridine	1:1
6	MSTFA II	pyridine	1:1

5.4.2. Experiment No. 2 – Reaction conditions

The reaction conditions, namely temperature and duration of the derivatization reaction, were the target parameters of this part of the study. Therefore, a variety of time and temperature combinations were tested in order to determine the optimal conditions. The combinations of three temperatures and three times were examined and are depicted in table 5.
The general derivatization procedure was conducted as described in chapter 5.4.1. MSTFA II was used as the derivatization agent. In contrast to experiment No. 1, however, 100 µL of sample and subsequently 100 µL of *n*-hexane for re-dissolution were used (instead of only 70 µL).

Table 5: Studied reaction conditions (time-temperature combinations)

No.	Temperature [°C]	Time [min]
1	55	30
2	55	60
3	55	90
4	65	30
5	65	60
6	65	90
7	75	30
8	75	60
9	75	90

5.4.3. Experiment No. 3 – Stability of derivatives

In the last part of the study, the stability of the derivatives during storage was investigated. Derivatization was performed with 100 µL of sample and 50 µL of MSTFA II at a temperature of 65°C for 60 minutes according to the procedure described in chapter 5.4.1. 100 µL of *n*-hexane were used for re-dissolving the analyte residues after derivatization and evaporation.

Two different storage conditions were examined. One part of the samples was stored in the refrigerator at a temperature of +4°C. The other half of the samples was kept in the freezer at a temperature of -17°C. Storage times of 0, 1, 4, 15 and 40 days before GC-MS analysis were examined.

5.5. GC-MS analysis

All samples containing the analyte derivatives and obtained from the various experimental setups were finally analysed by gas chromatography coupled to mass spectrometry after re-dissolution in *n*-hexane.

For the measurements, a Trace GC Ultra gas chromatograph coupled to an ITQ 1100 mass spectrometer (ThermoFisher Scientific, MA, USA) was used.

The chromatographic separation was performed on a ZB-5 MS capillary column (Phenomenex®, CA, USA) with a length of 30 m, an inner diameter of 0.25 mm and a film thickness of 0.25 µm. The stationary phase of the GC-column was composed of 5% phenyl-arylene and 95% of dimethylpolysiloxane. Helium served as the carrier gas and was operated at a constant flow rate of 1 mL/min.

Injection occurred in the splitless mode. The inlet temperature was set to 275°C, the transfer line temperature was adjusted to 250°C. The following temperature program was applied:

Table 6: GC-temperature program

Ramp	Rate [°C/min]	Final temperature [°C]	Hold time [min]
Initial T= 130°C			1.00
1	25.00	235	0.00
2	2.00	265	0.00
3	5.00	290	0.00
4	10.00	305	8.00

A positive ion mode was applied for mass analysis. The mass analyser was operated in the full scan mode with an m/z mass range of 50 to 650 Da and a solvent delay of 6.50 minutes. The ion source was maintained at a temperature of 200°C.

Quantification of the GC-MS data obtained from the sample measurement was performed via an external calibration in combination with an internal standard.
Thus, a calibration series had to be prepared. Calibration standards featuring concentrations of 0.5 µg/mL, 2 µg/mL, 5 µg/mL, 10 µg/mL and 30 µg/mL of each of the three analytes and an ISTD-concentration of 30 µg/mL were used.
These calibration standards were derivatized with the reagent MSTFA II at a temperature of 65°C for 60 minutes according to the above described procedure.

6. Results and discussion

6.1. Chromatographic separation of the analyte derivatives

The chromatographic separation of the individual analytes and the internal standard was successful. Sufficient separation could be achieved for all compounds.

A sample chromatogram can be found in the appendix (Image 10). It was obtained from the measurement of experiment No. 2 at a derivatization temperature of 55°C and a time of 90 minutes.

In table 7, the order of elution and the approximate retention times of the analysed steroid derivatives as obtained for the analysis of the above mentioned experiment chromatogram are listed.

Table 7: Retention times of the studied derivatives

Peak	Derivative of	Retention time [min]
1	ADD	14.6
2	Boldenone	14.9
3	17β-Estradiol	15.2
4	Cholesterol	24.6

Another peak could be obtained at a retention time of approximately 21.7 minutes as shown in the chromatogram in the appendix (Image 10). This peak refers to the derivative of cholecalciferol, a compound which was used as an alternative internal standard but was not considered for data evaluation.

6.2. Mass spectra of the analysed steroid derivatives

In the appendix, the mass spectra of the trimethylsilyl (TMS) derivatives of the three steroid analytes and the internal standard cholesterol are depicted. These spectra were obtained from the same measurement as the above illustrated chromatogram (Experiment No.2: derivatization at 55°C for 90 minutes).

Typical fragmentation patterns could be observed for the steroid derivatives. The mass-to-charge ratios of the major ions are listed in table 8. Their relative abundances are given in percent (%), the molecular ion is marked as $[M]^+$. The ion $[M-15]^+$ featuring an m/z of 15 Da lower than the molecular ion occurred due to the loss of a methyl group from the derivative.
The characteristic ions depicted in the mass spectra were used for identification of the individual analyte derivatives.

Table 8: Selected ions of the studied derivatives

Derivative of	m/z of selected ions [Da] (relative abundance [%])
ADD	428 $[M]^+$ (53%), 413 $[M-15]^+$ (26%), 323 (47%), 222 (40%), 206 (100%), 191 (33%)
Boldenone	430 $[M]^+$ (25%), 415 $[M-15]^+$ (12.5%), 325 (32%), 206 (100%), 191 (22.5%)
17β-Estradiol	416 $[M]^+$ (86.5%), 401 $[M-15]^+$ (4%), 326 (55%), 285 (100%), 232 (25%)
Cholesterol	458 $[M]^+$ (50%), 443 $[M-15]^+$ (13%), 368 (100%), 353 (36.5%), 329 (47%)

6.3. Results of quantitation

The quantitation of the measurement results was based on an external calibration in combination with the internal standard which had been added to both the samples and the calibration standards. A calibration curve was determined for each analyte for each of the three experimental setups by plotting the concentrations of the calibration standards against the ratio of the integrated areas of the analytes and the ISTD. The plots of the calibration curves can be found in the appendix (Images 1-9).

6.3.1. Experiment No.1 – Derivatization agents

The following tables illustrate the results obtained for the three analytes during the first experiment series. The results are given as the average yield calculated from the individual measurement values of the three samples prepared for each derivatization condition.

Table 9: Results of experiment No. 1 for derivatives of ADD

Reagent	Average yield [%]	Relative standard deviation [%]
MSTFA + pyridine	0	0
BSTFA + pyridine	0	0
MSTFA I	47.32	2.49
MSTFA II	72.21	7.78
MSTFA III	0	0
MSTFA II + pyridine	0	0

Table 10: Results of experiment No. 1 for derivatives of boldenone

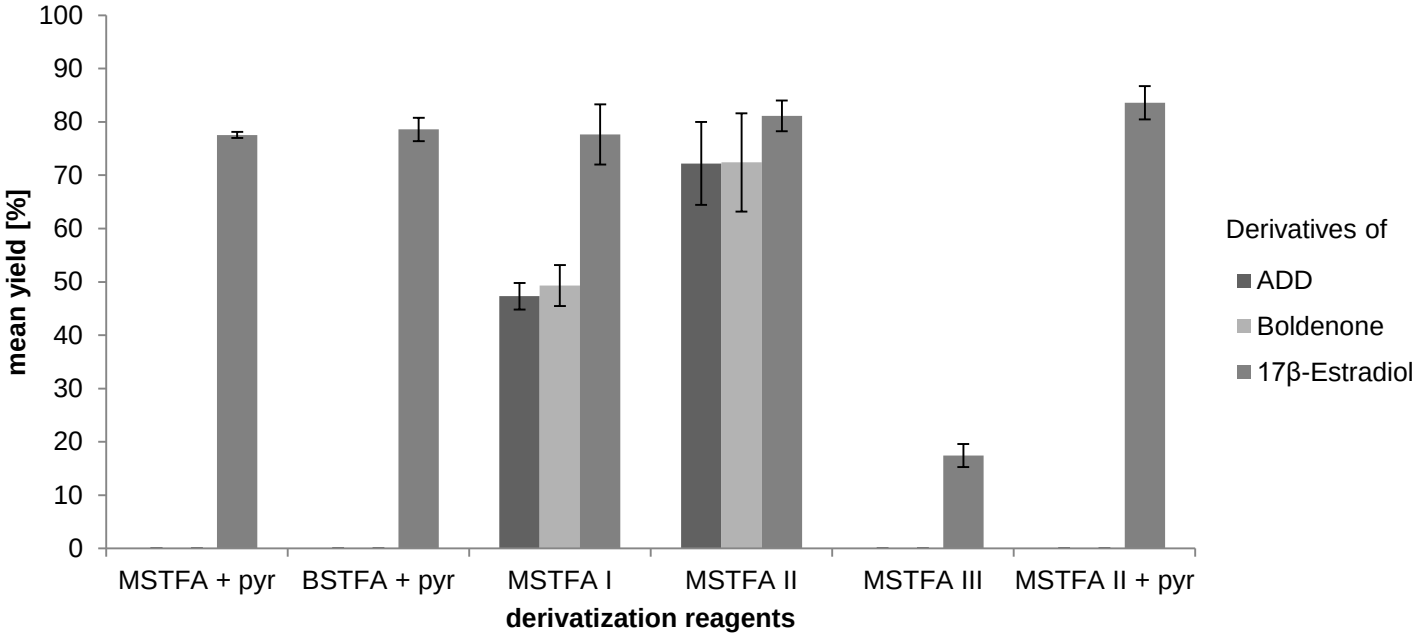
Reagent	Average yield [%]	Relative standard deviation [%]
MSTFA + pyridine	0	0
BSTFA + pyridine	0	0
MSTFA I	49.30	3.83
MSTFA II	72.41	9.22
MSTFA III	0	0
MSTFA II + pyridine	0	0

Table 11: Results of experiment No. 1 for derivatives of 17β-estradiol

Reagent	Average yield [%]	Relative standard deviation [%]
MSTFA + pyridine	77.54	0.57
BSTFA + pyridine	78.60	2.19
MSTFA I	77.66	5.66
MSTFA II	81.14	2.89
MSTFA III	17.41	2.15
MSTFA II + pyridine	83.57	3.12

A comparison of the results from all three analytes is represented graphically in figure 11.

Figure 11: Comparison of derivatization reagents used for the enolisation-silylation reaction



The results clearly show that successful derivatization of all three analytes could only be achieved by two reagents, namely MSTFA I and MSTFA II. The compounds ADD and boldenone could not be derivatized using any other reagent. ADD and boldenone both feature keto functional groups in comparison to 17β-estradiol which possess only hydroxyl groups in the positions of interest. Therefore, it can be considered obvious that derivatization of compounds containing keto groups can only be achieved when using the reagents MSTFA I or MSTFA II.

For the third analyte, 17β-estradiol, on the other hand, positive results could be obtained from all six reagent mixtures. The yield of the obtained trimethylsilyl derivatives of 17β-estradiol was comparable for all examined reagent treatments, except for MSTFA III. This method yielded in a significantly lower derivative amount, namely about 17%, whereas yields of approximately 80% could be obtained from the other reagents for 17β-estradiol. The significance could be proven via statistical analysis ($F = 8.59$; $p < 0.001$. Tukey HSD test).

A rather high difference between the yields obtained from the use of the two possible reagents, MSTFA I and MSTFA II, could be identified for the derivatives of ADD and boldenone. The two analytes showed the same trend of results. Yields of almost 50% could be obtained from MSTFA I for ADD- as well as boldenone-derivatives. Use of MSTFA II resulted in an average value of about 72% for both analytes. Therefore, it can be said that treatment with MSTFA II generally led to a significantly higher amount of derivatives than the method using the MSTFA I for both compounds (ADD: $F = 27.84$; $p < 0.01$. Tukey HSD and boldenone: $F = 16.10$; $p < 0.05$. Tukey HSD).

Ding et al. recorded recoveries between 78 and 102% in their study concerning the derivatization of estrogenic compounds using mainly trimethlsilylating agents [35]. This range is in accordance with the yield of 17β-estradiol derivatives of around 80% obtained for most of the reagents tested in this study. MSTFA activated with 2-(trimethylsilyl)ethanethiol (as used in MSTFA II) and ammonium iodide (as used in MSTFA I) was applied as derivatization agent on a variety of steroid hormones, including 17β-estradiol and cholesterol, in another study by Foreman et al. Recoveries between 60% and 120% were reported [18].

All results obtained for any steroid derivative or derivatization reagent in this study can be considered as conclusive, lying within a reasonable range of standard deviation.
MSTFA II could be identified as the most suitable reagent for the keto-group-containing steroids ADD and boldenone. For 17β-estradiol most reagents achieved similar results and thus, also MSTFA II yielded satisfactory amounts of derivatives for analysis.
Summing up, it can therefore be said that MSTFA II might be the derivatization agent of choice among the ones tested for the optimization of the enolisation-silylation reaction.

6.3.2. Experiment No.2 – Reaction conditions

The results obtained for the second experiment concerning the reaction conditions are shown in tables 12 to 14.

Table 12: Results of experiment No. 2 for derivatives of ADD

Temperature [°C]	Time [min]	Average yield [%]	Relative standard deviation [%]
55	30	85.61	2.82
55	60	90.60	4.66
55	90	95.47	10.69
65	30	88.47	2.51
65	60	77.06	0.51
65	90	90.58	8.63
75	30	67.53	14.61
75	60	80.79	10.59
75	90	75.94	13.61

Table 13: Results of experiment No. 2 for derivatives of boldenone

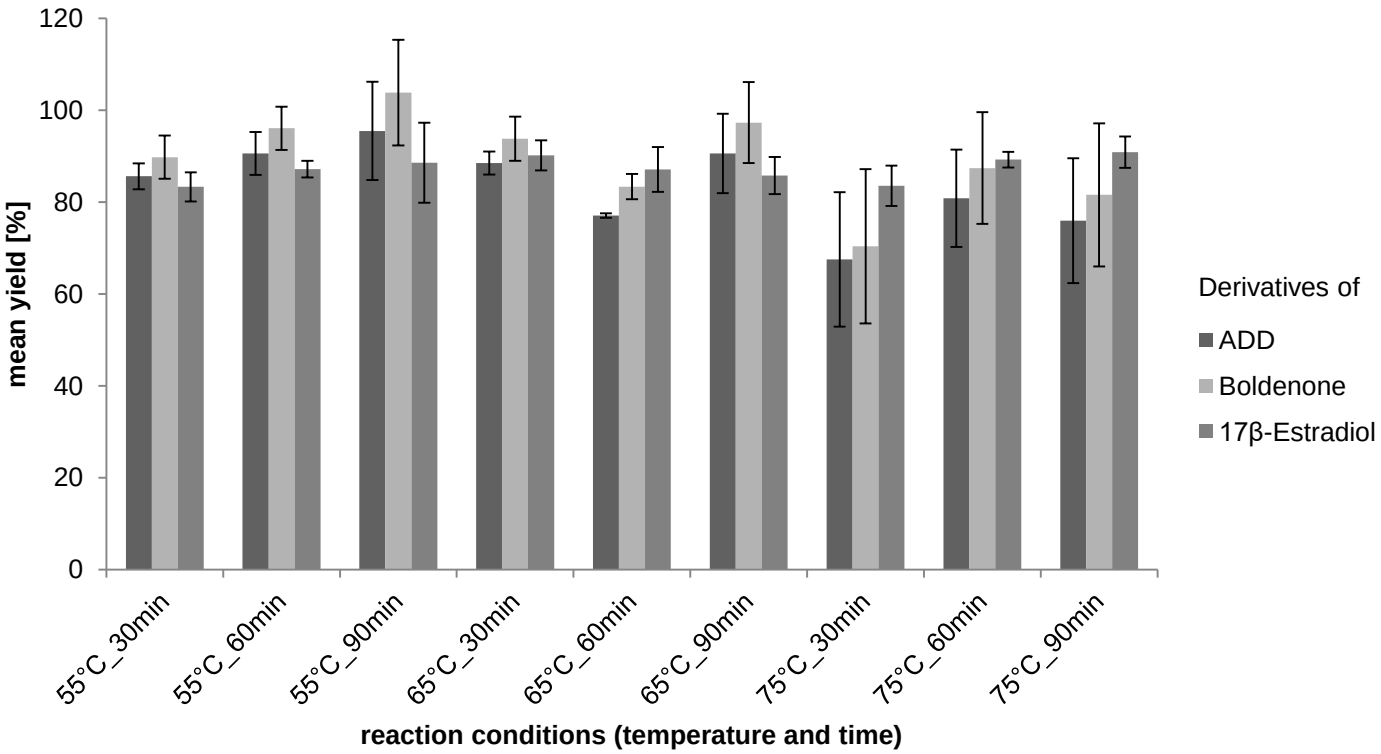
Temperature [°C]	Time [min]	Average yield [%]	Relative standard deviation [%]
55	30	89.77	4.97
55	60	96.05	4.73
55	90	103.81	11.51
65	30	93.78	4.81
65	60	83.34	2.76
65	90	97.27	8.82
75	30	70.36	16.79
75	60	87.40	12.18
75	90	81.57	15.58

Table 14: Results of experiment No. 2 for derivatives of 17β-estradiol

Temperature [°C]	Time [min]	Average yield [%]	Relative standard deviation [%]
55	30	83.30	3.17
55	60	87.18	1.80
55	90	88.54	8.72
65	30	90.16	3.28
65	60	87.07	4.88
65	90	85.78	4.04
75	30	83.53	4.41
75	60	89.23	1.70
75	90	90.87	3.43

All results are summarized and illustrated in figure 12.

Figure 12: Comparison of the time-temperature reaction conditions present during derivatization



The determination of the optimal reaction conditions for the enolisation-silylation process was much less explicit than defining the most suitable derivatization reagent.

For 17β-estradiol, average derivatization rates between 83 and 91% were obtained. Statistical analysis, however, did not reveal any significant difference in the yield for the derivatives of 17β-estradiol. Hence, it can be concluded that the reaction conditions, namely the different combinations of reaction time and temperature tested in this experiment, do not influence the outcome.

For ADD and boldenone, the range of average derivative yields obtained from the measurement was broader. Values between about 67 and 95% could be observed for ADD derivatives, for boldenone derivatives values of above 70% were achieved. The trends for these two compounds were again similar with boldenone leading to slightly higher results. A significant difference, however, could only be observed between the procedure conducted at 55°C for 90 minutes and the one at 75°C for 30 minutes (ADD: $F = 2.69$; $p < 0.05$. Tukey HSD and boldenone: $F = 2.81$, $p < 0.05$. Tukey HSD). For both analytes, the highest yields were obtained for the first treatment (55°C, 90 minutes), leading to values around 95% and 103% for the derivatives of ADD and boldenone respectively. The treatment at 75°C for 30 minutes, in contrast, resulted in the lowest yields of about 70% for both steroid analytes.

In a study from 2006 by Shareef et al., it was shown that full derivatization of the estrogen steroids estrone and 17α-ethinylestradiol could be achieved at temperatures between 45°C and 105°C for the reagents MSTFA, BSTFA and MTBSTFA in different solvents. TMS-derivatization at 60°C for 30 minutes served as the standard method. Moreover, it could be observed that during derivatization with MSTFA or BSTFA in pyridine a breakdown of the estradiol-derivative to estrone occurred at temperatures above 75°C [19]. Derivatization at lower temperatures could therefore be recommended. In order to avoid degradation, experiments at exceeding temperatures were not performed in the course of this bachelor thesis.

The highest yield of derivatives obtained in this study was achieved for the procedure performed at 55°C for 90 minutes. Accordingly, these reaction conditions might be considered as the optimum. However, the elapsed time period required for sample preparation and experimental work should be kept at a minimum when optimizing a method, but is particularly long for the above mentioned procedure. The time-temperature combination of 30 minutes and 65°C was therefore chosen as the optimal condition for the derivatization reaction. The yields obtained at this temperature are sufficient for all analysed derivatives and a reaction time of only 30 minutes is considerably lower than that of the previously mentioned method.

6.3.3. Experiment No.3 – Stability of derivatives

Evaluation of the data for the third part of the study revealed that some measurement results were rather inconclusive. Several low values were detected and in some cases quantification could not be executed due to values below the detection limit. These values, however, occurred in contrast to reasonable values. Deviations occurred within one set of measurement, i.e. for some of the three samples prepared and measured for each condition.
The reason for these divergences, unfortunately, is unclear. It can only be guessed that some sample vials might not have been properly closed and analyte derivatives might have been lost due to volatilization.
As a consequence, outliers were excluded when only one of the three measurement results was affected. The calculation of the average yield was then based on the remaining two values. Those figures are marked with a star (*) in tables 15 to 17. Due to lack of 2 or even three values for one sample triplicate, some measurement sets could not be quantified or evaluated at all.

The results, if obtained, are presented in the following tables.

Table 15: Results of experiment No. 3 for derivatives of ADD

Storage temperature	Storage time [days]	Average yield [%]	Relative standard deviation [%]
Fridge (+4°C)	0	80.64	2.74
Fridge (+4°C)	1	79.25	3.44
Fridge (+4°C)	4	68.58	0.40
Fridge (+4°C)	7	-	-
Fridge (+4°C)	15	62.55*	2.91*
Fridge (+4°C)	40	60.99	16.76
Freezer (-17°C)	0	77.90	2.14
Freezer (-17°C)	1	73.08	7.80
Freezer (-17°C)	4	72.94*	10.28*
Freezer (-17°C)	7	87.55*	17.01*
Freezer (-17°C)	15	-	-
Freezer (-17°C)	40	55.11	2.79

Table 16: Results of experiment No. 3 for derivatives of boldenone

Storage temperature	Storage time [days]	Average yield [%]	Relative standard deviation [%]
Fridge (+4°C)	0	85.05	2.63
Fridge (+4°C)	1	80.93	5.30
Fridge (+4°C)	4	69.76	1.21
Fridge (+4°C)	7	-	-
Fridge (+4°C)	15	68.77*	0.13*
Fridge (+4°C)	40	59.64	15.42
Freezer (-17°C)	0	80.28	0.70
Freezer (-17°C)	1	76.92	5.50
Freezer (-17°C)	4	77.45*	7.63*
Freezer (-17°C)	7	90.90*	18.83*
Freezer (-17°C)	15	-	-
Freezer (-17°C)	40	54.23	3.47

Table 17: Results of experiment No. 3 for derivatives of 17β-estradiol

Storage temperature	Storage time [days]	Average yield [%]	Relative standard deviation [%]
Fridge (+4°C)	0	81.37	3.97
Fridge (+4°C)	1	78.59	8.98
Fridge (+4°C)	4	86.66	6.23
Fridge (+4°C)	7	88.07*	3.42*
Fridge (+4°C)	15	79.91	4.91
Fridge (+4°C)	40	81.60	3.22
Freezer (-17°C)	0	79.02	2.79
Freezer (-17°C)	1	80.99	4.95
Freezer (-17°C)	4	83.96	4.74
Freezer (-17°C)	7	89.05	10.02
Freezer (-17°C)	15	70.91	3.67
Freezer (-17°C)	40	84.27	2.21

The results of each of the two storage methods are summarized and compared in the graphical illustrations of figures 13 and 14.

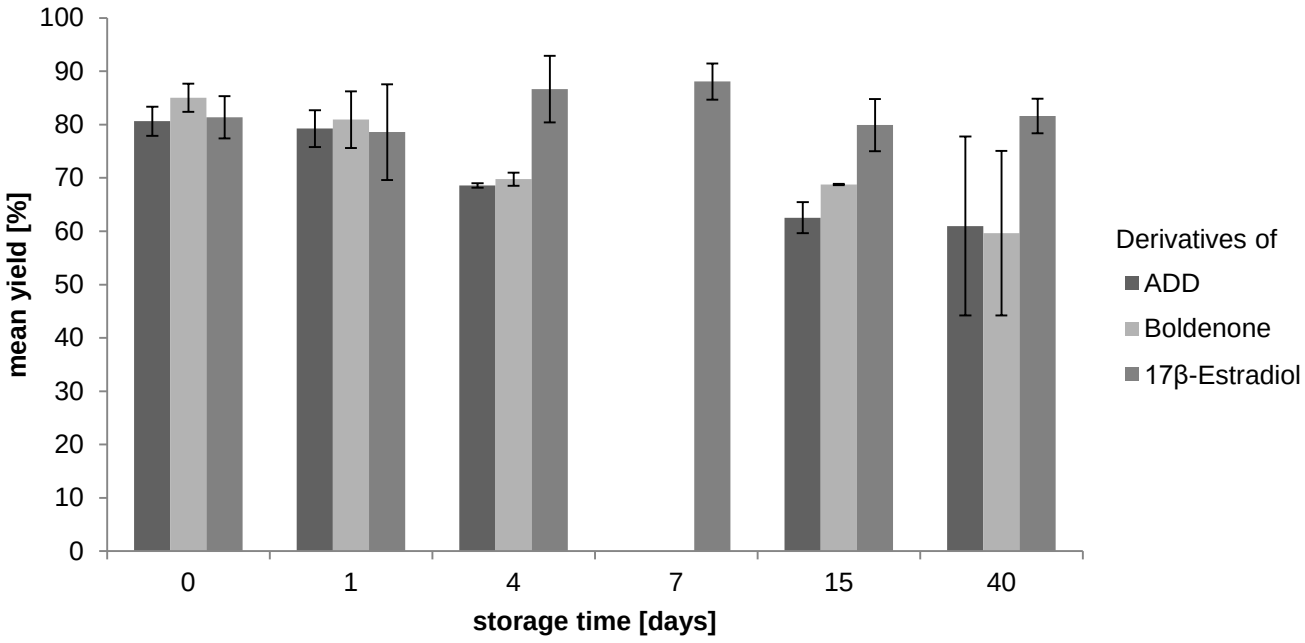


Figure 13: Stability of steroid derivatives during fridge storage (+4°C)

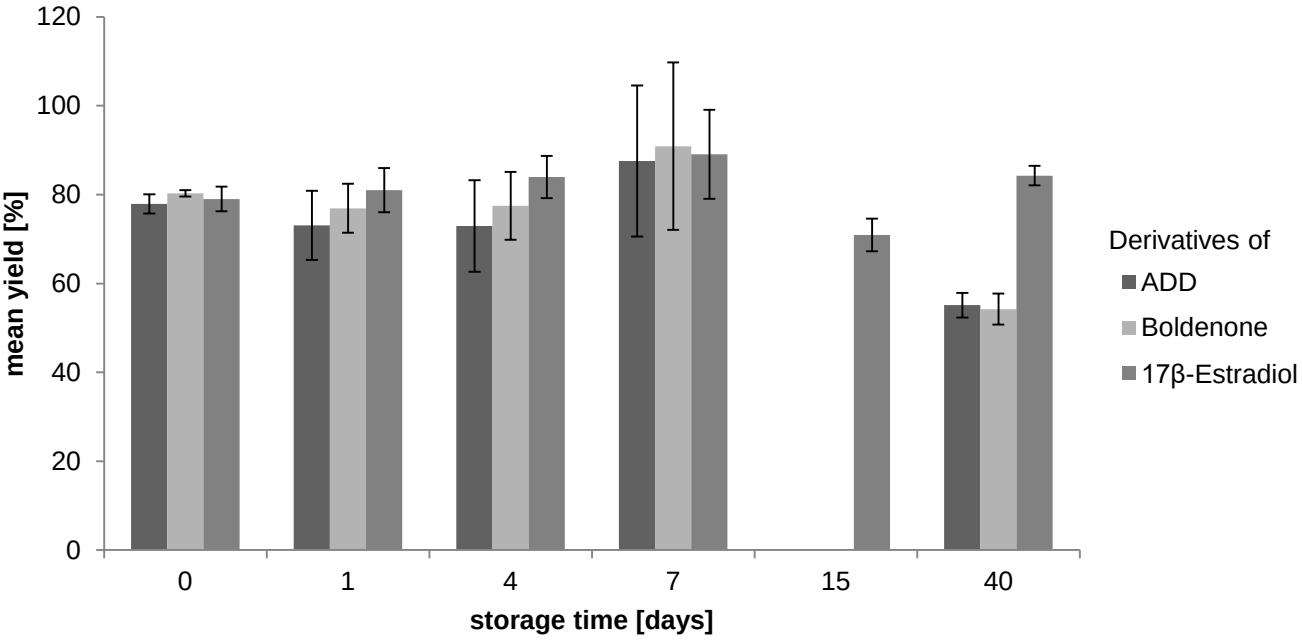


Figure 14: Stability of steroid derivatives during freezer storage (-17°C)

The graphs illustrate that no results could be obtained for ADD and boldenone when the derivatives were either stored at +4°C in the fridge for 7 days or when kept in the freezer at -17°C for 15 days due to the lack of sufficient measurement values.
The results from day 7 were consequently excluded for the statistical analysis since no results could be obtained for ADD and boldenone stored in the refrigerator and the values from the freezer storage were characterized by high standard deviations (17.01% and 18.83%). Due to the lack of data from ADD and boldenone derivatives, the results from day 15 were neglected for the analysis of storage at freezer conditions as well.

Comparison of the evaluation results revealed that no significant difference between the two storage temperatures could be found for any of the steroid analytes.
On the other hand, differences in results could be detected concerning the duration of storage.

In the following graphs the stability of all TMS-derivatives is again illustrated for the storage times selected for the statistical analysis.

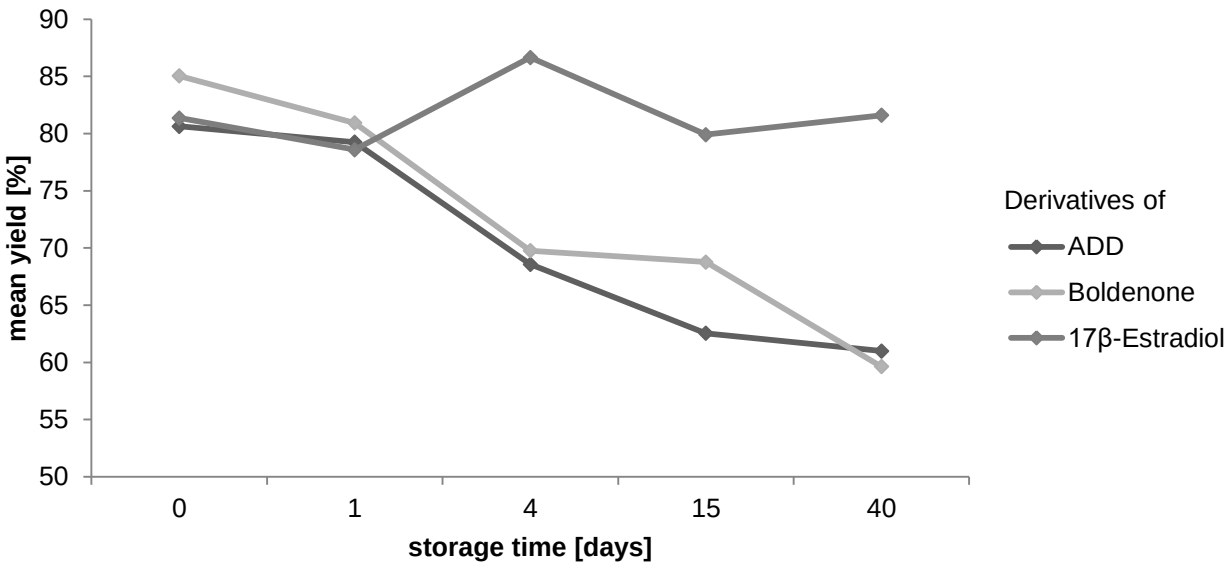


Figure 15: Stability trends of derivatives during storage in fridge (+4°C)

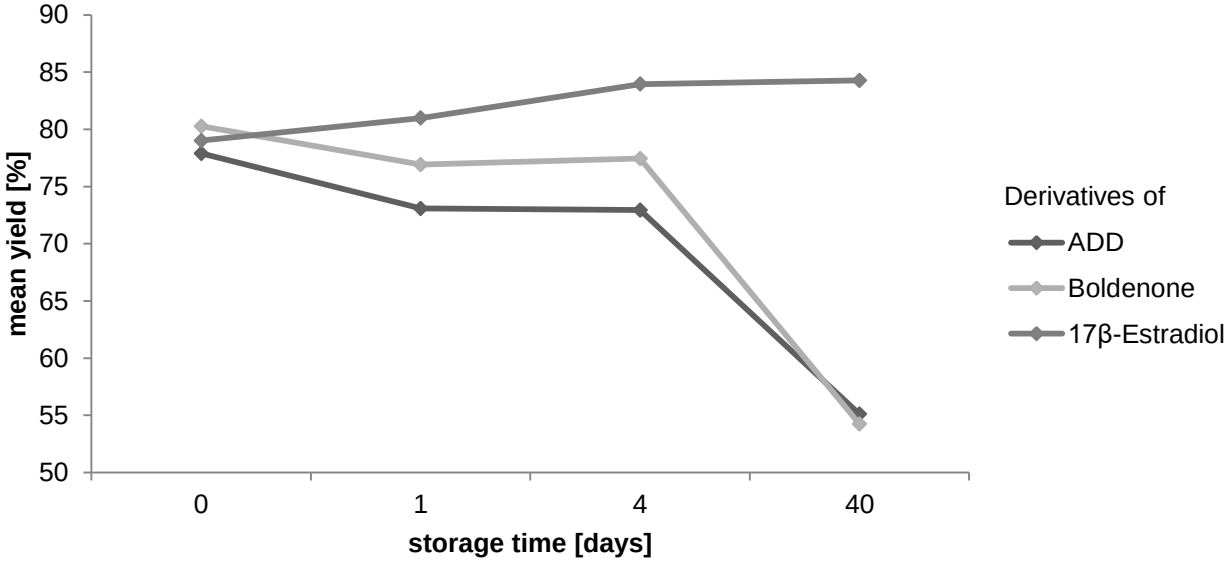


Figure 16: Stability trends of derivatives during storage in freezer (-17°C)

The graphs indicate that in the case of 17β-estradiol not only the storage temperature but also the storage time seems to have no effect on the stability of the derivatization products. For ADD and boldenone, however, a general decrease of analyte amount could be observed indicating a continuous decay of the derivatives during the course of the experiment. Similar degradation rates occurred for the derivatives of ADD and boldenone. The decrease reached approximate amounts of around 30%.

The stability trends depicted in the graphs demonstrate that the derivatives of the keto group containing steroids already start to degrade after 1 to 2 of storage in the fridge. Their stability is increased during storage at -17°C. No significant decrease in analyte amount occurs in the first days. The derivatives may therefore be kept in the freezer for at least 4 days before degradation becomes significant.

A comparable study by Shareef et al. on the analysis of steroid hormones has revealed that the steroids exhibit a stability of 8 days when stored at +4°C. Frozen analytes (storage temperature: -15°C) might even be stable for up to 56 days [19]. The stability of the actual compounds is therefore considerable higher than that of their derivatives, which could be determined to be a few days at a maximum in the previously described experiment of this bachelor thesis. Fang et al. suggest a stability of 48 hours for the derivatives of various steroids [36].

7. Conclusion

In the course of this study, three differently designed experiments were conducted in order to examine the derivatization of three steroid compounds. The method applied for derivatization was enolisation-silylation. For the optimization of the derivatization procedure different reagents were tested as well as a variety of reaction conditions. Subsequently, the stability of derivatives during storage at two temperatures was studied. Quantitation of all derivatives was performed via GC-MS analysis.

The results from the first experiment revealed that only two of the tested reagents are capable of successful derivatization of all three compounds of interest. For 17β-estradiol, however, most reagents were suitable and similar values were calculated. The choice of the optimal reagent was therefore based on the results from the other two compounds, ADD and boldenone. The highest yield of these two compounds was achieved by derivatization with MSTFA II. Values comparable to the results for 17β-estradiol were obtained. MSTFA II, the reagent activated by 2-(trimethylsilyl)-ethanethiol, can thus be concluded as the reagent of choice for this experiment.
For the second experiment, the influence of reaction time and temperature on the derivatization process was examined. Rather similar values were obtained for all conditions. This is especially true for the derivatives of 17β-estradiol for which no significant difference between the various results could be determined. Derivatization of ADD and boldenone is also hardly affected by the reaction conditions. A significant difference could only be

observed between the values from derivatization at 55°C for 90 minutes (giving the highest yield) and the procedure performed at 75°C for 30 minutes (leading to the lowest yield obtained). Despite yielding the highest amount of derivatives, the derivatization temperature of 55°C and the duration of 90 minutes were not chosen as the optimum reaction conditions due to the extensive reaction time. Derivatization at 65°C for 30 minutes was favoured as similar yields could be obtained and the duration of the procedure is significantly reduced.

The derivatives' stability during storage was tested in the course of the third and last experiment. Potential variations in the amount of TMS-derivatives over certain periods of time were analysed at two different storage temperatures. The derivatives of 17β-estradiol seemed to be rather stable, whereas those of ADD and boldenone exhibited degradation over the course of storage. Although no significant differences between the storage temperatures could be found, a certain trend could be observed for ADD and boldenone derivatives. The results indicate that these keto group-containing compounds might be kept in the freezer for at least four days without significant decay, but have got a stability of only 1 to 2 days when stored at higher temperatures in the fridge. The derivatives of 17β-estradiol, the steroid compound only featuring hydroxyl groups, were not affected by the storage temperature. In general, however, the immediate analysis of the TMS-derivatives following sample preparation is recommended due to their possible instability and definite decay in the course of long-time storage.

The results obtained from the individual procedures suggest that the experiments are reproducible. For the vast majority of successful measurements derivatization rates between 60% and 105% could be achieved. These values are generally consistent with the recovery range of 70% to 110% recommended by the U.S. Environmental Protection Agency (EPA) [37] and correspond to the AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals which suggest to avoid recoveries below 60-70% and above 110% [38]

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9. Appendix

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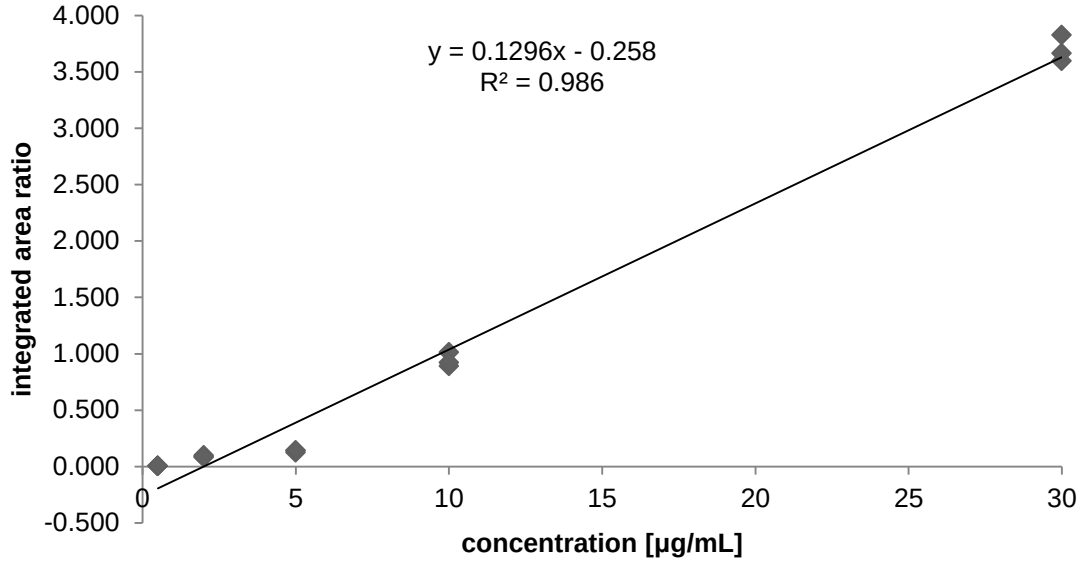

Image 1: Calibration curve of the TMS-derivative of ADD for experiment No. 1

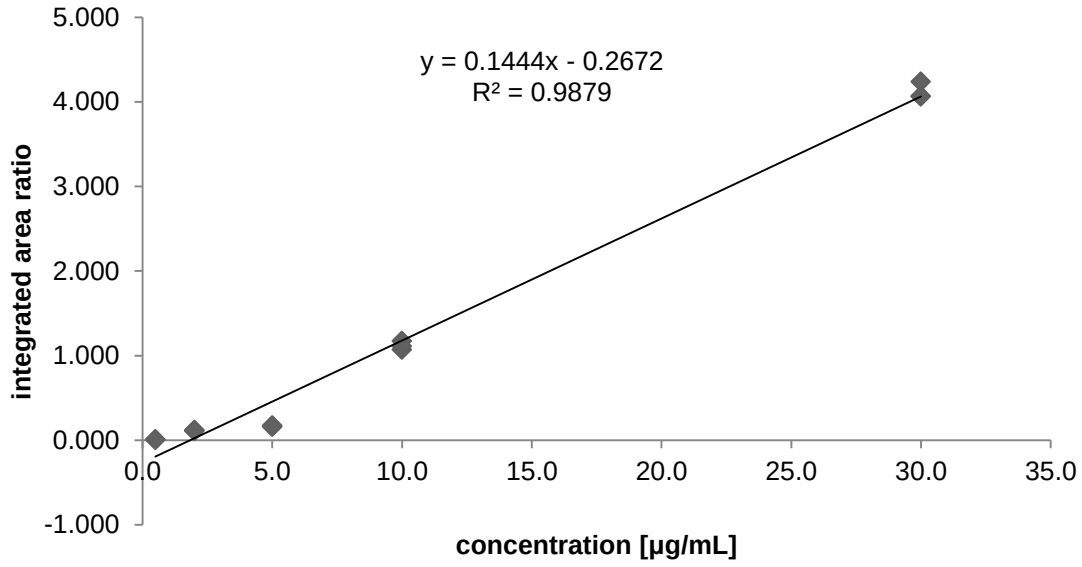

Image 2: Calibration curve of the TMS-derivative of boldenone for experiment No. 1

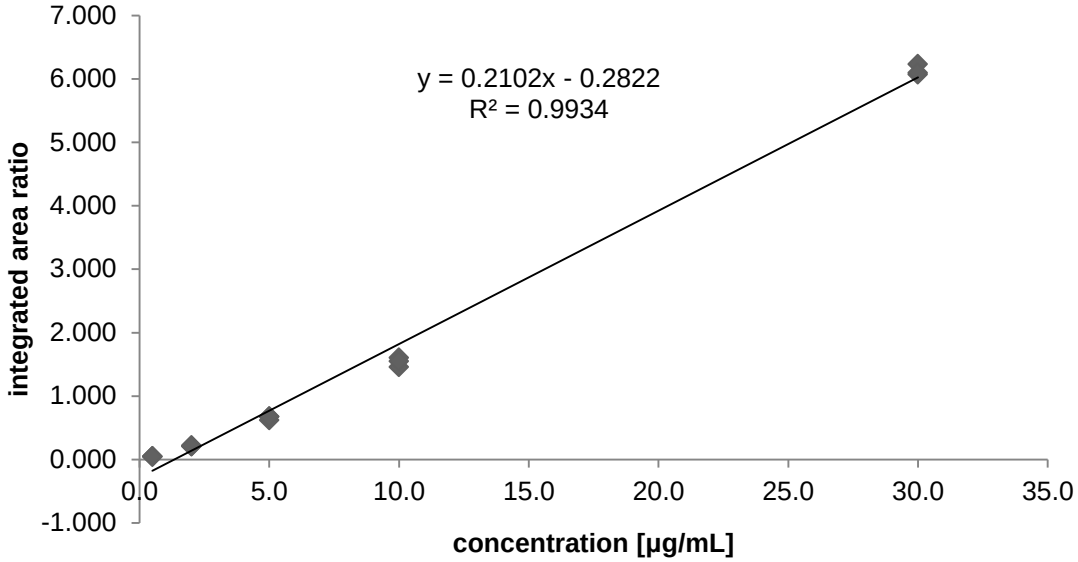

Image 3: Calibration curve of the TMS-derivative of 17β-estradiol for experiment No. 1

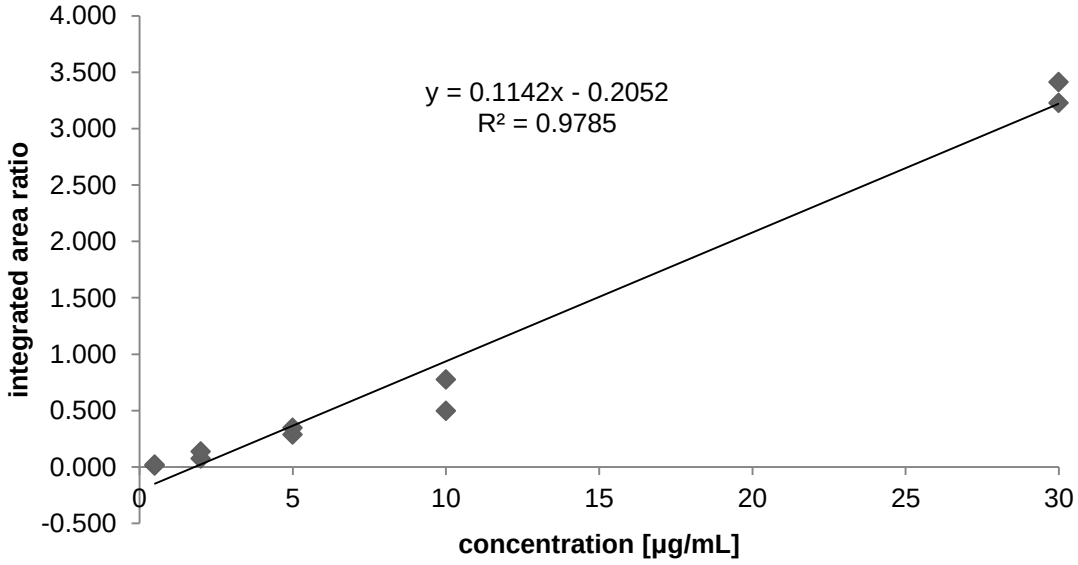

Image 4: Calibration curve of the TMS-derivative of ADD for experiment No. 2

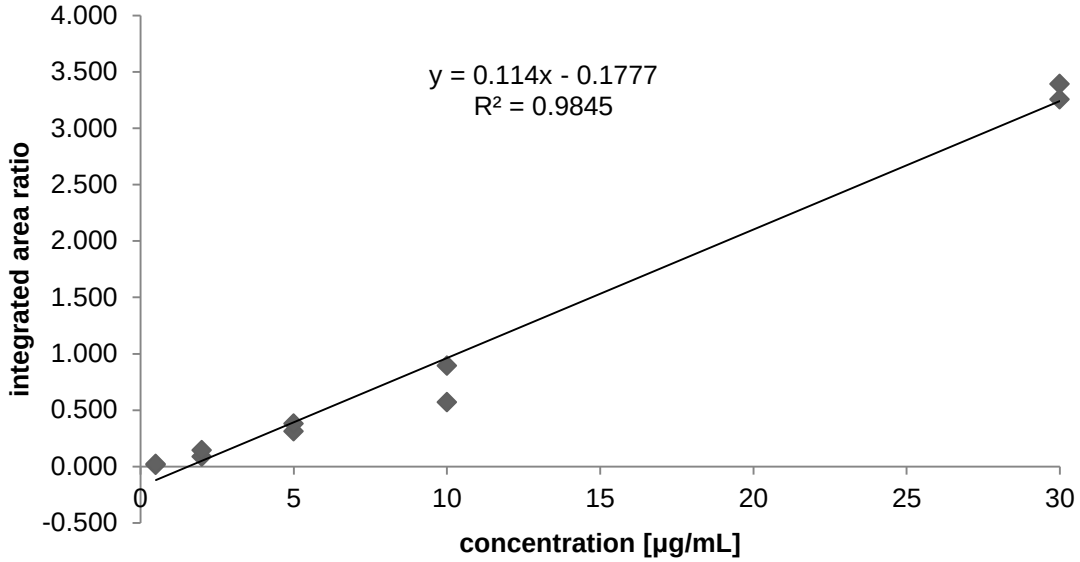

Image 5: Calibration curve of the TMS derivative of boldenone for experiment No. 2

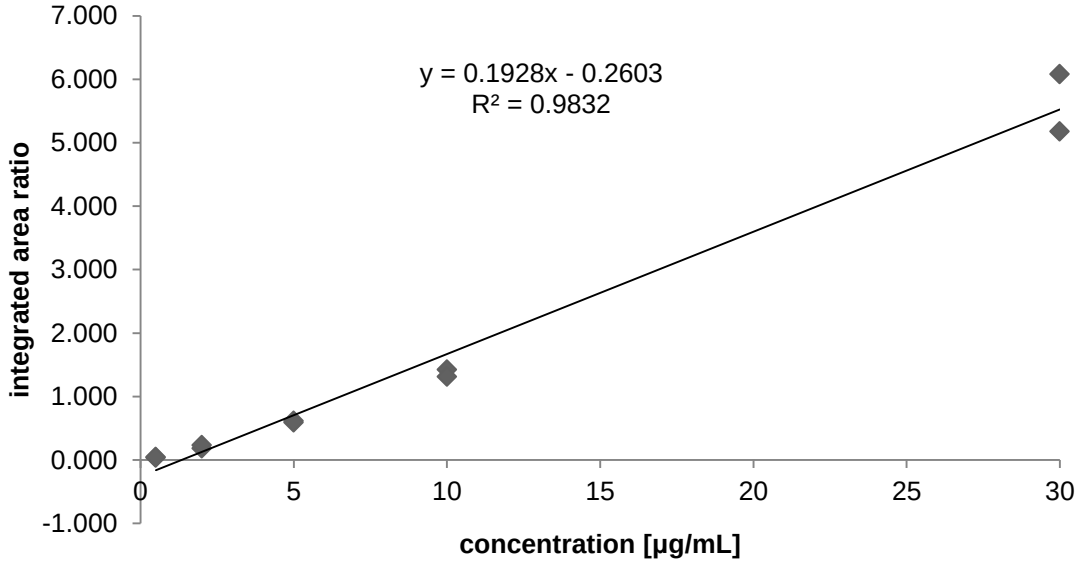

Image 6: Calibration curve of the TMS-derivative of 17β-estradiol for experiment No. 2

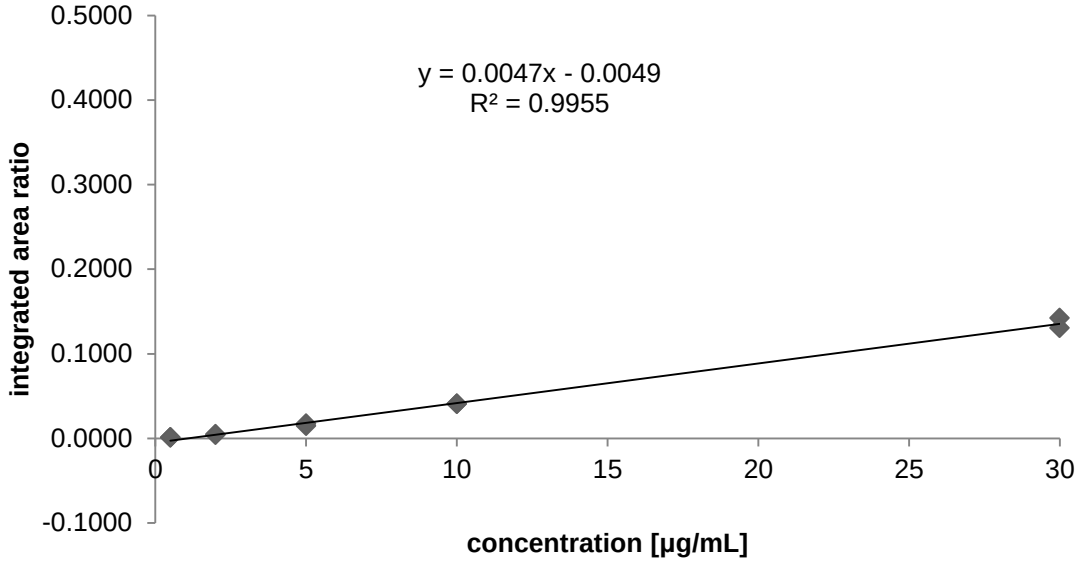

Image 7: Calibration curve of the TMS-derivative of ADD for experiment No. 3

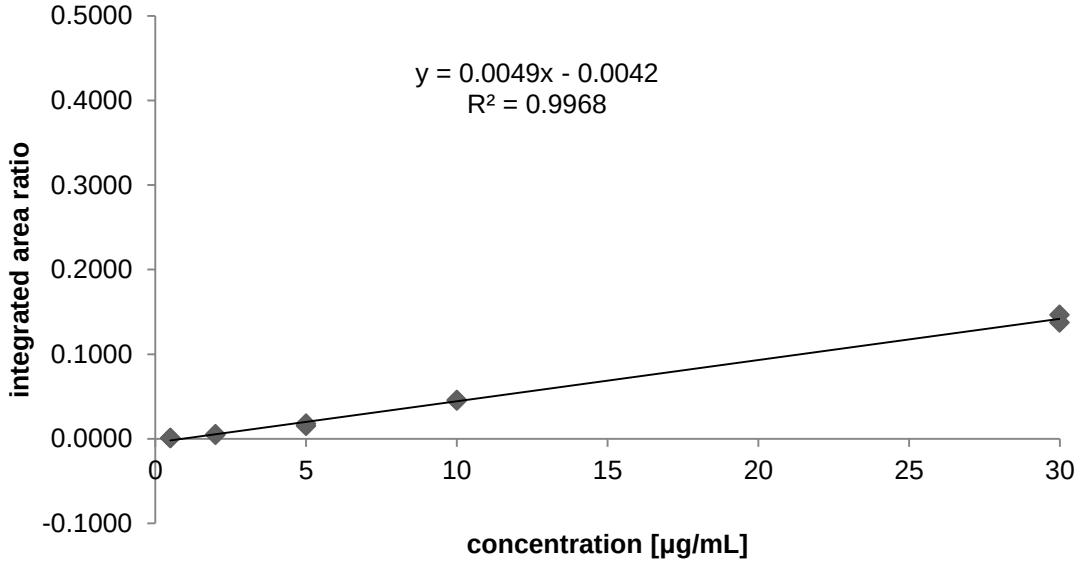

Image 8: Calibration curve of the TMS-derivative of boldenone for experiment No. 3

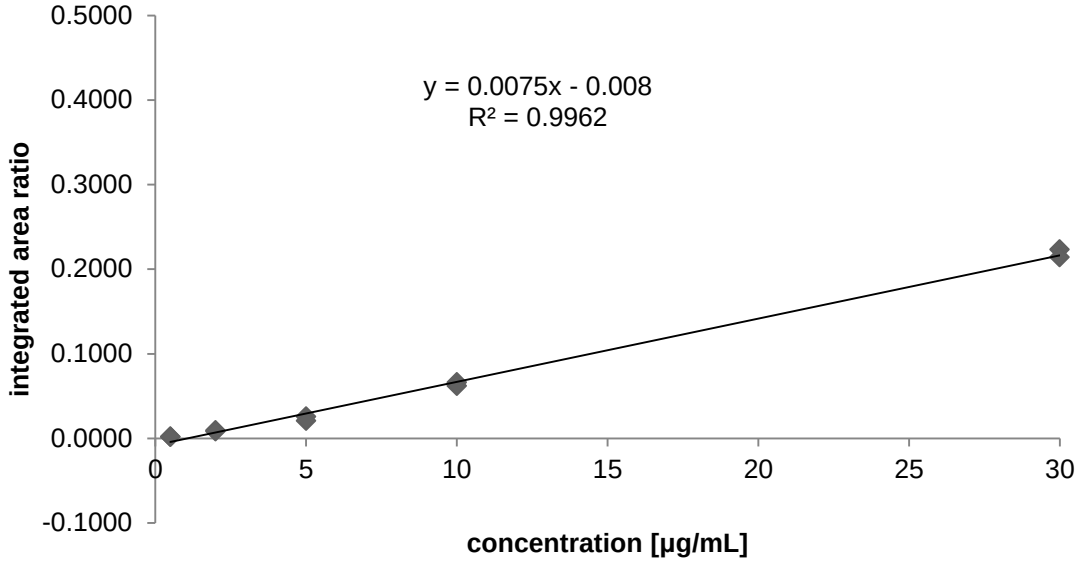

Image 9: Calibration curve of the TMS-derivative of 17β-estradiol for experiment No. 3

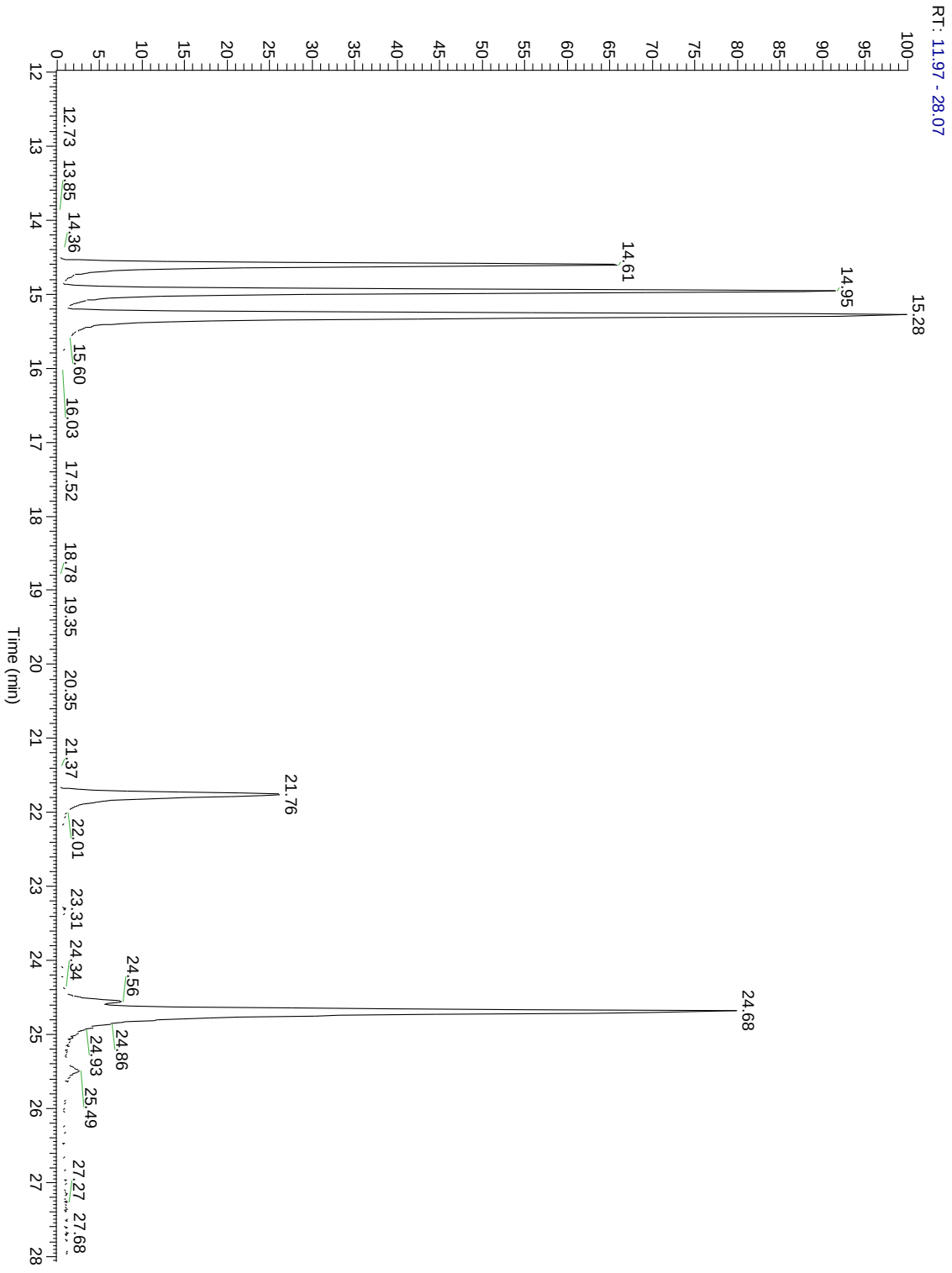

Image 10: Chromatogram (Experiment No. 2, derivatization at 55°C for 90 minutes)

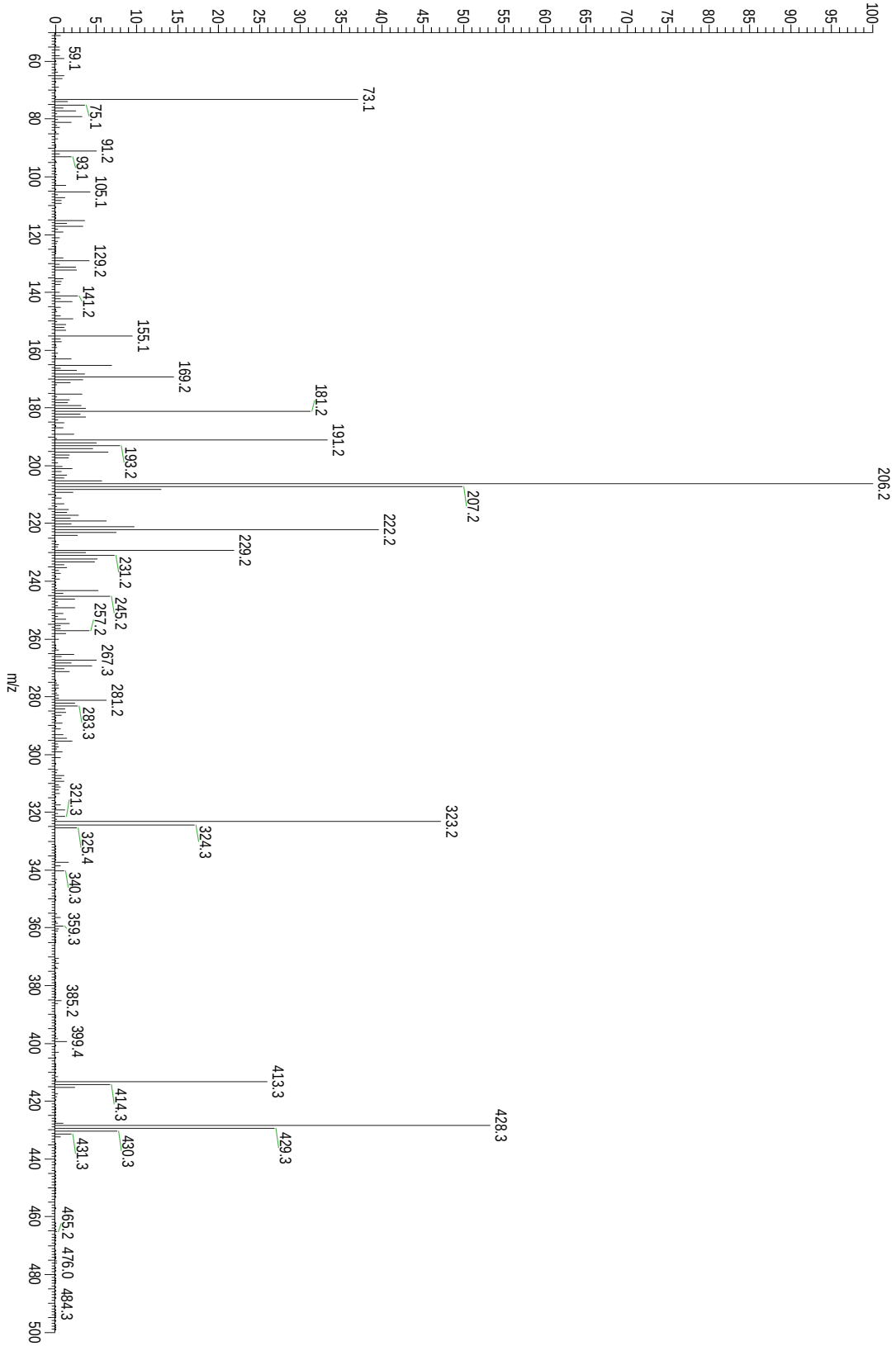

Image 11: Mass spectrum of the TMS-derivative of ADD

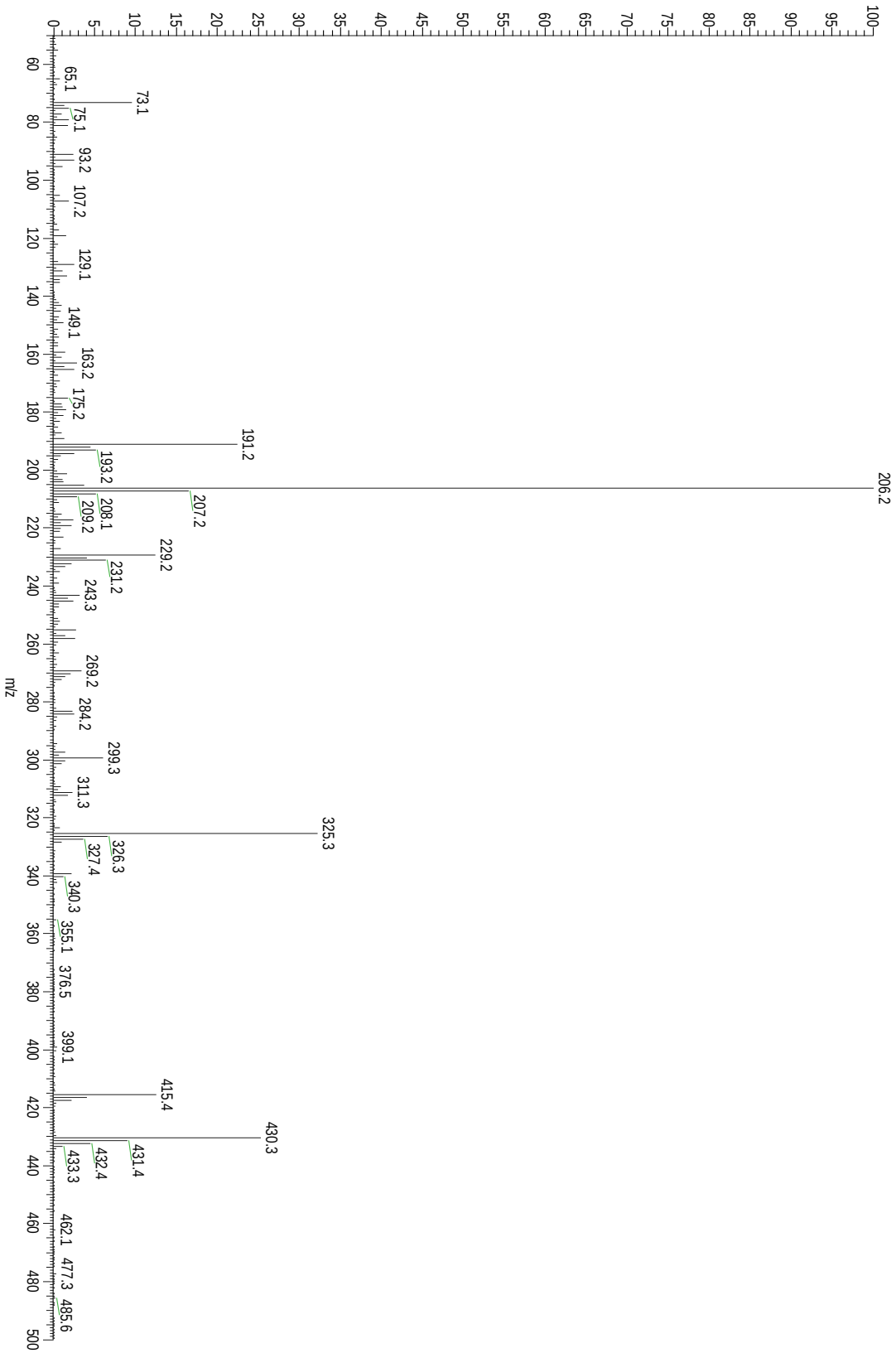

Image 12: Mass spectrum of the TMS-derivative of boldenone

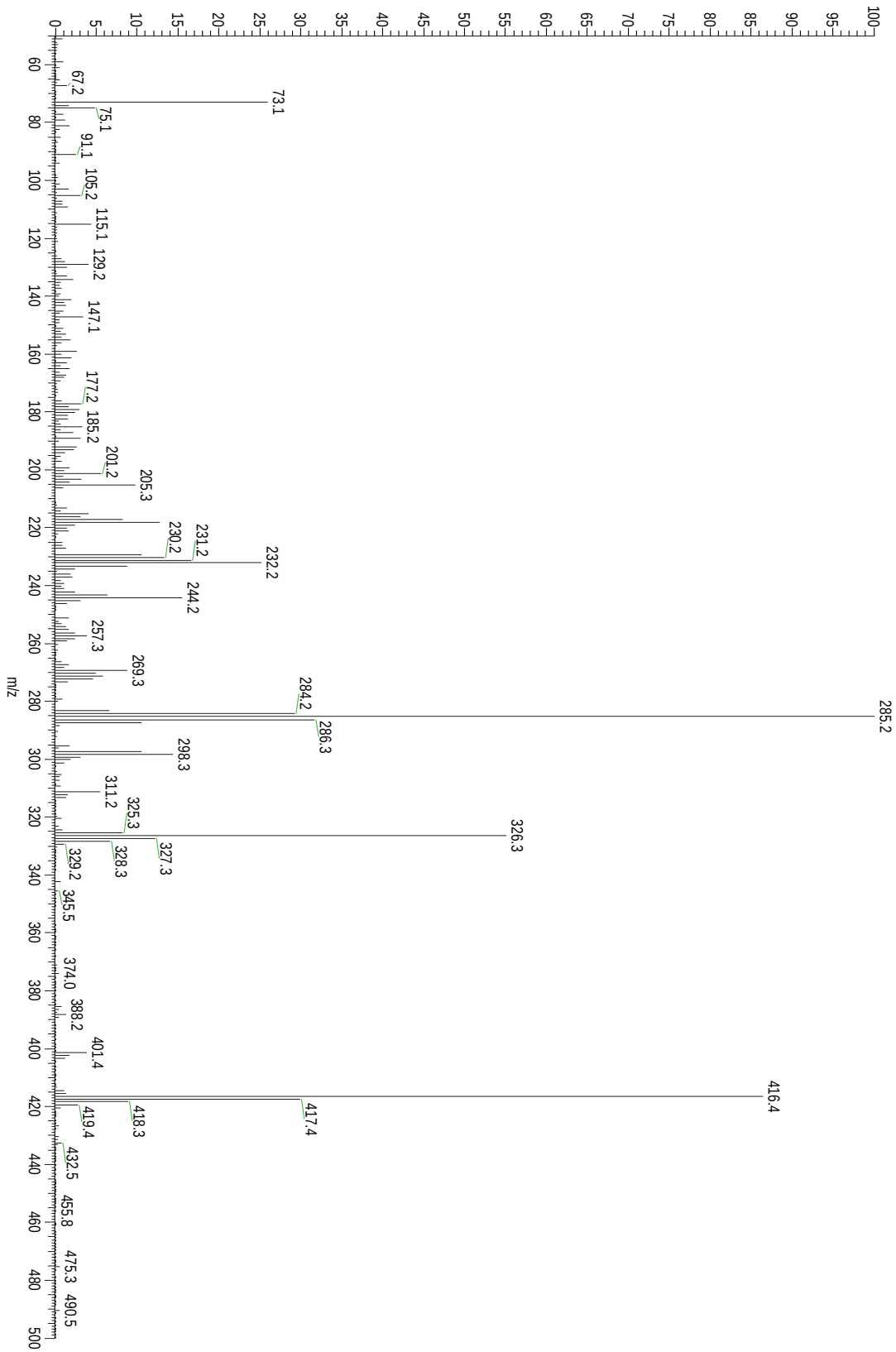

Image 13: Mass spectrum of the TMS-derivative of 17β-estradiol

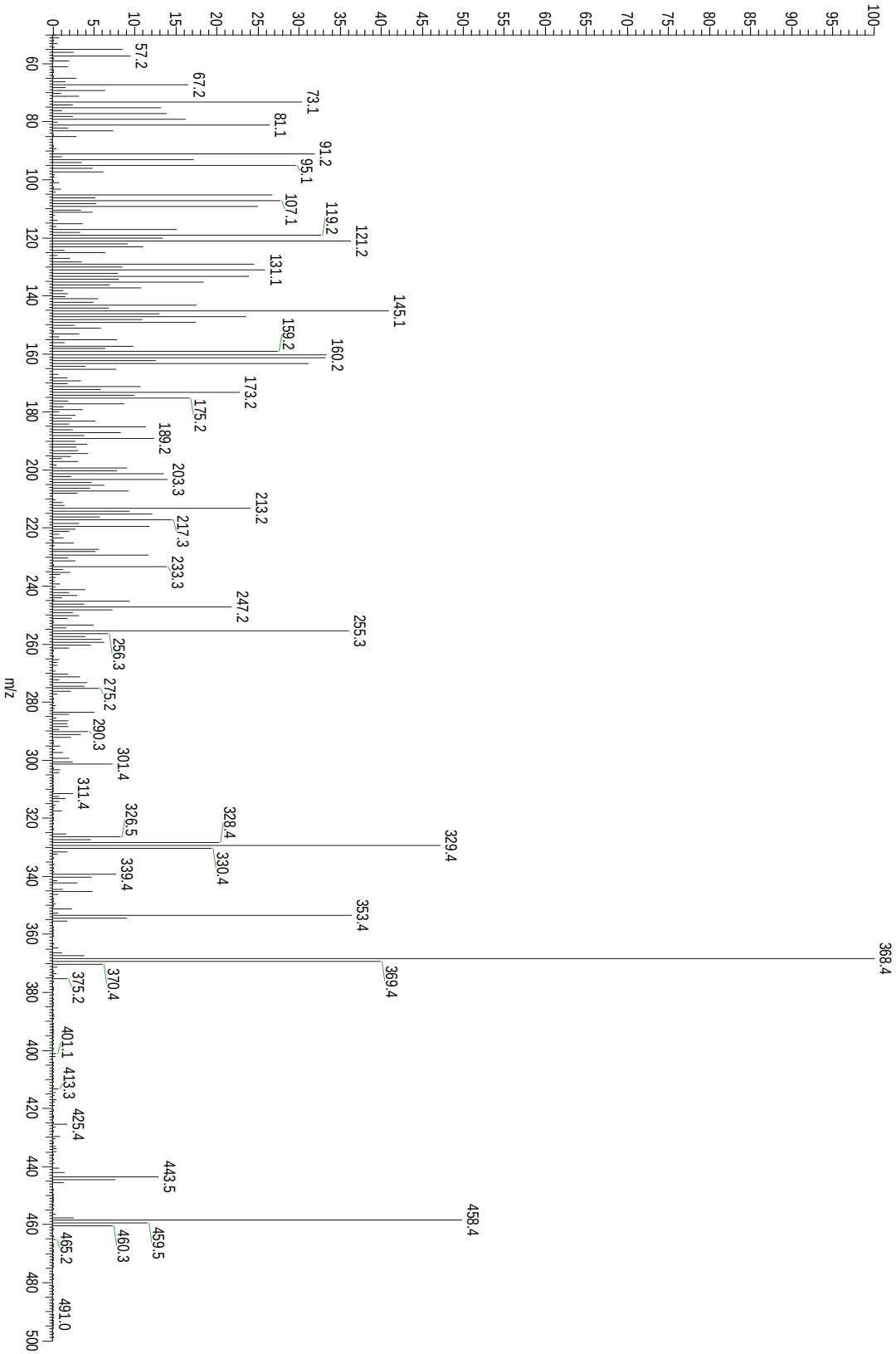

Image 14: Mass spectrum of the TMS-derivative of cholesterol