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Profiling of steroid conjugate metabolites in body fluids of pregnant women by liquid chromatography-mass spectrometry (LC-MS)

Bachelor Thesis

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Annotation

The aims of this thesis were to optimize the sample preparation for the determination of steroid conjugates in common samples as urine and plasma. The following analysis by liquid chromatography under different conditions was also examined.

Affirmation

I hereby declare that I have worked on the submitted bachelor thesis independently. All additional sources are listed in the bibliography section.

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full form to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages. Further, I agree to the electronic publication of the comments of my supervisor and thesis opponents and the record of the proceedings and results of the thesis defence in accordance with aforementioned Act No. 111/1998. I also agree to the comparison of the text of my thesis with the Theses.cz thesis database operated by the National Registry of University Theses and a plagiarism detection system.

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Abstract

Steroid hormones and their conjugates are highly important for the correct body function and might serve as indicators for natural body processes. During pregnancy, the levels of those compounds are closely observed in order to discover possible abnormalities. In this study, different approaches were examined in order to ease the future analysis of those compounds in biological samples. The liquid chromatography method is gaining great importance in biochemical and metabolomical analysis and thus it was used as the main tool for the steroid conjugates analysis. Variety of mobile phases for HPLC was examined in order to determine the optimal one and methanol with addition of 5mM ammonium formate buffer showed the best fitting results for the wide range of conjugates. Concerning the purification of the samples the SPE method was approached with variable combinations of HLB and NH_2 columns. Unfortunately the extraction results appeared to be problematic concerning the elution. In summary, the conjugates open many opportunities for the future research, but additional concern should be focused on the successful purification to avoid matrix effects.

List of Abbreviations

16-OH-DHEA-S	16α-Hydroxydehydroepiandrosterone Sulphate
17-OH-P	17α Hydroxyprogesterone
17-OH-Pan-G	17α-Hydroxypregnanolone Glucuronide
7-OH-DHEA-S	7α-Hydroxydehydroepiandrosterone Sulphate
A-G	Androsterone Glucuronide
A-Pan-S	Allopregnanolone Sulphate
APCI	Atmospheric-Chemical Ionization
APPI	Atmospheric Pressure Photoionization
A-S	Androsterone Sulphate
Cort-G	Cortisol Glucuronide
DHEA	Dehydroepiandrosterone
DHEA-G	Dehydroepiandrosterone Glucuronide
DHEA-S	Dehydroepiandrosterone Sulphate
DHP-G	17α, 20β-Dihydroxyprogesterone Glucuronide
DHP-S	17α, 20β-Dihydroxyprogesterone Sulphate
DHT-G	Dihydrotestosterone Glucuronide
E1	Estrone
E1-G	Estrone Glucuronide
E1-S	Estrone Sulphate
E2	17β-Estradiol
E2-17G	17β-Estradiol 17-Glucuronide
E2-17S	17β-Estradiol 17-Sulphate
E2-3G	17β-Estradiol 3-Glucuronide
E2-3S	17β-Estradiol 3-Sulphate
E2SS	17β-Estradiol Disulphate
E3	Estriol
E3.17S	Estriol 17-Sulphate
E3-17G	Estriol 17-Glucuronide
E3-3G	Estriol 3-Glucuronide
E3-3S	Estriol 3-Sulphate
EA-G	Epiandrosterone Glucuronide
EA-Pan-diol-S	Epiallopregnanediol 3-Sulphate
EA-Pan-S	Epiallopregnanolone Sulphate

EA-S	Epiandrosterone Sulphate
EDTA	Ethylenediaminetetraacetic Acid
E-Pan-S	Epipregnanolone Sulphate
epiT-S	Epitestosterone Sulphate
ESI	Electrospray Ionization
Etiochol-S	Etiocholanolone Sulphate
FA	Formic Acid
GC	Gas Chormatography
HLB	Hydrophilic-Lipophilic Balance
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
LLE	Liquid – Liquid Extraction
MeCN	Acetonitrile
MeOH	Methanol
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrometry
P4	Progesterone
Pan-S	Pregnanolone Sulphate
Pen-G	Pregnenolone Glucuronide
Pen-S	Pregnenolone Sulphate
SPE	Solid Phase Extaction
T-G	Testosterone Glucuronide
T-S	Testosterone Sulphate
TSQ	Triple Stage Quadrupole
UGT	Uridine Diphosphate-Glucuronosyltransferase
W	Water

Contents

1. Theoretical Part

1.1. Physico-chemical Properties of Steroid Hormones

Steroid hormones are biologically active substances affecting behavior, reproduction body functions and development. They are divided into five basic groups according to similarity in structure and function. The groups are mineralocorticoids, glucocorticoids, progestins, androgens and estrogens. They are formed from common precursor cholesterol by complex pathways resulting into final molecules of steroid hormones. All steroid hormones contain four-ring structure of the sterol nucleus and chemically are derivatives of cyclopentanoperhydrophenanthrene. Its structure and ring indication are shown below.

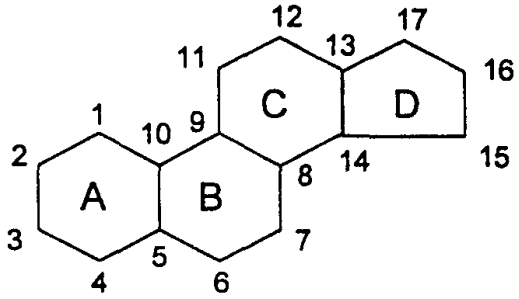


Figure 1: Structure and ring indication of cyclopentanoperhydrophenanthrene

Steroids are water insoluble meaning that in the body they are transported in blood forming complex with glycoprotein transcotin and occasionally albumin. Passage through the membranes of their target cells proceeds spontaneously. Entering cytosol, steroids bind their cognate receptor [1].

Mineralocorticoids and glucocorticoids are both originated in adrenal cortex and take important part in establishing hormonal balance in the body. The main function of mineralocorticoids is maintaining balance in metabolism of salt and water and its excretion out from the body. Glucocorticoids affect metabolism in many ways including resistance to stress and managing inflammation.

Progestins are produced in ovaries and placenta and the function of this hormone helps to mediate menstrual cycle and also maintain pregnancy. That makes them one of our points of interest in this thesis.

Estrogens and androgens are originated in gonads and play important role in maturation and development of secondary signs. Estrogens occur particularly in females developing theirs secondary sex organs and supporting the development. On the other hand androgens play

similar role in development of males. They are also responsible for male sexual differentiation.

Even though all steroid hormones share similar structural formula, their properties differ in function and we take main interest in progestins and estrogens as they take place in important changes during pregnancy. They appear either in original or in conjugated form in the body fluids of pregnant women. The levels of those hormones indicate important changes in the metabolism during the pregnancy. As the body goes through intense changes in this process, body needs to adapt to the actions proceeding in the body. Hormone levels in blood establish a balance for the mothers system accepting the baby and form their hormonal relationship.

1.2. Classification of Steroids and Their Conjugated Forms

1.2.1. C18 steroids

C18 steroids are naturally occurring estrogens. The main representatives of this group are estradiol, estrone and later formed estriol. They can be characterized by aromatic A ring and phenolic hydroxyl group at C3. Another characteristic sign is presence of either hydroxyl group (estradiol) or ketone group (estrone). The most significant C18 steroid hormone is estradiol. Conversion of estriol and estrone is necessary, as they are only weakly reactive in this form. Formation of estradiol is performed to fulfill the estrogenic potential.

The main function of estradiol is regulation of gonadotropin secretion and formation of secondary sexual characteristics in women as uterine growth and thickening of vaginal mucosa.[4]

1.2.2. C19 Steroids

C19 steroids include DHEA, androstenedione and testosterone. The major steroid in this group is androstenedione due to its possible pathway creating estradiol. [4] Importance of this group is minor regarding the topic.

1.2.3. C21 Steroids

The main representatives of C21 group are pragnenolone, progesterone and 17α hydroxyprogesterone. The importance of pragnenolone is great due to the fact; it is the main precursor for production of all other steroid hormones. It is produced directly from cholesterol. Compared to C18 steroids, C21 have three extra carbons. One in position 19 as simple methyl group, and another two bound to position 17 as ketone group with following

methyl group. In position 3 on A ring, possibilities are either ketone group (progesterone) or hydroxyl group (pragnenolone). [4]

The main function of progesterone is implantation of the fertilized ovum and maintenance of pregnancy. It also plays important role in development of breast glands, inhibition of contractions and many other important processes connected with pregnancy. 17α hydroxyprogesterone is just weakly active.

1.3. Conjugation of Steroid hormones

Conjugation of steroid hormones is an important part of steroid metabolism and results in the inactivation of the compound. Conjugation itself often produces hydrophilic molecules, increasing water solubility of the starting compound and simplifying its transport and excretion from the body. The most common conjugation metabolites of steroid hormones are sulphates and glucoronides. Majority of compounds excreted from the body are present in its conjugated form.

Enzymes catalyzing biotransformation of endobiotics (including steroids) can proceed in two types of reaction, each responsible for different functional groups. First type of reaction includes hydrolysis, oxidation and reduction, when second types are considered conjugation reaction including glucoronidation, sulphation, acetylation and methylation. [7]

1.3.1. Glucuronidation

The main function of glucuronidation is to increase solubility of the substance by addition of negative charge.[5] The family of enzymes catalyzing glucuronidation is the uridine diphosphate-glucuronosyltransferase (UGT) family. Those enzymes are in cells present on endoplasmatic reticulum and cytoplasm, most commonly in liver and kidney cells.[5] The glycosyl group of a nucleotide sugar is transfered to an acceptor compound at the nuclephilic functional group of oxygen, nitrogen, sulphur of carbon.

According to sequence identities, UGT has been divided into two groups, UGT1 and UGT2. UGT1 situation shows UGTs resulting from alternate splicing of multiple first exons and share common exons 2-5. [7] In the case of UGT2, mRNA is transcribed from individual genes.[7] Family UGT2 is responsible for glucoronidation of steroids, our main interest.

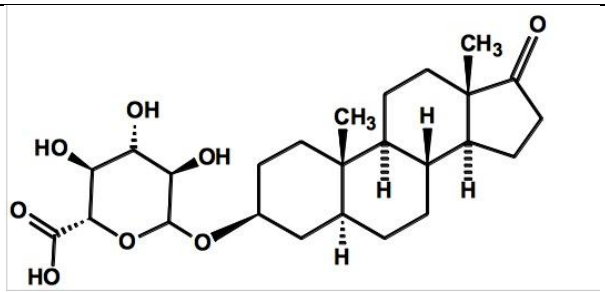
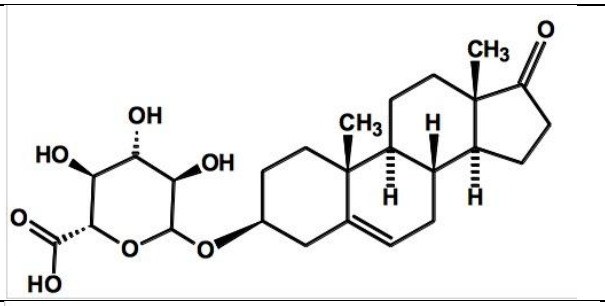
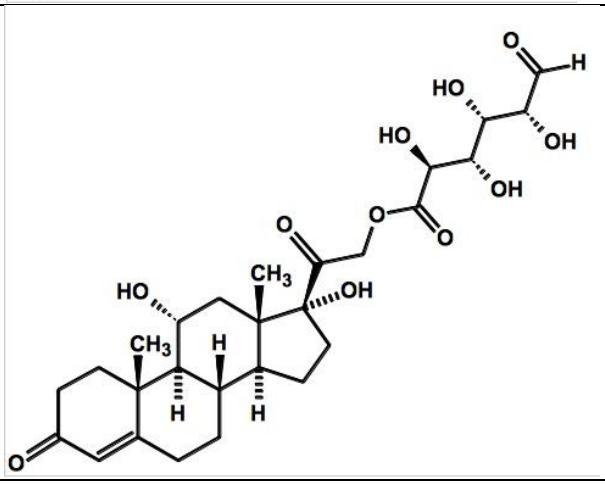
1.3.2. Sulphation

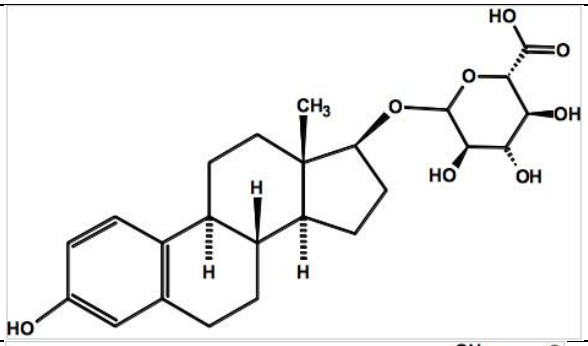
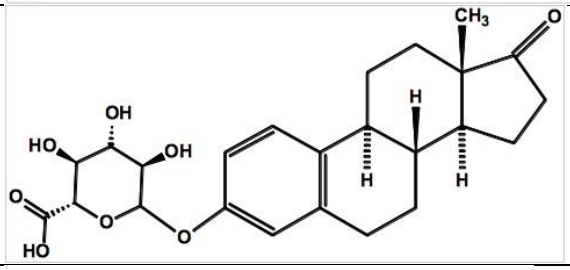
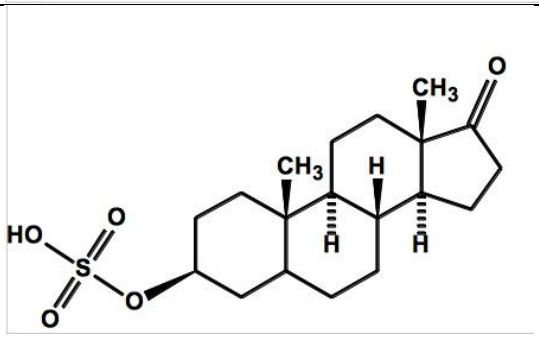
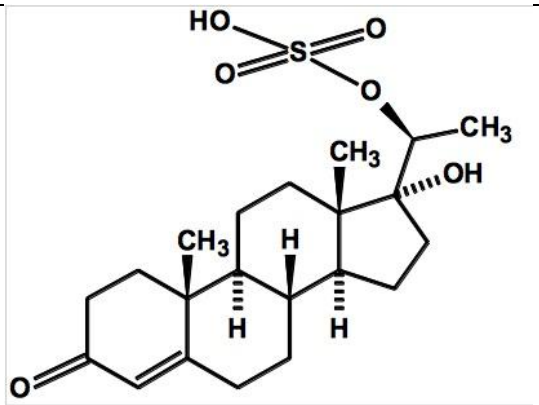
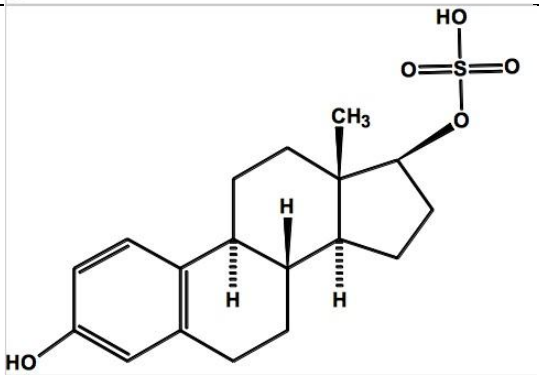
The process of sulphation includes attachment of sulphate group to the multiple ring structure attacking functional group of oxygen. The reaction is catalyzed with sulphokinases, present in

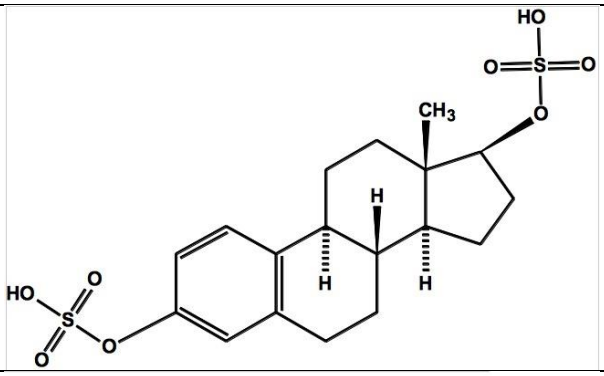
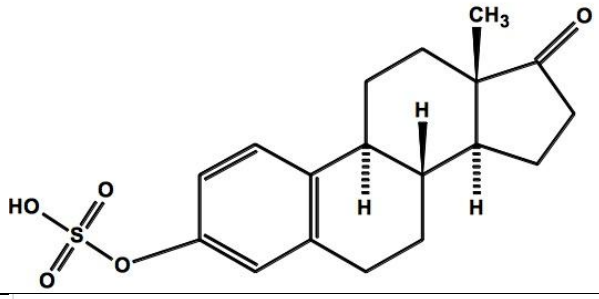
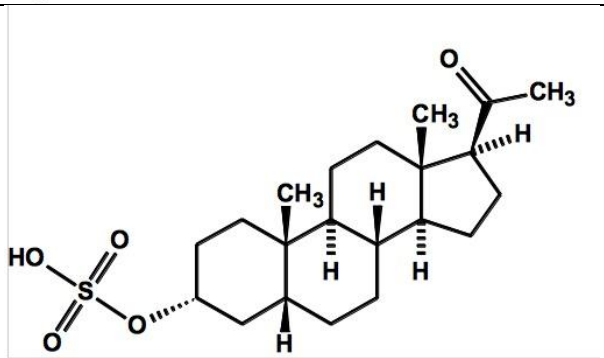
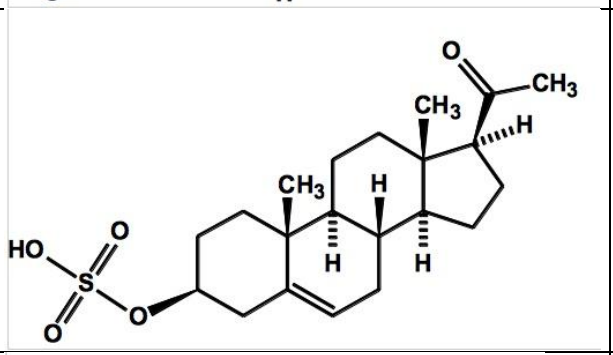
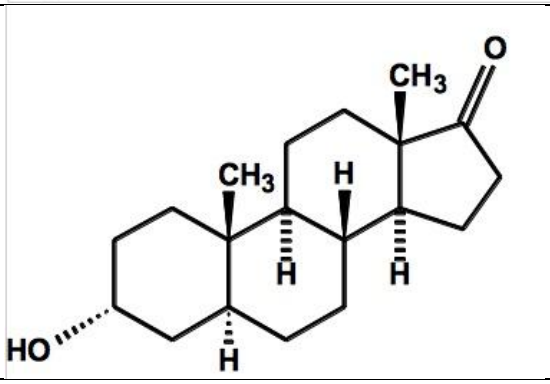
cytosol of cells of liver and fetal tissue. [8] Sulphation of a steroid hormone causes its inactivation and can serve as reservoir of the hormone, which gets released in the case of need. The example of this situation is estrone sulphate, being the most abundant estrogen in blood but not physiologically active. It is presumed to be a reservoir of estrone in different tissues including target tissue of estrogen. [4]

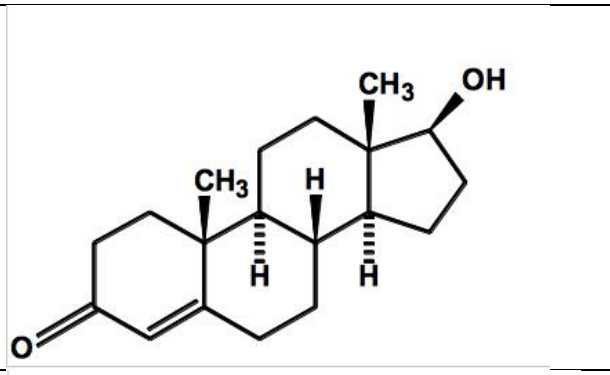
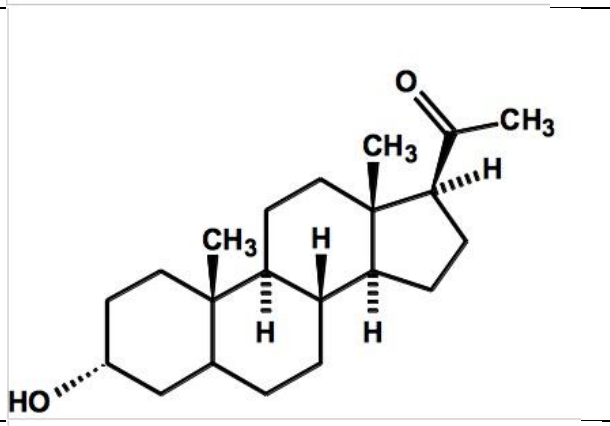
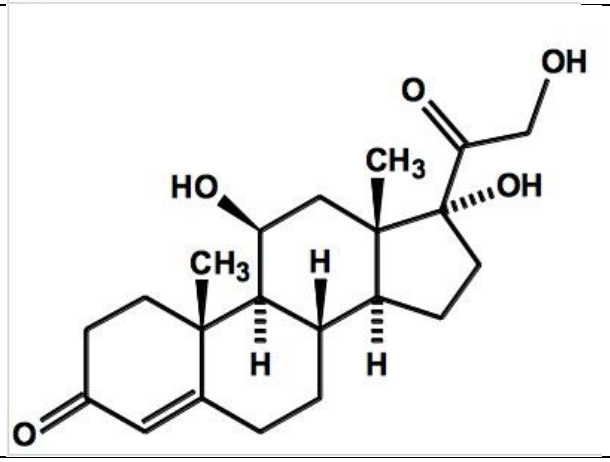
Most common steroid hormones, its related conjugated form and its formulas can be found in Table 1.

Table 1: Main steroid conjugates, glucoronides and sulphates, its structural formula, molecular formula and monoisotopic weight.

Name	Structural Formula	Molecular Formula	Monoisotopic mass [Da]
Epiandrosterone glucuronide (5-α-androstan-3β-ol-17-one glucuronide)		$C_{25}H_{38}O_8$	466.25667
DHEA glucuronide (5-androsten-3β-ol-17-one glucuronide)		$C_{25}H_{36}O_8$	464.24102
Cortisol glucuronide (4-pregnen-11β,17,21-triol-3,20-dione-21-glucosiduronate)		$C_{27}H_{38}O_{11}$	538.241415

Estradiol 17-glucuronide (1,3,5-estratrien-3,17β-diol 17-glucuronide)		$C_{24}H_{32}O_8$	448.20972
Estrone glucuronide (1,3,5-estratrien-3-ol-17-one glucuronide)		$C_{24}H_{30}O_8$	446.19407
Epiandrosterone sulphate (5-α-androstan-3β-ol-17-one sulphate)		$C_{19}H_{30}O_5S$	370.181396
Dihydroxy progesterone sulphate (4-pregnen-17,20β-diol-3-one, 20-sulphate)		$C_{21}H_{32}O_6S$	412.191961
Estradiol-17-sulphate (1,3,5-estratrien-3,17β-diol, 17-sulphate)		$C_{18}H_{24}O_5S$	352.134446

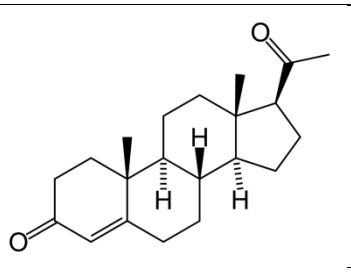
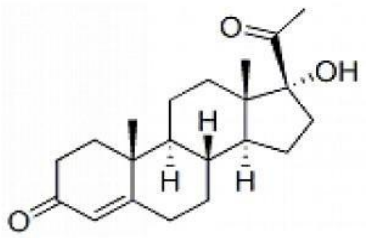
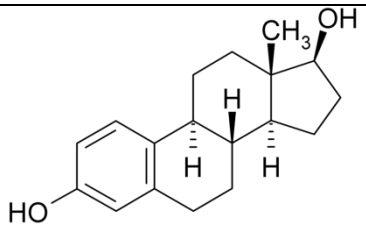
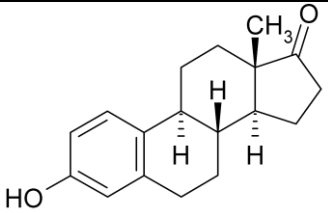
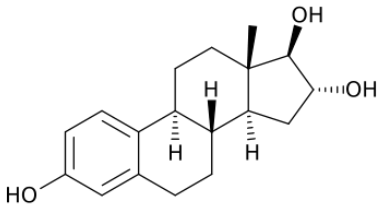
Estradiol disulphate (1,3,5-estratrien-3,17β-diol, 3,17-disulphate)		$C_{18}H_{24}O_8S_2$	432.091262
Estron sulphate (1,3,5-estratrien-3-ol-17-one sulphate)		$C_{18}H_{22}O_5S$	350.118796
Pregnanolone sulphate (5β-pregnan-3α-ol-20-one sulphate)		$C_{21}H_{34}O_5S$	398.212696
Pregnenolone sulphate (5-pregnen-3β-ol-20-one sulphate)		$C_{21}H_{32}O_5S$	396.197046
Androsterone (5-α-androstan-3α-ol-17-one)		$C_{19}H_{30}O_2$	290.22458

Testosterone (4-androsten-17β-ol-3-one)		$C_{19}H_{28}O_2$	288.20893
Pregnanolone (5β-pregnan-3α-ol-20-one)		$C_{21}H_{34}O_2$	318.25588
Cortisol (4-pregnen-11β,17,21-triol-3,20-dione)		C21H30O5	362.209325

1.4. Steroid Hormones Occurring in Pregnant Women

The production of steroid hormones is greatly increased during the pregnancy. The main hormones produced during pregnancy are progesterone, 17α hydroxyprogesterone, estradiol, estrone and estriol [2]. Their structures and used abbreviation is stated in Table 2.

Table 2: Common pregnancy steroid hormones and their structures

Name	Abbreviation	Structural Formula
Progesterone	P4	
17α Hydroxyprogesterone	17-OH-P	
17β Estradiol	E2	
Estrone	E1	
Estriol	E3	

Syntheses of those hormones differ during pregnancy from the pathways in non-pregnant women as the placenta is forming and taking over production of progesterone to maintain the pregnancy. Body of a pregnant woman goes through a lot of changes during pregnancy and endocrinological balance needs to be modified for this process.

The ratio of polar conjugates to unconjugated steroid hormones express a balance between activities of enzymes responsible for sulfonation and glucuronidation of steroid hormones. General increasing trend of those values was noticed with advancing gestation. [9]

The amount of excreted estrogens is multiplied regarding non-pregnant women. The biggest increase is visible at E3 values. Non-pregnant women show usually negligible concentrations of estriol, in pregnant women, levels are significantly increased and dominate in the circulation. The number of estriol can rise up to 78% of unconjugated and 95% of conjugated estrogens. [10]

The levels of common steroid hormones in plasma from umbilical artery in pregnant woman from 28^{th} to 41^{st} week of gestation are for progesterone 745 nmol/l, 17α hydroxy progesterone 30 nmol/l, estrone 17.4 nmol/l, estradiol 3.50 nmol/l and estriol 53 nmol/l. For other important steroids the values are following: testosterone 1.25 nmol/l, DHEA 8.1 nmol/l and pregnenolone 25.8 nmol/l. [20]

Also during the pregnancy, complex mixture of isomeric metabolites of progesterone is formed, including pregnanolone sulphates, pregnanediol mono- and disulphates and pregnandiolone and pregnantriol mono and disulphates. Levels of those compounds is highly increasing during the pregnancy [11]

Analysis of hormones in pregnancy is an important part of prenatal examination as it can show some important abnormalities and disorders. Common samples can be urine, blood, plasma or amniotic fluid. Steroid hormones and their metabolites show potential for prediction of reproductive disorders, misbalance of hypothalamic–pituitary–adrenal axis, and impaired insulin sensitivity. [6]

1.5. Current Methods for Steroid Conjugates Analysis

1.5.1. Body fluids used for steroid conjugate analysis

Samples for determination of steroid hormones and their conjugates can differ, mostly collected in form of urine, blood, serum or amniotic fluid of the mother as well as body fluids of the fetus. [14]

In case of urine, we cross many advantages as simple and non-invasive process of sample collection, low cost and accurate analytical methods focused on its analysis. [12] The main disadvantage of this sample form is certain instability in longer time scale that might cause the violated result. [13]

1.5.2. Biological factors influencing the analysis

Sampling of blood serum and plasma can be affected by many factors including fasting or elevated hormone levels due to stress related to the sampling. The most common biological

influencing factors are age, sex, pregnancy, stage of the menstruation and mental of physical condition.

Fertility steroids show clear dependency on pubertal age. The ranges of those hormones change significantly during adolescence and result from many changes responsible for development of secondary sexual characteristics. In adult females the phase of menstrual cycle or possible pregnancy needs to be taken into account, when it would show variation of levels of estradiol, testosterone and progesterone. Also levels of those hormones will visibly drop after menopause. Body mass and other physical factors can play an important role in steroid levels in healthy subjects. Critical illness show increasing trend in corticoids excretion, also lowered levels of testosterone was found in men with severe diseases. Another important factor is fasting and its influence of hormone levels. In blood sampling in women, estrogens do not seem to be influenced by fasting, yet progesterone showed elevated values after meal. Also adrenal steroids including cortisol and DHEA-S levels increase after ingestion of meal. The character of nutrition seems to affect steroid hormones values to some point as well. This error can be eliminated by usage of urine samples and their collection for 24 hours. This procedure would also prevent influence of circadian rhythms. [15]

1.5.3. Technical factors influencing the analysis

High number of technical factors influencing steroid hormone levels is experienced and can lead to possible errors in the analysis. Serious problem can be caused by interfering substances and proper procedure of sample preparation is required to avoid those problems. Another important aspect is stability of the sample. Steroid compounds show relatively good stability against pH and molarity changes in biological fluids. Urine steroids appear to be relatively stable, as testosterone and epitestosterone remained unchanged for at least 22 months. Stability of serum and plasma samples is also relatively high, as the testosterone and cortisol levels appeared stable for over 40 years at -25°C. But the use of the serum is preferred over the use of plasma as sodium EDTA showed significant rise of pragnenolone compared to serum or heparin plasma. [15]

1.6. Sample Preparation

The sample usually needs some kind of special pretreatment before the analysis due to many interfering molecules with more or less similar properties and chemical structure that may display a conflict with desired structures. Mostly with urine we face a difficulty with a large

sample volume. In that case, pre-concentration is necessary to achieve suitable analytical sensitivity. Next step usually contains extraction and later possibly hydrolysis and derivatization. [16]

1.6.1. Extraction methods

The most common form of extraction used for polar steroid conjugates is Solid Phase Extraction (SPE). This method employs stationary phase usually in a form of a disc or column which is first preconditioned with methanol or other solvent. After that, sample is applied and bound to the stationary phase. The impurities are also weakly bound in the phase and removed by subsequent elution by weak solvents. In the last step, the desired sample is eluted into separate vial and this step is usually followed by evaporation of the solvent and redisolving of the pure extracted sample. [17]

Another possible extraction method is Liquid – Liquid Extraction (LLE) based on a polarity of conjugated steroids. The separation was performed using plasma sample extracted in diethyl-ether when conjugated compounds remain in polar phase. This step is then followed by hydrolysis. Another possible reagent for the LLE is hexane instead of the diethyl-ether. This approach might be used in case of possible interference.

1.6.2. Hydrolysis of steroid conjugates

Steroid conjugates are usually processed in a form of hydrolysis to transform them into unconjugated form. The hydrolysis technique is commonly used for the GC-MS as the detection of the conjugates is problematic. For the LC-MS the hydrolysis si not necessary.There are two types of hydrolysis currently used consisting of either chemical of enzymatic treatment. The chemical approach can be facilitated using for example acid or in some cases elevated temperature. The disadvantage of the chemical approach is decreased specificity compared to the enzymatic hydrolysis and may cause product degradation. Enzymatic hydrolysis is usually employing enzymes β-glucuronidase / arylsulfatase isolated from *Helix pomatia*. Thanks to the exact enzyme usage, the approach is much more specific and thus more widely used.[16]

1.6.3. Derivatization methods

In prior to gas chromatography, derivatization step usually needs to be carried out to increase volatility of the sample and also improve its thermal stability as well as the chromatographic separation and detection. The preferred technique used in that case is mostly silylation

causing formation of alkylsilyl derivatives by replacing originally active protons of –OH and –NH groups. In some cases acylation can be used. [16]

The advantages of derivatization as sample preparation for liquid chromatography are to enhance quantification limits and also improve specificity. But we can see some important disadvantages that need to be taken into account as decreased precision due to extra steps in the procedure. Also accuracy can be violated due to possible hydrolysis of conjugates and following increased values caused by deconjugation. This may be problem mostly in estradiol compounds, when derivatization caused 10 – 20% increase in estradiol levels in laboratories using this method compared to those who did not. Although values of testosterone and progesterone remained unchanged regarding derivatization. [18]

In prior to gas chromatography, derivatization step needs to be carried out to increase volatility of the sample and also improve its thermal stability as well as the chromatographic separation and detection. The preferred technique used in that case is mostly silylation causing formation of alkylsilyl derivatives by replacing originally active protons of –OH and –NH groups. In some cases acylation can be used. [16]

The derivatization process itself changes both chemical and physical properties of the compounds and that may resolve into changes in efficiency of the ionization, fragmentation, chromatographic retention and potential of ion suppression. [17] The setup should always be adjusted for suitable method and all possible consequences should be taken into account. As we see progress in analytical methods, the detection limits and sensitivity are increasing and this allows us to decrease usage of derivatization.

1.7. Instrumental methods for analysis of steroid conjugates

Determination of steroid hormones and their conjugated forms is accomplished with variety of different analytical methods, when each method has its advantages and disadvantages and together they form a very useful tool for full analysis of steroid hormones.

1.7.1. Immunoassay

Different types of immunoassay are used daily in routine clinical practice as radioimmunoassay of enzyme-linked immunosorbent assay. Those methods are rather considered preliminary testing, especially in positive results, when the outcome was confirmed usually by chromatographic method. The most relevant advantages of this method are the low cost and the rapidity. This allows the most clinical laboratories to use this method

on daily basis. Common disadvantages of immunoassay methods are possible cross-reaction with compounds with similar structure giving false positive or false negative results. Other drawbacks of this technique are limited analytical range, lack of specificity and common matrix effects. Also determination of several samples and its metabolite is not allowed lowering the specificity. [16, 19]

1.7.2. Gas chromatography – mass spectrometry (GC-MS)

Gas chromatography is a separation method that requires its sample to be volatile and thermally stable as mentioned above. Those requirements also show the main disadvantage of this technique in steroid hormone conjugates as the conjugated form is not suitable for the analysis. The pretreatment of the sample needs to provide suitable derivatization step and most importantly hydrolysis of the conjugates. The recent approaches through this disadvantage are the analysis once with hydrolysis and again without presence of the conjugated sulphates and glucoronides resulting into ratio values. On the other hand this method is able to offer very high chromatographic resolution and very successful separation of the molecules with slight limitation concerning separation of steroid isomers with almost identical fragmentation. [20] Additionally, the sample volumes are very small and require high level of purity that employs sample pretreatment as intensive cleaning and preconcentration. Also the analytic runtimes can be very long exceeding an hour. Gas chromatography allows us to operate in the scan mode, which provides non-targeted profiling of steroid hormones and an opportunity to discover new compounds that can later serve as biomarkers. [16]

1.7.3. Liquid chromatography – mass spectrometry (LC-MS)

Liquid chromatography is lately considered main analytical tool for separation of steroid hormones in biological samples. Common detection method used after the separation is mass spectrometry in either standard form or also in form of tandem mass spectrometry employing ion trap. The chromatographic resolution and isomer separation is not so high when compared to GC but the sample pretreatment is not as difficult and also the analysis runtimes are lowered. Compared to gas chromatography, which is more suitable for novel steroid profiling, liquid chromatography is the best choice for targeted steroid profiling [16]

Most of the LC-MS methods are used as quantitative target analysis employing triple quadrupole mass spectrometry. The data usually contain three dimensions, retention time, mass to charge ratio of precursor ion and mass to charge ratio of product ion. The fundamental properties of the molecule as molecular weight and structure are presented in

these dimensions. Multiple reaction monitoring is typically performed, where mass analyzer transmits only ions characteristic for the target analyte and remove most of the chemical noise. This procedure allows one of the highest achievable analytical specificities and sensitivities. [17]

Different ionization procedures are used in steroid determination where the most popular still remains electrospray ionization (ESI). Two other methods are also commonly used in LC-MS and these are atmospheric-pressure chemical ionization (APCI) and atmospheric-pressure photoionization (APPI). Each method chooses form variety of ionization techniques to achieve the highest sensitivity. The APCI and APPI remain popular in determination of neutral steroid hormones as electrospray is more suitable for conjugated compounds. [21] The ESI procedure can be performed in either positive or negative ionization mode. For positive ion mode are usually used either formic of acetic acid to enhance protonation and increase sensitivity, as for the negative mode ammonium hydroxide is used to support deprotonation and as well increase sensitivity. Negative ion mode was found more suitable for both sulphate and glucuronide compounds showing higher stability than positive ion mode. [22]

Coupling mass spectrometry with chromatographic methods show many benefits as it is very sensitive, fast and reproducible method. But to validate its use in clinical laboratories, other testing needs to be carried out to prove accuracy and diagnostics utility, in this case immunoassay can take place but its reliability is limited as was mentioned above. The use of liquid chromatography and mass spectrometry opens new possibilities in early diagnostics and shows significant progress in metabolomics analysis.

2. Aims of the Thesis

- to review current extraction and LC-MS methods for the steroid conjugates in body fluids
- to optimize the sample preparation methodology and HPLC mobile phase effect for the determination of the steroid conjugates

3. Experimental Part

3.1. Used chemicals

Ammonium formate, formic acid acetic acid and ammonium hydroxide were purchased at Sigma Aldrich (Praha, Czech Republic). Methanol and acetonitrile were purchased at Fisher Scientific (Pardubice, Czech Republic). All used steroid conjugates were purchased from Steraloids (Newport, USA).

3.2. Laboratory materials and equipment

For the SPE the VisiprepTM Solid Phase Extraction Vacuum Manifold (Supelco, Bellefonte, Pennsylvania, USA) was used with water pump N86KT.18 (KNF Neuberger, Freiburg, Germany), the used extraction cartridges were Waters Oasis HLB 3cc (60 mg) or 1cc (30 mg) (Waters Corporation, Milford, Massachusetts, USA), Strata NH2/WAX 55 µm 10 mg/1 ml (Phenomenex, Torrance, CA, USA).

The analysis was performed using LC-MS system: TSQ Quantum Ultra (Thermo Scientific) equipped with ESI, Rheos Allegro HPLC pump (Flux Instruments, Basel, Switzerland), CTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland). The used HPLC column was Geminy C18 3µ 110A, 150 x 2mm (Phenomenex, Torrance, CA, USA).

3.3. Stock solutions and working solutions

For all the experiments the standard mixture of all glucuronides and sulphates stated in tables 3 and 4. The standard solution was available in the concentration of 200 pmol/µl for each compound. Prior to the analysis 25µl of the mixture were evaporated and redissolved in 50µl of MeOH to achieve the final concentration to be 100pmol/µl. The samples applied to the SPE columns were filled up to the volume of 1ml with water and acetic acid to the final pH to be 3. For the mobile phases of HPLC 5mM solution of ammonium formate in methanol and 5mM solution of ammonium formate in 80% acetonitrile in water were prepared. Also mobile phase

of 5mM solution of ammonium formate in MeOH or 80% MeCN with 0.1% formic acid were prepared.

3.4. Sample Preparation

For the study the SPE procedure was performed according to [23, 24]. Similar approach is also used in [3] and the experiment 5 was inspired by this article. For the experiments the standard solution mixture of sulphates and glucuronides was used. Compounds present in the mixture are stated in tables 3 and 4. The sample was always applied in volume of 1ml.

To explore multiple possibilities of SPE, five different approaches were examined with a primary base of Waters Oasis HLB column with two variations of either 3cc (60 mg) or 1cc (10 mg). Another part of our experimental setup was Strata NH_2 (10mg/1ml) column with pore size of 55μm. Different combinations of the two columns were examined. For each experiment, the procedure was also performed with blank solution to discover possible interferences.

For the first experiment the 3cc HLB column was connected to the NH_2 column using tubing thus the conditioning and sample application was performed subsequently. The connected columns were conditioned with 3ml of methanol and washed with 3ml of water. Next the sample was applied and the columns were washed with 3ml of water. This was followed by drying for 1 hour. After drying the elution step took place, first with 3ml of MeOH to elute the free form of the steroids and then with 3ml of 10% NH_4OH in MeCN to elute the conjugated form.

The second experiment was performed with the same setup but the NH_2 column was connected after the conditioning of the HLB, the sample application and the washing. Thus the HLB column was conditioned, sample applied, washed and dried as previously described and followed by the attachment of the NH_2 column. The elution was performed similarly as in the first experiment with MeOH for free steroids and then 10% NH_4OH in MeCN for the conjugates.

For the third and fourth experiment the 1cc HLB column was used in combination with the NH_2 column. The similar setup as in first two experiments was used with smaller volumes due to the change in the volume of the HLB column. For the third experimental setup the column were connected through the whole experiment. The conditioning and washing was identical to the first experiment with change of volume to 1ml. After the drying the free steroids were eluted with 1ml of MeOH and then the conjugates with 10% NH_4OH in MeCN. The fourth setup followed the procedure of the second experiment with conditioning and washing

volumes of 1ml. After the drying the free steroids were eluted with 1ml of MeOH and then the conjugates with 10% NH_4OH in MeCN.

Concerning the fifth experiment, only the 1cc HLB column was used with conditioning with 1ml of MeOH and 1ml of water followed by sample application and washing with 1ml of water. The column was dried for 1 hour and the elution was performed with 1ml of MeOH for free steroids and followed by 1ml of 10% NH_4OH in MeCN for the conjugated steroids.

After the SPE were all the samples as well as the blank solutions evaporated and redissolved in 50µl of MeOH to maintain the concentration of 100pM/µl for the further analysis by LC-MS.

3.5. LC-MS Conditions

The analysis was performed using HPLC method with flow rate of the mobile phase 300µl/min and the sample volume 5µl. For the mobile phase optimalization, variety of mobile phases was used to determine the most fitting criteria concerning the ionization and separation of the conjugates. Those mobile phases were divided into two groups with either methanol or acetonitrile as the main component of the phase. In both cases four versions of the mobile phase were investigated; pure main component, a solution acidified with formic acid, a solution with ammonium formate buffer and the buffered version with addition of formic acid. The detection was provided by the TSQ Quantum Ultra mass spectrometer.

After the analysis, the obtained peaks were integrated using Thermo Scientific X-calibur system to determine the intensity of each measured steroid in given mobile phase.

4. Results and discussion

4.1. Mobile Phase Optimalization for HPLC

To determine the most fitting conditions for the HPLC analysis as separation and possible signal shift the optimalization of the mobile phase was performed. Two main possibilities of either methanol or acetonitrile was examined with possible variations of pure main component, addition of formic acid for acidification, addition of ammonium formate buffer and acidified buffered version. For the analysis the conjugates stated in tables 3 and 4 were examined and evaluated.

4.1.1. Methanol mobile phase

The methanol mobile phase is generally more common for the reversed phase liquid chromatography thanks to the many factors including different elution factors, peak shapes and also price which is in favor. Through the optimalization procedure, different versions of the methanol mobile phase were used and many different effects on the final chromatogram were observed. The mobile phase of methanol with added ammonium formate buffer was evaluated to be the best fit and the most universal for the needs of the upcoming experiments.

MeOH

Concerning the pure methanol, the results were not as coherent as expected. Both glucoronides and sulphates were showing many false signals that failed to match the standard solutions and thus would interfere in more complex analysis. Most peaks appeared as wide signals with low intensity. The most promising appeared to be results for estrogens as estrone glucuronide showed single peak at 4.97 min. for estradiol 3-glucuronide and 17-glucuronide the main peak was present at 2.68 min but 3 other comparable peaks were also present in the later retention times. For the estriol 3-glucuronide the peak seemed to be slightly unsymmetrical appearing at 1.11min. Another large peak was also present around 10 min, thus not fully interfering but might be problematic in future. Concerning other glucoronides some of the peaks failed to appear at all as cortisol glucuronide, pragnenolone glucuronide and testosterone glucuronide. For the other glucuronides the signals were recorded but the recognition of the correct signal is difficult as the many conflicting peaks are present.

For the sulphates the results were similarly unsuitable for further analysis. The most stable signal appeared for estrone sulphate showing single peak at 2.77 min and dihydroxyprogesterone sulphate at 7.20 min. Some of the peaks appeared to be rather wide

with possibility of two peaks fusing into one. Also for the epitestosterone sulphate, testosterone sulphate, dehydroepiandrosterone and both hydroxy DHEA sulphates the signals were shifted so violently to elute after 10 min. As the common measuring time is around 10 min this fact would cause the loss of the signal and thus requirement of longer measuring period and inconvenience connected to that.

MeOH + 0.1% FA

For the acidified version of the methanol, the measurement was conducted. Concerning the glucuronides the results appeared to be quite nice, showing symmetrical peaks mostly in the mid-region of the measured time period. Some exceptions were also recorded as the testosterone glucuronide, DHEA glucuronide and estriol 17-glucuronide. Those three samples showed blurry double peaks with other interfering signals with high intensities.

The measured sulphates showed signals at quality incomparable to the glucuronides. The finest peak was present for estradiol disulphates at 7.01 min and estriol 3-sulphate at 4.67 min. most of the other signals showed strong shift towards 10^{th} min of the retention time and the peaks were wide and possibly fused. The separation of the signals was decreased as well as the intensity of the signals of those peaks.

MeOH + 5 mM NH_4COOH

The ammonium formate buffer was added into the methanol for the next series of measurement. The glucuronide results appeared to be very solid with nicely symmetric shape and lack of interfering signals for most of the compounds. In case the multiple signals occurred during the measurement the intensities were low. Compared to the acidified version the slight shift towards the start occurred possibly allowing the shorter measuring time period. The separation seemed to be an issue in some compounds as glucuronides of testosterone, estrone and both estradiols revealed small additional double peak.

Concerning the sulphate outcome, generally the signals were symmetrical, slightly wider than desired but situated around 3^{rd} minute of the measuring time and with very good separation. The problematic is the detection of epiandrosterone sulphate with almost no signal and estrone 3-sulphate with small peak at 2.20 min and two more intense peaks around 4^{th} minute. The rest of the measurements showed very small or none interference.

This measurement was determined to be the most fitting the desired criteria and thus was evaluated as the most fitting concerning the future experiments. For illustration the obtained chromatograms are shown in figure 2 and 3.

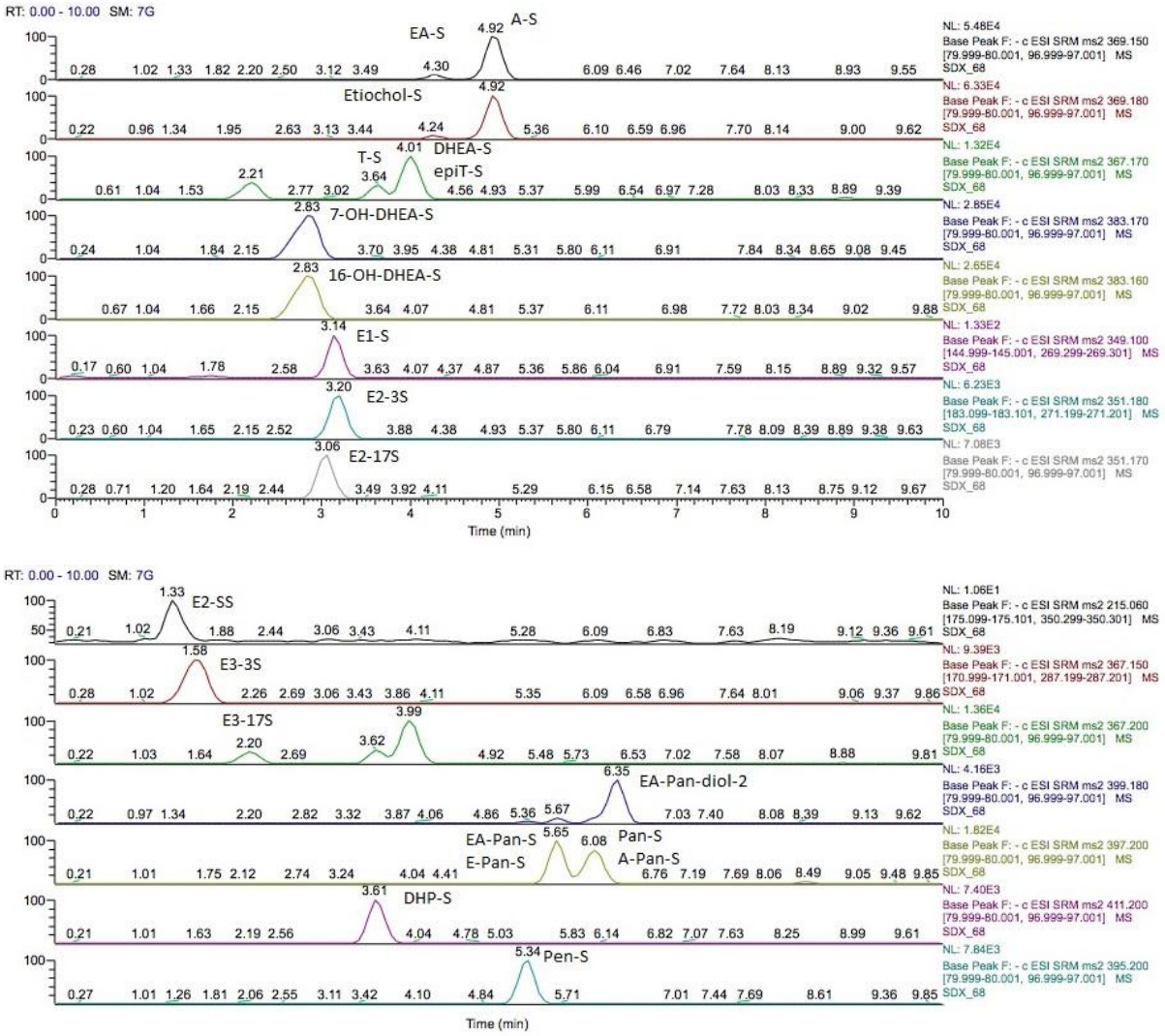


Figure 2: The chromatogram of measured steroid sulphates with compound indication next to each peak and right panel showing the used ESI mode and data of SRM monitoring

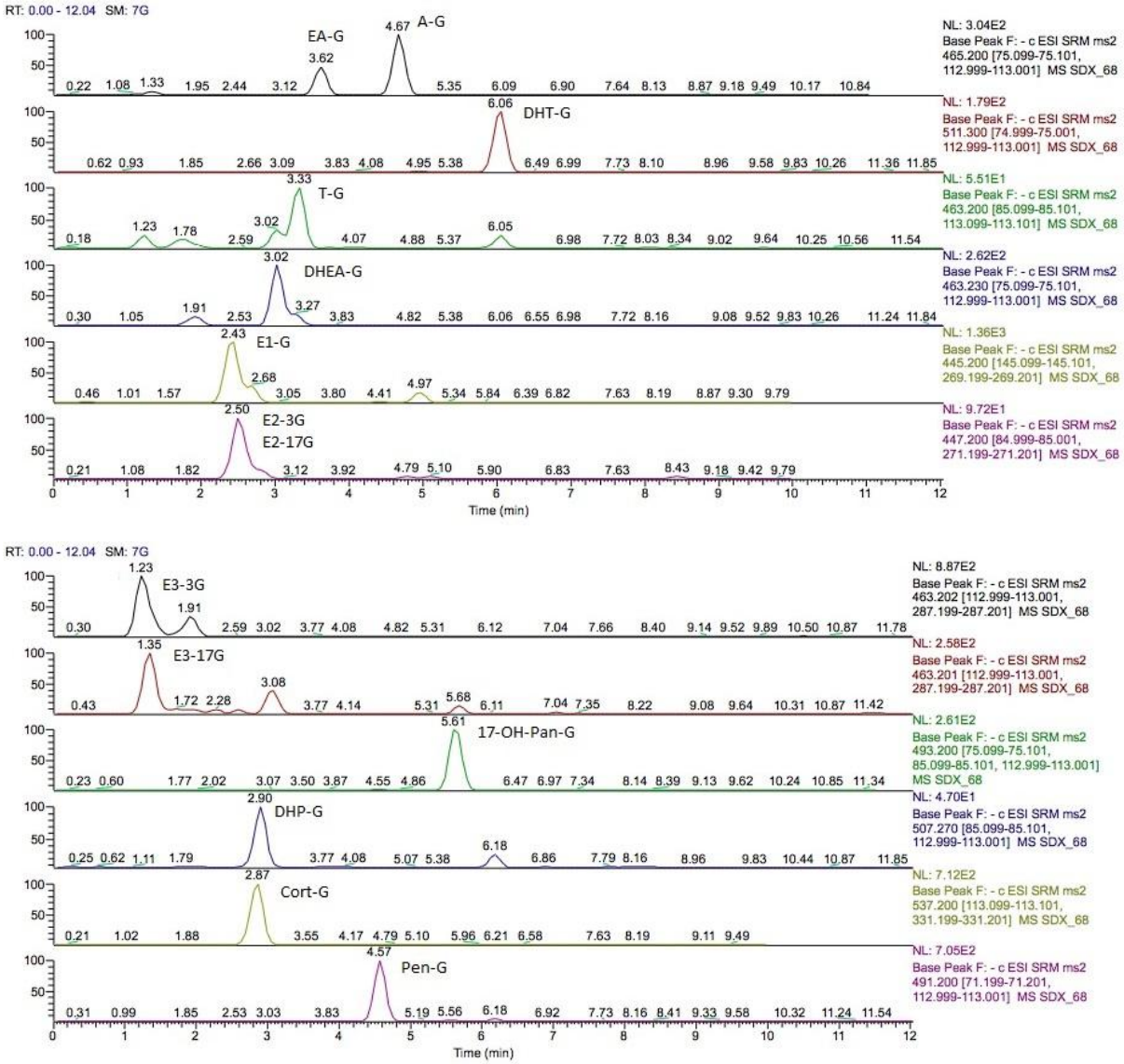


Figure 3: The chromatogram of measured steroid glucuronides with compound indication next to each peak and right panel showing the used ESI mode and data of SRM monitoring

MeOH + 5 mM NH_4COOH + 0.1% FA

As the last the possibility the acidified mobile phase with addition of buffer was investigated to determine any deviation to the previous measurements. The glucuronides showed solid intense signals with exception of epiandrosterone glucuronide that lack the signal. The dihydrotestosterone and testosterone glucuronides showed extra signal with significant intensities in addition to their proper signals. The signals appear to be slightly shifted towards the start compared to the non-acidified phase. Also the small double peaks were absent compared to this phase resulting from better separation.

For the sulphates the results seemed to be comparable to the non-acidified version with only small divergences. The estrogens showed sharper peaks with exception of estradiol disulphate where a small double peak occurred. The epiandrosterone sulphate exhibited increased intensity whereas the androsterone on the other hand showed small and rather round peak.

4.1.2. Acetonitrile mobile phase

The acetonitrile mobile phase was also investigated to discover possible differences in separation, shift and change in peak signals. Unfortunately the results were not as fitting as with methanol mobile phase and those mobile phases were not used in following experiments.

90% MeCN / W

The mobile phase of pure acetonitrile with water showed relatively nice signals for glucuronides especially the estrogens. The estriol glucuronides exhibited a few signals appearing after the retention of the original compound but the separation was good. Also glucuronides of dihydroxyprogesterone and pragnenolone showed sharp peaks but some compounds failed to occur at all. Dihydrotestosterone glucuronide didn't show any relevant signal and identification of epiandrosterone, androsterone and dehydroepiandrosterone glucuronides was fairly difficult due to the many interfering signals. All the signals were severely shifted towards the 12^{th} minute and prolonging of the measured time period was necessary up to 16 min.

The measurement of sulphates was not as successful and resulted in many wide peaks and problematic signals. The signals for epiandrosterone, androsterone and etiocholanolone sulphates were absent completely. The sulphates of 7 and 16 hydroxy dehydroepiandrosterone produced a signal with a width over 4 min. this might cause problems with integration and the

result would be biased. Other peaks tend to be wide as well with a general shift towards the end of the measurement.

90% MeCN / W + 0.1% FA

The acidified version of the acetonitrile solution was examined and the main difference compared to the non-acidified version is the shift of the glucuronides signals. The peaks are mostly situated around 2nd minute in contrast of the 12th minute significant for the non-acidified version. The signals of dihydroxyprogesterone glucuronide, cortisol glucuronide and pragnenolone glucuronide appear to be sharp and convenient as well as the signals of estrone glucuronide and both estradiol glucuronides. The estriol glucuronides signals seem to be buried in other interferences and dihydrotestosterone failed to exhibit signal coming out of the background.

The sulphate measurement seemed to be more successful compared to the non-acidified version as almost all the compounds were successfully recorded and identified. Compared to the glucuronide measurement no general significant shift is present and most of the signals occur in the mid-region. The signals for epiandrosterone and androsterone sulphate failed to separate forming only one peak. On the other hand both estriol sulphates exhibited nice separation and sharp, intense peaks which previously seemed to be slightly problematic.

90% MeCN / W + 5mM NH_4COOH

The ammonium formate buffer was added to the mobile phase and the effect was examined. Concerning the glucuronides, the signals were relatively sharp and intense with exception of epiandrosterone glucuronide which was only minor compared to the androsterone. The signal of estrone glucuronide was followed by wide triple peak which might be causing complications in the future analysis. Generally the signals were appearing in the region after 1st minute with exception of 17 hydroxy pregnanolone and dihydrotestosterone glucuronides. Their peaks appeared at 4.18 min and 4.26 min respectively.

The peaks for sulphates were slightly more unattractive as the width was mostly increased and occasionally double peaks occurred. The peaks for both 7 and 16 hydroxy dehydroepiandrosterone sulphates were very wide and non-symmetrical. Generally the signals were located in the middle of the chromatogram allowing to decrease the measuring tine up to 10 minutes.

90% MeCN / W + 5mM NH_4COOH + 0.1% FA

The acidified version of the acetonitrile mobile phase with buffer addition produced rather nice signals of the glucuronides located mostly on the region around 4th minute and with minority of the interfering signals. The separation of the epiandrosterone and androsterone glucuronides is adequate with their intensities being comparable. The interesting is appearance of unidentified signal accompanying both estriol glucuronides at 3.95 min suggesting possible side product.

Concerning the sulphates the signals were mostly sharp and intense with only mild background. The location of the signals is again in mid-region of the 13 minute measuring period. The separation of the epiandrosterone and androsterone sulphate seems to be a bit problematic causing the formation of the double peak. Apart from that the signals seem to be reasonably attractive.

4.2.Extraction Method Results

4.2.1. Compounds of interest

The series of experiments including different kinds of SPE extraction were performed to determine the best possible clean up method for the steroid conjugates. The conjugates of our interest are stated in tables 3 and 4 below. For the determination of the success rate of the SPE the LC-MS analysis was performed.

Table 3: Glucuronides used for the measurement, their trivial name, commonly used abbreviation and molecular weight

Conjugate	**Trivial name**	**Abbrev.**	**MW**
5α-Androsan-3α-ol-17-one Glucosiduronate	Androsterone Glucuronide	A-G	466.27
5α-Androstan-3β-ol-17-one Glucosiduronate	Epinadrosterone Glucuronide	EA-G	466.27
5α-Androstan-17β-ol-3-one Glucosiduronate	Dihydrotestosterone Glucuronide	DHT-G	466.27

4-Androsten-17β-ol-3-one Glucosiduronate	Testosterone Glucuronide	T-G	464.24
5-Androsten-3β-ol-17-one Glucosiduronate	Dehydroepiandrosterone Glucuronide	DHEA-G	464.24
1, 3, 5(10)-Estratrien-3-ol-17-one Glucosiduronate	Estrone Glucuronide	E1-G	446.19
1, 3, 5(10)-Estratrien-3, 17β-diol 3-Glucosiduronate, Sodium Salt	Estradiol 3- Glucuronide	E2-3G	448.21
1, 3, 5(10)-Estratrien-3, 17β-diol 17-Glucosiduronate	17β-Estradiol 17- Glucuronide	E2-17G	448.21
1, 3, 5(10)-Estratrien-3, 16α, 17β-triol 3- Glucosiduronate, Sodium Salt	Estriol 3- Glucuronide	E3-3G	464.21
1, 3, 5(10)-Estratrien-3, 16α, 17β-triol 17- Glucosiduronate	Estriol 17- Glucuronide	E3-17G	464.21
5β-Pregnan-3α, 17-diol-20-one Glucosiduronate, Sodium Salt	17α-Hydroxypregnanolone Glucuronide	17-OH-Pan-G	510.28
4-Pregnen-17,20β-diol-3-one-20-Glucosiduronate	17α, 20β-Dihydroxyprogesterone Glucuronide	DHP-G	508.6
4-Pregnen-11β,17,21-triol-3,20-dione 21 Glucosiduronate	Cortisol Glucuronide	Cort-G	538.6
5-Pregnen-3β-ol-20-one 3-Glucosiduronate	Pregnenolone Glucuronide	Pen-G	492.27

Table 4: Sulphates used for the measurement, their trivial name, commonly used abbreviation and molecular weight

Conjugate	**Trivial name**	**Abbrev.**	**MW**
5α-Androstan-3α-ol-17-one 3- Sulphate	Androsterone Sulphate	A-S	370.18
5α-Androstan-3β-ol-17-one Sulphate	Epinadrosterone Sulphate	EA-S	370.18
5β-Androstan-3α-ol-17-one Sulphate	Etiocholanolone Sulphate	Etiochol-S	370.18
4-Androsten-17α-ol-3-one Sulphate	Epitestosterone Sulphate	epiT-S	368.17
4-Androsten-17β-ol-3-one Sulphate,	Testosterone Sulphate	T-S	368.17

5-Androsten-3β, 7α-diol-17-one Sulphate	7α-Hydoxy Dehydroepiandrosterone Sulphate	7-OH-DHEA-S	384.16
5-Androsten-3β, 16α-diol-17-one Sulphate	16α-Hydroxy Dehydroepiandrosterone Sulphate	16-OH-DHEA-S	384.16
5-Androsten-3β-ol-17-one Sulphate	Dehydroepiandrosterone Sulphate	DHEA-S	368.17
1, 3, 5(10)-Estratrien-3-ol-17-one Sulphate	Estrone Sulphate	E1-S	350.12
1, 3, 5(10)-Estratrien-3, 17β-diol 3-Sulphate	17β-Estradiol 3-Sulphate	E2-3S	352.13
1, 3, 5(10)-Estratrien-3, 17β-diol 17-Sulphate	17β-Estradiol 17-Sulphate	E2-17S	352.13
1, 3, 5(10)-Estratrien-3, 17β-diol Disulphate	17β-Estradiol Disulphate	E2-SS	432.09
1, 3, 5(10)-Estratrien-3, 16α, 17β-triol 3-Sulphate	Estriol 3-Sulphate	E3-3S	368.13
1, 3, 5(10)-Estratrien-3, 16α, 17β-triol 17-Sulphate	Estriol 17-Sulphate	E3-17S	368.13
5α-Pregnan-3β, 20β-diol-3-Sulphate	Epiallopregnanediol 3-Sulphate	EA-Pan-diol-S	400.23
5α-Pregnan-3α-ol-20-one Sulphate	Allopregnanolone Sulphate	A-Pan-S	398.21
5α-Pregnan-3β-ol-20-one Sulphate	Epiallopregnanolone Sulphate	EA-Pan-S	398.21
5β-Pregnan-3α-ol-20-one Sulphate	Pregnanolone Sulphate	Pan-S	398.21
5β-Pregnan-3β-ol-20-one Sulphate	Epipregnanolone Sulphate	E-Pan-S	398.21
4-Pregnen-17, 20β-diol-3-one,20-Sulphate	17α, 20β-Dihydorxyprogesterone Sulphate	DHP-S	412.19
5-Pregnen-3β-ol-20-one 3-Sulphate	Pregnenolone Sulphate	Pen-S	396.2

4.2.2. LC-MS analysis results

The experiment 5 showed results different from those in experiments 1-4. In all the experiments both eluted fractions were analyzed in order to discover possible leakage or unexpected elution. For experiments 1-4 the first fraction didn't show any significant eluted conjugates but for experiment 5 the results were different. The first fraction was supposed to elute only the free steroids but concerning the 5th experiment the amount of eluted conjugates was surprisingly high and most of the conjugates occurred to be eluting at this step. The second fraction was analyzed as well but the amounts tend to be relatively low. Due to this fact, the analysis of the 5th experiment included two measurements of the first fraction and one measurement of the second fraction. For experiments 1-4 the second fraction was measured twice for reproducibility and reliability. For the first four experiments the results for the glucuronides are stated in table 5 and for sulphates in table 6. The data for the fifth experiment are stated in table 7. The results were evaluated as a percentage out of original sample amount before the extraction and analysis. The original value was 100pmol/µl.

Table 5: the glucuronides analyzed after the SPE experiments 1-4 and the amount detected by LC-MS (%)

Experiment #	1	1	2	2	3	3	4	4
EA-G	14.8	9.1	37.7	51.9	248.2	34.7	20.8	139.8
A-G	1.1	38.3	13.2	36.2	60.0	16.4	34.1	140.9
DHT-G	0.0	0.0	0.0	0.0	0.0	5.1	0.0	0.0
T-G	2.8	7.5	62.8	23.1	65.2	20.8	47.4	40.2
DHEA-G	1.6	4.7	48.4	25.5	25.7	80.9	13.0	32.4
E1-G	31.2	4.6	17.7	39.3	76.7	60.8	38.5	45.9
E2-3G, E2-17G	4.5	7.4	21.5	19.6	73.4	81.9	50.3	45.2
E3-3G	123.4	14.1	0.0	41.9	0.0	116.2	26.4	75.5
E3-17G	4.4	5.6	4.6	71.4	19.3	24.3	76.1	136.0
17-OH-Pan-G	10.6	1.8	14.5	120.0	5.0	166.7	14.3	46.8
DHP-G	9.8	13.6	4.2	2.9	8.5	4.9	2.6	10.9
Cort-G	17.1	5.3	1.5	1.6	45.2	16.0	31.4	20.9
Pen-G	0.0	12.6	49.1	47.6	43.9	18.7	87.2	71.6

Table 6 the sulphates analyzed after the SPE experiments 1-4 and the amount detected by LC-MS (%)

Experiment #	1	1	2	2	3	3	4	4
EA-S	41.3	46.8	68.2	70.3	61.2	70.6	68.2	72.6
A-S, Etiochol-S	27.6	25.7	53.2	49.7	33.5	37.0	43.6	44.3
T-S	44.3	36.6	38.0	81.8	69.9	76.4	54.3	62.0
epiT-S, DHEA-S	43.9	44.8	48.7	68.6	44.2	48.4	54.0	65.8
7-OH-DHEA-S, 16-OH-DHEA-S	22.9	24.7	41.5	45.5	35.3	39.4	30.8	39.8
E1-S	23.4	201.2	66.0	281.9	35.5	20.0	107.7	40.2
E2-3S	35.7	46.2	79.5	83.6	58.0	54.5	55.2	46.3
E2-17S	39.7	55.3	77.7	72.5	51.3	66.5	76.8	58.3
E2-SS	0.0	1.0	0.0	0.0	16.7	1.9	0.0	0.0
E3-3S	35.1	27.5	53.5	57.0	56.9	59.0	42.1	57.8
E3-17S	41.9	46.1	91.6	104.0	78.1	98.8	52.3	73.8
EA-Pan-diol-S	43.6	26.4	64.9	59.6	48.6	42.1	59.2	69.4
E-Pan-S, EA-Pan-S	56.5	50.2	75.5	75.4	46.3	56.7	66.4	63.3
Pan-S, A-Pan-S	39.4	31.1	67.1	69.4	47.9	43.0	53.2	64.9
DHP-S	42.6	34.7	58.1	51.5	50.0	58.6	44.1	42.9
Pen-S	41.1	46.9	77.5	75.3	56.7	47.1	67.7	77.4

Table 7: the glucuronides and sulphates analyzed after the fifth SPE experiment and the relative amount detected by LC-MS (%)

Fraction	1	1	2
Glucuronides			
EA-G	163.0	156.0	0.0
A-G	44.2	112.6	0.0
DHT-G	0.0	0.0	0.0
T-G	45.7	7.3	6.5
DHEA-G	51.7	86.9	0.0
E1-G	92.4	129.4	0.0
E2-3G, E2-17G	129.5	117.1	0.0
E3-3G	171.8	116.5	0.0
E3-17G	127.6	110.4	0.0
17-OH-Pan-G	91.4	144.8	0.5
DHP-G	74.9	73.5	2.6
Cort-G	45.9	92.2	0.0
Pen-G	56.6	66.1	0.0
Sulphates			
EA-S	117.4	99.6	0.0
A-S, Etiochol-S	133.2	313.6	0.2
T-S	93.9	92.2	5.4
epiT-S, DHEA-S	93.8	100.0	1.7
7-OH-DHEA-S, 16-OH-DHEA-S	116.5	98.9	0.1
E1-S	150.7	170.6	1.7
E2-3S	109.8	123.9	0.3
E2-17S	112.6	120.0	0.0
E2-SS	48.7	43.0	0.0
E3-3S	82.4	92.5	3.1
E3-17S	136.0	118.3	0.0
EA-Pan-diol-S	105.1	97.5	0.1
E-Pan-S, EA-Pan-S	106.0	109.8	0.0
Pan-S, A-Pan-S	141.5	182.1	0.0
DHP-S	82.2	108.0	0.0
Pen-S	107.5	119.3	0.4

As the results show, the values seem to be not as accurate as it was hoped. Many values were found to be reaching over 100% which might be caused by the matrix effect or by other interferences.. Thus the results cannot be used for further analysis and the error is expected. Generally the results for sulphates seem to be more reasonable and the yields are higher than for glucuronides This fact might be caused by more intense binding of the glucuronides to the SPE cartridge and the unsuccessful elution.

5. Conclusion

A large scale of common glucuronides and sulphates was investigated through this thesis and the complexity of the sample was very high. For the future analysis of the steroid conjugates this fact needs to be taken into account as it might be more suitable to divide such a large scale into smaller groups of conjugates with similar properties.

The mobile phase optimalization for the HPLC analysis showed interesting results. Two different approaches were examined with either methanol or acetonitrile.The mobile phase consisting of methanol with 5mM ammonium formate showed the best separation and most suitable shift and intensities of both glucuronides and sulphates being the best fit for the analysis of those compounds. For those reasons this mobile phase was used for the following experiment.

Concerning the extraction experiments the results did not show expected coherence and the reliability of the measured data is questionable. The SPE approach seems to be problematic even on the level of standard solutions, thus the analysis of more complex biological sample with possible matrix effect should be addressed after this issue is overcome. In the future, different purification method should be considered and tested.

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