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EVALUATING METHODS FOR THE ANALYSIS OF GLUCOCORTICOIDS IN ANIMAL FATS

Master thesis submitted in fulfillment of the requirements for the academic degree “Diplom-Ingenieur” at the University of Natural Resources and Life Sciences, Vienna

Submitted by Larissa Fleischanderl, BSc

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Abstract

Glucocorticoids are fat soluble steroid hormones produced by animals that occur naturally in animal fats. In this work, a reproducible method for the preparation and analysis of glucocorticoids in fat was developed and evaluated to be applied to any animal fat derived food sample or cosmetic product. This is necessary for food and consumer safety reasons, since there are regulations to be followed. The selected target corticoids were cortisone, hydrocortisone, corticosterone, Reichstein's substance S, 21-hydroxyprogesterone, 17α-hydroxyprogesterone and progesterone. First, corticoid standard substances were used for method optimization, later also real animal fat samples were investigated. Optimal extraction column for solid phase extraction (SPE) was a DSC-NH_2 tube with cyclohexane and methanol as eluents. Analysis was performed with gas chromatography/mass spectrometry (GC/MS) in selected ion monitoring (SIM) mode with specific molecular masses occurring in certain time ranges, which were improved in the course of optimization. Therefore, target substances were derivatized beforehand. The ideal gas chromatographic column and an appropriate heating program of the GC/MS for good separation of the substances were identified. In order to obtain recovery values for the analysis method, calibration of corticoids was carried out to determine, to what extent spiking and measuring corresponded. Concentrations of 6 to 30 ppm of corticoids in fat show recovery values ranging from 51 to 138 %, mean value is 84 %. Limits of detection and quantification (LOD, LOQ) were defined as well. They ranged from 0.5 to 4.2 ppm as detection limit and 1 to 8.3 ppm as quantification limit, depending on each corticoid. Results of the investigated animal fat ointments and fox fat tissue were beyond detection limit.

Keywords: glucocorticoids, animal fat, steroid hormones, GC/MS, SPE

Zusammenfassung

Glucocorticoide sind körpereigene fettlösliche Steroidhormone, die in tierischem Fettgewebe vorkommen. In der vorliegenden Arbeit wurde eine reproduzierbare Methode für die Aufbereitung und Analyse von Glucocorticoiden in Fett entwickelt und bewertet, um sie auf beliebige aus tierischem Fett gewonnene Lebensmittelproben oder Kosmetika anwenden zu können. Dies ist notwendig, um für den Zweck der Lebensmittel- und Konsumentensicherheit auf mögliche Steroide zu prüfen, ob gesetzliche Richtlinien eingehalten werden. Die ausgewählten Zielcorticoide waren Cortison, Hydrocortison, Corticosteron, Reichsteins Substanz S, 21-Hydroxyprogesteron, 17α-Hydroxyprogesteron und Progesteron. Zuerst wurden Reinstandards für die Methodenoptimierung verwendet, später auch echte Tierfettproben. Die optimale Extraktionssäule für Festphasenextraktion (SPE) war eine DSC-NH_2-Säule mit Cyclohexan und Methanol als Eluenten. Analysiert wurde mit Gaschromatographie/Massenspektrometrie (GC/MS) im SIM-Modus (selected ion monitoring) mit in bestimmten Zeiträumen vorkommenden spezifischen Molekularmassen, die im Laufe der Optimierung angepasst wurden. Dafür wurden die Zielkomponenten vor der Analyse derivatisiert. Auch die ideale Gaschromatographiesäule und ein passendes Temperaturprogramm für die GC/MS wurden ermittelt, um eine gute Trennung der Substanzen zu erhalten. Zur Erlangung der Wiederfindungsraten der Analysenmethode musste eine Kalibrierung der Corticoide durchgeführt werden, um zu sehen, inwieweit bereitete und gemessene Ergebnisse übereinstimmten. Konzentrationen von 6 bis 30 ppm Corticoide in Fett ergaben Wiederfindungsraten von 51 bis 138 % mit einem Mittelwert von 84 %. Detektions- und Bestimmungsgrenze (LOD, LOQ) wurden auch definiert. Je nach Corticoid betrug das Detektionslimit 0,5 bis 4,2 ppm und das Quantifikationslimit 1 bis 8,3 ppm. Ergebnisse der untersuchten Tierfettsalben und des Fuchsfettgewebes lagen unter dem Detektionslimit.

Schlüsselwörter: Glucocorticoide, Tierfett, Steroidhormone, GC/MS, SPE

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Content

1 Introduction

Glucocorticoids are familiar to people, as they are commonly used in daily life. An example for this is hydrocortisone – which is also known as cortisol – that it is applied on skin for anti-inflammatory and immunosuppressive effects in mild eczema, insect bites or small sun burns. It is known that glucocorticoids are secreted by the adrenal gland as a classic endocrine response to stress (Sapolsky *et al.*, 2000). They belong to the group of fat soluble steroid hormones which occur naturally in animal fats. Residues thereof are usually investigated by sampling either feces, urine or fat tissue.

Starting point of method development was a study by Wagner and Nusser (1988) who determined glucocorticoids in healing salves made from fat of marmot and badger. They already mentioned the parallels of corticosteroids containing fat of badger/marmot and 0.1 to 2 % hydrocortisone containing salves used for treating eczema. The two German scientists did fraction preparation using column chromatography and solid phase extraction with subsequent HPLC analysis. Amounts of substance were found in the range of 5 to 10 ppm per corticoid. Based on this thirty-year-old study, seven of the corticoids targeted in the previous study were also chosen for evaluation in animal fat in the present study, according to their availability on the market (see Figure 1 – Figure 7). The question was, whether there is a simple and more up-to-date method for detection of these corticoids in other wild animal fats, including preparation and analysis using GC/MS.

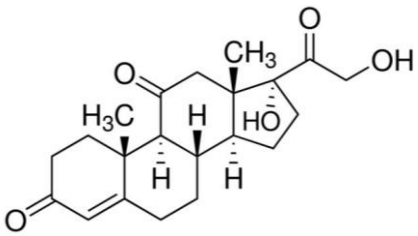

Figure 1: Molecular structure of cortisone

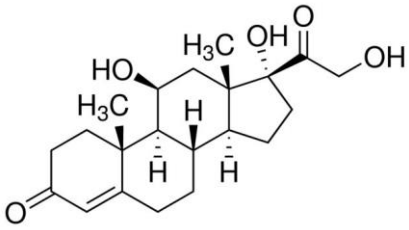

Figure 2: Molecular structure of hydrocortisone

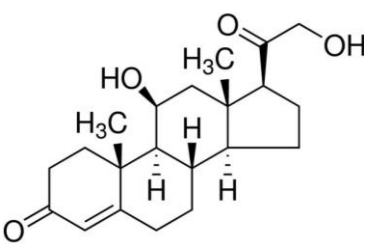

Figure 3: Molecular structure of corticosterone

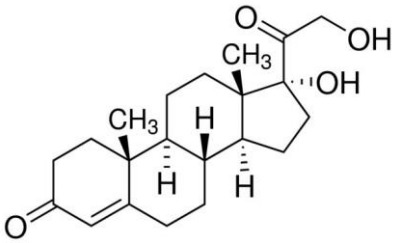

Figure 4: Molecular structure of Reichstein's substance S

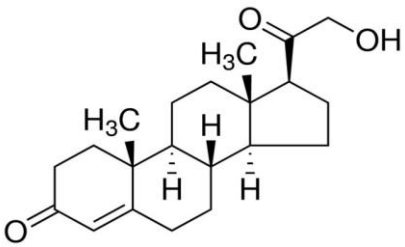

Figure 5: Molecular structure of 21-hydroxyprogesterone

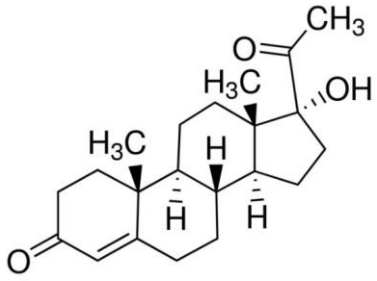

Figure 6: Molecular structure of 17α-hydroxyprogesterone

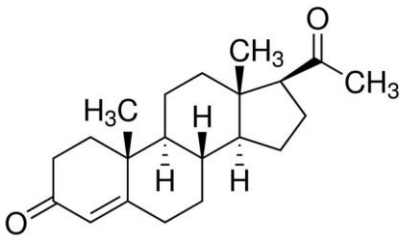

Figure 7: Molecular structure of progesterone

Other scientific work done in that field of interest was collected by Haugen *et al.* (2011) focusing on the pheromone steroid androstenone which has a similar molecule structure to corticoids. In this review, sample storage, treatment, separation and quantification were discussed. Concerning sample storage, it is said that conditions barely affect measured amounts of analyte, since androstenone is relatively stable. This is the same with hydroxyl groups containing steroids such as hydrocortisone (Kley and Rick, 1984). Something that probably does, however, affect the measured amount of analyte is the exact sampling location of fat tissue from the animal, but this is usually not defined by researchers.

When it comes to sample treatment, Haugen *et al.* (2011) name many different preparation methods. Extraction of steroids is mostly done either using liquid-liquid extraction, supercritical fluid extraction or solid phase extraction (SPE).

Among these three extraction techniques, SPE is a method which only requires simple equipment for extraction of samples from liquid matrices. Different SPE phases are recommended by Sigma-Aldrich Co. LLC. (2017, phase-selection), depending on type of sample matrix and analyte characteristics.

For sample preparation, saponification is commonly used. Since extremely high pH values and temperatures are needed for this kind of treatment, chromatographic methods are preferred instead. Furthermore, derivatization is a preparation method which is used to enhance detection of analytes, required prior to GC/MS analyses. As numerous other scientists have stated before, Shimada *et al.* (2001) explain that popular derivatization methods include trimethylsilyl (TMS) or other alkylsilyl ethers for silylation of hydroxy functions and O-methyloxime for oximation of ketonic functions.

For separation and detection techniques, Haugen *et al.* (2011) mention chromatography, such as liquid or gas chromatography, in combination with mass spectrometry as suitable approaches for detection.

The studies reviewed by Haugen *et al.* (2011) dealt with androstenone in fat tissue, which is similar but not fully comparable to corticoids. Numerous studies investigate steroids in urine – in particular testosterone – but little research has been conducted on glucocorticoids in fat. Therefore, there is a big potential to devote oneself to the research and development of a new method for detection of corticoids in animal fat.

Nowadays, liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have become the preferred type of analysis in clinical diagnostics. This is because they are very selective and sensitive. With these methods, very low concentrations of steroids, such as cortisone and cortisol occurring in saliva can be measured. Magda *et al.* (2017) for example present lower limits of quantification of cortisol being as little as 5 pg/ml.

For the purpose of developing a method, standard corticoids are necessary, which are tested for successful derivatization. Since the analytes are extracted from fat, a suitable eluent and the right column for solid phase extraction are evaluated via thin-layer chromatography. The GC/MS method is tailored to the masses of each corticoid for detection in SIM mode. Finally, detection and quantification limits are assessed to provide defined detection parameters for a verified method. As a first application of the developed method, it is used on real fat samples, such as ointment of badger, ibex and brown bear, as well as fat tissue of fox.

For future application of the method, it can be used in food quality control for animal fat intended for consumption. This is an important measure to ensure food and consumer safety. Concerning foodstuffs of animal origin, the Commission Regulation of the European Union states maximum residue limits of pharmacologically active substances (Commission Regulation (EU) No 37/2010, 2009). It is specified that the corticosteroid hydrocortisone aceponate, which is the ester of hydrocortisone with acetic and propionic acid, has a maximum residue limit of 10 µg/kg in the target tissue milk of the animal species ruminants and Equidae. Also, it is only allowed for intramammary use. In this case, marker residues are the sum of hydrocortisone and its esters after alkaline hydrolysis, expressed as hydrocortisone. Hydrocortisone itself exhibits no maximum residue limit for any food producing species and it is allowed for topical use only.

Regarding cosmetic products, glucocorticoids are listed as substances prohibited in cosmetics according to the regulation of the European Parliament and of the Council (Regulation (EC) No 1223/2009 of the European Parliament and of the Council, 2009).

2 Objectives

The aim of this project was to develop a reproducible and easy method for analyzing glucocorticoids in fat tissue of animals. This includes extraction of pure fat from fat tissue and further extraction of the steroid hormones from fat. Analysis of the corticoids should be conducted with an improved method for gas chromatography/mass spectrometry. This whole method is developed to be readily used in food safety monitoring, applied on animal fats for evaluating the amount of a selected group of glucocorticoids for ensuring their absence and therefore the safety of foodstuffs and cosmetics for humans.

3 Materials and Methods

3.1 Laboratory Equipment

3.1.1 SPE

All solid phase extractions with Supelclean LC-Si SPE or Discovery DSC-NH_2 tubes were performed with a SUPELCO visiprep vacuum extraction manifold, 12 port model.

3.1.2 Gas Chromatograph/Mass Spectrometer

GC/MS analysis was performed with a Thermo ELECTRON CORPORATION gas chromatograph Trace GC ultra (Model K01300730000070, Thermo Electron S.p.A., Strada Rivoltana, 20090 Rodano, Milan, Italy) coupled to a Thermo ELECTRON CORPORATION Thermo SCIENTIFIC DSQ II quadrupole mass spectrometer (Model Trace DSQ - mass spectrometer). A Thermo Finnigan AS 2000 autosampler (Model AS 2000 C.U., Strada Rivoltana, 20090 Rodano, Milano, Italy) was connected.

3.1.3 Gas Chromatograph/Flame Ionization Detector

The gas chromatograph, which was used for a few analyses, was a HRGC 5300 mega series, Carlo Erba instruments, MFC 500, EL 580, coupled to a flame ionization detector (FID).

3.2 Standard Substances

3.2.1 Reference Glucocorticoids

Cortisone ≥ 98 %, hydrocortisone (cortisol) ≥ 98 % (HPLC), corticosterone ≥ 92 %, Reichstein's substance S ≥ 98 %, 21-hydroxyprogesterone (deoxycorticosterone) ≥ 97 % (HPLC), 17α-hydroxyprogesterone ≥ 95 % and progesterone ≥ 99 % – all crystalline – were obtained from SIGMA-ALDRICH Chemie GmbH, Schnelldorf, Germany. The powdered glucocorticoid standards were stored at room temperature in the dark.

3.2.2 Betulin

Betulin (Lup-20(29)-ene-3β,28-diol) 1 g ≥ 98 %, crystalline, was used as an internal standard (IS) for GC/MS. It was chosen because the triterpene with its carbon network structure of 5 rings has a similar structure to the 4 rings of steroids (see Figure 8). It possesses two OH-groups, which some of the investigated corticoids do as well. The chemical substance was obtained from SIGMA-ALDRICH Chemie GmbH, Schnelldorf, Germany, and was stored in the refrigerator at 4 °C.

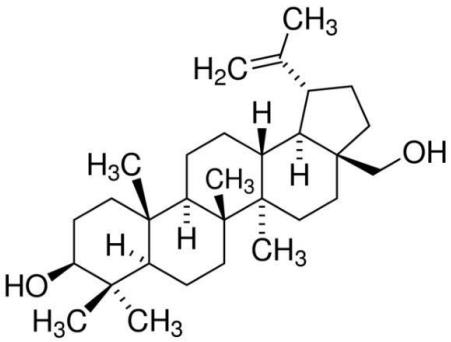

Figure 8: Chemical structure of betulin

3.2.3 Cholestanone

In later experiments, also 5α-cholestan-3-one, crystalline, was used as an internal standard for SPE to evaluate the behavior of betulin as analyte in fat extraction. It has a 4-ring carbon structure, similar to the basic structure of steroids and has one carbonyl group but no hydroxyl groups (Figure 9). It was purchased from SIGMA-ALDRICH Chemie GmbH, Schnelldorf, Germany.

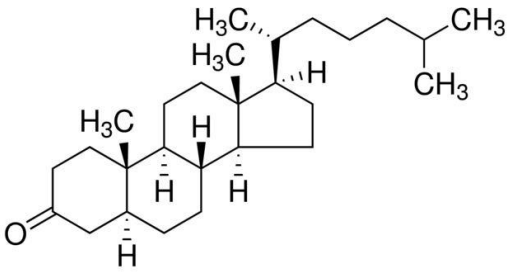

Figure 9: Chemical structure of 5α-cholestan-3-one

3.2.4 Stock Solutions

Standard stock solutions of glucocorticoids, betulin and cholestanone were prepared. This was done for easy portioning into small amounts for sample preparation. For the preparation of the stock solution of each standard the dry powder was weighed in with analytical precision and dissolved in acetone. Acetone was chosen as a solvent since it is very commonly used and all the substances dissolved easily. The stock solutions were kept in tightly screw-capped Pyrex vials at 4 °C in an explosion-proof refrigerator.

3.3 Standard Fat

The reference pork fat used for preparing fat samples with known amounts of corticoids of interest was “Radatz Schmalz”, 500 g, by Radatz GmbH, Erlaaer Straße 127, A-1230 Wien, bought at a local supermarket. The ingredients are pork fat from bacon, emulsifier E471 and E472c. Examination showed that it was free from naturally occurring glucocorticoids. It was kept at 4 °C in the refrigerator in its original packaging (plastic container).

3.4 Real Fat Samples

The animal fat samples for analysis were received from Dr. Teresa Valencak, Unit of Physiology, Pathophysiology, and Experimental Endocrinology, University of Veterinary

Medicine, Vienna. The ointments of real fat of badger, ibex and brown bear were kept at 4 °C in the refrigerator in their original packaging (small screw-capped round glass containers) until transferred into screw-capped Pyrex vials. The fox fat tissue samples were stored in the freezer at -24 °C and later at 4 °C in the refrigerator until use.

3.5 Reaction Solutions

3.5.1 Oximation Reagent

Hydroxylamine hydrochloride (HAH) from SIGMA-ALDRICH Chemie GmbH, Schnelldorf, Germany, was used as oximation reagent. Therefore, a stock solution of 2.5 % hydroxylamine hydrochloride in pyridine of the corticoids was prepared with weighing in the dry crystals and dissolving in pyridine. The solution was kept in a screw-capped Pyrex vial at 4 °C in the refrigerator.

3.5.2 Silylation Reagents

The silylation reagents BSTFA + TMCS, 99:1 (Sylon BFT) from SUPELCO, Bellefonte, USA, and 1-(trimethylsilyl)imidazole (TMSI) from SIGMA-ALDRICH Chemie GmbH, Schnelldorf, Germany, were stored in the refrigerator at 4 °C.

3.6 Sample Preparation

3.6.1 Real Fat Sample Preparation

- cut fat tissue into small pieces with a knife
- extract fat in a 10 ml-vial according to an adapted protocol of Bligh and Dyer (1959):
  - add 1 ml H_2O, 4 ml methanol and 2 ml chloroform to approximately 1 g sample
  - mix
  - add 2 ml chloroform and 2 ml H_2O
  - shake well
  - transfer liquid with 602 H ½ fluted filter and glass funnel into a separatory funnel
  - let settle overnight
- take lower phase with fat and evaporate chloroform in rotary evaporator
- add approximately 5 ml of acetone, evaporate again
- put in flow of nitrogen

3.6.2 Derivatization

Prior to GC/MS, derivatization of the polar functional groups of the glucocorticoids needed to be performed. Hereby, the carbonyl groups of the corticoids had to be transformed into ketoximes with reaction of hydroxylamine, leaving a hydroxyl side chain instead of each carbonyl group on the molecule (see Figure 10). Further, all the hydroxyl groups had to be transformed into non-reactive silyl groups, to enhance mass spectrometric properties for GC/MS analysis (Figure 11). With silylation, the molecules become more volatile, less polar and better thermally stable (Sigma-Aldrich, 2008). In Figure 12, the changes of the functional groups during derivatization of cortisone with oximation of the crude molecule to silylation of the created molecule are pictured as an example.

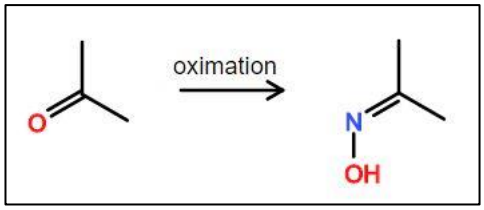

Figure 10: General oximation of a molecule with hydroxylamin hydrochloride

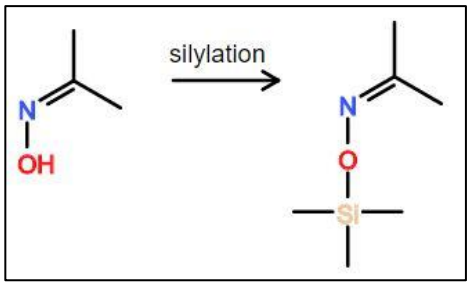

Figure 11: General silylation of a molecule with TMSI

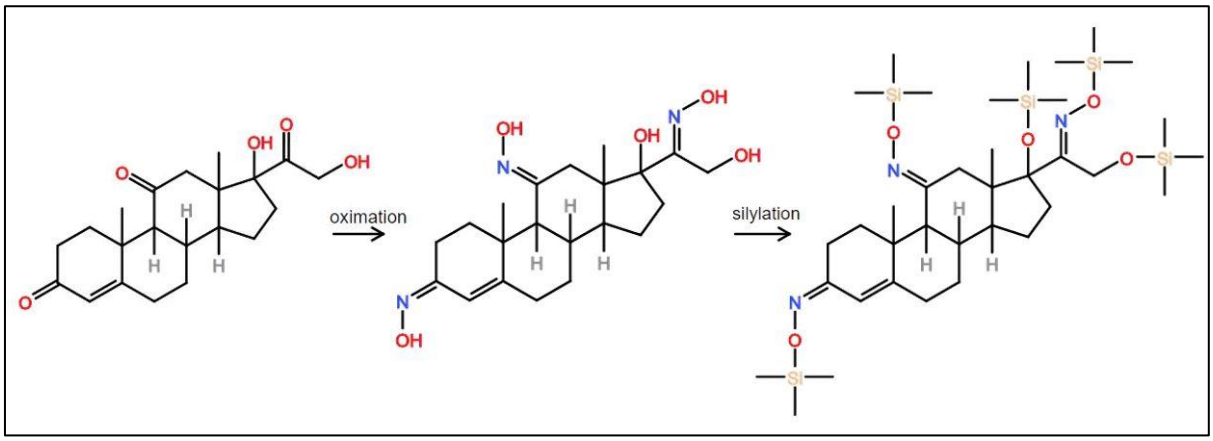

Figure 12: Changes of functional groups during derivatization of cortisone

3.6.3 Oximation

- evaporate solvent of sample with nitrogen at 40 °C till dry
- add 0.35 – 2.5 ml stock solution of 2.5 % hydroxylamine hydrochloride solution in pyridine (adapted to the amount of corticoids in the sample) to up to 35 mg of corticoids in the sample
- heat in the oven at 75 °C for 30 min

3.6.4 Silylation

For removing all hydroxyl side chains – also the ones added by oximation – silylation had to be performed after oximation. In the first attempts of sample preparation, silylation was conducted with the reagent BSTFA + TMCS, 99:1. Later, the reagent 1-(trimethylsilyl)imidazole was used since the first reagent was not able to silylate the structure of “hydroxyl groups on highly hindered steroids” according to Thermo Fisher Scientific Inc. (2012), protocol 2. When no oximation was performed prior to silylation, pyridine was additionally added in the same amount as silylation reagent.

3.6.4.1 Silylation with BSTFA + TMCS, 99:1

- (add 100 µl pyridine)
- add 100 µl BSTFA + TMCS, 99:1 to the sample
- heat in the oven at 60 °C for 30 min

3.6.4.2 Silylation with TMSI

- (add 100 µl pyridine)
- add 0.1 – 1.5 ml of TMSI to the sample
- keep in the oven at 100 °C for 2 h

3.6.5 Solid Phase Extraction

For separation of the samples from fat, the thought was to let the relatively high amount of fat through a column in which the relatively small amount of steroids would be retained and could be simply eluted later. Therefore, the use of a polar normal phase column was the type of choice. An apolar solvent was used as a first eluent to mix in the sample and for washing of the column. Methanol was chosen as a final eluent, since it is very polar and all of the glucocorticoids would be eluted with it from the column. First tests were performed with a self-made silica column, then commercially available columns were used. All of these columns were normal phase columns used for polar to moderately polar compounds (Sigma-Aldrich Co. LLC., 2017, phase-selection).

3.6.5.1 Self-prepared Silica Column

A column was built with a Pasteur pipette, glass wool and silica gel 60. 1 g of fat, spiked with 10 ppm of standard was dissolved in 10 ml of a mix of cyclohexane/ethyl acetate (1:1) and extracted through the column. 10 ml of the mobile phase were used for washing the column afterwards. It was expected that the polar corticoids would create a hydrogen bond with the free OH-groups of the surface of the silica gel and were retained, while the nonpolar fat would elute through the stationary phase. The corticoids were then eluted with 10 ml of the polar solvent methanol. The collected eluents were evaporated with a rotary evaporator for volume reduction.

3.6.5.2 Commercial LC-Si Silica Tube

Other experiments were performed with commercially available SPE tubes. Therefore, a solvent of cyclohexane/ethyl acetate in different concentrations was used to dissolve the sample of either fat containing standard corticoids or only standard without fat. Other than before, SUPELCO visiprep vacuum extraction manifold, 12 port model was used for accelerated extraction of the eluent through the column. SUPELCO Supelclean LC-Si SPE Tubes 3 ml, Bellefonte, PA, USA, were used as extraction columns.

The LC-Si silica tube has a stationary phase of silica, similar to the self-prepared column before, but was purchased ready for use. Cyclohexane/ethyl acetate was the solvent used to flush the tube first. An amount of methanol was taken to elute the steroids from the solid phase.

- use 10 ml of cyclohexane/ethyl acetate (4:1) to dissolve 1 g of fat sample/corticoid standards
- extract the sample with an LC-Si silica tube by using a vacuum box, then use 10 ml of solvent only for washing and finally extract with 10 ml of methanol as eluent
- collect the cyclohexane/ethyl acetate eluate in 1 ml-fractions and the methanol eluate in two fractions of ~ 5 ml each
- dry the fractions under a flow of nitrogen

3.6.5.3 Commercial DSC-NH_2 Tube

Cyclohexane was used as a first eluent and methanol was the eluent for the steroids.

- use 10 ml of cyclohexane to dissolve 1 g of spiked fat sample/corticoid standards or real animal fat sample
- extract the sample with a SUPELCO Discovery DSC-NH_2, 3 ml Tube, 500 mg, Bellefonte, PA, USA, by using a vacuum manifold, then use 10 ml of solvent only and finally extract with methanol as eluent
- collect the cyclohexane eluate in 1 ml fractions and the methanol eluate in two fractions (later only collect fractions 2 II – 10 II and methanol fractions)
- add 0.5 or 1 mg betulin as internal standard to each fraction

3.6.6 Acidic Transmethylation

After solid phase extraction, it can be necessary to do a transmethylation to exchange the hydroxy groups of the fatty acids with methyl groups (see Figure 13), in order to prevent strong bonding of the fatty acids with the polar groups of the column and to prevent it from taking damage. It was performed according to the following protocol described by Christie (2011). For the acidic transmethylation to work the absence of water before initiation of the reaction is important.

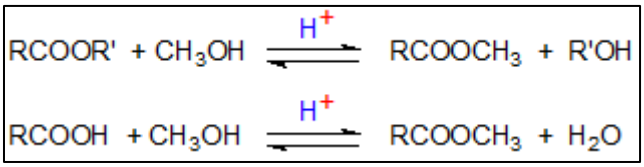

Figure 13: Reaction equation of an acidic transmethylation in general (obtained from Christie, 2011)

- evaporate hexane with nitrogen from the extract containing the corticoids
- weigh in approximately 30 mg of the sample containing fats
- add 3 ml toluene (no internal standard was added)
- add 3 ml methanol/HCl
- exchange air with nitrogen, cap tightly
- put in water bath at 70 °C for 1 h: transmethylation takes place; shake in-between
- let cool down slightly
- add 5 ml K_2CO_3 6 %
- centrifuge at 1100 rpm for 5 min
- prepare one spatula of sodium sulfate in new vial
- transfer upper organic phase into prepared vial, shake to remove water until solution is clear
- take upper phase, evaporate toluene, replace with hexane and inject in GC/MS

3.6.7 Basic Transmethylation

Base catalysed transmethylation – which is simpler than acidic transmethylation – was performed according to the protocol of Christie (2011). The reaction can be seen in the reaction equation of Figure 14.

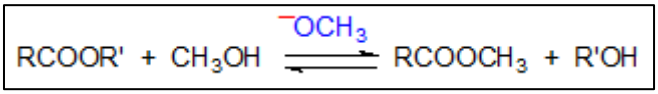

Figure 14: Reaction equation of a basic transmethylation in general (obtained from Christie, 2011)

- add 2 ml of C17 standard (1.02 mg/ml) in heptane
- add 2 ml of 2 M potassium hydroxide/methanol
- vortex 1 min
- centrifuge 2 min at 1100 rpm
- take organic phase and transfer to vial with Na_2SO_4
- fill clear solution into microvials and analyze with GC/MS

3.6.8 Preparation for GC/FID or GC/MS Analysis

The treated samples, either derivatized or not, were dissolved in approximately 1 ml of solvent and transferred into a microvial for chromatographic analysis. N-hexane or heptane were used as a solvent for the prepared samples, as they are commonly used due to their nonpolar and volatile characteristics.

3.7 Sample Analysis

3.7.1 Gas Chromatography/Mass Spectrometry

3.7.1.1 GC/MS Parameter Settings

Ionization was performed in the electron ionization mode at 70 eV. The program used for visualizing chromatograms on the computer was “Excalibur”. GC/MS of the first samples performed with an HP-5 column (length 30 m, inner diameter 0.25 mm, film thickness 0.25 µm) having 5 % phenyl 95 % methylpolysiloxane as a stationary phase. The column flow (carrier gas: helium) of the first column used was kept constant by electronic pressure control at 1.5 ml/min from the first sample until the first runs with splitless mode, then flow was set to 0.5 ml/min.

Sample injection of 1 µl (2 µl by autosampler) was done in split mode first (ratio 1:50 or 1:10) and later also in splitless mode (0.5 min). An autosampler was used for auto-injection, in case manual injection was not used or a big number of samples had to be analyzed.

The ion source temperature was set to 220 °C. The oven temperature program used in split and splitless mode for the first analyses was: initial temperature 100 °C, hold time 2 min, 30 °C/min to 270 °C, hold time 1 min, 3 °C/min up to 320 °C, held for 1 min. It was later optimized to a final setting of temperatures: initial temperature 100 °C, hold time 2 min, 30 °C/min to 270 °C, hold time 1 min, 3 °C/min up to 325 °C, held for 1 min.

The HP-5 column was later replaced by an SLB-5ms column (Sigma Aldrich) with the same dimensions and same type of stationary phase.

3.7.1.2 Preparation of GC/MS for Analysis

Automatic tuning prior to a set of analyses was performed. The method of choice was selected on the computer and sent to the machine for a warm-up, before the sample dissolved in solvent could be injected.

3.7.1.3 Cleaning of the GC/MS

Some parts of the GC/MS had to be cleaned – not regularly, but sometimes – particularly after suspiciously misshapen peaks in the chromatograms. Therefore, connector of ionization

chamber with column was disassembled and polished with a cotton bud and aluminum oxide to remove developed black spots. It was put into the ultrasonic bath in methanol first, later in chloroform. The oven at 200 °C dried the parts for an immediate use. Also, the septum of the injector and the injection liner were changed regularly.

3.7.2 Gas Chromatography/Flame Ionization Detection

The detector type on classical GC was a flame ionization detector. The program used for visualizing the chromatograms on the computer was “Chrom-Card”. A DB-5 test column from Restek, length 15 m, inner diameter 0.25 mm, was used. The carrier gas was H_2. Split was adjusted to 1:20 and carrier gas was kept at a constant pressure of 30 kPa. For applying splitless mode, a normal split method was selected but the left split valve was closed with a rubber gasket for approximately 10 seconds after injecting the sample. Method temperatures were set like GC/MS method temperatures: start temperature for the GC/FID was set to 100 °C; ramp 1: 20 °C/270 °C; ramp 2: 10 °C/320 °C.

3.7.3 General Injection Procedure for GC/FID or GC/MS

- wash syringe a few times with solvent, waste on paper towel
- wash syringe a few times with sample, waste
- load 0.8 µl of solvent, 0.2 µl air bubble and 1 µl of sample, pull back piston until another air bubble is visible
- when GC is ready for sample uptake, penetrate septum with syringe, wait 5 seconds, inject fast and smooth, wait 5 seconds and take out syringe
- wash syringe a few times with solvent

As solvent n-hexane, heptane or acetone were used.

3.7.4 Thin-Layer Chromatography

10x10 normal phase silica plates were prepared for the run by drying them in the incubator at 120 °C for about 30 min, then letting them cool down in the desiccator. This procedure was necessary for providing a water-free environment for the substances travelling on the plates. Per substance, a few drops of sample of interest were applied with a Pasteur pipette. When the solvent front was about 2 cm below the top, travelling time was over, the plates were taken away from the mobile phase and allowed to air dry. 10 % H_2SO_4/methanol was sprayed all over the plate and air dried again. Afterwards the plates were placed in the incubator for 10 min at 120 °C and could finally be evaluated. The retardation factor (R_F) was calculated by dividing the travelled distance of the product by the travelled distance of the solvent front measured from the initial spotting site (Equation 1).

$$R_F = \frac{d_P}{d_S}$$

Equation 1: Calculation of the retardation factor (R_F): travelled distance of the product (d_P) divided by the travelled distance of the solvent front (d_S)

3.7.5 Calculation for Calibration of Standards

After creating calibration functions of each standard, the parameters of the calibration equations could be used to calculate the concentration of corticoids found in a sample. This

was done based on the measured peak area of a substance (Az), the peak area of the internal standard betulin (Ab) and the amount of betulin added (Kb) (see Equation 2).

$$Kz = (Az/Ab - d)/k * Kb$$

Equation 2: Calculation of the amount of standard (Kz) using slope (k) and ordinate intercept (d) from the calibration functions

4 Results

4.1 Early Tests with GC/MS

4.1.1 Betulin Silylation

A first attempt of silylation was performed with betulin, to see if it works and if it can be evaluated in the chromatogram with the on-hand settings. For GC/MS analyses a HP-5 column was used for the first experiments. The molecular mass peak of 586 g/mol for silylated betulin was detected, showing successful silylation. Betulin was chosen as an internal standard. Best would have been to have deuterium labelled corticoids as IS, since isotope-labelled standards usually produce the most accurate results. However, they are very costly and their benefit is not significant on many cases. Androstanone was used in many papers as IS, but it was not commercially available as a high purity standard for the present study. Instead, betulin was already available in the lab, so it was used and it was effective. Also, it is not expected to be found in animal fat, which ensures it is detected only in the amount in which it was added. A bonus feature of the use of betulin is that its TMS derivative elutes after all the investigated corticoids and excellently distances itself from the other substances on the chromatogram (Dolan, 2012).

4.1.2 Standard Corticoid Silylation

The next experiment was silylation of the selected corticoids with the method described in 3.6.4.1 and analysis with GC/MS. Here it is noticeable that the corticoids did not dissolve but crystallize in hexane, when they were suspended for injection. Reasons for crystallizing in the solvent could be insolubility in hexane or incomplete silylation. Silylated betulin with two TMS groups and one carbonyl group has a similar structure like some of the corticoids and is soluble in hexane. Compared to that, the corticoids are expected to dissolve in hexane as well. Therefore, it is possible that silylation was not complete as there is a chance that underivatized corticoids crystallized after evaporation of the solvent and the crystals had no chance to crystallize completely.

The supernatant of all samples was taken anyway to analyze with GC/MS. After the supposed derivatives of cortisone and hydrocortisone did not show any relevant peaks, the maximum temperature of the method for the oven program was increased from 300 to 320 °C. Among the rest of the analyzed samples, only 21-hydroxyprogesterone derivative showed a peak, the program library confirmed deoxycorticosterone.

After the solvent of the vials with sample was evaporated and replaced with acetone, analysis showed some more peaks. Since some of the derivatized substances were identified in their underivatized state and others could not be detected at all, it was confirmed that silylation was unsuccessful.

4.1.3 Corticoid Acetylation

Acetylation seemed like an easier way than silylation to remove the hydroxyl groups, present on the glucocorticoids (except progesterone). It was used in a study by Aguilera *et al.* (1996) with urine samples, where the pure extract of the steroid testosterone was acetylated prior to GC/MS analysis. In the experiment, pyridine and acetic anhydride were added to the sample and left to incubate at room temperature. It was decided not to do this kind of derivatization

since it was considered too dangerous by the chemistry laboratory technician. Therefore, another technique with a less dangerous chemical had to be found.

4.1.4 Derivatization with Oximation

Rodríguez-Sánchez *et al.* (2010) describe an experiment with derivatization, including a proceeding oximation reaction. In their study, they did oximation with hydroxylamine hydrochloride and silylation with TMSI of low molecular weight carbohydrates. Due to the exhibition of hydroxyl and carbonyl groups as functional groups on both, carbohydrates and glucocorticoids, which they have in common, the described methods were given a try. Similarly, in the review of Ruiz-Matute *et al.* (2010), hydroxylamine hydrochloride for oximation of carbohydrates was mentioned as a general way of derivatization of carbonyl groups.

Therefore, derivatization was performed accordingly. First, the standard 21-hydroxyprogesterone was oximated. Silylation was performed with the silylation reagent BSTFA + 1 % TMCS instead of BSTFA + 10 % TMCS which was used in the mentioned study. Neither immediate analysis nor analysis after storing at 4 °C overnight according to the protocol gave any results. A possibility for an explanation of bad results could be that some parts of the GC have accumulated sample waste deposit, which could lead to false results and ugly chromatograms. Therefore, the connector between ionization chamber and column was dismounted and cleaned. The experiment was repeated with 21-hydroxyprogesterone in two preparations: one sample was oximated and silylated according to the previously used protocol, the other one was only silylated. Since the silylation seemed to be working, the oximation was not. This was a hint that the used hydroxylamine hydrochloride reagent was probably too old and a new reagent was bought and used for the next analyses.

All the corticoids available at that time (cortisone, hydrocortisone, corticosterone, Reichstein's substance S, 21-hydroxyprogesterone and 17α-hydroxyprogesterone) were oximated according to the previously used protocol but with a newly purchased hydroxylamine hydrochloride reagent. For the following silylations, 1-(trimethylsilyl)imidazole was chosen because it was used in some of the mentioned publications above. It was also successfully applied for steroid profiling by Casals *et al.* (2013), but in contrast to the present study they made use of methyloxime derivative formation prior to silylation. According to Thermo Fisher Scientific Inc. (2012), protocol 2 was followed "for the silylation of hydroxyl groups on highly hindered steroids".

For GC/MS analysis, the upper phase of readily prepared samples – where the corticoids were supposed to be – was taken to inject. Also, analysis of two of the standards was additionally done with the lower phase, to prove, that the sample of interest was actually in the organic phase. Smaller or bigger peaks with the correct mass fragments showed that both oximation as well as silylation were successful and the derivatives were present in the hexane phase. For the next measurement, all of the derivatized samples were analyzed as a mixed sample. The chromatogram of the scan mode showed three sharp and high peaks per substance and a number of small peaks hidden in noise, derived from the substances. One significant peak for each corticoid was among them, which could be identified according to the masses of the derivatized molecules.

It was noticeable that in these first analyses of successful oximation and silylation of the standard corticoids, the molecular ion of cortisone could not be detected in the mass spectrum. Since cortisone has two carbonyl and three hydroxyl groups, finding a derivatized

product where two oximations and five silylations were carried out was expected. Instead, the mass spectrum of the newly formed molecule suggested that only one oximation and further four silylations took place. Research showed that steroid treatment with methoxyamine hydrochloride by Casals *et al.* (2014) “derivatizes all ketone functions except for those in position C11”. Looking at the position numbers of the C-atoms in a steroid molecule proves, that one of the ketones in cortisone is on position 11. This strongly suggests, that oximation with hydroxylamine hydrochloride cannot react with the carbonyl group on C-position 11 to form an oxime either.

Spiltless injection was done to transfer the entire sample into the column and find even low amounts of sample. At the same time, the column flow rate was decreased from 1.5 to 0.5 ml/min. It was found that single components eluted in multiple peaks due to the formation of chiral centers on course of derivatization. Therefore, each standard component had to be separately injected for identification. New oximation and silylation of each corticoid were performed according to previous procedures. 100 µg of each standard was prepared and analyzed with GC/MS. Every sample had 3 peaks, except corticosterone had 5 peaks. For an overview of the obtained peaks of each standard, Table 1 shows the corticoids with corresponding peak masses.

Table 1: GC/MS of 100 µg of oximated (HAH) and silylated (TMSI) corticoids: retention time of each peak and corresponding main masses

Standard corticoid	Retention times (min)	Masses (m/z)
Cortisone	16.09	411.36, 501.43, 590.46
	16.24	499.41, 589.46, 678.49
	16.83	499.41, 589.46, 678.49
Hydrocortisone	16.55	575.48, 664.52
	16.69	663.52, 752.55
	16.88	663.52, 752.55
Corticosterone	15.86	485.61, 574.53, 576.51
	16.23	432.40, 575.52, 664.56
	16.31	432.40, 664.57
	16.46	664.54
	16.57	575.57, 664.54
Reichstein's substance S	14.71	397.35, 487.45, 578.55
	15.06	521.45, 575.50, 664.54
	15.52	485.44, 575.51, 664.57
21-Hydroxyprogesterone	14.97	473.46, 576.55
	15.19	473.44, 576.51, 578.51
	15.38	473.43, 561.46, 576.52
17α-Hydroxyprogesterone	13.30	399.41, 488.48
	13.65	433.41, 487.47, 576.53
	14.01	433.41, 487.47, 576.53

Concerning progesterone, 10 µg of it were oximated and silylated according to the previous protocols and analyzed with GC/MS. The chromatogram showed no distinct peaks for progesterone only. Therefore, 100 µg of underivatized progesterone were analyzed in splitless mode to find a high peak that could clearly be recognized as progesterone.

4.1.5 TLC with Derivatized Standards

This TLC was done to see, if the derivatized standards are travelling the same distance as the underivatized ones and to visualize reaction solutions. The plot in

Figure 15 shows that derivatized samples travel close with the solvent front. Also, the standards are tightly packed with one of the chemicals at the top of the solvent. The upper

circle of the second lane originates from the oximation solution. Since these standards had already been prepared for GC/MS injection, the volatile pyridine was already evaporated at this step. So, it is assumable that the upper circle marks hydroxylamine hydrochloride (HAH). The other white circular area, a bit retarded on the same lane, is from the silylation reagent. In the third lane, a smaller spot from the diluted standards is visible but corresponding derivatization reagents are not. In the fourth lane, HAH is a big, widely stretched spot on the solvent front. Also, pyridine is observable as an egg shaped white circular area in the lower third part of the lane. TMSI in the last lane dissolved the silica gel after applying. The mobile part of it is barely distinguishable from the background but it is possible to see a big white area that travelled a bit further than to the middle part of the lane.

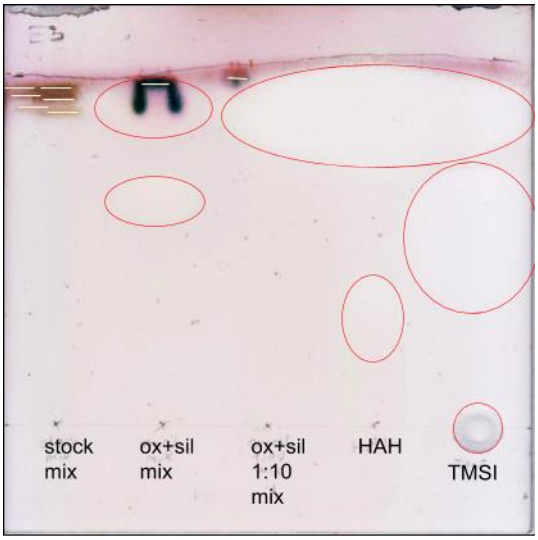

Figure 15: Solvent: chloroform:methanol:water 70:30:1; travel time: 30 min; from left to right: stock mix: mixture of underivatized standards 1-6; ox+sil mix: mixture of oximated and silylated standards 1-6; ox+sil 1:10 mix: one tenth of the amount of the previous lane, same substances; HAH: pure oximation reagent 2.5 % hydroxylamine hydrochloride in pyridine; TMSI: pure silylation reagent 1-(trimethylsilyl)imidazole;

4.1.6 Isomer Formation of Derivatives

It was always observed that oximated and silylated standards created up to five peaks per standard substance (see Table 1 above). Makin *et al.* (1995) summarized the problem: “A disadvantage in the use of oxime derivatives is the formation of isomeric *syn* and *anti* isomers which are generally separated by gas chromatography and therefore increase the apparent complexity of mixture analysis.” Also, Horning *et al.* (1967) who worked with methoxime derivatization already stated the isomer yield of derivatization of some kinds of steroids such as cortisone, hydrocortisone and progesterone, which requires consideration when employed. This means that oximation increases the complexity of the analysis but it is still possible to assign the different peaks according to their retention times of their originating substance.

4.1.7 Corticoid Methoxylation

Derivatization of steroids – in particular androstenone – was also done by Edelhäuser (1989) where a solution of 0.5 % of the reagent O-methylhydroxylamine hydrochloride (methoxyamine hydrochloride) in pyridine was used. This was a potential protocol that could

be applied on glucocorticoids which feature two to three carbonyl groups. The sum of the added side chains would increase the molecular weight of each molecule with 30 g/mol per carbonyl group only. Additionally, 72 g/mol for each hydroxyl group would be added by silylation. Compared to the oximation derivatization with subsequent silylation, which adds 15 g/mol to each carbonyl and another 72 g/mol to each new or persisting hydroxyl group, the methoxylation is the “lighter” choice. Also, Makin *et al.* (1995) state that preparation of methyl oxime and TMS ether derivatives is the most widely used method for the preparation of steroids to be analyzed by GC/MS. This is a fact even though the formation of isomers increases the complexity of steroid mixture analysis.

Nevertheless, it was decided to proceed with using the reaction to TMS oximes for all following experiments, since it showed success.

4.2 Runs with GC/FID

A similar column to the HP-5 column used for GC/MS was tried in the gas chromatograph to see, if there was a difference in molecular separation. This was an attempt to group the isomers together to make one clear, single peak. The column was a DB-5 (5 %-Phenyl)-methylpolysiloxane test column which is non-polar, has low bleed and offers a high temperature limit (Agilent Technologies, 2017). Approximately the same temperature settings were chosen on the GC/FID as they were used with the GC/MS. Underivatized and silylated samples were measured with the GC/FID to find out if keto groups were actually a problem when the molecules were separated in the column. Also, the aim was to possibly receive just one single peak per substance, rather than a few peaks. It appears that the two keto groups of underivatized progesterone do not cause any problems in GC/FID analysis and the sample even created one peak only. Molecules with two keto groups and one silylated hydroxyl group, such as 21-hydroxyprogesterone and 17α-hydroxyprogesterone, turn out to create a high peak surrounded by a number of small ones that are about one tenth of the peak area of the main peak. The gas chromatograph experiments show that it was still necessary to oximate prior to silylation, in order to obtain a few peaks per standard only.

4.3 Advanced experiments with GC/MS

The column in the GC/MS was changed to a SLB-5ms capillary non-polar column, since this was an unused one compared to the one used for GC/MS before. For the temperature program, the settings were the same as before but the temperature for ramp 2 was decreased from 320 °C to 316 °C in split mode, to decrease analysis time down to 24 min. All of the standard corticoids were analyzed in full scan split mode, either silylated only or both oximatied and silylated to see the difference between those two treatments and to be able to find out when which peaks appear. The silylated corticoids showed one main peak each and too many small peaks to evaluate their origin.

The question was whether this could be caused by standard impurities. As purities ranged from ≥ 92 up to ≥ 99 %, and a 99 % pure standard gave twice as many peaks as a 92 % pure one, this was probably not the reason. However, oximated and silylated samples created only either 2 or 3 peaks with the new setup. This finding was more important than the results of only silylated samples.

Since not all corticoid derivative isomers could be separated fully by chromatographic means, it was decided to measure the components in SIM mode, selecting the most appropriate masses for each substance. In Table 2, the peak retention times with their

corresponding masses are listed. The masses with the strongest signals were recorded for each of the highest peaks. Each mass distinct for a substance within a time range of peaks of more than one substance is highlighted in bold. Depending on peak times, specific masses for scanning of each corticoid were only set to a certain time frame, in order to maximize scanning resolution. Starting with 6.00 min of analysis time and onwards, full scan was recorded. From 16.00 min, a SIM at 344 and 567 m/z was additionally enabled. Then, 473 and 664 m/z were measured from 17.20 min and lastly 432, 678 and 753 m/z were measured from 18.70 min. This was the foundation for detection of a standard in selected-ion monitoring mode.

Table 2: Glucocorticoids both oximated and silylated with corresponding peak retention times and masses; masses in bold are selected for measurement of the respective substances in SIM mode

Standards	Purity	Supposed masses (m/z)			Peak time (min)	Masses (m/z)
		underivatized	silylated	ox+sil	oximated+silylated	
Cortisone	≥98 %	360.45	504	765	19.75	
					20.37	**678**, 589, 499
Hydrocortisone	≥98 % (HPLC)	362.46	578	752	20.07	
					30.34	**753**, 663, 419
Corticosterone	≥92 %	346.47	562	664	19.72	664, 575, **432**
					19.99	
Reichstein's substance S	≥98 %	346.47	402	664	18.35	
					18.82	**664**, 575, 485
21-Hydroxyprogesterone	≥97 % (HPLC)	330.461	402	576	18.32	
					18.51	576, **473**, 344
					18.78	
17α-Hydroxyprogesterone	≥95 %	330.46	402	576	16.77	
					17.15	**576**, 487, 433
Progesterone	≥99 %	314.47	314	488	16.74	
					16.87	488, 399, **344**

For the following GC/MS analyses, the derivatized standards were mixed and it was evaluated if the standards with their two to three peaks could be distinguished from each other. The selected masses for the SIM mode had to be revised to separate the peaks of the different substances from each other even more. Also, the change of degrees of Celsius per minute was decreased and therefore the elution time was increasing again. Finally, the chromatogram indicated suitable parameters for a successful separation of the corticoids (Figure 16). The final temperature settings decided on were initial temperature of 100 °C with a hold time of 2 min, then 30 °C/min until 270 °C with a hold time of 1 min and final rate of 3 °C/min until 325 °C and a hold time of 1 min and are summarized in Table 3.

Table 3: Final oven temperature program of GC/MS

	Rate (°C/min)	Temperature (°C)	Hold time (min)
Initial		100	2.00
Ramp 1	30	270	1.00
Ramp 2	3	325	1.00

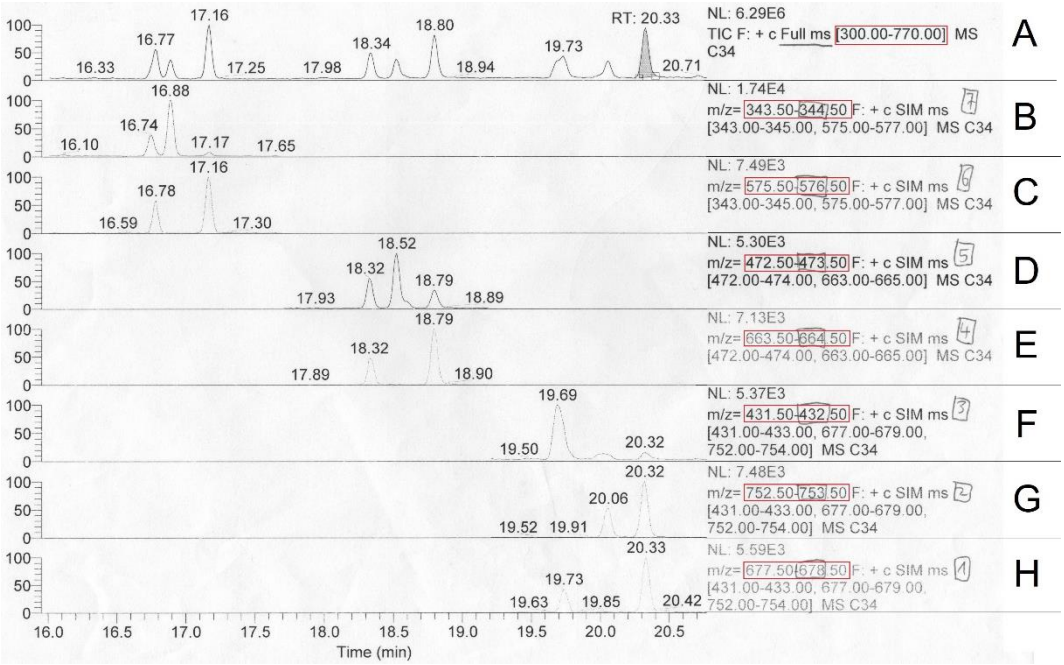

Figure 16: Chromatogram of a mixed sample with all corticoids of interest; for each time range the selective SIM mass of a substance is framed; lane A: full scan from 300–770 m/z, lane B: SIM mode at 344 m/z of progesterone, lane C: SIM mode at 576 m/z of 17α-hydroxyprogesterone, lane D: SIM mode at 473 m/z of 21-hydroxyprogesterone, lane E: SIM mode at 664 m/z of Reichstein's substance S, lane F: SIM mode at 432 m/z of corticosterone, lane G: SIM mode at 753 m/z of hydrocortisone, lane H: SIM mode at 678 m/z of cortisone

4.3.1 TLC for Solvent Evaluation

In order to determine whether the standard substances would move through silica when they were extracted from fat using a solid phase column, a preliminary experiment was performed. Another goal was to find a solvent which transports lipids fast and retains the corticoids on the stationary phase. This is desired in order to be able to use small columns instead of a system where lipids would be retained in a big column and corticoids eluted first. Based on the suggested TLC systems by Bhawani *et al.* (2010), some mobile phases were chosen to be combined with the silica gel as stationary phase.

4.3.1.1 Diethyl Ether:Hexane 1:1 and Acetic Acid 1 %

The first plate (Figure 17) with solvent diethyl ether:hexane 1:1 and acetic acid 1 % (similar to Vingler *et al.*, 1999) where standards 1–6 were applied show no movement of any of the substances and they appear circular.

Figure 17: Diethyl ether:hexane 1:1, acetic acid 1 %; travel time: 20 minutes; from left to right: 1: cortisone, 2: hydrocortisone, 3: corticosterone, 4: Reichstein's substance S, 5: 21-hydroxyprogesterone, 6: 17α-hydroxyprogesterone

4.3.1.2 Chloroform:Methanol:Water 65:35:4

When the standards were travelling in chloroform:methanol:water 65:35:4 (Figure 18) which was similar to the mobile phase used by Fenske and Schönheiter (1991), a movement of all standards – with small variations in travel distance – could be obtained. Each of the applied corticoid standards was concentrated in one line due to travelling.

Figure 18: Chloroform:methanol:water 65:35:4, travel time: 22 min; from left to right: 1: cortisone, R_F = 0.94; 2: hydrocortisone, R_F = 0.90; 3: corticosterone, R_F = 0.94; 4: Reichstein's substance S, R_F = 0.96; 5: 21-hydroxyprogesterone, R_F = 0.96; 6: 17α-hydroxyprogesterone, R_F = 0.98

4.3.1.3 Chloroform:Methanol:Water 70:30:1

Since the corticoids were travelling well in the previously used solvent, it was tested whether they would also travel similarly in a slightly changed concentration of mobile phase (Figure 19). Two types of fat, phospholipids present in egg lecithin "E 80", and triglycerides present in rapeseed oil "Rapso", were chosen to be compared with some of the standards in the same run. Applied were hydrocortisone, corticosterone and 21-hydroxyprogesterone which

were slowest, medium and fastest in the previous run respectively, compared to the rest of the standards. Hydrocortisone with its two carbonyl and three hydroxyl groups is more polar, compared to corticosterone with four polar groups and 21-hydroxyprogesterone with three polar groups. The three corticoids travelled again in the same manner as in the previous TLC. In similar solvent systems (containing silica and hexane) phospholipids stay at the origin and travel only, if methanol as polar solvent is also present (Christie, 2017). Therefore, spots 1 and 2 of lane “LEC” are assumed to be phospholipids, spot 3 represents other lipids. One small drop of rapeseed oil shows highly overloaded triglyceride, which travelled to the solvent front.

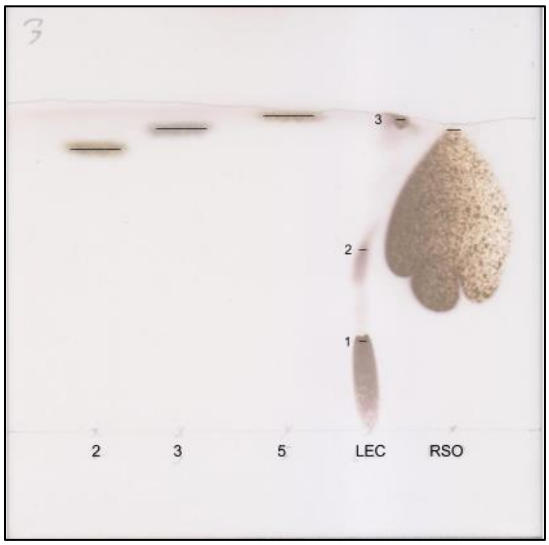

Figure 19: Chloroform:Methanol:Water 70:30:1; 2: hydrocortisone, 3: corticosterone, 5: 21-hydroxyprogesterone, LEC (1,2,3): egg lecithin, RSO: rapeseed oil

4.3.1.4 Cyclohexane/Ethyl Acetate 1:1

Pork fat (triglycerides) was spiked with about the same amount of hydrocortisone and progesterone and it was applied along with fat, hydrocortisone and progesterone on another silica plate. The TLC (Figure 20) was developed in cyclohexane/ethyl acetate (1:1), as it was used by Edelhäuser (1989) for the gel chromatographic cleanup of animal fat.

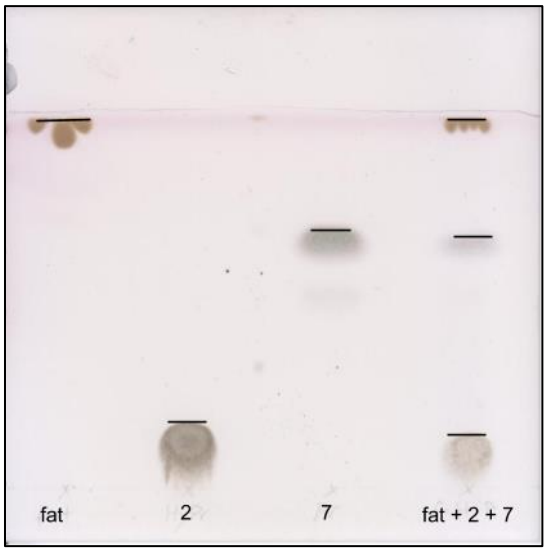

Figure 20: Cyclohexane/ethyl acetate (1:1); travel time: 30 min; from left to right: fat: pure pork fat, 2: hydrocortisone, underivatized; 7: progesterone, underivatized; fat+2+7: mixture of pork fat, hydrocortisone and progesterone

The TLC experiment shows that the triglycerides of fat travel faster than the least polar substance of all standards but the standards follow quite closely. It would be better if progesterone stayed further behind like hydrocortisone does, which travelled at around ⅕ the speed of fat. This shows that there should be some changes in solvent composition which causes the standards to be retained by the stationary phase a little longer.

4.3.1.5 Cyclohexane/Ethyl Acetate 4:1

Another TLC (Figure 21) with a more apolar solvent shows a more desirable result in terms of standard movement. The new setup with the changed composition of the solvent shows that the two standards are retained much stronger, as hydrocortisone does not move at all and progesterone moves 50 % less in distance than in the previous TLC. This is what was aimed at. The fatty acid standard C17 is not visible in the plot, which means it was probably applied with too little concentration.

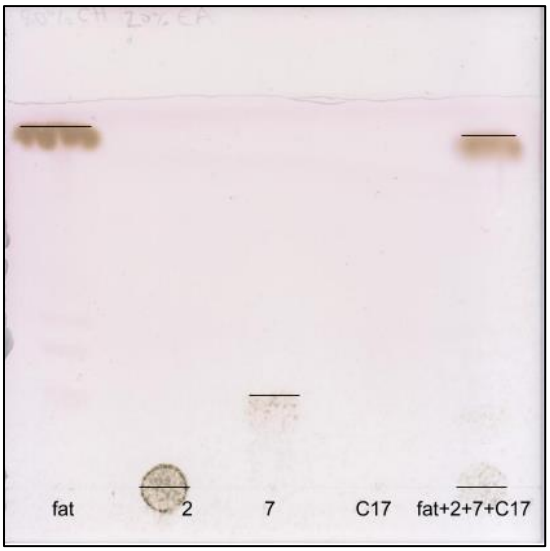

Figure 21: Cyclohexane/ethyl acetate (4:1); travel time: 31 min; from left to right: fat: pure pork fat, 2: hydrocortisone, underivatized; 7: progesterone, underivatized; C17: fatty acid standard; fat+2+7+C17: mixture of pork fat and the 3 mentioned standards

4.3.2 SPE of Spiked Fat

4.3.2.1 General Assessment

Since 1 mg of fat sample was dissolved in 10 ml of solvent which was applied on the SPE tube and another 10 ml followed to wash the tube, those two extraction cycles with apolar solvent were named I and II. The single fractions of 1 ml eluate collected of each cycle were named 1–10 in order of elution.

Of all the analyzed SPE fractions each obtained peak size of a standard was set in relation to the peak size of betulin. According to that, the relative amount of a standard was calculated by setting each relative peak area in relation to the respective sum (100 %).

4.3.2.2 Cyclohexane/Ethyl Acetate 1:1

For the first tests of preparing a spiked fat sample, known amounts of homogenous pork fat and standard corticoids were mixed. Extraction was based on a method performed by Edelhäuser (1989) who extracted androstenone from boar fat. Therefore, a silica gel column was built. The spiked fat sample was extracted by the column with cyclohexane/ethyl acetate (1:1) and methanol. Fat was expected to be eluted with the first eluent, but there was also some fat in the methanol eluate. The supposed corticoids were oximated with HAH and silylated with TMSI according to previously used protocols. Also, an acidic transmethylation was performed. GC/MS analysis showed that progesterone eluted with the cyclohexane/ethyl acetate mobile phase and not with methanol. There was a chance that water was present in the extract and then silylation would not have worked. This result suggested that the proportion of the apolar to the polar solvent (cyclohexane:ethyl acetate) could be increased.

Another spiked fat sample was extracted with the same eluents. 1 ml fractions were collected in separate vials and either the whole sample or if they contained fat, one drop as an aliquot of the fat was taken to perform basic transmethylation, which is less sensitive to present water. The GC/MS results showed that fatty acids elute within the first 10 minutes and do not

interfere with the peaks of the standards. Other than that, the standards could not be recognized in the chromatogram of any of the extracted fractions.

4.3.2.3 Cyclohexane/Ethyl Acetate 4:1 with LC-Si Tube

As the TLC with cyclohexane/ethyl acetate (4:1) (Figure 21) above showed a better separation of the standard corticoids from fat it was also used for the next vacuum manifold SPE with a commercial LC-Si tube.

An SPE was done with another spiked fat sample with standard corticoids. A majority of apolar lipids visibly eluted already in quite equal amounts in the here not analyzed vials with 1 ml collected eluates of the first 10 ml of extraction with the apolar eluent (1 I–10 I, one milliliter each) as well as in the first milliliter of washing with the same eluent (1 II). These lipids are assumed to be triglycerides which are apolar due to the three hydrophobic fatty acid chains they contain. The rest of the fractions from the washing cycle (2 II–10 II) did not really contain any visible fat which were therefore used for GC/MS analysis without further treatment. Only the first methanol fraction showed a little bit of fat, too. Methanol fractions were analyzed in any case, since it is the solvent that is supposed to release the corticoids from the column.

Cyclohexane/ethyl acetate fractions starting from the second milliliter fraction of washing cycle with apolar eluent (2 II) up to the methanol fractions were analyzed to find out how much of added progesterone could be found with GC/MS analysis. Actually, 82 % of the progesterone occurring in the investigated fractions was found in the early washing fractions (2 II–4 II), only a little bit more than 1 % could be found in the methanol fractions (Figure 22). This means that progesterone elutes in the washing cycle with apolar solvent already which is earlier than desired.

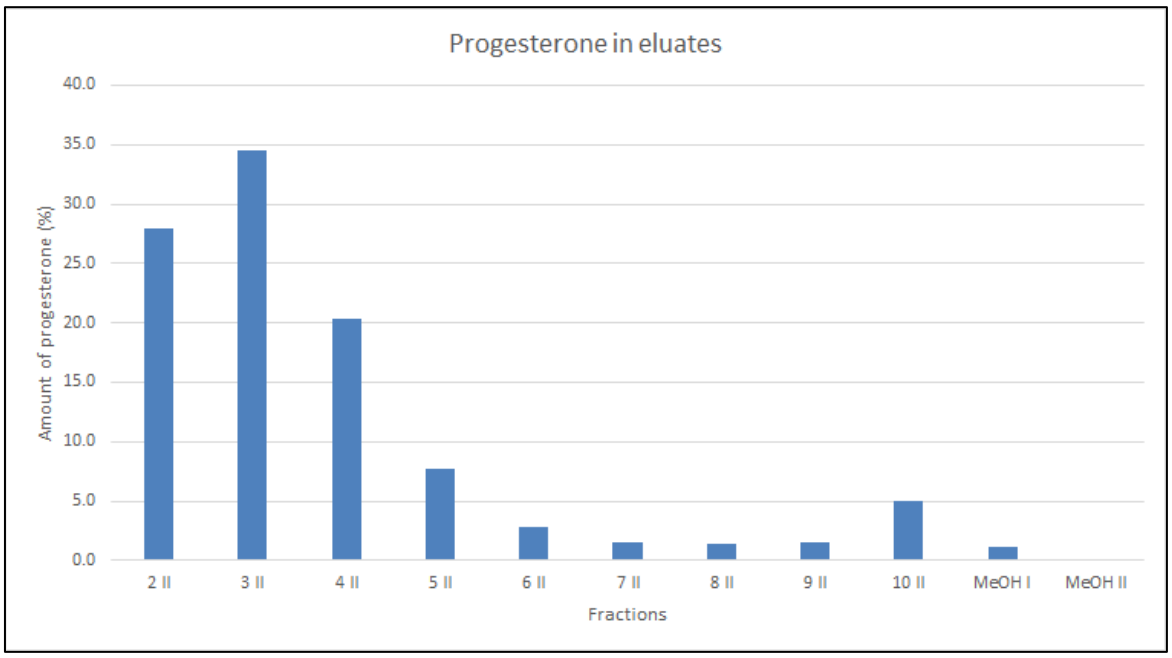

Figure 22: Eluents: cyclohexane/ethyl acetate (4:1) and methanol; Fractions 2 II to 10 II of washing solvent cyclohexane/ethyl acetate and the two methanol fractions MeOH I and MeOH II with their corresponding amounts of containing progesterone, related to the occurrence of progesterone in the investigated fractions

Also, the distribution of occurring lipids in fractions 2 II to 10 II and the methanol fractions of the same solvent system as used above were investigated and are visualized in Figure 23.

This result is based on the peaks arising before the standard peaks in the chromatogram of each fraction. It can be seen that about 50 % of the lipids are eluted with the first 5 ml of methanol (MeOH I), the rest is distributed quite equally in the other fractions.

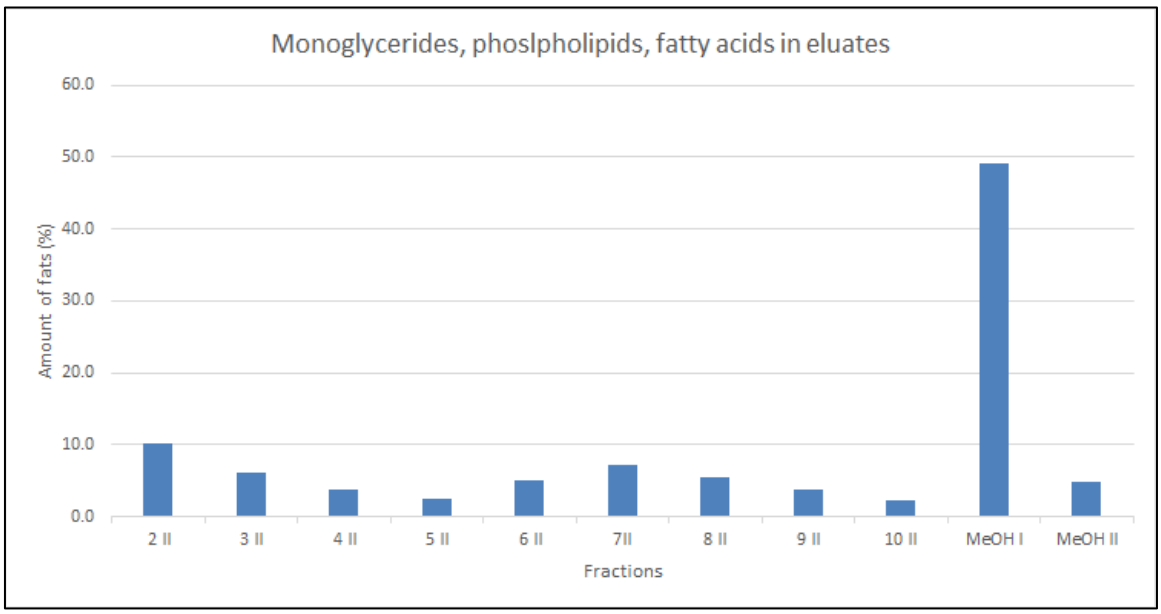

Figure 23: Eluents: cyclohexane/ethyl acetate (4:1) and methanol; fractions 2 II to 10 II of washing cycle with solvent cyclohexane/ethyl acetate and fractions of methanol (MeOH I, MeOH II) with their corresponding amounts of lipids, related to the occurrence of the fats in the investigated fractions

Since most of the triglycerides eluted already earlier in the first extraction cycle, the plotted amounts of fats in Figure 23 include other fatty acids containing lipids and only little amounts of triglycerides, if any. Other rather apolar lipids shown here are monoglycerides and diglycerides, eluted in the fractions 2 II–10 II of the washing cycle. These 50 % of another lipid class is very certain to be phospholipids, eluted with methanol. This can be stated, since the phospholipids from egg lecithin applied on the TLC plate in Figure 19 showed to move only, because methanol was added to the mobile phase.

The majority of progesterone could already be found in fractions 2 II–10 II. Analyzing only these fractions meant to exclude the earlier eluting fractions 1 I to 10 I and 1 II that contained 90 % of the weight of the prepared fat and avoiding the step of transmethylation.

As a verification that no significant amount of standards was disregarded in the first eluates of SPE, one run of extraction with a spiked fat sample was performed where the first fractions of cyclohexane were extracted a second time in a new run of SPE with a new tube. The results showed that it is legitimate to exclude the first fractions containing most of the fat, since they contain only a fractional amount of the corticoids compared to the other fractions.

4.3.2.4 Cyclohexane and DSC-NH_2 Tube

It was decided to use another type of polar column that possibly retains the corticoids better than the previously used column. Also, the solvent was changed to pure cyclohexane to have a very apolar environment for eluting the fat more easily.

A spiked fat sample with progesterone was extracted with SPE (Figure 24). With these parameters, almost all of the progesterone found by GC/MS eluted in the first methanol fraction (first 5 ml). This result shows the desired release of the corticoid with the polar solvent after washing of the column.

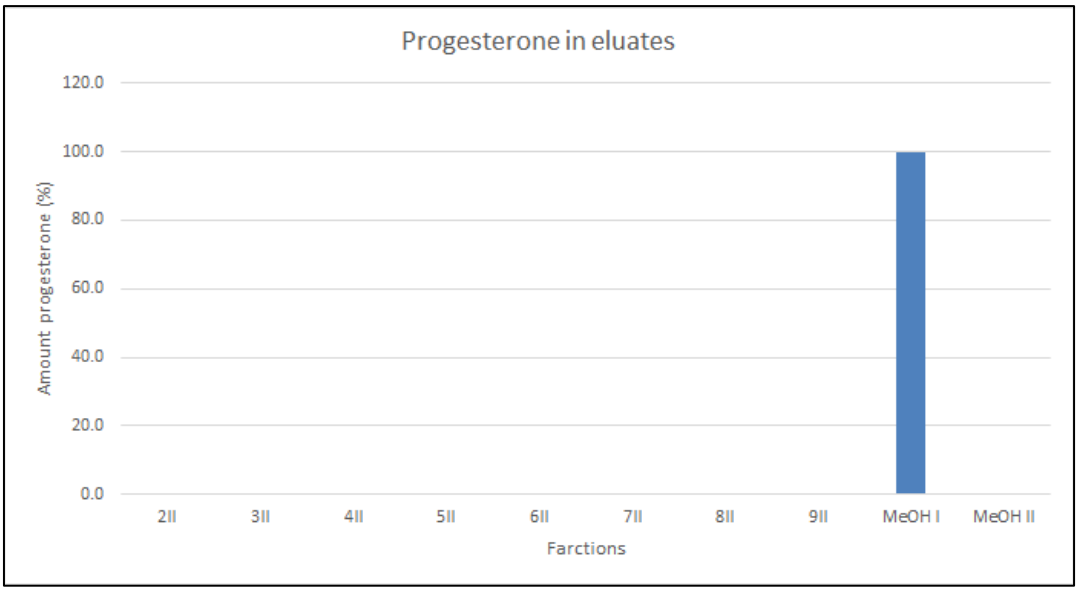

Figure 24: Eluents: cyclohexane and methanol; most of progesterone eluted with methanol, almost nothing (0.2 %) in the earlier fractions

An experiment with a sample of 10 mg of each corticoid in 1 g of fat extracted with SPE using cyclohexane showed that progesterone was the only standard present not only in the methanol fractions but also in the cyclohexane washing eluate. The relative amount of progesterone found in the analyzed fractions is 50 % only for the first methanol fraction (Figure 25). The rest of progesterone is found in slightly decreasing amounts from about 8 % in the first analyzed washing fraction 2 II to 2.5 % in the last washing fraction (9 II). Peaks of the rest of the standard corticoids are only found in the last methanol fraction, where also progesterone could still be found.

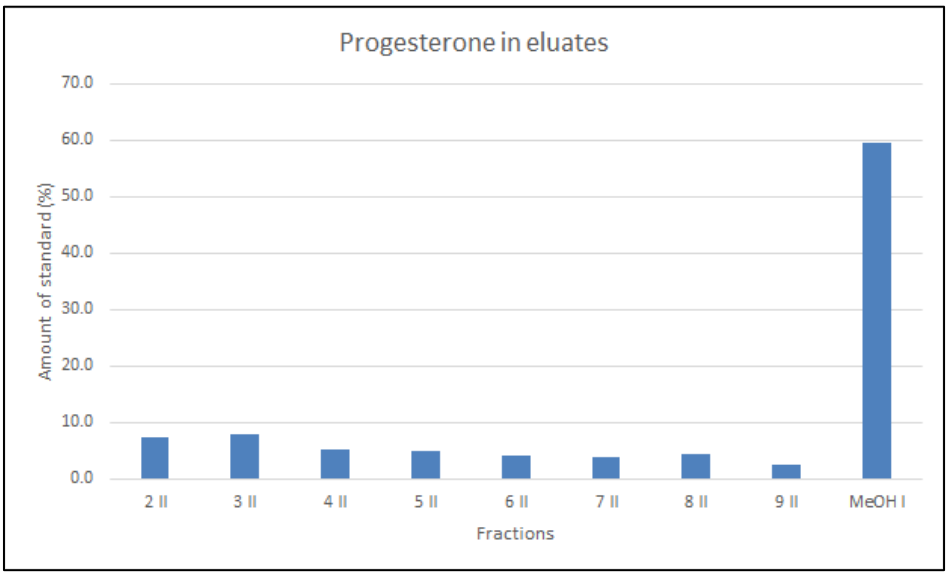

Figure 25: Eluents: cyclohexane and methanol; the relative amount of the standard progesterone in cyclohexane washing fractions 2 II–9 II and methanol fractions I and II

It is worth mentioning that there is a possibility of underestimation of the measured corticoids in the derivatization step. This is plausible, if the corticoid derivatives reach saturation earlier than the derivative of the internal standard betulin, such as Haugen *et al.* (2011) emphasize in the same manner but with androstanone as internal standard for measurements of androstenone. Therefore, longer derivatization time could potentially lead to an increase of the peak of internal standard, whereas the peak of the corticoids would stay the same.

4.4 Method Evaluation

4.4.1 Betulin Evaluation in Fat

Fat was spiked with 10 mg of each corticoid and 10 mg of betulin prior to SPE to see how betulin behaves as analyte in fat extraction. After extraction, 5α-cholestan-3-one was added as a comparing substance prior to oximation and silylation. The new internal standard cholestanone elutes earlier than the corticoids.

Looking at the diagram in Figure 26 of progesterone elution, it is noticeable that progesterone elutes with about 50 % at the beginning of the washing fractions and decreasing amounts elute until the end of the washing cycle. This is unlike the earlier experiments with progesterone in the same circumstances, where most of the identified progesterone eluted in the methanol fractions.

80 % of the other corticoids (including some of the progesterone) elutes in the first, the rest in the second methanol fraction (not shown in diagram).

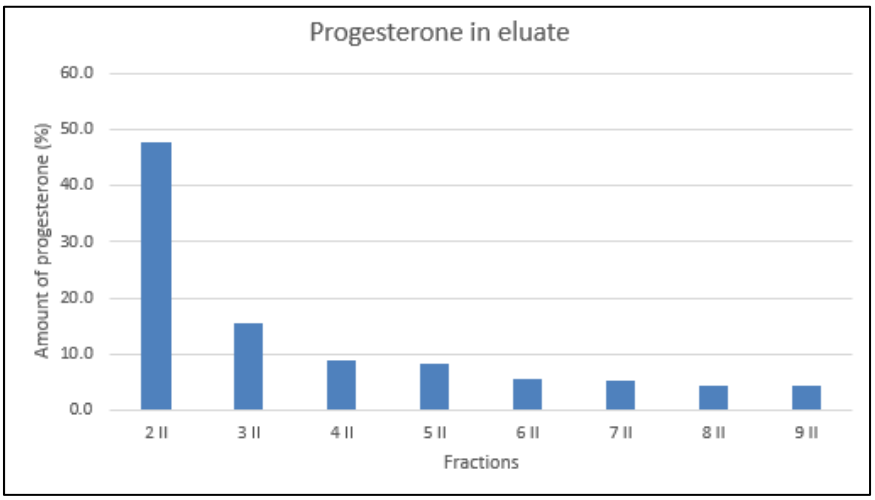

Figure 26: Eluents: cyclohexane and methanol; the relative amount of the standard progesterone in cyclohexane washing fractions 2 II–9 II

Figure 27 shows a very variable elution pattern of betulin. 1.5 % to 12.5 % of it can be detected from the first evaluated fraction (2 II) to the last washing fraction (9 II). Two thirds of betulin eluted with methanol at the end. Since only those fractions have been analyzed – as it was handled in earlier analyses – it is unknown whether betulin eluted in earlier fractions already and is therefore not visible anymore or not.

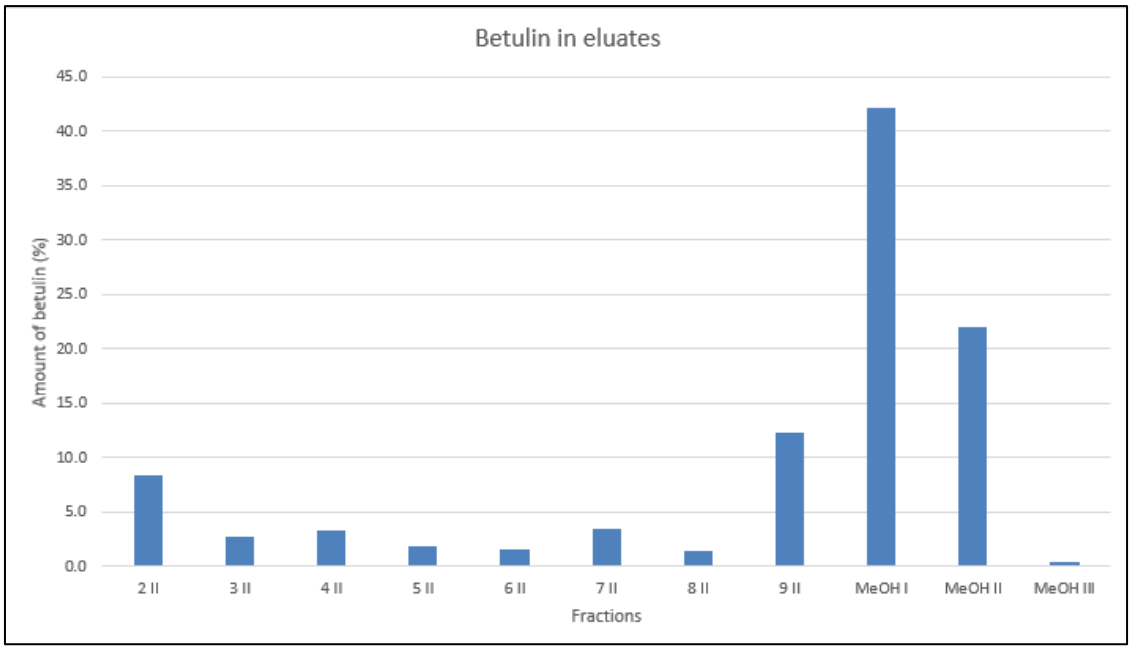

Figure 27: Eluents: cyclohexane and methanol; the relative amount of betulin in cyclohexane washing fractions 2 II–9 II and methanol

The unexpected behavior of progesterone and betulin, as well as the high amount of used standards indicate that the SPE column was most probably overloaded. This means, that free binding sites were occupied and even attracted molecules could not be retained anymore causing early elution of polar molecules in an unsteady pattern.

4.4.2 Standard Corticoid Calibration

In order to relate the amounts of standard to their peak area in the chromatograms, a calibration has to be done. It was decided to do a calibration without a fat matrix, since it is the common approach and an easier way with less treatment steps involved.

Therefore, amounts of 0.5 mg to 0.0005 mg of each corticoid standard were prepared, derivatized and analyzed with GC/MS. This was done to obtain the recovery rate of the concentrations found in the standard addition experiment (see below). The peak areas of each standard at the given amounts were recorded (Table 4). Then the relations of the amounts of standards to the amount of added betulin were calculated as shown in Table 5. Also, the relations of the peak areas of the standards to the peak area of betulin were assessed and can be seen in Table 6. Based on the standard amount ratio and the area ratio, calibration functions of each standard were created (Figure 28 – Figure 34) and the calibration equations used for further standard addition calculations.

Table 4: Peak areas of corticoid standards (Az) at certain amounts (Kz) for calibration[1]

1,2,4,6,7 (µg/ml)	500	100	10	5	1	0.5	Kz
3,5 (µg/ml)	416.6	83.3	8.33	4.2	0.833	0.42	
BETULIN	3280559	3135069	4484838	3918821	5218341	4844482	Ab
Progesterone (7)	5585136	947857	84503	25241	5762	2448	Az
17α-Hydroxyprogesterone (6)	3759896	681793	68820	22770	5962	3658	
21-Hydroxyprogesterone (5)	1609329	254156	23170	7338	1058	1257	
Reichstein's substance S (4)	3729635	628247	57863	18276	4598	1833	
Corticosterone (3)	1414814	201425	16640	5996	2065	578	
Hydrocortisone (2)	3905141	600286	53093	16319	3952	1623	
Cortisone (1)	2208990	374331	37270	11326	2954	1242	

Table 5: Relations of amounts of corticoid standards (Kz) to amount of betulin (Kb, 0.5 mg)[1]

1,2,4,6,7 (µg/ml)	500	100	10	5	1	0.5	Kz
3,5 (µg/ml)	416.6	83.3	8.33	4.2	0.833	0.42	
Progesterone (7)	1.000	0.2000	0.02000	0.01000	0.00200	0.00100	Kz/Kb
17α-Hydroxyprogesterone (6)	1.000	0.2000	0.02000	0.01000	0.00200	0.00100	
21-Hydroxyprogesterone (5)	0.833	0.1666	0.01666	0.00840	0.00167	0.00084	
Reichstein's substance S (4)	1.000	0.2000	0.02000	0.01000	0.00200	0.00100	
Corticosterone (3)	0.833	0.1666	0.01666	0.00840	0.00167	0.00084	
Hydrocortisone (2)	1.000	0.2000	0.02000	0.01000	0.00200	0.00100	
Cortisone (1)	1.000	0.2000	0.02000	0.01000	0.00200	0.00100	

Table 6: Relations of peak areas of corticoid standards (Az) to peak area of betulin (Ab) (see Table 4)[1]

1,2,4,6,7 (µg/ml)	500	100	10	5	1	0.5	Kz
3,5 (µg/ml)	416.6	83.3	8.33	4.2	0.833	0.42	
Progesterone (7)	1.702	0.302	0.019	0.0064	0.0011	0.0005	Az/Ab
17α-Hydroxyprogesterone (6)	1.146	0.217	0.015	0.0058	0.0011	0.0008	
21-Hydroxyprogesterone (5)	0.491	0.081	0.005	0.0019	0.0002	0.0003	
Reichstein's substance S (4)	1.137	0.200	0.013	0.0047	0.0009	0.0004	
Corticosterone (3)	0.431	0.064	0.004	0.0015	0.0004	0.0001	
Hydrocortisone (2)	1.190	0.191	0.012	0.0042	0.0008	0.0003	
Cortisone (1)	0.673	0.119	0.008	0.0029	0.0006	0.0003	

[1] numbers indicate which amount of substance applies

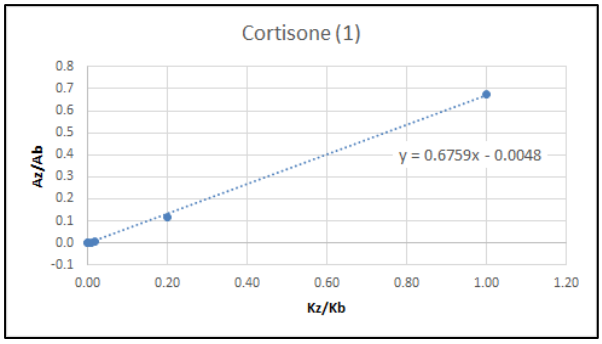

Figure 28: Calibration curve of cortisone

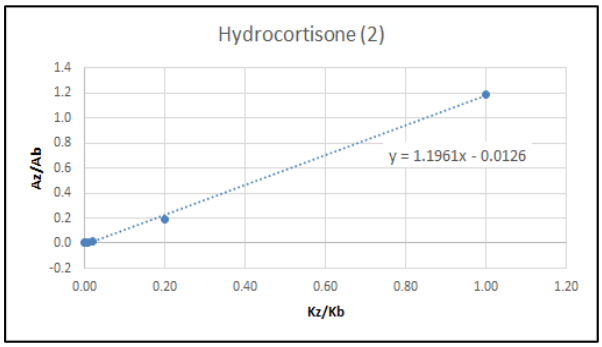

Figure 29: Calibration curve of hydrocortisone

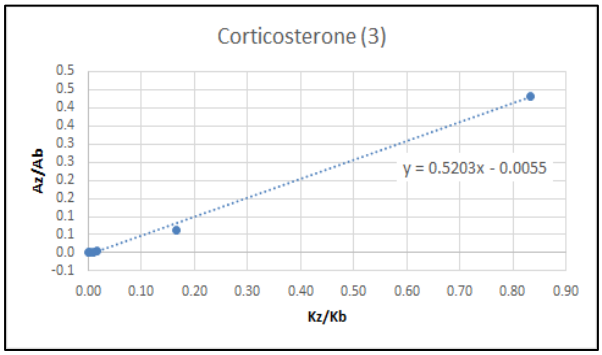

Figure 30: Calibration curve of corticosterone

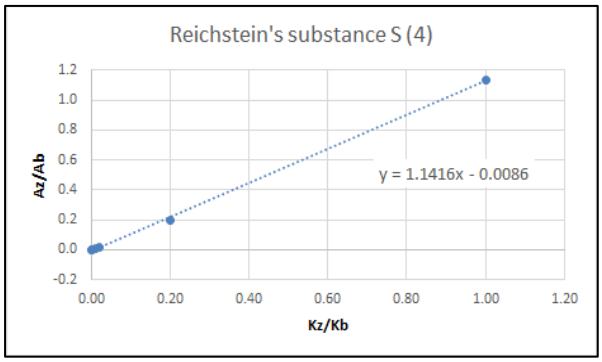

Figure 31: Calibration curve of Reichstein's substance S

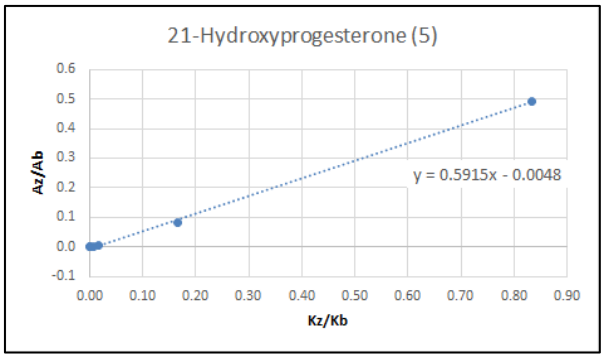

Figure 32: Calibration curve of 21-hydroxyprogesterone

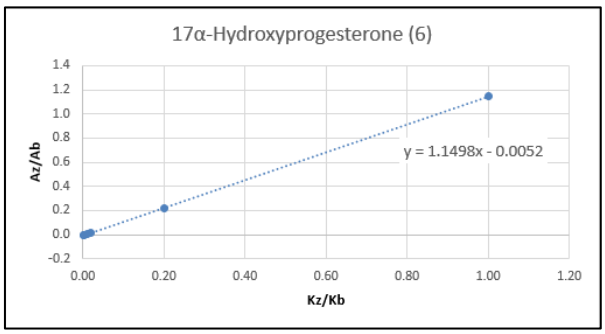

Figure 33: Calibration curve of 17α-hydroxyprogesterone

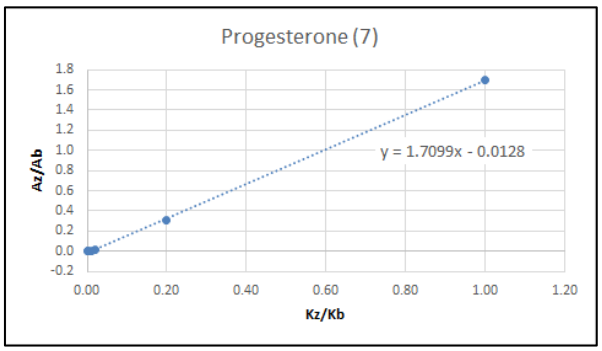

Figure 34: Calibration curve of progesterone

4.4.3 Standard Addition Method in Fat

To mask matrix effects, standard addition experiments with two different concentrations of all corticoid standards in fat were performed. It was possible to do this kind of method, because it can be seen in Figure 28 – Figure 34 that the corticoid amounts have a linear response behavior. Therefore, fat was spiked with 10 ppm (0.01 mg/g fat) on the one hand and 1 ppm (0.001 mg/g fat) on the other hand. Both of the fat samples were spiked with 1, 5, 10 and 20 ppm additionally to their initial spiking. An SPE with a DSC-NH_2 tube and cyclohexane as solvent was done each, the eluent was derivatized and analyzed with GC/MS. Table 7 shows the amounts of each standard (Kz) using Equation 2 for calculation. The results are then set in relation to the actually added amounts of corticoids for standard addition to create the recovery rate. Table 8 reveals that the recovery of very small amounts of standards are very unreliable. This means that a sample containing 2 ppm in total cannot be quantified to a reliable extent, since recovery of a sample containing 1 + 1 ppm of a compound has recovery values from 160–333 %, which suggests a 3 times higher amount of target compound than actually present. These values should not be taken into account, since they fell below determination limit.

Table 7: Fat with 1 ppm of standards spiked with 1 to 20 ppm

Calibration formula	Standard	Kz (mg) 1 ppm + 1	Kz (mg) 1 ppm + 5	Kz (mg) 1 ppm + 10	Kz (mg) 1 ppm + 20		d	k
y = 0.6759x - 0.0048	**Cortisone**	0.004	0.006	0.008	0.018		-0.005	0.68
y = 1.1961x - 0.0126	**Hydrocortisone**	0.006	0.008	0.010	0.019		-0.013	1.20
y = 0.5203x - 0.0055	**Corticosterone**	0.006	0.007	0.008	0.014		-0.006	0.52
y = 1.1416x - 0.0086	**Reichstein's substance S**	0.005	0.006	0.009	0.018		-0.009	1.14
y = 0.5915x - 0.0048	**21-Hydroxyprogesterone**	0.004	0.005	0.006	0.014		-0.005	0.59
y = 1.1498x - 0.0052	**17α-Hydroxyprogesterone**	0.003	0.005	0.007	0.015		-0.005	1.15
y = 1.7099x - 0.0128	**Progesterone**	0.004	0.005	0.006	0.014		-0.013	1.71

Table 8: Recovery of fat with 1 ppm of standards spiked with 1 to 20 ppm[2]

Recovery (%) 1 ppm	1 ppm + 1	1 ppm + 5	1 ppm + 10	1 ppm + 20
Cortisone	221.5	102.8	73.9	84.6
Hydrocortisone	306.4	128.8	89.7	90.1
Corticosterone	332.9	138.1	86.5	81.2
Reichstein's substance S	239.4	108.2	77.6	86.2
21-Hydroxyprogesterone	263.3	97.8	63.3	82.0
17α-Hydroxyprogesterone	163.7	79.0	61.9	72.1
Progesterone	208.8	84.6	51.2	68.8

The results of the spiked fat sample with a basic amount of 10 ppm in Table 9 and are better compared to the ones with 1 ppm. Amounts of measured and calculated standards are closer to the real amount added to the sample when the basis amount of spiked corticoids is higher. This is visualized in the table for recovery (Table 10), where the measured amounts were set in relation to the added amounts. Recovered amounts all together vary between 61 and 105 %, which shows the precision achievable with this method. These are acceptable percentages since the mean variation of the measurement is quite wide. Here, autotuning and calibration prior to measurement are crucial for accurate results.

[2] percentages are marked with colors from the lowest to the highest values (green, orange, red)

Table 9: Fat with 10 ppm of standards spiked with 1 to 20 ppm

Calibration formula	Standard	Kz (mg) 10 ppm + 1	Kz (mg) 10 ppm + 5	Kz (mg) 10 ppm + 10	Kz (mg) 10 ppm + 20	d	k
y = 0.6759x - 0.0048	Cortisone	0.010	0.011	0.017	0.025	-0.005	0.68
y = 1.1961x - 0.0126	Hydrocortisone	0.011	0.012	0.016	0.024	-0.013	1.20
y = 0.5203x - 0.0055	Corticosterone	0.009	0.010	0.018	0.021	-0.006	0.52
y = 1.1416x - 0.0086	Reichstein's substance S	0.010	0.012	0.016	0.024	-0.009	1.14
y = 0.5915x - 0.0048	21-Hydroxyprogesterone	0.007	0.010	0.014	0.021	-0.005	0.59
y = 1.1498x - 0.0052	17α-Hydroxyprogesterone	0.009	0.011	0.015	0.025	-0.005	1.15
y = 1.7099x - 0.0128	Progesterone	0.009	0.009	0.013	0.020	-0.013	1.71

Table 10: Recovery of fat with 10 ppm of standards spiked with 1 to 20 ppm[2]

Recovery (%) 10 ppm	10 ppm + 1	10 ppm + 5	10 ppm + 10	10 ppm + 20
Cortisone	92.0	76.2	84.6	83.7
Hydrocortisone	100.6	78.9	82.0	79.6
Corticosterone	96.5	79.1	105.1	83.3
Reichstein's substance S	93.1	76.7	78.3	80.8
21-Hydroxyprogesterone	78.3	78.9	85.8	83.6
17α-Hydroxyprogesterone	78.9	74.6	73.8	83.7
Progesterone	79.0	61.0	67.3	67.3

Recovery values of progesterone are particularly low compared to the other corticoids (see Table 8 and Table 10). This is explained by the early elution of progesterone during SPE with the apolar solvent in the washing cycle, where approximately 40 % of this corticoid is eluting before application of methanol (see Figure 25).

4.4.4 Limit of Detection and Quantification

To define whether a substance is found in a quantifiable amount, in traces or if it cannot be detected, it is necessary to determine detection and quantification limits for each corticoid. This was done with derivatized samples of all corticoids and betulin as internal standard without a fat matrix. Decisions were made according to the signal to noise ratio (S/N) of the highest appearing peak of each corticoid, with which it could be decided whether a sample was within a range or not. A peak in the chromatogram of the used program for visualization was marked after it was decided to be the highest of a corticoid and the S/N ratio was calculated automatically. The values are shown in Table 11. The downside and potential source of error of this method is that peaks for S/N have to be decided on manually. Also, different approaches to define limit of quantification (LOQ) and limit of detection (LOD) are possible, as LOD is sometimes fixed to an S/N value of 5 (Wisconsin Department of Natural Resources, 1996).

In the present case, it was decided that a substance cannot be called “detected”, if its highest peak features a signal to noise ratio of 3 or less. If the S/N ratio of a substance is less than 10 but more than 3, it can be stated to be found in traces. Therefore, limit of detection is the S/N value of 3, limit of quantification is an S/N of 10. The actual amounts of LOD and LOQ in milligrams can be found in Table 12. The values of LOD and LOQ for different corticoids range from 0.5 to 4.2 ppm as detection limit and 1 to 8.3 ppm as quantification limit. These limits correspond to GC/MS injection concentrations of 0.5 to 4.2 µg/ml and 1 to 8.3 µg/ml.

Table 11: S/N ratio of the substances at a certain corticoid concentration (in µg/ml injection volume) was calculated automatically by the used program[3]

1,2,4,6,7 (µg/ml)	500	100	10	5	1	0.5	0.1
3,5 (µg/ml)	416.6	83.3	8.33	4.2	0.833	0.42	0.0833
BETULIN	2284	2330	3064	2950	3911	3603	2764
Progesterone (7)	2560	571	69	33	10	2	0
17α-Hydroxyprogesterone (6)	1350	421	54	24	6	3	0
21-Hydroxyprogesterone (5)	80	35	19	7	2	1	0
Reichstein's substance S (4)	1153	573	102	53	11	7	0
Corticosterone (3)	639	116	11	3	1	1	2
Hydrocortisone (2)	1391	666	102	40	11	3	0
Cortisone (1)	851	376	71	22	7	3	0

Table 12: Actual concentrations of detection limit (LOD) and quantification limit (LOQ) of each standard in µg/ml of injection volume; LOD and LOQ were evaluated according to the S/N ratio of the highest peak of each substance

Standard	LOD	LOQ
	for each substance (µg/ml)	
Progesterone	1	1
17α-Hydroxyprogesterone	0.5	5
21-Hydroxyprogesterone	4.2	8.33
Reichstein's substance S	0.5	1
Corticosterone	4.2	8.33
Hydrocortisone	0.5	1
Cortisone	1	5

The calculated LOD and LOQ show that lower values for detection or quantification are different for each substance. Progesterone shows to have a quantification limit equal to the detection limit, because at the concentration of 1 µg/ml the S/N value was 10 already (see Table 11), which is above the LOQ. In contrast to these results, the experiment with standard addition indicates in a general manner that concentrations of standards with a minimum of 6 ppm are reliable.

4.5 Real Samples

4.5.1 Badger, Ibex and Brown Bear Ointments

No sample preparation was necessary here since the ointment was a homogenous clean fat in a glass container that could easily be handled for sample preparation. Two SPEs with 1 g of ointment were performed and analyzed per animal sample. GC/MS results of badger sample gave no hint for the presence of corticoids, since no peaks for any of the desired substances were visible. Ibex ointment was a little more difficult to handle since it was very solid at room temperature. SPE had to be performed very fast after heating column and solvent to a temperature of around 60 °C. Also, this sample showed a negative result as no peaks of corticoids were found in the chromatogram. Brown bear ointment was very soft at

[3] numbers indicate which amount of standard applies; values below LOD (S/N = 3) are colored in lime, values below LOQ (S/N = 10) are dark green

room temperature and sample preparation was not that difficult. But even in this type of sample no relevant peaks were obtained. Therefore, a result below detection limit had to be recorded for the ointment samples (Table 13).

4.5.2 Fat Tissue of Fox

Samples of fat tissue of an older male fox and an older female fox were also analyzed. After sample preparation with fat extraction from tissue, an SPE and subsequent oximation and silylation the samples were analyzed in the GC/MS. As with the samples before, no corticoids were detected. This also meant a result below detection limit (Table 13).

4.5.3 Results of Real Samples

Since no peaks for any of the investigated glucocorticoids in neither the ointments nor the fat tissue could be found in the chromatograms, the results are below detection limit and can be found in Table 13.

Table 13: Amounts of corticoids are below detection limits in ointments of badger, ibex and brown bear or fat tissue of fox; concentrations are in µg/ml injection volume

Corticoids in real samples	Concentration (µg/ml)
Progesterone	< 1
17α-Hydroxyprogesterone	< 0.5
21-Hydroxyprogesterone	< 4.2
Reichstein's substance S	< 0.5
Corticosterone	< 4.2
Hydrocortisone	< 0.5
Cortisone	< 1

It can be questioned whether the investigated corticoids were instable or might have gotten lost with extraction. Since fat tissue and ointment have always been stored appropriately, corticoids would have been conserved – as long as fat was present – if any had been present initially. A chance for escape of the corticoids could have been when sample was evaporated to dryness without any fat present. But this was not the case, as experiments with added standard corticoids were also performed and these showed no indication for a loss.

It was expected to find each corticoid to an approximate extend of 5 ppm, as Wagner and Nusser (1988) demonstrated amounts of glucocorticoids in badger and marmot in the range of 5 to 10 ppm per corticoid. Since no data about the fat of the here investigated animals and the content of corticoids was found in literature there are no disproving studies to that finding.

4.6 Final Method

An overview of the approach from raw fat tissue to corticoid concentrations as well as LOD and LOQ determination using the final developed method can be viewed in the flow chart below (Figure 35).

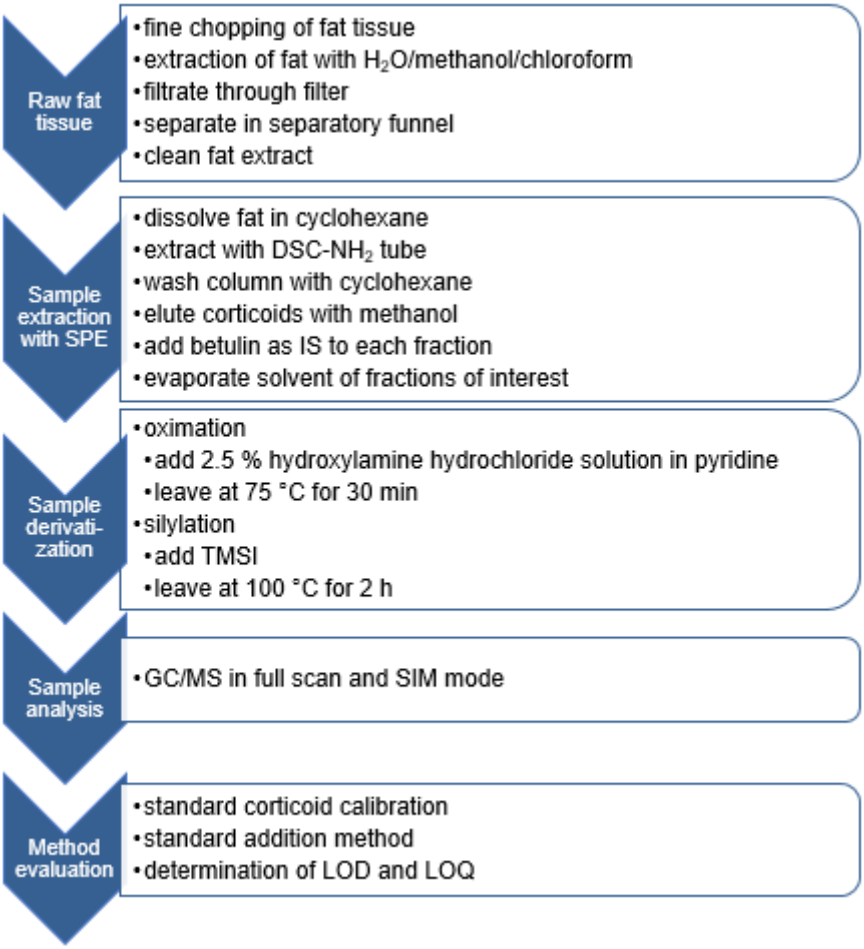

Figure 35: Flow chart of final method

5 Conclusion

To ensure safety for the consumer, corticoids in foodstuffs and cosmetics need to be determined analytically. Animals produce different corticoids, which can then be found as residues in the animals' fat tissue. Therefore, methods for extraction and analysis of selected corticoids have been developed and improved on that account.

The extraction method was designed considering that fat tissue samples of animals were used as raw material. The corticoids had to be separated from the fat and then derivatized for gas chromatography/mass spectrometry analysis.

The best suitable eluent for solid phase extraction had to be determined by experiments with thin-layer chromatography, in which behavior of corticoids and fat in different solvents could be observed. SPE showed the best results using a DSC-NH_2 tube, with cyclohexane as first eluent. For release of the retained corticoids, methanol was a simple and effective second eluent. Derivatization as preparation for analysis of each corticoid was done using 2.5 % hydroxylamine hydrochloride in pyridine for oximation and subsequent silylation with 1-(trimethylsilyl)imidazole. Analysis was conducted in SIM mode with the GC/MS, using specific molecular masses for each corticoid.

Calibration of the corticoids was performed to relate the amounts of standard in a sample to their peak area in the chromatograms. The calibration equations were then used for a standard addition experiment. Based on the calibration equation, recovery could be calculated to determine to what extent spiking and measuring corresponded. 6 to 30 ppm of corticoids in fat show good recovery values ranging from 51 to 138 %, with a mean value of 84 %. These values are reasonable, since the mean variation of the measurement is quite wide because of different peak qualities which depend on temporary peak settings due to autotuning.

Based on the signal to noise ratio of the highest peak of a corticoid, limits of detection and quantification were determined. The corticoids of the measured real fat samples (ointments, fat tissue) were beyond detection limits.

Standard addition and limits of detection and quantification show two different guide values for each corticoid for assessing results. The former indicates in a general manner that concentrations of standards with a minimum of 6 ppm are reliable, LOD and LOQ give specific values for different corticoids ranging from 0.5 to 4.2 ppm as detection limit and 1 to 8.3 ppm as quantification limit. Comparing these two statements, both are quite adequate considering that 6 ppm is located in the upper range between LOD and LOQ, which allow to specify trace amounts.

After recapping all the work carried out and seeing what results could have been obtained by it, some recommendations for future research arise.

It is remarkable that already in 1988, Wagner and Nusser could detect 6 to 14 ppm in animal fat samples, whereas with the here performed standard addition method 6 ppm was already the lower limit still showing acceptable recovery values. With the current analyzing methods and equipment, one would expect that much better results with lower detection and quantification limits should be possible. One way to achieve this is to use less solvent for dilution of readily prepared samples for GC/MS injection, so higher peaks for lesser amounts of corticoids can be obtained. Then smaller amounts of substances are more concentrated and can be detected more easily. Concerning real animal samples, more fat can be prepared, so possibly contained corticoids are more concentrated and the chance of exceeding detection limit is higher.

Regarding SPE, it can be said that it does not have unlimited binding sites. It would be helpful to empirically determine the rough amount of the capacity. Then, no uncertain explanations about overloaded columns due to unsteady elution patterns would be made. In order to be able to read out actual milligram amounts of corticoids and not only relative amounts, standard and internal standard in known amounts have to be derivatized (without preceding SPE) and analyzed in GC/MS. Preferably, this should be done in a series of analyses with the samples of interest to eliminate variations of measurements and peak qualities. Only then it can be concluded that relations of certain peak areas and their concentrations are proportional in milligram amounts.

All in all, similar studies dealing with corticosteroids in animal fat still need to be conducted. This is necessary to gain more data about this issue and to be able to compare results with each other.

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7 List of Tables

8 List of Figures

9 List of Abbreviations

BSTFA + TMCS, 99:1	N,O-bis(trimethylsilyl)trifluoroacetamide + 1 % trimethylchlorosilane
EC	European Commission
EU	European Union
GC/FID	gas chromatography/flame ionization detection
GC/MS	gas chromatography/mass spectrometry
HAH	hydroxylamine hydrochloride
HPLC	high-performance liquid chromatography
IS	internal standard
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
m/z	mass-to-charge ratio
ppm	parts per million
SIM	selected ion monitoring
SPE	solid phase extraction
S/N	signal to noise
TMS	trimethylsilyl
TMSI	1-(trimethylsilyl)imidazole

Curriculum Vitae

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Internships and experience

September 2016 – February 2017: 5 *months:*
Wageningen University – *The Netherlands*

Mandatory semester abroad as a part of the Master's program

October 2013 – present: **IAESTE – The International Association for the Exchange of Students for Technical Experience:** Voluntary member

- 2015–2016: Executive board member for Exchange Outgoing in the national committee of IAESTE Austria
- 2014–2015: Executive board member for Exchange Outgoing of IAESTE BOKU at my University
- Managing language tests, assisting students with administrative matters in the process of going on an internship abroad

---

July/August 2015: *6 weeks:* **Narodne Lesnicke Centrum** (National Forest Center) **– *Banská Štiavnica, Slovakia***

- Supervising and evaluating a laboratory experiment with beetles and fungi as natural pesticide
- Collecting *Agrilus spp*. (metallic wood-boring beetle) from sticky prism traps located in East Slovakia

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July 2014: *2 weeks:* **Summer School** Euroleague for Life Sciences at the **Czech University of Life Sciences Prague (CULS) – *Czech Republic***

"Chemical, microbiological and sensory analysis of food"

September/October 2013: *6 weeks:* **Machland Obst- und Gemüsedelikatessen GmbH – *Naarn, Austria***

Manufacture of fruit and vegetable products

- Working in the analytical laboratory
- Taking and maintaining retention samples
- Hygiene checks in the production, supervision of production lines (CCPs, packaging, labelling, …)
- Quality control and analysis of greenware and finished goods
- Documentation and management of daily checking lists, working with MS Access

---

July/August 2013: *8 weeks:* **Яхмоси 33** (Yakhmosi 33) – ***Khujand, Tajikistan***

Manufacture of ice cream, soft drinks, frozen products

- Responsibility: quality control, monitoring soft drink production
- Analyzing water hardness, acidity, CO_2, Brix etc.
- Assisting in several stages of the production line
- Contributing to product development of ice cream

Education

2015 – present
"Safety in the Food Chain", English Master's program, University of Natural Resources and Life Sciences, Vienna
Estimated completion: Oct. 2017

2010 – 2015
"Food Science and Biotechnology", Bachelor's program, University of Natural Resources and Life Sciences, Vienna
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2005 – 2010
HTBLuVA Waidhofen/Ybbs (secondary education institution), department: industrial engineering
Graduation: June 2010

2001 – 2005
BRG Waidhofen/Ybbs (secondary school)

Skills and abilities

Language skills

- **German**: mother tongue
- **English**: excellent knowledge oral and written
- **Norwegian**: basic knowledge

IT skills

MS Office (Word, Excel, PowerPoint, Access, Project)

CAD, Autodesk Inventor

Driving license – Category B

Interests

Nutrition, languages, foreign cultures

Hobbies

Travelling, cooking, climbing, music

Vienna, September 2017