# Targeted analysis of bioactive steroids and oxycholesterols

## Method development and application

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# Abstract

Peripheral steroids and oxycholesterols are important lipid compounds controlling various functions in the human body. Steroid analysis of biological samples is routinely employed in the clinical environment as an essential source of information on endocrine and metabolic disorders. It has been reported that stress related neurosteroids have been implicated in the development and prognoses of neurodegenerative disorders such as Alzheimer's disease (AD). These compounds have been identified as possible biomarkers in the diagnosis of AD and other neurodegenerative disorders. Therefore, methods for the simultaneous analysis of steroids from the four major classes (estrogens, androgens, progestogens and corticosteroids) are vital in providing useful and more comprehensive data.

Homeostasis of cholesterol in the brain is maintained primarily by metabolism to oxysterols, including oxycholesterols. These oxycholesterols act as a transport form of cholesterol as it readily navigates the blood-brain barrier. Oxycholesterols are generally more bioactive than cholesterol and is of interest in pathophysiology. Moreover, if their production in cells and tissues and/or their introduction with dietary animal fat are excessive, oxycholesterols could indeed contribute to the pathogenesis of various disease processes.

The first study in this thesis focuses on a novel supercritical fluid chromatography–tandem mass spectrometry method for targeted analysis of eighteen peripheral steroids. The method is simple and fast. It has sufficient sensitivity for quantification of 18 different steroids in small volume human plasma. Therefore, this novel method can be applied for screening many steroids within 5 minutes providing the possibility to use for routine healthcare practice. The second study involves the quantification of three adrenal steroids in plasma from domesticated White Leghorn (WL) chickens and Red Junglefowl (RJF) birds. The domestication effects on stress induced steroid secretion and adrenal gene expression in chickens are evaluated. The third study focuses on determination of more than ten oxycholesterols in biological samples with a gas chromatography–mass spectrometry method and a supercritical fluid–tandem mass spectrometry method.

*To my family*

*Education is the most powerful weapon which you can use to change the world.*

*Nelson Mandela*

# List of Papers

This thesis is, in part, based on the following papers, which are referred to in the text by their Roman numerals.

I **De Kock, N.**, Ubhayasekera, S.J.K.A., Bergquist, J. A novel targeted analysis of peripheral steroids by supercritical fluid chromatography hyphenated to tandem mass spectrometry. *In manuscript*

II Fallahsharoudi, A., **De Kock, N.**, Johnsson, M., Ubhayasekera, S.J.K.A., Bergquist, J., Wright, D., Jensen, P. (2015) Domestication effects on stress induced steroid secretion and adrenal gene expression in chickens. *Scientific Reports*, 5, 15345. (DOI: 10.1038/srep15345)

**Author's contribution**

**Paper I:** Planning of the research project with the co-authors, execution of analytical work, evaluation of the results and writing of the manuscript.

**Paper II:** Performed the hormonal analysis and wrote part of the paper.

# Contents

# Abbreviations

| | |
|---|---|
| AD | Alzheimer’s disease |
| ALD | aldosterone |
| AND | androstenedione |
| APCI | atmosperic pressure chemical ionization |
| AS | androsterone |
| B | corticosterone (also CORT) |
| BBB | blood–brain barrier |
| BHT | butylated hydroxytoluene |
| cPREG | $^{13}C_2$-$d_2$-pregnenolone |
| αCE | α-epoxycholesterol |
| βCE | β-epoxycholesterol |
| CNS | central nervous system |
| $CO_2$ | carbon dioxide |
| COP | cholesterol oxidation product (oxycholesterol) |
| CORT | corticosterone (also B) |
| cT | $^{13}C_3$-testosterone |
| CT | cholesteroltriol |
| dAS | $d_2$-androsterone |
| dB | $d_8$-corticosterone |
| dDHEA | $d_2$-dehydroepiandrosterone |
| dE1 | $d_4$-estrone |
| dF | $d_4$-cortisol |
| 11DeoxyB | 11-deoxycorticosterone |
| 11DeoxyF | 11-deoxycortisol |
| DHEA | dehydroepiandrosterone |
| DHT | dihydrotestosterone |
| dP | $d_9$-progesterone |
| d17OHP | $d_8$-17α-hydroxyprogesterone |
| E | cortisone |
| E1 | estrone |
| ECN | etiocholanolone |
| EI | electron impact |
| ESI | electrospray ionization |

| | |
|---|---|
| F | cortisol |
| GC | gas chromatography |
| 4βHC | 4β-hydroxycholesterol |
| 7αHC | 7α-hydroxycholesterol |
| 7βHC | 7β-hydroxycholesterol |
| 20αHC | 20α-hydroxycholesterol |
| 24HC | 24(S)-hydroxycholesterol |
| 25HC | 25-hydroxycholesterol |
| 27HC | 27-hydroxycholesterol |
| IS | internal standard |
| 7KC | 7-ketocholesterol |
| LC | liquid chromatography |
| LLE | liquid-liquid extraction |
| LOQ | limit of quantification |
| MO | methoxyamine/methoxime |
| MRM | multiple reactions monitoring (also SRM) |
| MS | mass spectrometry |
| MS/MS | tandem mass spectrometry |
| MTBE | *tert*-butyl methyl ether |
| 17OHP | 17α-hydroxyprogesterone |
| 17OHPREG | 17α-hydroxypregnenolone |
| P | progesterone |
| PONE | pregnanolone |
| PREG | pregnenolone |
| $R^2$ | correlation coefficient |
| RJF | Red Junglefowl |
| RT | retention time |
| SFC | supercritical fluid chromatography |
| SIM | selected ion monitoring |
| SRM | selected reactions monitoring (also MRM) |
| T | testosterone |
| TMS-ether | trimethylsilyl ether |
| $UPC^2$ | ultraperformance convergence chromatography |
| WL | White Leghorn |

# 1. Introduction

Endogenous steroids and oxycholesterols form part of the lipidome in the animal and human body. Lipids are biological compounds with hydrophobic or amphipathic characteristics and are generally soluble in organic solvents.[1,2] The study of the lipidome can be divided into two categories: global and targeted lipidomics. In this study, the focus will be on the latter. Targeted lipidomics is defined as the quantitative analysis of a single or several selected lipids within a specific lipid class.[3] Endogenous steroids and oxycholesterols are classified as sterol lipids[4] and are naturally occurring physiologically important compounds controlling different functions in the animal and human body as a part of the endocrine, neuronal and immune systems.[5]

Endogenous steroids control many physiological processes, including reproduction, maturation, gene expression and neurological functions. During the last two decades, there has been an increased focus on the application of steroids as biomarkers in healthcare practice.[6-12] Steroids have been implicated in the development and/or progression of many diseases, such as breast cancer, ovarian cancer, prostate cancer, endometrial cancer, osteoporosis, cardiovascular disease, obesity, and neurodegenerative disorders.[5] Depletion of steroid hormones with age is a well-known fact and has been implicated in the development of Alzheimer's disease (AD).[13,14] Endogenous steroids have been identified as possible biomarkers in the diagnosis of neurodegenerative conditions such as AD.[13]

The analysis of steroids in biological samples such as plasma, serum, urine, etc. is routinely used in clinical diagnosis as an essential source of information on endocrine and metabolic disorders,[6-9,12] and in neurodegenerative disorders.[10,11] Therefore, an accurate analysis of steroids in biological tissues has become important for contemporary medicine – even if troublesome, especially due to the low concentration levels in biological samples.[8] Steroid profiles, generated by the simultaneous determination of steroids from the four major classes (estrogens, androgens, progestogens and corticosteroids), provides useful data in the clinical environment.[9]

Endogenous oxycholesterols are more readily disposed by cells than cholesterol. Therefore, an oxygen function, such as a hydroxyl, epoxide or ketone group, is introduced to the sterol ring or side chain of cholesterol to make it

more polar. These oxycholesterols are generally more bioactive than cholesterol and is of interest in pathophysiology. Moreover, if their production in cells and tissues and/or their introduction with dietary animal fat are excessive, oxycholesterols could indeed contribute to the pathogenesis of various disease processes.[15]

As steroids and oxycholesterols are present in both normal and pathological conditions of the body, it is of high importance to develop analytical methods to determine and quantify these compounds in biological samples.

In this thesis, the development of sensitive methods for quantification of steroids and oxycholesterols in plasma samples, e.g. from patients with neurodegenerative disorders, which are manifested in humans, is of particular interest. Furthermore, a similar method for steroid analysis was applied to investigate the role of endogenous steroids in endocrinological stress responses in the domesticated White Leghorn chicken versus the ancestral Red Junglefowl.

## 1.1 Endogenous steroids and oxycholesterols

Endogenous steroids and oxycholesterols are derived from cholesterol which is absorbed through the diet or synthesized *de novo* in various tissues and cells.[16] The central nervous system (CNS) consists of the brain and the spinal cord. The blood-brain barrier (BBB) is formed by capillary endothelial cells and act as a physiological and biochemical barrier. The BBB precludes the uptake of cholesterol from the periphery and consequently cholesterol is produced *de novo* in the brain.[16] Cholesterol constitutes 2% of the wet weight of the human brain and 25% of cholesterol in the body is located in the brain.[17]

Steroids that are produced or biologically active in the CNS are referred to as neurosteroids.[16] Steroids can cross the BBB with ease and therefore be formed in the periphery. Dehydroepiandrosterone (DHEA) is a neurosteroid formed locally in the CNS, while corticosterone (B), aldosterone (ALD), and testosterone (T) are formed in the periphery. Pregnenolone (PREG) and progesterone (P) are produced in both the CNS and periphery.[16,17]

Homeostasis of cholesterol in the brain is maintained primarily by metabolism to oxysterols, including oxycholesterols. Oxycholesterols act as a transport form of cholesterol as it readily navigates the BBB.[17]

### 1.1.1 Biosynthesis of endogenous steroids

Steroids are formed from cholesterol during steroidogenesis through a series of enzyme controlled reactions (Figure 1). These enzymes belong to two major classes of protein: the cytochrome P450 proteins (CYP11, CYP17, CYP19, CYP21, etc.) and the hydroxysteroid dehydrogenases (3βHSD, 11βHSD, 17βHSD, etc.).[16] The cytochrome P450 enzymes are products of a single gene, while the HSDs are products of distinct genes. Steroids have different primary production sites due to variation in distribution of these enzymes between tissues, including the brain, adrenal glands, gonads, and placenta, resulting in biosynthesis of many of the steroids in more than one tissue.[16]

Conversion of cholesterol to pregnenolone (PREG) is the rate-limiting step in the biosynthetic pathway. Pregnenolone is formed on the inner membrane of mitochondria. Conversions of pregnenolone to other steroids occur through further enzymatic reactions during back and forth transfers between the mitochondria and endoplasmic reticulum.[16]

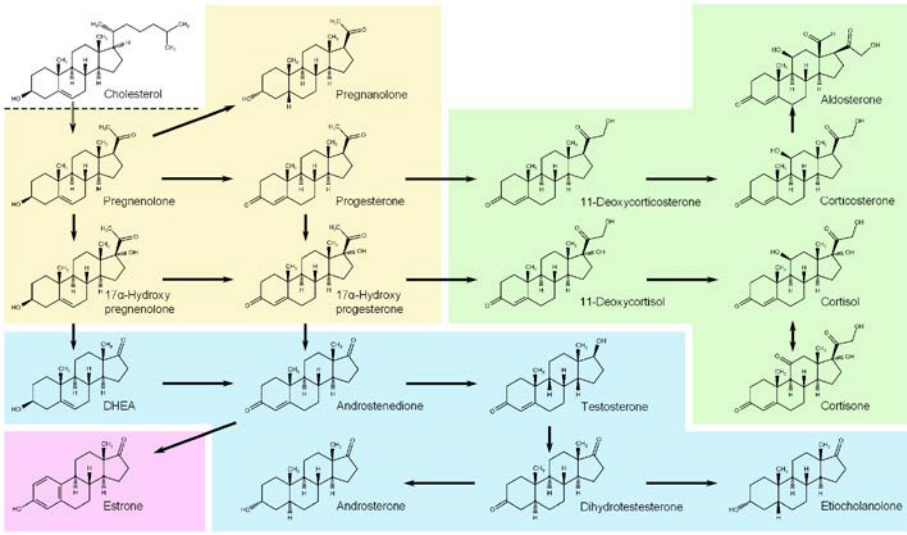


*Figure 1.* Biosynthesis of steroids in the cholesterol pathway.

Steroids bind to steroid receptors on the surface of target cells to initiate their physiological effect. Steroids are divided into four major classes or groups, namely estrogens, androgens, progestogens, and corticosteroids, depending on the type of receptor to which they bind.[16]

### 1.1.2 Formation of oxycholesterols

Oxycholesterols are formed by enzymatic and non-enzymatic oxidation of cholesterol. Non-enzymatic oxidation includes autoxidation and photoxidation. The sterol ring of cholesterol is most often oxidized by non-enzymatic mechanisms and leads to products such as 7-ketocholesterol (7KC), 7β-hydroxycholesterol (7βHC), 5α,6α-epoxycholesterol (αCE) and 5β,6β-epoxycholesterol (βCE), while 7α-hydroxycholesterol (7αHC) is formed by enzymatic oxidation. All of the side chain oxidations of cholesterol follows an enzymatic mechanism and produces oxycholesterols like 24-hydroxycholesterol (24HC), 25-hydroxycholesterol (25HC) and 27-hydroxycholesterol (27HC).[15] Some common oxycholesterols are listed in Table 1.

Free radicals or triplet oxygen initiates autoxidation of lipids, generating a series of autocatalytic free radical reactions (Figure 2). Oxidation products are formed during the breakdown of lipids by the autoxidation reactions. Cholesterol, an unsaturated lipid (RH), is subjected to the free radical ($R^{\bullet}$) chain reaction which includes three processes: initiation, propagation and termination. A peroxy radical reaction with another sterol molecule yields a sterol hydroperoxide and a sterol radical, thus altering the number of sterol radicals in the reaction sequence.[18-20]

Table 1. *Nomenclature and abbreviations of some common oxycholesterols.*

| Trivial name | Abbreviation | Systematic name |
|---|---|---|
| 7α-Hydroxycholesterol | 7αHC | Cholest-5-en-3β,7α-diol |
| 7β-Hydroxycholesterol | 7βHC | Cholest-5-en-3β,7β-diol |
| α-Epoxycholesterol | αCE | 5,6α-Epoxy-5α-cholestan-3β-ol |
| β-Epoxycholesterol | βCE | 5,6β-Epoxy-5α-cholestan-3β-ol |
| Cholesteroltriol | CT | 5α-Cholestan-3β,5,6β-triol |
| 7-Ketocholesterol | 7KC | 3β-Hydroxycholest-5-en-7-one |
| 4β-Hydroxycholesterol | 4βHC | Cholest-5-en-3β,4β-diol |
| 20α-Hydroxycholesterol | 20αHC | Cholest-5-en-3β,20α-diol |
| 24(S)-Hydroxycholesterol | 24HC | Cholest-5-en-3β,24-diol |
| 25-Hydroxycholesterol | 25HC | Cholest-5-en-3β,25-diol |
| 27-Hydroxycholesterol | 27HC | Cholest-5-en-3β,27-diol |

Initiation: $RH \rightarrow R^{\bullet} + H^{\bullet}$

Propagation: $R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$
$ROO^{\bullet} + RH \rightarrow ROOH + L^{\bullet}$

Termination: $ROO^{\bullet} + H^{\bullet} \rightarrow ROOH$
$R^{\bullet} + H^{\bullet} \rightarrow RH$

*Figure 2.* Lipid autoxidation pathway.

Cholesterol autoxidation usually starts at C-7 by the abstraction of a hydrogen atom following the addition of an oxygen atom forming primary oxycholesterols, isomers of 7-hydroperoxycholesterols (Figure 3). These 7-hydroperoxycholesterols can further convert into 7α-hydroxycholesterol and 7β-hydroxycholesterol. In addition, 7-ketocholesterol can be formed by the dehydration of isomeric 7-hydroxycholesterols (Figure 3). The side chain oxidation occurs at C-20, C-24, C-25 and C-26 with free radical attacks at these positions resulting in the production of relevant hydroperoxides, which can be further converted into 20α-hydroxycholesterol, 24-hydroxycholesterol, 25-hydroxycholesterol and 26-hydroxycholesterol.[18]

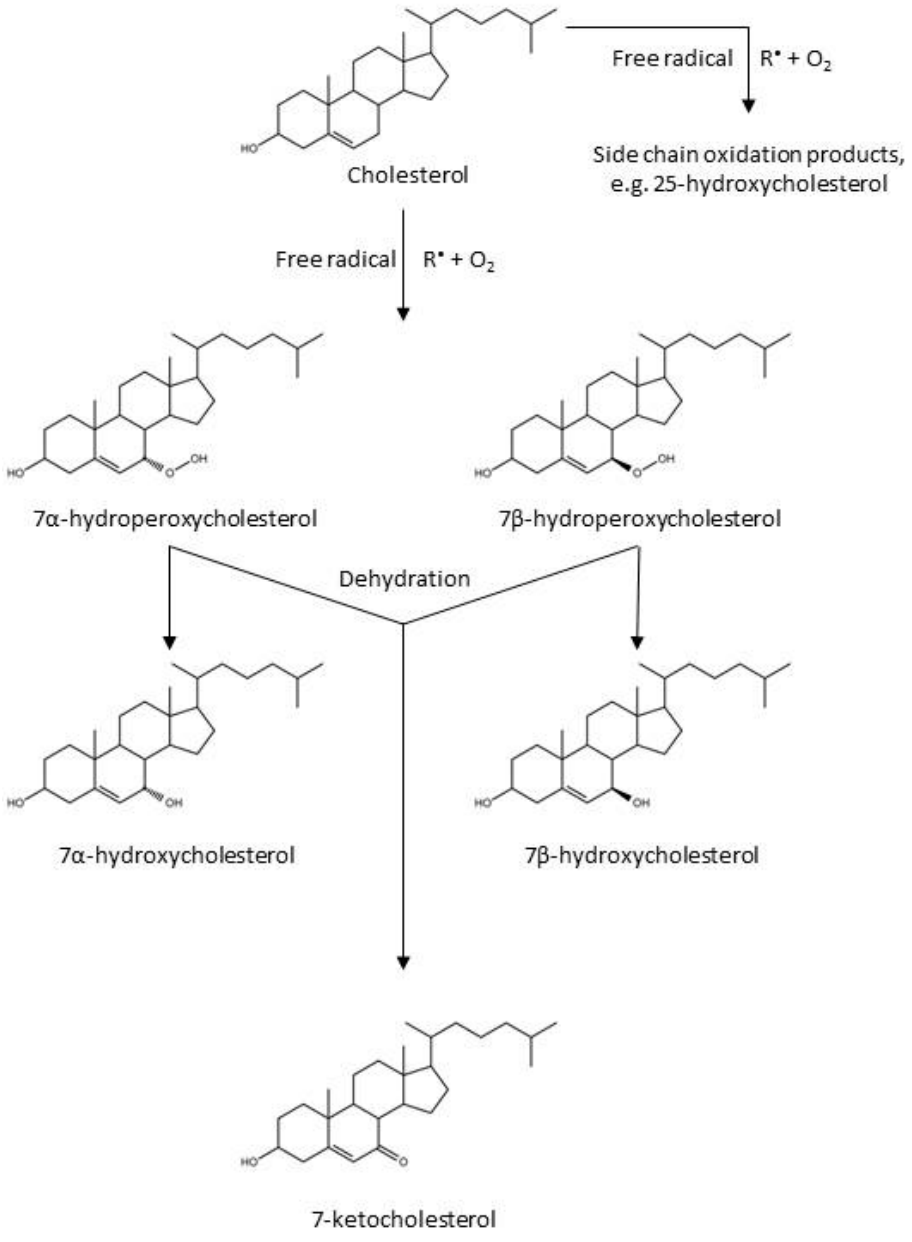


*Figure 3.* Autoxidation of cholesterol.

The formation of isomeric epoxycholesterol occurs due to interaction between cholesterol molecules and hydroxy radicals (Figure 4) and these epoxy compounds can be further hydrolyzed in an acidic medium converting them into cholesteroltriol.[18]

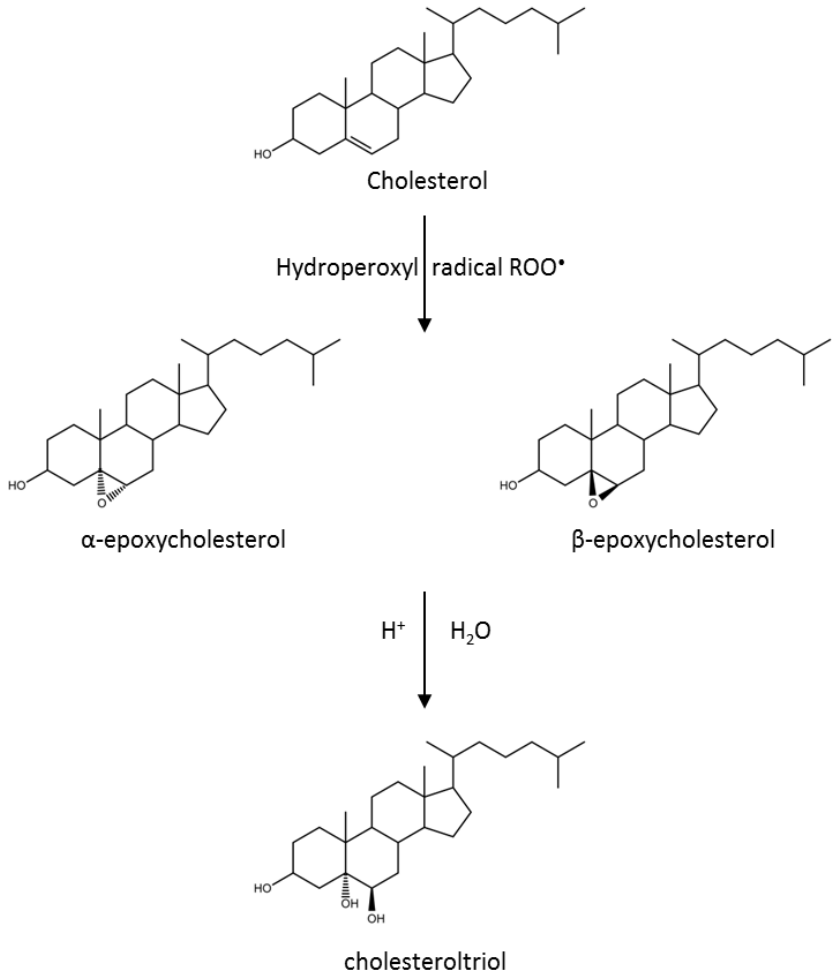


*Figure 4.* Formation of epoxycholesterols and cholesteroltriol.

## 1.2 Analysis of endogenous steroids

Several techniques are used for the quantification of steroids. The most common methods of steroid quantification in clinical practice include immunoassays such as radioimmunoassay or enzyme immunoassay. The main disadvantages of immunoassay techniques are the cross reactivity of the antibodies used in the assay with the related steroids, and being prone to matrix effects.[8,9] Separation methods like liquid and gas chromatography (LC and GC) rely on modern mass spectrometry (MS) techniques. These high-tech methods are practical and offer tremendous value in obtaining useful structural information on individual steroids and their metabolites.[7] These methods are compared and evaluated for factors such as sensitivity, specificity, limit of detection and quantification, etc.[9] Furthermore, the progress of analytical method development has a tremendous impact with the use of biomarkers for disease diagnostics,[10] e.g. AD.[11]

Analysis of steroids and their metabolites in biological samples with GC–MS is usually accompanied by different chemical derivatization methods.[21] The derivatization of steroids for GC–MS analysis aids in the enhancement of volatility, stability, ionization properties, and fragmentation behavior of these analytes in the electron ionization (EI) mode.[21] With the recent developments in MS, GC has been hyphenated with many different types of mass spectrometers, including triple quadrupole (TQ, tandem MS),[22] quadrupole ion trap (QIT–MS),[23,24] and time-of-flight (TOF–MS),[25] in order to improve the sensitivity of the steroid analysis. Likewise, LC has been coupled to different MS systems with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) as the most common ionization techniques.[9] Analysis of steroids without derivatization by LC–MS/MS is well documented and is also widely used in the clinical practice.[8,9] The advantages of LC–MS/MS are less sample preparation and shorter analytical time in comparison to GC–MS/MS, with the latter providing better chromatographic resolution.[7]

Supercritical fluid chromatography (SFC) is an important leading “green technology” method used for highly efficient separation with shorter analytical durations for ionizable analytes. SFC is similar to LC; however, SFC typically uses carbon dioxide as the main mobile phase. SFC is essentially a normal-phase chromatographic technique with inherent high speed and efficiency due to its mobile phase. The key factors for SFC method development are a stationary phase to ensure good resolution and the addition of an appropriate co-solvent for analyte solvation. Moreover, SFC improves the separation of isomers and enantiomers compared to LC.[26,27]

Coupling of SFC with MS/MS provides several advantages related to sensitivity and specificity. SFC has been coupled with both ESI and APCI as ionization sources for MS detection.[27] Yet, the application of ESI–MS in steroid analysis is limited. This is due to low proton affinity of the carbonyl and hydroxyl groups.[21] Therefore, chemical derivatization of steroid analytes is also a useful step prior to their SFC–ESI–MS analysis. The chemical addition to the corresponding steroid (carbonyl and/or hydroxyl groups) allows formation of derivatives with enhanced sensitivity compared to the underivatized form of steroids.[8]

## 1.3 Analysis of endogenous oxycholesterols

A comprehensive review on the analysis of oxysterols was recently published by Griffiths *et al*.[28] A short summary of the review pertaining to oxycholesterol analysis is presented here.

Quantification of oxycholesterols has been performed by several analytical techniques. Oxycholesterol analysis is classically done by GC–MS and considered the "gold standard" method. It was described over twenty years ago by Dzeletovic *et al*.[29] The method uses deuterated internal standards for quantification and is known as isotope dilution–MS. Sample preparation for oxycholesterol analysis by GC–MS usually includes the saponification, extraction, enrichment, and derivatization of the oxycholesterols. Silylation reagents are mostly used to form trimethylsilyl ether (TMS-ether) derivatives of the oxycholesterols. Detection by MS is mostly performed in selected ion monitoring (SIM) mode.[28]

LC–MS analysis of oxycholesterol has been performed with and without derivatization. Sample work-up usually includes extraction, hydrolysis and enrichment for most of the reviewed literature. McDonald, Russell and coworkers have analyzed ten oxycholesterols, without derivatization, together with sterols and secosteroids.[30] Detection was performed with ESI and multiple reaction monitoring (MRM).[28]

Analysis of underivatized oxycholesterols relies on chromatographic separation of isomeric oxysterols and the availability of authentic standards. This is a consequence of $[M+H]^+$ ions of isomeric oxysterols giving a similar MS/MS spectrum. In addition, oxysterols are neither basic or acidic, hence do not readily form $[M+H]^+$ or $[M-H]^-$ ions and do not give strong signals in ESI, APCI or other desorption ionization methods. In an effort to improve signal, derivatization methods have been designed which in some cases also provide added structural information.[28]

Different derivatization reagents have been used to derivatize oxycholesterols to picolinyl esters,[31] nicotinyl esters,[32] N,N-dimethylglycine esters,[33] oximes,[34,35] and Girard hydrazones[36] prior to LC–MS/MS analysis.[28] The majority of the LC–MS/MS methods with derivatization used ESI[31-36] instead of APCI[37] as ionization source and detection in MRM mode.[28]

# 2. Aims of the thesis

Steroids and oxycholesterols are present in both normal and pathological conditions of the body. These bioactive compounds might be possible biomarkers for the detection of neurodegenerative disorders, such as AD. Furthermore, the expression of steroids as a consequence to stress restraint can act as a measure of an organism's response to stress. Therefore, it is of high importance to develop analytical methods to determine and quantify these bioactive compounds in biological samples to aid in the collection of information for identification of possible biomarkers in AD and evaluate endocrinological stress responses.

In this thesis, the development of analytical methods for determination of steroids and oxycholesterols in plasma samples is discussed.

The main objectives of this work have been:

- To develop a simple, rapid and sensitive SFC–MS/MS method for the simultaneous analysis of steroids from the four major classes and to apply the method to analysis of AD and control samples.
- To develop a method in order to measure three steroid hormone levels in White Leghorn (WL) and Red Junglefowl (RJF) birds.
- To assess, optimize and compare the separation of a mixture of more than ten oxycholesterols by using GC–MS and SFC–MS/MS as analytical methods.

# 3. Materials and methods

This section provides a summary of the materials, work plan and methodologies used in this work. A brief description of the materials is shown below. Details of the materials and analytical procedure are stated in the attached **Papers I–II**.

## 3.1 Materials

### 3.1.1 Chemicals

All chemicals used in **Papers I** and **II** were analytical or chromatographic grade. Standard samples of 7αHC, 19HC (IS), 7βHC, 4βHC, βCE, αCE, 20HC, TC, 24HC, 25HC, 7KC and 27HC were purchased from Steraloids Inc. (Newport, RI, USA); 5α-cholestane, TMS reagent, and highest purity solvents and chemicals were obtained from Sigma-Aldrich (Stockholm, Sweden), unless otherwise stated.

### 3.1.2 Preparation of standard solutions and steroid-free plasma

Stock solutions of 1 mg/mL were prepared for all steroid and oxycholesterol analytes using methanol as solvent. All solutions were stored at –80 ºC. Plasma free of steroids was prepared as described by Aburuz *et al*.[38] in order to have a matrix similar to the true samples.

## 3.2 Sample preparation procedures

### 3.2.1 Procedures for steroids

The procedure for the determination of steroids is similar for **Papers I–II** with minor differences in the amount of plasma used (50 µL in **Paper I** versus 200 µL in **Paper II**) and concentration of the steroids in **Paper II**. The sample preparation involved two procedures, namely extraction and derivatization of steroids, as illustrated in Figure 5A. In short, steroids were

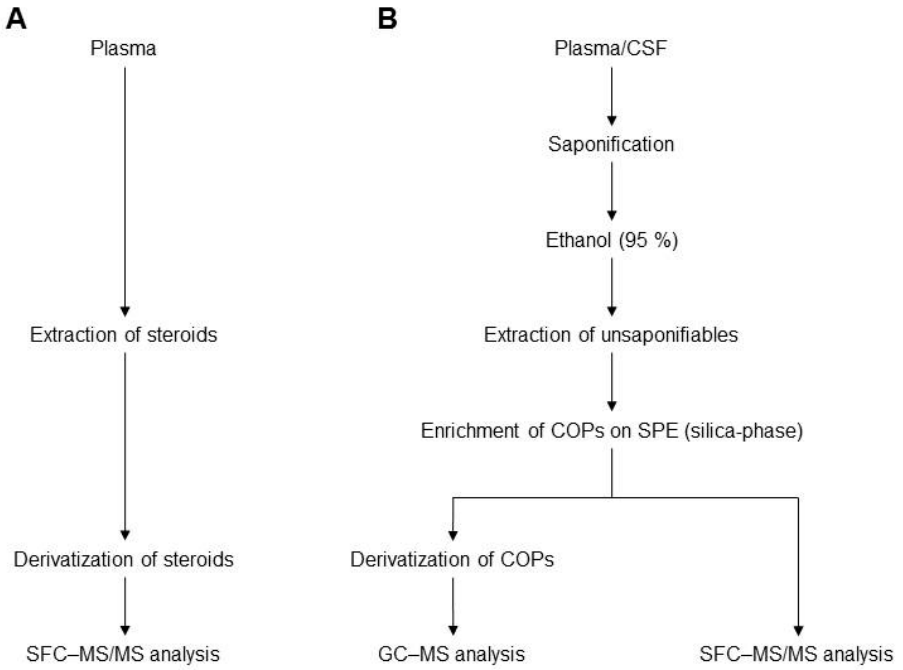


*Figure 5.* Flow chart of the experimental design of the methods of analysis of (A) steroids and (B) cholesterol oxidation products (COPs).

extracted from plasma with 2 mL *tert*-butyl methylether (MTBE), vortexed for 10 min and centrifuged at 1000 g for 10 min. The supernatant was collected and dried under a gentle stream of nitrogen gas before adding methoxyamine hydrochloride as derivatization reagent. The steroids were allowed to react with the derivatization reagent for 45 min at 60 °C to form oxime derivatives. The excess reagent was evaporated under nitrogen and the oxime derivatives were dissolved in methanol. Samples were kept at −20 °C prior to the analysis by SFC–MS/MS.

### 3.2.2 Procedures for oxycholesterols

The experimental design for the determination of oxycholesterols is presented as a flow chart in Figure 5B. The sample work-up prior to GC–MS analysis of COPs involved four steps: saponification, extraction, enrichment, and derivatization. Analysis of COPs by SFC–MS/MS analysis was preceded by the same steps, excluding derivatization.

#### 3.2.2.1 *Saponification*

Aliquots of 100 µL of plasma and 2 µL of a 20 µg/mL internal standard mixture (5α-cholestane and 19-hydroxycholesterol) were saponified with 500 µL

of 10 % KOH in 99 % ethanol in a glass tube at room temperature. The mixture was kept in the dark for approximately 18 hours. The reaction was stopped by the addition of 500 µL of saturated NaCl solution.

#### 3.2.2.2 *Extraction of unsaponifiables*

The unsaponifiable compounds were extracted twice with 200 µL of hexane. The extract was washed with 500 µL of saturated NaCl solution. The hexane phase were evaporated under a stream of nitrogen gas and the unsaponifiable fraction was dissolved in 50 µL of n-hexane:diethyl ether (75:25, *v*/*v*).

#### 3.2.2.3 *Enrichment of oxycholesterols by solid-phase extraction*

A silica cartridge was pre-equilibrated with 3 mL of hexane. The dissolved unsaponifiable fraction was loaded onto the cartridge. First, 3 mL of n-hexane:diethyl ether (75:25, *v*/*v*) was used to elute the first fraction. The second fraction was eluted with 3 mL of n-hexane:diethyl ether (60:40, *v*/*v*). The addition of 4 mL of acetone:methanol (60:40 *v*/*v*) was used to elute the oxycholesterols. The last fraction was dried under a stream of nitrogen gas. The dried enriched extract was either dissolved in methanol for SFC–MS/MS analysis, or derivatized to form trimethylsilyl ether (TMS-ether) derivatives for GC–MS analysis.

#### 3.2.2.4 *Preparation of TMS-ether derivatives of oxycholesterols*

A mixture of hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) in anhydrous pyridine (3:1:9) was used as silylation reagent. To the dried enriched extract 100 µL of HMDS/TMCS/Pyridine was added. The reaction mixture was incubated for 30 min at 60 °C. The excess reagent was dried under a stream of nitrogen gas and dissolved in 100 µL of hexane before it was injected into the gas chromatograph for GC–MS analysis.

## 3.3 Instrumentation and analytical methods

### 3.4.1 Supercritical fluid chromatography–tandem mass spectrometry (SFC–MS/MS)

The SFC used for chromatographic separation of steroids and oxycholesterols was a Waters ACQUITY® UPC$^{2}$™ (Milford, MA, USA) system. The columns used for human steroid analysis (**Paper I**) and chicken steroid analysis (**Paper II**) were Acquity UPC$^{2}$ BEH columns (Waters, Milford, MA, USA) with dimensions of 150 mm × 3.0 mm × 1.7 µm and 100 mm × 3.0 mm × 1.7 µm, respectively. Detailed conditions for steroid separation are described in **Papers I–II**.

Separation of oxycholesterols was performed on an Acquity UPC$^2$ CSH fluorophenyl column (100 mm × 3.0 mm × 1.7 µm; Waters, Milford, MA, USA) held at 55 °C. A gradient elution (eluent A, $CO_2$; eluent B, methanol) was performed with a mobile phase flow rate maintained at 3.0 mL/min. The gradient was programmed as follows: 2% of component B at the start, a linear gradient from 2% B to 17% B for 2.01 min, a linear gradient down to 2% B for 1.0 min, and held for 1.0 min at 2 % B for ionic liquids to elute from the instrument. The back pressure was set at 1800 psi and the injection volume was 1.0 µL. The make-up solvent was set to a flow rate of 0.4 mL/min and consisted of 0.1% ammonium hydroxide in methanol:water (97.5:2.5, *v*/*v*).

A Waters Xevo triple-quadrupole (TQ-S) mass spectrometer (Milford, MA, USA) was used as the detector for all analyses performed by SFC. The TQ-S was equipped with an ESI ion source for steroid analysis (**Papers I–II**) and an APCI ion source for oxycholesterol analysis. Detection conditions for steroid identification are reported in **Papers I–II**.

Data acquisition for the identification of oxycholesterols was performed in APCI+ mode with a range of *m*/*z* 354–402. The desolvation temperature was set to 500 °C and the desolvation gas (nitrogen) maintained at a flow rate of 750 L/h. The source temperature was kept at 150 °C, the cone gas flow rate set to 150 L/h and the nebulizer gas was maintained at a pressure of 7 bar. The collision gas was argon at a gas flow of 0.25 mL/min. The corona needle voltage and cone voltage was set to 1.5 kV and 50 V, respectively. MS data were collected using two scan functions both set at 5 eV collision energy, which enabled parent to parent ion detection as a double focusing mechanism and noise reduction measure. The scan time for each function was automatically optimized by the software. Data acquisition and analysis were performed with Waters MassLynx v4.1 software.

### 3.4.2 Gas chromatography–mass spectrometry

A Bruker 451-GC gas chromatograph (Billerica, MA, USA) with a split/splitless (S/SL) inlet and a GC PAL autosampler (Billerica, MA, USA) was used for separation of oxycholesterols. The GC was equipped with a 20-m DB-5MS analytical column (0.18 mm, 0.18 µm; Agilent, Kista, Sweden). Helium was used as the carrier gas at a flow rate of 1.5 mL/min. The inlet temperature was set to 300 °C and an injection split ratio of 100:1. A gradient oven temperature program was used and started at 250 °C for 0.5 min, increased to 290 °C at a rate of 50 °C/min for 0.8 min, followed by an increase to 295 °C at a rate of 0.5 °C/min for 10 minutes. Finally the temperature was raised to 315 °C at a rate of 50 °C/min and held for 2 min to obtain a total analytical time of 13.7 min.

Detection was performed by a Scion TQ triple quadrupole mass spectrometer (Bruker, Bremen, Germany). The mass spectra were recorded at electron energy of 70 eV with the electron impact (EI) ion source temperature and transfer line temperature set to 220 °C and 270 °C, respectively. The spectra were scanned in the range *m*/*z* 50–600. The COPs were identified by comparing retention time (RT) and the mass spectra with those of standards. Data acquisition and analysis were performed with Bruker Daltonics MS Workstation v8.2 software.

# 4. Results and discussion

## 4.1 Paper I

The main goal in **Paper I** was to develop a simple, rapid and sensitive SFC–MS/MS method for the simultaneous analysis of steroids from the four major classes. The application of this method to analysis of steroids in minimum blood volume as a diagnostic tool might become important for early detection of AD. SFC enables fast and high resolution separation of polar, non-polar and ionizable analytes due to the lower viscosity of supercritical fluid ($CO_2$) compared with solvents used in LC and requires considerable less sample preparation than methods using GC.

We have successfully achieved a fairly resolved separation of 18 different peripheral steroids in 5 min. This method is novel and there is a lack of literature for us to compare. The most techniques reported are GC–MS/MS or LC–MS/MS methods focused on the determination of only a few steroids within one class or a few classes.[9] Furthermore, methods analyzing steroids by SFC–MS have received limited attention over the past decade. According to our knowledge there are only two reported studies of analysis of steroids and their metabolites from one class (estrogens)[39] and two classes (estrogens and androgens),[40] respectively.

Initial screening of steroid standards revealed that certain steroids, e.g. PREG, were not detectable and others, e.g. testosterone and DHEA, could only be observed at concentrations above 1 ng/mL. Therefore, a derivatization step was added to the sample work-up procedure which included extraction of the steroids from plasma. Liquid-liquid extraction (LLE) with acetonitrile and MTBE was evaluated with MTBE found to be the best for a satisfactory extraction. Purification or enrichment methods were not performed prior to derivatization. Derivatization with methoxyamine (MO) which reacts with carbonyl groups to form the corresponding oximes of the steroids provided improved selectivity and detection sensitivity of the steroid analytes.

Derivatization resulted in mono-MO derivatives for nine of the steroids and di-MO derivatives for the other nine. Additionally, the derivatization resulted in the formation of two isomers for eleven different steroids and both peaks were used during quantification of these eleven steroids. Those corresponding peaks of geometric syn- and anti-isomers of oximes show baseline separation.[21] Also, before reporting the data we have optimized the incubation condition of the MO derivatization.

Mass spectrometric conditions were optimized and the best results were obtained using ESI in positive mode for all eighteen steroids. Methanol with the addition of 0.1 % formic acid enhanced the ionization efficiency.

The column selectivity was assessed by different stationary phases with the BEH column providing the best peak shapes and resolution of the isomeric/isobaric pairs of steroids such as testosterone/ dehydroepiandrosterone, androsterone/etiocholanolone, corticosterone/11-deoxycortisol, and 17α-hydroxyprogesterone/11-deoxycorticosterone. The addition of low concentration of formic acid as an additive in the mobile phase increases the solubility of derivatized steroids and thereby results in symmetric peak shapes.[26] Six co-solvents were evaluated with 0.1 % formic acid in 50/50, *v*/*v*, methanol:isopropanol providing the best results. The flow rate, column temperature, back pressure, and make-up solvent conditions were optimized by additional tests.

The validation was performed by determining the linear range, accuracy, precision, limit of quantification and recovery of the steroids. The linear range of the method was determined from calibration curves and yielded correlation coefficients ($R^2$) above 0.998. The results obtained for intraday and interday precisions were between 0.8 % and 10 % for most of the steroids and the accuracy was within ±15 %. The quantitative recovery of steroids in plasma was evaluated with mean recoveries in the range of 81–107 %. The limit of quantification (LOQ) for most of the steroids was less than 0.1 ng/mL with a few exceptions.

This SFC–MS/MS method is novel and provides simultaneous analysis of eighteen peripheral steroids from all four major classes in 5 min.

## 4.2 Paper II

The main aim for our contribution to **Paper II** was to develop a method in order to measure three steroid hormone levels in White Leghorn (WL) and Red Junglefowl (RJF) birds. As part of the study, the plasma levels of three central steroids with adrenal origin, pregnenolone (PREG), dehydroepiandrosterone (DHEA), and corticosterone (CORT) were compared at baseline and after restraint stress (ten minutes of physical restraint in a hanging net) in six weeks old domesticated WL and ancestral RJF birds in order to evaluate the effects of domestication on acute stress sensitivity of chickens at hormonal levels.

The method development procedure is not reported in **Paper II** as it was an application paper and is summarized here. A SFC system coupled to a triple quadrupole MS provided an opportunity to develop and apply a novel method for the analysis of steroids. Sample preparation included extraction and derivatization of the steroids prior to analysis by SFC–MS/MS. Steroid standards of DHEA, PREG and CORT and their internal standards (IS) $d_2$-DHEA, $^{13}C_2$-$d_2$-PREG and $d_4$-cortisol (dF) were used for method development and optimization. MS conditions and SRM transitions were optimized for each steroid and IS (Table 2). Separation of the steroids was evaluated by a 3 min generic screening gradient from 2–20 % methanol as modifier to $CO_2$ on four Waters Acquity UPC$^2$ columns (BEH, BEH 2-EP, CSH fluorophenyl and HSS C18 SB). All columns had dimensions of 100 mm × 3.0 mm × 1.7 µm. The BEH stationary phase provided the most promising resolution. Methanol as co-solvent proved sufficient in obtaining the required separation with the gradient elusion and make-up solvent optimized to the conditions reported in **Paper II**.

Triplicate calibration curves with a concentration range as reported in Table 3 were prepared by adding 100 µL of a mixture dilution of the steroids to 200 µL of steroid-free plasma. A mixture of the IS was prepared in methanol at a concentration of 1 ng/mL and 100 µL added to each sample.

Table 2. *Separation and mass spectrometric parameters for the identification of the oxime derivatives of steroids.*

| Compound | Derivative | RT (min) | Precursor ion (m/z) | Product ions (m/z) | Collision energy (eV) | Dwell time (s) |
|---|---|---|---|---|---|---|
| DHEA | DHEA-MO | 0.91 | 318.3 | 110.2/253.2/286.2 | 25/17/18 | 0.025 |
| PREG | PREG-MO | 0.83 | 346.2 | 100.1/300.1 | 23/26 | 0.028 |
| CORT | CORT-diMO | 1.07 | 405.1 | 343.1 | 28 | 0.112 |
| dDHEA | dDHEA-MO | 0.91 | 320.2 | 112.2/255.2/288.2 | 27/17/18 | 0.025 |
| cPREG | cPREG-MO | 0.83 | 350.3 | 104.3/304.3 | 27/21 | 0.028 |
| dF | dF-diMO | 1.41 | 425.2 | 288.0/363.0 | 28/29 | 0.112 |

RT: retention time.

Table 3. *Parameters for steroid quantification.*

| Compound | IS | Calibration equation | $R^2$ | Linear range (ng/mL) | LOQ (ng/mL) |
|---|---|---|---|---|---|
| DHEA | dDHEA | 0.711x + 0.0079 | 0.9989 | 0.05–2 | 0.05 |
| Pregnenolone | cPREG | 1.7005x – 0.034 | 0.9999 | 0.05–10 | 0.05 |
| Corticosterone | dF | 1.5892x – 0.1699 | 0.9995 | 0.05–25 | 0.05 |

IS: internal standard; $R^2$: correlation coefficient; LOQ: limit of quantification.

Plasma samples from the birds were provided by collaborators at Linköping University. In total 48 samples from 12 birds of each breed and sex were used in the study. Sample preparation of all bird plasma samples were performed individually. Each sample was analyzed in triplicate in SFC–MS/MS to test the repeatability, and a CV < 10% was accepted.

The results indicated that the basal levels of PREG and DHEA were significantly higher in RJF but there was no significant difference between WL and RJF in baseline levels of CORT. Ten minutes of physical restraint led to significant elevation of the CORT levels in both WL and RJF, with the levels after restraint being higher in RJF (Figure 6). With the exception of basal CORT, there was no significant effect of sex on the levels of the measured hormones. The results supported previous observations that endocrinological stress responses, and the associated breed levels of steroid hormones, have been modified by domestication in chickens.

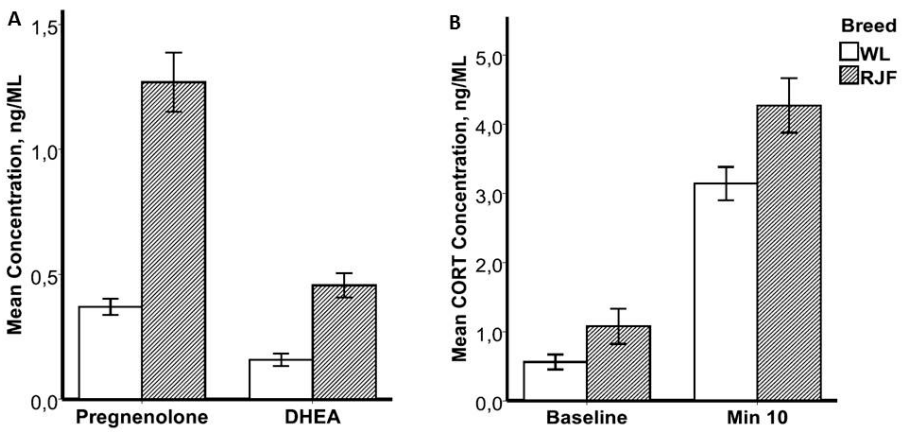


*Figure 6.* Serum concentrations of (a) baseline pregnenolone and DHEA and (b) baseline and post-restraint levels of corticosterone in domestic White Leghorn (WL; n=24) and wild Red Junglefowl (RJF; n=24). The values are given as mean ±SEM. The statistics for the figures is presented in **Paper II** (Table 1).

## 4.3 Oxycholesterols

The oxycholesterols analyzed in this study were sterol ring and side chain oxygenated cholesterols, epimeric epoxycholesterols and cholesteroltriol. The separation and identification were conducted by SFC–MS/MS and GC–MS. Sample preparation included saponification, extraction and enrichment prior to analysis by SFC–MS/MS with the addition of a derivatization step after enrichment for GC–MS. Oxycholesterol standards were used for method development.

### 4.3.1 SFC–MS/MS method development

Standard solutions of eleven oxycholesterols were introduced into the ion source by direct infusion using IntelliStart™. APCI in positive mode produced the best results for all tested oxycholesterols. Due to the stability of the molecular ion and a lack of product ion formation of oxycholesterols in APCI, the $[M+H]^+$, $[M–H_2O+H]^+$ and/or $[M–2H_2O+H]^+$ ions were selected for most of the oxycholesterols to construct the SRM method with parent to parent ion monitoring. Methanol:water (97.5:2.5, *v*/*v*) with the addition of 0.1 % ammonium hydroxide as make-up solvent enhanced the ionization efficiency. The optimized MS conditions are described in Section 3 and the SRM parent to parent ion monitoring conditions reported in Table 4.

Table 4. *A list of analyzed COPs with corresponding SRM transition ions.*

| Name | Abbreviation | Precursor ion | Product ion | Dwell time (s) | Collision energy (eV) |
|---|---|---|---|---|---|
| 7α-Hydroxycholesterol | 7αHC | 385.1 | 385.1 | 0.121 | 5 |
| | | 367.2 | 367.2 | | |
| 7β-Hydroxycholesterol | 7βHC | 385.1 | 385.1 | 0.079 | 5 |
| | | 367.2 | 367.2 | | |
| α-Epoxycholesterol | αCE | 385.1 | 385.1 | 0.121 | 5 |
| | | 367.1 | 367.1 | | |
| 7β-Epoxycholesterol | βCE | 385.1 | 385.1 | 0.122 | 5 |
| | | 366.9 | 366.9 | | |
| Cholesteroltriol | CT | 384.9 | 384.9 | 0.076 | 5 |
| | | 367.0 | 367.0 | | |
| 7-Ketocholesterol | 7KC | 401.2 | 401.2 | 0.121 | 5 |
| | | 383.2 | 383.2 | | |
| 20α-Hydroxycholesterol | 20αHC | 385.0 | 385.0 | 0.078 | 5 |
| | | 367.0 | 367.0 | | |
| 24(S)-Hydroxycholesterol | 24HC | 385.1 | 385.1 | 0.121 | 5 |
| | | 367.1 | 367.1 | | |
| 25-Hydroxycholesterol | 25HC | 385.1 | 385.1 | 0.121 | 5 |
| | | 367.1 | 367.1 | | |
| 26-Hydroxycholesterol | 26HC | 385.1 | 385.1 | 0.122 | 5 |
| | | 367.1 | 367.1 | | |
| 19-Hydroxycholesterol (IS) | 19HC | 385.1 | 385.1 | 0.121 | 5 |
| | | 354.9 | 354.9 | | |

Four columns with different stationary phases were screened for their ability to separate the 11 oxycholesterols:

1. Acquity UPC$^2$ BEH (100 mm × 3.0 mm × 1.7 µm);
2. Acquity UPC$^2$ BEH 2-EP (100 mm × 3.0 mm × 1.7 µm);
3. Acquity UPC$^2$ CSH fluorophenyl (100 mm × 3.0 mm × 1.7 µm); and
4. Acquity UPC$^2$ HSS C18 SB (100 mm × 3.0 mm × 1.7 µm).

Separation of oxycholesterols was achieved with the CSH fluorophenyl stationary phase. Different column temperatures were investigated and the best resolution was obtained at 55 °C. The mobile phase modifier, flow rate, gradient program and back pressure were evaluated. A decrease in flow rate resulted in broadening of the peaks, whilst an increase above 3 mL/min was avoided due to overpressure of the system. An increase in the back pressure required a decrease in the flow rate and/or the percentage of modifier used. The chromatographic separation of the oxycholesterols is presented in Figure 7.

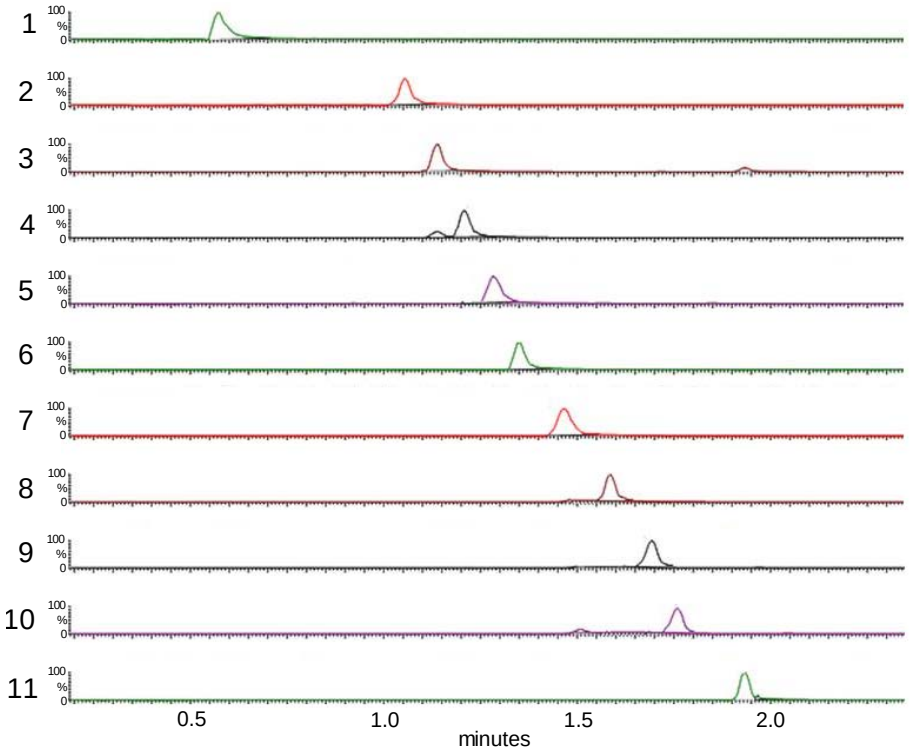


*Figure 7.* Resolution of standard mixture of COPs under the analytical conditions described in Section 3. Peak identification: (1) 20αHC; (2) βCE; (3) αCE; (4) 25HC; (5) 24HC; (6) 26HC; (7) 19HC (IS); (8) 7KC; (9) 7αHC; (10) 7βHC; (11) CT.

### 4.3.2 GC–MS method development

A mixture of eleven oxycholesterols and two standards were prepared, derivatized and analyzed by GC–MS in full scan mode. A generic temperature program starting at 80 °C and raised at 15 °C/min to 250 °C followed by an increase of 10 °C/min to 300 °C was used to evaluate five different columns for separation of the analytes in the mixture, namely

1. 15-m Rxi-5Sil MS (0.25, 0.25; Restek)
2. 20-m DB-5MS (0.18, 0.18; Agilent)
3. 30-m DB-5MS (0.25, 0.25; Agilent)
4. 20-m DB-35MS (0.18, 0.18; Agilent)
5. 20-m DB-17MS, (0.18, 0.18; Agilent)

The most promising separation was obtained with the two DB-5MS columns. Different temperature gradient programs were assessed and a faster run time was acquired with the 20-m DB-5MS column. Separation was achieved within 5 min. The mass spectra for the TMS-ether derivatives of oxycholesterols were used in identifying the different eluting peaks. The most abundant ions were selected to construct a SIM method. The separation conditions are reported in the material section with the separation data listed in Table 5. A depiction of the chromatographic separation is presented in Figure 8.

Table 5. *Ions for selected ion monitoring (SIM), retention times (RT) and relative retention times (RRT) of the TMS-ether derivatives of standard samples of oxycholesterols (COP).*

| COP | SIM ions | RT | RRT[a] | RRT[b] |
|---|---|---|---|---|
| 5αCh | 217.3/357.7/372.7 | 2.08 | 0.64 | 1.00 |
| 7αHC | 129.2/456.7/546.5 | 2.83 | 0.88 | 1.36 |
| 19HC | 129.2/353.6/456.7 | 3.24 | 1.00 | 1.55 |
| 7βHC | 129.2/456.7/546.5 | 3.42 | 1.06 | 1.65 |
| 4βHC | 129.2/366.6/456.7 | 3.57 | 1.10 | 1.72 |
| βCE | 129.1/145.2/474.7 | 3.62 | 1.12 | 1.74 |
| αCE | 129.1/145.2/474.7 | 3.70 | 1.14 | 1.78 |
| 20αHC | 129.1/281.2/456.7 | 3.97 | 1.23 | 1.91 |
| CT | 129.2/456.8 | 4.11 | 1.27 | 1.97 |
| 24HC | 129.1/145.2/456.6/545.9 | 4.36 | 1.35 | 2.09 |
| 25HC | 131.2/456.8 | 4.50 | 1.39 | 2.16 |
| 7KC | 129.1/367.6/472.7 | 4.60 | 1.42 | 2.21 |
| 26HC | 129.2/456.7/546.7 | 4.95 | 1.53 | 2.38 |

[a] Retention time relative to 19HC.
[b] Retention time relative to 5αCh.

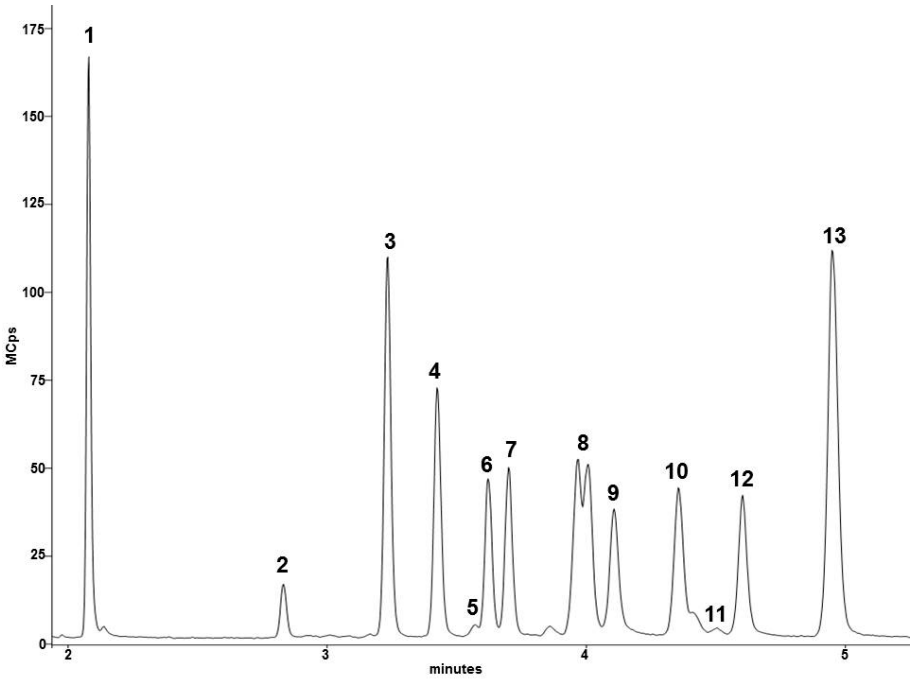


*Figure 8.* GC–MS full scan trace (TIC) of TMS derivatives of COPs standard mixture under the analytical conditions described in Section 3. Peak identification: (1) 5αCh (IS2); (2) 7αHC; (3) 19HC (IS1); (4) 7βHC; (5) 4βHC; (6) βCE; (7) αCE; (8) 20αHC; (9) CT; (10) 24HC; (11) 25HC; (12) 7KC; (13) 26HC.

# 5. Conclusions and future perspectives

Developing fast, reliable and sensitive analytical techniques is pivotal in determining and evaluating bioactive steroids and oxycholesterols in biological samples.

As described in **Paper 1**, we have developed and optimized a SFC–MS/MS method for targeted analysis of 18 steroids from the four major classes, namely estrogens, androgens, progestogens and corticosteroids. Inclusion of a derivatization step prior to analysis improved sensitivity of detection and outweighed the drawback of an increased analytical time. Different stationary phases, mobile phase co-solvent systems, and gradient elusions were assessed after MS detection conditions were established. The validation data suggests that it is possible to identify and quantify these steroid analytes in small sample volumes. The performance of the validated method was tested with plasma samples collected from AD (n = 10) and control patients (n = 10) (Uppsala biobank). The novel data will be published in a separate manuscript in the near future.

A study on the domestication effects on stress induced steroid secretion and adrenal gene expression in chickens required a sensitive method for determining steroid levels in plasma (**Paper II**). A similar SFC–MS/MS method to the one used in **Paper I** was successfully developed and applied to the analysis of three adrenal steroids in plasma from domesticated White Leghorn (WL) chickens and Red Junglefowl (RJF) birds. Results obtained from quantification of the steroids revealed that plasma levels of pregnenolone and dehydroepiandrosterone were significantly higher in the juvenile RJF in comparison with the domesticated WL chickens, but the baseline levels of corticosterone were similar between the breeds. However, restraining stress caused a significantly larger increase in corticosterone in RJF than in WL. Steroid hormone levels in domesticated WL and RJF were determined for a second study with a much larger number of individual birds (233 birds) and the results will be published in the near future.

Two methods for the determination of more than ten oxycholesterols in biological samples have been described. Separation of the oxycholesterols has been achieved on SFC–MS/MS and GC–MS. On both systems, various columns were investigated and MS conditions established. Despite reasonable

resolution obtained with the SFC–MS/MS method, detection sensitivity should be improved. Derivatization of the oxycholesterols together with using ESI as ion source will be investigated next. A combination of the GC columns in series will be attempted to increase the resolution of the compounds.

All of the main objectives have been achieved in this work. Further optimization of the methods used for the oxycholesterol determination is needed. However, all of these methods could find application in the clinical environment for assessing endocrine, metabolic and neurodegenerative diseases. We will develop and improve on methodological approaches such as dried blood spot (DBS), which is economical and easy to use in the clinical environment.

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