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VITAMIN D AND HAIR: A REVIEW OF CURRENT METHODS AND VALIDATION OF A QUANTITATION METHOD TO MEASURE 25(OH)D_3 FROM HAIR

SCHOOL OF MEDICINE

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Publications

- A systematic review by P. Maher, M. Healy, E. Laird, W. Gao, J. Marunica Karšaj, L. Zgaga titled “The Determination of Endogenous Steroids in Hair and Fur: A Systematic Review of Methodologies” has been submitted to The Journal of Steroid Biochemistry and Molecular Biology and was published in February 2025.
- A poster describing the findings from the systematic review titled “Sample preparation, extraction and quantification of endogenous steroids from hair and fur: A systematic review” by P. Maher, E. Laird, M. Healy and L. Zgaga was presented by P. Maher at the 2023 Society of Hair Testing annual conference in Lisbon, Portugal. 7-9th June 2023.
- A presentation of validation and sample testing results was given at the 2024 Irish Mass Spectrometry Society annual conference. The title was “vitamin D analysis using LC-QQQ Mass spectrometry” by P. Maher, E. Laird, M. Healy and L. Zgaga. This was presented by P. Maher in University College Dublin, Ireland on the 15th of May 2024.

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Figures and Tables

List of Abbreviations

%RE	Percentage of Relative Error
%CV	Percentage coefficient of Variation
1, 25$(OH)_2D_3$	1,25 dihydroxy-vitamin D_3
17-OHP	17- Hydroxyprogesterone
25$(OH)D_3$	25 hydroxy-vitamin D_3
APCI	Atmospheric Pressure Chemical Ionisation
BMI	Body Mass Index
c9-c10	Carbon bond between carbon 9 and carbon 10
CINAHL	Cumulative Index to Nursing and Allied Health Literature
CLIA	Chemiluminescent Immunoassay
DEQAS	Vitamin D External Quality Assessment Scheme
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone Sulphate
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay
EMBASE	Excerpta Medica Database
FDA	Food and Drug Administration
GCMS	Gas Chromatography Mass Spectrometry
INAB	Irish National Accreditation Board
IPA	Isopropanol
IQR	Interquartile Range
IRMS	Isotope Ratio Mass Spectrometry
LC-MS/MS	Liquid Chromatography tandem Mass Spectrometry
LCTOFMS	Liquid Chromatography Time-Of-Flight Mass Spectrometry
MeOH	Methanol
MRM	Multiple Reaction Monitoring
ms	Millisecond
NIST	National Institute for Standards and Technology
nmol/L	Nanomole per Litre
NMR	Nuclear Magnetic Resonance

pg/mg	Picogram per Milligram
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
RIA	Radioimmunoassay
RMP	Reference Measurement Protocol
rpm	Revolutions Per Minute
SIM	Selected Ion Monitoring
SoHT	Society of Hair Testing
TRFIA	Time-Resolved Fluorescence Immunoassay
UHPLCMS	Ultra-High-Pressure Liquid Chromatography Mass Spectrometry
UHPLC-QTOF-MS	Ultra-High-Pressure Liquid Chromatography Quadrupole Time-Of-Flight Mass Spectrometry
UV	Ultraviolet
UVB	Ultraviolet Blue

Abstract

Introduction: Hair has emerged as a promising matrix for assessing endogenous steroid hormone levels, yet its potential for the quantitation of vitamin D remains unexploited. Vitamin D status is typically assessed by measuring the circulating concentration of 25-hydroxyvitamin D_3 (25(OH)D_3) from a blood sample. 25(OH)D_3 is the most abundant, "storage" form of vitamin D3 and serves as the biomarker for vitamin D status. This study aimed to develop and validate a liquid chromatography mass spectrometry (LC-MS/MS) method for measuring 25(OH)D_3 in human and animal hair.

Method: A systematic review was conducted on 180 studies which describe endogenous steroid hormone analysis (cortisol, testosterone, androstenedione, dehydroepiandrosterone sulfate, 17-hydroxyprogesterone and vitamin D) in hair of humans and animals. Current methodologies employed in hair analysis were identified, to inform method of 25(OH)D_3 assessment. The validation of a method to measure 25(OH)D_3 in hair employed five validation parameters to evaluate suitability. A standard calibration curve to check linearity, limit of quantitation and limit of detection of the assay, accuracy, precision and comparison between hair preparation and effect on results. This method was then be applied to human and animal hair samples.

Results: Review findings suggest that while hair is a viable matrix for steroid measurement, further refinement is needed before it can be routinely applied in clinical settings as clinical requirements are more stringent. Guidelines for steroid testing in hair should standardise practices for sample collection, storage, pre-treatment, and analysis, alongside validation protocols, all of which were found to be varied among studies. The validation of the proposed method for quantifying 25(OH)D_3 in hair was completed and provided a highly sensitive, selective and reliable method. This validated method was used for hair samples from human, bovine, chimpanzee and lemurs. The difference in levels from human (Mean; 146 pg/mg) to animal (Mean; 1585 pg/mg) varied largely. Animals are routinely supplemented when in captivity and do not seem to be affected by toxicity at the same levels as humans.

Conclusion: The future of hair analysis of 25(OH)D_3 and other endogenous steroid hormones could be a useful tool in clinical and research settings. Further testing with larger sample size of varying age, sex and hair type is required in subsequent studies.

Lay Abstract

Vitamin D levels are measured from a blood sample taken at your local doctor or when in hospital. Measurement is usually carried out in a hospital laboratory and shows vitamin D levels in blood at the time the sample was taken. To measure vitamin D from hair could be an alternative. Hair has been used regularly to detect illegal drugs and monitor levels of drugs of abuse in a patient.

This research begins by looking at all work done until now in established areas of hormone monitoring in hair, such as cortisol in monitoring stress. All work published in the past thirty years will be reviewed. As part of this research we created a method to measure vitamin D from hair using the same method which is used for blood measurement. This method was then be used to assess human and animal hair samples received from research colleagues in Europe.

The review of past work in hair analysis describing how hormones are measured from hair was carried out. The results show there should be a better agreement between different laboratories and how they measure their markers. In doing this would allow each lab to see how they compare to each other and take action to improve if required. A method to measure vitamin D from hair was developed and test results showed the test to be accurate and precise when measuring vitamin D from hair. The method was used on human and animal hair samples and detected vitamin D in all samples. Animal hair samples, particularly monkeys had a much higher concentration of vitamin D when compared to humans. This could be explained by them being given vitamin D supplements while in captivity.

The validation of a method to measure vitamin D from hair could be a useful tool to monitor vitamin D status. Further testing on hair from people with varying age, gender and hair type will be needed in future studies.

Aims and Objectives of the project

Aims

- The primary aim of this research is to validate a method for quantifying vitamin D concentration from hair samples.

Objectives

- Conduct a systematic review to compare previously published methods for analysing endogenous steroids in hair, focusing on those structurally similar to vitamin D and commonly analysed in hair. The review will provide insights into hair sample collection, storage, preparation, and analysis.
- Develop and validate a method for measuring vitamin D in hair, evaluating its accuracy and precision by following established hair analysis validation protocols and assessing it against five key validation parameters.

Chapter 1: Introduction

1. Vitamin D

Vitamin D plays a major role in the absorption and metabolism of calcium and phosphorus which are in turn critical for normal bone homeostasis (1, 2). Vitamin D_3 is a secosteroid, also known as cholecalciferol, which is synthesised in the skin of both humans and animals. Vitamin D_2 , also known as ergocalciferol, is synthesised by plants and fungi (3).

The structure of secosteroids share numerous similarities with steroid hormones such as cortisol or testosterone, the active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), has hormone like properties, interacting and activating a specific vitamin D binding receptor (4).

The term 'vitamin' has been suggested as inaccurate because of the ability of skin to synthesise the molecule secondary to sunlight exposure. A true vitamin can only be obtained through food sources. However, food sources of vitamin D_3 and D_2 are scarce, particularly in the Western diet. Food sources of vitamin D_3 and D_2 are egg yolk, oily fish, red meat, mushrooms and vitamin D fortified food (5). Furthermore, synthesis can be adversely affected by age, body mass index (BMI), clothing choice, sunscreen use, an indoors oriented lifestyle, ethnicity and skin colour (6, 7). Therefore many populations are vulnerable to the risk of low vitamin D status.

Sources of vitamin D_3 for humans are exposure to sunlight resulting in dermal synthesis (8-12) and supplementation (13). The actions of a factor related to sunlight and certain foods was discovered in the 1920's. The structure of vitamin D was determined in the 1930's (14). Vitamin D_3 deficiency is of serious concern worldwide with almost one billion estimated to be deficient (< 30 nmol/L) (15-17). The lifestyle choices we make such as living and working the majority of time indoors, wearing protective clothing and skin products (which block UVB light) as well as pollution could all contribute to lessening skin synthesis of vitamin D (18, 19).

2. Vitamin D synthesis

Vitamin D is a pre-prohormone that is unique in that it is synthesised in the skin by the actions of solar radiation, specifically ultraviolet blue ((UVB) 280-315 nm) light on 7-dehydrocholesterol (20). The secosteroid family, of which vitamin D is a member, is distinctive due to an activation reaction leading to the C9-C10 bond being opened in ring B of the steroid structure caused by the absorption of UV light (3).

UVB at 280-315 nm wavelength enters the skin and induces photolysis of 7-dehydrocholesterol to pre-vitamin D_3 in the deep cellular layer of the epidermis. Under the influence of temperature (optimal 37°C), pre-vitamin D_3 is isomerised to vitamin D_3 (cholecalciferol) and subsequently released into the circulation.

After ingestion or synthesis in the skin of vitamin D_3, the liver hydroxylates vitamin D_3 to 25-hyroxyvitamin D_3 ($25(OH)D_3$) catalysed by the enzyme, 25-hydroxylase (CYP2R1). This is the most abundant form of vitamin D in the human body and is used as storage form and also as a biomarker of vitamin D status because of its long circulating half-life (3-4 weeks) (21).

In the kidney, $25(OH)D_3$ serves as a substrate for 1-alpha-hydroxylase (CYP27B1) yielding 1,25-dihydroxycholecalciferol ($1,25(OH)_2D_3$), the biologically active hormonal form of vitamin D_3. This reaction may be seen in **Figure 1**.

Presence of UVB depends on the angle between the sun and earth's surface, and both latitude and altitude. At high latitudes (>50° North or South), meaningful productive synthesis only occurs during the spring and summer months resulting in an increased dependence on dietary sources during the winter (22). Latitudes higher than 45-50° (e.g: France and Ireland) have sufficient UVB present for just 5-6 months, April to September (23). The higher the altitude, the less atmosphere there is available to absorb UV radiation. With every 1000 m in altitude, UV levels increase by approximately 10% (24, 25).

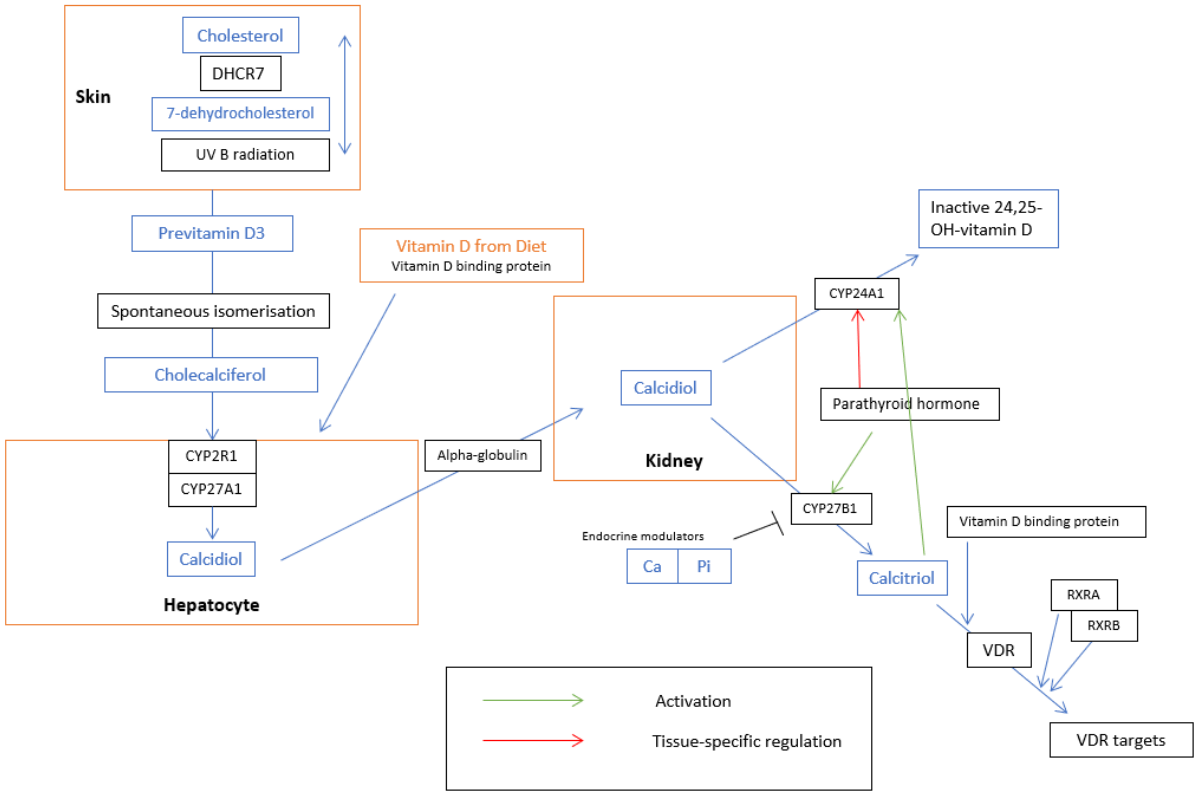


Figure 1 Synthesis of vitamin D_3 in the body.

Beginning on the surface of the skin and influenced by diet, Cholecalciferol is hydroxylated in the liver to $25(OH)D_3$, then hydroxylated again in the kidneys to $1,25(OH)_2D_3$

3. The importance of vitamin D for health

3.1 Phosphate homeostasis

Vitamin D controls phosphate and calcium balance within the body by promoting calcium and phosphate absorption from intestines and kidneys. If deficient in vitamin D, only 10% of calcium and 50% of phosphate may be absorbed from foods (1).

3.2 Rickets and Osteomalacia

Rickets is a clinical syndrome in children resulting in softening and weakening of bones as a result of the delay/ failure of mineralisation of the growth plate of growing bones (21). Vitamin D deficiency rickets is most prevalent in children under 2 years of age with its peak between 18 months and 3 years. Because $25(OH)D_3$ may be readily transported through the placenta, few cases are identified in infants. Adding vitamin D to the diet generally alleviates the bone problems as a consequence of rickets (26). Osteomalacia is a similar condition in adults and may be reversed with a vitamin D supplement over weeks/months (27).

3.3 Vitamin D and Immune function

The role of vitamin D in the regulation of immune cell proliferation, differentiation and responsiveness has been more closely studied in recent times (28, 29). It has been shown that supplementation with vitamin D or its precursors pre- and post-transplant reduces the risk of rejection (30).

Vitamin D increases the efficacy of the innate and adaptive immune system (29, 31). Monocytes and macrophages produce $1,25(OH)_2D_3$ with intracellular antimicrobial effects and can also interact with and control the cytokine profile of activated T and B cells (31). This production of $1,25(OH)_2D_3$ is dependent on sufficient serum concentrations of $25(OH)D_3$.

3.4 Cardiovascular effects and vascular calcification

Vitamin D plays a bidirectional role in human health. Insufficiency and over-supplementation (toxicity) may compromise cardiovascular health (32). Vitamin D

insufficiency is associated with significant undesirable changes in cardiovascular physiology and structure (33). There have been studies showing the possibility that vitamin D supplementation may reduce incidence of major cardiovascular events (34, 35). About 25% of people over 60 have a decline in renal function, awareness of chronic renal insufficiency is not well known. Renal function impairment may impact the production of the active form of vitamin D and result in loss of control of calcium homeostasis, causing calcification of the vascular network of the body (36).

3.5 Toxicity

Vitamin D toxicity is rare but may lead to hypercalcemia which is life-threatening if not identified early (37). Excessive consumption of vitamin D supplement is most common with exogenous supplementation (38, 39). The publications stating vitamin D's benefit in numerous clinical scenarios for bone and cell health have increased and with that the administration rate of vitamin D will increase, possibly increasing the prevalence of vitamin D toxicity (39).

4. Vitamin D status

Vitamin D status is typically assessed by measuring the circulating concentration of 25-hydroxyvitamin D_3 (25(OH)D_3) and 25-hydroxyvitamin D_2 (25(OH)D_2) in plasma or serum extracted from the blood sample (40). 25(OH)D_3 is the most abundant, “storage” form of vitamin D_3 and serves as the biomarker for vitamin D_3 status. 25(OH)D_2 is present in small amounts in comparison to 25(OH)D_3 in humans unless supplementation occurs (41).

4.1 Measurement of vitamin D

25(OH)D_3 is often measured in circulation by ELISA (40) immunoassay although this method can lack specificity and accuracy (42). The recognised gold standard method is liquid chromatography-mass spectrometry (LC-MS/MS) which has excellent sensitivity and specificity and is superior compared to other methods (17, 43-45).

4.2 Collection of blood for measurement

The collection of blood samples is routine; however it is nonetheless a multi-step process which has some disadvantages. Blood collection may cause patients discomfort as it is invasive. Healthcare staff who carry out collection must have appropriate training, adequate equipment together with appropriate reagents, and a hygienic environment to ensure patient safety. The collected blood sample is a potential biohazard and must be handled carefully by trained staff to prevent spread of infection. Transport of samples can be problematic, due to analyte stability in blood and required storage environment (4°C) (46). Because of this, collection of blood samples for analysis may be restricted to when required. Furthermore, each sample represents a single point in time measurement of circulating vitamin D, but factors such as illness and diurnal rhythms could result in daily variability of up to 20%, in addition to seasonal variability (47, 48). In summary, traditional measurement of 25(OH)D_3 yields only information on status at the time of blood draw and therefore gives little information on longer term vitamin D status (48).

Analysis in hair could be an alternative. The use of hair analysis has become an increasingly widespread tool for the detection and quantification of a variety of compounds including steroid hormones and drugs, both illegal and therapeutic. For example, testosterone in monitoring of transitioning patients, or exogenous steroid detection in professional sport (49).

The beginning of hair analysis originated in 1858, when Hoppe et al. published a study on the detection of arsenic in the hair of a human corpse exhumed after eleven years buried (50, 51). Subsequent advances in technology and research have resulted in the development of assays for drugs of abuse, performance enhancing drugs, environmental contaminants, alcohol, trace elements and stress related hormones (52-55). Many of these tests have become important for forensic evidence in judicial cases (56, 57).

5. Hair anatomy and physiology

Hair covers the majority of the surface of the human body with the exception of palms, soles and mucosal regions of lips and external genitalia (58, 59). The primary purposes of body hair is to protect skin surface from injury, sensory, and to aid in regulating the body's temperature (60). There are different types of hairs which grow on the surface of the body depending on the region, with thicker and longer hair on the scalp, termed terminal hairs, and fine colourless hairs which cover most of the body, vellus hairs (61).

Humans have approximately five million hair follicles and one million are on the scalp (58, 62). Hair may be looked at as having two distinct parts, the follicle, the living part below the skin, and the hair shaft, which is the fully keratinised, non-living part, above the skin. Human head hair has previously been divided into three subgroups: African, Asian and European. More recently human head hair has been classified into eight different subgroups based on three parameters: curl diameter, curl index and number of waves (63).

The follicle is located 3-4 mm below the surface of the skin and is central to hair growth. The follicle is surrounded by a sebaceous gland, which is believed to be the source of stem cells responsible for hair follicle development. At the base of the hair bulb is the dermal papilla which contains the blood supply, responsible for nutrient delivery, waste elimination and growth (64).

The hair which can be seen above the scalp is referred to as the hair shaft and is composed of tightly packed keratinised cells with an outer protective layer, the hair cuticle. The cuticle is exposed to the environment and is susceptible to damage from various sources such as heat, chemical treatments (bleaching, perming), light exposure and physical damage (65, 66). This damage can be particularly evident at the distal end where damage can expose the inner layers resulting in split-ends.

The majority of the inner structure of hair is composed of cortical cells forming the cortex, which in turn contains cuticle cells where melanin, the pigment in hair, is located (67). The central structure of the hair inside the cortex is the medulla (**Figure 2**). The medulla may be continuous along the length of the hair, discontinuous or not present.

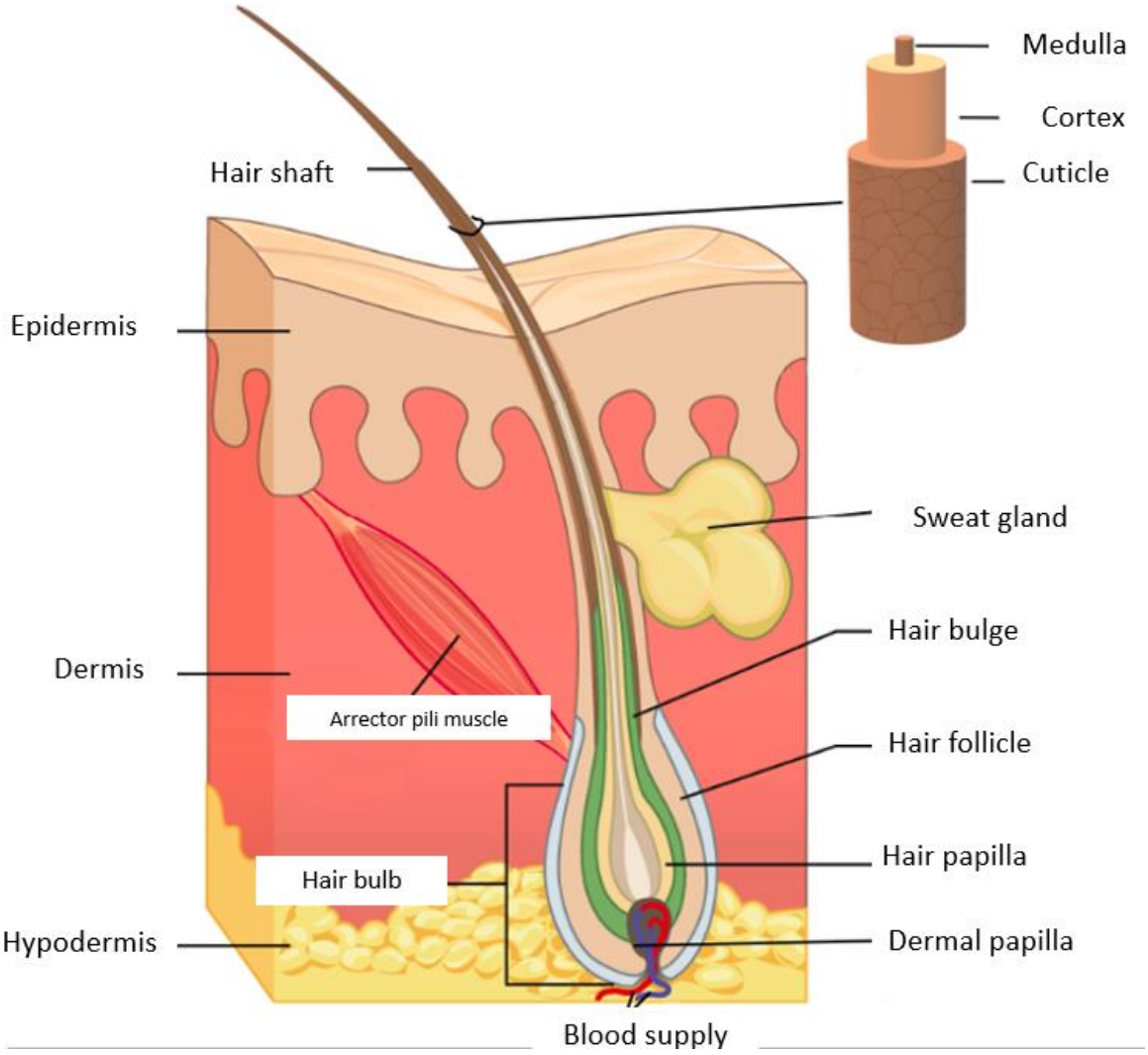


Figure 2 Hair anatomy and physiology.

Showing structure of the hair root, arrector pili muscle, sweat gland, cuticle, cortex and medulla. Source; Https://commons.wikimedia.org/wiki/File:Human_hair_ku.png

6. Hair growth

Numerous elements can have an impact on hair growth including age, stage of development, sex, genetic or metabolic disorders, nutrition and seasonal changes (68-70). There are three recognised growth phases in hair: the anagen, catagen and telogen phases, exogen is a fourth phase of hair cycle and results in shedding (71, 72).

The anagen is the growth phase and ~85% of hairs are in this phase. It can last for several years and is characterised by the emergence of a hair shaft from the skin (73). The catagen phase comes next: cell division stops, hair becomes fully keratinised, and the bulb begins to degenerate. The telogen phase follows and includes 10-15% of all hairs, hair is easily removed during this phase, this then becomes the exogen phase, the loss of hair (74).

After the exogen phase, the growth phase recommences after a short time. The growth rate of hair on different regions of the body varies considerably with 1 cm/month the average for scalp hair and 0.3 cm/month the average for body hair (75, 76). It is with this in mind that it is important to consider that it can take up to two weeks for current circulating analyte/s of interest in blood to deposit in hair follicle and a subsequent 7-10 days active growing before reaching skin surface (77).

This means for a comparison between a blood concentration and hair, blood would be collected six weeks before the 1 cm of hair close to scalp to ensure the 1 cm of hair would represent the time at which the blood was sampled (78, 79). A 3 cm piece of hair represents 3 months of growth, and when segmented into 1 cm segments, provides an historical record of the systemic status of hormone concentration in the circulation over that period. 25(OH)D_3 analysis in hair reflects its physiological status over an extended period depending on the length of hair used for assay. This can be valuable for long-term assessment of supplementation and compliance.

7. Incorporation into hair

The exact means by which incorporation of drugs and other analytes of interest are incorporated into hair along with issues affecting stability are not yet fully understood. There are three recognised routes by which analytes may be incorporated into the hair: by direct blood supply from vessel attached to hair follicle, from sebum/sweat surrounding the hair, or external contamination (80, 81).

Hair has varying affinities and binding abilities depending on the molecules being incorporated. Factors such as pH, structure, size, polarity, lipophilicity, keratin protein binding ability and melanin affinity can affect binding within the hair structure (82). The lipid solubility of the analyte of interest is an essential determinant in the transition from the blood across the cell membrane to the hair bulb (83).

8. Advantages of hair measurement

The advantages of testing using hair over traditional matrices is that collection of the sample is non-invasive, the sample is easy to store, does not present biohazard risk, is stable over long periods of time without sample preparation or refrigeration, and as a consequence of this, transportation of samples is more convenient (57, 73).

In addition, hair samples provide a longitudinal view of the biomarker levels, which would be beneficial in monitoring 25(OH)D_3 over a longer period, from a single sample. Hair does not re-model after growth and therefore a specific hair segment potentially provides a cumulative record of the 25(OH)D_3 status of an individual unaffected by the fluctuations of blood concentrations over the time of growth of that segment.

Hair analysis, therefore, gives a retrospective assessment of 25(OH)D_3 over extended periods of time and may yield information on the association of extended low 25(OH)D_3 with bone trauma, disease, chronic disease and autoimmune 'flare up's'. The concept of this idea is similar to other widely used health measures such as HbA1C for the longitudinal monitoring of glucose in diabetes and red cell folate for measuring long-term folate status (84-86).

In the case of vitamin D_3, in 2019 a proof-of-concept study showing that it was possible to extract and quantify vitamin D from human hair was published by Zgaga, Laird and Healy (87).

8.1 Hair stability

Interestingly, because of the excellent long-term stability of hair samples both anthropological and archaeological studies of vitamin D_3 can be performed in historical and ancient human hair samples (88, 89). This could yield invaluable archaeological data which could be used to assess the vitamin D_3 status of individuals of differing age, sex and socio-economic groups, from a range of ancient populations across time, maybe even offering new insights related to vitamin D status from the distant past.

From an archaeological and anthropological point of view skeletal remains show bone deformations synonymous with vitamin D_3 deficiency across the millennia - from ancient Rome to Victorian London (90, 91). These manifests themselves as rickets in children and osteomalacia in adults.

To date, however, it has not been possible to estimate the vitamin D status of individuals from these eras. Analysis of vitamin D_3 in hair samples from historical human remains may be able to address this shortfall and allow researchers to develop an overall picture of vitamin D_3 biology in these time periods. It will also help answer the question whether the current prevalence of low vitamin D_3 status is a modern symptom (due to indoors oriented lifestyle and higher BMI) or a phenomenon that has been present throughout human history.

8.2 Animal hair

In addition to human hair samples, zoological hair specimens can yield valuable information on animal vitamin D_3 status helping us understand vitamin D metabolism and its evolution. Vitamin D is essential for normal musculoskeletal health and the ability to assess long-term vitamin D_3 status from a few centimetres of hair from, for example, chimpanzee's, can aid in the optimisation of bone and muscle physiology in these animals.

9. Vitamin D measurement in hair

To our knowledge, the first report detailing 25(OH)D_3 extraction and quantitation from hair using LC-MS/MS was published by Zgaga, Laird and Healy in 2019 "*25-Hydroxyvitamin D measurement in human hair: results from a proof-of-concept study*" (87). They were successful in extraction and quantitation of 25(OH)D_3 from hair.

Shah et al. subsequently confirmed the analysis of 25(OH)D_3 in hair in 2021 but failed to detect 1,25$(OH)_2D_3$ "*A Non-Invasive Hair Test to Determine Vitamin D_3 Levels"* (92).

A report published in 2022 demonstrated no correlation between serum and hair 25(OH)D_3 concentrations in samples taken at the same time, Gáll et al., "*Vitamin D status assessment: lack of correlation between serum and hair 25-hydroxycholecalciferol levels in healthy young adults*" (93).

10. Hair testing in other fields

10.1 Chronic stress

The most commonly measured endogenous steroid in hair is cortisol (94-97). Cortisol is a biomarker of stress and is synthesised in the adrenal cortex in response to stress-induced hypothalamic pituitary adrenal (HPA) axis activity (77). The circadian rhythms which occur daily in the human body as well as levels of stress strongly influence the circulating levels of cortisol and cause major fluctuation (98, 99). Blood measurements taken during the periods of these fluctuations can give very variable results.

Cumulative incorporation of cortisol in hair over several months gives a better reflection of cortisol production in that period compared to serum analysis which can be acutely influenced by daily stressors. Hair grows at 1 cm per month on average. Cortisol measurement in the 1 cm of hair nearest the scalp represents total cortisol secretion in the previous month and may function as an indicator of chronic stress over an extended period of time (72, 100-102).

10.2 Diabetes mellitus, Chronic alcohol abuse, Drug use detection, Postmortem investigations, Exposure to environmental toxins

A method to detect levels of chromium in hair was developed in 1979 and analysed glycation of hair proteins to allow long term Diabetes Mellitus monitoring and establishment of a hair glycation index to differentiate between normal and hyperglycaemic individuals (103) (104, 105). The monitoring of alcohol abuse using hair is well established (106). Ethyl glucorinide is a stable metabolite of ethanol which is used to detect alcohol consumption and monitor abstinence. Hair testing has become very popular in drug detection because of the ease of collection of samples, in both recreational drug use and in professional sport (49, 107-109). Hair testing is now capable of accurately measuring various illegal drugs including amphetamines, opiates, cocaine, cannabis, to name a few (110, 111). Postmortem samples may need to be investigated to determine drug use pre-death (112). The receipt of samples may be challenging as samples may not have been stored correctly and root end may not have been highlighted (113). The exposure and accumulation of environmental toxins may lead to health problems (114). Toxins such as heavy metals, pesticides and polycyclic aromatic hydrocarbons may be detected in hair after exposure to xenobiotic agents (115).

11. Future directions in Hair Analysis

The detection alone of these exogenous substances in hair is sometimes the goal, such as presence of recreational drugs in employees as part of workplace drug testing, in other instances it is important to have the ability to quantify to ascertain the ingestion level, such as monitoring the level of a drug in therapeutic drug monitoring as part of medical care. The ability to test for the presence of recreational drugs, alcohol or poisons is useful in areas such as workplace testing, judicial processes and post-mortem testing where detection but not quantitation is sufficient.

Clinical applications of hair analysis in monitoring biomarkers such as vitamin D status in various populations would be a major advantage to clinicians and researchers but would require the ability to quantitate levels as vitamin D is an endogenous steroid which is continually being produced within the body. This would require the establishment of

reference ranges of when values indicate clinically significant levels. This would allow longitudinal studies of vitamin D status over time, exploring the correlation between hair and serum vitamin D levels and the variation in vitamin D in hair of different hair types and populations.

The objective of this research is to investigate and systematically review current employed methods in the analysis of endogenous steroids in hair. The next step will then be to validate the method for vitamin D_3 testing in hair.

Chapter 2: The determination of Steroids in Hair or Fur

A systematic review of methodologies

1. Introduction

Steroid hormones are essential for optimal biochemical functioning and health. They contribute to the control of multiple physiological processes including metabolism, electrolyte and water balance, development of sexual characteristics and immune function (116). Endogenous steroids are produced within the body through an array of chemical reactions, and cholesterol is the precursor molecule from which various steroid hormones within the body are synthesised (117, 118).

The monitoring of steroids as key biomarkers in blood, urine, cerebrospinal fluid (CSF) and saliva has long been established and is widely used in clinical and research settings. While these matrices provide a valuable insight into physiological conditions at the time of sampling, the readings are influenced by circadian rhythms, disease status, medication, and other varying factors. Indeed, the nature of these molecules and how their circulating levels fluctuate throughout the day and over time has been extensively described (119-122). To enable characterisation of this variability over time, numerous repeated sample collections are necessary.

More recently, methods focused on the detection and quantification of endogenous steroid hormones in hair have been proposed. Steroids are most commonly thought to be deposited in hair following the multi-compartment model (114). The expectation is that the amount of analyte incorporated into the hair matrix will be proportional to the concentration of analyte in the circulation at the time of hair growth. This has been supported on by Stalder et al. (123) who reported a strong intra-individual stability of hair cortisol concentrations in healthy individuals, whilst other studies have reported the ability to capture changes in cortisol concentrations introduced by psychological stress (124, 125).

By analysing endogenous steroids in longer hair segments, potential insights into average concentrations over the period of specific segment's growth can be obtained, thereby capturing cumulative long-term levels over an extended time. In addition, because hair

grows on average 1 cm per month (58, 126), segment-by-segment analysis can theoretically provide insight into changes over time.

The benefits of being able to assess average concentrations over a longer period while using a single, non-invasive sample is clearly relevant for cortisol, given its major daily fluctuations; (127) however, other steroids are increasingly being investigated, including most recently 25-hydroxyvitamin D_3, the seasonally-changing biomarker of choice for assessing vitamin D status (87, 92).

To-date, there has not been a systematic review of the methodology used for endogenous steroid analysis in hair. Therefore, the aim of this study is to systematically review methodologies employed to assess the subset of commonly measured endogenous steroids in hair, focusing on methods relating to the collection, preparation, and analysis of hair and fur samples.

2. Methods

The analytes which were selected are routinely monitored biomarkers in a clinical laboratory, including cortisol, testosterone, androstenedione, 17-hydroxyprogesterone (17O-HP), dehydroepiandrosterone sulfate (DHEAS) and 25-hydroxyvitamin D_3 ($25(OH)D_3$). The systematic review was conducted in accordance with the preferred reporting items for systematic reviews and meta-analysis (PRISMA) statement (128).

2.1 Search strategy

Ovid Medline, CINAHL, Psychinfo, and EMBASE were searched. The filters employed were publication date from 1st January 1990 to January 10th, 2023. Additionally, we also searched our institutional library website (https://www.tcd.ie/library/) and Google Scholar (from 1st January 2019- 10th January 2023) to uncover work submitted more recently. The following keywords were included: hair, fur, extraction, quantification, steroid, cortisol, DHEAS, 17-OHP, testosterone, androstenedione, vitamin D ($25(OH)D_3$).

2.2 Inclusion and exclusion criteria

Studies were included if they analysed one or more of the selected endogenous steroids in humans or animals, in hair and/or fur. Only studies published in English were included. Review articles were excluded but their references were screened.

2.3 Study selection

Covidence, a collaborative online systematic review manager, was used to assist with the review (129). First, one reviewer assessed the retrieved records for relevance based on titles and abstracts. Next, full texts of selected records were reviewed by two authors, and if eligible included in synthesis. Disagreements were discussed, and consensus was reached between reviewers and in the discussion with a fourth researcher.

2.4 Data collection

Data extraction process was conducted through Covidence (129) and a custom data extraction template was prepared (**Figure 3**). It included 34 input fields to capture relevant information from each article, including: first author, publication year, number of participants, steroid being extracted, details regarding hair sample (collection area, weight, preparation [whole, minced, powdered], storage and washing), and details on extraction and quantification procedures and protocols and analyte concentration found. In identifying the referenced methodology protocol, it was first searched within the text for a direct reference to a specific paper. If this was not available, the first relevant reference in the citation list was chosen. Data from the first 15 studies were extracted in duplicate by two researchers and compared. The agreement was complete so extraction from the remaining studies was completed by one researcher, with data from a random subset of 18 studies was verified by another researcher once data extraction was completed.

2.5 Quality and risk of bias assessment

Our review focuses on describing the methodological approaches and not on association analysis and statistical inference, therefore a formal quality assessment of reviewed studies was not necessary and therefore was not conducted.

2.6 Analysis

We first presented and summarised general information for included studies. Second, we provide information regarding sample acquisition and preparation of samples for analysis. Third, we evaluated analysis and post-analysis: quantitation method used, assessment of inter-assay and intra-assay precision, accuracy studies ran conjunctively within assays, quality controls implemented, levels detected and repeatability of results.

In majority of the analyses, we present results from human and animal studies separately. Various units were used in original publications to report the concentration of analyte in hair.

Figure 3 Data extraction template

- Year published
- Journal
- Title
- Author and contact details
- Abstract
- Country where it was conducted
- Human or animal (explain)
- Analyte being studied
- Methods;
    1. objective
    2. inclusion criteria
    3. exclusion criteria
- Participants;
    1. sample size
    2. country of origin
    3. cohort or study name
    4. mean age
    5. gender distribution
- Referenced protocol;
    1. author
    2. follow protocol exactly
    3. changes (if any) to referenced Protocol
    4. hair weight used (mg)
    5. hair collection area
    6. hair preparation (powdered, segmented, whole)
    7. storage conditions
- Cleaning solution (ml)
- Extraction solvent (ml)
- Study funding sources
- Conflict of interest
- Method of recruitment of participants
- Quantitation method
- CV reported?
- Inter/Intra assay results

3. Results

Most studies (N=148, 82%) were published in the last 8 years (**Figure 4**). A total of 116 studies (64%) investigated steroids in human hair, four studies investigated human and animal hair (3%) (130-133), and remainder studied animal hair only (60, 33%). The subjects included in human studies varied by age, gender, race and disease status. Animal studies investigated cattle (N=15), rodent (guinea pig, squirrel, mice, chipmunk, ferret, bat and rat) (N=12), monkey (rhesus macaques, barbary macaques, marmosets, chimpanzee; N=9), horse or deer (N=7), pig (N=5), bear (polar and brown; (N=4)), sheep (N=4), seal (N=4), dog (N=3), cat (N=3), and koala (N=2). Some animal studies included more than one type of animal. Four studies that measured both human and animal samples have been included with animal studies in the summary analysis below. The most used unit was pg/mg (76%), so where possible reporting unit was converted to pg/mg for all.

In total, 1,071 records were found (**Figure 5**). One hundred and forty-three duplicates were removed before screening of the 928 unique records. Four hundred and one records were deemed irrelevant as they did not describe hair analysis and 527 full texts were assessed for eligibility. From this, 347 studies were excluded as they did not describe hair analysis methods. Finally, 180 studies were included in data extraction and synthesis for this review (87, 96, 100, 131, 132, 134-308).

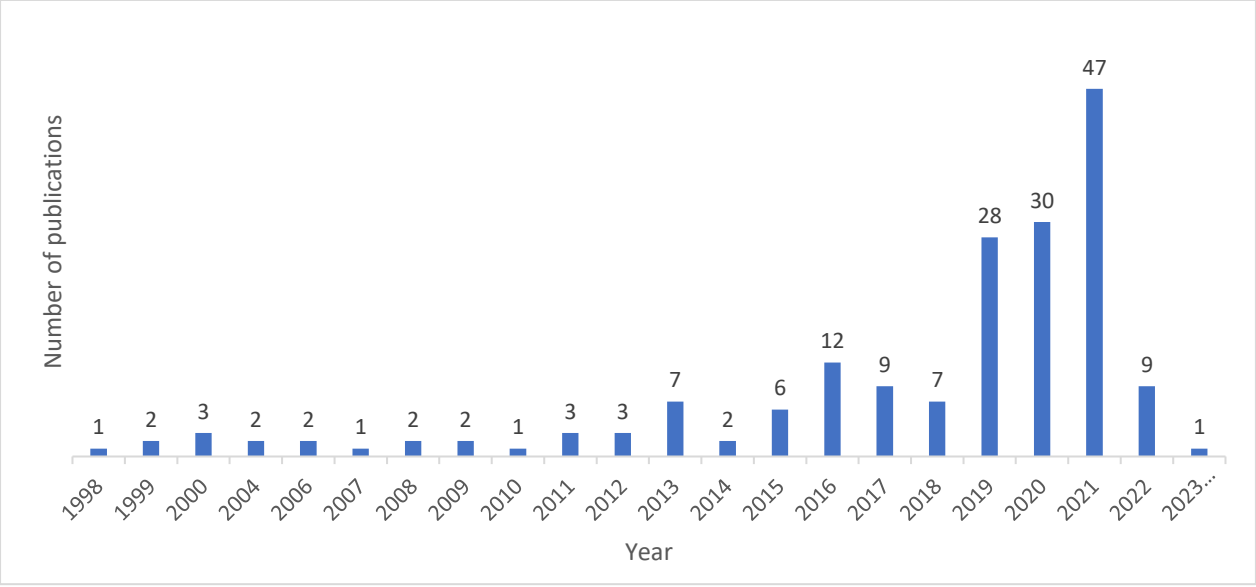


Figure 4 The number of publications investigating endogenous steroid hormone levels in hair

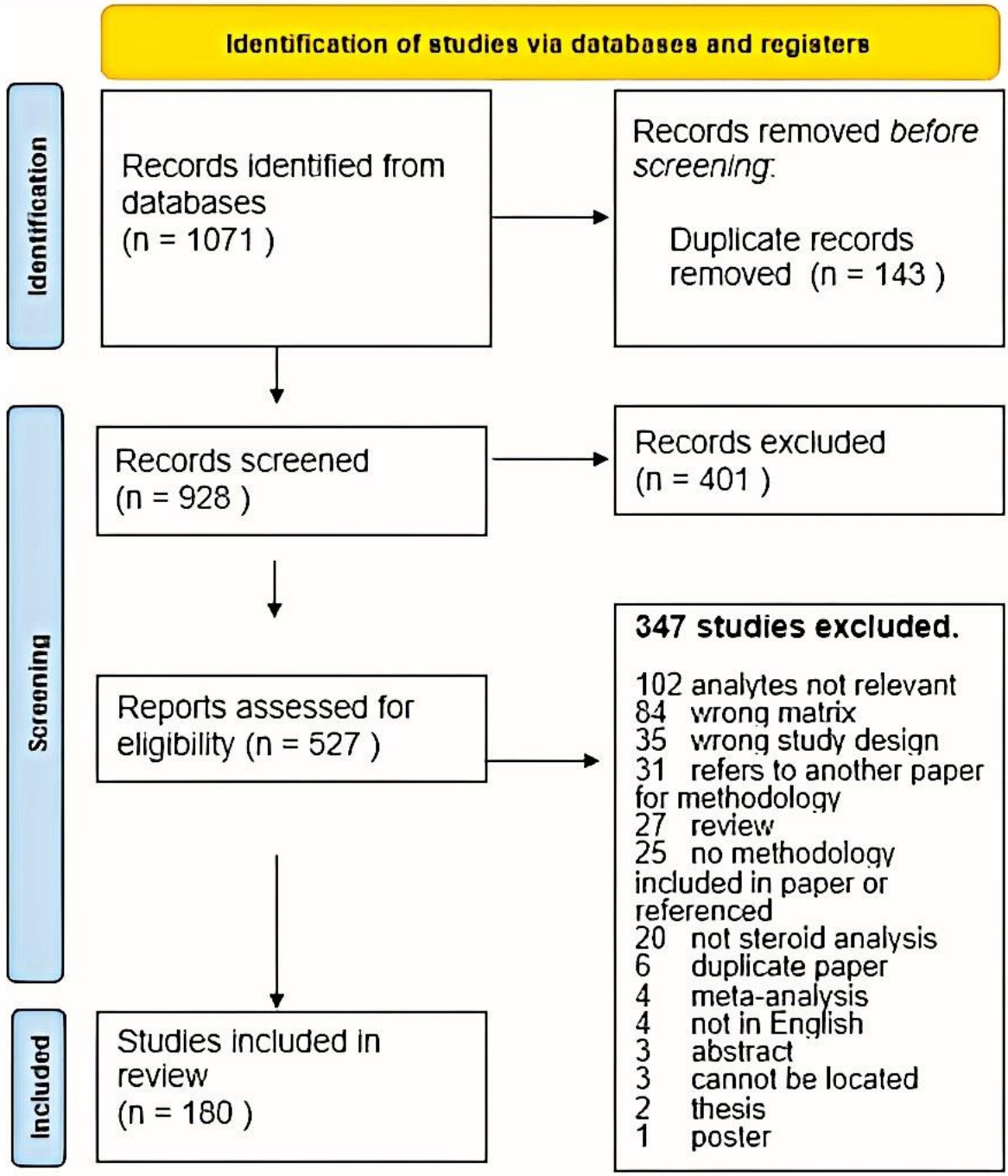


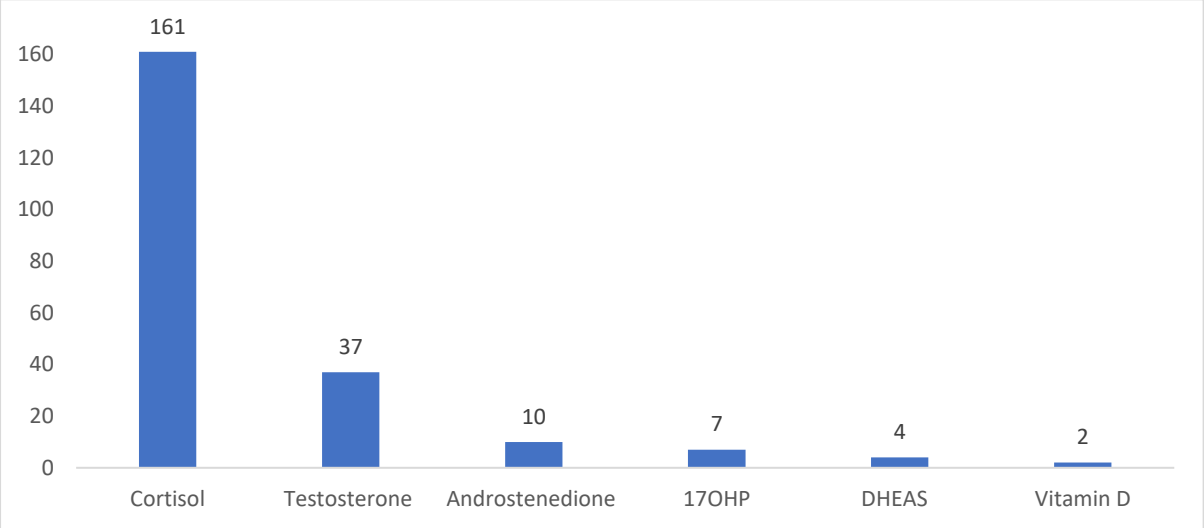
Figure 5 The PRISMA flow chart

This flow chart depicts the publications that were initially identified, screened and included in the review.

3.1 Studied steroid analytes

Cortisol was the most studied analyte (**Figure 6**) (total N=161/180 [89%]; in human studies this was 106/116 [91%] and in animal studies 56/64 [88%]), followed by testosterone (total: 37 [21%]; human: 22 [19%], animal: 15 [23%]), androstenedione (total: 10 [6%], human: 9 [8%], animal: 1 [2%]), 17O-HP (total: 7 [4%], human: 5 [4%], animal: 2 [3%]), DHEAS (total: 4 [2%], human: 2 [2%], animal: 2 [3%]) and vitamin D (only human: 2/116 [2%]). Majority of studies investigated a single analyte (N=156, 87%). Twelve studies investigated two, eight studies investigated three, three studies investigated four and one investigated five analytes.

Figure 6 The number of publications that investigated selected analytes



3.2 Participant sample size and location

The median number of participants in human studies was 73, interquartile range [IQR]: 34-165 (range: 3-5,883), and in animal studies 46, IQR: 17-68 (range: 1-540). Studies were carried out in 39 different countries (**Table 1**).

3.3 Referenced protocol

The most commonly referenced author was Davenport (171) with 23 citations, Gao (185) had 14 references, Meyer (132) and Sauvé (153) had 9, Noppe (309) had 8, Russell (310) had 6, Kintz (214, 311) and Koren (89, 312, 313) had 5 references each. One paper sent their samples to Viaguard Accu-Metrics, Toronto, Canada for analysis, and three papers sent samples to Dresden LabService GmbH for analysis. Wash and extraction procedures were carried out according to Kirschbaum et al. (314). The results from all the 180 studies general information may be seen in **Table 2**.

Table 1 Number of published studies reporting on analysis of selected endogenous steroids by country.

Country	No. of studies
USA	32
Germany	15
Netherlands	15
China	14
Canada	10
Spain	10
Australia	9
Italy	9
United Kingdom	7
France	6
Japan	6
Switzerland	6
Finland	4
Denmark	4
Brazil	3
Slovakia	3
Luxembourg	2
Austria	2
Iran	2
Ireland	2
Korea	2
Lithuania	2
Argentina	1
Greece	1
India	1
Iraq	1
Israel	1
Kenya	1
Nigeria	1
Poland	1
Portugal	1
Republic of Korea	1
South Africa	1
South Korea	1
Sweden	1
Türkiye	1
United Arab Emirates	1

Table 2 General information for 180 included studies.

Study ID	Year	Title	Human or Animal	Cortisol	17-OHP	Testosterone	Androstenedione	DHEAS	25(OH)D	Participants sample size	Referenced protocol author
Brendgen	2023	Link Between Peer Victimization in College and Cortisol Secretion: Roles of Genetic Vulnerabilities and Social Support	Human	x						812	Ouellet-Morin, 2016
Peng	2022	Profiling steroid and thyroid hormones with hair analysis in a cohort of women aged 25 to 45 years old	Human	x	x		x	x		196	Appenzeller, 2022
Doom	2022	Differential associations of parental harshness and parental disengagement with overall cortisol output at 15 years: Implications for adolescent mental health	Human	x						171	Gao, 2013
Koskivuori	2022	A quantitative ultra-performance liquid chromatography high-resolution mass spectrometry analysis of steroids from human scalp hair	Human	x	x	x	x			85	Hakkinen, 2018
Moody	2022	Impact of hair type, hair sample weight, external hair exposures, and race on cumulative hair cortisol	Human	x						259	Koren, 2002
Puglisi	2022	Limited Role of Hair Cortisol and Cortisone Measurement for Detecting Cortisol Autonomy in Patients with Adrenal Incidentalomas	Human	x						148	Salamone, 2012
Scholz	2022	Single sample preparation for the simultaneous extraction of drugs, pharmaceuticals, cannabinoids and endogenous steroids in hair	Human	x		x				1	Voegel, 2020

Zhu	2022	Simultaneous LC-MS/MS quantification of glucocorticoids, melatonin and its metabolites in hair	Human	x						65	Russell, 2012
Broeks	2021	An exploratory study of perinatal hair cortisol concentrations in mother-infant dyads with severe psychiatric disorders versus healthy controls	Human	x						120 (73 mothers, 47 infants)	Noppe, 2015
Brossaud	2021	Hair cortisol and cortisone measurements for the diagnosis of overt and mild Cushing's syndrome	Human	x						132	Manenshcijn, 2012
Bryson	2021	Hair cortisol in mother-child dyads: examining the roles of maternal parenting and stress in the context of early childhood adversity	Human	x						922	Bryson, 2019.
Cajachagua-Torres	2021	Parental cannabis and tobacco use during pregnancy and childhood hair cortisol concentrations	Human	x						2577	Rippe, 2015
Chen	2021	Propensity scores matching evaluation of psychological stress and hair cortisol among people living with HIV in China	Human	x						220	Russell, 2015
Damen	2021	Long-term cortisol levels in hair of children and adolescents with Prader-Willi Syndrome	Human	x						40	Noppe et al., 2015; de Kruijff et al., 2020
Duan	2021	Simultaneous Determination of Cortisol, Cortisone, and Multiple Illicit Drugs in Hair among Female Drug Addicts with LC-MS/MS	Human	x						40	Russell, 2015
Gherlone	2021	Hair cortisol concentrations among urban and rural-dwelling mother-child dyads, La Romana, Dominican Republic	Human	x						175 (88 mothers, 87 children)	Sauve, 2007
Izawa	2021	A validation study on fingernail cortisol: correlations with one-month cortisol levels estimated by hair and saliva samples	Human	x						23	Sugaya ,2020

Mazgelyte	2021	Association between hair cortisol concentration and metabolic syndrome	Human	x						163	Raul, 2004
Muentner	2021	Getting under the skin: Physiological stress and witnessing paternal arrest in young children with incarcerated fathers	Human	x						123	Kapoor et al., 2014
Ney	2021	Chloroform-based liquid-liquid extraction and LCMS/MS quantification of endocannabinoids, cortisol and progesterone in human hair	Human	x						28	Ney, 2020
Rajcani	2021	Stress and hair cortisol concentrations in nurses during the first wave of the COVID-19 pandemic	Human	x						67	Kirschbaum, 2009
Reid	2021	Validity and reliability of method used to analyse hair cortisol concentration	Human	x						85	Parker & Bristow, 2020
Tuladhar	2021	Infant diurnal cortisol predicts sleep	Human	x						86	Meyer, 2014
Vehmeijer	2021	Associations of hair cortisol concentrations with cardio-metabolic risk factors in childhood	Human	x						2598	Noppe, 2015
Voegel	2021	Simultaneous quantification of steroid hormones and endocannabinoids (ECs) in human hair using an automated supported liquid extraction (SLE) and LC-MS/MS - Insights into EC baseline values and correlation to steroid concentrations	Human	x		x	x			58	Binz, 2018
Wu	2021	Evidence of the Moderating Role of Hair Cortisol and Hair Cortisone in the Relationship Between Work Stress and Depression Symptoms Among Chinese fishermen	Human	x						229	Chen, 2019
Zhang	2021	Greater Pain Severity is Associated with Higher Glucocorticoid Levels in Hair Among a Cohort of People Living with HIV	Human	x						431	Gao, 2013

Shah	2021	A Non-Invasive Hair Test to Determine vitamin D_3 Levels	Human						x	70	Zgaga, 2019
Bevan	2021	Maternal separation in childhood and hair cortisol concentrations in late adulthood	Human	x						4460	Stalder, 2012
Cruickshank	2021	Hair and salivary cortisol and their relationship with lifestyle, mood and cognitive outcomes in premanifest Huntington's disease	Human	x						40	Meyer, 2014
Hobo	2021	Analysis of hair steroid hormone concentrations at different parts of the head by liquid chromatography-tandem mass spectrometry	Human	x		x	x			8	Hobo, 2020
Marceau	2021	Within-person changes of cortisol, dehydroepiandrosterone, testosterone, estradiol, and progesterone in hair across pregnancy, with comparison to a non-pregnant reference group	Human	x		x				70	Wang, 2019
Mažeikienė	2021	The association between endogenous hair steroid hormones and social environmental factors in a group of conscripts during basic military training	Human	x		x				185	Gao, 2013
Skoluda	2021	HOME vs. LAB hair samples for the determination of long-term steroid concentrations: a comparison between hair samples collected by laypersons and trained research staff	Human	x						60	Gao, 2013
Spikman	2021	Coping with stress before and after mild traumatic brain injury: a pilot hair cortisol study	Human	x						57	Savas, 2019
Zhang	2021	Association between hair cortisol, hair cortisone, and fatigue in people living with HIV	Human	x						425	Gao, 2013
Buse	2021	Hair cortisol-a stress marker in children and adolescents with chronic tic disorders? A large European cross-sectional study	Human	x						579	Dettenborn, 2012

Simon	2021	Socioeconomic factors, stress, hair cortisol, and white matter microstructure in children	Human	x						158 (94 parents, 64 children)	Meyer, 2014
Behnke	2021	hair based biomarkers in women with major depressive disorder glucocorticoids, endocannabinoids, n-acetyethanolamines, and testosterone	Human	x		x				48 (females with major depressive disorder)	Krumbholz, 2013
Romero-Gonzalez	2021	Hair cortisol levels in pregnancy as a possible determinant of foetal sex: a longitudinal study	Human	x						108 (first trimester of gestation)	Romero-Gonzalez, 2018
Castro-Vale	2020	Hair Cortisol as a Marker of Intergenerational Heritage of War? A Study of Veterans and Their Offspring	Human	x						59	Noppe, 2015
Dion Nist	2020	Protocol to Measure Hair Cortisol in Low Mass Samples from Very Preterm Infants	Human	x						29	Hoffman, 2017
Ferro	2020	Hair cortisol concentration mediates the association between parent and child psychopathology	Human	x						92	Vaghri, 2012
Grova	2020	Ultra-performance liquid chromatography - tandem mass spectrometer method applied to the analysis of both thyroid and steroid hormones in human hair	Human	x		x				15	Noppe, 2015
Hagaman	2020	Association of maternal depression and home adversities with infant hypothalamic-pituitary-adrenal (HPA) axis biomarkers in rural Pakistan	Human	x						1154	Gao, 2013
Hobo	2020	Measurement of steroid hormones by liquid chromatography-tandem mass spectrometry with small amounts of hair	Human	x		x	x			8	Pragst, 2006
Lob	2020	Persistent depressive symptoms, HPA-axis hyperactivity, and inflammation: the role of cognitive-affective and somatic symptoms	Human	x						4761	Gao, 2013

Kluczniok	2020	Early life maltreatment and depression: mediating effect of maternal hair cortisol concentration on child abuse potential	Human	x						127	Davenport, 2006
Lanfear	2020	Hair cortisol measurement in older adults: Influence of demographic and physiological factors and correlation with perceived stress	Human	x						42	Binz, 2016
Leppanen	2020	Association of screen time with long-term stress and temperament in preschoolers: results from the DAGIS study	Human	x						631	Davenport, 2006
Ling	2020	Does hair cortisol really reflect perceived stress? Findings from low-income mother-preschooler dyads	Human	x						35	Meyer, 2014
Musana	2020	Association of differential symptoms of stress to hair cortisol and cortisone concentrations among pregnant women in Kenya	Human	x						133	Wennig, 2000
Schmidt	2020	Reconsidering the construct validity of self-reported chronic stress: A multidimensional item response theory approach	Human	x						Sample 1: 5379 Sample 2: 504	Gao, 2013
Ertekin	2020	Examining the role of socioeconomic status and temperament in the hair cortisol levels of infants	Human	x						49	Flom, 2017
Tarullo	2020	Cortisol and socioeconomic status in early childhood: A multidimensional assessment	Human	x						173 (children)	Davenport, 2006
Gomez-Gomez	2020	Determination of steroid profile in hair by liquid chromatography tandem mass spectrometry	Human	x		x	x			44	Society of Hair Testing
Troller Renfree	2020	Infants of mothers with higher physiological stress show alterations in brain function	Human	x						188 (94 infant-mother dyads)	Davenport, 2006

Caparros-Gonzalez	2019	Maternal and neonatal hair cortisol levels and psychological stress are associated with onset of secretory activation of human milk production	Human	x						120	Sauve, 2007
De la Rubia Orti	2019	Cortisol and IgA are Involved in the Progression of Alzheimer's Disease. A Pilot Study	Human	x						49	Feller, 2014
Ferro	2019	The role of hair cortisol concentration in the association between parent and child psychopathology: A sex-based analysis	Human	x						92	Vaghri, 2012
Galbally	2019	Trans-generational stress regulation: Mother-infant cortisol and maternal mental health across the perinatal period	Human	x						241	Noppe, 2015
Garcia Leon	2019	Resilience as a protective factor in pregnancy and puerperium: Its relationship with the psychological state, and with Hair Cortisol Concentrations	Human	x						151	Russell, 2015
Gonzalez	2019	Hair Cortisol Measurement by an Automated Method	Human	x						286	Sauve, 2007
Hardy	2019	Chronic stress and ovarian function in female childhood cancer survivors	Human	x						24	Kirschbaum, 2009.
Jahangard	2019	Prenatal and postnatal hair steroid levels predict post-partum depression 12 weeks after delivery	Human	x						100	Gao, 2013
Kim	2019	Feasibility of Hair Cortisol as a Biomarker of Well-being in Older Adults with Dementia	Human	x						13	Feeney, 2020
Molenaar	2019	Prenatal maternal psychopathology and stress and offspring HPA axis function at 6 years	Human	x						2546	Rippe, 2016
Mustonen	2019	Maternal prenatal hair cortisol is associated with prenatal depressive symptom trajectories	Human	x						689	Sauve, 2007

Savas	2019	Hair glucocorticoids as a biomarker for endogenous Cushing's syndrome: validation in two independent cohorts	Human	x						384	Noppe, 2015
Schepers	2019	Effects of 5-HTTLPR genotype and cognitive rumination on long-term cortisol reactivity measured in human hair	Human	x						54	Kirschbaum, 2009
Wang	2019	Hair testing for cortisol by UPLCMS/MS in a family: External cross-contamination from use of cortisol cream	Human	x						12	Dettenborn, 2010
Xu	2019	Hair cortisol levels and symptoms of anxiety and depression in Chinese adolescents: Comparison between incarcerated and community populations	Human	x						62	Li, 2012
Zgaga	2019	25-Hydroxyvitamin D Measurement in Human Hair: Results from a Proof-of-Concept study	Human						x	3	Gao, 2016
Shapero	2019	The interactive association of proximal life stress and cumulative HPA axis functioning with depressive symptoms	Human	x						58	Stalder, 2012
Abdulateef	2019	Assessment of hair cortisol in euthyroid, hypothyroid, and subclinical hypothyroid subjects	Human	x						124	Sauve,2007
Aryaie	2018	Association of Maternal Hair Cortisol Level with Neonatal Facial Pain Expression Based on Gender of Newborns	Human	x						105	Davenport, 2006
Balagova	2018	Importance of methodological details in the measurement of cortisol in human hair	Human	x						hair pool	Xiang, 2016
Binz	2018	Systematic investigations of endogenous cortisol and cortisone in nails by LC-MS/MS and correlation to hair	Human	x						120	Binz, 2016
Muehlhan	2018	HPA axis stress reactivity and hair cortisol concentrations in recently detoxified alcoholics and healthy controls with and without childhood maltreatment	Human	x						130	Gao et al. 2013

Van Aken	2018	Hair cortisol and the relationship with chronic pain and quality of life in endometriosis patients	Human	x						62	Gow, 2010
Dong	2017	A UHPLC-MS/MS method for profiling multifunctional steroids in human hair	Human	x	x	x				22	Gao, 2013
Hollanders	2017	Interpretation of glucocorticoids in neonatal hair: A reflection of intrauterine glucocorticoid regulation?	Human	x						107	Noppe, 2015
Kristensen	2017	Hair dyeing, hair washing and hair cortisol concentrations among women from the healthy start study	Human	x						266	Van uum, 2008
Slezak	2017	Testosterone-like immunoreactivity in hair measured in minute sample amounts - a competitive radioimmunoassay with an adequate limit of detection	Human			x				20	Manenschijn, 2011
Ramirez	2017	Evaluation of cortisol and telomere length measurements in ethnically diverse women with breast cancer using culturally sensitive methods	Human	x						100	Sauve, 2007
Gaudl	2016	Liquid chromatography quadrupole linear ion trap mass spectrometry for quantitative steroid hormone analysis in plasma, urine, saliva and hair	Human	x						93	Gao, 2013
Mwanza	2016	Simultaneous HPLC-APCI-MS/MS quantification of endogenous cannabinoids and glucocorticoids in hair	Human	x						473	Krumbholz et al, 2013
Olstad	2016	Hair cortisol levels, perceived stress and body mass index in women and children living in socioeconomically disadvantaged neighbourhoods: the READI study	Human	x						100	Sauve, 2007
Papafotiou	2016	Hair cortisol concentrations correlate with daily salivary levels and are increased in obese prepubertal girls	Human	x						50	Olstad, 2016
Turner	2016	The importance of hair cortisol levels and perceived stress to body mass index in women and children living in socioeconomically disadvantaged neighbourhoods	Human	x						100	Sauve, 2007

Wester	2016	Long-term glucocorticoids measured in hair predict weight loss during a lifestyle intervention for obese patients	Human	x						175	Manenschijn, 2011
Andrade	2016	Hair cortisol in drug-naive first-episode individuals with psychosis	Human	x						51	Van Uum, 2008
Noppe	2015	LC-MS/MS-based method for long-term steroid profiling in human scalp hair	Human	x	x	x	x	x		5	Gao, 2013
Quinete	2015	Highly selective and automated online SPE LC-MS3 method for determination of cortisol and cortisone in human hair as biomarker for stress related diseases	Human	x						33	Sauve, 2007
Xiang	2015	A modified and cost-effective method for hair cortisol analysis	Human	x						12	Davenport, 2006
Slominski	2015	Methodological Considerations for Hair Cortisol Measurements in Children	Human	x						34	Russell, 2012
Gao	2013	Quantitative analysis of steroid hormones in human hair using a column-switching LC-APCI-MS/MS assay	Human	x		x	x			30	Stalder et al, 2012
Hoffman	2013	Hair cortisol and foetal adrenal ultrasound, potential non-invasive markers of preterm birth	Human	x						92	D'anna hernandez, 2011
Krumbholz	2013	Diagnostic value of concentration profiles of glucocorticosteroids and endocannabinoids in hair	Human	x						1	Krumbholz et al, 2013
Xing	2013	Study on dissolution mechanism of cortisol and cortisone from hair matrix with liquid chromatography-tandem mass spectrometry	Human	x						19	Russell, 2012
Deshmukh	2012	Detection of testosterone and epitestosterone in human hair using liquid chromatography-tandem mass spectrometry	Human			x				75	N.Deshmukh,2010.

Van Rossum	2012	Long term cortisol in bipolar disorder: Associations with age of onset and psychiatric comorbidity	Human	x						295	Dettenborn, 2010
Dominguez-Romero	2011	Screening and quantitation of multiclass drugs of abuse and pharmaceuticals in hair by fast liquid chromatography electrospray time-of-flight mass spectrometry	Human			x				6	Cirimele, 1996
Jung	2011	Gas chromatography mass spectrometry-based hair steroid profiling may reveal pathogenesis in hair follicles of the scalp	Human	x	x	x	x			37	M. H. Choi, 1999
Xie	2011	Correlation of cortisol in 1-cm hair segment with salivary cortisol in human: Hair cortisol as an endogenous biomarker	Human	x						32	Raul, 2004
Gao	2010	HPLC-FLU detection of cortisol distribution in human hair	Human	x						40	Davenport, 2006
Zhang	2008	Fiber-packed SPE tips based on electrospun Fibers	Human	x						4	Qi, 2008
Sauve	2007	Measurement of cortisol in human hair as a biomarker of systemic exposure	Human	x						39	Yamada, 2007
Wheeler	2006	Measurement of androgens	Human			x				Not stated	Kintz, 1999
Raul	2004	Detection of physiological concentrations of cortisol and cortisone in human hair	Human	x						44	Bevalot, 2000
Bang	2004	Comparative studies on level of androgens in hair and plasma with premature male-pattern baldness	Human			x				31	Choi, 1999
Cirimele	2000	Identification of ten corticosteroids in human hair by liquid chromatography-ion spray mass spectrometry	Human	x						3	Kintz, 1996

Kintz	2000	Dehydroepiandrosterone (DHEA) and testosterone concentrations in human hair after chronic DHEA supplementation	Human			x				3	Kintz, 1999
Bevalot	2000	Analysis of corticosteroids in hair by liquid chromatography-electrospray ionization mass spectrometry	Human	x						19	Kintz, 1995
Kintz	1999	Identification of testosterone and testosterone esters in human hair	Human			x				26	Kintz, 1999
Scherer	1998	Determination of testosterone in human hair by gas chromatography-selected ion monitoring mass spectrometry	Human			x				12	Hold, 1996
Akefe	2017	Cortisol Levels as a Determinant of Disease and Health Status in Animals	Human and Animal	x						10 species (sheep, goat, cow, horse, camel, monkey, cat; 10 samples each)	Ito, 2005
Binz	2016	Development of an LC-MS/MS method for the determination of endogenous cortisol in hair using (13)C3-labeled cortisol as surrogate analyte	Human and Animal	x						2 humans, 25 cows	Henriksen, 2005
Meyer	2014	Extraction and analysis of cortisol from human and monkey hair	Human and Animal	x						46 (human and monkey)	Meyer, 2012
Hold	1999	Detection of nandrolone, testosterone, and their esters in rat and human hair samples	Human and Animal			x				4 (rat)	Hold, 1996
Braun	2022	Hair cortisol concentrations in different breeds of cows: Comparison of hair from unshorn and previously shorn areas and from various regions of the body	Animal	x						151 (cow)	Binz, 2016

Otten	2022	The age of hair matters - the incorporation of cortisol by external contamination is enhanced in distal hair segments of pigs and cattle	Animal	x						30 (pig and cow)	Heimburge, 2020
Pokharel	2021	The tail-tale of stress: an exploratory analysis of cortisol levels in the tail-hair of captive Asian elephants	Animal	x						6 (Asian elephants)	Yamanashi, 2013
Santymire	2021	Using hair cortisol analysis to understand the biological factors that affect black-footed ferret (Mustela nigripes) stress physiology	Animal	x						345 (black-footed ferret)	Loeding , 2011
Contreras	2021	Evaluation of hair and nail cortisol concentrations and associations with behavioral, physical, and environmental indicators of chronic stress in cats	Animal	x						52 (cat)	Davenport, 2006
Van Eerdenburg	2021	The relation between hair-cortisol concentration and various welfare assessments of Dutch dairy farms	Animal	x						10 (cow)	Davenport, 2006
Amin	2021	In utero accumulated steroids predict neonate antipredator response in a wild mammal	Animal	x		x				185 (deer)	Koren , 2008
Begall	2021	Life expectancy, family constellation and stress in giant mole-rats (Fukomys mechowii)	Animal	x						37 (giant mole rats)	Davenport, 2006
Olvera-Maneu	2021	Hair cortisol, testosterone, dehydroepiandrosterone sulfate and their ratios in stallions as a retrospective measure of hypothalamic pituitary adrenal and hypothalamic pituitary gonadal axes activity: Exploring the influence of seasonality	Animal	x		x		x		10 (horse)	Tallo-Parra, 2015
Arena	2021	Assessment of horses' welfare: Behavioral, hormonal, and husbandry aspects	Animal	x						56 (horse)	Accorsi, 2008
Charalambous	2021	Physiological stress in rescued wild koalas (Phascolarctos cinereus) being held in a rehabilitation sanctuary: A pilot study	Animal	x						3 (koala)	Charalambous, 2019

Di Francesco	2021	Qiviut cortisol reflects hypothalamic pituitary adrenal axis activity in muskoxen (Ovibos moschatus)	Animal	x						31 (muskoxen)	Mastromonaco, 2014
Sandoval-Herrera	2021	Inter-and intra-specific variation in hair cortisol concentrations of Neotropical bats	Animal	x						18 (neotropical bats)	Acker, 2018
Wiechers	2021	Analysis of hair cortisol as an indicator of chronic stress in pigs in two different farrowing systems	Animal	x						61 (pig)	Davenport, 2006
Hein	2021	Analysis of hair steroid hormones in polar bears (Ursus maritimus) via liquid chromatography tandem mass spectrometry: Comparison with two immunoassays and application for longitudinal monitoring in zoos	Animal	x						13 (polar bear)	Kirschbaum, 2009
Van der Walt	2021	Measuring adrenal and reproductive hormones in hair from Southern Beaufort Sea polar bears (Ursus maritimus)	Animal	x		x				141 (polar bear)	Davenport, 2006
Cordeschi	2021	Environmental variability and allostatic load in the Eurasian red squirrel Sciurus vulgaris	Animal	x						50 (red squirrel)	Peric et al., 2013
Keogh	2021	Whiskers as a novel tissue for tracking reproductive and stress-related hormones in North Pacific otariid pinnipeds	Animal	x		x				36 (seal)	Macbeth, 2010
Otsuki	2021	Testosterone levels in hair of free-ranging male northern fur seals (Callorhinus ursinus) in relation to sampling month, age class and spermatogenesis	Animal			x				57 (seal)	Otsuki, 2020
Alon	2021	Higher cortisol and testosterone levels in sheep with larger litter sizes	Animal	x		x				40 (sheep)	Koren, 2008
Weaver	2021	Chronic elevation of plasma cortisol causes differential expression of predominating glucocorticoid in plasma, saliva, fecal, and wool matrices in sheep	Animal	x						12 (sheep)	Davenport, 2006
Park	2020	Impact of a cattle brush on feedlot steer behavior, productivity and stress physiology	Animal	x						51 (cow)	Moya, 2013

Peric	2020	Comparison of AlphaLISA and RIA assays for measurement of wool cortisol concentrations	Animal	x						89 (lamb)	Peric, 2013
Calamari	2020	Hair as an alternative non-invasive matrix: sources of variation in testosterone levels	Animal			x				28 (male dogs of poodle breed)	Baumgartner, 1979
Garber	2020	Life in a harsh environment: the effects of age, sex, reproductive condition, and season on hair cortisol concentration in a wild non-human primate	Animal	x						61 (marmoset)	Meyer, 2014
Chu	2020	LC-APCI + -MS/MS method for the analysis of ten hormones and two endocannabinoids in plasma and hair from the mice with different gut microbiota	Animal			x	x			60 (mice)	Chu, 2018
Heimburge	2020	Within a hair's breadth - Factors influencing hair cortisol levels in pigs and cattle	Animal	x						46 sows 40 cows	Meyer, 2014
Elmi	2020	Quantification of hair corticosterone, DHEA and testosterone as a potential tool for welfare assessment in male laboratory mice	Animal			x				56 (mouse)	Bacci, 2014
Lopez-Arjona	2020	Measurement of cortisol, cortisone and 11-hydroxysteroid dehydrogenase type 2 activity in hair of sows during different phases of the reproductive cycle	Animal	x						32 (pig)	Davenport, 2006
Montillo	2020	Steroids in pig hair and welfare evaluation systems: combined approaches to improve management in pig breeding?	Animal	x				x		86 (pig)	Bergamin, 2019
Colding-Jorgensen	2020	Hair glucocorticoids are not a historical marker of stress exploring the timescale of corticosterone incorporation into hairs in a rat model	Animal	x						36 (rat)	Albar, 2014
Sergiel	2020	Do follicles matter? Testing the effect of follicles on hair cortisol levels	Animal	x						30 free-ranging Scandinavian brown bears	Macbeth, 2010

Lubcker	2020	What's in a whisker? High-throughput analysis of twenty-eight C 19 and C 21 steroids in mammalian whiskers by ultra-performance convergence chromatography-tandem mass spectrometry	Animal	x	x	x				70 (southern elephant seals, Antarctic fur seal and subantarctic fur seal)	Vanaelst, 2013
Schillaci	2019	Hair cortisol and stable carbon and nitrogen isotope ratios in barbary macaques (Macaca sylvanus) from Gibraltar	Animal	x						65 (Barbary macaques)	Meyer, 2012
Colombo	2019	Supplementing an immunomodulatory feed ingredient to improve thermoregulation and performance of finishing beef cattle under heat stress conditions	Animal	x						128 (cow)	Moya, 2013
Maxwell	2019	Hair cortisol concentrations, as a measure of chronic activity within the hypothalamic-pituitary-adrenal axis, is elevated in dogs farmed for meat, relative to pet dogs, in South Korea	Animal	x						170 (dogs, 84 pets, 86 former farm)	Accorsi, 2008
Karpovich	2019	Examination of relationships between stable isotopes and cortisol concentrations along the length of phocid whiskers	Animal	x						68 (earless seals)	Macbeth, 2010
Santangeli	2019	Hair cortisol concentration in Siberian flying squirrels is unrelated to landscape and social factors	Animal	x						192 (flying squirrels, in total tested. sixty-nine study sites, each site includes 1-5 nest boxes)	Mastromonaco, 2014
Nejad	2019	Comparing hair cortisol concentrations from various body sites and serum cortisol in Holstein lactating cows and heifers during thermal comfort zone	Animal	x						70 (47 Holstein cows and 23 Holstein heifers)	Davenport, 2006
Placci	2019	Natural horse boarding vs traditional stable: A comparison of hormonal, hematological and immunological parameters	Animal	x						47 (horse)	Tamanini, 1983

Charalambous	2019	Cortisol Measurement in Koala (Phascolarctos cinereus) Fur	Animal	x						1 (koala)	Davenport, 2006
Nejad	2019	Methodological validation of measuring Hanwoo hair cortisol concentration using bead beater and surgical scissors	Animal	x						9 cattle (Korean Hanwoo cattle; mature cows, heifers and calves)	Davenport, 2006
Sharma	2019	Hair cortisol in sheltered cows and its association with other welfare indicators	Animal	x						540 (Shetland cows from 10 shelters)	Davenport, 2006
Phillips	2018	Quantification of hair cortisol concentration in common marmosets (Callithrix jacchus) and tufted capuchins (Cebus apella)	Animal	x						16 (monkey)	Meyer, 2014
Kapoor	2018	Radiolabel validation of cortisol in the hair of rhesus monkeys	Animal	x						7 (rhesus monkey)	Davenport, 2006
Nedic	2017	Cortisol concentrations in hair, blood and milk of Holstein and Busha cattle	Animal	x						61 (cow)	Snoj, 2012
Tallo-Parra	2017	Prediction of Cortisol and Progesterone Concentrations in Cow Hair Using Near-Infrared Reflectance Spectroscopy (NIRS)	Animal	x						52 (cow)	Tallo-Parra, 2015
Kwok	2017	Detection of anabolic and androgenic steroids and/or their esters in horsehair using ultra-high performance liquid chromatography-high resolution mass spectrometry	Animal		x	x				55 (horse)	Cooper, 2012
Yamanashi	2016	Analysis of hair cortisol levels in captive chimpanzees: Effect of various methods on cortisol stability and variability	Animal	x						72 (chimpanzee)	Yamanashi, 2013
Fourie	2016	Sources of variation in hair cortisol in wild and captive non-human primates	Animal	x						13 (non-human primates)	Davenport, 2006

Weisser	2016	A novel method for analysing key corticosteroids in polar bear (Ursus maritimus) hair using liquid chromatography tandem mass spectrometry	Animal	x						4 (polar bear)	Davenport, 2006
Tallo-Parra	2015	Hair cortisol detection in dairy cattle by using EIA: protocol validation and correlation with faecal cortisol metabolites	Animal	x						17 (dairy cattle)	Davenport, 2006
Mastromonaco	2014	Validation and use of hair cortisol as a measure of chronic stress in eastern chipmunks (Tamias striatus)	Animal	x						23 (eastern chipmunks (Tamias striatus))	Munro and Lasley, 1988
Peric	2013	hair cortisol concentrations in Holstein-Friesian and crossbreed F1 heifers	Animal	x						295 (145 from Holstein-Fresian and 150 from crossbreed F1 heifers)	Koren, 2002
Gray	2013	Investigations into the feasibility of routine ultra-high performance liquid chromatography-tandem mass spectrometry analysis of equine hair samples for detecting the misuse of anabolic steroids, anabolic steroid esters and related compounds	Animal			x				7 (horse)	Popot, 2003
Keckeis	2012	Hair cortisol: a parameter of chronic stress? Insights from a radiometabolism study in guinea pigs	Animal	x						8 (guinea pig)	Davenport, 2006
Aqai	2009	Effect of sample pre-treatment on the determination of steroid esters in hair of bovine calves	Animal			x				10 (bovine calves; 4 treated once; 4 treated three times; 2 untreated control animals)	Nielen, 2006
Shen	2009	Simultaneous determination of anabolic androgenic steroids and their esters in hair by LC-MS-MS	Animal			x				30 (male guinea pigs)	Xiang , 2006
Accorsi	2008	Cortisol determination in hair and faeces from domestic cats and dogs	Animal	x						27 cats, 29 dogs	Koren, 2002

Davenport	2006	Analysis of endogenous cortisol concentrations in the hair of rhesus macaques	Animal	x						20 (rhesus macaques)	Thieme, 2000

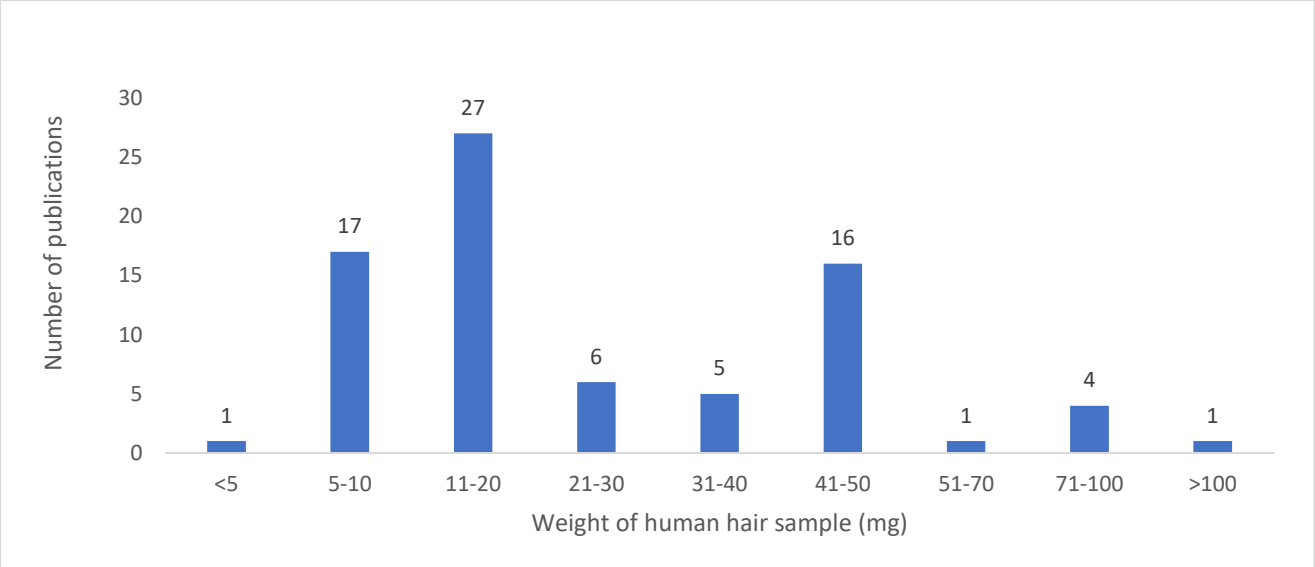
3.4 Pre-extraction

3.4.1 Hair sample weight

In the analysis of human hair, the lowest and the highest sample weights reported were 1.25 mg (152, 184, 236) and 200 mg (92). Three studies used infant hair with some samples weighing less than 5 mg (2%) (152, 158, 283). Twenty-one (13%) studies used samples with weight in the 5-10 mg range, twenty-nine (18%) in the 11-20 mg range, 10 (6%) in the 21-40 mg range and 17 (11%) studies in the 41-50 mg range, and six studies used 100-200 mg hair (4%) (**Figure 7**). Thirty-one (19%) studies did not mention weight of hair used.

In animal studies, weight of hair/fur tended to be higher, the lowest sample weights reported were 5 mg (228, 304), and the highest 500 mg (315) and 1000mg (295). Eight studies out of 64 used 5-10 mg (13%), nine studies used 11-20 mg (14%), nineteen studies used 21-50 mg (30%) and eleven studies used 51-70 mg (17%), six (9%) studies used more than 70 mg of hair, and finally eleven (17%) studies did not mention weight of hair used. Animal size did not often correlate with reported sample weight used (data not shown).

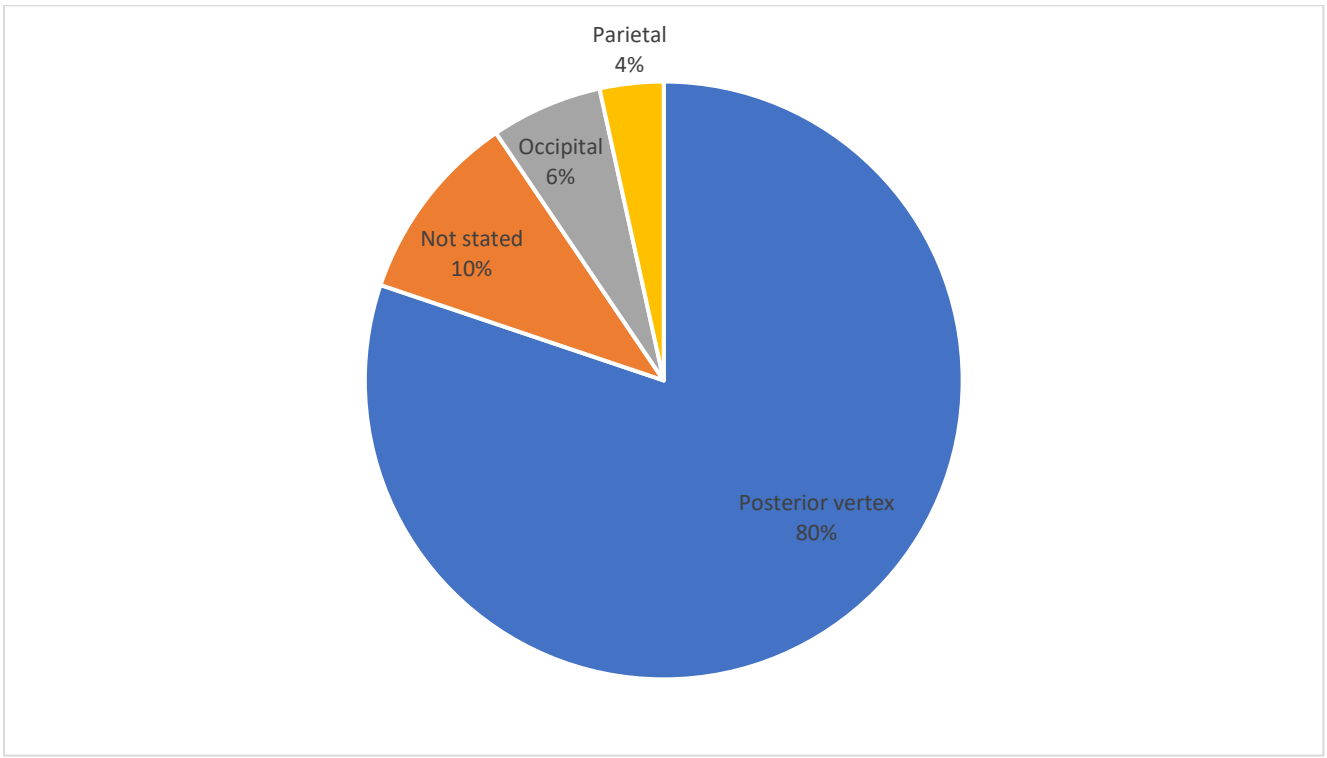
Figure 7 Weight of human hair used in analysis in studies



3.4.2 Hair collection area

The most common sampling site for humans (93/116 papers, 80%) was the posterior vertex region of the skull. Other sites were the occipital region (7/116, 4%) and the parietal region (4/116, 3%). There were two papers that additionally collected hair from the beard of a participant (87, 316). Twelve papers (7%) did not explicitly state a collection area (**Figure 8**). Animal hair samples were harvested from the back (N=16, 25%), neck (13, 20%), head (8, 13%), tail (6, 9%), rump (6, 9%), leg (6, 9%) and whiskers (2, 3%). One study used a hair collection trap in a public place to collect red squirrel hair (1.5%) (168). Seven papers did not explicitly state the collection site (11%).

Figure 8 Collection area of human hair samples



3.4.3 Storage conditions

Samples were predominantly stored at room temperature (86 studies, 48%) though 17 studies reported storage at -20°C (9%) and two studies each stored at -40°C and -80°C respectively (1%). However, for a large proportion of studies the storage conditions were not discussed (N=75, 42%). Only 10 papers (6%) specifically mentioned storing their samples in dark conditions, either a light-protected container or dark room/cabinet. Drying was mentioned in 45 studies (25%) as a necessity to store hair for any period prior to analysis. In terms of a storage container, 33 studies (18%) used aluminium foil to store samples, 28 (16%) used paper envelopes; both predominantly coupled with the storage at room temperature. 20 studies (11%) used biohazard/plastic bags and 10 (6%) used plastic tubes; these storage containers were mostly used when samples were refrigerated or frozen. Eighty-nine studies (49%) did not mention how they stored samples. Weight of hair used in extraction, hair collection area, hair preparation, storage medium and temperature are shown in **Table 3**.

Table 3 Overview of procedures relating to collection, preparation and storage of samples

Study ID	Year	Hair weight used (mg)	Hair collection area	Hair preparation	Storage temperature	Storage medium
Brendgen	2023	50	Posterior vertex	Powdered ball bill	Room temperature	Plastic bag
Peng	2022	50	Posterior vertex	Pulverised (ball mill)	Room temperature	Aluminium foil, within paper envelope
Doom	2022	10	Posterior vertex	Whole	Room temperature	Aluminium foil (taped to the foil with scalp ends together and clearly labelled. Foil was folded with-out folding any hair)
Koskivuori	2022	10-15	Posterior vertex	Whole	Ambient temperature (+22 ± 2 ◦C)	Aluminium foil and stored in the dark at ambient temp. (+22+/-2)
Moody	2022	5-15	Parietal region	Powdered	Room temperature	Aluminium foil
Puglisi	2022	50	Posterior vertex	1-2mm segments	Room temperature	Dry, away from sunlight
Scholz	2022	20	Posterior vertex	Powdered with ball mill	Not explicitly stated	Not explicitly stated
Zhu	2022	20	Occipital region	1-2mm segments	Not explicitly stated	Not explicitly stated
Broeks	2021	Not explicitly stated	Posterior vertex	Minced	Room temperature	Taped to paper, scalp end marked, in envelope
Brossaud	2021	Not explicitly stated	Posterior vertex	Milled	Room temperature	taped to paper, scalp end marked, in envelope

Bryson	2021	Not explicitly stated	Posterior vertex. when children's hair was not sufficient also collected from other sites on head (temporal, parietal, combined temp.+ pari.).	Mechanically crushed	Not explicitly stated	Not explicitly stated
Cajachagua-Torres	2021	30 (Noppe)	Posterior vertex	Cut into small pieces	Not explicitly stated	Not explicitly stated
Chen	2021	20	Posterior vertex	Not explicitly stated	Not explicitly stated	Aluminium foil
Damen	2021	30 (Noppe)	Posterior vertex	Not explicitly stated	Room temperature	Paper envelope
Duan	2021	20	Posterior vertex	cut into 5x 1cm pieces, finely minced to 1-2mm pieces	Not explicitly stated	Not explicitly stated
Gherlone	2021	Not explicitly stated	Posterior vertex	Not explicitly stated	Room temperature	Paper envelope and plastic bag
Izawa	2021	50	Posterior vertex	Powdered using cell disruptor	Room temperature	Aluminium foil
Mazgelyte	2021	20-50	Not explicitly stated	Cut into small fragments with scissors	Room temperature	Paper envelope
Muentner	2021	Not explicitly stated	Posterior vertex	Powdered	Room temperature	Aluminium foil
Ney	2021	50	Posterior occipital bone position	Not explicitly stated	Not explicitly stated	Not explicitly stated
Rajcani	2021	7.5	Occipital region	Not explicitly stated	Not explicitly stated	Aluminium foil
Reid	2021	25	Study 1: Temporal and occipital regions. Study 2: Posterior vertex	Pulverised (ball mill)	Room temperature	Aluminium foil

Tuladhar	2021	15-30	Posterior vertex	Powdered	-20°C	Plastic vials
Vehmeijer	2021	10-30	Posterior vertex	Finely cut	Room temperature	Hair taped to paper with scalp-end marked and placed in envelope
Voegel	2021	20	Posterior vertex	Cut into snippets	Not explicitly stated	Not explicitly stated
Wu	2021	20	Posterior parietal region	Finely cut	-20°C	Dry sealed bags
Zhang	2021	10	Posterior vertex	Whole	Not explicitly stated	Aluminium foil with distal end labelled with ID number
Shah	2021	200	Posterior vertex	Pulverised (ball mill)	cold and dark place	plastic bag
Bevan	2021	10	Posterior vertex	Whole	Not explicitly stated	Not explicitly stated
Cruickshank	2021	50	Posterior vertex	Powdered	-20°C	Aluminium foil
Hobo	2021	Not explicitly stated	9 different areas on head	Pulverised (ball mill)	Not explicitly stated	Not explicitly stated
Marceau	2021	15	Posterior vertex	Pulverised (ball mill)	Room temperature	Aluminium foil in paper envelope
Mažeikienė	2021	20	Posterior vertex	Not explicitly stated	Room temperature in dark conditions	Aluminium foil
Skoluda	2021	7.5	Posterior vertex	Finely chopped	Dark dry place	Tied with string, in aluminium foil
Spikman	2021	20	Posterior vertex	1cm pieces	Not explicitly stated	Not explicitly stated

Zhang	2021	20	Posterior vertex	Cut finely	Not explicitly stated	Aluminium foil (study number labelled)
Buse	2021	7.5	Below cranial bone	Whole	Not explicitly stated	Not explicitly stated
Simon	2021	15	Posterior vertex	Powdered	-20°C	Not explicitly stated
Behnke	2021	9.7-10.2	Posterior vertex	Pulverised (ball mill)	Not explicitly stated	Not explicitly stated
Romero-Gonzalez	2021	Weight not explicitly stated. 150 strands taken initially	Posterior vertex	Pulverised (ball mill)	Room temperature	Not explicitly stated
Castro-Vale	2020	Not explicitly stated	Posterior vertex	Cut into 1cm segments	Room temperature	taped to a piece of paper, and the scalp end was then marked
Dion Nist	2020	Not explicitly stated	Nape of neck, 1-2cm from hairline	Pulverised (ball mill)	Room temperature and dark room	Not explicitly stated
Ferro	2020	30-35	Not explicitly stated	Pulverised (ball mill)	Not explicitly stated	Paper clipped to cardstock indicating the direction of growth.
Grova	2020	50	Not explicitly stated	Powdered and minced with scissors	-20°C	Aluminium foil
Hagaman	2020	Not explicitly stated	Posterior vertex	Not explicitly stated	Not explicitly stated	Not explicitly stated
Hobo	2020	Not explicitly stated	Posterior vertex	Pulverised (ball mill)	Not explicitly stated	Not explicitly stated
Lob	2020	10 (minimum)	Posterior vertex	~3cm length	Not explicitly stated	Not explicitly stated

Kluczniok	2020	7.5 (minimum)	Posterior vertex	Not explicitly stated	Not explicitly stated	Not explicitly stated
Lanfear	2020	Not explicitly stated	Posterior vertex	Cut into snippets	Not explicitly stated	Aluminium foil
Leppanen	2020	Not explicitly stated	Posterior vertex	Not explicitly stated	Not explicitly stated	Aluminium foil and plastic bag
Ling	2020	20	Posterior vertex	Pulverised (ball mill)	Room temperature	Aluminium foil
Musana	2020	5	Occipital region	Pulverised using an omni bead ruptor tissue homogeniser	Not explicitly stated	Aluminium foil (end closest to the scalp was marked with tape), placed in a plastic bag
Schmidt	2020	7.5	Posterior vertex	Pulverised (ball mill)	Not explicitly stated	Not explicitly stated
Ertekin	2020	Not explicitly stated	Not explicitly stated	Not explicitly stated	Not explicitly stated	Not explicitly stated
Tarullo	2020	15-30 or 5 when hair sample limited	Posterior vertex	Pulverised (ball mill)	-20°C	Not explicitly stated
Gomez-Gomez	2020	50	Posterior vertex	Comparison studies on milled v minced	Room temperature	Not explicitly stated
Troller Renfree	2020	15	Posterior vertex	Pulverised (ball mill)	-40°C	Not explicitly stated
Caparros-Gonzalez	2019	infant min : 5	Posterior vertex	Powdered	Room temperature	wrapped in aluminium foil and stored in paper envelope
De la Rubia Orti	2019	Not explicitly stated	Nape of neck	Not explicitly stated	Not explicitly stated	Not explicitly stated

Ferro	2019	30-35	Posterior vertex	Pulverised (ball mill)	Not explicitly stated	Paper clipped to cardstock indicating the direction of growth.
Galbally	2019	5	Posterior vertex	Cut into 1cm pieces	Not explicitly stated	Not explicitly stated
Garcia Leon	2019	Not explicitly stated	Posterior vertex	Pulverised (ball mill)	Room temperature	Aluminium foil
Gonzalez	2019	50	Posterior vertex	Cut into small pieces with scissors	Room temperature	Aluminium foil
Hardy	2019	20	Posterior vertex	Not explicitly stated	Not explicitly stated	Aluminium foil
Jahangard	2019	7.5	Posterior vertex	Whole	Not explicitly stated	Aluminium foil
Kim	2019	10-20	Posterior vertex	Minced with scissors	Room temperature	Aluminium foil
Molenaar	2019	Not explicitly stated	Posterior vertex	Minced	Room temperature	Paper envelope, taped with the scalp end marked
Mustonen	2019	5-15	Posterior vertex	Minced using surgical scissors	Dark dry place	Aluminium foil
Savas	2019	20	Posterior vertex	Cut into 1 cm pieces	Room temperature	Not explicitly stated
Schepers	2019	Not explicitly stated	Posterior vertex	Whole hair sent to Dresden (first 3 cm of hair used)	Not clear but presumably room temperature	Not explicitly stated
Wang	2019	10	Posterior vertex	Pulverised (ball mill)	Room temperature	Not explicitly stated
Xu	2019	Not explicitly stated	Posterior vertex	Pulverised (ball mill)	-20°C	Dry tubes

Zgaga	2019	20-44	Posterior vertex	Cut into 1.25cm segments	Room temperature and in dark conditions	Not explicitly stated
Shapero	2019	7.5	Posterior vertex	Whole	Room temperature	Not explicitly stated
Abdulateef	2019	20-50	Posterior vertex	Cut into 2-3mm segments	Room temperature and a dark place	Aluminium foil
Aryaie	2018	50	Not explicitly stated	Pulverised (ball mill)	Not explicitly stated	Not explicitly stated
Balagova	2018	12.5, 25 and 50	Not explicitly stated	Pulverised	Not explicitly stated	Not explicitly stated
Binz	2018	20	Posterior vertex	3cm segments	Not explicitly stated	Not explicitly stated
Muehlhan	2018	Not explicitly stated	Inion of the Os occipitalis.	Not explicitly stated	Not explicitly stated	Not explicitly stated
Van Aken	2018	17.2	Posterior vertex	Not explicitly stated	Room temperature	plastic tube
Dong	2017	30	Posterior vertex	1cm segments	Not explicitly stated	Not explicitly stated
Hollanders	2017	Not explicitly stated	Posterior vertex	Not explicitly stated	Not explicitly stated	Not explicitly stated
Kristensen	2017	10-20	Posterior vertex	Minced with scissors	Not explicitly stated	Aluminium foil
Slezak	2017	5.2	Posterior vertex	Comparison between pulverised, non-pulverised and digested	Room temperature	Not explicitly stated
Ramirez	2017	10	Base of skull	Minced 1 mm pieces of the hair used	Room temperature	Not explicitly stated

Gaudl	2016	Not explicitly stated	Collection of hair samples followed standardized protocols of the LIFE Child study centre	Pulverised (ball mill)	Not explicitly stated	Not explicitly stated
Mwanza	2016	20	Posterior vertex	Not explicitly stated	-20°C	Not explicitly stated
Olstad	2016	15-20	Posterior vertex	Cut into 1cm segments then finely chopped	Room temperature	Paper envelope
Papafotiou	2016	20	Posterior vertex	Cut into 1cm segments	Room temperature	Paper envelope
Turner	2016	15-20	Posterior vertex position	Cut finely	Room temperature	Taped to paper slip, then placed in paper envelope
Wester	2016	15	Posterior vertex	Cut finely	Not explicitly stated	Not explicitly stated
Andrade	2016	10 (per 1 cm hair)	Posterior vertex	Minced	Not explicitly stated	Not explicitly stated
Noppe	2015	10-30	Posterior vertex	Whole	Not explicitly stated	Not explicitly stated
Quinete	2015	50	Posterior vertex	Minced	Not explicitly stated	Not explicitly stated
Xiang	2015	Not explicitly stated	Posterior vertex position	Pulverised (ball mill) and minced	Not explicitly stated	Not explicitly stated
Slominski	2015	50	Posterior vertex	Comparison between pulverised and non-pulverised hair	Room temperature	Not explicitly stated
Gao	2013	10	Posterior vertex	Pulverised (ball mill) and minced	Not explicitly stated	Not explicitly stated
Hoffman	2013	5-20	Posterior vertex	Pulverised (ball mill)	Not explicitly stated	Aluminium foil

Krumbholz	2013	50	Posterior vertex	Pulverised (ball mill)	Not explicitly stated	Not explicitly stated
Xing	2013	30	Posterior vertex	Pulverised (ball mill)	Room temperature	Dry tubes
Deshmukh	2012	50	Posterior vertex	Minced to 1mm segments using scissors	Not explicitly stated	Paper envelopes (labelled, sealed)
Van Rossum	2012	10	Posterior vertex	Cut into small pieces	Not explicitly stated	Paper (taped)
Dominguez-Romero	2011	20	Posterior vertex	cut into 1mm	Room temperature	Paper envelope
Jung	2011	Not explicitly stated	Posterior vertex	Cut into 1-2mm lengths	Room temperature	Not explicitly stated
Xie	2011	20	Posterior vertex region	Pulverised (ball mill)	-20°C	Dry tubes
Gao	2010	50	Close to scalp	Pulverised (ball mill)	Room temperature	Dry tubes
Zhang	2008	100	Posterior vertex	Not explicitly stated	Not explicitly stated	Not explicitly stated
Sauve	2007	150 strands (approx. 20)	Posterior vertex	Cut into small segments	Room temperature	Not explicitly stated
Wheeler	2006	50	Not explicitly stated	Cut into 5mm lengths	Not explicitly stated	Not explicitly stated
Raul	2004	30-100	Vertex Posterior	Pulverised (ball mill)	Room temperature	Not explicitly stated
Bang	2004	50	Posterior vertex and occipital region	Cut into 1-2mm segments with scissors	Not explicitly stated	Not explicitly stated
Cirimele	2000	50	Posterior vertex	Pulverised (ball mill)	Room temperature	Dry tubes

Kintz	2000	100	Not explicitly stated	1cm section	Not explicitly stated	Not explicitly stated
Bevalot	2000	50	Posterior vertex	Pulverised (ball mill)	Not explicitly stated	Not explicitly stated
Kintz	1999	100	Not explicitly stated	Minced into 1mm pieces	Room temperature	Tubes
Scherer	1998	100	States "head hair".	Pulverised (ball mill)	Room temperature	Not explicitly stated
Akefe	2017	10 (minimum)	Posterior vertex	Minced (scissors)	Room temperature in dry place	Not explicitly stated
Binz	2016	20	Posterior vertex from humans. Shoulder region, 1 x 1 cm square from cow.	4 different methods (snippets, pulverised(powder))	Not explicitly stated	Not explicitly stated
Meyer	2014	50	Human; Posterior vertex Monkey; Neck	Pulverised (ball mill)	-20°C and, if necessary, shipped overnight at ambient temperature.	Aluminium foil, Paper envelope, or a 15 ml screw-cap polypropylene centrifuge tube
Hold	1999	10-50	Back of rats close as possible to scalp for humans	Not explicitly stated	Not explicitly stated	Not explicitly stated
Braun	2022	20	Neck area and forehead	Snippets	Room temperature	Aluminium foil and stored in airtight plastic bags until analysis.
Otten	2022	50	Back (caudo-dorsal); Pig Proximal tail hair; Cow	Powdered with ball mill	Room temperature	dried, and stored protected from light
Pokharel	2021	50	Tail hair	Pulverised (ball mill)	Not explicitly stated	Not explicitly stated
Santymire	2021	Not explicitly stated	Not explicitly stated	Not explicitly stated	Not explicitly stated	Not explicitly stated

Contreras	2021	20 and 40	Lumbosacral area	Powdered	Room temperature	Aluminium foil
Van Eerdenburg	2021	35	Flank, a square of 2x2cm	Powdered	Room temperature and in dark conditions	Not explicitly stated
Amin	2021	Not explicitly stated	Belly	Not explicitly stated	Not explicitly stated	Not explicitly stated
Begall	2021	50	Dorsal part of the body with a small-animal shaver	Powdered	-20°C	Not explicitly stated
Olvera-Maneu	2021	50	Body location chosen to prevent contamination with urine or faeces	Pulverised (ball mill)	Room temperature	Paper envelope
Arena	2021	65	Not explicitly stated	Cut to 1-3mm length	-20°C	Plastic bag
Charalambous	2021	60	Not explicitly stated	Powdered	-18°C	plastic bag
Di Francesco	2021	50	Rump (Experiment 1), neck, shoulder and rump (Experiment 2)	cut into 5mm pieces	Not explicitly stated	Paper envelopes
Sandoval-Herrera	2021	3-10 Minimum of 3 needed	Scapular region on the back of the bats	Whole	Not explicitly stated	Not explicitly stated
Wiechers	2021	7.5	Between neck and shoulder blades	Not explicitly stated	Room temperature in dark and dry conditions	Plastic bag
Hein	2021	15	Guard fur	cut into <4mm segments	Room temperature in dark conditions	Paper envelope
Van der Walt	2021	133	Rump and back	Pulverised (ball mill)	Room temperature	Plastic bag
Cordeschi	2021	Not explicitly stated	Collected by hair trap	Not explicitly stated	Not explicitly stated	Not explicitly stated

Keogh	2021	Not explicitly stated	Not explicitly stated	pulverised to powder	Room temperature	Paper envelope or plastic bag
Otsuki	2021	10	Ventral side	Pulverised (ball mill)	-20°C	Not explicitly stated
Alon	2021	72 (wool)	Mid-side	Whole	Not explicitly stated	Paper bags
Weaver	2021	100	Mid-side of the sheep	Powdered	Room temperature	Plastic bag
Park	2020	20	Tail switch	Pulverised (ball mill)	-80°C	Not explicitly stated
Peric	2020	60	Not explicitly stated	Whole. Hair not powdered or scissors cut	Room temperature	Not explicitly stated
Calamari	2020	50	head, torso and limbs	Minced using scissors	Room temperature	Paper bag
Garber	2020	Not explicitly stated	Back of neck	Not explicitly stated	Not explicitly stated	Paper envelope and stored in dry container
Chu	2020	20	Back/leg region	1-2mm segments	Not explicitly stated	Not explicitly stated
Heimburge	2020	50	Back and tail tip on both, from neck on pigs and shoulder on cows using electric shaver as close to skin a possible	Not explicitly stated	Room temperature and dark	Not explicitly stated
Elmi	2020	60	Back area, neck to base of tail	Not explicitly stated	Room temperature in dark conditions	Biohazard transport bags
Lopez-Arjona	2020	60	Rump region	Powdered in a homogeniser	Room temperature	Plastic bag (individually marked)
Montillo	2020	60	Dorsal area of neck, behind the ears	Not explicitly stated	Room temperature in dark conditions	Paper envelope

Colding-Jorgensen	2020	Not explicitly stated	Back	Pulverised (ball mill)	Room temperature	Cellulose bag inside a plastic tube
Sergiel	2020	25	Guard hairs between the shoulder blades	Pulverised according to Macbeth paper	Room temperature	Not explicitly stated
Lubcker	2020	Not explicitly stated	Whiskers, did not include sections of the root	Powdered in mortar and pestle after freezing with liquid nitrogen	Room temperature	Plastic bag
Schillaci	2019	0.69 -3.67	Not explicitly stated	Powdered	Not explicitly stated	Not explicitly stated
Colombo	2019	20	Close to the skin	Pulverised (ball mill)	-20°C	Not explicitly stated
Maxwell	2019	5	Thigh	Cut with scissors to 2-5mm pieces then powdered after freezing in liquid N2	Room temperature	Plastic bag
Karpovich	2019	0.7-15.5	Whiskers	fine powder	Room temperature	Paper envelopes
Santangeli	2019	10	Tail	Pulverised (ball mill)	Room temperature	Not explicitly stated
Nejad	2019	50	3 sites; Forehead, Withers (right side), and Rump (right side)	Minced with scissors	Not explicitly stated	Not explicitly stated
Placci	2019	60	Mane	Trimmed horsehair	-20°C	Not explicitly stated
Charalambous	2019	60	Nape of neck	Pulverised (ball mill)	-20°C. In transit, the fur was kept at ambient temperature, and on arrival to the laboratory, the fur was stored at -80°C	Aluminium foil

Nejad	2019	50	Forehead	Comparison between pulverised and scissors cutting	Room temperature	Not explicitly stated
Sharma	2019	50	Tail switch	Pulverised manually (pestle and mortar)	Room temperature	Not explicitly stated
Phillips	2018	50	Thigh	Pulverised (ball mill)	Room temperature in dark conditions	15 mL screw-cap polypropylene centrifuge tubes
Kapoor	2018	Not explicitly stated	Back area, through shave and reshave	Pulverised (ball mill)	Not explicitly stated	Not explicitly stated
Nedic	2017	Not explicitly stated	Tail	Pulverised (ball mill)	-20°C	Plastic bag
Tallo-Parra	2017	50	Ventrolateral region of the neck	Pulverised (ball mill)	Room temperature	Not explicitly stated
Kwok	2017	50	Mane	Pulverised (ball mill)	Not explicitly stated	Not explicitly stated
Yamanashi	2016	5	Arm hair	Powdered	Room temperature	Not explicitly stated
Fourie	2016	10	Tail, thigh and deltoid. inter scapular region from wild animals	Minced	Lab temperature	Paper envelopes (labelled)
Weisser	2016	1000	Not explicitly stated	Cut into <3mm pieces using scissors	Not explicitly stated	Not explicitly stated
Tallo-Parra	2015	50	Forehead	Minced into <2 mm pieces	Room temperature	Not explicitly stated
Mastromonaco	2014	Not explicitly stated	Right hindlimb	Cut into 5mm pieces	Room temperature	Labelled 1.5ml Eppendorf tube
Peric	2013	60	Forehead	Trimmed hair	Room temperature	Not explicitly stated

Gray	2013	100	Mane hair samples were obtained using the mane pulling technique, commonly employed during routine grooming	Cut into short sections of 3mm or less	4°C	Plastic bag (sealed)
Keckeis	2012	19-95	Back area, shave and reshave	Not explicitly stated	-20°C	Plastic bag
Aqai	2009	500	Back. 20 cm from pour-on area	Pulverised (ball mill)	Not explicitly stated	Not explicitly stated
Shen	2009	50	Back	not powdered or minced	Room temperature	Not explicitly stated
Accorsi	2008	60	Ischiatic region was manually shaved to the level of the skin 1-2 days before commencement of the study to eliminate old-growth hair. The same patch of skin was resampled at the end of the sampling period.	Minced (scissors (1-3 mm))	Room temperature	Not explicitly stated
Davenport	2006	50	Posterior vertex of neck	Pulverised (ball mill)	Not explicitly stated	aluminium foil

3.5 Sample preparation

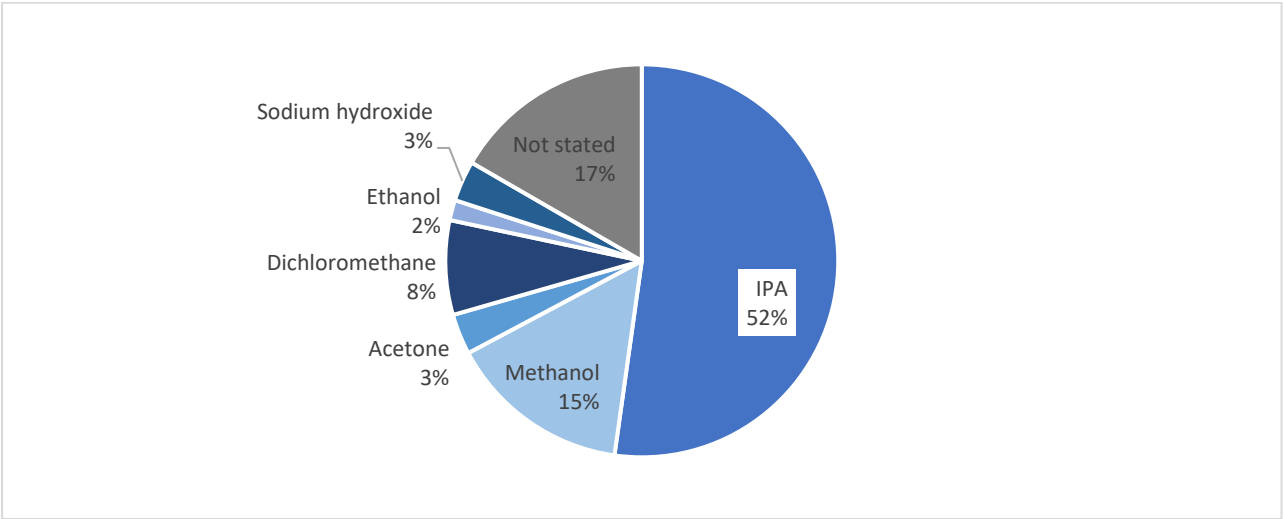
3.5.1 Cleaning solution and conditions

A total of 94 studies (52%) used isopropanol, 27 studies (15%) used methanol as their cleaning solution. Acetone (N=6), ethanol (N=3), sodium hydroxide (N=6) or dichloromethane (N=14) were used to wash hair in a small number of studies (N=29, 16%). For use of a washing solution, thirty studies (17%) did not mention using one (**Figure 9**).

Thirty-two studies (18%) mentioned room temperature as their chosen cleaning temperature, 33 studies (18%) cleaned at 40°C, and one study set 95°C as their cleaning condition (1%). A total of 114 did not mention what temperature cleaning was carried out under (63%). The duration of washes varied, but all wash protocols were reasonably short (≤30 min.) with long drying times for samples afterward (16-24 hours). Seventy-six studies (42%) did not describe washing method.

Fourteen studies rotated samples during washing (8%), twenty-three studies used shaking (13%), including 5 studies which shook by hand, seven studies which used a vortex and 11 studies which used a shaking table. Four studies used an ultrasonic bath (2%). One hundred and thirty-nine studies (77%) did not mention if they rotated/rolled, shook, or sonicated samples. Twenty-five studies (14%) had wash durations of 2 minutes or less, 40 studies (22%) had 3-minute wash durations, 11 studies (6%) had washes between 5 and 15 minutes, one study (1%) washed for 30 minutes. One hundred and three studies (57%) did not mention a duration of washing.

Figure 9 Washing solution used in studies



3.5.2 Hair preparation

Seventy-eight studies out of 180 studies (43%), powdered their hair samples by means of either a ball mill (73/78), homogeniser (2/78), cell disruptor (1/78), or mortar and pestle (2/78). A further 60 (33%) segmented their hair samples using a scissors or other means, and 11 (6%) used whole hair in their analysis. Thirty-one (17%) papers did not mention hair preparation.

3.5.3 Extraction solution and conditions

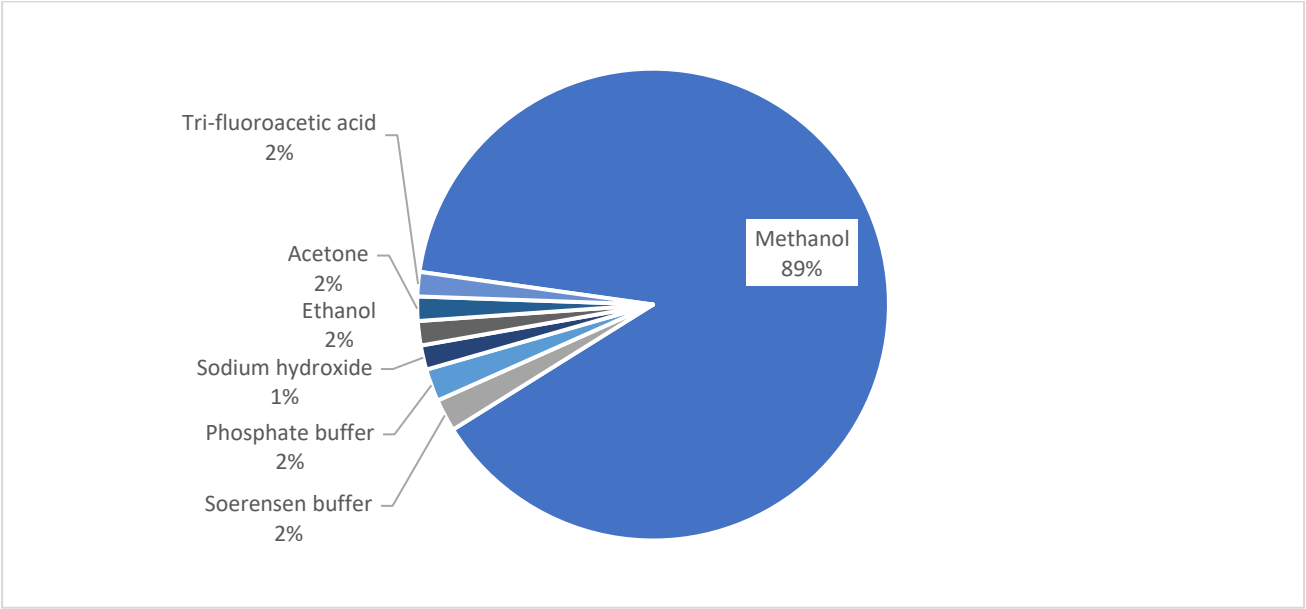
The most used solvent when carrying out extraction was methanol, in 160 out of 180 studies (88%). Four studies (2%) used Soerensen's buffer, 4 phosphate buffer (2%), 3 ethanol (2%), 3 acetone (2%), 3 sodium hydroxide (2%), and 3 tri-fluoroacetic acid (2%) (**Figure 10**).

Thirty-three studies (18%) also spiked their methanol extraction solvent with an internal standard of the analyte being investigated (e.g: cortisol, testosterone, etc.) as a means of minimising the effects of random and systematic errors during analysis.

The extraction conditions and duration varied. Ninety of the publications (50%) carried out extraction for 18-24 hours. Forty-two studies (23%) had extraction times shorter than 18 hours. Five studies (3%) carried out extraction between 25 and 72 hours, and forty-three studies (24%) did not describe their extraction time. The publications which had shorter extraction times had adjusted variables (i.e., temperature at which extraction was carried out, use of ultrasonic bath, inversion, rolling).

Forty studies (22%) shook their samples during extraction, twenty-seven studies (15%) used rotation, nineteen studies (11%) used sonication. Ninety-four studies (52%) did not mention any of these extraction conditions.

Figure 10 Extraction solution used in studies



3.6 Quantitation method

Hair steroid measurements were made in 63 studies (35%) by Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS), forty-five (25%) by Enzyme-Linked Immunosorbent Assay (ELISA), twenty-six (14%) by Enzyme Immunoassay (EIA), thirteen (7%) by Radioimmunoassay (RIA), fourteen (8%) by Ultra-High Performance Liquid Chromatography Mass Spectrometry (UHPLCMS), six (3%) by Gas Chromatography Mass Spectrometry (GCMS), nine (5%) by Chemiluminescence Immunoassay (CLIA), two (1%) by AlphaLISA, and one each by Time-Resolved Fluorescent Immunoassay (TRFIA), Liquid-Chromatography Time-Of-Flight Mass Spectrometry (LCTOFMS) and isotope-ratio mass spectrometry (IRMS) assay methods (N=3, 2%)(**Figure 11**).

The mean concentrations for each analyte and reported values for each publication are shown in **Table 4**. Sample preparation details for all 180 studies are described in **Table 5.**

Figure 11 Quantitation method used in studies

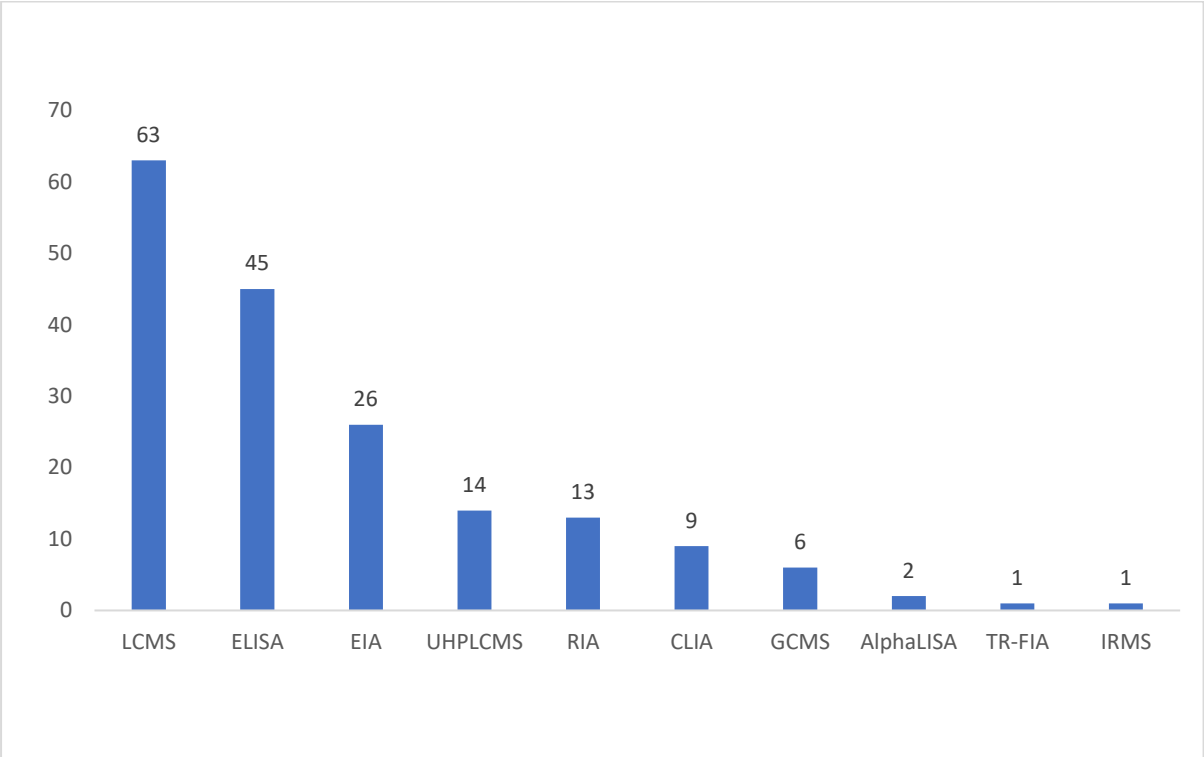


Table 4 The mean (range) concentrations (pg/mg) in hair for the selected endogenous steroids

Mean of reported means/medians is shown.

	Human	Animal
Cortisol	57 (0.252-1166)	275.6 (0.32-3500)
17-OHP	15.4 (0.219-66)	5.9*
Testosterone	8.2 (0.259-67.5)	14.6 (2-67.5)
Androstenedione	15.5 (1.6-80)	4.6*
DHEAS	31.1 (2.27-60)	4.5 (1.4-7.66)
Vitamin D	237.2 (197.6-276.7)	n/a

Footnote: 17-OHP: 17-hydroxyprogesterone; DHEAS: dehydroepiandrosterone sulfate. Values based on a single publication are highlighted with an asterix (*). Some studies were not included because it was not possible to convert reported concentrations to pg/mg: Cruickshank (2021), Jahangard (2019), Schepers (2019), Arvaie (2018), Slominski (2015), Akefe (2017), Begall (2021), Arena (2021), and Kapoor (2018). We also excluded extreme outliers: Van der Walt (2021), Santangeli (2019) and Maxwell (2019).

Table 5 Overview of sample preparation procedures

Study ID	Year	Volume (ml) Cleaning solution	Cleaning conditions	volume (ml) Extraction solvent	Extraction conditions	Assay quantitation method
Brendgen	2023	Isopropanol (2.5ml)	gently mixed on an overhead rotator. After decanting, the wash cycle was repeated and the hair was left to dry overnight	Methanol (1.5 ml 100%)	Rotated slowly for 24 hours	CLIA
Peng	2022	SDS	(SDS(5%)) in water for 5 min., followed by Milli-Q water for 1 min. and Methanol for 5 min. at room temperature	Methanol (2ml) and internal standard (10ul)	Incubated for 24 hours @40°C with gentle shaking. After centrifugation (10 min., 4000 g, room temperature), 1.6 mL of clear supernatant was transferred into a new glass tube resuspended in MeOH (1 mL of 5%) in water and subsequently purified by solid-phase extraction (SPE) using a Bond Elut C18 cartridge preconditioned with 1 mL of MeOH followed by 1 mL of Milli-Q water. After loading the sample, the SPE cartridge was washed with 1 mL of 10% MeOH in water, vacuum dried for 30 min, and eluted three times with 0.7 mL of methanol each.	LC-MS/MS
Doom	2022	Isopropanol (2.5ml)	3 min. at Room temperature. Allowed dry in fume hood for 12 hours	Methanol (1.8ml) and internal standard (50ul)	Incubated for 18 hours @RT. Samples were spun in a centrifuge at 10,000 rpm for 2 min. and 1 mL of the clear supernatant was transferred into a new 2ml tube. The alcohol was evaporated at 65°C under a constant stream of nitrogen until the samples were completely dried	LC-MS/MS
Koskivuori	2022	Isopropanol (1.5ml)	Washed for 2 min. and dried under a stream of nitrogen at 40°C	Methanol (1.5ml) and Internal standard (20ul)	Shaking for 18-20 hours in ambient temperature (+22 ±2°C)	UPLC-HRMS
Moody	2022	Isopropanol (5ml HPLC grade)	Rotating	Methanol (1.5ml)	Inversion at room temperature for 24 hours.	EIA
Puglisi	2022	Dichloromethane (1.5ml) and methanol (1.5ml)	Once with each, Vortex mixed for 3 min.	Methanol (1ml) and Internal standard (25ul)	Vortexed for 5 min, centrifuged at 4,000 rpm for 3 min., to ensure the complete immersion of the matrix into the solvent, and final incubation at 55°C for 15 hours	UPLCMS
Scholz	2022	Deionised water (15ml) and Acetone (10ml) and hexane (10ml)	Washed for 2 min. each with slight shaking	Methanol (1ml) and internal standard (0.1ml)	Ultra-sonification for 2 hours at 55°C	LC-MS/MS
Zhu	2022	Methanol (2ml)	Washed twice	Methanol (950ul) and internal standard (50ul)	Vortexed for 30 seconds and incubated at 27°C for 24 hours	HPLC-MS

Broeks	2021	Not explicitly stated	Not explicitly stated	Methanol	25°C for 18 hours with internal standard	LC-MS/MS
Brossaud	2021	Not explicitly stated	Not explicitly stated	Sodium hydroxide (NaOH (0.77 mg/mL 0.1N))	Incubated for 2 hours. Supernatant then extracted by Liquid-liquid extraction with dichloromethane	LC-MS/MS
Bryson	2021	Not explicitly stated	Not explicitly stated	Methanol	Reconstituted in phosphate buffered saline	ELISA
Cajachagua-Torres	2021	Isopropanol (LC grade)	Room temperature, left dry for at least two days	Methanol (LC grade)	for 18 hours at 25°C in slightly shaking water basin. Transferred to glass tube, centrifuged @4300xg	LC-MS/MS
Chen	2021	Methanol (3ml)	Twice for 2 min.	Methanol (1ml) with 1ng internal standard	24 hours at 25°C. Centrifuged @12000 rpm for 5 min.	LC-MS/MS
Damen	2021	Not explicitly stated	Not explicitly stated	Not explicitly stated	Not explicitly stated	LC-MS/MS
Duan	2021	Methanol (1ml)	Three times	Methanol (950ul) and internal standard (50ul)	At 40°C for 24 hours. After incubation, vortexed for 5 seconds and centrifuged @12000 rpm for 5 min.	LC-MS/MS
Gherlone	2021	Isopropanol	Three times	Methanol (1ml HPLC grade)	Overnight at room temperature. Methanol decanted and dried at 37°C in vacuum centrifuge for at least 1.5 hours. Extracts reconstituted in 250ul PBS ph8 and vortexed.	ELISA
Izawa	2021	Isopropanol (2.5ml)	Three times	Methanol (1.5ml)	48 hours with slow rotation. samples then spun in centrifuge @10000 rpm for 2 min., 0.5ml supernatant evaporated @60°C until completely dry. Resuspended in 50ul assay diluent before analysis	EIA
Mazgelyte	2021	Isopropanol (5ml)	Twice	Soerensen's buffer (1.5 mL pH 7.6)	16 hours at 40°C, in the presence of 10 ng of 6-Î±methylprednisolone as internal standard. Each sample then was transferred to solid-phase extraction Discovery DSC-18 column, which was previously equilibrated (3 mL MeOH followed by 1.5 mL of water). The subsequent steps were the following: washing with 0.5 mL of water followed by 0.5 mL of acetone/water(1:4, v/v), 0.25 mL of hexane, and elution with 1.5 mL of diethyl ether. The eluates were evaporated under a stream of nitrogen gas and resuspended with 100uL of acetonitrile/water(1:1, v/v).	UHPLC
Muentner	2021	Isopropanol	Twice	Methanol and internal standard	Incubated overnight. Following incubation, tubes were vortexed and centrifuged, the supernatant was removed and run through solid-phase, followed by liquid-phase extraction.	LC-MS/MS
Ney	2021	Isopropanol/hexane mixture(4:1,v/v) (2ml)	3 min.	Methanol (1.6ml) and internal standard (50ul)	Incubated overnight (18h). The following day the hair was removed before the samples were dried under nitrogen.	UPLC MS

Rajcani	2021	Isopropanol (2.5ml)	Twice	Methanol (1.5ml)	Not explicitly stated	CLIA
Reid	2021	Isopropanol (2ml)	shaking for 3 min., twice	Methanol (2ml)	shaking horizontally for 24 hours	ELISA
Tuladhar	2021	Isopropanol	Twice	Methanol	Evaporated, and the residue was reconstituted in assay buffer.	EIA
Vehmeijer	2021	Isopropanol (LC-grade)	2 min. at room temperature	Methanol (LC-grade)	18 hours at 25°C, in the presence of deuterated steroids	LC-MS/MS
Voegel	2021	Deionised water (15ml) followed by Acetone (10 ml)	Water for 3 min. , followed by Acetone for 2 min. , Tubes were shaken by hand during this process. The washing solutions were decanted and disposed.	Methanol (1ml) and internal standard (50ul)	Briefly shaken and placed in a sonication bath (35 kHz, 600 W) for 4 hours at 55°C. Centrifugation for 5 min. at 9000 g, the methanolic extract was transferred into a column rack. Sample extracts loaded onto SLE columns and allowed to absorb for 5 min. Analytes were then eluted two times with 2.5 mL ethyl acetate with a wait time of 5 min in between. The extracts were dried and resuspended in 60uL MeOH and 140 uL reconstitution solution	LC-MS/MS
Wu	2021	Methanol (3ml)	2 min. shaking	Methanol (950ul) and internal standard (50ul)	Incubated at 25°C for 24 hours. After, 800ul of the incubation solution was taken out and dried at 50°C, protected by nitrogen, and then resuspended in 100ul tri-distilled water and 1ml methanol. Then the mixture was extracted using a SPE C18 column. Afterwards, the eluate was dried again and dissolved in 50ul mobile phase for HPLC analysis.	LC-MS/MS
Zhang	2021	Isopropanol (2.5ml)	3 min. at room temperature	Methanol (1.8ml) and internal standard (100ul)	Incubated for 18 hours @RT. Samples were spun in a centrifuge at 10,000 rpm for 2 min. and 1 mL of the clear supernatant was transferred into a new 2ml tube. The alcohol was evaporated at 65°C under a constant stream of nitrogen until the samples were completely dried (20 min.). The dry residue was resuspended using 250uL distilled water, 200uL of which were used for LC-MS/MS analysis.	LC-MS/MS
Shah	2021	Methanol/water	Dried at room temperature under gentle stream of nitrogen	Methanol/water (2ml, 50:50) and internal standard	Sonicated for 2 hours, followed by centrifugation at 1250g for 15 min. Top layer was collected and filtered through a syringe filter PTFE membrane (0.45um) into new test tubes and then dried at 40°C under a gentle stream of nitrogen.	UHPLC MS
Bevan	2021	Isopropanol (2.5ml)	3 min. at room temperature	Methanol (1.8ml) and internal standard (100ul)	Incubated for 18 hours @RT. Samples were spun in a centrifuge at 10,000 rpm for 2 min. and 1 mL of the clear supernatant was transferred into a new 2ml tube. The alcohol was evaporated at 65°C under a constant stream of nitrogen until the samples were completely dried (20 min.). The dry residue was resuspended using	LC-MS/MS

					250uL distilled water, 200uL of which were used for LC-MS/MS analysis.	
Cruickshank	2021	Isopropanol	Twice, left to air dry	Methanol	Incubated overnight (16 hours) at room temperature	ELISA
Hobo	2021	Isopropanol (0.5ml)	Twice	Tri-fluoroacetic acid (0.5 mL 0.1 M) and acetonitrile solution (50%)	Incubated at 40°C for 1 hour. Next, Methyl tert-butyl ether (4ml) was added to extract in organic phase the analytes and the mixture was shaken. The organic layer was then separated and dried with a centrifugal evaporator. An aliquot of methanol (0.5ml) and water (1ml) were sequentially added to the residue before purifying it with the OASIS MAX cartridge, a mixed-mode type solid-phase extraction (SPE) column. The steroid fraction was then eluted and dried with a centrifugal evaporator, and the fraction derivatized with picolinic acid was then purified on the HyperSep SI cartridge, which is a normal phase SPE column.	LC-MS/MS
Marceau	2021	Isopropanol (5ml)	Twice, Rotating for 3 min.	Methanol (1.5ml)	Inversion at room temperature for 24 hours.	EIA
Mažeikienė	2021	Isopropanol (3ml)	Gently shaking for 3 min. at room temperature	Methanol (1.4ml) and internal standard (20ul)	Incubated at 50°C for 4 hours.	UHPLC
Skoluda	2021	Isopropanol (2.5ml)	3 min. at room temperature	Methanol (1.8ml) + Internal standard (50ul)	Incubated 18 hours at room temperature. 1ml supernatant transferred to clean tube and dried . Resuspended in 250ul distilled water, 200ul used for LCMS analysis	LC-MS/MS
Spikman	2021	Not explicitly stated	Not explicitly stated	Methanol (1.4ml (LCMS-grade)) and internal standard (100ul)	Incubated for 18 hours , then SPE carried out	LC-MS/MS
Zhang	2021	Isopropanol (2.5ml)	3 min. at room temperature	Methanol (1.8ml) + Internal standard (50ul)	Incubated 18 hours at room temperature. spun 10000 rpm for 2 min. and 1ml supernatant transferred to clean tube and dried . Resuspended in 250ul distilled water, 200ul used for LCMS analysis	LC-MS/MS
Buse	2021	Isopropanol (2.5ml)	3 min. rotating at room temperature	Methanol (1ml)	24 hours with slow rotation	CLIA
Simon	2021	Isopropanol (5ml)	3 min. repeated inversion on rotator, twice	Methanol (1ml)	18-24h at room temp. with constant inversion on a rotator	ELISA
Behnke	2021	No cleaning	Not explicitly stated	Methanol (1.5ml)	Ultrasonic bath at 50°C for 6 hours, then centrifuged (3100 rpm for 10 min.)	LC-MS/MS
Romero-Gonzalez	2021	Isopropanol	Not explicitly stated	Methanol	Not explicitly stated	ELISA

Castro-Vale	2020	Isopropanol (2ml)	for 2 min.	Methanol(1.4ml) with Internal standard (100ul)	18 hours at 25°C being gently shaken. Transferred to glass vial then centrifuged @4500 rpm @4°C for 5 min.	LC-MS/MS
Dion Nist	2020	Isopropanol (250ul hplc grade)	Inverted on shaking table for 5 min., Isopropanol poured off and repeated.	Methanol (550ul HPLC grade)	24 hours at room temperature on shaking table. Centrifuged 5000xg for 5 min.	ELISA
Ferro	2020	Isopropanol (12ml)	Shaken for 2 min. by hand, Isopropanol then discarded. Twice	Ethanol (1ml 1%)	Shaken by hand and rotated at 45 rpm on mixer for 24-72 hours at room temperature. vortexed for 5 seconds then centrifuged @2800 rpm for 15 min. 1ml supernatant air dried for 48 hours. supernatant reconstituted with 150ul assay diluent, vortexed for 5 seconds and centrifuged for 10 min., assayed in duplicate	ELISA
Grova	2020	SDS and then methanol	Three washes; SDS (5%) in water, Samples agitated for 5 min. in shaker incubator and aqueous phase removed. Second washing performed by adding water again to eliminate foam from SDS, Aqueous phase removed again. Methanol added and solution agitated for 5 min. in shaker/incubator.	Methanol (2ml)	50 mg of powdered hair, in the presence of 2ng of cortisol-D4, THE-D5, Testosterone-D3, estradiol-D5, 13C6-T3 and 13C6T4 (40 pg/mg of hair). The mixture was agitated for 24 hours at 40°C in an incubator. The liquid phase was separated from the solid phase by centrifugation at 2800xg for 10 min. at room temperature. A second step of solid-liquid extraction was then applied on the residue by adding 2mL of a mixture of H2O-MeOH(50:50,v/v). The mixture was shaken 2 hours at room temperature. The supernatant was then transferred into a tube and evaporated to dryness under nitrogen flow (N2) at 37°C . Then, acidic hydrolysis was carried out by adding 1mL of 0.1M of HCl into the extract residue and left to react at 90°C for 1 hour. The sample was cooled before addition of 100uL of NaOH solution at 1N and 900 µL of phosphate buffer (pH=7.0,0.1M)and shaken vigorously for 2min. For the next purification stage, an SPE-C18 purification column conditioned sequentially. The hormones were then eluted with two successive volumes of 1mL methanol. The eluates were then evaporated to dryness under nitrogen flow at 37°C and suspended again in 100uL of mobile phase composed of methanol and water with 0.1% HCOOH(50:50;v/v).	UPLC MS
Hagaman	2020	Not explicitly stated	Not explicitly stated	Not explicitly stated	Not explicitly stated	LC-MS/MS
Hobo	2020	Isopropanol (10ml)	Twice	Trifluoroacetic acid (5 mL 0.1 M) and acetonitrile solution (50%)	Incubated at 40°C for 1 hour. After adding 4mL of methyl tert-butyl ether to the extract, the mixture was shaken. The organic layer was then separated and dried with a centrifugal evaporator. To the residue, 0.5 mL of methanol and 1 mL of water were sequentially added to get it dissolved which was applied into the cartridge, a mixed-mode type solid-phase extraction (SPE) column. The SPE	LC-MS/MS

					column was washed with 1 mL of 1% acetic acid solution, 1 mL of 45% methanol solution, and 1 mL of 1 M sodium hydroxide solution. Androgen, progestogen and glucocorticoid fraction was then eluted with 1 mL of methanol. Dried with a centrifugal evaporator. After the eluent was evaporated, the residue was dissolved in 100 uL of 40% acetonitrile solution.	
Lob	2020	Isopropanol (2.5ml)	3 min. at room temperature	Methanol (1.8ml) + Internal standard (50ul)	Incubated 18 hours at room temperature. spun 10000 rpm for 2 min. and 1ml supernatant transferred to clean tube and dried. Resuspended in 250ul distilled water, 200ul used for LCMS analysis	LC-MS/MS
Kluczniok	2020	Repeated washing	Not explicitly stated	Methanol (100%)	Steroid extraction with pure methanol, evaporation of methanol at 50 °C	LC-MS/MS
Lanfear	2020	Not explicitly stated	Not explicitly stated	Methanol	Not explicitly stated	LC-MS/MS
Leppanen	2020	Isopropanol (5ml)	Rotator at room temperature for 3 min. per wash	Methanol (1ml)	24 hours with slow rotation	CLIA
Ling	2020	Isopropanol	3 min., Twice	Methanol	Incubation with methanol for 18-24 hours. Methanol extracts were evaporated, and the cortisol was reconstituted in assay buffer. Particulate matter in the reconstituted samples was removed by filtration, and then the cortisol content was analysed in duplicate by enzyme immunoassay.	EIA
Musana	2020	Isopropanol (2.5 ml)	Not explicitly stated	Methanol and internal standard	Incubated for 2 hours at 37°C. Extraction was repeated twice by vortexing for 15 minutes with methanol at 37°C. Supernatants from each extraction were combined and evaporated. The extract was resuspended in 2% methanol and cleaned by solid phase extraction. The final SPE eluate was evaporated, and the resulting extract was resuspended in 100mL 15% methanol solution with 0.1% formic acid and 2Mm ammonium acetate.	LC-MS/MS
Schmidt	2020	Isopropanol	Not explicitly stated	Methanol	Not explicitly stated	LC-MS/MS
Ertekin	2020	Isopropanol (2.5ml)	In a 10ml glass, twice	Methanol (1.5ml)	18 hours, then spin the samples for 2 min. in a microcentrifuge at 10,000 rpm, 1ml of the clear supernatant was added to a new 2ml glass.	CLIA
Tarullo	2020	Isopropanol	Not explicitly stated	Methanol	Not explicitly stated	EIA
Gomez-Gomez	2020	Dichloromethane	10 min., Twice	Methanol	Sonication for 1 hour, twice. Further extraction of methanolic extract performed.	LC-MS/MS

Troller Renfree	2020	Isopropanol	Not explicitly stated	Methanol	Not explicitly stated	EIA
Caparros-Gonzalez	2019	Not explicitly stated	Not explicitly stated	Methanol (HPLC grade)	72 hours at room temperature in dark while under constant inversion using a rotator.	ELISA
De la Rubia Orti	2019	Not explicitly stated	Not explicitly stated	Methanol (1ml)	Shaking lightly at 34°C for 36 hours	ELISA
Ferro	2019	Isopropanol (12ml)	Shaken for 2 min. by hand, Isopropanol then discarded. Twice	Ethanol (1ml 1%)	Shaken by hand and rotated at 45 rpm on mixer for 24-72 hours at room temperature. Vortexed for 5 seconds then centrifuged @2800 rpm for 15 min., 1ml supernatant air dried for 48 hours. Supernatant reconstituted with 150ul assay diluent, vortexed for 5 seconds and centrifuged for 10 min., assayed in duplicate	ELISA
Galbally	2019	Isopropanol (LCMS grade)	At room temperature	Methanol (1.4 ml) and internal standards (100ul)	1.4 mL methanol and 100ul internal standards were added and the hair was incubated for 18 hours at 25°C. Samples were centrifuged at 4302g(4500 rpm), 4°C for 5 min, and 1ml of the clear supernatant was transferred into a new glass tube. The methanol was evaporated at 50°C under a constant stream of nitrogen until the samples were completely dried. The dry residue was resuspended using 1ml 2% methanol and vortexed for 1 min. Hair extracts were subsequently cleaned by solid-phase extraction	LC-MS/MS
Garcia Leon	2019	Isopropanol	Twice	Methanol (HPLC grade)	72 hours at room temperature in the dark with constant inversion on a rotor. After incubation, supernatant evaporated and extract reconstituted in 150ul PBS at ph8, sample then frozen at -20°C for later analysis.	ELISA
Gonzalez	2019	Not explicitly stated	Not explicitly stated	Methanol (4ml) with emery boards	Incubated 3 hours on a shaker at room temperature and then overnight at 50°C. Spun for 1 min. and 1.2ml supernatant collected and dried at room temperature.	CLIA
Hardy	2019	Not explicitly stated	Not explicitly stated	Not explicitly stated	Not explicitly stated	LC-MS/MS
Jahangard	2019	Isopropanol (2.5ml)	3 min.	Methanol (1.8ml)	18 hours at room temperature. Spun in a centrifuge and 1ml clear supernatant transferred to new tube. Evaporated under nitrogen and reconstituted with 225ul water, 50ul injected into HPLCMS with online SPE for purification	LC-MS/MS
Kim	2019	Isopropanol	Three times	Methanol (1ml)	Methanol added to a glass vial containing the minced hair and incubated for 16 hours at 52°C rotated at 100 rpm for steroid extraction. Next, the methanol was cooled at room temperature and transferred to a test tube. The methanol was evaporated under a gentle stream of nitrogen gas at 50°C. 250mL of phosphate buffered saline (PBS) solution was added.	ELISA

Molenaar	2019	Isopropanol (LC-grade)	2 min. at room temperature	Methanol (LC-grade)	18 hours at 25°C gently shaking in water basin. The extract was then transferred to a glass tube, centrifuged at 4300g, and evaporated to dryness at 37 °C under a constant flow of nitrogen. After reconstitution in 1 ml 2% LC-grade methanol, the extract was loaded on an offline solid phase extraction plate , washed with 1 ml 30% LC-grade methanol, and eluted twice in 300ul 100% LC-grade methanol. The extract was then evaporated to dryness at 50°C under a constant flow of nitrogen and stored at 4°C until further analysis. Prior to analysis, the samples were reconstituted in 100ul eluent, mixed using a vortex mixer	LC-MS/MS
Mustonen	2019	Isopropanol	3 min., Three times	Methanol (1.5ml)	Incubated at 55°C for 24 hours. After centrifuging at 10,000 rpm for 2 min., the supernatant was transferred to a new vial. Methanol was evaporated at 60°C under a constant stream of nitrogen until samples were dried completely. Finally, 0.15 ml of phosphate buffer was added and 50ul of each sample was analysed	ELISA
Savas	2019	Isopropanol	Not explicitly stated	Methanol	Not explicitly stated	LC-MS/MS
Schepers	2019	Isopropanol (5ml)	Rotator at room temperature for 3 min. per wash	Methanol (1ml)	24 hours with slow rotation	CLIA
Wang	2019	Isopropanol (1ml)	Washed twice with water (0.5ml), shaken for 1 min., placed in 1mL of Isopropanol (1ml) without shaking.	Methanol (500ul) and internal standard (25ul)	Incubated @37°C for 4 hours and then centrifuged again. The methanol was transferred to a glass tube and evaporated under a stream of nitrogen at 37°C. The residue was reconstituted in 150ul mobile phase A/B (v/v,60/40) and mixed for 1 min. The extract was filtered through a PTFE filter and 10uL was injected for analysis	UPLC MS
Xu	2019	Methanol	Twice	Methanol	Not explicitly stated	LC-MS/MS
Zgaga	2019	Isopropanol (2ml)	Vortexing for two min. at room temperature	Methanol (1.4 ml MS grade)	and mixing on a roller mixer for 18 hours. Following extraction, samples were spun at 1500g for 15 min. The methanol extracts were transferred to new tubes and evaporated under nitrogen stream at 50°C until dry. Samples were resuspended in 120ul methanol for assay by LC-MS/MS.	LC-MS/MS
Shapero	2019	Not explicitly stated	Not explicitly stated	Methanol (1.8ml)	Not explicitly stated	CLIA
Abdulateef	2019	No cleaning described	Not explicitly stated	Methanol (1.5ml)	Not explicitly stated	ELISA

Aryaie	2018	Isopropanol (2.5 ml)	3 min on roller, repeated 3 times.	Methanol (1.5ml)	Rotating overnight, then centrifuged @ 10000 rpm for 2 min.	EIA
Balagova	2018	Isopropanol (5ml)	3 times	Methanol (2ml)	Overnight on rotating shaker at room temperature, centrifuged @ 2000 rpm for 10 min.	ELISA
Binz	2018	Water (15ml) then Acetone (10ml)	Human-Water (15ml) 3 mins., then Acetone (10ml) 2 mins.) Cow-(Water (15ml) at 40°C for 1 hour followed by Acetone (10ml) for 2 min.)	Methanol (5ml) spiked with 50 uL internal standard	Ultrasonic bath for 16 hours at 55°C. The supernatant was evaporated under nitrogen (N2) at 35°C and reconstituted in 150ul methanol and 350ul reconstitution solution (1 M ammonium formate, 0.1% formic acid)	HPLC MS
Muehlhan	2018	Isopropanol (2.5ml)	3 min. at room temperature	Methanol (1.8ml) + Internal standard (50ul)	Incubated 18 hours at room temperature. spun 10000 rpm for 2 min. and 1ml supernatant transferred to clean tube and dried. Resuspended in 250 µL distilled water, 200 µL used for LCMS analysis	LC-MS/MS
Van Aken	2018	Not explicitly stated	Not explicitly stated	Not explicitly stated	Not explicitly stated	ELISA
Dong	2017	Plant shampoo	Not explicitly stated	Methanol	Incubation for 18 hours	UHPLC MS
Hollanders	2017	Not explicitly stated	Not explicitly stated	Methanol (LC grade)	At 25°C for 18 hours., centrifuged, cleaned using SPE and then tested on LCMS	LC-MS/MS
Kristensen	2017	Not explicitly stated	Not explicitly stated	Methanol	Not explicitly stated	ELISA
Slezak	2017	Not explicitly stated	Not explicitly stated	Methanol	Not explicitly stated	RIA
Ramirez	2017	Methanol	Not explicitly stated	Methanol	Not explicitly stated	ELISA
Gaudl	2016	Water (1ml) and Acetone (1ml)	Not explicitly stated	Methanol (1.4ml)	24 hours rolling. 1ml methanol removed, evaporated and residue reconstituted in 120ul water	LC-MS/MS
Mwanza	2016	Methanol (2ml)	3 min.	Methanol (1ml) and internal standard	Incubated for 24 hours, the mixture underwent centrifugation at 12,000 rpm for 5 min. after which, an 800ul supernatant was drawn and transferred into clean tubes. Next, the solution was dried in nitrogen gas at a temperature of 50°C. Reconstitution was achieved by the addition of 50ul methanol and 1mL deionised water and thereafter, a solid phase extraction process was carried out. The	LC-MS/MS

					supernatant was loaded onto the SPEC-18 column and let to drain. The remaining deposit on the SPE column was then rinsed with 3ml of deionised water to dislodge any soluble impurities that could be present on the column. From then on, a vacuum pump was used to rid the column of any solvent. The analytes retained on the column were then eluted with 1mL of methanol. The resulting eluate was dried in nitrogen gas at 50°C. The eluate was reconstituted in 50ul mobile phase for analysis. 20ul was injected into the HPLC machine for analysis	
Olstad	2016	Isopropanol	In a Hoffman screw compressor clamp	Methanol (1ml)	Incubated on an orbital shaker at 150 rpm for 16 hours at 52°C. Methanol was transferred to a glass Kimble tube and evaporated under a nitrogen gas stream at 50°C. The remaining residue in the tube was reconstituted in 250ul of phosphate buffered saline (pH 8.0) and vortexed for 60 seconds	ELISA
Papafotiou	2016	Isopropanol (2ml LCMS grade)	Not explicitly stated	Methanol (1.4ml LCMS grade) and internal standard (100ul)	18 hours @25°C. After centrifugation, the clear supernatant was evaporated under a nitrogen stream. Samples were then reconstituted in 1ml of 2% methanol and purified using solid phase extraction.	LC-MS/MS
Turner	2016	Isopropanol	Not explicitly stated	Methanol (1ml)	Incubated on an orbital shaker at 150 rpm for 16 hours at 52°C. Methanol was transferred to a glass Kimble tube and evaporated under a nitrogen gas stream at 50°C. The remaining residue in the tube was reconstituted in 250ul of phosphate buffered saline (pH 8.0) and vortexed for 60 seconds.	ELISA
Wester	2016	Not explicitly stated	Not explicitly stated	Methanol (1ml)	At 52°C for 16 hours. After extraction, methanol was transferred into glass tubes, and evaporated under constant nitrogen stream. Next, 250ul of phosphate buffered saline (PBS, pH 8.0) were added. Samples were vortexed prior to analysis	ELISA
Andrade	2016	No cleaning	Not explicitly stated	Methanol (1ml)	Sonication for 30 min. at 50°C, Overnight incubation (16 hr at 52°C)	ELISA
Noppe	2015	Isopropanol (2ml LCMS grade)	Gently shaking at room temperature	Methanol (1.4ml) and internal standard (100ul)	Incubated for 18 hours at 25°C. Samples were centrifuged (4500 rpm), 4°C for 5 min, and 1 ml of the clear supernatant was transferred into a new glass tube. The Methanol was evaporated at 50°C under a constant stream of nitrogen until the samples were completely dried. The dry residue was resuspended using 1 ml 2% methanol and vortexed for 1 min. Hair extracts were subsequently cleaned by solid-phase extraction conditioned with 1 ml of methanol followed by 1 ml Milli-Q. Samples were evaporated to dryness at 50°C under a constant flow of nitrogen and stored at 4°C until analysis. Before injection, the dry residue was resuspended in the initial mobile phase.	LC-MS/MS

Quinete	2015	Isopropanol (2.5ml)	2 min. in a 5ml polypropylene tube	Methanol (2ml)	24 hours at room temperature. Samples were centrifuged at 4500 rpm for 10 min. and 500uL of clear supernatant was transferred to a LC vial.	LC-MS/MS
Xiang	2015	Isopropanol (5ml)	Placed into a 50-ml Falcon tube and washed 3 times with 5 ml aliquots of isopropanol and allowed to dry for at least 12 hours	Methanol (1.5ml)	Slowly rotated on an Orbital Shaker for 24 hours at either room temperature or 50°C for steroid extraction. Samples were spun at 1000 rpm for 2 min., and 1 ml of the supernatant was transferred into a new tube. To identify whether nitrogen gas is required to optimise the quantity of cortisol recovered from the extraction process, the methanol was evaporated at either 50°C under a stream of nitrogen or with room air. The tube containing the remaining white residue was capped and stored at -80°C for future analysis. Prior to analysis, the white residue was reconstituted with 250ul of phosphate-buffered saline and gently swirled until fully dissolved.	EIA
Slominski	2015	Not explicitly stated	Not explicitly stated	Methanol/Acetone	Not explicitly stated	ELISA and LC-MS/MS comparison
Gao	2013	Isopropanol (2.5ml)	3 min. at room temperature	Methanol (1.8ml) + Internal standard (50ul)	Incubated 18 hours at room temperature. spun 10000 rpm for 2 min. and 1ml supernatant transferred to clean tube and dried. Resuspended in 250ul distilled water, 200ul used for LCMS analysis	LC-MS/MS
Hoffman	2013	Isopropanol (100%)	Three times	Methanol (1ml)	Overnight. Centrifuged and supernatant removed and dried in fresh tube under stream of nitrogen. Reconstituted with assay diluent based on hair weight	EIA
Krumbholz	2013	Methanol/water (35/65;v/v (5ml))	1 min.	Methanol (2.5ml)	After addition of 2.5ng cortisol-d4(d4-F)as internal standard, extraction with 2.5mL methanol was carried out in an ultrasonic bath at 50°C for 4 hours. The liquid phase was separated after centrifugation. Ethylene glycol (30mL) was added before evaporation under nitrogen stream at 60°C. After reconstitution with 0.5mL water the solution was transferred to SPE columns. SPE columns were preconditioned with 3mL methanol and 1mL water. After loading, columns were washed with 2mL methanol /2% NH4OH-solution(40/60;v/v) and eluted with 2mL methanol/water(90/10;v/v). After addition of 30mL ethylene glycol SPE-eluates were evaporated under nitrogen stream at 60°C.	LC-MS/MS
Xing	2013	Methanol and water	At 25°C	Methanol and water	The incubated hair samples were dried with pure nitrogen and cut into pieces with iron scissors and re-incubated at 25°C again for another 48 hours in Methanol (1ml) to extract the remaining hair cortisol. The reincubation medium was separated by vortex and 600ul supernatant was transferred to a dry tube and 50ul 10 ng/ml cortisol-d4 was added in the supernatant, then was evaporated to	LC-MS/MS

					dryness and the dry residue was redissolved in 50ul methanol for LC-MS/MS analysis.	
Deshmukh	2012	Dichloromethane	Not explicitly stated	Sodium hydroxide (1M)	At 95°C for 10 min., Neutralised with HCL (1M) and phosphate buffer (0.2M) followed by Liquid-Liquid extraction using pentane.	LC-MS/MS
Van Rossum	2012	Not explicitly stated	Not explicitly stated	Methanol (1ml)	Overnight incubation (16 hours) @28°C gently shaking. Methanol transferred to a clean glass vial and evaporated under nitrogen stream. Samples resuspended in phosphate buffered saline	ELISA
Dominguez-Romero	2011	Shampoo and deionised water, then rinsed with acetone.	Not explicitly stated	Methanol (4ml)	Ultrasonic bath for 8 hours at 50°C then incubated at room temperature overnight. The extract was centrifuged, and the supernatant was evaporated under nitrogen stream until almost dry. The residue was reconstituted with 1ml of methanol/water (1:1) and filtered through a 0.45um PTFE syringe filter and then transferred to an analysis vial. 20uL of the extract was analysed with the LCTOFMS system	LC-TOF MS
Jung	2011	Water and Isopropanol	Not explicitly stated	Methanol or methanol/dichloromethane (1:2 or2:1)	30 mg samples were incubated with 0.5 mL of methanol in an ultrasonic bath for 1 hour at 50°C. After cooling to room temperature, 5.5 mL of sodium acetate buffer(pH 5.2) was added to the mixture and vortexed for 20 seconds. The samples were extracted with an SPE cartridge coupled to a peristaltic pump at a low flow rate (<1 mL/min). For SPE, an Oasis HLB cartridge was preconditioned with 3 mL of methanol followed by 3 mL of deionised water. After loading a sample onto a cartridge, the cartridge was washed with 5mL water and eluted twice with 2 mL of methanol. The combined methanolic eluates were evaporated using a nitrogen evaporator and dried further in a vacuum desiccator over for at least 30 min., The dried residue was derivatized with 30uL of MSTFA/NH4I/DTE (500:4:2,v/w/w) for 20 min. at 60 °C and 20uL of the resulting mixture was analysed by GC/MS analysis in selected ion monitoring (SIM) mode.	GCMS
Xie	2011	Methanol (5 mL)	2 min. at room temperature and dried under nitrogen, Washed again by the same procedure	Methanol (1ml)	Incubated at 40°C for 24 hours. The incubation medium was separated by centrifugation at 9000g for 10 min. and the supernatant was transferred to a dry tube and then evaporated to dryness by pure nitrogen. The extraction was resuspended with 50ml methanol and 1 mL water, and then transferred to the SPE C18 column which was activated with 3 ml methanol and washed with 3 mL deionised water prior to use. The deposited on the activated SPE column was rinsed with a sequence of 1 ml 2:8 (v/v) acetone/deionized water, 1 mL deionised water, and 1 mL hexane, and dried for 30 min and eluted successively with 0.5 ml methanol	LC-MS/MS

					three times. The eluate finally obtained was evaporated to dryness and the dry residue was resuspended using 50uL mobile phase for LC/MS/MS analysis	
Gao	2010	Methanol (5ml)	2 min. at room temperature, twice	Soerensen, HCL or NaOH (1ml)	16 hours at 40°C. Centrifuged at 12000 rpm for 10 min. and supernatant transferred to dry tube. Acetic ether added to extract cortisol from solution with incubated specimens, evaporated to dryness by nitrogen. Eluate evaporated after SPE and resuspended in 50ul methanol for HPLC analysis.	LC-MS/MS
Zhang	2008	Chloroform bath	2 min. with soft agitation at room temperature, Three times	Methanol (2ml)	Ultrasonic bath for 2 hours at 37°C. After centrifuging for 20 min. twice, the supernatant was transferred to a clean vial and evaporated under a stream of nitrogen at 50°C. The residue was dissolved in 4 mL water. Extraction using fiber packed SPE tips the packed tip contained poly nanofibers whose average diameter was 242 nm. The Fibers were activated with 50uL methanol followed by 50uL water. A 1mL aliquot of residue solution was then deposited on the tip and eluted with 50uL methanol via the 12-port vacuum SPE manifold. Finally, 20uL of the eluted solution was analysed by high-performance liquid chromatography. Solid-phase extraction AC18SPE column was first activated with 4 mL methanol followed by 2 mL water, and then 2 mL residue solution was applied onto it. The column was washed with 1 mL water and dried by passing air through for 20 min. The analytes were eluted with two volumes of 1 mL methanol.	HPLC
Sauve	2007	No wash	Not explicitly stated	Methanol	Not explicitly stated	ELISA
Wheeler	2006	Ethanol (3ml)	In small glass beaker, vortex for 20 seconds.	Sodium hydroxide (NaOH (1 mL 1M))	In glass boiling tube in boiling water bath for 10 min., allowed to cool before adding 5ml diethyl ether. Extract for 1 min. on a rotamixer or 10 min. on a multi-tube vortex mixer. Allow two phases to separate. Transfer 3 mL of the solvent mixture into clean glass tubes. Evaporate to dryness	RIA
Raul	2004	Methylene Chloride	Not explicitly stated	Soerensen buffer	Incubated for 16 hours at 40°C, extracted using C18 columns, eluted off using methanol. Evaporated. Resuspended in 500 uL 0.2N NaOH. Liquid-liquid extraction with diethylether. Evaporated and resuspended in 25ul LC mobile phase.	LC-MS/MS
Bang	2004	Ethanol, distilled water and acetone	Not explicitly stated	Phosphate buffer, hydrochloric acid and N-pentane	Phosphate buffer (1ml pH7) added after hydrolysis, pH adjusted to 10-11 by adding 2M Hydrochloric acid. Liquid-liquid extractions were made with N-pentane (3ml)	GCMS
Cirimele	2000	Methylene chloride (5ml)	Twice	Soerensen buffer (1ml)	16 hours at 40°C. Further purified by SPE.	HPLC IS MS

Kintz	2000	NaOH (1mL of 1mol/L) in the presence of 1ng of testosterone-d3 (internal standard)	Incubated for 15 min. at 95°C. After cooling, homogenate was neutralized with HCl (1 mL of 1mol/L) and phosphate buffer (2 mL of 0.2 mol/L (pH7.0)) was added.	Methanol (3ml) and deionised water (2ml)	Extracted by solid-phase extraction. The Isolute C18 columns were conditioned with 3mL of methanol, followed by 2mL of deionised water. After sample addition, the columns were washed twice with 1mL of deionised water. After column drying, analyte elution occurred with the addition of two 0.75-mL aliquots of methanol. The eluant was evaporated to dryness under nitrogen at 40°C, and the residue was reconstituted in 1mL of 0.2 mol/L phosphate buffer (pH 7.0). A further purification step was achieved by addition of 100 mg of Na2CO3/NaHCO3(1:10, by weight) and 2mL of pentane. After agitation and centrifugation, the organic phase was removed and evaporated to dryness. The residue was derivatised by the addition of 50mL of N-methyl-N-trimethylsilyltrifluoroacetamide/NH4I/2-mercaptoethanol(1000:2:5, by volume), and then incubated for 20 min. at 60°C. A 4mL aliquot of the derivatised extract was injected into the column of a Hewlett Packard gas chromatograph via a Hewlett Packard autosampler. The flow of carrier gas (helium; purity grade N55) through the column was 1.0 mL/min. The injector temperature was 270°C, and splitless injection was used with a split valve off-time of 1.0 min, using the pulsed mode. The column oven temperature was programmed to rise from an initial temperature of 150°C, maintained for 1 min., to 295°C at 30°C/min, and was maintained at 295°C for the final 8 min. The detector was a Hewlett Packard 5973 operated in the electron impact mode. The electron multiplier voltage was set at 600V above the electron impact-tune voltage.	GCMS
Bevalot	2000	Chloroform bath	for 10 min., twice	Methanol (1ml)	Ultrasonic bath for 2 hours, then centrifuged (750xg for 10 min.)	LC-MS/MS
Kintz	1999	Methylene chloride (5ml)	2 min. at room temperature, twice	NaOH (1 mL 1M)	Incubated for 15 min. at 95°C in 1ml 1M NaOH in the presence of 1 ng of testosterone-d3 (prepared in methanol) used as internal standard. After cooling, the homogenate was neutralised with 1mL 1M HCl, and 2 mL of 0.2M phosphate buffer (pH 7.0) were added. The Isolute C18 columns were conditioned with 3 mL of methanol, followed by 2 mL of deionised water. After sample addition, the columns were washed with 2 x 1 mL of deionized water. After column drying, the analytes were eluted by the addition of 2 x 0.75 mL of methanol. The eluant was evaporated to dryness under nitrogen flow at 40°C and the residue was reconstituted in 1 mL of 0.2M phosphate buffer (pH 7.0). A further purification step was achieved by addition of 100 mg of Na.CO3PNaHCO 3 (1:10,	GCMS

					way) and 2 mL of pentane. After agitation and centrifugation, the organic phase was removed and evaporated to dryness. The residue was derivatized by 50 IJL MSTFA/NH4I/2-mercaptoethanol (1000:2:5, v/v/v) for 20 min. at 60°C	
Scherer	1998	Acetone and Methanol	Not explicitly stated	Ethyl acetate	Not explicitly stated	GCMS
Akefe	2017	Not explicitly stated	Not explicitly stated	Methanol (1ml)	Rocking for 16 hours at 52°C	HPLC MS, RIA
Binz	2016	Water (15ml) then Acetone (10ml)	Handshaking with water for 3 min., Then acetone for 2 min.	Methanol (5ml) spiked with 50 uL internal standard	Ultrasonic bath for 16 hours at 55°C. The supernatant was evaporated under nitrogen (N2) at 35°C and reconstituted in 150ul methanol and 350ul reconstitution solution (1 M ammonium formate, 0.1% formic acid)	LC-MS/MS
Meyer	2014	Isopropanol (5ml HPLC-grade)	15 ml screw-cap polypropylene centrifuge tube followed by repeated inversion for 3 min. using a rotator. Twice	Methanol (1.5ml HPLC grade)	Incubate the sample for 18-24 hours at room temperature with constant inversion using a rotator. Centrifuge the tubes at 10,000 rpm for 5 min. at room temperature to pellet the powdered hair. Dry down the methanol using either a vacuum evaporator or a stream of nitrogen gas. Following removal of the methanol, reconstitute the extract in an appropriate volume of assay buffer	EIA
Hold	1999	Not explicitly stated	Not explicitly stated	NaOH (1 mL of 1N)	At 70°C for 15 min., After digestion, the tubes were cooled in a freezer for 10 min., the pH of the samples was adjusted to 6.0 with 1N HCI, and 1 mL 100mM phosphate buffer (pH 6.0) was added. 5ml of ethyl acetate was then added, and analytes were extracted by mechanical shaking for 30 min. The tubes were centrifuged at 2500 rpm for 10 min. The organic phase was transferred to a silanized tube and evaporated to dryness at 40°C in a water bath under air.	GCMS
Braun	2022	Water (15ml) and Acetone (10ml)	Handshaking in tubes water for 3 min. followed by washing with acetone for 2 min., then dried at room temperature for 4 hours.	Methanol (5ml) and 50ul Internal standard	Ultrasonic bath at 55°C for 16 hours	LC-MS/MS
Otten	2022	Isopropanol	Washed twice and dried at room temperature for 2 days.	Methanol (1ml HPLC grade)	2ml microcentrifuge tube incubated at room temperature for 18-24 hours with slow shaking	ELISA
Pokharel	2021	Isopropanol	Not explicitly stated	Methanol (80%)	Not explicitly stated	EIA

Santymire	2021	Methanol (90%)	Not explicitly stated	Methanol	Not explicitly stated	ELISA
Contreras	2021	Isopropanol (5ml)	Rotator at room temperature for 3 min. per wash	Methanol (1ml)	24 hours with slow rotation	EIA
Van Eerdenburg	2021	Isopropanol	Not explicitly stated	Methanol	Centrifuge the samples, 600uL of the supernatant transferred to a new tube. The extracts were dried with medium temperature for 2.5 to 3 hours. The precipitate was then dissolved in 100ul of phosphate buffer for 24 hours on a shaker set at 300 rpm	EIA
Amin	2021	Isopropanol	Twice	Methanol	Sonicated for 30 min. and incubated overnight @50°C with gentle shaking.	ELISA
Begall	2021	Isopropanol (4ml hplc grade)	3 min. on shaker at room temperature. ,5 times	Methanol (1.5ml HPLC grade)	Incubated at room temperature for 24 hours on an orbital shaker. Samples then centrifuged (8600xg for 5 min.)	ELISA
Olvera-Maneu	2021	Isopropanol (2.5ml)	Vortexed at 145g for 2.5 min., then the supernatant was separated by decantation. The samples were washed 3 times.	Methanol (0.5ml)	Incubated in an orbital shaker at 36°C and 100 rpm 18 hours for the steroid extraction. Samples were then centrifuged at 9500g for 5 min., and 0.75 mL of the supernatant were transferred into a new microtube until complete evaporation. Once the process was completed, the dried extracts were reconstituted with 0.2 mL enzyme immunoassay (EIA) buffer provided by the commercial kit and stored at -20°C until assay analysis.	EIA
Arena	2021	Not explicitly stated	Not explicitly stated	Methanol (5ml)	Gentle shaking at 50°C for 18 hours.	RIA
Charalambous	2021	Not explicitly stated	Not explicitly stated	Methanol (1ml 90%)	Overnight @4°C , samples then vortexed and centrifuged, then placed in laminar flow chamber for 24 hours to evaporate methanol. 1ml assay buffer added to each tube for assay	ELISA
Di Francesco	2021	Methanol (4ml 100%)	Vortexing for 10 seconds and immediately removing all methanol with pipette.	Methanol ((100%) @0.01g/ml)	Directly after washing, Rotated for 24 hours. Centrifuged for 5 min. @2400g	EIA
Sandoval-Herrera	2021	Methanol	Not explicitly stated	Methanol	Not explicitly stated	EIA
Wiechers	2021	Isopropanol (2.5ml)	In a 10ml glass vial rotating for 3 min. at room temperature, twice	Methanol (1.5ml)	18 hours	CLIA

Hein	2021	Isopropanol (3-6ml)	In glass jars shaking for 3 min. at room temperature , twice	Methanol (1.8ml LCMS grade)	Incubated at room temperature	LC-MS/MS, EIA and CLIA
Van der Walt	2021	Methanol (HPLC-grade)	Three times	Methanol (3ml)	Vortexed it for 1 min., then placed on a slow vortexer for 24 hours.	RIA/EIA
Cordeschi	2021	Isopropanol (5ml)	3 min. at room temperature, twice	Methanol (3ml)	Not explicitly stated	RIA
Keogh	2021	Deionised water	Sonicating for 10 min., the outer and inner root sheaths removed when present. Further cleaned with Kimwipe moistened with 2:1 chloroform/methanol solution	Methanol (1ml 100%)	Rotated slowly on a benchtop tube rotator (13 rpm) for 24 hours at room temperature. Samples then centrifuged @10500g for 13 min. and the supernatant was transferred to a new polypropylene tube.	EIA
Otsuki	2021	Distilled water and isopropanol	Water wash until the water became clear. Then hair was washed with Isopropanol (70%) for 2 min., Three times	Methanol (1ml 99.8%)	Sonicated in a water bath at 60°C for 10 min.	TR-FIA
Alon	2021	Water and then IPA	Twice with water, then twice with IPA	Methanol (6ml)	Sonicated for 30 min., Then incubated overnight at 50°C with gentle shaking.	ELISA
Weaver	2021	Isopropanol (12ml)	In a 15 ml conical tube and placed on a rotary mixer for 15 min., Twice	Methanol (5ml 100%)	Incubated for 24 hours on a rotary mixer at slow speed. Tubes were then centrifuged at 2,000g for 10 min. at 4°C. Supernatant was collected and evaporated in a 65°C water bath. The dried extract was then reconstituted in 250uL EIA buffer on the day of analysis.	EIA
Park	2020	Isopropanol	Not explicitly stated	Methanol	Not explicitly stated	ELISA
Peric	2020	Isopropanol	Not explicitly stated	Methanol	Not explicitly stated	RIA and AlphaLISA
Calamari	2020	Not explicitly stated	Not explicitly stated	Methanol (3ml)	Vortexed for 60 seconds, then sat at room temperature for 48 hours, Vortexed again for 60 seconds. Contents transferred to new tube.	RIA
Garber	2020	Not explicitly stated	Not explicitly stated	Not explicitly stated	Not explicitly stated	ELISA

Chu	2020	Methanol (3ml)	Twice	Methanol (950ul) and internal standard (50ul)	Vortexed then incubated at 40°C in water bath. vortexed then spun @12000 rpm for 5 min.	LC-MS/MS
Heimburge	2020	Isopropanol (5ml)	15-ml screw-cap polypropylene tube, gently mixed on a rotator at room temperature for 3 min., Twice	Methanol (1ml HPLC grade)	Approximately 50 mg of pulverized hair was weighed out and transferred into a 2-ml microcentrifuge tube. 1ml methanol was added, and the tubes were incubated at room temperature for 18-24 hours with slow shaking to extract the steroids.	ELISA
Elmi	2020	Deionised water and Isopropanol	Not explicitly stated	Methanol (2ml)	Added to 10mg powdered hair and incubated overnight on rotation. after centrifugation @10000xg for 30 min., 600ul collected and evaporated to dry under air stream in fume hood. dried extracts reconstituted with 250ul of assay buffer and analysed immediately.	RIA
Lopez-Arjona	2020	Isopropanol (5mL)	In polypropylene tube, mixed at room temperature, centrifuged (1500g,1 min.) and Isopropanol discarded. Twice	Methanol	Incubated with 1mL of methanol for 18 hours at room temperature with continuous gentle agitation for steroid extraction. Samples were then centrifuged (2000g,5min)and 0.6mL of each methanol extract was aliquoted into a new Eppendorf tube. The samples were evaporated to dryness in a Concentrator. The dry extracts were reconstituted with 0.1mL of phosphate buffer saline (PBS) and stored at -80°C until analysis.	AlphaLISA
Montillo	2020	Not explicitly stated	Not explicitly stated	Methanol (3ml)	Incubated at 37°C for 16 hours. Next, the liquid in the vial was evaporated to dryness at 37°C under an airstream suction hood, and the remaining residue was dissolved in 0.8mL of 0.05 M phosphate buffered saline (PBS) of pH 7.5	RIA
Colding-Jorgensen	2020	Isopropanol	Twice	Methanol (50ml/g)	Overnight. Then centrifuged, first at 3000g for 20 min., and then at 10000g at 15 min.	ELISA
Sergiel	2020	Methanol	Not explicitly stated	Methanol	Not explicitly stated	ELISA
Lubcker	2020	Distilled water (10ml) , Followed by Isopropanol (10ml)	Distilled water for 3 min. at room temperature while shaking. Followed by Isopropanol, twice	Methanol (10 mL 100%)	The dried subsamples were then spiked with four internal standards at a concentration of 0.1 ng/mL (D711OHA4,D9PROG,D7A4)and0.01ng/mL(D2T)before extraction. Steroids were extracted using 10mL 100% MeOH with sample incubation at 37°C and shaking for 24-hours at 1250 rpm. Thereafter, the suspension was centrifuged at 4500 rpm on a bench centrifuge and the supernatant aspirated. The pellet was washed with 1mL 100%MeOH for 15 min. by shaking at 1500 rpm, centrifuged at 4500 rpm, and the supernatant added to the initial supernatant. Extracted steroids were dried under a gentle stream	UPLC MS

					of nitrogen at 40°C until completely dry. The sides of the test tubes were rinsed with 1mL methanol and vortexed gently to ensure that all the steroids concentrated at the bottom of the test tubes, before being transferred to 2mL LC-MS/MS vials and dried at room temperature. The dried steroid residue was resuspended in 100 uL 50% MeOH/Water, which was gently vortexed (ca. 40 s) before being transferred to LC-MS/MS vial inserts and stored at -20°C until analyses	
Schillaci	2019	Isopropanol	Not explicitly stated	Methanol	Not explicitly stated	IRMS
Colombo	2019	Water then Isopropanol	Water @37°C for 30 min., dried at room temperature for 24 hours. Then isopropanol	Methanol (1ml)	Sonicated for 30 min. and incubated for 18 hours at 50°C and 100 rpm.	ELISA
Maxwell	2019	Ethanol (20ml, 100%)	For 20 seconds, twice	Methanol (2ml 100% HPLC grade)	5mg of each powdered hair sample was transferred to a glass tube vial and 2ml of 100% HPLC grade methanol added. Vials were capped and placed into a heated orbital shaker at 53°C, 200 rpm, for 18 hours. Samples were then centrifuged, and a 375ul aliquot of the supernatant was removed, placed in a clean glass tube and dried down.	ELISA
Karpovich	2019	Methanol (100%)	Sonicated in deionised water for 30 min.	Methanol (0.5ml HPLC grade)	Adding 0.5 mL of HPLC-grade methanol to each powdered sample (0.7-15.5 mg) and placing samples on a slow rotator for 24 hours at room temperature. Samples were then centrifuged for 15 min. at 2,150 g at 20°C and the supernatant was collected. To ensure all extracted cortisol was recovered, the powdered samples were rinsed twice more with 0.5 mL methanol, vortexed for 1 min., centrifuged, and the supernatant from each methanol rinse was combined in the same tube and dried under a gentle stream of nitrogen gas at 38°C. The extract was reconstituted with 0.2 mL phosphate buffer for 24 hours at 4°C.	ELISA
Santangeli	2019	Methanol	Not explicitly stated	Methanol (90%)	Not explicitly stated	EIA
Nejad	2019	Isopropanol (5 ml)	3 min., Twice	Methanol (1ml)	Incubated @RT for 24 hours with slow rotation. Samples were spun for 30 seconds in a microcentrifuge, and a 0.6 mL aliquot of the methanolic extract was added to a new tube and dried at 38°C. The dried extracts were reconstituted with 0.4 mL of phosphate buffer.	ELISA
Placci	2019	No wash step detailed	Not explicitly stated	Methanol (5ml)	Not explicitly stated	RIA

Charalambous	2019	Isopropanol (1ml HPLC grade)	Not explicitly stated	Ethanol (1.5ml), Methanol (1.5ml), then Isopropanol (1.5ml)	Not explicitly stated	ELISA
Nejad	2019	Isopropanol (5 ml)	Not explicitly stated	Methanol (1ml)	Not explicitly stated	ELISA
Sharma	2019	Isopropanol and water	Not explicitly stated	Methanol	Not explicitly stated	EIA
Phillips	2018	Isopropanol	Three times	Methanol (1ml)	24 hours. Samples were then centrifuged for 1 min. at 14,000 rpm to pellet the powdered hair. A 600ul sample of the supernatant was evaporated for 45 minutes using a nitrogen evaporator. Samples were then reconstituted with 200ul of assay buffer and diluted 1:40	EIA
Kapoor	2018	Isopropanol	Twice	Methanol (2ml)	Incubated with inversion rotation overnight	Spectrophotometer (Analytic liquid scintillation)
Nedic	2017	Not explicitly stated	Not explicitly stated	Methanol (1.1ml 55%)	Shaken at 500 rpm for 30 min., then centrifuged at 2500 g for 20 minutes at 4°C. The supernatant was collected and stored in plastic tubes at -20°C until analysis.	ELISA
Tallo-Parra	2017	Isopropanol	Not explicitly stated	Methanol	Not explicitly stated	EIA
Kwok	2017	Anionic detergent (i.e.,0.1% SDS solution), followed by deionised water and acetonitrile (ACN)	Not explicitly stated	Liquid-liquid extraction(LLE), with 0.5mL phosphate buffer (0.1M;pH9.5) and 1mL hexane/ethylacetate (EA)(7:3,v/v)	d3-Testosterone(2.5ng),d3-testosteronephenyl propionate and d3-stanozolol(2.5ngeach) were spiked into the hair sample as the internal standards. 2mL microcentrifuge tube and centrifuged for 5 min. at approximately 20,000g. The organic layer was pipetted into a 5mL reaction vial and evaporated to dryness under nitrogen at 40°C. The hair extract was reconstituted with 3mL phosphate buffer(0.1M;pH6)/MeOH(9:1,v/v)before loading onto a cartridge ,previously conditioned with 2mL of methanol, followed by 2mL of deionised water and 2mL of phosphate buffer(0.1M;pH6). The cartridge was then washed with 2mL of phosphate buffer (0.1M;pH6) and 2mL of acetic acid (1M). The target analytes were eluted into two fractions using 3mL of dichloromethane(DCM)/EA(4:1,v/v) (fraction 1 for acidic and neutral drugs) and 3mL of EA/DCM/isopropanol(5:4:1,v/v/v)with 2%ammonia (fraction 2 for basic drugs). The two fractions were then combined and dried under nitrogen at 40°C. The extract was reconstituted with 50uL MeOH for instrumental analysis	LC-MS/MS
Yamanashi	2016	Isopropanol (5ml)	Shaking for 2 min., Three times	Methanol (1ml)	Shaking in tubes for 24 hours at ambient temperature. Following extraction, the samples were centrifuged, and 0.6ml of	EIA

					supernatant was aliquoted into different tubes and evaporated by vacuum oven. Samples were reconstituted using phosphate buffer	
Fourie	2016	Isopropanol (3ml)	Twice	Methanol (4ml)	Incubated in sonicating water bath 50hz at 55°C , then incubated on plate shaker for remainder of 24 hours. centrifuged @2500xg for 5 min. and 3.5ml supernatant dried under air in a fume hood. extract reconstituted and dried with successively smaller volumes of methanol (2,1 and 0.5ml) and finally reconstituted with 500ul phosphate buffer from assay kit.	ELISA
Weisser	2016	Isopropanol (20ml/g of hair)	In 70ml glass vials for 3 min. ,with rotation (300 rpm) @RT. Hair rinsed with milli-Q water after.	Acetone: methanol(1:1,v/v)	Pressurised liquid extraction (PLE) followed by solid phase extraction (SPE)	HPLC MS
Tallo-Parra	2015	Isopropanol	Not explicitly stated	Methanol	Not explicitly stated	EIA
Mastromonaco	2014	Methanol (100%)	Vortexing in a tube for 10 seconds and immediately removing all the methanol using a pipettor.	Methanol and water	Immediately after cleaning, 80% methanol in water (v:v) was added to the samples, at a ratio of 0.001/0.005g/ml, and samples were vortexed for 5-10 seconds. Samples were then mixed for 24 hours on a plate shaker. After 24 hours, the vials were centrifuged for 10 min. at 2400g. The supernatants were pipetted off into clean glass vials and dried down under air in a fume hood. The dried extracts were stored at -20°C until analysis. Samples were removed from the freezer and brought to room temperature on the laboratory bench prior to analysis. Reconstitution of the dried-down extracts was done by adding assay buffer and sonicating for 20 seconds followed by vortexing for 10 seconds.	EIA
Peric	2013	Isopropanol	Not explicitly stated	Methanol	Not explicitly stated	RIA
Gray	2013	Methanol (20%)	Ultrasonic bath for 15 min.	Phosphate buffer (2ml 0.1M @ph9.5)	Ultrasonic bath for 60 min. liquid extraction performed 3 times by adding 4mL of hexane: ethylacetate(7:3,v:v) rotary mixing for 20 min. and then centrifuging at 3000 rpm for 5 min., remove the organic layer to a fresh tube. evaporated to dryness under nitrogen at 50°C before redissolved in methanol. 3ml reagent grade water added, and tube whirly mixed before passed through a SPE column, eluted with acetonitrile followed by 2ml of ethyl acetate.	UHPLC MS
Keckeis	2012	N-hexane (5ml 100%), Then Methanol (5 ml 100 %)	N-hexane for 10 min., Twice. Then Methanol using a vortex, twice	Methanol (5ml 100%)	washed hair samples were immersed with 5 ml 100 % methanol and incubated at room temperature for 24 h with gentle rotation. After centrifugation (15 min at 2,500g) the methanol supernatant was removed, evaporated to dryness and reconstituted with 1 ml	EIA

					of 100 % methanol. A 20 µL aliquot of each sample was measured in the liquid scintillation counter. Following extraction and removal of the methanol supernatant, the hair samples were moistened with 100 µL water for 20 min and solubilized for 24 h with 1 ml of Biolute S. The samples were then neutralized with 1 ml 1M HCl and 18 ml scintillation fluid for measurement of radioactivity (30 min).	
Aqai	2009	MeOH/water ratios used, Also SDS +/- 100 ml water	10 min. cold water ultra-sonification followed by 100 ml acetone 10 min. ultra-sonification	Hydrochloride (TCEP (2ml 25mM)). Followed by Methanol (4ml) and water (4ml)	Not explicitly stated	LC-MS/MS
Shen	2009	Water and acetone	Not explicitly stated	Methanol	Not explicitly stated	LC-MS/MS
Accorsi	2008	No cleaning	Not explicitly stated	Methanol (5ml)	Incubated at 50°C for 18 hours	RIA
Davenport	2006	Isopropanol (5ml)	Rotator at room temperature for 3 min. per wash	Methanol (1ml)	24 hours with slow rotation	EIA

* Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS), Enzyme-Linked Immunosorbent Assay (ELISA), Enzyme Immunoassay (EIA), Radioimmunoassay (RIA), Ultra-High Performance Liquid Chromatography Mass Spectrometry (UHPLCMS), Gas Chromatography Mass Spectrometry (GCMS), Chemiluminescence Immunoassay (CLIA), AlphaLISA, Time-Resolved Fluorescent Immunoassay (TRFIA), Liquid-Chromatography Time-Of-Flight Mass Spectrometry (LCTOFMS), isotope-ratio mass spectrometry (IRMS).

3.7 Validation and precision

Eighty-five (47%) studies reviewed provided standard deviation values, results of repeated measurements as well as inter- and intra-assay precision for their studies. Ninety-five (53%) studies did not report a percentage coefficient of variance (%CV) result. Intra-assay %CV was <10% in 67 studies, and of those, 28 studies had intra-assay %CV <5%. Sixteen papers reported intra-assay precision between 10% and 25%, and 97 studies did not report intra-assay precision. Inter-assay %CV was less than 10% in 56 studies, and of those 12 studies had inter-assay %CV of < 5%. Twenty-six papers showed inter-assay %CV > 10% but < 20%. Ninety-eight studies did not report inter-assay precision. In **Table 6** it can be seen the average reported concentrations for each analyte included in the review.

Table 6 Reported concentration of selected analytes in included manuscripts.

Black is mean, Green is median. Units were converted to pg/mg where possible (Red indicates units which could not be converted). NS= Not Stated.

Study ID	Year	Human/ Animal	Cortisol	17-OHP	Testosterone	Androstenedione	DHEAS	Vitamin D	Units
Brendgen	2023	Human	NS						ug/dl
Peng	2022	Human	3.37	2.34		3.2	2.27		pg/mg
Doom	2022	Human	794.3						pg/mg
Koskivuori	2022	Human	1166	66	32	80			pg/mg
Moody	2022	Human	588.8						pg/mg
Puglisi	2022	Human	3.4						pg/mg
Scholz	2022	Human	14		NS				pg/mg
Zhu	2022	Human	2.8						pg/mg
Broeks	2021	Human	18.5						pg/mg
Brossaud	2021	Human	27.3						pg/mg
Bryson	2021	Human	NS						pg/mg
Cajachagua-Torres	2021	Human	1.7						pg/mg
Chen	2021	Human	28.14						pg/mg
Damen	2021	Human	1.9						pg/mg
Duan	2021	Human	6.28						pg/mg

Gherlone	2021	Human	4.71						pg/mg
Izawa	2021	Human	9						pg/mg
Mazgelyte	2021	Human	61.11						pg/mg
Muentner	2021	Human	175.5						pg/mg
Ney	2021	Human	0.62						pg/mg
Rajcani	2021	Human	6.11						pg/mg
Reid	2021	Human	7.73						pg/mg
Tuladhar	2021	Human	301.2						pg/mg
Vehmeijer	2021	Human	7.86						pg/mg
Voegel	2021	Human	3.6		0.7	1.6			pg/mg
Wu	2021	Human	11.7						pg/mg
Zhang	2021	Human	7.06						pg/mg
Shah	2021	Human						276.7	pg/mg
Bevan	2021	Human	19.65						pg/mg
Cruickshank	2021	Human	3.37						nmol/L
Hobo	2021	Human	21.43		11.9	21.6			pg/mg
Marceau	2021	Human	10.1		1.88				pg/mg
Mažeikienė	2021	Human	5.22		0.65				pg/mg
Skoluda	2021	Human	4.22						pg/mg
Spikman	2021	Human	0.67						pg/mg
Zhang	2021	Human	7.6						pg/mg
Buse	2021	Human	3.36						pg/mg
Simon	2021	Human	25.2						pg/mg
Behnke	2021	Human	9		6.45				pg/mg
Romero-Gonzalez	2021	Human	132.7						pg/mg
Castro-Vale	2020	Human	4.3						pg/mg
Dion Nist	2020	Human	23.74						pg/mg
Ferro	2020	Human	12.3						pg/mg
Grova	2020	Human	1.56		1.57				pg/mg
Hagaman	2020	Human	28.15						pg/mg
Hobo	2020	Human	5.55		6.95	8.77			pg/mg
Lob	2020	Human	7.76						pg/mg
Kluczniok	2020	Human	11.75						pg/mg
Lanfear	2020	Human	10.5						pg/mg
Leppanen	2020	Human	11.8						pg/mg
Ling	2020	Human	6.97						pg/mg
Musana	2020	Human	6.11						pg/mg
Schmidt	2020	Human	22.4						pg/mg
Ertekin	2020	Human	15.11						pg/mg
Tarullo	2020	Human	53.38						pg/mg
Gomez-Gomez	2020	Human	15.51						pg/mg
Troller Renfree	2020	Human	1.1						pg/mg
Caparros-Gonzalez	2019	Human	1.1						pg/mg

De la Rubia Orti	2019	Human	7.76						pg/mg
Ferro	2019	Human	12.3						pg/mg
Galbally	2019	Human	1.23						pg/mg
Garcia Leon	2019	Human	566.93						pg/mg
Gonzalez	2019	Human	152.5						pg/mg
Hardy	2019	Human	19.94						pg/mg
Jahangard	2019	Human	8.57						nmol/L
Kim	2019	Human	16.29						pg/mg
Molenaar	2019	Human	1.6						pg/mg
Mustonen	2019	Human	21.2						pg/mg
Savas	2019	Human	7.7						pg/mg
Schepers	2019	Human	8.94						nmol/L
Wang	2019	Human	3.9						pg/mg
Xu	2019	Human	17.9						pg/mg
Zgaga	2019	Human						197.6	pg/mg
Shapero	2019	Human	91.55						pg/mg
Abdulateef	2018	Human	19.91						pg/mg
Aryaie	2018	Human	0.97						ug/dl
Balagova	2018	Human	5.49						pg/mg
Binz	2018	Human	7						pg/mg
Muehlhan	2018	Human	6.5						pg/mg
Van Aken	2018	Human	44.35						pg/mg
Dong	2017	Human	0.25	0.219	0.26				pg/mg
Hollanders	2017	Human	62.25						pg/mg
Kristensen	2017	Human	138						pg/mg
Slezak	2017	Human			2.04				pg/mg
Ramirez	2017	Human	55						pg/mg
Gaudl	2016	Human	4.1						pg/mg
Mwanza	2016	Human	13.3						pg/mg
Olstad	2016	Human	120.4						pg/mg
Papafotiou	2016	Human	2.6						pg/mg
Turner	2016	Human	120.4						pg/mg
Wester	2016	Human	15.9						pg/mg
Andrade	2016	Human	8.09						pg/mg
Noppe	2015	Human	2.7	0.75	0.7	1.7	60		pg/mg
Quinete	2015	Human	12.7						pg/mg
Xiang	2015	Human	21.16						pg/mg
Slominski	2015	Human	33.13						ng/ml
Gao	2013	Human	7.06		1.48	4.14			pg/mg
Hoffman	2013	Human	2.1						pg/mg
Krumbholz	2013	Human	5.2						pg/mg
Xing	2013	Human	6.3						pg/mg
Deshmukh	2012	Human			2.15				pg/mg

VanRossum	2012	Human	33						pg/mg
Dominguez-Romero	2011	Human			NS				pg/mg
Jung	2011	Human	10.96	7.53	2.79	3.19			pg/mg
Xie	2011	Human	16.31						pg/mg
Gao	2010	Human	11.8						pg/mg
Zhang	2008	Human	NS						Not stated
Sauve	2007	Human	46.1						pg/mg
Wheeler	2006	Human			NS				Not stated
Raul	2004	Human	18						pg/mg
Bang	2004	Human			8.9				pg/mg
Cirimele	2000	Human	NS						ng/mg
Kintz	2000	Human			2.13				pg/mg
Bevalot	2000	Human	NS						ng/mg
Kintz	1999	Human			2.2				pg/mg
Scherer	1998	Human			2.45				pg/mg
Akefe	2017	Human and Animal (sheep, goat, cow, horse, camel, monkey, cat)	H: 26.35 A: 6.8						ug/Dl
Binz	2016	Human and Animal (cow)	H: 3.2 A: 1.1						pg/mg
Meyer	2014	Human and Animal (monkey)	H: 36 A: 75.8						pg/mg
Hold	1999	Human and Animal (rat)			H: 67.5				pg/mg
Braun	2022	Animal (Cow)	0.75						pg/mg
Otten	2022	Animal (Pig and Cow)	80						pg/mg
Pokharel	2021	Animal (Asian elephants)	38.6						pg/mg
Santymire	2021	Animal (black-footed ferret)	5.4						pg/mg
Contreras	2021	Animal (cat)	4.2						pg/mg
Van Eerdenburg	2021	Animal (cow)	20.5						pg/mg
Amin	2021	Animal (deer)	8		2				pg/mg
Begall	2021	Animal (Giant mole rats)	9.3						ng/ml

Olvera-Maneu	2021	Animal (horse)	4.47		2.7		1.4		pg/mg
Arena	2021	Animal (horse)	0.6						pg/ml
Charalambous	2021	Animal (koala)	0.53						pg/mg
Di Francesco	2021	Animal (muskoxen)	11.35						pg/mg
Sandoval-Herrera	2021	Animal (Neotropical Bats)	2025.12						pg/mg
Wiechers	2021	Animal (pig)	2						pg/mg
Hein	2021	Animal (polar bear)	3.8						pg/mg
Van der Walt	2021	Animal (polar bear)			63100				pg/mg
Cordeschi	2021	Animal (red squirrel)	45.4						pg/mg
Keogh	2021	Animal (seal)	14.4		5.96				pg/mg
Otsuki	2021	Animal (seal)			42.6				pg/mg
Alon	2021	Animal (sheep)	3.68		7.7				pg/mg
Weaver	2021	Animal (sheep)	4.6						pg/mg
Park	2020	Animal (cow)	3.5						pg/mg
Peric	2020	Animal (Lambs)	5.66						pg/mg
Calamari	2020	Animal (male dogs of poodle breed)			5.9				pg/mg
Garber	2020	Animal (marmoset)	1750						pg/mg
Chu	2020	Animal (mice)			2.1	4.6			pg/mg
Heimburge	2020	Animal (pig + cow)	74.1						pg/mg
Elmi	2020	Animal (mouse)			6.42				pg/mg
Lopez-Arjona	2020	Animal (pig)	32						pg/mg
Montillo	2020	Animal (pig)	17.5				7.66		pg/mg
Colding-Jorgensen	2020	Animal (rat)	100						pg/mg
Sergiel	2020	Animal (Scandinavian brown bears (Ursus arctos))	3.28						pg/mg
Lubcker	2020	Animal (southern elephant seals, Antarctic fur seal (Arctocephalus gazella)	33.56	5.9	3.1				pg/mg

		and subantarctic fur seal(Arctocephalus tropicalis))							
Schillaci	2019	Animal (Barbary macaques)	155						pg/mg
Colombo	2019	Animal (cow)	3.26						pg/mg
Maxwell	2019	Animal (dog)	29820						pg/mg
Karpovich	2019	Animal (earless seals)	3.86						pg/mg
Santangeli	2019	Animal (flying squirrels)	4.8 (log)						pg/mg
Nejad	2019	Animal (Holstein lactating cows and heifers)	8.3						pg/mg
Placci	2019	Animal (Horse)	460						pg/mg
Charalambous	2019	Animal (koala)	1700						pg/mg
Nejad	2019	Animal (Korean Hanwoo Cattle)	3.2						pg/mg
Sharma	2019	Animal (Shetland cows)	1.43						pg/mg
Phillips	2018	Animal (monkey)	2581						pg/mg
Kapoor	2018	Animal (rhesus monkey)	20.67						uCi
Nedic	2017	Animal (cow)	181						pg/mg
Tallo-Parra	2017	Animal (Cow)	4.45						pg/mg
Kwok	2017	Animal (horse)		NS	NS				ng/mg
Yamanashi	2016	Animal (chimpanzee)	20.9						pg/mg
Fourie	2016	Animal (non-Human primates)	3500						pg/mg
Weisser	2016	Animal (polar bear)	0.32						pg/mg
Tallo-Parra	2015	Animal (dairy cattle)	2.47						pg/mg

Mastro-monaco	2014	Animal (eastern chipmunks (Tamias striatus))	115						pg/mg
Peric	2013	Animal (Holstein-Friesan and crossbreed F1 heifers)	4.89						pg/mg
Gray	2013	Animal (horse)			NS				pg/mg
Keckeis	2012	Animal (guinea pig)	0.75						ng/fr
Aqai	2009	Animal (Bovine calves)			NS				mg/kg
Shen	2009	Animal (male guinea pigs)			NS				ng/mg
Accorsi	2008	Animal (cat and dog)	2.71						pg/mg
Davenport	2006	Animal (rhesus macaques)	110.3						pg/mg

***** Green indicates median value reported. Red indicates not possible to convert to pg/mg.

NS=not stated

4. Discussion

This work provides the first comprehensive review of the methodological approaches used in the measurement of common endogenous steroids in hair. Published methodologies were found to be diverse, encompassing a variety of approaches to sample collection, preparation, storage, analyte extraction and analytical techniques. The absence of commonly used methods or standardised protocols was striking.

For humans, samples were almost exclusively harvested from the scalp, specifically the posterior vertex region which is in keeping with the Society of Hair Testing recommendations (317), as this area has been shown to have the least variation. Hobo et al. (318) compared nine different scalp regions and found some variation for several hormones (including androstenedione, cortisol and testosterone), which highlights the importance of harvest site standardisation (201).

The length of human hair analysed can also have an impact on levels of analytes held within the hair because of the washout effect in the more distal segments, as has been shown for cortisol levels (314). When scalp hair is not available (e.g: baldness) or suitable (e.g: hair bleached), hair from other sites could be harvested, such as beard or body hair, thus offering alternative sampling locations (319), although the rate of hair growth and hair type varies across sites (58, 320). Hair collection sites for animals were chosen based on areas which may be free from contamination of faeces or other organic matter (e.g: neck, back, shoulder). Interestingly, one study used a hair collection trap in a public place to collect red squirrel hair (168); this approach offers the possibility of studying analytes at a population level and might be appropriate in some settings.

Endogenous steroid hair testing seeks to determine the mass of the analyte incorporated in hair matrix. An ideal method would eliminate all externally acquired analytes (by washing) and extract the analyte from the hair matrix fully. Because endogenous steroids are excreted via sebum and sweat, they can also be found on the hair surface. This can result in inaccurate quantitation and thus washing is essential (321). The conditions under which washing is carried out – such as temperature, time, repeated cleaning, and inversion/rotation, are likely to have an impact on the degree of cleaning (322). The most common solution used for washing was isopropanol, a non-polar solvent. However,

several studies used a more polar solvent, methanol, which may lead to the washout of steroids from hair matrix prior to extraction.

The Society of Hair Testing recommends that hair samples are cut finely using scissors or ground to powder (323). The most common technique used among examined studies was to grind the hair to a fine powder using a ball mill. This procedure increases the surface area for the extraction solution to permeate and may result in a more complete extraction of the analyte from the keratin-bound hair structure, but some of the sample may be lost in the process. Alternatively, hair samples were finely minced (mainly using scissors) or used whole. Gao and Binz showed that cortisol extraction efficiency is only slightly increased when comparing snippets to ground hair in extraction (131, 324) while Monch showed greater values in samples which were ground (325).

If weighed sample transfer to an extraction tube is not complete due to, for example, the static forces acting upon them, the end concentration will be lower. None of the studies reviewed here considered the influence of static, which we experienced as a real issue in practice (326, 327). Currently, there are several antistatic instruments available (e.g: Haug Mettler-Toledo™ 1 Point Ionizer (Fisher Scientific, US)) to mitigate these effects but we could find no evidence for their use in any studies evaluated.

Generally, hair is a stable sample, particularly, when stored under appropriate environmental conditions: dry and out of direct sunlight. Hair is still available today from various historical samples that are often hundreds or thousands of years old (89, 328-330). This feature further increases the potential value of hair samples in archaeological biomarker analysis.

4.1 Method validation

During the international congress of the Society of Hair Testing in 2012 a comparison group to monitor cortisol levels in hair was established (331). This is an incredibly positive move in the hair testing field and has laid a blueprint for inter laboratory comparisons to be established for future validated methods in hair testing of various drugs and hormones.

The recommended validation parameters for a new assay have been described in an updated FDA guideline (*Q2(R2) Validation of Analytical Procedures Guidance for Industry. Available at Q2(R2) Validation of Analytical Procedures (fda.gov)*). The extent of validation

performed depends on the how the assay will be utilised. For studies which are research-use only, validation parameters including precision, accuracy, linearity, lower limit of detection and lower limit of quantitation would be appropriate. In assays developed for clinical diagnostic use validation requires a more stringent approach to determine if an assay is fit for purpose. These includes the establishment of reference ranges, demonstrating robustness and stability of the assay, matrix interference studies and the availability of quality control and proficiency testing schemes. The Society of Hair Testing (SoHT) published a short guideline in 2011 (updated in 2022) for drug testing in hair with an emphasis on hair sampling and handling in forensic samples but did not address more general validation procedures (332).

An examination of the papers selected for review demonstrates a considerable variation in approaches to validation of hair steroid analysis. Precision was most often determined (41% of papers) while linearity was mentioned in 28% of papers. 18% of papers determined all five of the most used parameters (precision, accuracy, linearity, limit of detection and limit of quantitation) when validating their assays.

With reference to proficiency testing the SoHT established a round-robin proficiency scheme for hair cortisol in 2015 (240). No proficiency scheme is available for testosterone, 17-OHP or DHEAS. There was no evidence that the cortisol scheme was availed of, where appropriate, in the papers included in this review.

Interestingly, there are commercially available hair cortisol assays marketed directly to the public or as aids to endocrinologists in diagnostic workups of diseases such as Cushing's syndrome or Addison's syndrome. Little information is provided, however, on assay validation, analysis methodologies or how reference ranges were established.

The lack of a standardised approach to the validation of hair steroid analysis makes it difficult to incorporate findings into clinical guideline strategies. An international strategy is required to improve assay harmonisation with a view to moving assays from research to clinical utility.

4.2 Advantages and limitations of hair sample analysis

The measurement of endogenous steroid hormones in hair is becoming increasingly popular due to the numerous advantages. Sampling requires simple equipment (namely scissors); an expert phlebotomist, array of consumables, and hygienic/sterile conditions are not a prerequisite. Sample storage and transport are simplified as samples do not need to be frozen, and biohazard concerns are negligible. The sampling procedure is painless which is particularly important for children and animals, and the measured concentration is not influenced by the sampling procedure itself: for example, cortisol level can increase acutely due to the stress of blood draw.

The unique aspect of hair measurement is that it can allow retrospective assessment of analytes over a period of several months or even years, depending on the length. If using blood or saliva, this can only be achieved via multiple sampling prospectively, which is in most settings inconvenient for the study participants and staff, time consuming and expensive. In some cases, the hair measurement might be important for establishing the diagnosis or monitoring the success of treatment. For example, the diagnosis of Cushing's syndrome is complicated if episodes of hypercortisolism are interspersed by periods of normal cortisol production (333).

Hair measurements is often referred to as a historical record of past exposures. There is some evidence to suggest that the rate of hair growth varies over time (75, 334, 335), dependent on season, nutritional status, age, gender and other (58), however average growth of human head hair is considered to be approximately 1cm per month (75). Thus, when analysing a proximal 3cm segment of hair, it should correspond to the most recent three-month period, during which circulating molecules have been deposited within the hair shaft. Some studies suggest 3-6cm can be reliably assessed (290, 333), and others allow for much longer periods (118, 151). By extension, hair samples could be cut into smaller segments (e.g: into 1cm pieces). Such segmental analysis has the potential to provide information on hormone concentrations over multiple past periods, yielding important insights into the dynamics of steroid metabolism (336).

Furthermore, hair growth cycle includes the growing phase (anagen), the regressing phase (catagen) during which hair follicle detaches from the blood supply and the resting phase (telogen). Consequentially the "recording" of circulating analyte levels is only happening

during the anagen phase (337, 338). It is estimated that at any time majority of the hairs (about 85%) are in anagen phase and 14% in telogen phase (58). To improve accuracy, the area to be sampled from can be shaved before the start of the study so that only growing hair is sampled (339, 340).

4.3 Strengths and Limitations of review

In this systematic review we extracted and analysed information from a total of 180 studies. Thus, to the best of our knowledge this is the largest review on endogenous steroid hair testing methodology. In terms of the limitations, sheep were included in this review despite "wool" not being included in the search terms, however there is a possibility some studies in sheep were missed. We only investigated DHEAS, and not its precursor DHEA. However, DHEA is synthesised mainly in the adrenal glands and is quickly sulfated to DHEAS; DHEAS circulates in concentrations 1,000 times greater than DHEA, making up 98% of circulating DHEA type molecule. Finally, in hospital labs, DHEAS is the preferred measurement.

5. Conclusion and Future Recommendations

Overall, hair is a viable matrix source in measurement of steroids in hair but will require further work before it can be reliably used in clinical practice. Guidelines for steroid testing should be developed and should include reference to the recommended sample collection site, storage, sample preparation, pre-treatment and analysis.

Chapter 3: The validation of a method to measure 25-hydroxyvitamin D_3 in hair

1. Introduction

The measurement of endogenous steroid hormones, such as glucocorticoids, in hair has been increasingly recognised as a useful monitoring tool in fields such as psychiatric and stress-related research (341, 342). This increase in research around the analysis of endogenous steroids in hair has not yet become a routine measurement tool in clinical or diagnostic scenarios.

The work presented here builds on a previously published study by Zgaga et al. (87) where vitamin D ($25(OH)D_3$) was detected for the first time from human hair using liquid chromatography-mass spectrometry (LC-MS/MS). Subsequently, two other research groups published studies on hair vitamin D analysis, Shah et al. (272) used LC-MS/MS in an attempt to identify and quantitate several different vitamin D metabolites. Gáll et al. (93) also used LC-MS/MS to investigate the relationship between serum and hair vitamin D ($25(OH)D_3$) levels.

Vitamin D is a seco-steroid hormone which plays a major role in bone remodelling as well as being linked with a wide range of diseases ranging from cancer to cardiovascular and metabolic diseases (33, 35, 343). Routine monitoring of vitamin D status measures the levels of the storage form of vitamin D_3, 25-hydroxyvitamin D_3 ($25(OH)\ D_3$), from a blood sample using most commonly either electrochemiluminescence binding assay (CLIA) or by LC-MS/MS.

The review results from chapter two will allow us to carry out hair analysis parameter testing as part of the validation process. This will inform the approach from site of collection of hair sample, sample storage and preparation and finally, quantitation of the analyte being investigated.

Development of hair analysis of $25(OH)D_3$ offers several advantages over traditional methods. These include much enhanced sample stability and the ability to track vitamin D status over extended periods of time.

The work presented here describes the development and validation of an assay for measurement of $25(OH)D_3$ in human hair. The benefits of using hair as a matrix are outlined.

2. Materials and Methods

The LC-MS/MS method used to quantitate 25(OH)D_3 was developed by Chromsystems GmbH, Munich, Germany (MassChrom® 25-OH-Vitamin D3/D2 in Serum/Plasma - LC-MS/MS). The assay is fully validated and calibrators are traceable to the National Institute of Standards and Technology (USA) accuracy standard, SRM 972a (344).

The samples were analysed in the Biochemistry Department of St. James's Hospital, Dublin, Ireland. The laboratory is ISO 15189 accredited. Assay quality was monitored using in-house and third-party quality controls and calibrators from Chromsystems Instruments & Chemicals GmbH, Munich, Germany and Roche Diagnostics GmbH, Mannheim, Germany. The laboratory also participates in the vitamin D External Quality Assessment Scheme (DEQAS). The respective inter- and intra-assay coefficients of variation in serum were 5.7% and 4.5%.

2.1. Sample preparation

Preliminary work identified a hair sample with significant amounts of 25(OH)D_3 present. This was subsequently used to perform the validation experiments.

2.1.1. Specimen collection and blank hair samples

The subject characteristics are as follows; Male, 68 years old and reported taking vitamin D supplements (1000 IU D_3 daily). Subject was found to be 25(OH)D_3 sufficient (circulating 25(OH)D_3 >50 nmol/L) from serum measurement on two occasions one year apart (90 nmol/L on 10/05/2023 and 108 nmol/L on 11/05/2024).

The subject provided informed consent to participate in the study; ethical approval was not required because they were involved with this study.

The hair collection procedure followed the consensus recommendations provided by the Society of Hair Testing (317). Hair sample provided for validation were cut at barbers using an electric trimmer as close as possible from posterior vertex region of the scalp and collected into a folded paper envelope.

Sample was ensured to be dry, labelled with name and date and stored in paper bag in a dry and dark environment at room temperature until ready for transport to the laboratory for analysis (336).

2.1.2. Extraction procedure

The first step in sample preparation involved washing, which decontaminates the outer surface of the hair (345). Washing also prevents analytical interferences from external sources of 25(OH)D_3 which may be present in sebum and sweat. For validation, the effect of washing versus no washing on the result was examined. Sample handling was carried out using a steel tweezers to transfer hair to and from tubes/weigh boat.

Samples were individually washed by placing them in a 12 mL Sarstedt plastic tube and vortexing in 1 mL LC/MS Grade isopropanol for two minutes at room temperature to remove contaminants. Washing solution was transferred to a clean labelled tube. Samples were subsequently dried using lint-free tissue and cut into 5-10 mm segments using scissors. Aliquots of the wash solution were taken for analysis to determine if 25(OH)D_3 leaching from the hair strands occurred.

Washed samples were weighed and placed in a 12 mL Sarstedt plastic test tube. 25(OH)D_3 was extracted by adding 1.0 mL LC/MS Grade methanol and mixing on a roller mixer for 18 hours at room temperature (25°C). Following the extraction period samples were spun at 3000 rpm for 10 minutes. 700 µL of the methanolic extract was pipetted to a deep well microtitre plate and evaporated under nitrogen stream at 40°C until dry (1 hour). Samples were re-suspended in 150 µL mobile phase A, they were then placed on a plate shaker at 1000 rpm for 2 minutes, then centrifuged for ten minutes at 3500 rpm. The resuspended samples are placed on the mass spectrometer for analysis. The standard operating procedure for quantification of 25(OH)D_3 from hair may be seen on the next page.

Standard operating procedure for quantification of 25(OH)D_3 from hair

1. Hair samples are cleaned by vortexing in LC/MS Grade isopropanol (IPA) for 30 seconds. IPA is discarded and hair samples are dried using lint-free wipes.
2. The dried hair samples are held with a steel tweezers over a weigh boat and a clean scissors are used to cut the hair segments directly into the weigh boat. The hair segments are weighed then transferred to a labelled clean 12 ml polypropylene tube.
3. 1 ml of LC/MS Grade methanol is then added to the tube. Tube is left rolling at room temperature for 18 hours.
4. The methanol (0.7 mL) from the tube is transferred to a well in a deep-well plate to be dried under nitrogen stream at 40°C for 1 hour or until dried.
5. After drying, sample is reconstituted in 150 µL of mobile phase A. Plate is shaken at 250 rpm for 2 minutes. Plate is then centrifuged at 3000 rpm for 5 minutes.
6. 200 µL of Chromsystems Internal standard is pipetted into a well of a 96-well microtitre filter plate.
7. 25 µL of Chromsystems precipitation reagent is added.
8. 100 µL of reconstituted sample is added.
9. Plate is then left shaking at 600 rpm at 4°C for 10 minutes.
10. Filter plate is placed on top of collection plate and placed in centrifuge for 8 minutes at 4000 rpm. Collection plate is sealed and placed on the mass spectrometer.
11. 25 µL of the eluate is injected into the LC-MS/MS system for measurement of 25(OH)D_3.

2.1.3 Weight of hair used in analysis

Having carried out a systematic review of methodologies used in hair analysis, one area that showed a lot of variability was the weight of hair used in the extraction of the analyte of interest. As part of this validation various weights of hair from the same collection were tested and their results were compared calculating the percentage coefficient of variance. There were twelve samples extracted in total for measurement of 25(OH)D_3, three each of 10, 20, 30 and 40 mg of hair and samples were extracted as described above.

2.2. Chemicals and reagents

25-hydroxyxvitamin D_3 (25(OH)D_3) internal standards and quality controls were obtained from Chromsystems Instruments and Chemicals GmbH, Munich. LC/MS Grade methanol was purchased from Fisher Scientific, Ireland. LC/MS Grade isopropanol was purchased from Fisher Scientific, Ireland. Distilled water was deionised using an Elix® 100 Water Purification System.

2.3. Preparation of standard solutions

Standard solutions were prepared as outlined in the instruction manual provided by Chromsystems. The levels of standard were 12.5, 79 and 151 nmol/L. Quality controls were also prepared which had target values of 38 and 80 nmol/L. Each standard/quality control sample was reconstituted in 1 mL deionised water. Standards were stored at 4°C when not in use. Quality controls were aliquoted into 250 µL aliquots and frozen at -20°C until use. Samples were rolled to thaw prior to use in assay.

2.4. Instrumentation

The LC-MS/MS system consisted of a Shimadzu LC-20AD HPLC unit, a Shimadzu SIL-20AC autosampler, a CBM-20A communications module, a DGU-20A degasser unit and a Shimadzu CTO-20AC column temperature oven (Shimadzu, Canby, OR, USA). This was coupled to a Sciex API 4000 triple quadrupole tandem mass spectrometer equipped with atmospheric pressure chemical ionisation (APCI) ion source (Sciex, Foster city, CA, USA). The system was controlled by Sciex Analyst software (version 1.6.1). A Chromsystems 25-OH-vitamin D_3/ D_2 Analytical column with a separate Trap column from Chromsystems (Chromsystems Instruments and Chemicals GmbH, Munich) were used in analysis. Both

columns remove possible interferents before samples were analysed in the mass spectrometer.

2.5. Chromatographic conditions

2.5.1. Trap column method

The chromatographic program was accomplished by an online trap column which aids in concentrating the analytes and separates interfering substances. A 10-port 2-position VICI external switch valve (VICI AG International, Switzerland)(**Figure 12**) controlled by the HPLC system was used to connect the trap column and allow flow to the analytical column and into the mass spectrometer, or alternatively, to waste. A binary mobile phase gradient with a single injection was used.

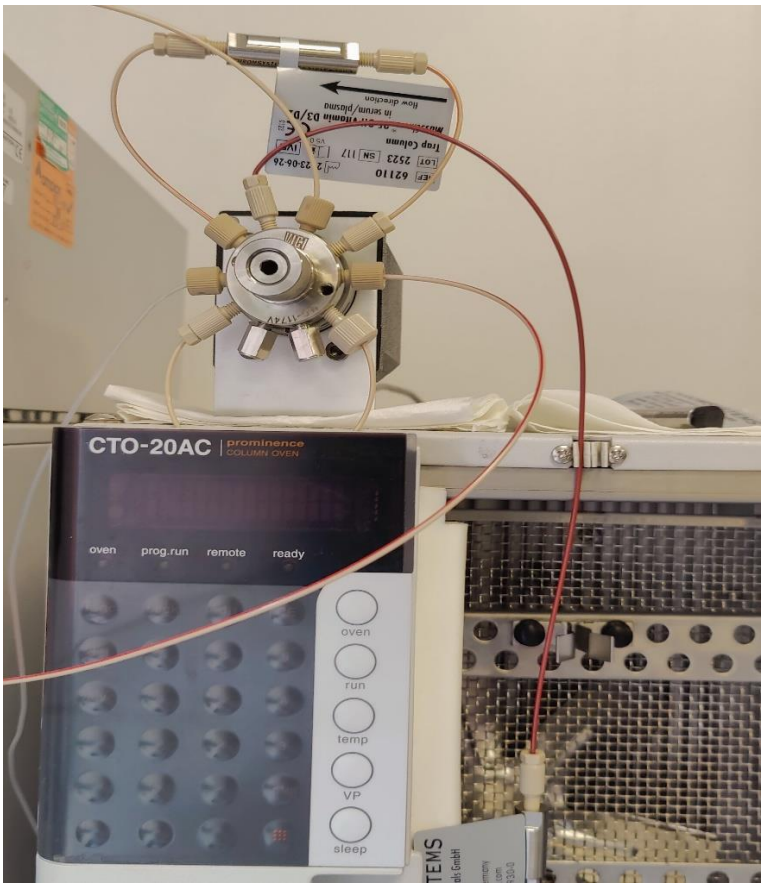


Figure 12 VICI 10-port 2-position external switcher valve

This valve is used to connect the HPLC to the trap column and analytical column.

2.5.2. Liquid chromatography methodology

Mobile phase A, Mobile phase B and rinsing solution are proprietary products provided by Chromsystems (Chromsystems Instruments and Chemicals GmbH, Munich). The total flow rate of the first binary gradient module was maintained at 1 mL/min, the flow rate of the second isocratic module was maintained at 0.6 mL/min. The column temperature was set at 30°C. The autosampler was cooled to 4°C. The injection volume was 25 µL.

2.6. Mass spectrometric conditions

The LC eluent was introduced into the mass spectrometer through a turbo ion spray probe (APCI). Multiple reaction monitoring (MRM) mode was utilised for the detection of target compound. The mass spectrometer was operated in the positive ionisation mode. The main operational parameters of the mass spectrometer are as follows: curtain gas at 25 psig, collision-assisted dissociation gas at 6 psig, nebuliser current at 4 µA, sheath gas at 30 psig, resolution at unit and temperature at 400°C. The MRM dwell time was 200ms.

2.7. Assay validation parameters

2.7.1. Standard calibration curve

Calibration curves were prepared by serially diluting a larger weight extraction of the validation hair. Hair was washed in 1 mL LC/MS Grade IPA, then 20 mg of hair was extracted in 2 mL LC/MS Grade Methanol for 18 hours on a roller. 700 µL of this extract was dried down in duplicate and then resuspended in 200 µL of mobile phase A. The resuspended sample was further serially diluted. The final concentrations were 0, 7, 15, 30, 90, 180, 380, 760 and 1500 pg/mg of 25(OH)D_3. To build calibration curves, the following equation was used: $y=Ax+B$, where x is the compound concentration and y is the ratio of the compound peak area to the internal standard peak area. Correlation coefficients for the determination of the linear fit of the curve were obtained by plotting the peak area ratios against the nominal concentration from each of the samples.

2.7.2. Limit of quantitation

The limit of quantitation (LOQ) was based on the criteria that variability in accuracy and precision was less than 20% and the corresponding signal/noise ratio was greater than 10. The signal/noise ratio was calculated based on the peak area of the compound versus the peak area of the background noise in blank samples in respective biological matrices. The

LOQ was determined by the lowest amount of the serially diluted samples which still provided a 10-fold or greater signal/noise ratio.

2.7.3. Conversion of 25(OH)D_3 result from nmol/L to pg/mg

The following equation from Gaudl et al. (346) was used to approximate the concentration (pg/mg) of 25OHD_3 in hair samples:

$$c\left(\frac{pg}{mg}\right)(hair) = \frac{c(calculated)\left[\frac{ng}{mL}\right] \times V1(extraction)[mL]}{\frac{V2(evaporation)[mL]}{V3(reconstitution)[mL]} \times m(sample)[mg]} \times 1000$$

where c (calculated) is the concentration of 25(OH)D_3 obtained by LC-MS/MS in nmol/L ÷ 2.5 (to convert to ng/mL) (347); and for example: V1 (extraction volume = 1.0 mL); V2 (evaporation volume = 0.7 mL); V3 (reconstitution volume = 0.10 mL).

2.7.4. Inter- and intra- day variation

Inter- and intra-day variation were determined by calculating precision and accuracy estimated for hair samples with three replicates on five separate days and ten samples on one day. Accuracy and precision were obtained by calculating the percentage of relative error (%RE) and the percentage coefficient of variation (%CV) respectively. Accuracy and precision batches were ran alongside serum assay controls and internal standard to ensure comparison between batches.

2.7.5. Comparison between whole, segmented and milled/pulverised hair.

As previous hair analysis protocols of endogenous steroids have frequently involved the milling of hair prior to incubation, we conducted a comparison between the above stated extraction method using whole non-milled hair, segmented (~5 mm segments cut with scissors) and milled hair. For this comparison, 15 different extractions on the hair sample for validation were used. Five of these were incubated as whole, non-milled hair, five were incubated after being segmented to 5 mm segments using scissors. Five hair samples were powdered using a SpexSamplePrep 6775 freezer/mill (8 cycles per second (CPS) for 10 min.) prior to incubation. Hair samples in all three conditions were extracted and analysed as normal following the protocol. To evaluate the quality of these different hair

preparation protocols, the accuracy and precision of each of the three groups, whole, segmented and milled hair samples, was calculated and compared.

2.7.6. Stability of sample post nitrogen dry-down

The investigation of the sample stability after it has been extracted and evaporated under nitrogen was conducted with five samples which were extracted and dried down in a single assay. One sample is analysed on day 1 and another is measured each 24 hours thereafter. After evaporation under nitrogen the sample is reconstituted in 150 µL mobile phase A for LC-MS/MS analysis. Samples were stored dry in the dark between analyses.

2.7.7. Verification of 25(OH)D_3 detection using Chemiluminescent immunoassay (CLIA) and Nuclear Magnetic Resonance (NMR)

LC-MS/MS is the current gold standard for detecting and quantitating 25(OH)D_3 in biological matrices. It demonstrates excellent sensitivity and specificity. Two other methodologies were used to confirm the presence of 25(OH)D_3 in hair extracts; immunoassay and nuclear magnetic resonance (NMR).

Immunoassay uses an antibody to detect and quantify 25(OH)D_3. It is not 100% specific due to cross-reactivity with other similarly structured molecules. Nevertheless, if detected, the assay demonstrates presence of 25(OH)D_3 in the sample. Extracted samples were assayed using the Roche CLIA assay (Roche Diagnostics UK, Vitamin D Total G3). Assays were performed on a Roche Cobas 8000 immunoassay analyser (Roche Diagnostics, UK). Samples were extracted using the same procedure as LC-MS/MS described elsewhere. Methanol was used for resuspension of the evaporated extract due to the hydrophobicity of free 25(OH)D_3 in the Roche aqueous buffer.

NMR, which is not quantitative in this case, identifies the molecular structure of 25(OH)D_3 by analysing the behaviour of its atomic nuclei in a strong magnetic field. Spectra generated are unique to the molecule being examined. NMR analysis is considerably less sensitive than LC-MS/MS or immunoassay and requires significantly higher weights of hair and extra sample clean-up to ensure sufficient amounts and purity for analysis. NMR was performed on a Bruker Ultrashield 400 Plus instrument in collaboration with a research group in the School of Pharmacy, Queen's University, Belfast. The following 25(OH)D_3

purification protocol was adapted from Shah et al. (348). A separate specimen from the same sample collection was extracted which will be analysed by LC-MS/MS to have a comparison level of pg/mg concentration.

Protein extraction and purification standard operating procedure for nuclear magnetic resonance testing

1. Cleaned hair specimens are held with a steel tweezers over a weigh boat, a clean scissors are used to cut the hair segments directly into the weigh boat. The final weight for LC-MS/MS analysis (tube 1) was 120 mg and for NMR analysis (tube 2) was 540 mg.
2. The hair segments are transferred to a labelled clean 15 ml polypropylene tube. 2 ml of HPLC-grade isopropanol (IPA) is added to the hair then vortexed for 30 seconds. The IPA is discarded.
3. 2 ml and 4 ml of HPLC-grade methanol is then added to tube 1 and tube 2, respectively. Both tubes are left rolling at room temperature for 20 hours.
4. The methanol from tube 1 is transferred to a 5 ml glass tube to be dried under nitrogen at 40°C. Tube 1 follows assay preparation protocol for LC-MS/MS analysis. Tube 2 has 3 ml of methanol removed to a light-protected clean tube to undergo further purification steps.
5. 3 ml hexane/dichloromethane (1:1,v/v) is added, then vortex briefly and centrifuge for 10 minutes at 40°C and 3500 rpm.
6. Two layers should be present, and 25(OH)D_3 is extracted in the hexane layer.
7. Transfer the hexane layer into a clean amber vial and dry under nitrogen at 40°C.
8. Reconstitute in 2 ml deuterated methanol for NMR testing.

3. Results and Discussion

3.1. Method validation

3.1.1. LC-MS/MS method parameters

For our 25-hydroxyvitamin D_3 mass spectrometry assay we used a proprietary kit (MassChrom® 25-OH-Vitamin D3/D2) developed by Chromsystems Instruments & Chemicals GmbH, Munich, Germany. The company validates the method according to the EU In-Vitro Diagnostic Medical Devices Regulation (2017/746/EU). Components of the assay are listed in the Product Information Leaflet on the Chromsystems website (www.chromsystems.com). The values of the calibrators determining accuracy are traceable to the National Institute of Standards and Technology (NIST) standard reference material 972a (344).

An API 4000 LC-MS/S instrument was used for the analyses (AB Sciex, USA). Sample ionisation was performed using atmospheric pressure chemical ionisation (APCI) and quantitation of 25-hydroxyvitamin D_3 is carried out using multiple reaction monitoring (MRM).

3.2. The effects of washing/non-washing on results

Ten samples were extracted in total, five were washed prior to extraction, with wash solution retained for testing, and five were not washed. The mean result from the tested IPA washing solution was 31.7 pg/mg (standard deviation was 6.6 pg/mg). When comparing the results of the five samples washed and not washed, the washed samples had a mean concentration of 776.5 pg/mg (standard deviation was 24.4 pg/mg) while the non-washed samples had a higher concentration of 1150 pg/mg (standard deviation was 140.2 pg/mg). The difference in these results is not detected in the washing solution where concentrations are relatively low (31.7 pg/mg) compared to the difference between the means of the five washed samples and five non-washed 25(OH)D_3 concentration (374 pg/mg). The results of these analyses may be seen in **Table 7**.

Conc. vitamin D_3 (pg/mg)	Washed	IPA discard	Non-washed
1	760.8	35.2	1188.6
2	812.5	33.4	1245.3
3	748.9	30.1	1310.1
4	774.5	21.2	1014.7
5	785.6	38.4	994.8
Mean	776.5	31.7	1150.7
SD	24.4	6.6	140.2
Range	749-813	21-38	995-1310

Table 7 25(OH)D_3 detection from washed, Isopropanol (IPA) discard and non-washed hair samples

3.3. Linearity, quantitation and detection limit

A linear response in the serially diluted validation hair samples was observed for 25(OH)D_3. Dilution was carried out directly after reconstitution of the dried extract. The calibration curve was y=0.002x + 0.0125, R value was 0.9998, indicating adequate linearity of the analytical procedure (**Figure 13**). The concentration of 25(OH)D_3 detected in the neat sample was 1289 pg/mg. Lower limit of quantitation (LOQ) was 6.5 pg/mg and had a linear range to 1400 pg/mg which is similar to a published method by Gáll et al. (93). The lower limit of detection (LOD) was found to be 4 pg/mg, which is lower than previously published methods investigating 25(OH)D_3 from hair. Shah et al. published a LOD of 10 pg/mg using UHPLC-MS system while Gáll et al. published a LOD of 5 pg/mg using UHPLC-QTOF-MS (92, 93). The chromatogram representing the neat and serially diluted samples used in calculation of linearity may be seen in **Figure 14-21**.

Figure 13 Linearity of extractions of 25(OH)D_3 from hair samples

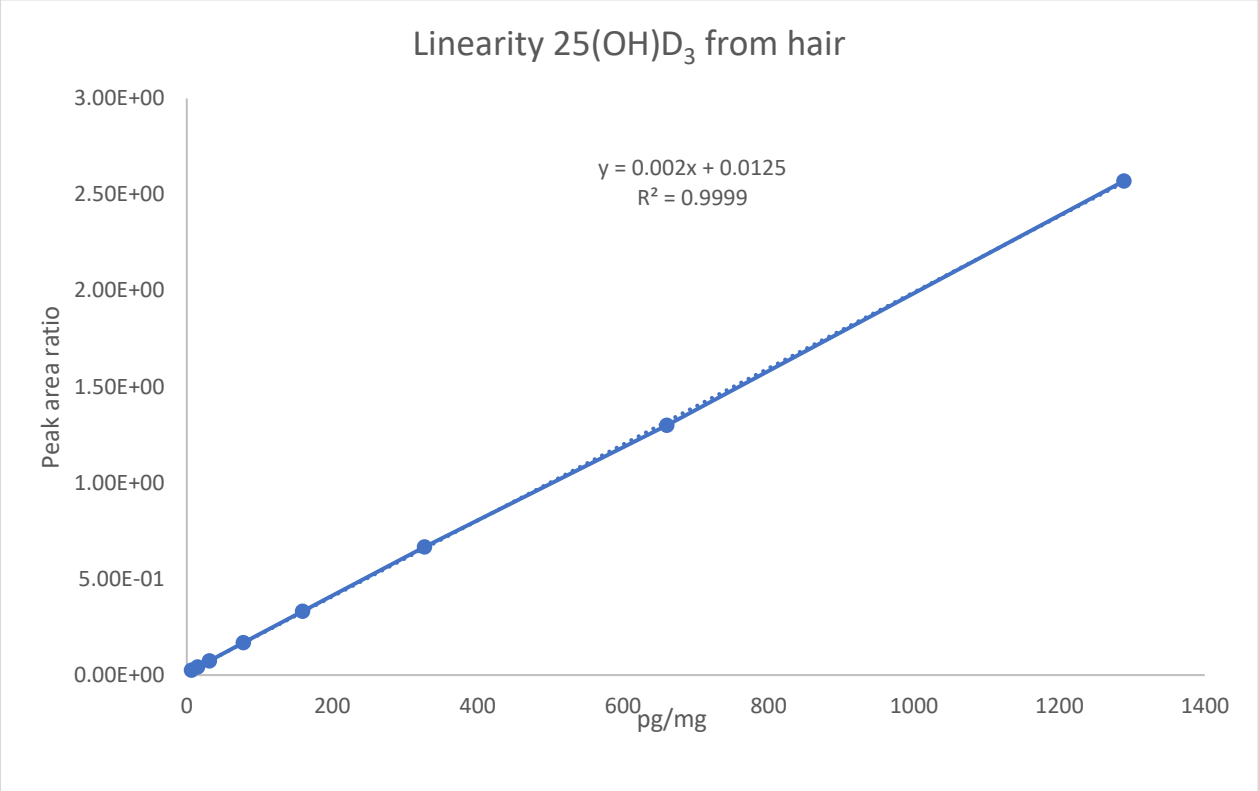


Figure 14 Neat sample for linearity

Red peak is the Internal Standard, Blue peak is detected 25(OH)D_3 from sample. Intensity (cps) is on the Y-axis, time (min.) is on the X-axis.

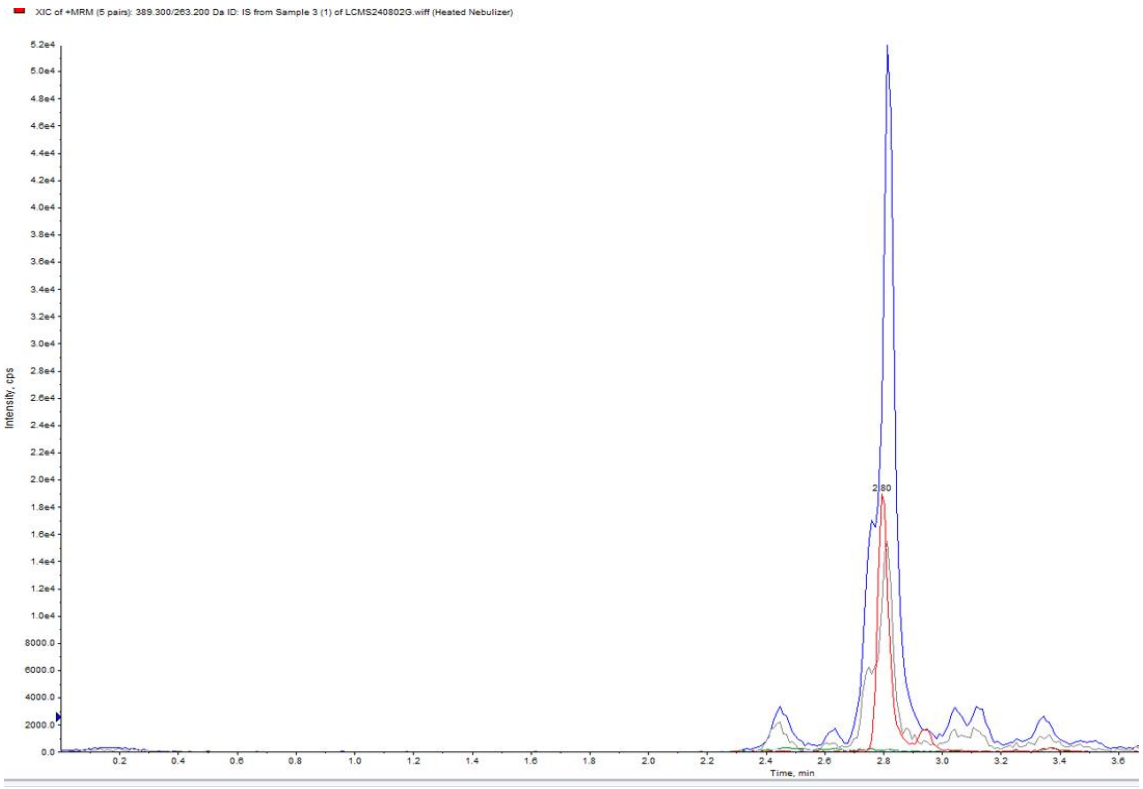


Figure 15 1:2 sample for linearity

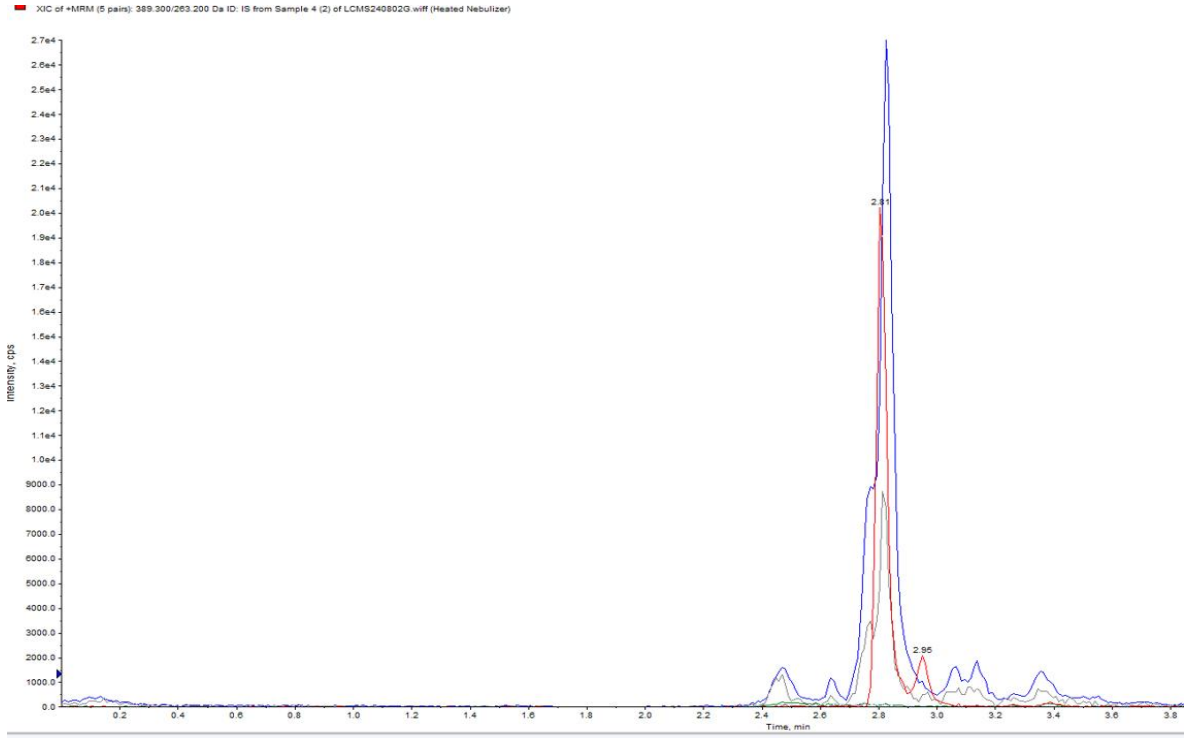


Figure 16 1:4 sample for linearity

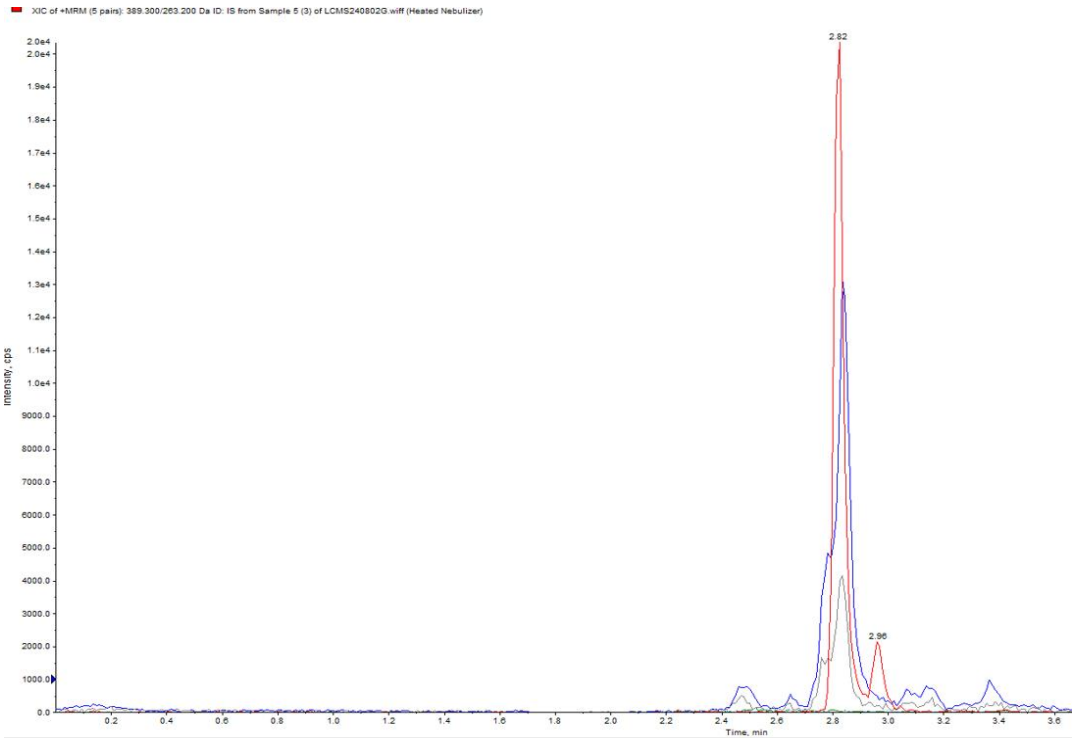


Figure 17 1:8 sample for linearity

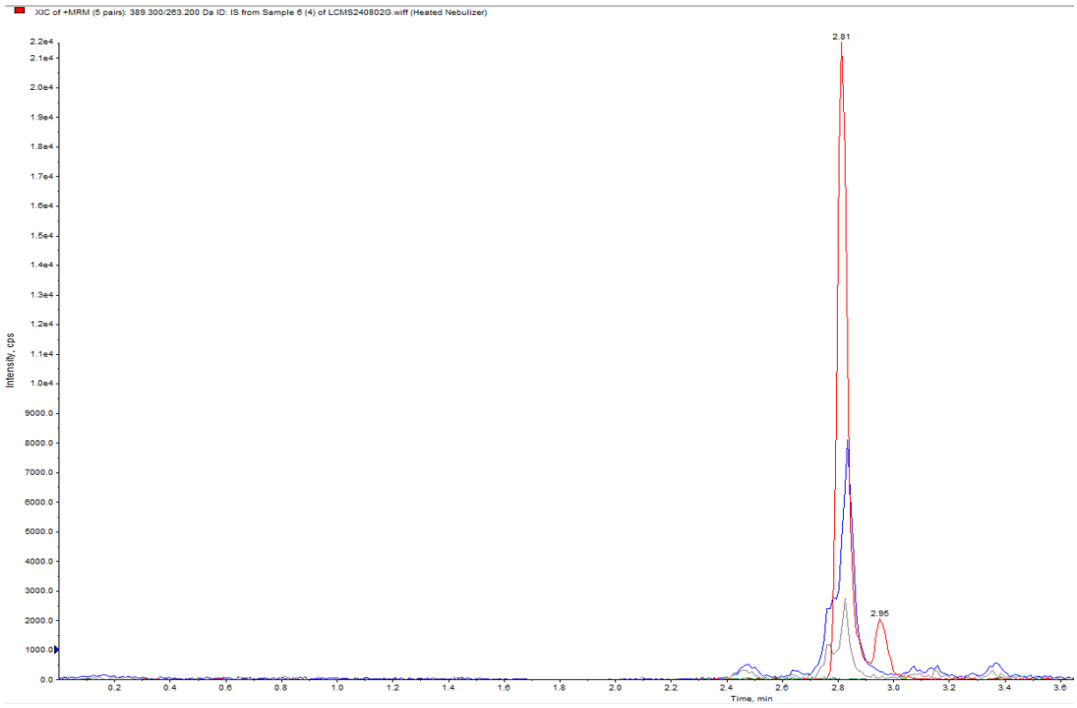


Figure 18 1:16 sample for linearity

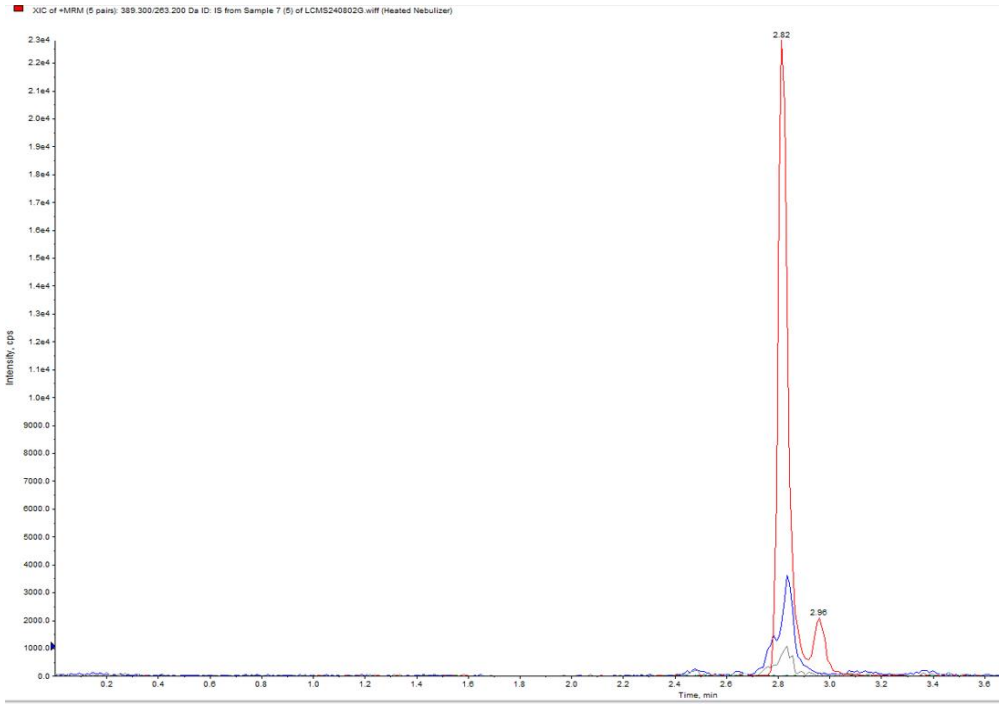


Figure 19 1:32 sample for linearity

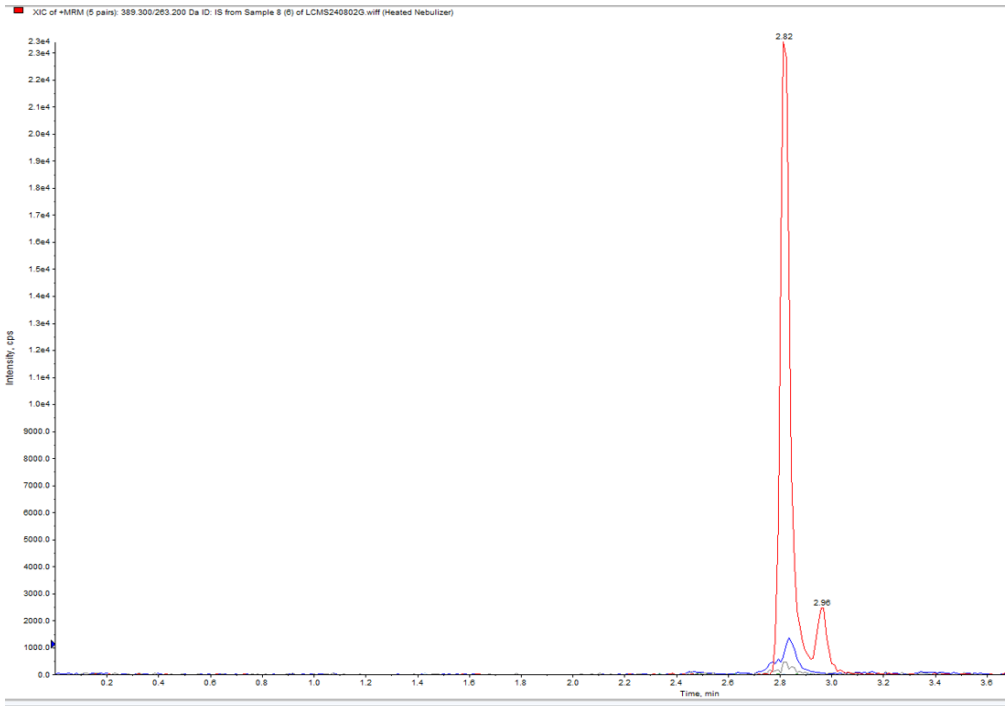


Figure 20 1:64 sample for linearity

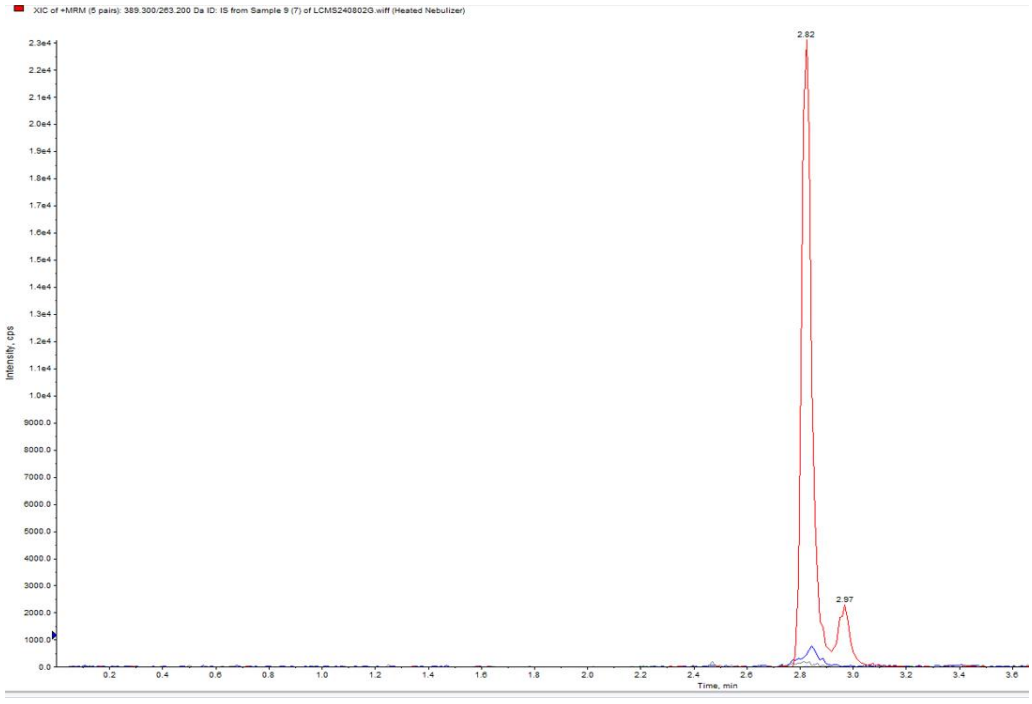
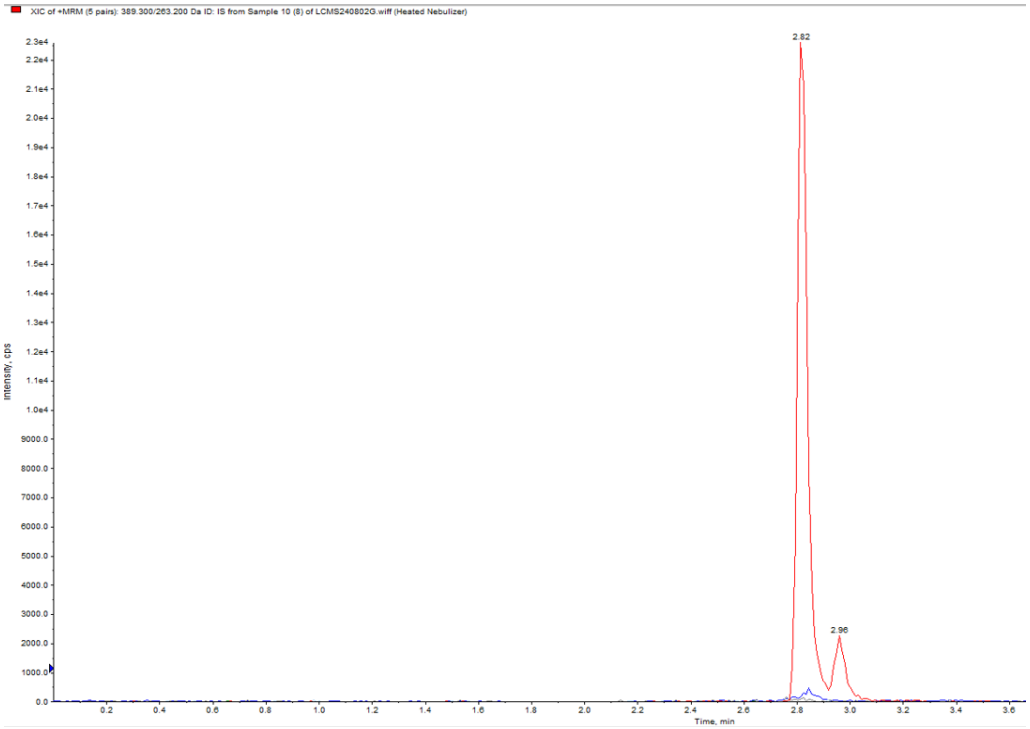


Figure 21 1:128 sample for linearity



3.4. Weight of hair used in analysis

The results from the test on various hair weights extracted and compared can be seen in **Figure 22**. The lowest %CV may be seen when extracting from 10 mg of hair (3.9%) and 40 mg hair (8%), 20 mg and 30 mg have %CV of 36% and 29%, respectively. A higher extraction efficiency of 25(OH)D_3 from hair may be observed with the 10mg of hair, it is the lowest weight of hair used in extraction but yielded the highest concentrations of 25(OH)D_3. This may be explained by the volume of extraction solution, at 1 mL it may have been sufficient to fully permeate all the hair in the 10 mg extractions but unable to do this with higher volumes of hair. This may suggest a ratio required for hair being extracted and volume of extraction solution required to efficiently extract bound analytes from the keratin matrix. The results from all ten extraction's comparing weight of hair used in analysis may be seen in **Table 8**.

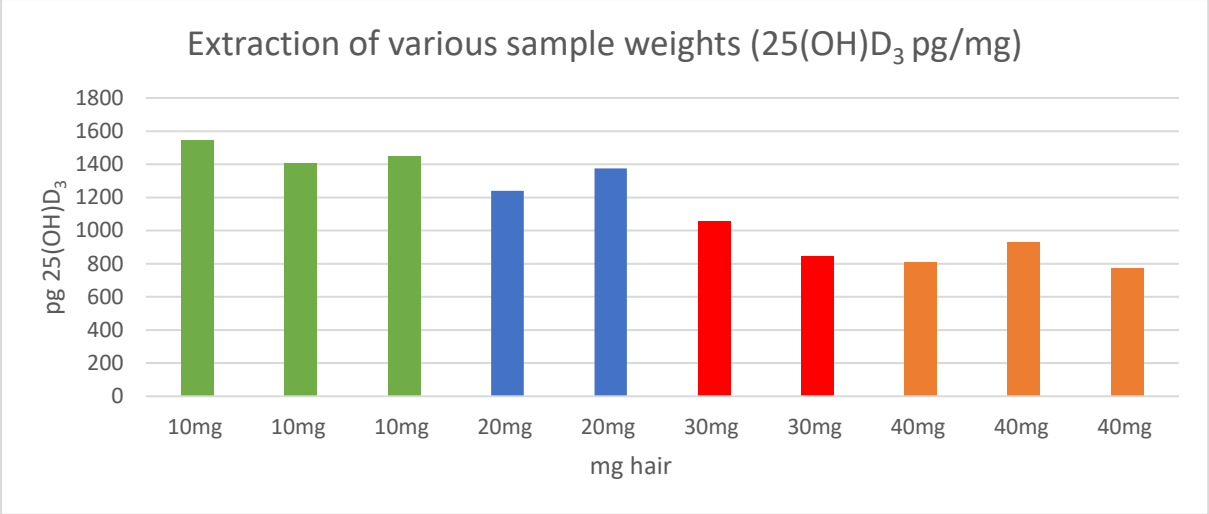


Figure 22 Comparison of different weight of hair for extraction of 25(OH)D_3

Weight (mg)	Mean Conc. vitamin D_3 (pg/mg)	SD	%CV
10	1467	71.5	3.9
20	1307	94.6	5.1
30	951	149.9	11.1
40	836	83.2	8.1

Table 8 Comparison of different weights of hair used in analysis of 25(OH)D_3

3.5. Inter- and Intra-day variation

The inter-day results from ten hair samples extracted on the same day had a mean of 792 pg/mg (standard deviation was 118.2 pg/mg), and a coefficient of variation of 14% (**Table 9**). The intra-day results from three hair samples extracted on five consecutive days had a mean of 739 pg/mg (standard deviation was 124.3 pg/mg), and a coefficient of variation of 16% (**Table 10**). As these results were within the acceptable range [CV <20%], the assay was considered suitable in terms of accuracy and precision (349, 350).

Sample ID	Weight (mg)	Conc. vitamin D_3 (pg/mg)
1	10	1017.1
2	10.1	882.6
3	9.8	740.5
4	10.2	666.6
5	10.1	667.6
6	9.9	836.9
7	10	662.8
8	10.3	882.1
9	10.6	835.5
10	9.9	727.2
	Mean	791.9
	SD	118.2
	%CV	14.2

Table 9 Inter-day precision results for validation of 25(OH)D_3 measurement from hair

Sample ID	Weight (mg)	Conc. vitamin D_3 (pg/mg)
1	10.5	740.1
2	10.2	801.1
3	10.8	841.2
4	10.2	801.1
5	10.1	667.6
6	11.4	631.5
7	9.6	558.3
8	10.6	544.4
9	10.8	629.6
10	12.5	690.2
11	10.2	773.1
12	9.6	666.6
13	10.1	933.5
14	10	914.2
15	9.8	886.2
	Mean	738.6
	SD	124.3
	%CV	16.3

Table 10 Intra-day precision results for validation of 25(OH)D_3 measurement from hair

The percentage of relative error (%RE) was calculated for the controls to show precision. For this experiment there were five different concentrations (very-low quality control (VLQC), low quality control (LQC), middle quality control (MQC), high quality control (HQC) and very-high quality control (VHQC)). The results of these may be seen in **Table 11**.

Sample	Inter-day %RE	Intra-day %RE
VLQC	0.8	0.8
LQC	3.9	1.9
MQC	1.9	1.9
HQC	3.7	8.7
VHQC	0.7	0.7

Table 11 Percentage of Relative Error (%RE) results for precision

3.6. Comparison of whole, segmented and milled hair

The comparison of results between the same weight of each hair preparation may be seen below in **Table 12**. Using identical weights of hair a comparison of extraction and quantitation of 25-hydroxyvitamin D_3 from whole, segmented or minced hair was performed (N=3 for each). The highest result was observed from the milled samples, this result was expected as the surface area is increased due to being powdered and allows methanol during extraction to fully permeate the hair and release more 25-hydroxyvitamin D_3. The most reproducible results were found for segmented samples. For the purposes of the thesis segmented samples were used throughout for extraction and chromatography.

Conc. vitamin D_3 (pg/mg)	Whole	Segmented	Milled
1	617.6	753.1	870
2	680.2	745.5	808.6
3	765.8	763.1	760.1
Mean	687.9	753.9	812.9
SD	74.4	8.8	55.1
%CV	10.8	1.2	6.8

Table 12 25(OH)D_3 comparative measurement in three different hair preparations

3.7. Stability of sample post nitrogen dry-down

The results from the stability test may be seen in **Table 13**. A linear depreciation of 25(OH)D_3 content within the extracted sample may be observed over 5 days. The results indicate an almost 8-fold deterioration in recovery of 25(OH)D_3 from extracted hair samples stored at room temperature over a five-day period. In practice, samples should be analysed on the same day as extraction.

Day	Conc. vitamin D_3 (pg/mg)
1	735.5
2	438.2
3	366.6
4	170.8
5	95.1

Table 13 Stability of 25(OH)D_3 in extract sample post dry-down under nitrogen stream.

3.8. Verification of 25(OH)D_3 detection using Chemiluminescent immunoassay and Nuclear Magnetic Resonance (NMR)

3.8.1. Immunoassay

The comparison between mass spectrometry and chemiluminescence immunoassay showed variable results. The result comparisons may be seen below in **Table 14**. The %CV is markedly better in LC-MS/MS detection. Detection was first attempted with the universal diluent supplied by Roche. This diluent is water-based and as vitamin D is a hydrophobic compound it would make sense that it would not reconstitute in this diluent. To overcome this the sample was reconstituted in MeOH and ran on the Roche 8000 immunoassay analyser. The objective of this exercise was to verify that it was 25(OH)D_3 that was extracted and detected from human hair. The results obtained by chemiluminescence immunoassay detection show that it is 25(OH)D_3 being extracted and detected. To the best of my knowledge this is also the first time that 25(OH)D_3, that has been extracted from hair, has been quantitated using CLIA. There is a significant difference between the two analysis methods, quantification is not accurate using the CLIA method when looking at the %CV of the results.

	Mean vit D_3 Conc. (pg/mg)	**SD**	**%CV**
CLIA	268.8	147.8	44.9
LC-MS/MS	1095.7	146.8	11.6

Table 14 Comparison of results between CLIA and LC-MS/MS analysis (N=8).

3.8.2. Nuclear Magnetic Resonance

In this experiment we attempted to utilise NMR technology to identify the molecular structure of 25-hydroxyvitamin D_3 in extracts of hair samples. NMR is several orders of magnitude less sensitive than mass spectrometry in identifying low molecular weight molecules. NMR analysers vary in their sensitivities from low to high. The instrument utilised for these experiments (Bruker Ultrashield 400 Plus) operates in the mid-range and was unable to detect the vitamin at the pg/ml amounts present in the analytical sample even though significant weights of hair were extracted (>500 mg). In addition, pre-NMR analysis, samples require significant clean-up to remove contaminating substances.

Further analyses using higher specification NMR analysers and sample purification would be required to determine if 25(OH)D_3 could be detected in hair extracts.

3.9. Analysis of human and animal hair samples

Following the validation, hair samples from subjects based in Ireland, the UK and Europe were analysed for 25(OH)D_3.

3.9.1. UK study on 25(OH)D_3 measurement in post-covid patient hair

Forty-five human hair samples were received from a research fellow, based at University Hospital of North Tees, United Kingdom. They were carrying out a study investigating if major histocompatibility complex (MHC) genes play a role in the severity of COVID-19 (351). Vitamin D deficiency was shown in some cases to result in more severe symptoms from the virus so hair testing for retrospective vitamin D levels would be advantageous.

The results when compared to the hair sample which was used in validation, which had a mean result of 792 pg/mg, are all lower except for four samples. The results from the forty-five human hair samples may be seen in **Figure 23**. The mean concentration of the samples was 146 pg/mg (standard deviation was 358 pg/mg), the median was 12.46 pg/mg (the interquartile range was 0.71-34.14 pg/mg). There were nineteen samples that had concentrations less than 10 pg/mg and there were four samples with concentrations above 900 pg/mg.

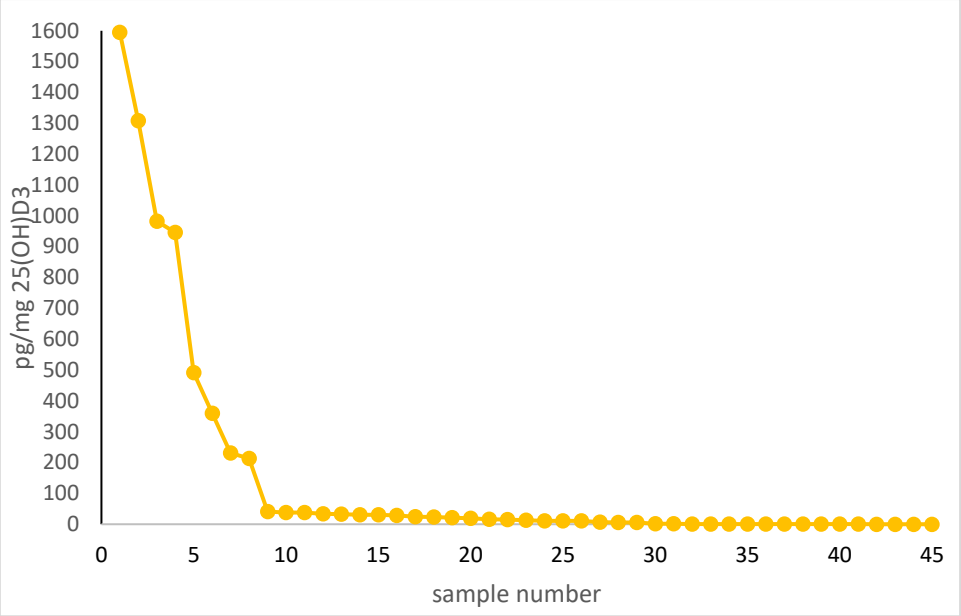


Figure 23 $25(OH)D_3$ results from forty-five human hair samples**.**

$25(OH)D_3$ was assessed using the validated method in 45 post-covid patients based in the UK.

Serum $25(OH)D_3$ values were not available for these patients. To speculate, if we look at our hair sample used in validation for comparison and say 792 pg/mg corresponds to 100 nmol/L. We know levels below 30 nmol/L are considered deficient in vitamin D. From the above comparison we can say that, in this case, 30 nmol/L would correspond to 237.6 pg/mg. There are thirty-nine of the forty-five samples analysed which have $25(OH)D_3$ concentrations below 237.6 pg/mg.

3.9.2. Ghent university and Ghent Zoo hair analysis

Collaboration with a research group in the University of Ghent, Belgium resulted in the investigation of 25(OH)D_3 levels in lemurs and a selection of new and old-world monkeys. These included ring-tailed lemurs (Lemur catta) (352), Moustached guenon (Cercopithecus cephus), Columbian spider monkey (Ateles fusciceps rufiventris), Bornean orangutan (Pongo pygmaeus), East Javan langur (Trachypithecus auratus), Red shanked douc (Pygathrix nemaeus) and chimpanzee (Pan troglodytes). similarly to the human population, vitamin D deficiency is an issue facing housed primates and monkeys worldwide. A comparison between housed animals with their wild counterparts would be an interesting future study similar to the research published by Ziegler et al. (353) on captive and wild baboons. Previous studies have examined serum 25(OH)D_3 variation in lemurs between summer and winter (354). The results presented here demonstrate for the first time that extraction and quantitation of 25(OH)D_3 from primate hair is possible.

The results of analysis may be seen in **Table 15**. The results obtained for lemur samples have remarkably high concentrations in comparison to human or other animal samples assessed, minimum result was 1171 pg/mg, and maximum was 5037 pg/mg. The mean was 2996 pg/mg (standard deviation was 1140 pg/mg), the median was 2916 pg/mg (interquartile range was 2167-3578 pg/mg). When in captivity, animals may be given vitamin D through diet and supplements. Toxicity does not seem to occur in lemurs and other primates at the same concentrations as humans (355, 356).

The analyses on the other samples received may be seen below, chimpanzee samples had two collection methods in some cases (352). Hair was plucked and snipped with scissors in four sample collections, the samples that were plucked have a higher concentration than the snipped samples in three out of four cases. This may be because plucked hair contains follicles which might contain extraneous 25(OH)D_3 before it enters the hair shaft. The chimpanzee subjects analysed in this study are residents in a safari park “Beekse Bergen”, in The Netherlands. They are supplemented with vitamin D_3 through diet and supplements. Diet consists of vegetables and pellets, pellets contain 8500 IU/kg of vitamin D_3, also supplemented with 0.7 ml of an oral 50,000 IU vitamin D_3 human oil supplement twice a month. Hair has several advantages over serum for analysis of 25(OH)D_3 status in animal populations. The procedure is both painless and non-invasive and does not require

needles or specialised personnel. From an epidemiological perspective hair analysis can provide longitudinal data on vitamin D status depending on the length of hair sampled (357). The mean concentration of 25(OH)D_3 in chimpanzees was 388 pg/mg (standard deviation was 236 pg/mg), the median was 335 pg/mg (interquartile range was 180-541 pg/mg). For the other species analysed the mean was 1372 pg/mg (standard deviation was 649 pg/mg), the median was 1253 pg/mg (interquartile range was 941-1906 pg/mg).

Sample ID	Weight (mg)	Conc. vitamin D_3 (pg/mg)	Sample ID	Weight (mg)	Conc. vitamin D_3 (pg/mg)
lemur 1	13.6	3360.4	Moustached guenon	31.6	477.4
lemur 2	10.5	5037.8	Columbian spider monkey 1	23.7	2314.8
lemur 3	12.1	4785.7	Columbian spider monkey 2	35.8	1252.8
lemur 4	10.8	2731.7	Columbian spider monkey 3	30.9	1109.6
lemur 5	11.8	3100.7	Columbian spider monkey 4	45.7	1992.7
lemur 6	7.6	4169.3	Columbian spider monkey 5	11.4	2382.1
lemur 7	11.6	1746.7	Bornean Orangutan 1	33.5	396.3
lemur 8	8.6	4712.5	Bornean Orangutan 2	48.5	842.7
lemur 9	10.4	2160.6	East Javan Langur 1	39.3	935.3
lemur 10	9.1	1171.2	East Javan Langur 2	40	1646.7
lemur 11	16.4	2242.4	East Javan Langur 3	41.9	1253.7
lemur 12	12	2168.4	Red shanked douc 1	11.1	957.6
lemur 13	15.1	1564.3	Red shanked douc 2	21.6	2146.2
lemur 14	10.7	3266.8	Red shanked douc 3	22.3	1500.7
lemur 15	14.2	1670.3	Chimpanzee 1 snip	44.4	431.4
lemur 16	9.6	3380.8	Chimpanzee 1 pluck	31.4	655.7
lemur 17	10.9	3171.8	Chimpanzee 2 snip	41.1	541.2
lemur 18	13.2	2618.7	Chimpanzee 2 pluck	31.6	798.7
lemur 19	4	2614.9	Chimpanzee 3	67.3	149.2
lemur 20	10.5	4249.6	Chimpanzee 4 snip	69.2	179.9
			Chimpanzee 4 pluck	47.9	335.1
			Chimpanzee 5 snip	39.1	246.5
			Chimpanzee 5 pluck	47.9	149.8

Table 15 Ghent University samples 25(OH)D_3 concentration

3.9.3. 25(OH)D_3 measurement in Holstein Friesian dairy cattle

Nine Friesian dairy cattle hair samples were received from the School of Agriculture and Food Science at University College Dublin. These samples were assessed for 25(OH)D_3 using our validated method and results may be seen in **Table 16.** Samples are annotated below according to sample ID and hair colour. Four of the samples assessed had levels of 25(OH)D_3 below the detectable limit. There did not seem to any observable difference in levels of 25(OH)D_3 in white or black hair. The mean and median levels of white hair are 76 pg/mg and 68 pg/mg (standard deviation was 79 pg/mg, interquartile range was 35-114 pg/mg), the mean and median levels in black hair are 140 pg/mg and 98 pg/mg (standard deviation was 173 pg/mg, interquartile range was 0.4-215 pg/mg). Dietary intake of vitamin D may be higher in vitamin D_2 (ergocalciferol) as their diet is mainly grass. Vitamin D deficiency is prevalent in cows due to modern farming practices. Animals spend considerable time indoors and are not exposed to sunlight needed for dermal synthesis of the vitamin. A recent study conducted at a commercial dairy in Slovenia showed vitamin D deficiency in all but one of twelve high-producing Holsten Friesian cattle (358, 359). The difference in 25(OH)D_3 levels between the Ghent primate and monkey samples compared to cattle may be caused by lack of supplementation with cattle, as well as physiological differences (360).

Sample ID	Conc. vitamin D_3 (pg/mg)
cow 1 white	68.37
cow 2 black	0.33
cow 3 black	0
cow 4 white	1.28
cow 5 black	0.7
cow 6 black	423.9
cow 7 black	221.7
cow 8 black	195.1
cow 9 white	158.9
Mean	118.9
Median	68.4
SD	146

Table 16 Results from 25(OH)D_3 measurement on nine Holstein Friesian dairy cattle hair samples**.**

3.10 Further discussion and future directions

The physiology of hair formation is complex and consists of 3 phases: Anagen (the active growth phase), Catagen (transition phase-hair stops growing) and Telogen (resting phase with eventual hair loss). This growth cycle lasts up to 10 years per strand of human hair. Approximately 85% of hair is in the anagen (growth) phase and strands grow at a rate of 0.3-0.4 mm per day or approximately 1.0 cm per month on average. Incorporation of molecules such as $25(OH)D_3$ into the hair shaft occurs at this stage. Variations on this growth rate can occur between both individuals and between different animal species.

Once deposited in hair the molecules remain at the site of deposition and do not migrate within the shaft. Hair growth is asynchronous i.e. the growth phase varies from strand to strand. How this impacts incorporation of metabolites is not clear currently and requires further investigation. In addition, although the uptake of metabolites into hair comes primarily from the circulation, other points of entry include diffusion from tissues surrounding the growing hair or glandular, sebaceous and sweat secretions. How these impact on the final level of $25(OH)D_3$ in hair is unknown although it is thought their contribution is small.

The weight of hair used in analyses was 10 mg. A comparison was conducted with larger weights of hair (20, 30, 40 mg) using the same volume of extraction solution (1 mL MeOH). The results of this showed a lower %CV with 10 mg of hair extracted in 1mL of MeOH. In a separate experiment a low weight of hair (5 mg) was assessed for $25(OH)D_3$ presence. The vitamin was detected because of the high concentration present. However, to offset potential sensitivity issues in subjects with vitamin D deficiency, a minimum weight of 10 mg was used for all extractions.

The human hair samples which were assessed as part of the UK study were samples which were self-collected by participants. Each participant received an information pack through the mail which included hair sample collection instructions and storage information. Samples were posted back to the laboratory for analysis. This process highlights the benefits of using hair for analytical purposes. These include the non-invasive and painless nature of the collection, self-sampling (no professional intervention needed), and easy storage and despatch of samples (361, 362).

Previous work by Zgaga et al. (87) demonstrated the feasibility of detecting and quantifying 25(OH)D_3 in hair in a proof-of-concept study. The work presented here corroborates these finding and validates the assay parameters employed. Since the Zgaga report, two other published methods by Shah et al. and Gáll et al. have supported hair as a matrix for analysis of hair 25(OH)D_3 (92, 93). Direct comparison of results is not possible because of differences in assay parameters employed.

The assay followed a published method for extraction of steroids from hair (185, 363) and a proprietary 25(OH)D_3 LC-MS/MS assay using calibrators traceable to National Institute of Standards and Technology (NIST) (Chromsystems, Munich, Germany) (344). Both Shah et al. and Gáll et al. use 'in-house' developed assays with no information on the traceability of standards used. In addition, their extraction, resuspension and chromatography conditions differed from each other and from the Zgaga et al. study. Finally, Shah et al. used pulverised hair for extraction while Gáll et al. and Zgaga et al. used whole hair. These observations along with evidence from the systematic review above, demonstrate the lack of a standardised approach to analysis of steroid or steroid-like molecules from hair.

The Society of Hair Testing has gone some way towards recommending standardised procedures for hair analysis in forensic science. However, there appears to be little consensus on agreed assay protocols for the clinical use of hair analysis. Published research in this area gives variable analytical information particularly regarding the validation of methodologies employed. This in turn makes it difficult to compare or combine results across publications. Recent years have seen big strides in the harmonisation of serum 25(OH)D_3 assays with reference method procedures (RMP's) published and traceable standards made available (364-366). Similar approaches will be necessary for hair analysis to improve comparability of results.

Analysis of hair 25(OH)D_3 by both LC-MS/MS and immunoassay demonstrated the presence of the vitamin in both cases. Correlation between the two sets of results was poor, however. 25(OH)D_3 is a hydrophobic compound whose physiochemical properties may be affected when attempting to assay the resuspended methanolic extract. Further work will be required to examine this more fully. Currently, mass spectrometry is considered the method most sensitive and specific for analysing 25(OH)D_3 both in research and clinical scenarios.

NMR analysis of 25(OH)D_3 did not result in spectra being identified. The sensitivity of the NMR analyser is critical in this process. The instrument used in this case had mid-range sensitivity which was insufficient to detect 25(OH)D_3 spectra despite the extraction of larger amounts of hair and further purification of the extract. Further investigations are required using more advanced NMR technology. Li et al. (367) have described a complex procedure using a combination of high-resolution NMR spectroscopy data and high sensitivity Time-of-Flight mass spectroscopy. They statistically correlate spectra generated by both instruments to improve identification of metabolites in metabolomics mixtures. While they encourage further development and use of this technique, its high cost and expertise requirements are outside the scope of most research and clinical laboratories.

3.11 Strengths and limitations of the study

3.11.1 Strengths

The results presented here confirm the proof-of-concept finding of hair as an alternative matrix for 25(OH)D_3 vitamin D analysis.

The LC-MS/MS assay employed here was Irish National Accreditation Board (INAB) accredited to ISO 15189 standard for serum analysis of 25(OH)D_3. The work presented here validates the method for quantitation of 25(OH)D_3 in hair. Validation parameters included linearity, precision, accuracy, lower limit of quantitation and lower limit of detection.

Mass spectrometry, used to quantify 25(OH)D_3 in hair, is regarded as the gold standard assay method for measuring 25(OH)D_3 in clinical samples. It has superior sensitivity, requires lower sample volumes and is not affected by cross-reactivity compared to immunoassay.

The assay used is industry developed and validated and uses traceable standards which, if adopted by other research groups, would facilitate comparison of results in relevant publications.

The assay uses segmented hair samples for analysis.

The assay demonstrated 25(OH)D_3 presence in humans and several animal species, including primates.

3.11.2 Limitations

The study focused primarily on two aspects: A systematic review of steroid extraction and analysis from hair, and validation and determination of the fitness of the analytical method for hair analysis of 25(OH)D_3. The study did not examine epidemiological aspects of hair 25(OH)D_3.

LC-MS/MS, although the gold standard methodology for analysing 25(OH)D_3 in biological samples, is expensive, is not readily available and requires expertise.

Correlation was not performed between serum and hair 25(OH)D_3.

The LC-MS method employed does not chromatographically resolve the epimers of 25(OH)D_3. Concentration of these is very low in adults and should not materially affect the levels of 25(OH)D_3 found.

4. Conclusion

The work presented here validates a mass spectrometry method for extraction and measurement of 25(OH)D_3 in hair. It is precise and accurate and can detect down to 6.5 pg/mg. The levels found in the subjects studied are considerably higher than this. For research purposes the method is fit for purpose. However, for use in a clinical diagnostic setting further validation steps are required as outlined in guidelines authored by the Food and Drug Administration (FDA), the European Medicines Agency and the International Council for Harmonisation (368-370).

The availability of a research-ready validated assay can aid in expanding wider investigations into the use of hair as an analytical matrix. For this to progress, however, some outstanding issues need to be addressed. The systematic review outlined in chapter two demonstrates the lack of comparability between publications regarding assay methodologies. A standardised approach to the various aspects of the pre- and post-analytical methods would ensure comparability between results independent of the laboratory they were produced in. Crucial to this would be the availability of standardised reference material traceable to a reference measurement protocol (RMP). Currently no such RMP exists for analysis of steroids or 25(OH)D_3 in hair. In addition, sample handling, extraction, resuspension and chromatography descriptions differ widely.

Separate from the methodological issues, there are numerous physiological factors associated with hair growth that have not been fully explored. These include differing hair growth rates between individuals, as well as gender, age, ethnicity, season and nutrition. Despite these variables, the accepted published consensus is that hair in general grows at a rate of approximately 1 cm per month.

Within the hair strands several issues might influence deposition and stability of metabolites. For example, prolonged exposure of hair to UV light may cause any 25(OH)D_3 present to degrade slowly over time (371). It is also possible that diffusion of steroids or 25(OH)D_3 into the hair shaft may occur from external environmental sources or via secretions via the hair follicle or scalp adding to the total 25(OH)D_3 detected (114). These latter influences are thought to be minor compared to the store of 25(OH)D_3 in the circulation.

It is difficult to identify a correlation between serum and hair 25(OH)D_3 levels because the circulating vitamin is in a dynamic state physiologically with concentrations rising and lowering according to metabolic demand, and intake through supplementation or nutrition (372). Medical conditions such as liver disease may also influence 25(OH)D_3 production (373). In addition, there is a temporal gap of approximately two weeks between circulating 25(OH)D_3 and deposition in the hair shaft (374). The lack of correlation between circulating metabolites and levels in hair is well recognised in the literature. Additional research is required to help resolve all of these issues.

Despite the issues described above, a substantial literature attests to the usefulness of measuring steroids (particularly cortisol) in hair (131, 134, 185). This is reflected in the systematic review performed as part of this thesis. Although 25(OH)D_3 measurement in hair samples is a new development it shares many of the advantages associated with hair steroid analysis. Each centimetre of hair reflects one month's growth and represents a timeline of 25(OH)D_3 status over several months depending on the length of hair strands studied (75). The maximum length of hair from the scalp that can be analysed is contentious. Several authors state that no more than 6 cm should be harvested (representing a retrospective 6-month timeline of 25(OH)D_3 status). Other research groups disagree and claim that significantly longer lengths can be studied (374).

As previously stated, a significant advantage of using hair for 25(OH)D_3 analysis is its ease of collection, non-complex storage and uncomplicated despatch to laboratories for assay. It is also an extremely stable biological sample (375, 376). These attributes are particularly useful where phlebotomy is not appropriate. They are also an advantage in animal studies where there can be significant difficulties in collecting blood samples especially from animals in the wild (377).

There are a number of issues that should be considered when sampling hair for analytical purposes. Standardised hair collection recommends sampling from the posterior vertex area of the scalp. Where hair is absent in this region, other areas of head hair can be used if available. Alternatively, hair can be collected from other body sites. It must be emphasised, however, that validation of extraction and analysis must be performed for different hair types utilised.

Hair treatments of any kind may influence recoveries of analytes of interest and must be considered prior to sampling. Passive external contamination of hair with environmental agents may yield false positive or negative results. The length of hair taken should be recorded, and, for examination of temporal changes in analytes, the scalp end should be clearly identified. Certain people, for cultural reasons, privacy concerns or concerns about the amount of sample required, may not be willing to provide hair samples. Particular hair styles, e.g., closely cropped hair, may not yield a sufficient sample for accurate analysis.

Low levels of $25(OH)D_3$ in hair are thought to reflect chronic deficits in the circulating vitamin. A current significant drawback in hair analysis, though, is the lack of available reference ranges for metabolites of interest including $25(OH)D_3$. It is also not possible, at present, to correlate serum and hair levels accurately (93). These are areas that needs further research.

Notwithstanding these issues hair analysis of $25(OH)D_3$ can yield valuable information. It can be useful in longitudinal and epidemiological research and can yield important long-term information in zoo and farm animals where frequent blood sampling is difficult (378). Also, because of its excellent stability, hair analysis could be very useful in anthropological, archaeological or paleontological studies where other tissues have long since completely degraded. Cortisol, for example, has been detected in hair samples thousands of years old and testosterone has been detected in Mammoth hair at least 20,000 years old (89, 329).

While there is significant further research required in the hair analysis field the results presented in this thesis show that $25(OH)D_3$ can be readily detected in hair. The validation data collected indicates that the assay examined can facilitate further research into the methodological and physiological aspects of hair as a biomarker for vitamin D studies. Hair extends the window of $25(OH)D_3$ detection beyond that of biological fluids although it does not reflect day to day changes in $25(OH)D_3$. Instead, it gives a long-term view of vitamin D status which could inform improved strategies for managing vitamin D deficiency in the short term, rather than analysing blood samples over an extended period of time.

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