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Analysing and quantifying chronic stress-associated endogenous steroids in hair samples

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Abstract

In previous studies, various steroids have been associated with stress and have therefore been quantified to investigate stress-related questions. Since the main stress-related steroid cortisol follows a circadian rhythm, often hair is analysed to quantify this steroid. Further, hair analysis gives the unique possibility of long-time monitoring by analysing a certain segment of hair, since hair grows on average 1 cm per month. Hair is a difficult matrix due to the complex sample preparation with many steps including washing and grinding, followed by various extraction steps. Additionally, steroids are endogenous and are therefore present in the hair matrix. Hence, no analyte free matrix is available, which is needed for the quantification via external calibrators. To overcome this problem, the so-called surrogate methods can be used, for which a $^{13}C_3$ labelled or deuterated reference compound of the steroid of interest is used for quantification. In the present study, a surrogate method was developed and fully validated for the quantitative analysis of seven steroids in human hair. Validation experiments showed that the method is further suitable for semi-quantitative analysis of estradiol. However, it is not suitable for the analysis of androsterone and DHEAS. The method was successfully used to analyse steroids in a comprehensive study of 360 adolescent hair samples, enabling research into stress markers.

KEYWORDS
cortisol, hair analysis, LC–MS/MS, steroids, stress

1 | INTRODUCTION

Stress is the body's nonspecific adaptive response to adjust to disruption, which can be psychological or physiological.[1] When quantifying stress via biomarkers, it is essential to differentiate between acute and chronic stress. In the present sub-study, chronic stress response in adolescent due to physical abuse by their parents was of interest, with the overall study aiming at investigating resilience trajectories of these adolescents.[2]

Different biomarkers have been associated with chronic stress in humans as well as in animals.[3,4] Especially cortisol and cortisone have been extensively evaluated in this context.[5–7] When a body undergoes physiologic duress, cortisol starts to mobilize energy stores and modulates the immune system. While cortisol is the commonly used glucocorticoids biomarker for stress in humans, non-human primates and larger mammals produce corticosterone as their primary stress hormone in other vertebrates such as rodents.[3] Another steroid associated with stress is dehydroepiandrosterone (DHEA) yet correlated with the

response to acute stress.[8,9] However, its water soluble sulfate metabolite dehydroepiandrosterone-sulfate (DHEAS) is a stable index of adrenocortical activity linked with chronic stress.[10,11] These steroids are produced in the adrenal cortex and regulated by the hypothalamic–pituitary–adrenalaxis (HPA).[12] HPA responds to stress by an increased release of stress hormones such as cortisol.

While blood samples depict the status of a patient at the time of sampling, urine depicts a longer detection window. However, as urine concentrations highly depend on the water content of a person, it is not a suitable matrix for quantification of these analytes. If long-time monitoring of a biomarker is desired, hair has established itself as the matrix of choice.[13] Exogenous compounds such as pharmaceuticals or drugs of abuse are implemented into hair after consumption,[14,15] but also endogenous compounds such as steroids will be incorporated into the hair shaft upon its growing.[13,16–18] The advantages of hair analysis are the possibility of long-term monitoring non-invasive sampling, protection of bodily integrity, easy storage and shipment of samples and the small amount of sample required for analysis (between 10–30 mg depending on the analytes and analysis).[19,20] Further, hair growth is relatively consistent with roughly 1 cm per month on average per person. This allows for retrospective analysis of a person's steroid profile and makes it possible to establish a timeline (if several segments are being evaluated).

However, it is crucial to consider wash-out effects when it comes to the analysis of hair segments, which have been associated with steroid analysis. These wash-out effects can result from hygienic hair washing,[21] hair treatment, swimming and light exposure.[22] Dong et al.[23] reported on the segmental analysis (1 cm) of hair samples from two females, which showed a gradual decrease in steroid concentration from the root-side end towards the distal side hair segments.

The overall conclusion is to analyse segments close to the scalp, which have naturally been less exposed to environmental stimulants, in order to avoid wash-out effects.[23] Dong et al. recommend to analyse not more than 3 cm of hair segment.

Different types of strategies are available to analyse steroids (e.g., cortisol), such as immunoassays with enzyme-linked immunosorbent assay (ELISA) or liquid-chromatography coupled to tandem mass spectrometry (LC–MS/MS).[13] Since steroids are endogenous compounds, no analyte free matrix is available. In order to circumvent this problem, different approaches have been proposed such as using surrogate matrix in combination with authentic analyte[24] or the removal of the endogenous analyte from the authentic matrix.[25] Also using standard addition for quantification instead of an external calibration has been applied in hair analysis.[26] However, this approach is labour and time consuming, especially when a larger number of samples has to be analysed. An alternative is the use of a surrogate analyte approach with authentic matrix. In order to do so, quantification is performed using $^{13}C_3$ or deuterated reference compound of the steroid of interest.[27] Binz et al. reported on such a method for the analysis of cortisol[16] as well as cortisone[17] or testosterone in hair and nails.[28] Further, they reported on different steroid sets (cortisone, cortisol, 11-deoxycortisol, androstenedione, 11-deoxycorticosterone, testosterone and progesterone) analysed in hair samples.[18] Essentially, calibration standards of $^{13}C_3$-labelled or deuterated reference compounds (surrogate analytes) are spiked to pooled hair matrix and this surrogate calibration is used for the quantification of the analytes in authentic samples. An important factor to consider is the possible different MS response of the steroid and its $^{13}C_3$-labelled or deuterated counterpart, which needs to be determined.[27]

To the best of the authors' knowledge, no validated method for the quantification of the steroids 17-α-hydroxyprogesterone, androsterone, corticosterone, cortisol, cortisone, dehydroepi-androsterone-sulfate (DHEAS), estradiol, estrone, progesterone and testosterone in hair has been published so far. Hence, the aim of the present work was to develop and validate a method for the quantification of 10 different steroids associated with stress in hair samples (see Figure 1). These particular steroids were chosen since we aimed to identify correlations between steroids present in the hair of adolescents and their resilience pathways with experience of physical family violence in an empirical study (https://data.snf.ch/grants/grant/185481).[2] The overarching project deals with resilience-trajectories of adolescents having experienced physical abuse by their parents. Due to an exploratory research focus, we are testing several steroids and their possible connection to resilience in adolescents. Hence, we included corticosterone in this study due to the fact that it is a metabolite of progesterone. Further, the main reproductive hormones progesterone and testosterone were included but also their metabolites 17-α-hydroxyprogesterone, estrone and estradiol and androsterone. Cortisol, cortisone and DHEAS were included due to their known associations with chronic stress. The different steroids are related to a variety of psychological areas in adolescence, as cognitive, academic, emotional and social development.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Water was produced in-house using a direct-Q water purification system from Millipore (Zug, Switzerland). Methanol in LC–MS/MS grade was obtained from Sigma-Aldrich (Buchs, Switzerland), as well as all reference standards and material, with the exception of testosterone-d_3, which was purchased from Lipomed (Arlesheim, Switzerland). All $^{13}C_3$ or deuterated reference standards with the exception of estradiol-d_5, and corticosterone-d_4, were obtained as solvents in concentrations of either 1 mg/mL or 100 µg/mL. All other reference compounds were purchased in powdered form and stock solutions of 1 mg/mL in methanol prepared. Pooled hair from volunteers were used as hair matrix for the preparation of calibrations standards and quality control samples (QC).

2.2 | Preparation of standard stock solutions

An 8-point calibration curve was prepared. For cortisol, corticosterone and cortisone, the calibration range was from 1.9 to 225 pg/mg, and for all other analytes, it was from 1.0 to 120 pg/mg analyte

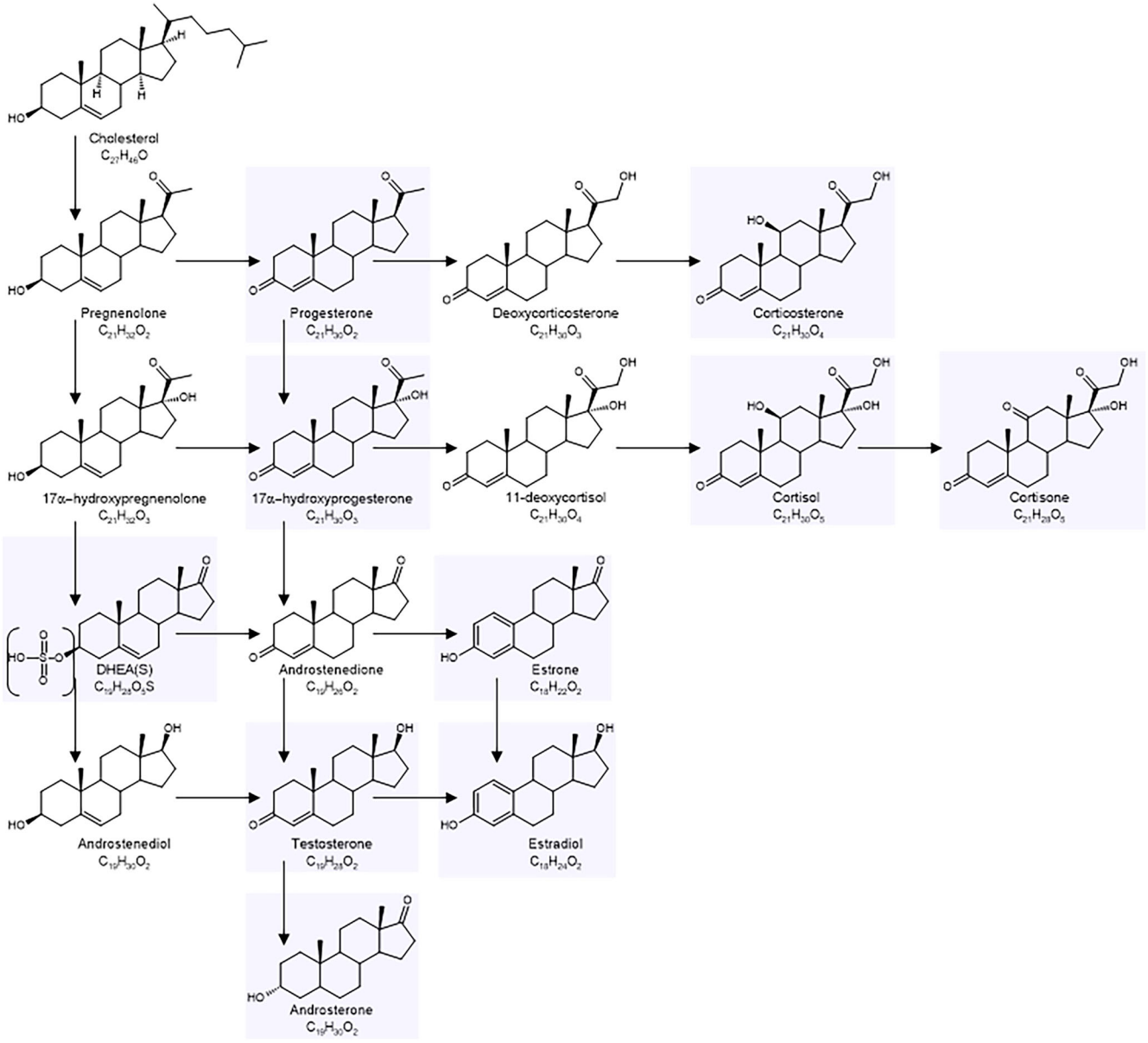

FIGURE 1 Human steroid metabolism including all 10 investigated steroids, which is indicated by purple boxes, in the present study.

concentration in hair. A stock solution mixture of all analytes was prepared in methanol with concentrations of either 1 µg/mL (cortisol, corticosterone and cortisone) or 0.533 µg/mL (all other analytes), which was serial diluted using methanol to obtain working solutions for spiking pooled hair matrix.[18]

QCs were prepared in three concentration levels (7.0, 98.6 and 200.0 pg/mg for cortisol, corticosterone and cortisone and 3.8, 52.5 and 106.5 pg/mg for all other analytes) from a stock solution mixture of all analytes.

For the preparation of calibration standards and QC samples in hair matrix, approximately 20 mg of hair pool sample (exactly weight in) was spiked with 30 µL working solution, let to dry and stored at −20°C before pulverisation for sample extraction. Calibration samples were then extracted analogously to the hair samples.

2.3 | Hair sample extraction

Before extraction, hair samples were first washed with ultrapure water followed by acetone and continuous shaking for 2 and 3 min, respectively. This combination of washing solutions has been previously evaluated and reported by Binz et al.[16] for hair steroid analysis. Samples were then let to dry overnight at room temperature. Hair segments of 3 cm were cut off per sample starting from the hair root, which was marked during the sampling process. These were then cut into small pieces, and approximately 20 mg of each sample was exactly weight in duplicates into Eppendorf tubes. Pulverisation of samples was conducted using two stainless-steel beads (5 mm diameter) at 30 Hz in three sessions of 1.5 min with a break of 1 min.

TABLE 1 MRM parameters of the investigated steroids, $^{13}C_3$ labelled and deuterated reference compounds.

Analyte (steroid, surrogate)	Sum formula	MW (g/mol)	$[M+H]^+$/ $[M-H]^-$ (m/z)	Quantifier qualifier (m/z)	Fragmentor (V)	CE (V)	Mode	RT (min)
17-α-Hydroxyprogesterone	$C_{21}H_{30}O_3$	330.2	331.2	109.1	137	30	+	5.0
				97.1		25		
17-α-Hydroxyprogesterone-d_8	$C_{21}H_{22}O_3D_8$	338.5	339.3	100.1	135	29	+	4.9
				113.1		32		
Androsterone	$C_{19}H_{30}O_2$	290.4	291.2	273.2	75	4	+	4.9
				255.1		12		
Androsterone-d_4	$C_{19}H_{30}O_2D_4$	294.5	295.3	259.0	85	12	+	6.5
				218.9		18		
Corticosterone	$C_{21}H_{30}O_4$	346.2	347.1	329.1	125	12	+	3.8
				121.0		23		
Corticosterone-d_4	$C_{21}D_4H_{26}O_4$	350.5	351.3	121.1	125	25	+	3.7
				97.1		38		
Cortisol (Hydrocortisone)	$C_{21}H_{30}O_5$	362.0	363.1	91.0	110	76	+	2.6
				121.1		25		
Hydrocortisone-2,3,4-$^{13}C_3$	$^{13}C_3C_{18}H_{30}O_5$	365.2	366.2	124.0	120	25	+	2.6
				91.0		90		
Cortisol-d_4	$C_{21}H_{26}D_4O_5$	366.5	367.2	331.1	134	15	+	2.4
				121.1		25		
Cortisone	$C_{21}H_{28}O_5$	360.4	361.1	163.1	129	25	+	3.7
				91.0		80		
Cortisone-$^{13}C_3$	$C_{18}{}^{13}C_3H_{28}O_5$	363.4	364.1	166.1	130	25	+	3.6
				124.0		35		
DHEAS	$C_{19}H_{28}O_5S$	368.5	367.0	96.8	140	40	−	3.2
				79.7		80		
DHEAS-d_5	$C_{19}H_{23}D_5O_5S$	373.0	372.2	97.9	170	40	−	3.1
				79.8		120		
Estradiol	$C_{18}H_{24}O_2$	272.4	271.2	145.0	180	44	−	4.4
				183.0		40		
Estradiol-d_5	$C_{18}H_{19}D_5O_2$	277.0	276.2	147.1	170	55	−	4.3
				145.1		65		
Estrone	$C_{18}H_{22}O_2$	270.2	269.0	145.0	145	40	−	4.2
				142.9		65		
Progesterone	$C_{21}H_{30}O_2$	314.5	315.2	109.1	122	28	+	5.8
				97.1		25		
Progesterone-d_9	$C_{21}D_9H_{21}O_2$	323.5	324.3	113.1	135	30	+	5.7
				100.1		25		
Testosterone	$C_{19}H_{28}O_2$	288.4	289.2	109.1	112	30	+	4.7
				97.1		23		
Testosterone-2,3,4-$^{13}C_3$	$^{13}C_3C_{16}H_{28}O_2$	291.4	292.2	100.1	125	25	+	4.7
				112.1		26		
Testosterone-d_3	$C_{19}H_{25}D_3O_2$	291.4	292.2	109.1	135	25	+	4.7
				97.1		25		

Extraction of the hair samples was performed according to an adjusted protocol previously published by Voegel et al.[18] Briefly, samples were extracted after the addition of 1 mL of methanol and 50 µL of internal standard solution (ISTD, cortisol-d_4 80 µg/mL and testosterone-d_3 110 µg/mL) for 2 h at 55°C in an ultra-sonic water path. Following extraction, samples were centrifuged for 10 min at 8°C and with 13,000 rpm (Eppendorf centrifuge 5425R, Schönenbuch, Switzerland) and the supernatant transferred to a new Eppendorf vial. Samples were dried using a Genevac EZ-2 (SP Scientific, Ipswich, UK) with a 70 min program consisting of 20 min to final stage and 50 min at the final stage at a temperature of 40°C under vacuum.

Further, sample clean-up was performed using liquid–liquid extraction (LLE) with ethyl acetate and water (v/v, 26:1). Samples were shaken before being stored at −20°C for 1 h followed by centrifugation for 5 min at 8°C with 13,000 rpm. A 1.1 mL of the organic supernatant was transferred to an autosampler vial and again evaporated to dryness using previous conditions. Finally, samples were reconstituted in 500 µL methanol and mobile phase (3:7 [v/v]).

2.4 | Quantification using LC–MS/MS analysis

Quantification of the steroids of interest was performed using an Agilent 1260 Infinity II coupled to an Agilent 6460 Triple Quadrupole (Basel, Switzerland) LC–MS/MS system. Separation was performed on an Agilent PoroShell 120 EC-C18 (2.1 × 50 mm, 2.7 µm) using water with 2 mM ammonium fluorid (A) and methanol (B) as mobile phases with a flow rate of 0.4 mL/min and an oven temperature of 50°C. The total run time was 10.5 min, with a 7.5 min gradient following 3 min post-time. The gradient had following conditions of B: 0–1 min 40% B, 1–6 min 80% B, 6.1–7.5 min 97.5% B. The injection volume was 10 µL, and the autosampler consistently cooled at 8°C. MS experiments were conducted in multi reaction monitoring (MRM) mode using transitions depicted in Table 1. Further MS conditions were the following: a source gas temperature of 350°C, a source gas flow of 11 L/min, source nebulize 60 psi, a capillary voltage of 3000 V and noozle voltage of 500 V.

2.5 | Method validation

The validation of the method was performed according to guidelines by the German Society of Toxicological and Forensic Chemistry (GTFCh).[29] Linearity, stability, limit of quantification, bias, inter- and intra-day precision, matrix effects, extraction efficiency and recovery were assessed.

The linearity was assessed with six replicate calibration curves in the above stated concentration range. Outlier detection was performed for each concentration using one-sided Grubbs-test ($\alpha = 0.05$), and variance homogeneity between the lowest and highest concentration was shown using *F*-test ($\alpha = 0.01$). The linear regression and R^2 were determined plotting the ISTD normalized data versus the concentration. A R^2 of 0.99 or higher was desired.

The bias was determined by measuring on days 1, 3 and 7 six newly prepared QC samples ($n = 18$) per level (low, mid and high) and calculating the difference to the respective nominal value relative to the nominal value in percentage. Inter-day precision and intra-day precision were determined on days 1, 2, 3, 4 and 7 using six replicates of the QC samples in low, mid and high concentrations. The autosampler stability was determined for 3 and 7 days, respectively, and was assessed using calibration curves reinjected on days 1, 2, 3, 4 and 7. Both one-way ANOVA and a visual comparison were performed to proof stability of the calibration curve over 7 days.

TABLE 2 Calibration range, limit of detection (LOD), limit of quantification (LOQ), regression coefficient for all $^{13}C_3$ labelled and deuterated compounds used for quantification.

Analyte	Calibration range (pg/mg)	LOD (pg/mg)	LOQ (pg/mg)	Regression coefficient R^2	ANOVA *F*	ANOVA *P*-value
Quantitative analysis						
17-Hydroxyprogesterone-d_8	1.00–80.00	0.20	1.00	0.993	2.22	0.08
Corticosterone-d_4	1.88–150.00	0.38	1.88	0.993	0.91	0.46
Cortisol-$^{13}C_3$	1.88–150.00	0.38	1.88	0.993	0.34	0.85
Cortisone-$^{13}C_3$	1.88–150.00	0.38	1.88	0.992	1.23	0.32
Estrone	1.00–80.00	0.20	1.00	0.988	1.03	0.40
Progesterone-d_9	1.00–80.00	0.20	1.00	0.990	1.22	0.32
Testosterone-$^{13}C_3$	1.00–80.00	0.20	1.00	0.994	1.04	0.40
Semi-quantitative						
Estradiol-d_5	0.2–80	0.20	1	0.926	0.61	0.65
Failed (unsuccessful)						
Androsterone-d_4	0.2–80	na	na	0.057	na	na
DHEAS-d_4	0.2–80	na	na	0.044	na	na

Note: Additionally, the ANOVA result from the comparison of the neat steroid versus the neat labelled reference compound for the establishment of the response factor.

The matrix effects, recovery and extraction efficacy of the sample preparation were evaluated according to Matuszewski et al.[30] Three different sets of samples (A, B and C) for each QC concentration level (cortisol, corticosterone and cortisone: 7.04, 98.6 and 200.0 pg/mg; all other analytes: 3.75, 52.5 and 106.5 pg/mg) were prepared. Set A consisted of pooled blank hair spiked with surrogate spiked prior to extraction. Set B was extracted hair samples spiked with surrogate before LC–MS/MS analysis, and Set C consisted of neat surrogate samples in mobile phase. The matrix effects are the ratio of Set B to Set C. The recovery is the ratio of Set A to Set C, and the extraction efficacy is the ratio of Set A to B. All three entities are expressed in percentage.

2.6 | Hair sampling of authentic hair samples for an explorative study

The authentic hair samples were collected in the cause of the SNF funded research project 100019_185481 'Understanding the resilience pathways of adolescent students with experience of physical family violence: The interplay of individual, family and school class risk and protective factors'. This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the School of Education, University of Applied Sciences and Arts Northwestern Switzerland.

TABLE 3 Validation results including bias, intra-day and inter-day precision, matrix effects, recovery and efficacy.

Surrogate-analyte	Level	Bias (%)	Intra-day precision (%)	Inter-day precision (%)	Matrix effect (%)	Recovery (%)	Efficacy (%)
Quantitative analysis							
17-α-Hydroxyprogesterone-d_8	Low	−7.8	20.1	24.6	167.8	117.7	70.2
	Mid	7.9	7.9	11.2	151.9	102.1	67.2
	High	18.1	4.4	8.5	153.8	102.3	66.5
Corticosterone-$^{13}C_3$	Low	−6.7	15.3	14.9	129.0	82.0	63.6
	Mid	8.5	9.9	9.4	116.0	83.9	72.3
	High	20.0	21.3	19.5	126.8	79.6	62.8
Cortisol-$^{13}C_3$	Low	−5.3	14.0	17.1	141.2	93.6	66.3
	Mid	9.2	4.8	5.1	136.1	92.8	68.2
	High	16.5	19.7	18.1	146.6	87.2	59.4
Cortisone-$^{13}C_3$	Low	15.6	14.4	21.3	151.1	91.4	60.5
	Mid	20.0	4.0	17.7	131.7	88.0	66.8
	High	26.0	24.0	26.3	142.2	83.0	58.4
Estrone	Low	16.3	28.9	33.7	203.8	102.4	50.3
	Mid	3.5	12.3	11.4	204.2	111.2	54.5
	High	12.1	12.1	11.2	183.7	90.5	49.3
Progesterone-d_9	Low	25.4	13.4	13.5	123.5	71.1	57.6
	Mid	13.3	14.6	18.9	114.4	73.9	64.6
	High	17.9	9.1	13.3	120.0	72.6	60.5
Testosterone-$^{13}C_3$	Low	14.1	11.2	15.3	135.1	96.4	71.3
	Mid	11.2	4.8	4.5	129.9	95.0	73.2
	High	16.9	4.7	4.7	136.6	93.9	68.8
Semi-quantitative							
Estradiol-d_5	Low	3.3	130.8	138.1	172.5	130.0	75.4
	Mid	15.8	27.7	38.5	181.4	105.6	58.2
	High	22.1	18.8	21.5	206.9	107.0	51.7
Failed (unsuccessful)							
Androsterone-d_4	Low	na	na	na	na	na	na
	Mid	na	na	na	na	na	na
	High	na	na	na	na	na	na
DHEAS-d_5	Low	na	na	na	na	na	na
	Mid	na	na	na	na	na	na
	High	na	na	na	na	na	na

Hair was sampled after informed consent of the adolescents (study participant), parents and teachers. Each sample was collected from the posterior vertex region, cut as close to the scalp as possible and stored wrapped in aluminium foil in a zip-log bag with silica gel to ensure integrity of the sample. Samples were then sent to the laboratory for analysis.

2.7 | Data evaluation and statistical analysis

Data were processed using MassHunter Workstation v.10.0.707.0 (Agilent, Basel, Switzerland), and the raw data were analysed using Microsoft Excel 365 MSO v.2309. Measured areas under the curve (AUC) were normalized using the AUC of the internal standards. Depending on the retention times, cortisol, cortisone, corticosterone and DHEAS were normalized with cortisol-d_4, and all other analytes were normalized using testosterone-d_3.

3 | RESULTS AND DISCUSSION

3.1 | Method validation

The results of the method validation are depicted in Tables 2 and 3. The linearity of the calibration curves of 17-α-hydroxyprogesterone-d_8, corticosterone-d_4, cortisol-$^{13}C_3$, cortisone-$^{13}C_3$, progesterone-d_9 and testosterone-$^{13}C_3$ showed high linearity with a determination coefficient (R^2) higher than 0.99 in the respective calibration range. For estradiol-d_5 ($R^2 = 0.92$) and estrone ($R^2 = 0.98$), the linearity was satisfactory. Unfortunately, the calibration curves of the analytes androsterone-d_4 ($R^2 = 0.057$) and DHEAS-d_4 ($R^2 = 0.044$) showed no linearity.

The limit of quantification was between 1.88 pg/mg for corticosterone, cortisol and cortisone and 1.00 pg/mg all other analytes, respectively (see Table 2).

The stability of the calibration samples stored in the autosampler for 7 days at 8°C could be shown. Two-way ANOVA (without replication) showed no significant differences between calibration 1 measured on days 1, 2, 3, 4 and 7 with F between 0.61 and 2.22 (F crit = 2.63, see Table 2). Further, a visual comparison also revealed no difference between the measured calibrations (see Figure 2). Carryover was not observed for any analyte after the injection of the highest calibration sample.

The bias for all analytes and levels were in an acceptable range between −7.8% and 26% (see Table 3). The intra-day precision was between 4.0% and 28.9% for the quantitative analytes for all three concentration levels. Further, the inter-day precision for the mid and highest concentrations were between 4.5% and 26.3% for all quantitative analytes. For the lowest concentration, the inter-day precision was between 13.5% and 33.7% for the quantitative analytes. These results are in alignment with previously reported validation data on steroids in hair.[17]

With the exception of cortisone (high QC), estrone (low QC) and progesterone (low QC) were all analytes in accepted range of ±20%.

The matrix effects, recovery and extraction efficacy for all 10 surrogates were determined at three concentration levels (see Table 3).

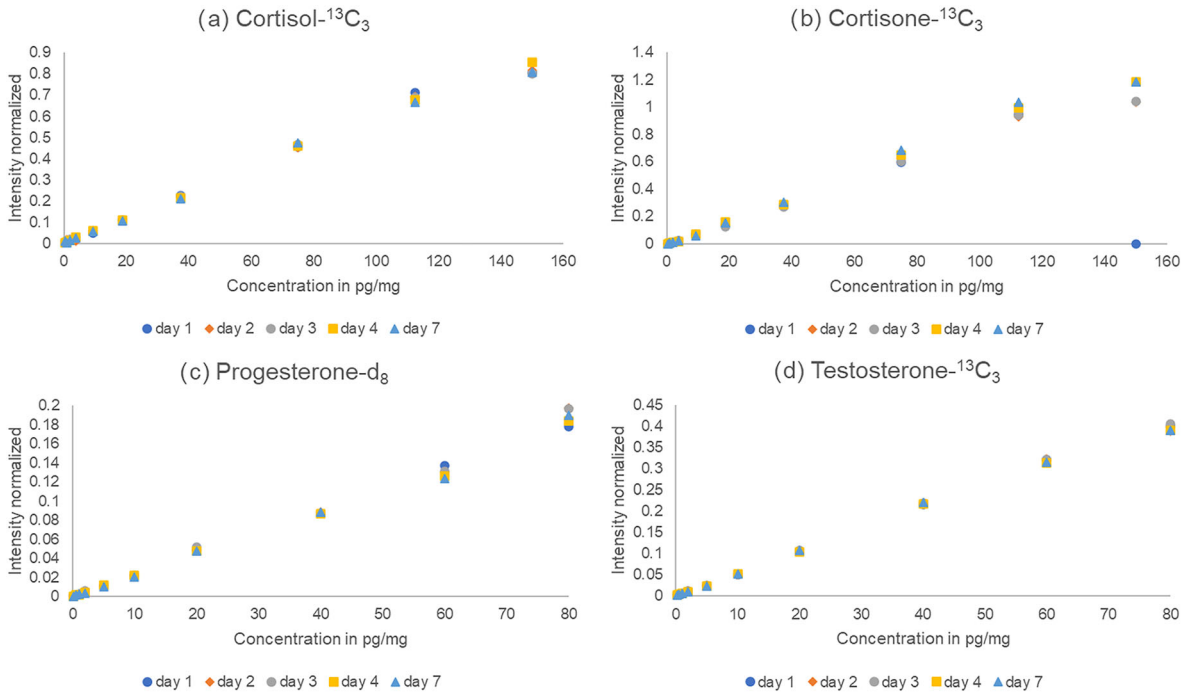

FIGURE 2 Visual comparison of calibration 1 measured on days 1, 2, 3, 4, and 7 in order to evaluate autosampler stability at 8°C over 7 days: (a) cortisol-$^{13}C_3$, (b) cortisone-$^{13}C_3$, (c) progesterone-d_8 and (d) testosterone-$^{13}C_3$.

For androsterone-d_4, very high matrix effects and recoveries were calculated making the method unsuitable for this analyte. Also, for DHEAS-d_4, estradiol-d_5 and estrone rather high matrix effects between 155% and 207% were determined. However, their recoveries were between 90% and 130%, which is in agreement with the recoveries of all other analytes (with the exception of androsterone-d4). The extraction efficacy of all analytes was between 49% and 75%, which is commonly observed in hair analysis.[17]

Validation experiments showed that the method is not suitable for androsterone-d_4 and DHEAS-d_4. From the pre-experiments (not shown), it can be concluded that the second extraction step of LLE did not extract these two analytes sufficiently. The direct analysis of the hair extract (before LLE) showed sufficient linearity for androsterone-d_4 and DHEAS-d_4. However, since more than 360 hair samples are included and analysed in the main study, we decided to opt for a more HPLC friendly approach, meaning that we aimed for as matrix-free/extracted samples as possible, in order to avoid clogging of the valves in the HPLC. Hence, we decided to conduct a second extraction step of LLE. Further, only semi-quantitative results could be obtained for estradiol-d_5, since the linearity was not sufficient (0.92).

3.2 | Response factor

Since steroids and their $^{13}C_3$-labelled or deuterated counterparts do not always behave exactly the same in the mass spectrometer (see Figure 3),[27] a response factor had to be determined for the correct determination of the concentration of steroid in hair.[28] For this, neat calibration standards of the steroids and the surrogates were treated as described in the method section. For each quantified steroid, a response factor was established (see Table 4), which is the ratio of the responses found for the steroid and respective surrogate. For cortisol, testosterone and cortisone response factors between 1.02 and 1.17 were determined meaning the steroid with the respective labelled standard had the same response (compare Figure 3). For all other analytes, a response factor between 1.31 and 1.44 was determined. The response factor was applied to correct for this difference in response

TABLE 4 Response factor determined from the ratio of the steroid calibration curve versus the respective surrogate calibration curve.

Steroid/surrogate	Response factor
17-α-Hydroxyprogesterone/17-α-hydroxyprogesterone-d_8	1.31
Corticosterone/corticosterone-d_4	1.37
Cortisol/cortisol-$^{13}C_3$	1.02
Cortisone/cortisone-$^{13}C_3$	1.17
Estradiol/estradiol-d_5	1.44
Estrone	n/a
Progesterone/progesterone-d_9	1.30
Testosterone/testosterone-$^{13}C_3$	1.14

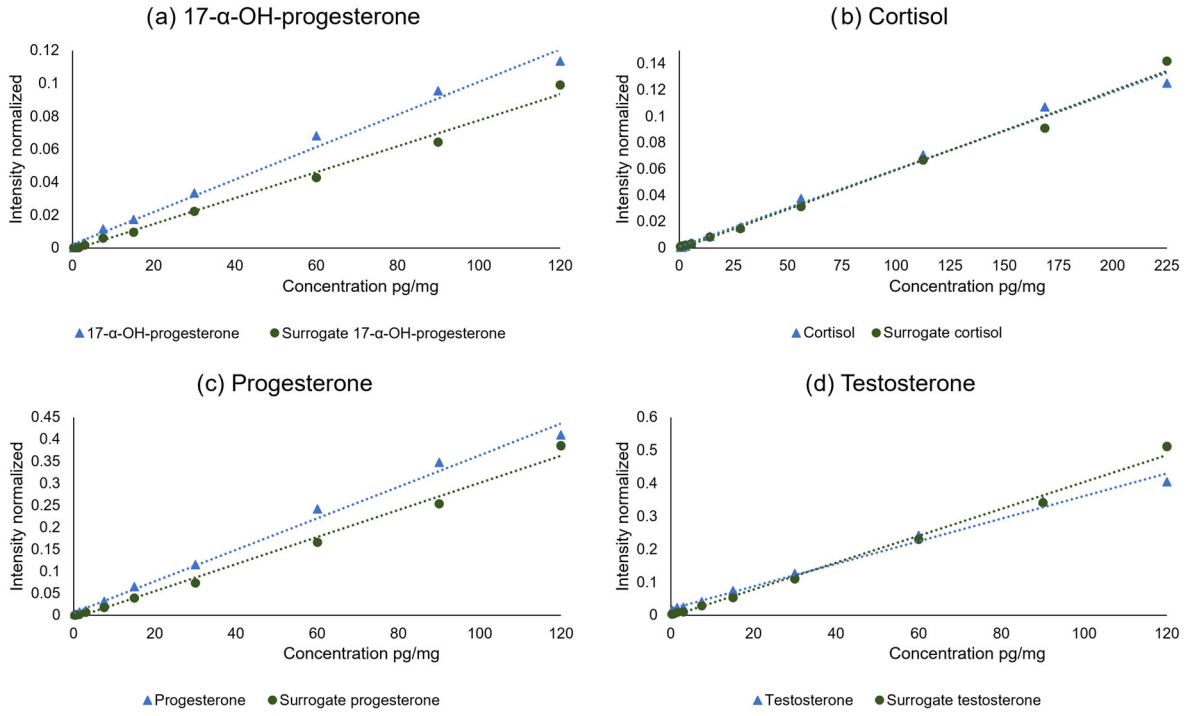

FIGURE 3 Visual comparison of the calibration curves of the neat steroid with their respective neat surrogate extracted analogous to the extracted hair samples: (a) 17-α-hydroxyprogesterone, (b) cortisol, (c) progesterone and (d) testosterone.

TABLE 5 Exemplary results of 10 authentic hair samples from an explorative study (F, female; M, male).

Sample number	Gender	17-Alphahydroxy (pg/mg)	Corticosterone (pg/mg)	Cortisol (pg/mg)	Cortisone (pg/mg)	Estradiol (pg/mg)	Estrone (pg/mg)	Progesterone (pg/mg)	Testosterone (pg/mg)
1	F	2.9	5.2	16.7	52.4	6.0		4.5	9.5
2	F	3.9	8.1	60.0	82.0	2.9		9.5	10.8
3	F	6.6	11.2	7.9	34.2	1.9		11.6	6.9
4	M	2.6	4.3	3.8	29.3	3.6		3.4	6.3
5	M	8.9	3.5	4.2	40.1	2.4		2.6	7.5
6	M	3.4	3.7	7.2	22.9	3.6		2.5	6.5
7	M	6.2	4.7	6.1	27.0	1.7		2.6	5.4
8	M	3.9	3.7	5.4	33.0	5.4		2.1	7.9
9	F	2.5	7.3	19.5	84.4	6.4		11.0	4.3
10	M	4.4	7.6	5.3	10.4	5.4		1.9	3.7

by multiplying the result obtained for each sample in order to get the correct result for the quantification of the endogenous analyte.

3.3 | Application to authentic hair samples

Following validation studies, the method was applied to 360 blinded hair samples from an explorative study (data published elsewhere).[2] Samples were measured in duplicates, and the average result is communicated. Table 5 lists the results of 10 representative samples from this study to show the applicability of this method. The measured concentration range was 3.8–60.0 pg/mg cortisol, 3.7–10.8 pg/mg testosterone and 1.9–11.6 pg/mg progesterone. Values determined for cortisol, cortisone, testosterone and progesterone were in good agreement with previously published data.[18] Out of the 10 samples listed in Table 5, subjects 1, 2 and 9 had elevated hair cortisol concentration of 16.5 pg/mg or higher. Subjects had to fill in a questionnaire in the overarching study,[2] and for subjects 1 and 2, their values for anxiety (subscale of the Hopkins scale) at the time of hair sampling was 2.70 and 3.2, respectively. Hence, both subjects had anxiety values above the average value (1.95) of the whole population at this time of sampling.

Studies have shown that adolescents experiencing neurodevelopmental disorders such as depression and dissociation[31] show also significantly higher cortisol levels.[32–34] Hypercortisolism or hypocortisolism is considered 'problematic as chronically higher or lower cortisol secretions are presumed to induce long-lasting alterations in brain structures involved in depression and anxiety disorders such as the amygdala, the hippocampus, and the prefrontal cortex'.[35]

Also, an increased progesterone value could indicate the gender of the subject. In the present study, subjects 1–3 and 9 were female, and also in those individuals, a progesterone hair concentration of 4.54 pg/mg or higher was quantified.

4 | CONCLUSION

In the present work, we successfully fully validated a method for the simultaneous quantification of seven steroids in hair samples according to an extended version of the guidelines by the GTFCh.[29] Additionally, estradiol could be determined semi-quantitative. Unfortunately, the method was not applicable for androsterone and DHEAS.

In the present approach, we used the so-called surrogate method, using $^{13}C_3$ or deuterated reference standards as calibration for the quantification of endogenous analytes in a complex matrix. In this context, it is important to ensure that the surrogate analytes behave in the same way as the target analytes in the LC–MS/MS. Therefore, the response factor was determined, which revealed that cortisol and testosterone have the same response as their $^{13}C_3$ labelled surrogate compound. However, for the other analytes, a difference was observed; hence, a response factor had to be applied for the evaluation of authentic samples.

The method was successfully applied to 10 hair samples from the explorative study in adolescents (https://data.snf.ch/grants/grant/185481), proofing the feasibility of the method. Building on this workflow, 360 hair samples were analysed for an explorative study.[2]

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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