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Highly selective and automated online SPE LC–MS^3 method for determination of cortisol and cortisone in human hair as biomarker for stress related diseases

Natalia Quinete [a,*], Jens Bertram [a], Marcus Reska [a,1], Jessica Lang [a], Thomas Kraus [a]

[a] Institute for Occupational and Social Medicine, Medical Faculty, RWTH Aachen University, Pauwelsstrasse 30, D-52074 Aachen, Germany

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ABSTRACT

Hair analysis has been increasingly used to establish long-term biomarkers of exposure to both endogenous and exogenous substances, with a special emphasis on steroidal hormones. Hair cortisol and cortisone have been associated to physiological and psychological strains, anxiety and depression. Hair is a very complex matrix, which might jeopardize analyte detection at low concentrations. A new, highly selective and sensitive method based on fragments of second order, MS^3 (MS/MS/MS), was developed and validated for the analysis of hair cortisol and cortisone. An online solid phase extraction was performed on a C8 restricted access material (RAM) phase following by separation on a reversed-phase C18 column using methanol and 0.02% ammonium hydroxide as mobile phase. The developed method required minimal sample preparation and the injection of only 50 μL of sample leading to a LOQ of 2 pg mg^{-1}. Good linear responses were observed in the range 2–200 pg mg^{-1} ($R^2 > 0.99$) and extraction recoveries ranged between 77–125% and 70–123% for cortisol and cortisone, respectively. Intra- and inter-assay coefficients of variation were between 1.4 and 14%. In order to evaluate the applicability of the method, preliminary tests ($N=33$) were conducted in 3 cm hair samples (close to scalp) of healthy volunteers with an age range of 4–63. Average concentrations in hair were 12.7 ± 14 pg mg^{-1} and 41.6 ± 42 pg mg^{-1} for cortisol and cortisone, respectively. Further investigations on cortisol and cortisone as biomarkers for chronic psychological strain will be assessed as a next step.

1. Introduction

Hair analysis has been increasingly used to monitor long-term exposure to both endogenous and exogenous substances, with a special emphasis on steroidal hormones [1,2]. Steroids have important physiological functions because of their anti-inflammatory, anti-allergic and immunosuppressive properties [3]. Natural corticosteroids, hydrocortisone (cortisol) and cortisone, in human hair have the potential to reflect the long-term response of the hypothalamus–pituitary–adrenal axis (HPA) [1,4], therefore, being considered a retrospective biomarker for various chronic physiological and psychological stress diseases, anxiety and depression [5–8]. Abnormal cortisol levels are an indication of a wide variety of diseases, such as Addison's disease, Cushing's syndrome, diabetes and other enzyme deficiencies [9].

Compared to serum, saliva and urine, which represents real-time levels of cortisol, hair samples would be able to accurately measure cortisol and cortisone over a longer time period of exposure, presenting the advantages of stress-free collection, independence from circadian rhythm, easy transportation and storage, traceability and stability [10].

Initial methods for the determination of steroids have primarily focused on immunoassay technologies [4,11] due to their high sensitivity, simplicity and speed [3,12]. However, immunoassays could quantify only one steroid at a time, showing high crossreactivity with other endogenous steroids and lack of specificity as a result of matrix interference, which could lead to overestimation of actual cortisol concentrations in hair samples [13,14].

Although gas chromatography–mass spectrometry (GC–MS) methods have shown good specificity and sensitivity, they are unsuitable to steroids, which are slightly volatile and could be easily denaturated with heat [15], requiring a derivatization step, which not only increases sample preparation time but is also possible for few corticosteroids [15,16].

More recently, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has been increasingly used for corticosteroids determination in hair samples [2,3,9,13,15,17,18]. Though LC–MS/MS methods provide the necessary specificity and sensitivity for the accurate determination of cortisol and cortisone in hair, they

* Corresponding author. Tel.: +49 241 8088865; fax: +49 241 8082587.
E-mail addresses: nsoaresquine@ukaachen.de, nataliaquinete@yahoo.com.br (N. Quinete).
[1] Current address: German Consulting Centre for Hospital Epidemiology and Infection Control (BZH), Schnewlinstr. 10, D-79098 Freiburg/Breisgau, Germany.

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still require pretreatment to separate the analyte from interfering substances [9]. In general, solid phase extraction (SPE) has been carried out to remove proteins and interferences from biological samples [3,17,19]. While SPE cleanup would represent an extra step in the analytical procedure decreasing sample throughput, the development of an online SPE method coupled to mass spectrometry has been shown to improve method sensitivity, reduce pretreatment and analysis times as well as increase the number of the samples that can be analyzed simultaneously [13,20,21].

Previous studies have observed that different ionization methods could be employed for the determination of cortisol and cortisone in hair, but that electrospray ionization (ESI) would be more suitable for the ionization of steroids, since they are low polar or even non-polar [3,22]. Although ESI in positive mode has been frequently used in most methods, interestingly, ESI in negative mode has shown low background noises [23] and to be more sensitive than in positive mode for low and non-polar materials [3,24]. At the same time, even though the use of multiple reaction monitoring (MRM) mode in tandem systems is more suitable for the assay of steroids in comparison to selected ion monitoring (SIM) mode in single MS systems, high background and/or potential interferences around the expected retention time could still interfere in the determination of the analytes in biological matrices.

Recently, another new technology available in some instruments improving quantitative results is the possibility to perform MRM^3 transitions, which reduces the fragmentation time, increases excitation efficiency and with faster scanning of up to 20,000 Da s^{-1} leads to a more sensitive and selective detection mode. Few studies have already shown promising results on applying LC–MS^3 (MS/MS/MS) methods in the quantitation of the pesticide malathion in fruits [25] and serotonin in plasma samples [26].

Due to the low concentrations of cortisol and cortisone and the complexity of the hair matrix, this novel, highly selective and sensitive method based on fragments of second order, MS^3, was developed and validated for the simultaneous identification and quantitation of cortisol and cortisone in human hair. Also, MRM transitions were monitored for comparison purposes. The developed method was tested in hair samples collected from healthy volunteers with an age range of 4–63 years.

2. Material and methods

2.1. Chemicals

Certified standards of cortisol and cortisone and their deuterated internal standard (cortisol-d^4 and cortisone d^7) were obtained from Sigma–Aldrich (Hamburg, Germany). Stock and working solutions were prepared with ultra-gradient HPLC grade methanol from JT Baker (Deventer, Netherlands) and stored at −20 °C. 2-Propanol and water gradient grade for chromatography (Lichrosolv) and ammonia solution 25% GR for analysis were purchased from Merck (Darmstadt, Germany).

2.2. Hair collection and sample preparation

Hair strands were cut with scissors as close as possible to the scalp from a posterior vertex position. The 3-cm hair segment (close to scalp), which based on an average hair growth rate of 1 cm $month^{-1}$ [27] represents hair grown over the three months period prior to hair sampling, was further used for analyses.

Hair samples were not pulverized, since previous studied indicated no significant difference on cortisol/cortisone extraction with this procedure [13,18,28]. 50 mg of minced hair was carefully weighed out in a 5 mL polypropylene tube (Sarstedt &Co, Nümbrecht, Germany), then washed by shaking with 2.5 mL of isopropanol for 2 min and dried overnight at room temperature. The washing procedures aimed to completely remove the contaminations and non-bloodborne cortisol coated on the outer surface of hair strands [2]. After the addition of the internal standards, cortisol-d^4 and cortisone-d^7, the hair samples were incubated in 2 mL methanol at room temperature for 24 h. Finally, samples were centrifuged at 4500 rpm for 10 min and 500 μL of clear supernatant was transferred to a LC vial.

2.3. Chromatographic and mass spectrometric conditions: SPE–LC–ESI–MS^3

Chromatographic separation was carried out on a LC system (Agilent Technologies1200 Infinity series) consisting of a degasser, two binary pump and an autosampler capable of performing up to 100 μL injections. A 50 μL volume of the extract was injected onto a restricted access material (RAM) phase, a LiChrospher® RP-8 ADS (25 μm) 24 × 4 mm RAM from Merck (Darmstadt, Germany), as pre-concentration and cleanup step prior to analytical separation by a Poroshell 120 EC-C18 (50 × 4.6 mm, 2.7 μm) from Agilent (California, USA). The scheme used for the analytical instrumentation with the online SPE setup is shown in Fig. 1.

The online procedure consists of a six-port divert valve on the autosampler which is programmed by the data system to control the flow direction of solvent into the two columns. In the Position A, 50 μL of sample was injected into the RAM phase (SPE column) by Pump 1 using 10% MeOH in water as mobile phase at a flow rate of 0.5 mL min^{-1}. The target compounds are retained in the RAM phase and the matrix that is not retained during the extraction process is directed to waste, while simultaneously Pump 2 equilibrates the analytical column in the starting gradient conditions. After 1.5 min, the valve was switched to Position B, the solvent flow through the RAM phase column is reversed, and the analytes were then backflushed onto the Poroshell C18 column for separation and quantitation by ESI–MS^3. After 15 min, the switching valve returned to Position A to allow the columns to be re-equilibrated with the starting conditions. The total run time per sample was 20 min. Valve

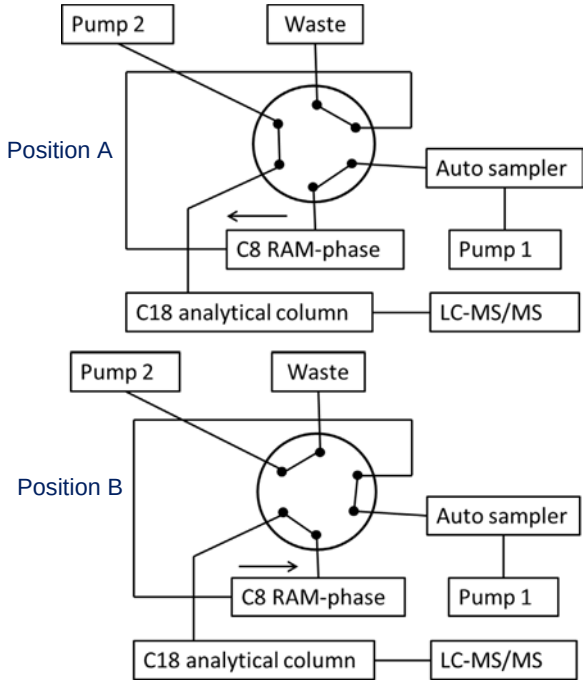

Fig. 1. Online SPE system. The Position A allowed the sample to pass through the RAM-phase washing the interferences into the waste and in the Position B the RAM-phase column is backflushed into the analytical column for analysis by LC–MS/MS.

switching events as well as the gradient programs are summarized in Table 1. The samples were kept at 10 °C in the autosampler.

Tandem mass spectrometry was carried out on a Q-Trap 5500 mass spectrometer (ABSciex, Darmstadt, Germany) equipped with electrospray ionization (ESI) interface. The instrument was operated in the negative ionization mode at −4500 V and a temperature of 450°C. MRM^3 transitions as well as multiple reaction–monitoring (MRM) transitions (for comparison purposes) were optimized for cortisol and cortisone (Table 2).

2.4. Method validation

Validation of the method was performed according to FDA international guidelines [29]. Blank hair samples are very difficult to obtain, therefore for the preparation of calibration and control samples, hair samples were taken from hair segments distant from the scalp of individuals with long hair, where due to "wash out" effects is expected to have very low concentrations of cortisol and cortisone [1].

Calibration samples were prepared in blank hair by spiking a mixture of standards at concentrations ranging from 2 to 200 pg mg^{-1}. Analytical curves were revalidated after every set of twenty samples. Quality control samples included a method/procedural blank, a spiked blank, matrix spike and a matrix spike duplicate with every sample set. Control matrix samples were spiked at 4 different concentrations (at the limit of quantitation, low, medium and high concentrations).

The method was validated for the following parameters: limits of detection and quantitation (LOD and LOQ), linearity, matrix effects, intra- and inter-day precision and accuracy.

Table 1
Valve switching and gradient program for LC pump 1 and 2. A1:0.02% ammonium hydroxide, B1:methanol, A1: water, B2:methanol.

	Pump 1			Pump 2			Valve position
Time (min)	A1 (%)	B1 (%)	Flow (mL min^{-1})	A2 (%)	B2 (%)	Flow (mL min^{-1})	
0	90	10	0.5	20	80	0.2	Position A
1	90	10	0.5	20	80	0.2	
1.5	90	10	0	20	80	0	Position B
2	20	80	0.2				
14.5	10	90	0.2				
15	5	95	0	20	80	0	Position A
15.5	0	100	0.5	0	100	0.3	
16.5	0	100	0.5	0	100	0.3	
17	90	10	0.5	20	80	0.2	
20	90	10	0.5	20	80	0.2	

A1:0.02% ammonium hydroxide, B1:methanol, A1: water, B2:methanol.

Table 2
MRM^3 and MRM optimized parameters for quantitation of cortisol and cortisone. DP: declustering potential, AF2: excitation energy, scan rate:10,000 Da s^{-1}, LIT fill time:250 ms, excitation time: 20 ms.

Analytes	MRM^3 transition	MRM transition	DP (V)	Collision energy (eV)	AF2 (V)
Cortisol	361.2→331.3→297.10	361.2→331.3	−63	−16	0.09
Cortisone	359.2→329.1→301.1	359.2→329.1	−55	−15	0.09
Cortisol d4	365.2→335.1→301.2		−90	−19	0.08
Cortisone d7	367.3→337.1→309.2		−98	−15	0.1

DP: declustering potential, AF2: excitation energy, scan rate:10,000 Da s^{-1}, LIT fill time:250 ms, excitation time: 20 ms.

LOD was defined as the lowest concentration of cortisol and cortisone that can reliably differentiate from background noise (three times higher than background) and LOQ was established as five times the response observed in the blanks.

Precision and accuracy were expressed as relative standard deviation percentage (RSD%) and bias percentage (%Bias) respectively, and were evaluated by blank hair samples spiked with a mixture of cortisol and cortisone at concentrations of 2, 4, 40 and 120 pg mg^{-1}. The intra-assay precision was assessed by analyzing these samples on the same day (five replicates of each sample), while the inter-assay precision was assessed over three different days (five replicates of each sample).

Recovery and matrix effects were determined by comparing the analytical results for extracted samples spiked at three concentrations (4, 40, and 200 pg mg^{-1} for matrix effects and 2, 4, 40, and 120 pg mg^{-1} for recoveries studies) with unextracted standards (without matrix) that would represent 100% recovery.

2.5. Statistics tests

Statistical tests were performed using SPSS version 21.0 for Windows (SPSS Inc, IBM, IL, USA.) Data distribution normality was examined using one-sample Shapiro–Wilks test. Non-normally distributed data were further correlated by Spearman Tests. *t*-Test was done for comparison between hair cortisol, cortisone and cortisol-to-cortisone ratio and ordinal variables (gender and hair color). The statistical significance was accepted at $p < 0.05$.

3. Results and discussion

3.1. Method development

The implementation of an effective cleanup procedure is imperative prior to analysis by LC–MS/MS in order to reduce the negative effects of the interfering substances present in biological samples. However in several cases, due to the structural and physical affinity of fatty acids, the classical sample cleanup/enrichment not always result in an appropriate increase of selectivity of the overall method [25].

When the chromatography is not enough for the complete separation of the analytes from the interferences, the use of tandem mass spectrometry (MS/MS, or MS2) in MRM mode normally eliminates interferences and increases the selectivity resulting in very low baselines and low LOQs.

In some cases of very complex matrix, such as hair, matrix interference cannot be eliminated using MRM, which leads to high background levels compromising the LOQ and therefore the accurate determination of low levels of the substance. While a more elaborate sample cleanup and/or chromatography to eliminate these interferences would implicate in a labor-intensive and long-time consuming sample preparation step and method, the addition of a third MS stage has been shown to greatly increase selectivity and eliminate the high baseline or chromatographic interference of complex matrices [25,30]. In this way, there is the possibility to perform MRM^3 transitions, which will provide higher selectivity due to one additional fragmentation step. The QTRAP® system when operated in MRM^3 mode first filter the first precursor ion in the Q1; then fragment in Q2 generating the product ions which are isolated and trapped in Q3 operating as a linear ion trap (LIT). Afterwards the LIT isolates the second precursor ion and generates the second generation of product ions, which are scanned out towards the detector [25].

In general MRM^3 shows better sensitivity than MRM transitions (Fig. 2). In the case of cortisol and cortisone in hair, although the quantifier MRM has sufficient sensitivity at lower levels, the second MRM transition required for identification (qualifier) showed a

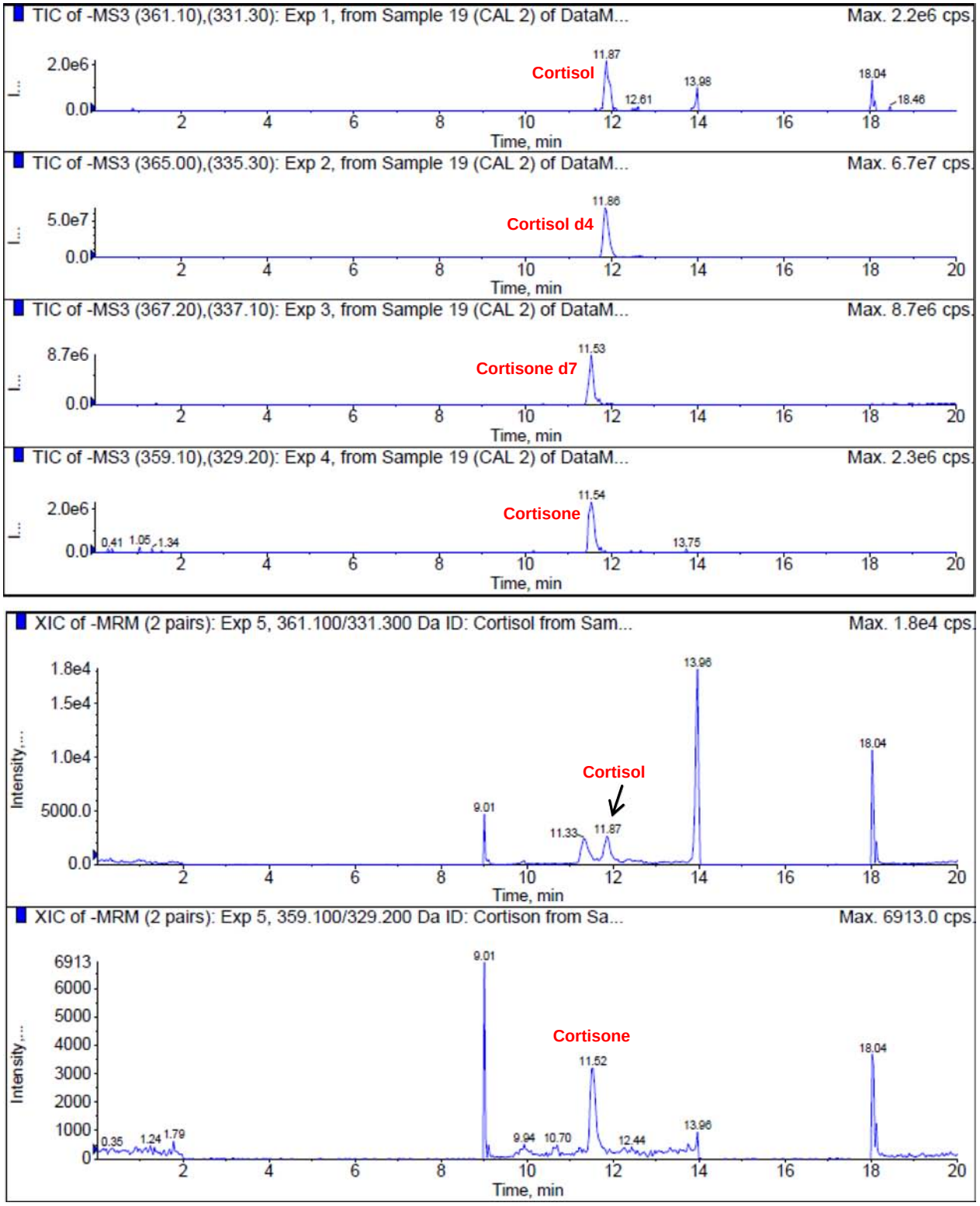

Fig. 2. Chromatograms of cortisol and cortisone spiked at 2 pg mg^{-1} in blank hair by MRM^3 (upper) and MRM (bottom) detection modes.

significant loss in signal to noise (S/N) due to the elevated background, which would increase the LOQ to higher than 4 pg mg^{-1} (data not shown). Consequently, the use of a specific MRM^3 transition would allow the accurate and precise determination of cortisol and cortisone in the hair matrix. A typical chromatogram of cortisol and cortisone in hair is shown in Fig. 2 comparing MRM and MRM^3 detection modes.

In addition, in order to increase sample throughput but maintaining the high selectivity of the method, an online SPE–LC method was implemented together with MS^3 shortening sample pre-treatment time while improving the detection and response in analytical analysis. Previously, Gao et al. [13] have also proposed a column switching strategy for online SPE followed by analyte detection on MRM positive mode, evidencing the advantages of an

online SPE–LC–MS/MS method for the simultaneous determination of steroid hormones in human hair.

3.2. Linearity and detection limit

A 7-point calibration curve was built with the area ratio (area of the analyte standard divided by area of the internal standard) as a function of the analyte concentration. A linear response was observed for cortisol and cortisone in the range of 2–200 pg mg^{-1} ($R^2 > 0.99$). The lower limit of quantitation achieved was 0.05 ng mL^{-1}, which corresponds to 2 pg mg^{-1}, although lower levels could still be detected by the method. In fact, results suggest a LOD lower than 0.4 pg mg^{-1}(0.01 ng mL^{-1}). Unfortunately, since the blank hair samples still had a small amount of the compounds (around five times lower than the established LOQ), these led to an increase of the LOQ, which could be lower taking into consideration that there is minimal background noise in the method. However, the LOQ values obtained were more than satisfactory for the determination of cortisol and cortisone in human hair. The results found in the present study were comparable to recent studies on cortisol and cortisone in hair [3,13], being more sensitive than previous LC–MS methods [15,17,31] or GC–MS methods [10,16]. More importantly, the developed method required small amount of extracted sample (50 μL) and no prior evaporation/pre-concentration step when compared to previous online and offline SPE methods [3,13].

3.3. Recoveries and matrix effects

Recovery and matrix effect analyses were performed in order to assess respectively the efficiency of the extraction procedure and the possible variation in response due to the presence of interferences in the matrix.

Matrix effects resulting from co-eluting matrix components can affect the ionization of the target compounds, causing either ion signal enhancement or suppression, which can severely compromise the quantitative analysis of biological samples [32]. The following equation was used to evaluate the influence of the matrix on the analysis:

$$\text{Matrix effects}(\%) = \left[\frac{(A_s - A_{us})}{A_0} - 1\right] \times 100\%,$$

where A_s is the peak area of analyte found in the spiked sample matrix, A_{us} is the peak area measured in unspiked sample matrix and A_0 is the peak area of directly injected standards at the same concentrations without matrix. Positive values indicate signal enhancement while negative values indicate signal suppression. As expected, although both methods are susceptible to the matrix effects, it's clear that the effects of ion suppression are more pronounced in MRM detection mode and higher at lower concentrations as seen in Table 3. Noteworthy, the values observed here are indeed similar or lower when compared to previous studies [3,13,15].

Recoveries were performed in five replicates at four different concentration levels (2, 4, 40, and 120 pg mg^{-1}) in blank hair samples and calculated by subtracting the amount of the target analyte found in non-spiked samples. Recoveries were in the range of 77–112% (mean:90 ± 9%), 70–113% (mean:89 ± 9%) for cortisol and cortisone, respectively in MRM mode, and ranged from 86 to 125% (mean:105 ± 12%) and 70–123% (mean:100 ± 15%) for cortisol and cortisone, respectively in MRM^3 mode.

Accuracy (Bias) was assessed by calculating the RSD between the true (spiked) concentration and the measured concentration in the spiked hair samples, with values ranging from 3.6 to 19% for intra and inter-days analysis. Reproducibility and repeatability expressed as RSD were lower than 15%, ranging from 1.4 to 14%, for intra-day and inter-day analysis for cortisol and cortisone in hair calculated from repeated injections ($N=5$) of 2, 4, 40 and 120 pg mg^{-1} spiked samples. Table 3 presents a summary of the validation results.

All validation parameters were satisfactory, with the developed method showing high sensitivity, good linearity, precision and accuracy, while having easy sample preparation steps, high sample throughput and low matrix interferences by employing an online SPE–LC method together with a highly specific MS^3 detection mode.

3.4. Method application to human hair samples

Hair samples from 33 healthy individuals (7 male/26 female, mean age: 37 years, age range: 5–63 years) were collected and analyzed in order to further evaluate the applicability of the developed online SPE–LC–ESI–MS^3 method. Cortisol and cortisone

Table 3
Matrix effects, extraction recoveries and intra- and inter-precision and accuracy variations.

Compound	Spiked concentration	Recovery ($N=5$)		Matrix effect ($N=5$)		Precision (RSD%)		Accuracy (Bias%)	
	pg mg^{-1}	Average (%)	RSD (%)	Average	± SD	Intra-days ($N=5$)	Inter-days ($N=5$)	Intra-days ($N=5$)	Inter-days ($N=5$)
Cortisol MRM	2	92	11			7.54	8.09	9.71	18.6
	4	88	8	−50.5	10.8	4.60	9.30	9.09	9.02
	40	87	6	−54.6	6.51	2.82	5.73	4.44	5.51
	120	94	7			6.44	8.10	4.43	9.34
	200			−25.2	32.5				
Cortisone MRM	2	90	13			7.77	6.87	11.0	18.1
	4	85	10	−42.2	15.3	6.75	10.5	12.3	13.2
	40	88	4	−44.6	9.28	4.10	8.75	6.92	6.02
	120	94	6			5.65	5.95	4.52	4.85
	200			−14.1	34.5				
Cortisol MRM^3	2	115	9			5.07	10.1	7.60	15.5
	4	113	9	−31.5	8.80	6.41	10.2	7.30	13.9
	40	92	4	−29.5	10.0	4.58	5.22	3.62	6.41
	120	115	9			4.86	8.37	3.69	9.82
	200			−13.8	20.5				
Cortisone MRM^3	2	100	15			14.0	11.2	5.99	19.3
	4	104	9	−15.9	24.9	8.36	13.1	11.3	18.0
	40	100	4	−23.3	9.54	2.64	7.40	8.11	11.9
	120	94	6			5.47	8.07	6.71	9.37
	200			−7.98	19.6				

were detected in all hair samples analyzed at concentrations higher than LOQ with few exceptions that were close or lower than LOQ. Average hair cortisol and cortisone concentrations were 12.7 ± 14 pg mg^{-1}, ranging from 1.9 to 72.2 pg mg^{-1} and 41.6 ± 42 pg mg^{-1}, ranging from 5.7 to 202 pg mg^{-1}, for cortisol and cortisone, respectively. The respective results are summarized in Table 4. A MRM^3 chromatogram of a real sample is also shown is Fig. 3.

Most samples were collected from adults older than 20 years, except three hair samples from children and youths (age 4 to 16 years). Hair cortisone values were higher than cortisol, being in the same range as previous results conducted in hair by LC–MS/MS methods [2,3,7,13,18,28]. Chen et al. [3] found cortisol ranging from 2.0 to 87.8 pg mg^{-1} and cortisone from 18.9 to 542 pg mg^{-1} at two different cohorts in China ($N=103$), while in another study carried out by Gao et al. [13] 30 volunteers had cortisol and cortisone levels of 1.62–17.64 and 6.07–52.74 pg mg^{-1}, respectively. In a study conduct in 8 years old girls, cortisol and cortisone ranged from 5 to 1330 (median: 8.80 pg mg^{-1}) and 5 to 70 pg mg^{-1}(median: 12.6 pg mg^{-1}), respectively [18]. However in posttraumatic stress disorder patients (PTSD), cortisone and cortisol results were significantly lower than in non-traumatized controls (7.5 and 5.5 vs 15 and 17 pg mg^{-1}) [7], corroborating previous evidence of hypocortisolism in PTSD [33].

Table 4
Cortisol and cortisone levels (pg mg^{-1}) and cortisol-to-cortisone ratio in 33 individual hair samples. F:female, M:male.

Gender	Age	Hair colour	Hair cortisol [pg mg^{-1}]	Hair cortisone [pg mg^{-1}]	Ratio [cortisol/ cortisone]
M	36	Brown	6.89	22.3	0.31
F	35	Brown	10.5	28.6	0.37
F	29	Blond	2.79	8.30	0.34
F	23	Dyed	4.41	11.2	0.39
F	63	Dyed	< LOQ	6.76	N/A
F	35	Brown	2.24	11.3	0.20
F	24	Brown	2.18	5.72	0.38
F	26	Blond	10.1	18.1	0.56
M	31	Brown	8.59	18.9	0.45
M	36	Brown	7.38	31.6	0.23
F	54	Grey	11.1	36.9	0.30
M	20	Brown	7.34	30.1	0.24
F	47	Dyed	7.68	21.6	0.36
F	57	Dyed	53.5	152	0.35
F	5	Brown	3.29	22.3	0.15
M	7	Brown	13.2	106	0.12
F	29	Blond	11.4	46.4	0.25
F	33	Black	4.34	24.5	0.18
F	32	Blond	7.38	31.6	0.23
M	29	Brown	10.6	43.0	0.25
F	44	Dyed	6.23	11.5	0.54
F	16	Blond	6.88	21.4	0.32
F	56	Dyed	12.1	36.7	0.33
F	48	Brown	19.2	71.4	0.27
F	53	Dyed	25.1	41.8	0.60
F	43	Blond	5.76	20.6	0.28
F	50	Black	12.7	42.7	0.30
F	45	Brown	22.6	81.3	0.28
F	47	Blond	6.68	30.8	0.22
M	36	Black	22.4	64.8	0.35
F	44	Dyed	72.2	202	0.36
F	47	Dyed	14.4	48	0.30
F	52	Dyed	9.0	23	0.39
Mean	37		13.1	41.6	0.32
Min	5		2.18	5.72	0.12
Max	63		72.2	202	0.60

F:female, M:male, N/A:not available.

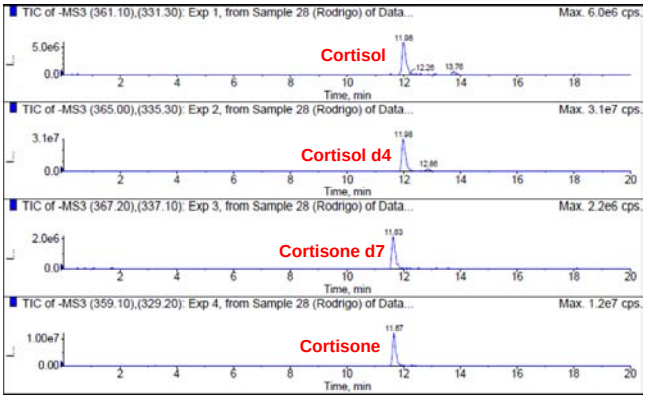

Fig. 3. MRM^3 chromatograms of cortisol and cortisone in a hair sample (Cortisol: 22 pg mg^{-1}, Cortisone: 65 pg mg^{-1}).

The ratio between cortisol and cortisone is an indirect measurement of the 11β-hydroxysteroid dehydrogenase enzymatic activity, which is responsible for mediating the intracellular conversion of cortisol into cortisone and vice-versa, thereby locally regulating glucocorticoid action and the cortisol–cortisone shuttle [18,34]. The ratio values observed in the present study were less than 1, suggesting increased conversion of cortisol to cortisone, which is in line with previous studies in hair, saliva and urine [18,35,36]. The importance of hair cortisone and cortisol-to-cortisone ratio measurement in stress related research has been reinforced by altered 11β-hydroxysteroid dehydrogenase enzymatic activity under stress and HPA activity [18,36,37].

One-sample Shapiro–Wilk test revealed that the levels of cortisol, cortisone and the cortisol/cortisone ratio were not normally distributed with all p-values less than 0.001. A positive correlation was observed between hair cortisol and hair cortisone (Spearman correlation coefficient: 0.880, $p < 0.001$) and a significant correlation was found between cortisol and age (Spearman coefficient: 0.380, $p=0.03$, $\alpha=0.05$), although the same was not observed with cortisone. The correlation cortisol and cortisone with age is still very controversial in the literature. Stalder et al. [28] found both hair cortisol and cortisone significantly increasing with age, while others found no correlation [3,31] or even cortisol higher in young children than in adults [38]. No gender differences were revealed for hair cortisol, cortisone or cortisol/cortisone ratio in the present study, which was also confirmed by other studies [3,31,39], even though some researchers found significantly higher cortisone levels in males than females and no correlation with cortisol [28] or higher cortisol levels in males [38].

Interestingly, independent t-test presented a significant difference between cortisol/cortisone ratios in natural hair (M: 0.28 ± 0.10) and in dyed hair (M: 0.38 ± 0.12) for $T(31)=2.27$, $p=0.03$, $\alpha=0.05$, although no differences were observed for cortisol or cortisone. This result suggests that the conversion of cortisol into cortisone is more pronounced in subjects with dyed hair, which could be due to oxidation processes caused by the hair coloring treatment. Previously, lower cortisol and cortisone levels have been reported by the use of hair treatments [4]. Despite that, the number of samples analyzed in the present study is still not sufficient to make substantial assumptions related to the influence of cosmetic hair treatment or even age in cortisol or cortisone levels in hair samples and further studies should be performed in order to confirm these findings.

4. Conclusions

A new, highly selective and sensitive automated online SPE LC–ESI–MS^3 was successfully developed and validated for the determination of cortisol and cortisone at low pg mg^{-1} levels in hair samples. To our knowledge, this is the first time that a method based on fragments of second order was considered for the identification and

quantitation of cortisol and cortisone in human hair, which together with an online SPE protocol allowed a fast, reliable and accurate measurement of very low concentrations of hair cortisol and cortisone with minimal matrix effects. All samples analyzed showed cortisol and cortisone levels higher than (or equal to) LOQ, with cortisol/cortisone ratio < 1, suggesting an increased 11β-hydroxysteroid dehydrogenase enzymatic activity towards the conversion of cortisol to cortisone. Even though the number of samples available in our pilot study was relatively small, results suggested a positive correlation of cortisol with age and a significant difference between cortisol/cortisone ratio in dyed hair and natural colored hair. Because of its ability to provide a long-term cortisol and cortisone exposure, hair cortisol analysis is becoming a useful tool for the diagnosis of clinical pathological syndromes and psychological status. Therefore, further investigations on cortisol and cortisone as biomarkers for chronic psychological strain will be assessed as a next step.

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