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Determination of thyroid hormones in human hair with online SPE LC–MS/MS: Analytical protocol and application in study of burnout

Wei Gao*, Marlene Penz, Magdalena Wekenborg, Andreas Walther, Clemens Kirschbaum

Department of Biological Psychology, TU Dresden, Germany

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ABSTRACT

Thyroid hormones (THs) play a key role within the endocrine system. Incorporated biomarkers in hair can reflect endogenous excretion patterns over several months. We present an online solid phase extraction-liquid chromatography-mass spectrometry (online SPE-LC–MS/MS) method for quantification of THs in human hair and test it in the volunteers suffering from different severity of burnout symptom. THs were extracted from 7.5 mg hair by methanol incubation. Extracts were analyzed with LC–MS/MS in positive electrospray ionization mode. Burnout symptoms were assessed with the Maslach Burnout Inventory-General Survey (MBI-GS). THs levels were determined in 208 hair samples from adults and related to individual MBI-GS score. Intra- and inter-day coefficients of variance were between 3.1% and 10.2%. The recoveries of this method were between 88.5% and 102.1%. Hair T_4 levels correlated significantly with total and free T_4 in plasma. Participants with high degree of burnout had significantly higher hair T_4 levels and lower T_3/T_4 ratio compared to those with no or moderate degree of burnout. A trend towards higher hair T_3 levels was observed in subjects with high burnout score. Hair T_4 levels showed a significant positive relationship with MBI-GS score, whereas no significant correlation emerged for hair T_3 levels. The negative correlation between T_3/T_4 ratio and MBI-GS score was also significant. We have developed an online SPE-LC–MS/MS method for measurement of THs in human hair, allowing high analytical specificity and sensitivity. The novel finding of hair THs levels from individuals suffering from chronic stress in burnout underscores the relevance of this method for medical and psychological research.

1. Introduction

Thyroid hormones (THs) are secreted by the thyroid gland, which mainly produces the prohormone L-3,5,3',5'-tetraiodothyronine (T_4) and a smaller fraction of the biologically active hormone L-3,5,3'-triiodothyronine (T_3). THs play a key role in the human endocrine system and participate in the control of overall body metabolism, protein synthesis, carbohydrate and fat metabolism, neural development, normal growth and maturation of bones, as well as cardiovascular and renal functions (Horn and Heuer, 2010; Mondal et al., 2016). The combined action of the hypothalamus, pituitary gland, and thyroid gland is known as the hypothalamus-pituitary-thyroid (HPT) axis that regulates thyroid hormone synthesis and production in the body (Lechan and Fekete, 2004). In humans approximately 0.3% of T_3 and only 0.02% of T_4 in the periphery are available in the free, i.e., biologically active form (Russell et al., 2008).

The association between thyroid function and psychiatric disorders, particularly mood disorders, has long been recognized (Hage and Azar, 2012). Several lines of evidence suggest that there may be abnormalities in thyroid hormone metabolism in patients with mood disorders (Bauer et al., 2008). Hypothyroidism and hyperthyroidism are accompanied by mood disorders like depression and anxiety. For instance, 60% of hyperthyroid patients suffer from anxiety disorders, while 31–69% have depression disorders (Kathol and Delahunt, 1986; Trzepacz et al., 1988). Although the majority of patients with depression do not show thyroid dysfunction with overt biochemical evidence (Vandoolaeghe et al., 1997), abnormalities of HPT axis in patients with depression has been frequently reported, including changes of T_3 or T_4 level in serum, blunted thyroid-stimulating hormone (TSH) response to thyrotropin-releasing hormone (TRH), abnormal diurnal TSH rhythm, and positive anti-thyroid antibodies (Berent et al., 2014; Brouwer et al., 2005; Hage and Azar, 2012). However, a possible causal role of thyroid hormones in mood disorders is still unclear. Moreover, there has long been an interest in using thyroid hormones to treat mood disorders due to the relationship between thyroid disease states and psychiatric symptoms (Bauer et al., 2008). Although thyroid hormone monotherapy is not an adequate treatment for patients with primary mood disorders, a series of open and controlled clinical trials have confirmed

* Corresponding author at: Department of Biological Psychology, Technische Universität Dresden, 01069, Dresden, Germany.
E-mail address: wei.gao@tu-dresden.de (W. Gao).

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the therapeutic value of adjunctive treatment with thyroid hormones in mood disorders (Altshuler et al., 2001; Bauer et al., 2008).

Many studies have measured THs in the peripheral blood of patients with depression with rather inconsistent results (Brouwer et al., 2005; Kirkegaard et al., 1990; Premachandra et al., 2006). Compared with healthy subjects or psychiatric control groups, serum T_4 levels in the upper range of normal or slightly elevated concentrations have been reported in depressed patients, which have been found to regress after successful treatment of depression (Chopra et al., 1990). Low levels of T_3 have been reported in patients with depression (Premachandra et al., 2006), as well as normal daily production of T_3 in unmedicated and moderately depressed patients (Kirkegaard et al., 1990). Indeed, heterogeneous findings exist as well, showing increased T_3 and decreased T_4 serum levels in depressed individuals (Brouwer et al., 2005; Hage and Azar, 2012). Multiple factors may contribute to these inconsistent results, including the heterogeneity of depression, antidepressant medication, and even in/outpatients status (Brouwer et al., 2005; Wei et al., 2014). Another important factor is the well-characterized circadian rhythm of serum THs concentration, which may mask an association between THs and depression if samples are collected within an unsuited time window (Russell et al., 2008; Wei et al., 2014).

Hair analyses of endogenous substances are increasingly used in biomedical and psychological research for investigations of biological mechanisms of chronic stress-associated poor mental health (Gao et al., 2016; Russell et al., 2012; Stalder and Kirschbaum, 2012). A prime advantage of hair sample collection is that it offers an easy, noninvasive and stress-free sampling. According to the free hormone hypothesis, only the unbound, free hormone fraction should be incorporated into hair (Russell et al., 2012; Stalder and Kirschbaum, 2012). Regarding the incorporation of a drug or hormone including THs into hair, the most commonly suggested hypothesis is based upon the complex multicompartment model that has been used to explain drug incorporation in hair (Boumba et al., 2006). Four general models have been proposed: active or passive diffusion from blood into growing cells in hair follicle, diffusion from body secretion (sweat, sebum) during formation of hair shaft, incorporation from deep skin compartments during hair shaft formation, and external environmental sources after hair shaft formation (Pragst and Balikova, 2006). By capitalizing on the continuous incorporation of lipophilic substances into the slowly growing hair matrix, hair analyses are assumed to provide a retrospective index of endogenous substances integrated over extended periods of several months prior to hair sampling (Kirschbaum et al., 2009; Stalder et al., 2012). A corresponding time period would have been virtually impossible to capture adequately using traditional methods (Stalder et al., 2017). Furthermore, due to their integrative and retrospective nature, hair analysis is not prone to situational influences or issues of noncompliance (Russell et al., 2012; Stalder and Kirschbaum, 2012). Therefore, previous blood methods only reflect short-term THs levels, which is given pulsatile secretion, while hair THs concentrations are assumed to reflect integrated THs secretion over periods of several months, which can mitigate the issue of intra- and inter-day THs fluctuations (Russell et al., 2012). Besides multiple steroid hormones, the presence of THs in hair has recently been reported (Wei et al., 2014). Human hair follicles are direct targets of THs, TRH and TSH, and studies have demonstrated that T_3 and/or T_4 modulate multiple biological hair parameters (Bodó et al., 2009; Van Beek et al., 2008; Vidali et al., 2014). Moreover, THs in hair may also be associated with mental disorder, including depression, as observed in a group of Chinese female patients (Wei et al., 2014; Yang et al., 2016).

Immunoassay (IA) methods are routinely used for THs determinations in serum, plasma, and even hair (Kirkegaard et al., 1990; Premachandra et al., 2006; Wei et al., 2014). Although IAs are sensitive, the specificity of the antibodies used in these assays limits selectivity (Kunisue et al., 2011). The College of American Pathologists reported that assay results of T_3 and T_4 varied widely, depending on the chosen antibodies (Soldin et al., 2004). Compared with IA, mass spectrometry (MS) is found to be highly sensitive, and superior in both, precision and accuracy (Sakai et al., 2015). Recently, gas chromatography-mass spectrometry (GC–MS) and liquid chromatography-mass spectrometry (LC–MS/MS) have been developed for the determination of THs in serum, plasma, brain, thyroid gland (Hantson et al., 2004; Kunisue et al., 2011; Yue et al., 2008), placenta (Leonetti et al., 2016), sea lamprey plasma, gill, kidney and liver (Bussy et al., 2017), and white whales blood (Hansen et al., 2017). liquid chromatography-quadrupole time-of-flight mass spectrometer LC-Q-TOF-MS has also been developed in mouse tissues and placenta (De Angelis et al., 2016; Li et al., 2018). Compared with LC–MS/MS, the sample preparation for GC–MS requires more processing steps, like derivatization and cleanup (Hantson et al., 2004). Hence, LC–MS/MS may be far better suited for high throughput determination of THs in biological samples (Sakai et al., 2015; Soldin and Soldin, 2011). Nevertheless, to our knowledge, no LC–MS/MS method has been reported to analyze THs in hair. Recently, the utility of online solid phase extraction (SPE), an extraction technique for hair steroids analysis, has been improved (Gao et al., 2016, 2013). The online SPE is a promising tool to improve method sensitivity and shorten pretreatment times as well as increase throughput (Gao et al., 2015, 2013).

We therefore developed an online SPE-LC–MS/MS method to determine THs in human hair samples. The utility of this method was investigated by measuring hair extracts from individuals suffering from burnout.

2. Materials and methods

2.1. Reagents and materials

T_3 and T_4 were purchased from Sigma–Aldrich (Hamburg, Germany). C13-labeled internal standard ($^{13}C_6$-T_4) was obtained from Toronto Research Chemicals (Canada). LC–MS grade methanol and water were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). Ammonium acetate, acetic acid and ethyl acetate of LC–MS grade were obtained from Sigma–Aldrich (Hamburg, Germany).

2.2. Standard solutions and quality control samples

T_3, T_4 and $^{13}C_6$-T_4 were prepared in methanol with 40% NH_4OH (v/v) at final concentrations of 1 mg/mL as respective stock solutions. Calibration solutions were prepared by diluting each stock solution with methanol to various concentrations ranging from 0 to 5 ng/mL, while T_4-C_6 was used as internal standard at level of 2 ng/mL. All stock solutions and working standard solutions were stored at −10 °C until required and were stable for a minimum of 3 months.

For validation of method, blank hair samples were required. For this, distal hair segments of long hair samples (more than 40 cm) from four normal individuals with no thyroid disease (according to self-report) were used. THs were undetectable in the distal hair segments of these long hair samples (i.e., below the limit of detection, LOD), while THs concentrations in the proximal segments were above the limit of quantification (LOQ). The possible reason of undetectable THs concentrations in distal hair segments was likely due to a leaching effect, where more distal hair segments had experienced greater environmental damage, compromising hair integrity (Russell et al., 2012). In line with this, chromatograms of blank hair samples did not show any relevant peaks which confirmed their suitability as blank samples (data was not shown here). The four hair samples were mixed as blank hair samples.

2.3. Hair samples collection and preparation

Hair strands were cut with scissors close to the scalp from posterior vertex position. A minimum of 10 mg hair from the 3 cm segment most proximal to the scalp was obtained from each participant in case of

material loss during preparation. THs concentrations were determined from the 3 cm hair segment. Based on an average hair growth rate of 1 cm/month (Wennig, 2000), that represents hair grown over the three months period prior to hair sampling.

Firstly, collected hair samples were washed by shaking them twice in 2.5 mL isopropanol for 3 min each at room temperature. Then they were moved into a fume hood to dry for at least 12 h. 7.5 mg of whole hair was carefully weighed and cut into small pieces. After this, 20 μL internal standard and 1.8 mL methanol was added, and the hair was incubated for 18 h at room temperature for THs extraction. 1.6 mL of clear supernatant was transferred into a new tube, and was evaporated at 50 ℃ under a constant stream of nitrogen until the samples were completely dried (duration: approximately 20 min). Finally, the dry residue was resuspended using 120 μL methanol/water (50:50, v/v), 100 μL of which was used for LC–MS/MS analysis.

2.4. Instrumental condition

The LC–MS/MS system consisted of Shimadzu Nexera XR ultra-high performance liquid chromatograph (Shimadzu, USA) and AB Sciex QTRAP 6500 mass spectrometer equipped with Electrospray Ionization (ESI) source (AB Sciex, USA). A 6-port switch valve in column temperature oven controlled by the HPLC system was used for online SPE which has been described in our previous method (Gao et al., 2015, 2013). The LC–MS/MS system was controlled by AB Sciex Analyst® software (version 1.6.2). Chromolith®Speed ROD RP-18e HPLC column (4.6-mm × 50-mm) from Merck KGaA (Darmstadt, Germany) was used as the online SPE column. The analytical column was a Shim-pack XR-ODS LC column (3.0-mm × 75-mm, 2.2-μm) from Shimadzu (Shimadzu, USA). Security guard columns (C18, 4-mm × 2.0-mm) were purchased from Phenomenex (Aschaffenburg, Germany).

Mobile phase A was water containing 2.0 mM ammonium acetate with 0.1% acetic acid, and Mobile phase B was methanol with 0.1% acetic acid. The total flow rate of the first binary gradient module was maintained at 0.4 mL/min. Mobile phase C-A was methanol/water (v/v = 10/90) while Mobile phase C-B was methanol. The flow rate of the second isocratic module was maintained at 3 mL/min. The column temperature was set at 40 ˚C. The injection volume was 100 μL.

The mass spectrometer was operated in positive ionization mode, while scheduled multiple reactions monitoring (MRM) mode was utilized for the detection of THs. The MRM transitions of the precursor ion to the product ion (Q1→Q3) with rich structure features were chosen for quantification. Two transitions were monitored for THs, while one transition was monitored for IS: *m/z* 651.7–605.6 and 507.9 for T_3; *m/z* 777.5–731.4 and 633.5 for T_4; and *m/z* 783.5 –737.4 for $^{13}C_6$-T_4. Since ^{13}C-labeled T_3 were not commercially available at the initiation of this study, and considering the similar chemical structures of T_3 and T_4, we quantified the two thyroid hormones by the internal standard $^{13}C_6$-T_4.

2.5. Optimization of extraction method

To evaluate the extraction procedure, hair samples from five participants were washed with the protocol specified above. 5 mL isopropanol from each hair sample washing was collected in a new tube and evaporated to dryness. The dry residue was resuspended using 120 μL methanol/water (50:50, v/v), 100 μL of which was used for LC–MS/MS analysis. The five hair samples were divided into six aliquots of 7.5 mg each and incubated with the protocol specified above for six different time periods: 2, 5, 10, 18, 24 and 48 h. The subsequent analytical procedures were the same as described above.

Here we also conducted a comparison between whole hair and milled hair. For this comparison, five different hair samples were used which were divided into two aliquots each. One was incubated as whole hair, and the other was powdered using a Retsch ball mill (5 min at 30 Hz) prior to extraction with methanol. No other changes were made to the protocol. To evaluate the quality of these different protocols, the ratio of whole hair THs level and milled hair THs level was used.

2.6. Method validation

This method was validated based on the evaluation protocols of the Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute, 2014).

Calibration standards were prepared by spiking 7.5 mg blank hair with 40 μL standard solutions at final concentrations of 0, 0.036, 0.072, 0.36, 0.72, 3.6, 7.2 and 36 pg/mg of hair samples for T_3 and T_4. Calibration linearity coefficients were obtained by plotting the peak area ratios against the nominal concentration. The limit of quantification (LOQ), which was defined as the lowest injected concentration that had the variability in accuracy and precision (%CV) less than 15% and the corresponding signal/noise ratio of 10, was determined by injecting serial diluted calibrators containing the thyroid hormones.

Method precision can be determined by calculating inter- and intra-day variation. Inter-day variation was evaluated with five replicate measurements of quality control (QC) samples on five separate days, while five replicates of QC samples were measured on one single day for intra-day variation. Precision was obtained by computing the percentage coefficient of variation (%CV). The QC samples were two natural hair sample and five blank hair samples spiked with standard samples at 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 final concentrations were 0.05, 0.1, 1, 10 and 20 pg/mg for T_3 and T_4 separately. Further assessment of method accuracy was obtained by measuring the recovery (R) from the QC samples of five different concentrations. The method recovery value was calculated as: $R\ (\%) = A/B \times 100$, where A is the value measured with the routine method and B is the true value of spiked standard sample.

To evaluate the matrix effect (ME), five blank hair samples were extracted with the routine method and spiked with pure standard samples of five different concentrations (identical to the VLOC, LQC, MQC, HQC and VHQC values specified above). The matrix effect value for each sample was calculated as: $ME\ (\%) = ((C/D) - 1) \times 100$, where C is the analyte peak area of hair spiked with the standard sample after extraction and D is the analyte peak area of the pure standard sample (i.e., in the absence of hair matrix). An ME value < 0 indicates ionization suppression and a value > 0 indicates ionization enhancement. Carryover was evaluated by running a blank sample after the highest calibrator.

2.7. Demonstration of utility – Burnout samples

Participants were a subsample of the Dresden Burnout study (DBS), a large-scale cohort study with annual study waves for biomarker sampling that was approved by the local ethics committee and conducted in accordance with the Declaration of Helsinki. The DBS is a longitudinal study with duration of 12 years, with annual sampling waves. To overcome shortages of previous studies that focused predominately on very specific subsamples of individuals, inclusion criteria for DBS participation is solely based on age (18–68 years) and language skills (sufficient to fill out German questionnaires). Exclusion criteria of this experiment were the diagnosis of thyroid disease, use of medication that affected the HPT axis' function in the past three months. Therefore, participants with different professional and socio-economic status and working area were recruited, independent of their individual work and stress history. Finally, hair samples and psychological data were collected from 207 participants (mean ± SD age: 41.5 ± 11.5 years; 18.4% male). Burnout severity was measured with the Maslach Burnout Inventory-General Survey (MBI-GS) (Schaufeli et al., 1996), which is considered the gold standard for assessing burnout. The MBI-GS cutoff score for high burnout degree was set as 3.5. Blood samples were also taken between 9–10 am at an

experimental lab for comparing THs levels in these spot samples with the incorporation into hair over prolonged periods. Plasma total THs were extracted with ethyl acetate and plasma free THs were prepared by using ultrafiltration with Microcon® YM-30 centrifugal filter devices (Merck Millipore, Tullagreen, IRL), then they were measured using the same method as for hair THs. According to the THs concentrations in plasma, the validation of the method for plasma THs was done with the same procedures of hair THs method.

2.8. Statistical analyses

Analyses were performed using SPSS version 23 (IBM® SPSS® Statistics). The critical p value for statistical significance was set to 0.05. THs levels in the text are reported in original units (pg/mg). THs levels of high burnout group (MBI-GS total score ≥ 3.5) and no/moderate burnout group (MBI-GS total score < 3.5) were compared using Mann-Whitney U test due to non-normally distributed values. Correlations between THs data and MBI-GS score data were calculated using Spearman's correlation ρ for non-normal distribution.

3. Results and discussion

3.1. LC–MS/MS method development

Mass spectra obtained by quantitative optimization showed a protonated molecular ion $[M+H]^+$ for T_3 (m/z 651.7) and T_4 (m/z 777.5). Fragmentation of these precursor ions yielded some product ions, the strongest ones were chosen for quantification. The first and second most abundant fragment ions in the MS/MS spectrum of the THs, $[M+H-HCOOH]^+$ (m/z 605.6 for T_3, m/z 731.4 for T_4) and $[M+H-IOH]^+$ (m/z 507.9 for T_3, m/z 633.5 for T_4), were selected for MRM transitions. Product ions $[M+H-HCOOH]^+$ were used for quantitative analysis of these substances in the MRM mode. Ratio of peak area between these two MRM transitions are shown in Table 1, which was calculated based on all the samples in the validation experiment for each thyroid hormones. The ratios were stable for each thyroid hormone. Hence, the assay was considered stable in terms of the chosen MRM transitions.

The HPLC conditions were optimized to achieve good baseline separation of the substrate and products and to provide good peak shape and resolution. By this also the chance that the substrate would interfere with the chromatogram of the product was reduced. To keep the total analysis time short, the column temperature was set to 40 °C. The mobile phase with 0.1% acetic acid was found to be best-suited for the formation of precursor ions in THs analysis and for avoiding potential peak tailing during separation. As seen from the chromatograms of a spiked and a natural hair sample in Fig. 1(A) and (B), low background noise from the hair matrix showed good selectivity and sensitivity for T_3, T_4 and the internal standard (IS). The retention times were 4.64 min for T_3 and 5.30 min for T_4. The column switching strategy for online SPE achieved an analysis time of 9 min/sample by allowing the simultaneous performance of sample cleaning and HPLC analysis. The current online SPE method was able to shorten sample preparation times and to increase analysis throughput. The LC–MS/MS with ESI positive mode and MRM mode provided a highly selective and sensitive method for the determination of T_3 and T_4 in hair.

Moreover, compared with a previous hair THs study, which used around 50 mg powdered hair (Tagliaro et al., 1998; Wei et al., 2014), the present protocol requires only 7.5 mg non-pulverized hair. This preparation protocol reduced overall processing time and hair sample weight needed for analysis.

3.2. Extraction method optimization

The T_3 and T_4 levels of the five washing solutions of isopropanol were all below LOD, which showed no detectable T_3 or T_4 was extracted in the step of hair cleaning with isopropanol. The result of the duration dependence of hair T_3 and T_4 during the incubation in methanol showed in Fig. 2. The increasing T_3 and T_4 content was observed with the increasing incubation time. Moreover, there was a sharp increase of T_3 and T_4 levels in the first four incubation time durations (2 h, 5 h, 10 h and 18 h) with asymptotic stable levels in the following two incubation time durations (24 h and 48 h). Therefore, the incubation time of 18 h was chosen in this study.

Table 1
Inter and intra-day variability, matrix effect and method recovery for thyroid hormones measurement in hair and plasma.

Matrix	Compound	Nominal	Intra-assay CV (%)	Inter-assay CV (%)	ME ± SD (%)	R ± SD (%)	RPA ± SD
Hair (pg/mg)	T_3	0.05	9.5	8.6	31.9 ± 5.3	98.2 ± 4.7	5.2 ± 0.3
		0.1	9.7	7.1	14.8 ± 6.4	95.5 ± 5.6	
		1	8.6	6.7	12.7 ± 3.8	102.1 ± 2.8	
		10	7.3	7.2	5.8 ± 6.2	92.7 ± 2.9	
		20	3.1	5.9	3.9 ± 2.4	89.7 ± 1.9	
		NH1 (0.31)	4.6	5.1			
		NH2 (0.09)	10.7	9.2			
	T_4	0.05	10.2	8.7	26.2 ± 7.5	94.6 ± 8.1	6.8 ± 0.6
		0.1	9.7	8.4	23.9 ± 5.9	93.7 ± 2.8	
		1	8.4	3.4	12.5 ± 6.3	88.5 ± 3.2	
		10	8.8	7.5	8.4 ± 3.6	92.2 ± 6.0	
		20	3.9	2.8	6.2 ± 4.4	91.5 ± 5.1	
		NH1 (0.12)	9.4	8.6			
		NH2 (0.84)	5.8	6.9			
Plasma (ng/mL)	T_3	0.1	10.7	10.3	−64.5 ± 6.1	91.4 ± 3.3	5.6 ± 0.1
		1	7.9	6.8	−51.8 ± 1.9	97.2 ± 4.8	
		10	4.2	8.2	−46.5 ± 5.1	96.6 ± 2.9	
		NP1 (0.66)	7.4	7.8			
		NP2 (1.23)	3.6	8.7			
	T_4	10	4.5	4.8	−53.7 ± 3.2	104.6 ± 5.2	7.1 ± 0.2
		60	5.2	7.9	−42.4 ± 1.6	94.2 ± 4.7	
		200	4.7	3.1	−50.8 ± 3.5	105.6 ± 3.3	
		NP1 (47.7)	5.2	4.1			
		NP2 (153.0)	4.4	4.6			

CV: Percentage coefficient of variation; ME: Matrix effect; R: Method recovery; SD: Standard deviation; RPA: Ratio of peak area between MRM of quantifier and MRM of qualifier; NH: Natural hair (Mean); NP: Natural plasma (Mean).

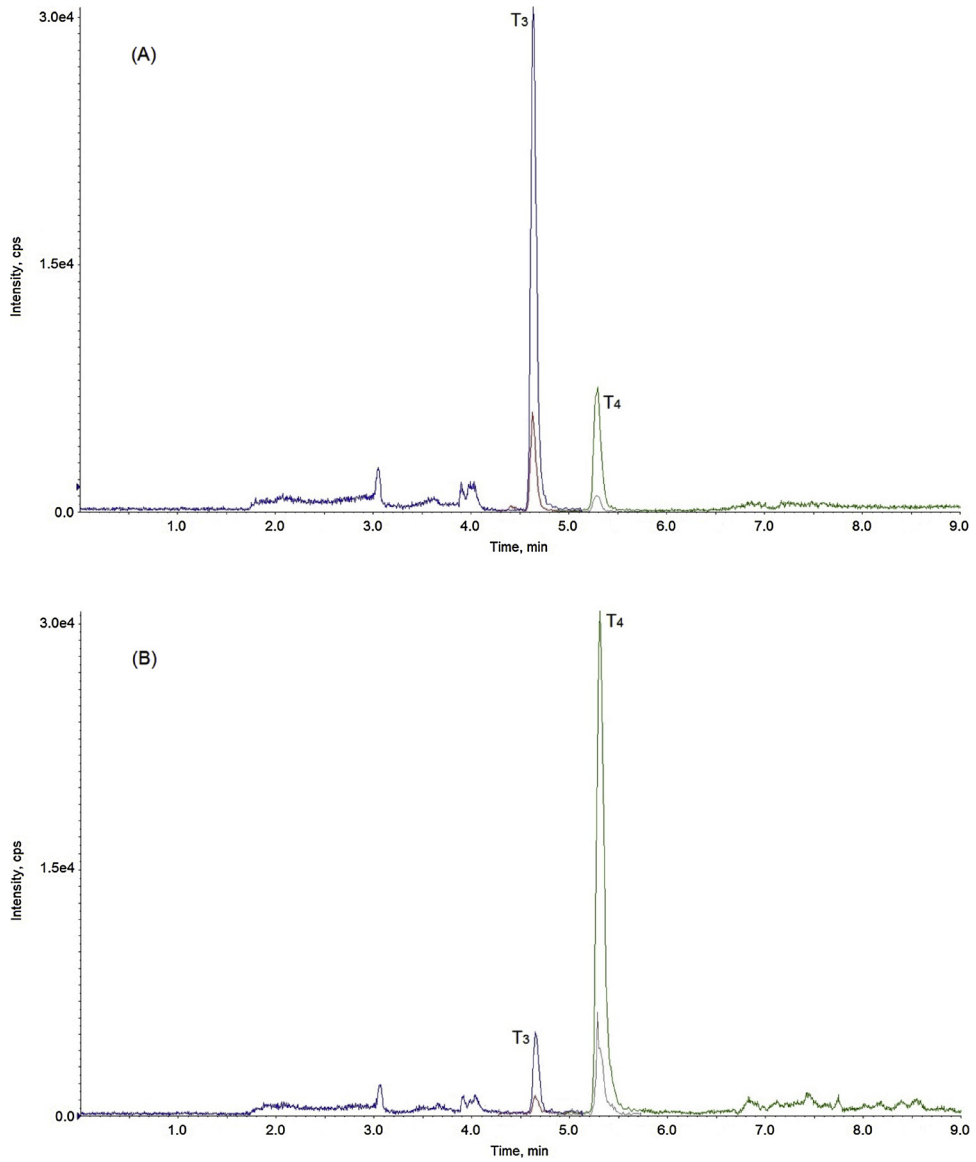

Fig. 1. An example of LC–MS/MS chromatogram of (A) a spiked blank hair sample (standard solutions added at final concentrations of 0.72 pg/mg for T_3 and T_4), (B) a natural hair sample (0.14 pg/mg for T_3 and 4.42 pg/mg for T_4). Colours of chromatogram: Blue represents MRM 651.7→605.6 for T_3; Red represents MRM 651.7→507.9 for T_3; Green represents MRM 777.5→731.4 for T_4; Gray represents MRM 777.5→633.5 for T_4 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The ratio of whole hair THs level and milled hair THs level ranged from 83.8%–103.8 % for T_3 (mean ± SD: 91.0 ± 7.5%), and ranged from 78.5%–106.9% for T_4 (mean ± SD: 93.9 ± 10.4%). The result indicated that THs were extracted somewhat more efficiently from milled hair than from whole hair. However, given that the pulverization of hair using a ball mill involves a considerable amount of manual sample handling which is thus likely to reduce the throughput of this method, the extraction of whole hair samples appears to be a valuable alternative strategy for the analysis of THs, specifically when a larger number of samples need to be processed.

3.3. Method validation

Calibration curves were constructed with linear regression ranging from 0 to 36 pg/mg for T_3 and T_4. The correlation coefficients were greater than 0.999 for each analyte, indicating adequate linearity of the analytical procedure. In addition, the results showed that LOQ value was 0.02 pg/mg for T_3 and 0.03 pg/mg for T_4. These LOQ values were considered highly suitable for the quantification of THs in hair.

The results of method precision and accuracy are shown in Table 1. The inter- and intra-day coefficients of variation (CVs) were below 11% for the natural hair samples and QC samples with hair matrix. Recovery ranged between 88.5% and 102.1%, and the CV of triplicate analyses was between 2.1% and 8.6% for T_3 and T_4, respectively. As these results were all within the acceptable range (CV < 15%) (Caruso et al., 2008), the assay was considered suitable in terms of precision and accuracy.

Results of matrix effect performed with the current protocol are provided in Table 1. Coeluting matrix components can interfere with the analytes in the MS interface, which may result in suppression or enhancement of the MS signal (Van Eeckhaut et al., 2009). For THs in hair, T_3 and T_4 showed a positive matrix effect behavior ranging from 3.9%–31.9%. The result suggests that the responses of T_3 and T_4 in hair matrix were higher than those in the mobile phase and that, hence, an enhancement effect of ionization was observed for these analytes in hair samples. The possible reason of higher matrix effect at low THs concentration in hair is that the degree of the matrix effect will depend on the concentration of the analyte being infused (Gosetti et al., 2010). If the concentration of analyte being infused is too high, matrix effects

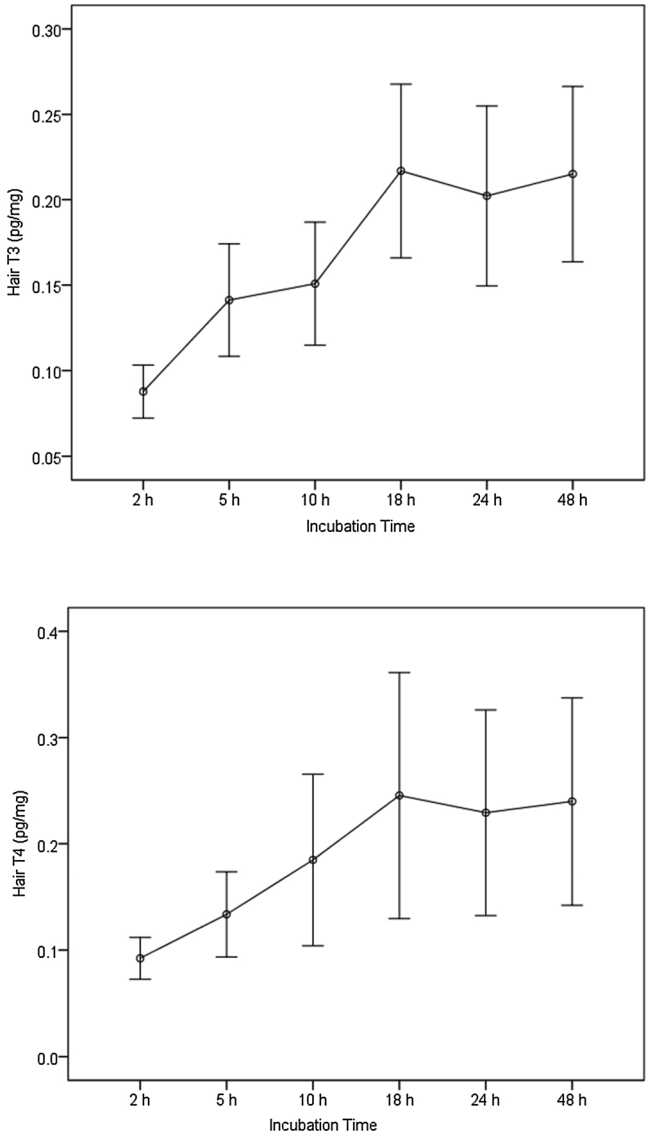

Fig. 2. Mean (± SE) hair T_3 and T_4 levels (pg/mg) after incubation for 2 h, 5 h, 10 h, 18 h, 24 h and 48 h (n = 5).

might be masked (Hall et al., 2012). The matrix effect originates from the competition between the analyte and co-eluting interfering species and causes a suppression or enhancement in ionization efficiency relative to the analyte eluting in the absence of the matrix component. But the exact mechanisms by which matrix components cause ionization suppression (or enhancement) is not yet fully understood (Gosetti et al., 2010). The possible reason is that the ionization reaction extent depends on the ionization energy and on the proton affinity of all the molecules present in the interface, so that the efficiency of formation of the desired ions also depends on the possible competition with the interfering species (Gosetti et al., 2010). However, by using internal standards the matrix effect could be corrected to 88.5–102.1% according to recovery rates. Matrix-matched calibration plots in blank hair samples with the internal standard were also built in order to compensate for the matrix effect. After the highest calibrator running, there was no T_3 or T_4 that could be detected in the following blank sample. Hence, there was no carryover for T_3 or T_4 within this analytical protocol.

The data on validation of the method for plasma THs measurement are given in Table 1. According to the results, this method was also considered suitable in measurement of THs in plasma samples.

3.4. Demonstration of utility – Burnout samples

Descriptive characteristics and hair THs concentrations for burnout participants are provided in Table 2. The ranges of hair THs concentrations were 0.034 − 0.355 pg/mg for T_3 and 0.030 − 0.712 pg/mg for T_4. And the ranges of plasma total THs concentrations were 0.587–2.350 ng/mL for T_3 and 38.9–159.0 ng/mL for T_4, while the ranges of plasma free THs concentrations were 0.368–2.416 pg/mL for T_3 and 8.3–50.0 pg/mL for T_4. It was observed that the concentrations of T_3 and T_4 in hair (pg/mg) were of similar magnitude to the concentrations of plasma free THs (pg/mL) instead of plasma total THs (ng/mL). The hair THs result is also in line with the data published from brain (Hantson et al., 2004; Kunisue et al., 2011; Yue et al., 2008). Based on the multiple mechanisms of THs synthesis, transport, regulation and the free hormone hypothesis, the most probable reason of this observation is that only unbound, free THs fraction in blood, rather than total THs, should be delivered into peripheral tissues such as brain and hair (Russell et al., 2012; Schroeder and Privalsky, 2014). T_3 and T_4 levels were significantly inter-correlated (Spearman's $\rho = 0.328$, $p < 0.001$) with no significant correlation between hair T_3 or T_4 levels or T_3/T_4 ratio and age or body mass index (Table 3). In addition, similar T_3 or T_4 levels or T_3/T_4 ratio were observed in hair samples from male and female study participants. Moreover, there was a significant correlation between hair T_4 levels and plasma total T_4 levels (Spearman's

Table 2
Demographic, clinical characteristics and hair thyroid hormones levels (pg/mg) of burnout population.

		Whole study cohort (N = 207)	BL group (N = 171)	BH group (N = 36)	Statistics
Sociodemographics					
Age (Mean ± SD)		41.5 ± 11.5	41.2 ± 11.5	43.3 ± 11.5	
Sex (Male/Female)		38/169	33/138	5/31	
BMI (Mean ± SD)		25.1 ± 4.6	24.9 ± 4.4	26.2 ± 5.7	
Clinical Characteristics					
MBI-GS score (Mean ± SD)		2.32 ± 1.14	1.97 ± 0.92	3.97 ± 0.39	
Hair Thyroid Hormones					
T_3	Range	0.034 − 0.355	0.034 − 0.355	0.045 − 0.303	$Z = -1.924$, $p = 0.054$
	Mean ± SD	0.142 ± 0.057	0.138 ± 0.057	0.156 ± 0.058	
T_4	Range	0.030 − 0.712	0.030 − 0.712	0.048 − 0.653	$Z = -3.094$, $p = 0.002$
	Mean ± SD	0.133 ± 0.120	0.121 ± 0.111	0.185 ± 0.148	
T_3/T_4	Range	0.15 − 8.13	0.15 − 8.13	0.15-3.86	$Z = -2.002$, $p = 0.045$
	Mean ± SD	1.58 ± 1.07	1.65 ± 1.11	1.28 ± 0.82	

BL: group of participants with low burnout score. BH: group of participants with high burnout score. BMI: body mass index. MBI-GS: Maslach Burnout Inventory-General Survey. MBI-GS cutoff score is 3.5.

Table 3
Spearman's correlation analysis between hair/plasma thyroid hormones levels and demographic/clinical characteristics in burnout population.

			Age	BMI	MBI-GS score
Hair THs	T_3	ρ	−0.030	0.028	0.123
		p	0.673	0.688	0.078
	T_4	ρ	0.022	0.066	0.234**
		p	0.755	0.351	0.001
	T_3/T_4	ρ	−0.047	−0.080	−0.153*
		p	0.500	0.260	0.028
Plasma Total THs	T_3	ρ	−0.192**	0.080	0.070
		p	0.006	0.257	0.319
	T_4	ρ	−0.178*	0.018	0.161*
		p	0.010	0.803	0.021
	T_3/T_4	ρ	0.019	0.117	−0.062
		p	0.782	0.098	0.372
Plasma Free THs	T_3	ρ	−0.179**	−0.044	0.001
		p	0.010	0.538	0.984
	T_4	ρ	0.087	0.059	0.217**
		p	0.212	0.402	0.002
	T_3/T_4	ρ	−0.199**	−0.094	−0.133
		p	0.004	0.184	0.057

ρ: Correlation coefficient; *p*: Significance (2-tailed).
* Correlation is significant at the 0.05 level.
** Correlation is significant at the 0.01 level.

ρ = 0.149, p = 0.032) and a weak significant correlation between hair T_4 levels and plasma free T_4 levels (Spearman's ρ = 0.134, p = 0.054), but no significant correlation between hair T_3 levels and plasma total T_3 levels (Spearman's ρ = −0.080, p = 0.250) or plasma free T_3 levels (Spearman's ρ = 0.081, p = 0.246) was observed (Table 4). The most probable reason is that the circadian rhythm of blood free T_3 is much stronger than blood free T_4 (Russell et al., 2008). Therefore, the plasma samples are subject to more physiological daily fluctuations of plasma T_3 concentrations, making the assessment of overall long-term systemic T_3 more difficult.

As shown in Table 2, group-level analyses revealed that participants with high burnout degree exhibited higher T_4 levels in hair (mean ± SD: 0.185 ± 0.148 pg/mg) compared to those with no or moderate burnout degree (mean ± SD: 0.121 ± 0.111 pg/mg; Z = −3.094, p = 0.002). Hair T_3 levels further tended to be higher in individuals with high burnout degree (p = 0.054). These results are inconsistent with the previous report on lower hair T_3 and T_4 levels detected among patients with depression in disease episode (Wei et al., 2014). However, they are consistent with elevated hair T_4 level and no significant difference of hair T_3 level in patients with depression in pre-disease episode in the same report (Wei et al., 2014). Hence, the possible reason is that the abnormal HPT axis function in burnout populations is more similar to it in depression patients in pre-disease episode instead of in the disease episode. Further studies are needed to provide more insights into the relationship between hair THs, HPT axis function and burnout.

We also found that high burnout degree group had significantly lower T_3/T_4 ratio (mean ± SD: 1.28 ± 0.82) as compared with the no or moderate burnout degree group (mean ± SD: 1.65 ± 1.11; Z = −2.002, p = 0.045). Analyses based on continuous burnout dimensions in Table 3 revealed that hair T_4 levels in particular showed a positive relationship with MBI-GS score (Spearman's ρ = 0.234, p = 0.001), whereas no significant associations emerged between hair T_3 levels and the MBI-GS score (p = 0.078). The negative correlation between T_3/T_4 ratio and MBI-GS score was also significant (Spearman's ρ = −0.153, p = 0.028). Consistent with hair THs result, there was also a positive relationship between plasma total/free T_4 levels and MBI-GS score, but no significant association was observed between plasma total/free T_3 levels and the MBI-GS score (Table 3).

Although the hair THs result is in line with the plasma THs result in this study, the correlation between the two specimens was weak. It is most probably because of the difference in the time frames that are reflected by the measurements: plasma THs levels reflect acute levels, which could be affected by circadian rhythm and acute stress, whereas hair THs levels represent an integral measure of THs output over the past 3 months.

The vast majority of studies investigating associations between psychiatric disorders and thyroid functioning have relied on peripheral blood THs levels with rather inconsistent results (Brouwer et al., 2005; Hage and Azar, 2012; Kirkegaard et al., 1990; Premachandra et al., 2006). One possible reason is may be the fact that THs in blood or brain samples reflect transient or short-term levels, which differ from hair THs level. In contrast to such spot measures, hair THs levels reflect an integral of THs secreted over weeks and months prior testing, which has already been reported to be associated with depression in a group of Chinese female patients (Wei et al., 2014; Yang et al., 2016). While THs, TSH, and TRH found in hair follicles may alter hair follicles functions directly (Bodó et al., 2009; Gáspár et al., 2010; Van Beek et al., 2008), the quantification of hair THs might be a most helpful and easy-to-assess long-term biomarker of HPT axis function. However, the effects of natural hair color, cosmetic treatments, hair washing and environment on hair THs concentrations are still outstanding questions and gaps in our knowledge that need to be addressed urgently in future studies.

4. Conclusions

In summary, we have developed a novel online SPE-LC–MS/MS method for the measurement of thyroid hormones in human hair. Due to the low concentrations of T_3 and T_4 in hair, a highly sensitive method is required. Our results indicate that the presented LC–MS/MS protocol is able to achieve these objectives, providing a highly sensitive, selective and reliable method for the quantification of T_3 and T_4 in human hair. Moreover, considering that THs analyses in hair will be increasingly used in psychoendocrinological research where often a larger number of samples have to be processed, a particular concern in this

Table 4
Spearman's correlation analysis between hair thyroid hormones levels and plasma thyroid hormones levels.

			Plasma Total THs			Plasma Free THs		
			T_3	T_4	T_3/T_4	T_3	T_4	T_3/T_4
Hair THs	T_3	ρ	−0.080	0.011	−0.055	0.081	−0.094	0.156*
		p	0.250	0.878	0.433	0.246	0.179	0.024
	T_4	ρ	0.111	0.149*	0.009	0.056	0.134	−0.044
		p	0.111	0.032	0.898	0.425	0.054	0.533
	T_3/T_4	ρ	−0.186**	−0.155*	−0.075	−0.001	−0.214**	0.161*
		p	0.007	0.026	0.283	0.991	0.002	0.021

ρ: Correlation coefficient; *p*: Significance (2-tailed).
* Correlation is significant at the 0.05 level.
** Correlation is significant at the 0.01 level.

method development was to increase throughput. Here, the coupling of the LC–MS/MS with an online SPE led to a considerable shortening of pretreatment and analysis times. This approach allowed sample throughput times of 9 min/sample as well as simultaneous sample cleaning and analysis.

In a proof-of-principle approach, we have applied the novel protocol to the measurement of THs in hair samples from individuals with varying degrees of burnout. We could show that a significant increase in T_4 deposition in hair and a significant decrease T_3/T_4 ratio in hair were present with increasing severity of burnout. A trend was found for also elevated incorporation of T_3. This demonstration of usefulness of hair THs analysis may spark the interest of basic researchers and clinicians alike to unravel the contributions of THs to human diseases.

In future research, ^{13}C-labeled T_3 internal standard will be incorporated for T_3 as they become commercially available, while the inactive forms of metabolites of T_3 or T_4, rT_3 and T_2, will be included in this method in order to completely understand THs homeostasis. And it is important to obtain more specific information on the effects of natural hair color, cosmetic treatments, hair washing and environment on hair THs concentrations. Finally, further studies are necessary to assess the relationship between hair THs, HPT axis function and mood disorders.

Conflicts of interest

The authors declare no competing financial interests.

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