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A new method for measuring thyroid hormones using nano-LC-MS/MS

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ABSTRACT

This paper describes a novel mass spectrometry based analytical method for analyzing thyroid hormones (THs). Thyroid hormones play a critical role in the regulation of many biological processes such as growth, metabolism and development. Several analytical methods using liquid chromatography-mass spectrometry (LC–MS) and tandem mass spectrometry (LC–MS/MS) have previously been developed to measure THs, especially in humans. For biomedical and toxicological research using small animal models, and in ecophysiological research using wild species where sample volume is limiting, sensitive methods are needed. In this study, we developed a nano-LC-MS/MS method enabling quantification of two key THs, thyroxine (T4) and 3,3′,5-triiodothyronine (T3). The method was tested with egg yolk samples. We used a low flow rate (300 nl/min) to obtain maximal sensitivity of the method. The limit of quantitation was 10.6 amol for T4 and 17.9 amol for T3. The method shows good linearity ($r > 0.99$), repeatability and reproducibility (CVs < 10%). We also re-analyzed yolk samples with radioimmunoassay for a comparison of the newly developed and previously used methods. Finally, we applied the methodology to measure hormones in egg yolk extracts in multiple avian species, and report interesting variation in maternal TH deposition. The newly developed nano-LC-MS/MS method is thus suitable for measuring THs in low concentrations and across species.

1. Introduction

Thyroid hormones (THs) control and regulate vital biological processes such as thermogenesis, growth, and metamorphosis [1]. The two most recognized THs, the prohormone thyroxine (T4) and the biologically more active form 3,3′,5-triiodothyronine (T3) are synthesized and secreted from the thyroid gland with the former produced in greatest amounts [1, 2, 31]. Circulating levels of T4 and T3 are maintained in a constricted range by negative feedback in the hypothalamic-pituitary-thyroid (HPT) axis [1, 2, 30]. THs are enzymatically transformed by deiodinases (DIO1, 2 and 3) in target tissues, importantly, T4 is deiodinated to form T3 by removing one iodine ion.

Traditionally concentrations of THs have been measured by radioimmunoassays (RIA) or other IA-based methods [3–6]. However, in the last two decades, bioanalytical chemistry has entered the era of liquid chromatography tandem mass spectrometry (LC–MS/MS) [7]. LC–MS/MS has numerous advantages compared with RIA: 1) in contrast to IA-based methods, LC-MS does not use antisera, thus cross-reactivity of antisera against unknown compounds in the sample does not interfere with the assay; 2) various THs and their metabolites can be quantitatively measured in one run, thus making the method more cost-efficient; 3) there is no need to use radioisotopes, which pose a health risk; 4) the method enables reaching potentially lower detection limits, reducing sample mass/volumes needed.

Previous studies have reported validation of LC–MS/MS methods for various THs and their metabolites, mostly for plasma, but also in some tissues [7–14]. Importantly, in biomedical and toxicological research using small animal models, and in ecophysiological research in wild species, the acquired sample volumes are likely to be very low and the small sample volume often has to be sufficient for multiple physiological measurements. Therefore methods with very low detection limits are needed. Richards et al. [7] recently summarized studies measuring plasma THs by LC-MS/MS (mostly in humans and rat/mouse) and associated pitfalls. For example, Hansen et al. [8] developed the simultaneous measurement of 11 TH metabolites in frog plasma, and Noyes et al. [9] validated the method in teleost fish. Kiebooms et al. [32] and Tanoue et al. [33] recently validated free T3 and T4 measurements in bovine serum. Kunisue et al. [10], Ackermans et al. [11],

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Saba et al. [12] and recently de Angelis et al. [13] reported methods measuring THs and their metabolites in rat and mice tissues while Bussy et al. [14] recently validated an UPLC method for tissues of sea lamprey. The detection limits in plasma ranged between 29–42 pg/ml for T3 and 24–70 pg/ml for T4 [8, 9] and recently 4.5–6 pg /ml for free T4 and 1.7–2.7 pg/ml for free T3 [32, 33]. In tissues, detection limits of 1 pg/ml [14], 0.33 ng/ml [10] and 0.20–0.39 pg on-column [11] have been reported. Reported quantitation limits in tissues range from 10 pg/ml (0.1 pg on-column) [14], 5–20 pg/ml (1.3–1.4 pg on-column) [11], 0.5–0.75 ng/ml (3.8–5.0 pg on-column) [13] to 1.1 ng/ml [10] for T3 and T4. On-column amounts are not always explicitly stated, even if that would make comparisons easier [7]. Some of the above-mentioned studies have used microflow scale LC-MS/MS methods. However, to our knowledge, the methodology has not been validated for nanoflow LC-MS/MS technique, which is increasingly common, e.g. in proteomics facilities. Nano-LC-MS/MS can provide similar quantitation limits as microflow [14, 15], but uses even smaller injection volumes.

We therefore developed a nano liquid chromatography tandem mass spectrometry method (nano-LC–MS/MS), to identify and quantify THs (T4 and T3) in low (amol) amounts. To our knowledge, this is the first validation of such a nanoflow method. We used a separate internal standard for T3 and T4 to reach higher accuracy, and further calculated recovery (extraction efficiency) for each sample using an additional internal standard. We reanalyzed samples with RIA for a comparison of the newly developed and previously used methods. Finally, we applied the methodology to measure THs in egg yolk across multiple avian species. The reasoning behind using egg yolk samples is the recent interest in the critical role of maternally transmitted THs, via placenta or the egg yolk, on offspring early development [16–19,34]. To our knowledge, no LC-MS method has been validated for yolk in any taxa. We applied our newly-developed method to measure maternal TH levels in five species that are important in physiological and evolutionary ecology research, and for which little or no previous data is available [20–23,34].

2. Material and methods

2.1. Chemicals and standards

Ammonia, formic acid (purity ~98%), and calcium chlorite dehydrate were purchased from Sigma. Organic solvents methanol (HiPerSolv grade) and chloroform (AnalaR Normapur) were from VWR and acetonitrile (LiChrosolv) from Merck. AG 1-X2 Resin was purchased form Bio-Rad. Ultrapure water was obtained from a MilliQ device. Labelled isotopes, $^{13}C_6$-L-Thyroxine (0.1 µg/µl) and $^{13}C_6$-3,3′,5-Triiodo-L-Thyronine (0.1 µg/µl) were purchased from Sigma, and $^{13}C_{12}$-L-Thyroxine (purity 97%) from Larodan. Non-labelled hormones L-Thyroxine and 3,3′,5-Triiodo-L-thyronine were purchased from Sigma.

2.2. Nano-LC–MS/MS conditions and instrumentation

The LC-ESI-MS/MS analyses were performed on a nanoflow HPLC system (Easy-nLC1000, Thermo Fisher Scientific, two parallel Easy-nLC units were used) coupled to a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher Scientific, San Jose, CA) equipped with a nano-electrospray ionization source. Samples were first loaded on a trapping column (100 µm i.d × 2 cm, ReproSil-Pur 5 µm 200 Å C18-AQ) with 12 µl of solvent A (0.1% formic acid) with constant pressure of 250 bar and subsequently separated inline on a 15 cm C18 column (75 µm × 15 cm, ReproSil-Pur 5 µm 200 Å C18-AQ, Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany). The flow rate was 300 nl/min. The mobile phase consisted of water with 0.1% formic acid (solvent A) or acetonitrile/water (80:20 (v/v) with 0.1% formic acid (solvent B). A linear 10 min gradient from 25% to 70% B, followed by 2 min from 70% to 100% B, and wash with 100% B for 8 min, was used to elute hormones. Wash solutions, 0.01% NH_3 and solvent B, were used for washing an autosample needle and a loop to reduce carry-over between samples.

Table 1
Transition list of the monitored compounds. Note that only T4, T3 and $^{13}C_{12}$-T4 were quantified.

Compound	Parent (m/z)	Product (m/z)	Collision (eV)
T4	777.7	731.7	25
13C6-T4	783.8	737.7	25
13C12-T4	789.7	743.7	25
T3	651.8	605.8	22
13C6-T3	657.8	611.8	22

The mass spectrometer was operating in a positive mode by applying 1600 V spray voltage, 270 °C capillary temperature, and collision gas pressure of 1.2 mTorr argon. Data was acquired in selected reaction monitoring (SRM) mode. The Q1 and Q3 peak width was set to 0.7 unit resolution (fwhm). Fragment ion transitions were measured with 0.1 s dwell time for each transition for a minimum of 10 data points per precursor. Transitions are shown in Table 1, following [11]. T3, T4 and $^{13}C_{12}$-T4 were quantified based on one internal standard for each compound. MS data was acquired automatically using Thermo Xcalibur software (Thermo Fisher Scientific) and analyzed using Skyline [24]. In the analyses, peak area ratios of sample to its internal standard (see below) were calculated.

2.3. Sample preparation

We performed full method validation on egg yolk samples. Egg yolk samples were acquired from adult laying hens (*Gallus gallus*, Laboratory of Comparative Endocrinology, KU Leuven, Belgium) and Japanese quails (*Coturnix japonica*, University of Zurich, Switzerland). Eggs of pied flycatcher (*Ficedula hypoleuca*), collared flycatcher (*F. albicollis*) and great tit (*Parus major*) were collected from wild populations in Finland and Sweden, under appropriate licenses. Eggs were collected on the day of laying and frozen in −20 °C until analyses. Yolks were dissected prior to analyses.

2.3.1. Sample pre-treatment: extraction of T4 and T3 from yolk

First, T4 and T3 were extracted from yolk following previously published methods [25]. In short, yolk was homogenized in 2 ml of methanol. Depending on species, 50–150 mg of fresh yolk was used. As an internal recovery tracer, a known amount of $^{13}C_{12}$-T4 (in methanol) was added to each sample. This allowed us to control for the variation in recovery (i.e. extraction efficiency) between individual samples. Four milliliters of chloroform was then added. After centrifugation (15 min, 1900*g*, +4 °C), supernatant was collected and the pellet was re-extracted in a mixture of chloroform and methanol (2:1). Back-extraction into an aqueous phase (0.05% $CaCl_2$) was followed by a re-extraction with a mixture of chloroform:methanol: 0.05% $CaCl_2$ (3:49:48) and this phase was further purified on Bio-Rad AG 1-X2 resin columns. The iodothyronines were eluted with 70% acetic acid, and evaporated to dryness under vacuum overnight. Blanks (plain reagents without any sample) were analyzed in each extraction batch to detect any contamination.

The extraction protocol was thereafter downscaled to 1/5 of the original [25] to facilitate measurements from very small samples. We extracted THs from 5 mg yolk from Japanese quails and pied flycatchers (N = 16). Samples were homogenized in 400 µl methanol and all reagents were downscaled to 1/5 of the original protocol. Protocol was conducted in 2 ml microcentrifuge tubes. See examples of chromatograms for 150 mg and 5 mg samples in Fig. 1.

2.3.2. Sample dilution

Dried TH extracts (from samples with 50–150 mg original sample mass) were initially dissolved in 150 µl 0.1% NH_3. Further dilutions,

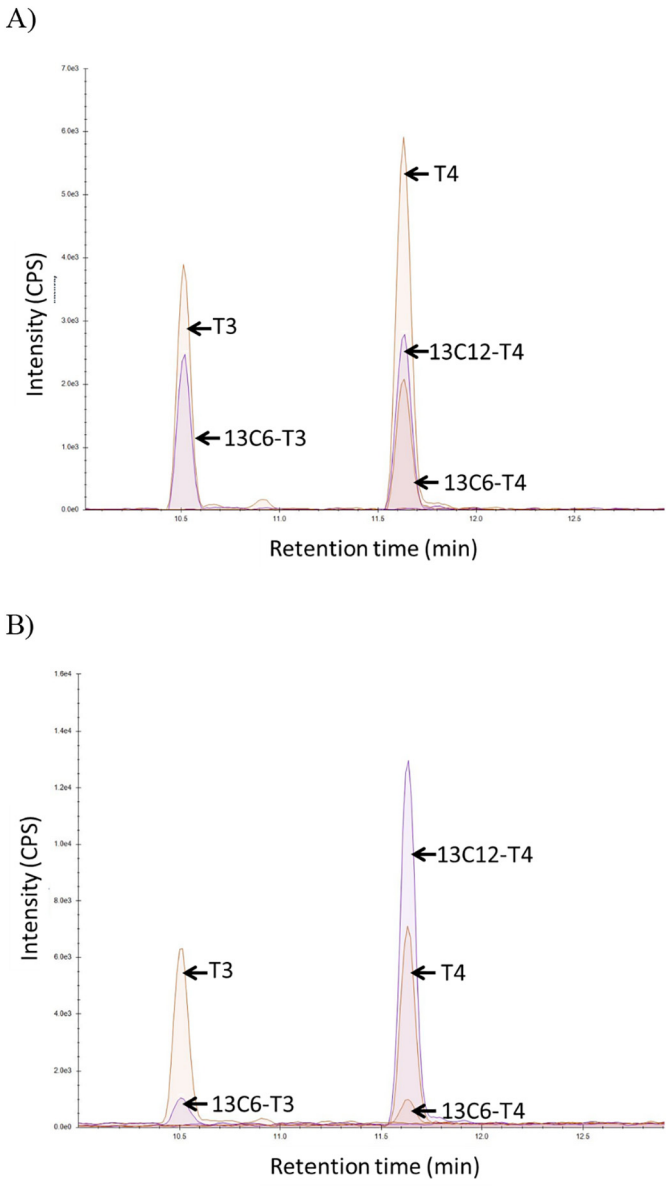

Fig. 1. Examples of chromatograms of T3 and T4 in A) a yolk sample from Japanese quail (*Coturnix japonica*) using 150 mg sample mass; B) a yolk sample from Japanese quail using 5 mg sample mass. Note that the peak of $^{13}C_{12}$-T4 and endogenous THs vary as different spiking volume and different dilution factors were used in each sample.

prepared on the day of measurement, were performed in 0.01% NH_3. The final dilution factor depended on the expected concentration of T3 and T4 in the sample and the sample starting mass. For species with existing data on yolk TH levels (chicken, Japanese quail and great tit), we used previous data from literature to estimate the dilution factor. For species where no prior data was available (pied and collared flycatcher), we tested two dilutions in a few samples find the correct dilution factor prior to running the rest of the samples. The final dilution factors for the different samples and species are the following: great tit yolk samples: 600×; pied and collared flycatcher yolk samples: 20,000×; chicken and quail yolk samples: 30,000×.

The extracts of 5 mg yolk samples from Japanese quail were dissolved in 100 µl 0.1% NH_3 and further diluted to reach a final dilution factor of 1000×. Internal standards, $^{13}C_6$-T4 and $^{13}C_6$-T3, were added to each sample upon dilution to reach a concentration of 10 amol/µl in the final dilution. All samples were stored at −20 °C.

Table 2
Repeatability and reproducibility of T3, T4 and $^{13}C_{12}$-T4 using standard points with sample matrix and actual samples.

Parameters	T3 and T4 Content (on column)	T3 CV%	T4 CV%	$^{13}C_{12}$-T4 CV%
Repeatability				
Standard with sample matrix (N = 3 per standard point)[a]	30 amol	9.6	6.5	2.1
	50 amol	7.1	2.9	7.9
	70 amol	5.1	4.4	4.9
	100 amol	5.8	3.9	4.7
	200 amol	0.7	3.3	1.6
Reproducibility				
Standard with sample matrix (N = 15, spread over 5 measurement sessions)[b]	50 amol	8.4	5.7	5.4
Samples				
Flycatcher yolk, sample mass 50-150 mg (N = 60, spread over 2 measurement sessions)[c]	40–200 amol	9.7	3.5	5.9
Japanese quail and flycatcher yolk, sample mass 5 mg (N = 32, spread over 2 measurement sessions)[d]	40–500 amol	8.9	5.2	5.0

[a] The standard curve was analyzed three times during one day.
[b] The 50 amol-standard point was analyzed for a total of 15 times, and the measurements were spread in 5 different measurement sessions which were conducted over 3 months.
[c] Yolk samples from pied and collared flycatchers (N = 30 samples) were analyzed in two measurement sessions that were separated by 2 weeks.
[d] Yolk samples from Japanese quail and pied flycatchers (N = 16 samples) were analyzed in two measurement sessions that were separated by one week.

2.4. Preparation of TH-depleted yolk sample

A TH-depleted sample was prepared to include a biological matrix in the standard. This is a common practice to control for potential matrix effects. The TH-depleted sample was prepared following Cao et al. [26]. The starting point was a chicken yolk sample that had been extracted and dried under vacuum as described above. The sample was first diluted in 1 ml of 0.1% NH_3. Thereafter resin (Bio-Rad AG 1-X2) was added 0.05 g/1 ml as suggested in Cao et al. [26]. The sample was then incubated for 5 h in RT (rocking) and thereafter centrifuged. The supernatant was further incubated in resin at RT for 18 h and final sample was acquired after centrifugation. The sample was dried and dissolved in 60 µl of 0.1% NH_3 and further dilutions were made in 0.01% NH_3 (see below).

2.5. Method validation

2.5.1. Quality control

Before the analysis of hormone samples with unknown concentrations, the performance of the nano-LC–MS/MS instrument was checked by analyzing an in-house quality control (QC) sample (a mixture of peptides from the bovine carbonic anhydrase 2 protein) by full MS and SRM mode. After that, 5 µl of TH QC sample was injected. It contained 50 amol of T3, T4, $^{13}C_6$-T3, $^{13}C_6$-T4 and $^{13}C_{12}$-T4, spiked in sample matrix in relevant concentration (see *Preparation of the calibration curve*). Peak intensities and retention times were manually checked. Procedural blanks (0.01% NH_3) were further included in every analysis batch.

2.5.2. Preparation of the standard curve

We prepared standard curves with the following standard points of light forms of T3 and T4 and $^{13}C_{12}$-T4 (per 5 µl on-column injection): 0, 10, 30, 50, 70, 100, 200 amol. Each standard point also included (1)

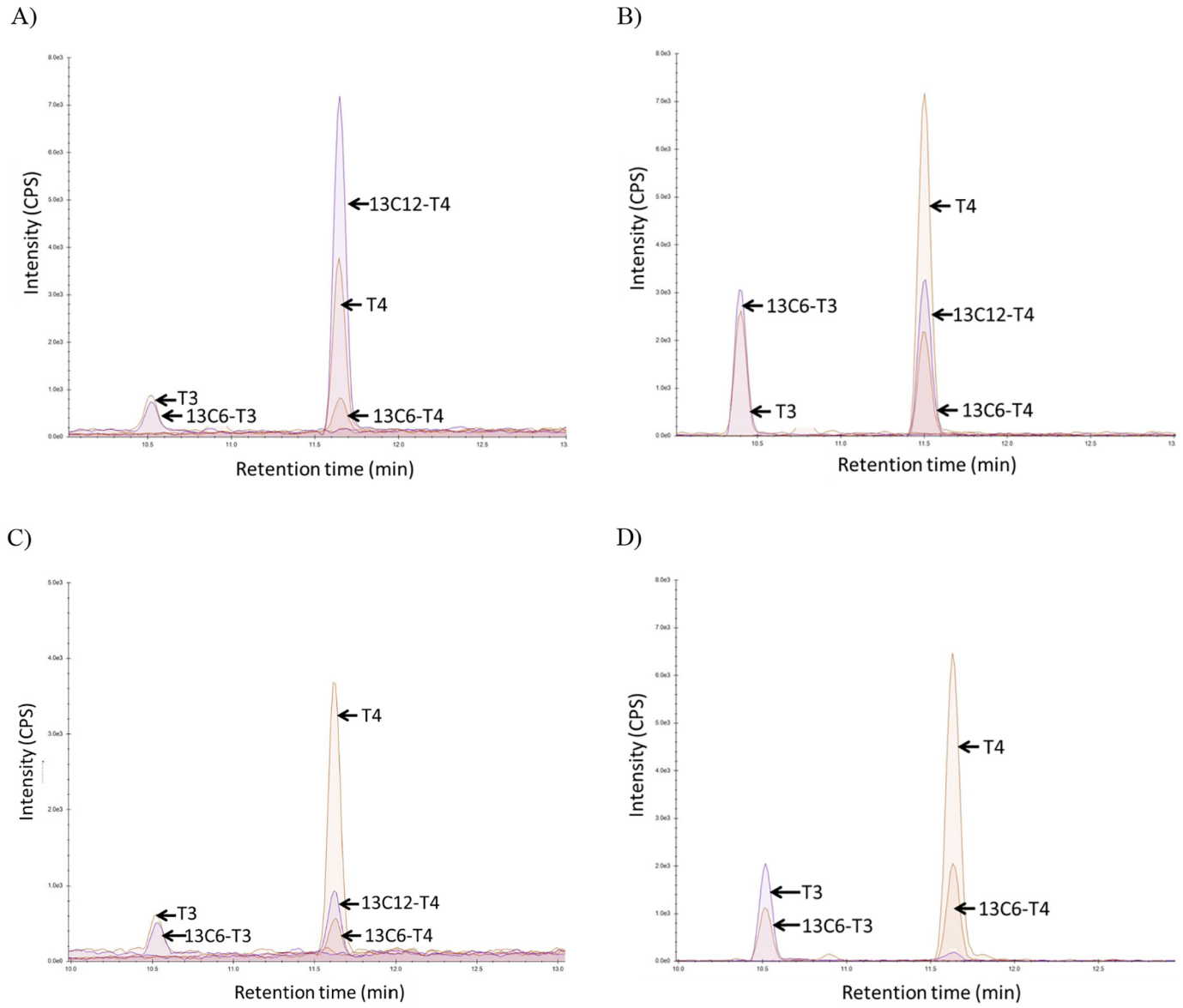

Fig. 2. Examples of chromatograms of T3 and T4 in yolk samples across species: A) pied flycatcher (*Ficedula hypoleuca*); B) collared flycatcher (*F. albicollis*), C) great tit (*Parus major*); D) chicken (*Gallus gallus*). In D $^{13}C_{12}$-T4 was not spiked before extraction. Note that the peak of $^{13}C_{12}$-T4 and endogenous THs vary as different spiking volume and different dilution factors were used in each sample.

Table 3
Average (± SD and min-max) values of T3 and T4 concentrations (pg per mg tissue) and T3/T4 ratios in yolk samples across avian species. N = sample size. See Figs. 1 and 2 for examples of the chromatograms.

Species	T3 pg/mg	T4 pg/mg	T3/T4 ratio	N
Japanese quail	3.44 ± 0.88	9.74 ± 2.20	0.37 ± 0.14	30
(*Coturnix japonica*)	2.10–6.58	5.91–14.15		
Collared flycatcher	1.97 ± 0.48	7.21 ± 0.99	0.28 ± 0.07	15
(*Ficedula albicollis*)	1.31–3.00	5.01–8.51		
Pied flycatcher	1.86 ± 0.56	5.72 ± 1.42	0.35 ± 0.15	15
(*Ficedula hypoleuca*)	1.03–3.17	3.81–9.24		
Great tit (*Parus major*)	0.14 ± 0.07	1.15 ± 0.42	0.13 ± 0.06	20
	0.06–0.28	0.61–2.53		

50 amol (per 5 µl on-column injection) of $^{13}C_6$-T4 as the internal standard for T4 and 50 amol of $^{13}C_6$-T3 as the internal standard for T3; and (2) matrix from a TH-depleted sample (see preparations above), to account for potential matrix effects: The dilution factor of the depleted sample in the final standard samples was 15,000×, which reflects the dilution factor in the samples.

Preparation of the standards started by diluting the original stock solutions (0.1 µg/µl) of T3 and T4 1:100 in 40% methanol with 0.1 M NH_3. Similarly, heavy-labelled internal standards, $^{13}C_6$-T4 and $^{13}C_6$-T3 (0.1 µg/µl, Sigma) were first diluted 1:100 with the same solution. One milligram of $^{13}C_{12}$-T4 (Larodan) was dissolved into 1 ml of methanol with 0.1 M NH_3. All diluted stocks were stored at −20 °C. Prepared stock solutions (T3, T4, $^{13}C_{12}$-T4) and the depleted sample (see Section 2.4), were further diluted with 0.01% NH_3 to gain final concentrations for the standard points (0, 10, 30, 50, 70, 100, 200 amol for T3, T4 and $^{13}C_{12}$-T4 per 5 µl injection). Every standard point included also 50 amol of $^{13}C_6$-T4 and $^{13}C_6$-T3 as an internal standard. The final standards were frozen in aliquots and thawed on the day of the measurements. In the analyses, peak area ratios of sample T4 to internal standard ($^{13}C_6$-T4) and peak ratios of sample T3 to internal standard ($^{13}C_6$-T3) were calculated.

2.5.3. Detection and quantification limits

Lower limits of detection and quantification (LLOD and LLOQ) were

determined by a method analyzing the standard deviation of the response and slope [27].

Lower limit of detection was calculated as:

$$LLOD = 3.3\,\sigma/S \quad (1)$$

where σ = standard deviation of the response, calculated as the standard deviation of the y-intercepts of the regression line, and S = slope of the calibration curve.

Limit of quantitation was calculated as:

$$LLOQ = 10\,\sigma/S \quad (2)$$

2.5.4. Precision and carryover

Repeatability (within a sample set) and reproducibility (across different measurement days/sessions) for both standards and yolk samples are reported using the coefficient of variation. Carryover was checked by running technical blank samples (0.01% NH_3) directly after the highest standard point and after samples with high concentration of T3 and T4.

2.5.5. Recovery

Previous studies have shown that extraction efficiency can vary significantly [2, 21, 25]. Accurate calculation of recovery for each sample was made possible by spiking $^{13}C_{12}$-T4 before the extraction, and using the calibration curve for $^{13}C_{12}$-T4. These results were then compared to '100% recovery sample' which included only spiking solution that was diluted similarly as the sample (in NH_3), to calculate the recovery percentage.

Based on previous RIA data, the recoveries of T4 and T3 appeared highly correlated (Darras and Ruuskanen, unpubl data, $r > 0.8$), T3 showing a slightly higher recovery than T4. Thus T3 recovery was calculated by using the known recovery of T4 as shown below.

The ratios of the peak areas of T3 and T4 are the product of differences in absolute recovery and a response factor in the MS, giving the formula:

Peak area T3: T4 ratio = Absolute T3: T4 ratio of recoveries in the sample × response factor

This can be converted to:

Absolute T3: T4 ratio of recoveries in the sample = peak area T3: T4 ratio/response factor

To calculate a correction factor for T3 recovery we performed two experiments.

Experiment 1: First, to calculate the peak area T3:T4 ratio, we spiked a known, equal amount of $^{13}C_6$-T3 and $^{13}C_6$-T4 to yolk samples before extraction. Samples were extracted as described above and analyzed in triplicate (final N = 27). We then measured the peak areas and calculated the average T3/T4 ratio. From the experiment above the average T3/T4 ratio of peak areas was 1.466.

Experiment 2: To determine the response factor, we performed a second experiment where we spiked a known, equal amount of $^{13}C_6$-T3 and $^{13}C_6$-T4 into extracted samples, which had no $^{13}C_6$-T3 or $^{13}C_6$-T4 spiked before extraction. These samples were analyzed in triplicate (final N = 9). The calculated average ratio of T3/T4, i.e. response factor was 1.363.

The 'absolute T3/T4 ratio of recoveries in the sample' was (1.466/1.363) 1.076. This means that T3 recovery is 1.076 times higher than T4 recovery, which supports the previous findings.

2.5.6. Comparison with radioimmunoassay (RIA)

Pools of yolk samples were analyzed using both RIA and the nano-LC-MS/MS. Briefly, 28 yolk samples (chicken eggs, 400 mg of yolk per sample) were extracted as described above. Before drying, all yolk extracts were pooled. An equal amount of the extract was divided into 20 tubes, which were thereafter dried in a vacuum centrifuge overnight, and stored at −20 °C. Note that when extracting these samples, $^{13}C_{12}$-T4 as an internal standard for recovery was not yet available, thus the measurements are uncorrected for extraction efficiency. Two yolk extract samples from these pools were analyzed for T4 and T3 with RIA (at KU Leuven, Belgium) following methods in van der Geyten et al. [28]. Antisera for the RIAs were purchased from Fitzgerald Industries International (US-Ireland, 20-TR40 for the T4 antibody and 20-TR45 for the T3 antibody). The T3 RIA had a detection limit of 2 fmol and an intra-assay variability of 2.2%. The T4 RIA had a detection limit of 5 fmol and an intra-assay variability of 2.8%. For the T3 RIA cross-reactivity with T4 was 0.1–0.5%, whereas for the T_4 RIA cross-reactivity with T3 was 3.5%. All samples were measured within a single RIA assay. Two yolk extract samples from the same pools were analyzed using the newly developed nano-LC-MS/MS. Again, $^{13}C_6$-T4 was added as an internal standard for T4 and $^{13}C_6$-T3 as an internal standard for T3 prior to LC-MS/MS analysis.

3. Results and discussion

3.1. Identification and retention time

Fig. 1 shows examples of chromatograms of yolk samples from Japanese quail. $^{13}C_6$-T4 and $^{13}C_{12}$-T4 have the same retention time as the endogenous T4 in the sample, which confirms the identity of the measured compounds. Similarly, T3 can be identified overlapping with the peak of $^{13}C_6$-T3. T3 and T4 can be detected and quantified in extracts starting with very small sample mass, 5 mg yolk (Fig. 1B). Overall, the developed method is relatively fast with retention times shorter than 13 min.

3.2. Linearity

Matrix-matched calibration curves, based on three replicates, yielded the following R^2 values: T3 $R^2 = 0.997$; T4 $R^2 = 0.9996$; $^{13}C_{12}$-T4 $R^2 = 0.9972$, in the concentration range 0–200 amol. When the range was increased to 1000 amol, linearity remained very good (T3 $R^2 = 0.9998$; T4 $R^2 = 0.998$; $^{13}C_{12}$-T4 $R^2 = 0.995$).

3.3. Limit of detection and quantification

Limit of detection, calculated as the standard deviation of the response and slope, was 5.9 amol (on-column) for T3 and 3.5 amol for T4. Limit of quantification was 17.9 amol (on-column) for T3 and 10.6 amol for T4. The corresponding LLOQs expressed as concentrations are 1.4 pg/ml for T4 and 2.3 pg/ml for T3. Compared to the previously reported detection and quantification limits in RIA (2–5 fmol) [29], our method is almost 100 times more sensitive. Using the recent UPLC-MS methods, LLOQ of 10 pg/ml for T4 and T3 has recently been reported [14] which is similar range as in this study. However, the on-column amounts are lower in nano-LC-MS/MS than in other previous methods (10–20 amol in our nano-LC-MS/MS vs 128–150 amol on-column in reference 14).

3.4. Precision and carryover

Repeatability and reproducibility are reported in Table 2. These parameters were determined for standards containing sample matrix, as well as real samples with a variable range of TH concentrations and variable amount of yolk sample, from 150 mg to very small, 5 mg samples. All CVs were lower than 10%.

No carryover was detected when blank samples (0.01% NH_3) were analyzed directly after high standard concentration and samples with high concentration of T3 and T4. This confirms that the applied wash protocol was efficient.

3.5. Recovery of T4

We calculated recovery (extraction efficiency) of T4 using $^{13}C_{12}$-T4

spiked into the samples prior to extraction. Recovery ranged from 40 to 80%, which was similar as reported previously for this extraction method [2, 25].

3.6. Comparison with RIA

The average (SD) concentration of chicken yolk T4 (pg/mg) was 3.06 (0.09) with nano-LC-MS/MS and 3.00 (0.11) with RIA (see Fig. 2D for an example of a chromatogram). The average (SD) concentration of chicken yolk T3 (pg/mg) was 1.02 (0.02) with nano-LC-MS/MS and 1.49 (0.07) with RIA. Thus, for yolk T4, both methods gave similar results. For T3 in yolk the values were in the same range (pg/mg) for both methods, but with some deviation. Such deviation between different methods has also been previously reported [30], and could be partly attributed to interferences from endogenous immunoglobulins/antibodies in the RIA.

3.7. Application study

After method validation, we applied the newly developed nano-LC-MS/MS method to determine maternally derived THs in egg yolks of various species of birds. Maternal hormones, via the placenta or egg yolk, critically affect offspring early development [15–17], but the naturally occurring variation has rarely been measured in vertebrates [19–23,34]. We measured maternally derived hormones in egg yolks of Japanese quails, collared and pied flycatchers and great tits. The Japanese quail is a common model in avian physiological and behavioral studies and the three passerine species are well-known models in avian ecological, behavioral, ecophysiological and evolutionary studies. Both T3 and T4 could be reliably quantified in eggs of all species. Figs. 1 and 2 show examples of the chromatograms for samples with 50–150 mg yolk, and for very small, 5 mg yolk samples. There was variation in TH concentrations across species and substantial variation within species (see minimum and maximum values, Table 3). Especially great tits had considerably lower levels of T3. The T3/T4 ratio also varied across species. Causes and consequences of variation in TH levels among and within species remain to be studied.

4. Conclusions

We developed a new and relatively fast method for measuring multiple THs. The methodology employed a nano-LC-MS/MS, with selective reaction monitoring (SRM) mode on a triple quadrupole mass spectrometer. Using a separate internal standard for T3 and T4, the method showed good linearity, repeatability and reproducibility. This method enables quantification of low TH content in the sample (LLOQ < 20 amol on-column) and is suitable for measuring yolk THs in small concentrations or small samples in various species. Therefore it can be applied to both biomedical research and ecophysiological work on wild vertebrates. We applied the methodology to measure maternally derived hormones in a number of avian species which are important models in physiological and evolutionary ecology research and report, for the first time, interesting variation across and within species.

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