Journal of Chromatography B 1226 (2023) 123803

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Determination of thyroid hormones in lake sturgeon (*Acipenser fulvescens*) tissues at different developmental stages using a sensitive liquid chromatography-mass spectrometry method

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ARTICLE INFO

Keywords:
T4
T3
rT3
Lake sturgeon
Developmental stages
Method validation

ABSTRACT

Thyroid hormones (TH) are known to play an important role in the growth and development of vertebrates. In fish species, TH regulates the larval-juvenile metamorphosis, and is crucial for development during early life stages. Monitoring the variations in TH levels at different life stages can provide insights into the regulation of metamorphosis and fish development. In this study, we developed an extremely sensitive method for the quantification of thyroxine (T4), triiodothyronine (T3), and reverse-triiodothyronine (rT3), in lake sturgeon (*Acipenser fulvescens*) tissues from eggs, free embryos, larvae, and juveniles. The target compounds were extracted by an enzymatic digestion method, followed by protein precipitation. Further cleanup was performed by liquid–liquid extraction (LLE) and solid phase extraction (SPE) using SampliQ OPT cartridges. The liquid-chromatography tandem mass spectrometry (LC-MS/MS) method used to quantify TH compounds showed remarkably high sensitivity with the limit of detection (LOD) and the limit of quantification (LOQ) ranging from $<$ 1 pg/mL to 10 pg/mL and linearity in the range of 10–50,000 pg/mL. This method was validated for tissue samples across several early developmental stages and was checked for intra- and inter-day accuracy (78.3−111.2 %) and precision (0.1−4.9 %), matrix effect (75.4−134.1 %), and recovery (41.2−69.0 %). The method was successfully applied for the quantification and comparison of T4, T3 and rT3 in hatchery raised lake sturgeon samples collected at unique time points (*i.e.,* days post fertilization dpf) including fertilized eggs (11 dpf), free embryos (14 dpf), larvae (22 dpf), juveniles (40 dpf) and older juveniles (74 dpf). With modifications, this method could be applied to other species important for agriculture or conservation.

1. Introduction

Thyroid hormones (TH) are tyrosine-derived liposoluble hormones responsible for metabolic regulation, growth, and other bodily functions in vertebrates. In amphibians and teleosts, these hormones play a crucial role in the early development at the embryonic and larval phases, reproduction, and adaptation to environmental changes [1,2]. However, the most important role attributed to TH in fish development is the transition between larval to juvenile life stages. TH present in the eggs and embryonic stages are of maternal origin [1]. Adult fish, unlike mammals, are characterized by scattered thyroid follicles distributed mainly along the ventral aorta [3], and heterotopically in head/ trunk, liver, kidney, heart, spleen, esophagus, and brain [3,4]. The prohormone thyroxine (T4) is the predominant hormone secreted by thyroid follicles [5]. It is converted to a more physiologically active form, triiodothyronine (T3) by outer ring deiodination, or degraded to an inactive form, reverse- triiodothyronine (rT3) by inner ring deiodination in the central [2] and peripheral tissues [1]. Therefore, activation and inactivation of TH is controlled by these deiodinases found in the brain, liver, kidney, and gills[3].

Radioimmunoassays (RIA) have been traditionally used to quantify TH in biological samples [6,7].However, due to the challenges related to specificity, accuracy, cross-reactivity, and the requirement of an exclusive work environment for handling radioisotopes, TH are increasingly

Abbreviations: T4, Thyroxine; T3, Triiodothyronine; rT3, reverse-triiodothyronine.

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https://doi.org/10.1016/j.jchromb.2023.123803
Received 28 March 2023; Received in revised form 12 June 2023; Accepted 15 June 2023
Available online 18 June 2023

being quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS) methods [8–10]. A previous study comparing the RIA and LC-MS/MS techniques for the measurement of TH in fish plasma showed that the T4/T3 ratios were mostly consistent in both techniques [11]. Although LC-MS/MS methods are highly sensitive and specific in quantifying these compounds, recent methods focus primarily on blood, serum, plasma, and specific organs [12–16]. Chen et al. described a method for the quantification of 5 TH in larval zebrafish exposed to TH-disrupting polychlorinated biphenyls[17]. Distribution of TH in fish is not limited to specific organs, and the concentrations vary significantly throughout development [18]. Therefore, it is important to have a method that can efficiently extract and quantify TH in different fish tissues including eggs, embryos, larvae, and whole fish. Fish eggs and whole fish are rich in proteins and lipids[19,20] which can interfere with the quantification of TH if they are not removed adequately during sample cleanup. Therefore, quantification of TH in fish tissues requires an extensive cleanup method while ensuring minimal loss of target compounds during sample extraction. The TH levels in sturgeon at early developmental stages [21], and in 2-year-old whole carcass, plasma, and tissues, and in the serum of spawning adults have been reported using RIA [22].

Lake sturgeon (*Acipenser fulvescens*) are fish species found in the Great Lakes, lakes of south Canada, Hudson-James Bay, and Mississippi River drainage [23]. The survival of this species has been threatened due to over-exploitation and the loss or degradation of habitat. In an effort to conserve the population, lake sturgeon are being reared in hatchery conservation programs [24]. These hatchery programs, which attempt to replicate natural environmental conditions and development, have a vested interest in understanding the effects of hatchery practices on growth and hormone production [25]. Since TH are known to regulate the growth and development in fish, hatchery reared fish provide an excellent opportunity to monitor TH levels under known time periods and controlled environmental conditions, including levels of nutrient uptake.

We developed a method for the extraction of TH from fish tissues using pronase enzyme digestion to release the hormones bound to the protein carriers [14,26], followed by a multi-step cleanup process involving protein precipitation, liquid–liquid extraction (LLE), and solid phase extraction (SPE) with SampliQ OPT cartridges. This method was validated by determining the linearity, limit of detection (LOD), limit of quantification (LOQ), intra- and inter-day accuracy and precision, recovery, and matrix effects. This method was successfully applied for the quantification of T4, T3 and rT3 in lake sturgeon samples collected at unique time points or days post fertilization (dpf) representing different developmental stages: fertilized eggs (11 dpf), free embryos (14dpf), larvae (22 dpf), juvenile fish (40 dpf), and older juvenile fish (74 dpf).

2. Materials and methods

2.1. Chemicals and reagents

Methanol, chloroform, citric acid, L-ascorbic acid, DL-dithiothreitol, glutathione, N-phenylthiourea, Tris and triton X-100 were purchased from Sigma- Aldrich Co., Saint Louis, MO, USA. Pronase enzyme (from *Streptomyces griseus*) was obtained from Roche Diagnostics, Indianapolis, IN, USA. Sampli Q OPT SPE cartridges, 60 mg, 3 mL were purchased from Agilent Technologies Inc., Folsom, CA, USA. Bead kits (2.8 mm ceramic, 2 mL) were procured from Omni International, Kennesaw, GA, USA. L-thyroxine (T4), L-thyroxine-$^{13}C_6$ (T4-$^{13}C_6$), 3,3′,5-triiodothyronine hydrochloride (T3), 3,3′,5-triiodothyronine-(tyrosine phenyl-$^{13}C_6$) hydrochloride(T3-$^{13}C_6$), 3,5,5′-triiodothyronine hydrochloride (rT3), and 3,5,5′-triiodothyronine-(tyrosine phenyl-$^{13}C_6$) hydrochloride (rT3-$^{13}C_6$) were also purchased from Sigma-Aldrich Co., Saint-Louis, MO, USA. Deionized water was generated using Thermo Scientific GenPure UV/UF water purification system (Waltham, MA, USA).

2.2. Preparation of standard solutions

Stock solutions for each TH standards were prepared at a concentration of 100 μg/mL using methanol containing 0.1 mol/L NH_3. The internal standards (IS) were purchased as 100 μg/mL solutions in methanol containing 0.1 mol/L NH_3. The stock solutions were stored at −20 °C in amber colored vials to avoid exposure to light. Methanol was used for all subsequent dilutions.. A 12-point calibration curve ranging from 10 to 50,000 pg/ml with 1000 pg/mL internal standard was prepared fresh before analyses.

2.3. Sample collection

Experimental animals were used with approval from the Michigan State University Animal Use and Care Committee (AUF PROTO202000023/AMEND202100062). Lake sturgeon were reared from eggs fertilized at the Black Lake Sturgeon Rearing Facility in Onaway, MI, USA, a flow-through streamside rearing facility (SRF) that utilizes ambient water from the Upper Black River. Eggs and sperms were collected from lake sturgeon in the Upper Black River on May 6, 2020. Eggs were fertilized within 8 hrs following standardized hatchery protocols [27,28]. Lake sturgeon were randomly sampled at different developmental stages represented by five unique time points (*i.e.,* days post fertilization (dpf): 1) fertilized eggs (11 dpf), 2) hatched free-embryos (14 dpf, ~ 10 mm total length), 3) exogenously feeding larvae (22 dpf, ~ 20 mm total length) – when individuals began feeding on brine shrimp (*Artemia* spp.), 4) juveniles (40 dpf, ~ 40 mm total length) – when individuals began feeding on blood worms (Diptera: Chironomidae), and 5) older juveniles (74 dpf, ~ 70 mm total length). At each time point, ten random fish were collected, euthanized in 500 mg/L tricaine methanesulfonate (Syndel, Ferndale, WA, USA), transferred to a 2.0 mL microcentrifuge tube, and snap-frozen in liquid nitrogen. Samples were then transferred to a −20 °C freezer and wrapped in a black bag to prevent light exposure prior to extraction.

2.4. Sample preparation

Whole -lake sturgeon eggs, free embryos, larvae, and juvenile were thawed in a refrigerator at 4 °C, and transferred into the ceramic bead kit and homogenized in a bead mill homogenizer (Bead Ruptor Elite, Omni International, Kennesaw, GA, USA) at 5 – 7 m/s for 30 s. A portion of the homogenized tissue (94.2 mg – 120.3 mg) was transferred to a new 15 mL polypropylene tube (Corning Inc., Corning, NY, USA). Pronase enzyme solution prepared in digestion buffer (153 mg glutathione, 1 mL Triton X-100, 1.21 g Tris, and 42.5 mg N-phenylthiourea in 100 mL deionized water) was added at 4 units per mg homogenized tissue. The enzyme solution was prepared at a concentration of 100 mg/mL and was added at 6 μL/ mg of homogenized tissue. The enzymatic digestion was facilitated by an overnight incubation at 37 °C in the dark, at 100 rpm. After incubation, 0.1 mL antioxidant solution (6 mg each of citric acid, L-ascorbic acid, and DL-dithiothreitol per mL deionized water), and 10 μL of IS mix containing 100 ng/mL each of T4-$^{13}C_6$, T3-$^{13}C_6$ and rT3-$^{13}C_6$ was added to each tube and vortexed for a few seconds.

2.5. Sample cleanup

The samples were cooled at 4 °C for about 30 min, followed by protein precipitation with 1 mL of ice-cold methanol. The tubes were stored in a −20 °C freezer for 30 min to allow complete protein precipitation. The supernatant was collected into a new 15 mL polypropylene tube after centrifugation at 3000 rpm for 10 min. The pellet was washed with an additional 1 mL of ice-cold methanol for a repeated protein precipitation and centrifugation step. The supernatants were pooled together and adjusted to 1:1 (methanol: water, v/v) ratio for a subsequent liquid–liquid extraction (LLE) with 1 mL chloroform, to separate out the fatty constituents in the matrix. After vortexing for a

few seconds, the supernatant was centrifuged again at 3000 rpm for 10 min. The topmost layer containing the TH was collected and freeze-dried under vacuum pressure using the Labconco freeze-drying system (Labconco Corporation, Kansas City, MO, USA). The dried supernatant was then reconstituted in 1 mL of 10 % methanol in water and was subjected to solid phase extraction (SPE) using SampliQ OPT SPE cartridges[29]. The cartridge was activated with 3 mL methanol, conditioned with 3 mL deionized water and loaded with the reconstituted sample. The sample tube was rinsed with an additional 1 mL of 10 % methanol in water solution which was also loaded into the cartridge. The cartridge was then washed with 3 mL of deionized water. Before the final elution step with 6 mL of methanol, the cartridges were dried under vacuum for 30 min. The eluent was freeze-dried under vacuum and reconstituted in 1 mL of 50 % methanol in water for LC-MS/MS analysis.

2.6. Instrumentation

A Waters Acquity H-Class UPLC system connected to a Xevo TQ-XS Triple Quadrupole mass spectrometer was used for the analyses. Separation was achieved by injecting 5 μL of the samples into a Waters BEH C18 column (2.1 × 100 mm; 1.7 μm particle size) attached to an Acquity column in-line filter kit (2.1 mm; 0.2 μm particle size). Milli-Q water containing 0.1 % formic acid was used as mobile phase A, and methanol was used as mobile phase B. The gradient was maintained as follows, 0–0.2 min (50 % B); 4–5.5 min (99 % B); 6–8 min (50 % B). TH were analyzed by electrospray ionization in the positive mode using multiple reaction monitoring (MRM), and the ESI-MS/MS parameters were set as follows, capillary voltage: 1.00 kV; source temperature: 150 °C; desolvation temperature: 400 °C; desolvation gas flow: 800 L/h (N2, 99.9 % purity). Argon (99.9 % purity) was introduced as the collision gas into the collision cell at a flow rate of 0.17 mL/min. MassLynx 4.2 software was used for data acquisition and data were processed using TargetLynx XS (Waters Corp., Milford, MA, USA).

2.7. Method validation and application

The method was validated in replicate samples of lake sturgeon eggs, free embryos, larvae, and juvenile fish. The method was tested for linearity, limit of detection (LOD), limit of quantification (LOQ), intra-day and inter-day accuracy and precision, recovery, and matrix effect. Method linearity was evaluated using mixtures of standard solutions in 50 % methanol in water. Although it is preferable to prepare the calibration curve in the sample matrix, due to the endogenous nature of the target analytes, neat solvent was used as the surrogate matrix. Isotopically labelled internal standards for each target compound were also used to account for the potential difference in signal intensities. The correlation coefficient was determined from the $1/\chi$ weighted calibration curve with serial concentrations on the X-axis and response [standard area*(IS area/IS concentration)] on the Y-axis. TargetLynx 4.2 software (Waters Corp., Milford, MA, USA) was used to generate the calibration curves and perform peak integration. The LOD and LOQ were determined using the signal to noise ratio of at least 3 and 10, respectively.

Accuracy, precision, matrix effect, and recovery were evaluated on pooled sample matrices or quality control (QC) samples using 5 replicates for 3 concentration levels, LQC, MQC and HQC. To prepare the QC samples, tissue samples from each developmental stage were homogenized and pooled together respectively, to obtain approximately 2 g to 4 g of homogenate. Apart from the 3 concentration levels, a set of 5 replicates of QC samples that were not spiked with the target compounds was also prepared as blank. Blank subtraction was performed before determining the accuracy, precision, matrix effect, and recovery. Accuracy was determined as the percentage ratio of the IS corrected measured concentration over the spiked concentration. Precision was measured as the percentage coefficient of variation (CV) between replicates. The matrix effect was calculated as the percentage ratio of the peak area in the matrix spiked post extraction minus the peak area in the blank sample matrix over the peak area in the standard solution. Recovery was calculated as the percentage ratio of the peak area in the matrix spiked pre-extraction over the peak area in the matrix spiked post-extraction [14].

The validated method was applied to extract and quantify TH in sturgeon tissues at different developmental stages. TH was analyzed in ten replicates each of hatchery raised fertilized eggs (11 dpf), free embryos (14 dpf), larvae (22 dpf), juvenile fish (40 dpf) and older juvenile fish (74 dpf).

2.8. Statistical analysis

The TH concentrations in different developmental stages are reported as means ± standard error of mean (SEM). The differences between groups were estimated using one-way ANOVA, followed by a Tukey test as a post hoc analysis using GraphPad Prism, version 7.0 (GraphPad Software, San Diego, CA). A p-value of < 0.05 was considered statistically significant.

3. Results and discussion

3.1. Sample extraction procedure

TH are known to play a crucial role in the survival, growth, and development of teleosts [30]. Fish tissues undergo extreme changes throughout the life cycle, starting from fertilized eggs hatching to free embryos, which develop into exogenously feeding larvae that eventually transition to sexually immature, juvenile fish. Therefore, it becomes extremely challenging to develop a method that can accurately extract, quantify, and compare TH levels in different developmental stages of fish, each with its unique tissue matrices. A multi-step sample extraction and cleanup method was developed to quantify T4, T3 and rT3 in sturgeon eggs, free embryos, larvae, and the whole body of juvenile fish. Samples were homogenized using ceramic bead kit in a bead mill homogenizer. A 30 s cycle at 5 m/s was sufficient for homogenization of softer tissues like eggs, free embryos, and larvae, but the juvenile fish required a 7 m/s for a 30 sec cycle for complete homogenization. An overnight pronase enzyme digestion using 4 units of enzyme per mg of homogenized tissue was performed to release the hormones bound to any TH binding proteins, and an antioxidant solution (6 mg each of citric acid, L-ascorbic acid, and DL-dithiothreitol per mL deionized water) was used to prevent the deiodination of TH during sample preparation [26]. Pronase enzyme digestion followed by protein precipitation with ice-cold methanol was reported to be most efficient in extracting T4, T3, and rT3 in sea lamprey tissues [14]. However, due to extremely low recoveries in sturgeon tissues, additional steps of LLE with chloroform to remove the fatty content of the matrix, and an SPE step with SampliQ OPT cartridges were necessary to recover quantifiable levels of TH.

3.2. LC-MS/MS method

The LC-MS/MS method used for the separation and identification of T4, T3 and rT3 was based on a previously published method for sea lamprey tissues and plasma [14]. Further optimization of MS/MS parameters was done on 3 different tandem quadrupole instruments, Xevo TQ-S, Xevo TQ-XS, and Xevo TQ-S Micro (Waters Corp., Milford, MA, USA). Xevo TQ-XS showed the highest sensitivity among all 3 instruments and was therefore chosen for further analyses. The MRM transitions used for the quantification of TH in positive ionization mode and other MS-MS parameters such as collision energy (CE) and cone voltage (CV) are shown in Table 1. The MRM chromatograms of the 3 target compounds detected in the sample matrices of lake sturgeon eggs, free embryos, larvae, and juveniles can be seen in Fig. 1 (a-d).

Table 1
MRM transitions, cone voltage (CV), collision energy (CE), and analytical performance including linearity range, r^2, limit of detection (LOD) and limit of quantification (LOQ).

	Precursor	Fragment	CV (V)	CE (V)	Linearity Range (pg/mL)	r^2	LOD (pg/ml)	LOQ (pg/ml)
T4	777.6	731.6	45	25	10–50,000	0.996	<1	5
T3	651.7	605.8	15	25	10–50,000	0.995	<1	5
rT3	651.7	605.8	15	25	10–50,000	0.997	<1	10
T4-$^{13}C_6$	783.6	737.7	35	25	NA	NA	NA	NA
T3-$^{13}C_6$	657.8	611.5	15	25	NA	NA	NA	NA
rT3-$^{13}C_6$	657.8	611.5	15	25	NA	NA	NA	NA

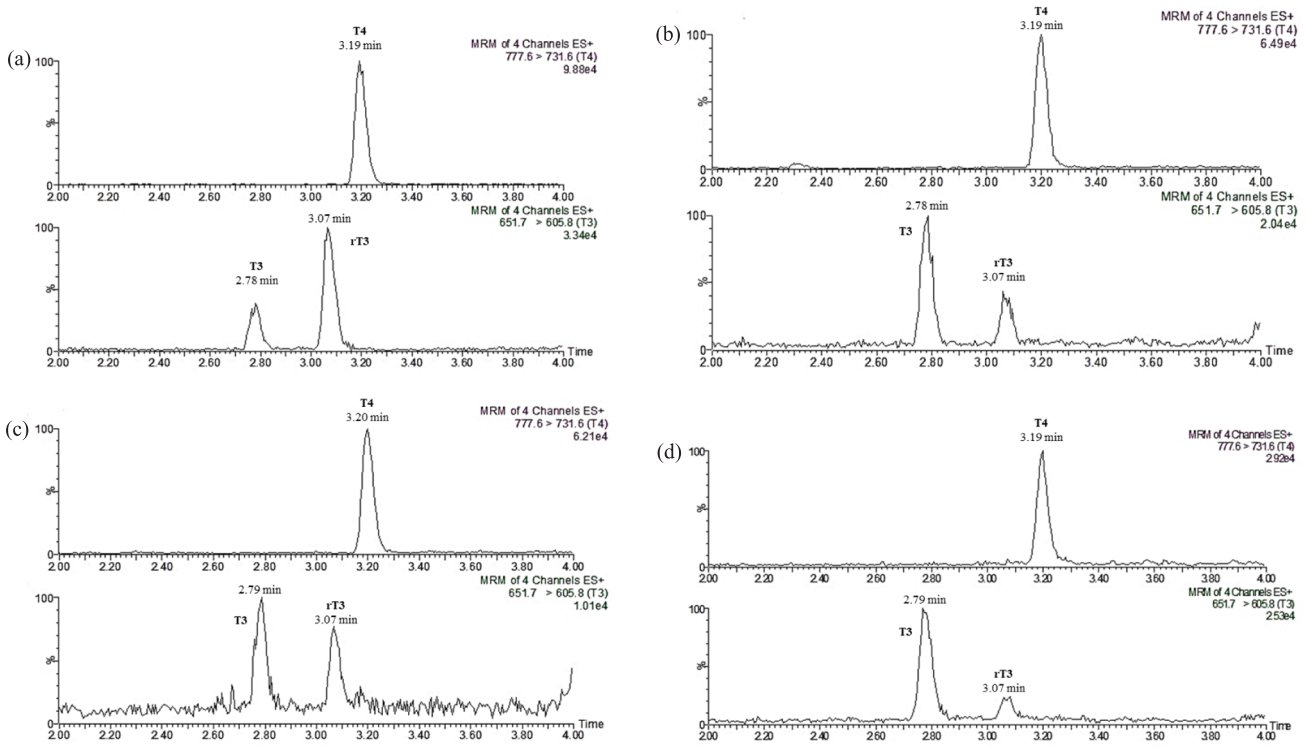

Fig. 1. MRM chromatograms of T4, T3, and rT3 in the lake sturgeon sample matrices of (a) eggs, (b) free embryos, (c) larvae, and (d) juveniles.

3.3. Method validation

The LC-MS/MS method developed for the quantification of 3 thyroid hormones, T4, T3 and rT3, was validated based on the guidelines provided by the FDA. Isotopically labelled internal standards, T4-$^{13}C_6$, T3-$^{13}C_6$, and rT3-$^{13}C_6$ were used for their respective target compounds to account for the potential loss during sample extractions and instrumental variability. The linearity of the calibration curves was determined by the correlation coefficients (r^2) greater than 0.99. All 3 compounds were found to have a linearity range of 10–50,000 pg/mL (Table 1). The LOD was found to be < 1 pg/ml for all 3 compounds (<0.005 pg on the column). The LOQ was determined to be 5 pg/mL (0.025 pg on the column) for T4 and T3, and 10 pg/mL (0.05 pg on the column) for rT3. The LOD of this method is more sensitive than the radioimmunoassay (0.12 ng/ml) [22]and that of previous LC-MS/MS method developed for zebrafish larvae (0.5–0.6 pg on the column)[17].

The intra-day and inter-day accuracy and precision were tested on QC samples spiked at 3 concentration levels, LQC (2.5 pg), MQC (25 pg), and HQC (250 pg), with 5 replicates for each level. The intra-day measurements were determined by injecting 3 sets of LQC, MQC, and HQC levels within the same day; and the inter-day measurements were obtained by injecting a set of LQC, MQC, and HQC replicates for 3 consecutive days. Overall, the method was found to have good accuracy and precision (Table 2). In the LQC level, the highest accuracy or concentration closest to the spiked concentration was seen for T4 in eggs (100.5 % intra-day, and 100.1 % inter-day). In the free embryos, T4 and rT3 (102.2–106.4 %) showed better accuracies than T3 in both intra-day and inter-day measurements. Similarly, lower accuracies were seen for T3 (78.3 % intra-day, and 79.1 % inter-day) in juvenile fish. In the MQC and HQC levels, all the observations were within 100 ± 15 % range. Better precision was seen for intra-day samples than for inter-day samples. However, both intra-day and inter-day precisions were below 5 % in all 3 QC levels indicating extremely low variability between samples. The highest variability of 4.9 % was seen for rT3 in eggs for inter-day observation in LQC. The precision values for MQC and HQC levels for all other samples ranged from 0.1–1.79 %.

Recovery and matrix effect were also analyzed in all the developmental stage QC samples at LQC, MQC, and HQC levels with 5 replicates for each level (Table 3). Despite the addition of multiple steps during sample cleanup, the observed recoveries (40.3–69.0 %) were better than previously reported values[14]. The highest recovery (69.0 %) was observed for T3 in LQC larvae, and the lowest recovery (40.3 %) was

Table 2
Intra-day and inter-day accuracy (Acc) and precision (CV) of T3, T4, and rT3 in sturgeon eggs, free embryo, larvae, and juveniles.

	LQC (n = 5)				MQC (n = 5)				HQC (n = 5)			
	Intra-day		Inter-day		Intra-day		Inter-day		Intra-day		Inter-day	
Developmental Stage	Acc%	CV%	Acc%	CV%	Acc%	CV%	Acc%	CV%	Acc%	CV%	Acc%	CV%
Eggs												
T4	100.5	0.8	100.1	1.3	105.6	0.6	105.5	0.6	108.9	0.3	108.9	0.2
T3	84.5	1.2	85.0	2.4	98.7	0.4	98.7	1.6	103.4	0.4	103.4	1.8
rT3	89.3	2.1	90.3	4.9	103.2	0.5	103.4	0.4	107.5	0.5	108.2	1.6
Free Embryo												
T4	103.8	4.5	104.1	2.2	94.5	0.8	94.5	0.8	107.4	0.1	108.2	0.8
T3	81.8	0.4	82.1	1.2	87.8	0.4	88.5	0.6	105.1	0.4	105.9	0.5
rT3	106.4	1.7	102.2	2.7	95.5	0.2	95.7	0.4	110.3	0.3	111.2	0.9
Larvae												
T4	86.0	2.7	89.6	1.2	94.3	0.5	96.0	1.1	102.7	0.2	103.7	0.8
T3	87.7	1.5	86.1	3.0	93.6	0.9	92.4	1.8	101.1	1.0	100.1	1.3
rT3	89.0	0.8	89.3	2.6	96.2	1.5	95.2	1.2	105.2	0.5	104.6	1.6
Juvenile												
T4	89.4	1.7	85.6	2.7	89.7	0.1	89.5	0.1	101.1	0.1	101.2	0.2
T3	78.3	0.3	79.1	1.3	86.5	1.5	87.2	0.8	97.5	0.4	98.2	0.4
rT3	85.5	0.5	85.5	0.2	91.6	0.1	91.3	0.9	103.1	0.3	103.0	0.5

Table 3
Matrix effect (ME)% and recovery (Rec)% of T3, T4, and rT3 in sturgeon eggs, free embryos, larvae, and juveniles.

	LQC (n = 5)		MQC (n = 5)		HQC (n = 5)	
	ME% (Mean ± SD)	Rec% (Mean ± SD)	ME% (Mean ± SD)	Rec% (Mean ± SD)	ME% (Mean ± SD)	Rec% (Mean ± SD)
Eggs						
T4	133.5 ± 5.5	46.6 ± 6.5	134.1 ± 3.5	43.3 ± 1.7	131.8 ± 3.3	40.3 ± 2.2
T3	117.1 ± 2.2	51.2 ± 3.1	113.7 ± 3.5	47.7 ± 1.6	110.1 ± 3.0	45.3 ± 2.9
rT3	105.5 ± 3.6	41.2 ± 4.3	126.6 ± 3.7	43.6 ± 1.1	120.9 ± 3.0	41.4 ± 1.1
Free Embryo						
T4	85.4 ± 15.7	65.3 ± 7.0	113.5 ± 2.5	55.4 ± 5.5	112.5 ± 6.3	54.1 ± 6.0
T3	87.0 ± 2.6	54.6 ± 2.1	100.2 ± 1.1	53.1 ± 2.4	98.7 ± 1.2	58.5 ± 4.5
rT3	86.8 ± 12.4	62.5 ± 4.3	106.8 ± 1.9	54.6 ± 0.9	108.0 ± 1.9	57.5 ± 3.3
Larvae						
T4	95.0 ± 10.6	66.2 ± 4.0	108.3 ± 7.3	55.6 ± 2.0	112.8 ± 5.7	55.0 ± 2.8
T3	88.6 ± 8.8	69.0 ± 4.1	93.1 ± 5.4	59.8 ± 2.4	96.1 ± 4.7	59.7 ± 3.1
rT3	85.3 ± 7.4	59.7 ± 5.2	103.9 ± 5.7	57.1 ± 1.5	102.3 ± 3.2	56.1 ± 5.0
Juvenile						
T4	81.8 ± 9.7	61.5 ± 5.1	96.4 ± 4.2	55.8 ± 2.8	100.7 ± 6.3	56.1 ± 6.0
T3	89.7 ± 7.9	54.5 ± 3.7	102.8 ± 3.0	54.2 ± 1.8	104.2 ± 2.7	56.3 ± 2.7
rT3	75.4 ± 2.1	54.2 ± 3.3	93.2 ± 2.7	55.9 ± 1.5	99.1 ± 4.2	50.6 ± 7.8

observed for T4 in HQC eggs. Lower recoveries were seen in eggs (41.2–51.2 %) compared to the other tissues (50.6–69.0 %). Similarly, enhanced matrix effect was observed for eggs compared to other tissues. The matrix effect for eggs ranged from 105.5 % for rT3 in LQC to 134.1 % for T4 in MQC. For free embryos and larvae, the matrix effect in all 3 QC levels were within 100 ± 15 % range. Juvenile fish in the LQC level showed slightly suppressed matrix effect of 75.4 % in rT3 and 81.8 % in T4.

3.4. Application of the method for TH quantification in sturgeon

The LC-MS/MS method was applied for the quantification of TH in hatchery raised sturgeon eggs (11 dpf), free embryos (14 dpf), larvae (22 dpf), juvenile fish (40 dpf), and older juvenile fish (74 dpf). The levels of T4, T3, and rT3 were analyzed in 10 replicates at each developmental stage. T4 was found to be the highest TH compared to T3 and rT3 in all stages (Fig. 2). The highest level of T4 was observed in eggs (8.9 ± 0.5 ng/g). A significant drop in T4 level was seen immediately post hatching in the free embryos (2.2 ± 0.2 ng/g) with a steady rise in the larval (3.6 ± 0.2 ng/g) and the 40 dpf juvenile stage (6.0 ± 1.0 ng/g). A second drop was observed in the T4 level between the 40 dpf juvenile and the 74 dpf older juveniles. Apart from the rT3 level in eggs (2.6 ± 0.2 ng/g), the T3 and rT3 levels in all developmental stages ranged from 0.2–0.6 ng/g.

These results are consistent with the timeline of thyroid gland development in sturgeons. Thyroid follicles were first observed at 3–4 days post hatch (dph) in stellate sturgeon (*Acipenser stellatus*) and at 4–5 dph in Russian sturgeon (*A. gueldenstaedtii*). The follicle diameter and follicular cells fast developed from 12–19 dph, and the lumen was filled with thyroglobulin [31]. Similarly, we observed higher amounts of TH in 40 dpf juvenile compared to free embryos and larvae in lake sturgeon. As sturgeon grow older (2–5 years old), they seem not to maintain high levels of circulating TH (0–6.9 ng/ml T3 and 0–3.6 ng/ml T4 in *Acipenser transmontanus*), and there seems to be a seasonal increase during the seaward migratory between February and April in white sturgeon [32]. In 2-year-old immature lake sturgeon held in fresh water and in serum of adults at spawning time from the Winnipeg River, circulating T4 (0.3 ng/ml) and T3 (0.2 ng/ml) levels were low [22]. In parallel, we observed a decrease of TH in 74 dpf compared to 40 dpf juvenile lake sturgeon.

4. Conclusion

We developed and validated a multi-step extraction and LC-MS/MS quantification method for the efficient extraction and quantification of TH from lake sturgeon tissues at different developmental stages, including eggs, free embryos, larvae, and juvenile fish. The combined approach of protein precipitation with LLE and SPE with SampliQ OPT cartridges contributed immensely to cleaning up the sample matrix and achieving greater recoveries of the target compounds. This method was successfully applied to monitor T4, T3, and rT3 levels in hatchery raised lake sturgeon from the fertilized eggs stage (11 dpf) to older juveniles (74 dpf) with a more sensitive LOD than previous methods. With slight modifications, this method can be used for other species with agriculture or conservation importance.

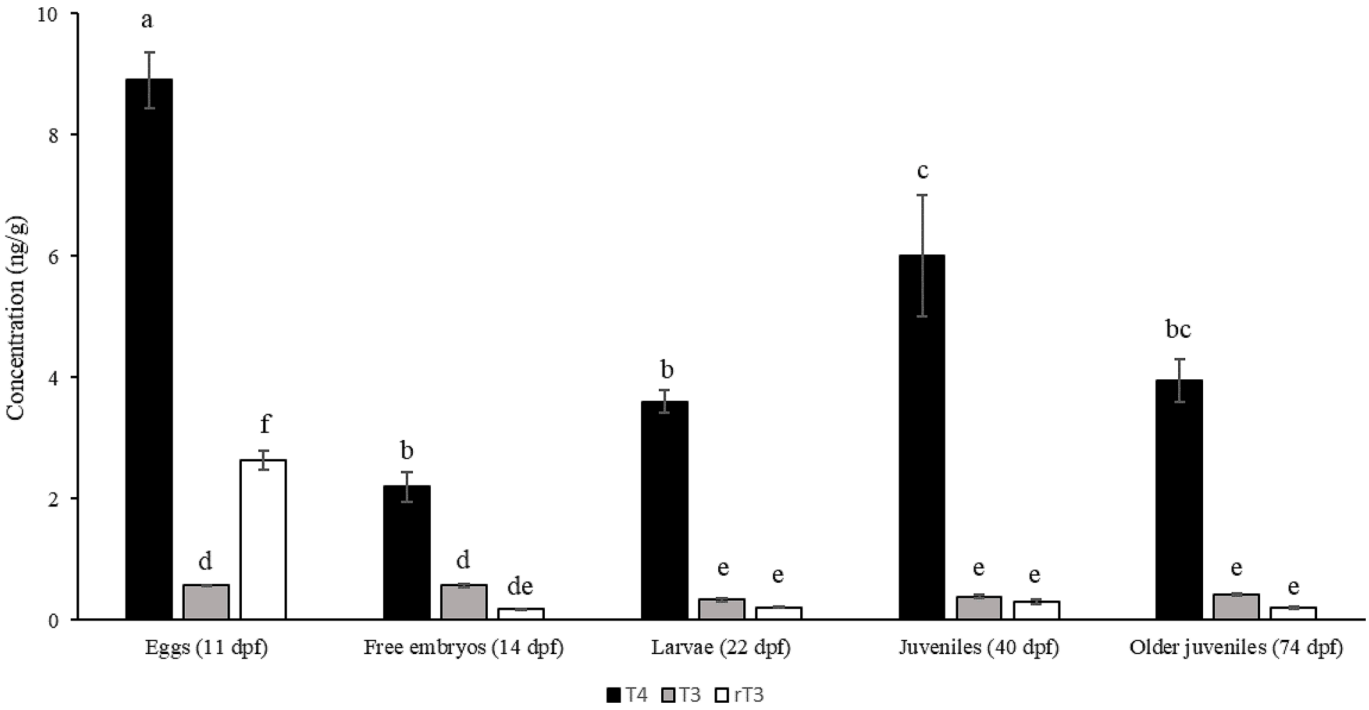

Fig. 2. Concentrations (ng/g) of T4, T3, and rT3 in lake sturgeon samples collected at unique time points or days post fertilization (dpf): eggs (11 dpf), free embryos (14 dpf), larvae (22 dpf), juveniles (40 dpf) and older juveniles (74 dpf). Different letters (a, b, c, d, e and f) on top of each bar represent statistically significant differences between life stages. A p-value of < 0.05 was considered statistically significant.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by the Great Lakes Fishery Trust [project ID 1785], the Great Lakes Fishery Commission (2019_LIW_540890), and Michigan Department of Natural Resources State Wildlife Grants Program (2017_KTS_T-10-T-8). We would like to thank the staff at the Mass Spectrometry and Metabolomics Core Facility of Michigan State University for their technical support. We would also like to thank Black River Sturgeon Facility staff for their assistance with fish husbandry and sample collection.

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