



Rapid determination of thyroid hormones in blood plasma from Glaucous gulls and Baikal seals by HybridSPE®-LC-MS/MS

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ABSTRACT

A rapid hybrid solid phase extraction (HybridSPE®) protocol tailored to liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) analysis, was developed for the determination of four thyroid hormones, *L*-Thyroxine (T₄), 3,3',5-triiodo-*L*-thyronine (T₃), 3,3',5'-triiodo-*L*-thyronine (*r*T₃) and 3,3'-diiodo-*L*-thyronine (T₂) in blood plasma from Glaucous gulls (*Larus hyperboreus*) and Baikal seals (*Phoca sibirica*). The use of target analyte specific ¹³C internal standards allowed quantification to be performed through the standard solvent calibration curves and alleviated the need to perform quantification with matrix match curves. The relative recoveries were 100.0–110.1 % for T₄, 99.1–102.2 % for T₃, 100.5–108.0 % for *r*T₃, and 100.5–104.6 % for T₂. The matrix effects ranged from –1.52 to –6.10 %, demonstrating minor signal suppression during analysis. The method intra-day precision (method repeatability, RSD %, *N* = 5, *k* = 1 day) and inter-day precision (method reproducibility, RSD %, *N* = 10, *k* = 2 days) at the 1 ng/mL concentration of fortification were 8.54–15.4 % and 15.4–24.8 %, respectively, indicating acceptable chromatographic peak stabilities for all target THs even at trace level concentrations. The method limit of detection (LOD) for T₄, T₃, *r*T₃ and T₂ was 0.17, 0.16, 0.30 and 0.17 ng/mL, respectively. The HybridSPE® protocol was simple and rapid (<1 min) upon application, while the HybridSPE® cartridge did not require (as in classical SPE cartridges) any additional equilibration nor conditioning step prior sample loading. A total of 46 blood plasma samples, 30 samples collected from Glaucous gulls and 16 samples collected from Baikal seals, were analyzed for thyroid hormones to demonstrate the applicability of the developed method in these wildlife species. The concentrations of T₄ and T₃ in blood plasma from the Glaucous gulls were 5.95–44.2 and 0.37–5.61 ng/mL, respectively, whereas those from Baikal seals were 3.57–46.5 and 0.45–2.07 ng/mL, respectively. In both species, *r*T₃ demonstrated low detection rate, while T₂ was not detected. Furthermore, cross-array comparison between the HybridSPE®-LC-MS/MS protocol and an established routine radioimmunoassay (RIA) kit-based method was performed for T₄ and T₃ concentrations from selected Baikal seal plasma samples.

1. Introduction

Thyroid hormones (THs) are endocrine molecules that regulate a wide range of biological processes in vertebrates, including growth, energy metabolism, neurodevelopment, protein synthesis and mitochondrial action [1,2]. *L*-thyroxine (T₄) is the major TH synthesized in

the thyroid gland and transferred through the bloodstream to the organs before it is biotransformed into 3,3',5-triiodo-*L*-thyronine (T₃) [1,3]. A minor percentage of T₃, which varies depending on the species, is also formed in the thyroid gland [1,3]. Furthermore, T₄ can be converted to 3,3',5'-triiodo-*L*-thyronine (*reverse* T₃; *r*T₃), while T₃ and *r*T₃ can be converted to 3,3'-diiodo-*L*-thyronine (T₂) through deiodination [3].

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Disruption of normal thyroid function in animals is linked to pollution exposures [1,4], and the concentration profiles of blood circulating THs are commonly used to diagnose actual thyroid disorders [4,5]. Increased serum rT_3 concentrations, but also specific concentration profiles of THs are linked to adverse health effects [5,6]. Traditionally, immunoassay (IA)-based methods were the main choice for bioanalysis of THs, and thus far, they remain the predominant technique of analysis [6–8]. Currently, THs measurements in clinical routines are regularly performed by the radioimmunoassay technique (RIA) [6,8], which is a sensitive and rapid technique, but it has limitations with respect to specificity and accuracy [9–12]. As liquid chromatography (LC)-tandem mass spectrometry (MS/MS) technique evolved during the past 20 years, an increased number of bioanalytical LC-MS/MS methods were developed for THs analysis [9,13]. The LC-MS/MS technique allowed for THs quantification with enhanced specificity, reproducibility, and sensitivity [14–16]. A few gas chromatography–mass spectrometry (GC-MS) methods are also reported, but with limited applicability due to often laborious derivatization processes [16].

The THs are mostly determined in blood serum or plasma and they are bound to proteins (e.g., 99.9 and 99.7 % of T_4 and T_3 , respectively), while a small percentage (< 0.5 %) of those remain free (unbound) [16]. For the measurement of total concentrations of THs (bound and unbound hormone chemical species), the protein-TH binding sites are disrupted through protein precipitation methods, which destroy the binding ability of the proteins through denaturation [13,16]. Strong acid reagents (e.g., HCl), salts and organic solvents are reported to yield clear supernatants with effective denaturation of proteins for the determination of THs [13,16]. However, the major bioanalytical challenge for THs analysis is the light, heat and chemical stability, which can be significantly impacted when exhaustive sample preparation protocols are applied [13,17]; it is documented that T_4 can be degraded or bio-transformed (due to remaining deiodinase activity in the samples) to T_3 and rT_3 , while T_3 can be further converted to T_2 [3,6,18]. In particular, the transformation of T_4 to T_3 has a strong impact on T_3 concentrations, which are significantly (even 2 orders of magnitude) lower than those of T_4 in the blood of vertebrates [19]. Moreover, another major challenge in LC-MS/MS bioanalysis of THs is the uncertainty of quantification due to matrix effects that derive mainly from the high endogenous protein and phospholipid content of serum and plasma in contrast to the natural occurring trace concentrations of THs [13,16].

In this study, a rapid methodology that used the hybrid solid phase extraction (HybridSPE®) technique tailored to LC–electrospray (ESI+)–MS/MS was developed for the analysis of the total concentrations of four THs, T_4 , T_3 , rT_3 and T_2 . Blood plasma concentrations and profiles of those THs were determined in actual samples from Glaucous gulls (*Larus hyperboreus*) and Baikal seals (*Phoca sibirica*). The method performance characteristics and the applicability of the HybridSPE® method was extensively demonstrated. The advantage of the HybridSPE® technique is that the extracted biological matrix is rapidly processed, and the gross amounts of proteins and phospholipids are removed, significantly decreasing the impact of the matrix during analysis [20]. In this context, three different sample preparation techniques, including liquid–liquid (LLE), solid phase (SPE) and HybridSPE® extraction were assessed in ultrapure water and bovine blood serum. The T_4 and T_3 concentrations that were determined in the blood plasma from Baikal seals by the HybridSPE® method, were also cross-array compared with those concentrations determined by an established routine RIA kit-based method to further demonstrate measurement validity.

2. Experimental

2.1. Materials and chemicals

Methanol (MeOH) and acetonitrile (ACN) of LC-MS grade, ethyl acetate (MS *SupraSolv*) and ammonium acetate (98 % w/w) were purchased from Merck (Darmstadt, Germany). Formic acid (98 % v/v),

hydrochloric acid (HCl), ammonium formate (99 % w/w) and ammonium hydroxide were purchased from Sigma-Aldrich (Steinheim, Germany). Water was purified with a Milli-Q grade water purification system (Qoption, Elga Labwater, Veolia Water Systems LTD, U.K.).

Standards of T_4 (≥ 98 %, 5gr), T_3 (≥ 95 %, 5gr), rT_3 (≥ 95 %, 50 mg), T_2 (98 %, 10 mg), *L*-thyroxine- $^{13}C_6$ ($^{13}C_6$ - T_4 ; certified reference material, 100 μ g/mL in MeOH with 0.1 N NH_3 , ampule of 1 mL, Cerilliant), 3,3',5-triiodo-*L*-thyronine- $^{13}C_6$ ($^{13}C_6$ - T_3 ; ≥ 95 %, 5 mg), 3,3',5'-triiodo-*L*-thyronine- $^{13}C_6$ ($^{13}C_6$ - rT_3 ; certified reference material, 100 μ g/mL in MeOH with 0.1 N NH_3 , ampule of 1 mL, Cerilliant), and 3,3'-diiodo-*L*-thyronine-(phenoxy- $^{13}C_6$) ($^{13}C_6$ - T_2 ; 97 %, 5 mg) were purchased from Sigma-Aldrich (Steinheim, Germany). Individual stock solutions of 1000 μ g/mL in (1:1 v/v) MeOH:Milli-Q water (ammonium hydroxide 0.1 % (v/v)) were prepared for T_4 , T_3 , rT_3 , T_2 , $^{13}C_6$ - T_3 and $^{13}C_6$ - T_2 . The 1000 μ g/mL stock solutions of the target THs were used to prepare the working standard mixture solution of 100 μ g/mL. The 1000 μ g/mL stock solutions of $^{13}C_6$ - T_3 and $^{13}C_6$ - T_2 were mixed with the 100 μ g/mL standard solution of $^{13}C_6$ - T_4 and $^{13}C_6$ - rT_3 to prepare the working internal standard mixture solution of 10 μ g/mL. Both working standard mixture solutions of the target THs and their internal standards, but also the calibration standard solutions were prepared in 1:1 (v/v) MeOH:Milli-Q water (ammonium hydroxide 0.1 % (v/v)). All standard solutions were stored in the darkness at -20 °C.

Strata™-X-CW Polymeric RP 200 mg/3 cc (Phenomenex; Værløse, Denmark), Oasis® HLB 200 mg/3 cc (Waters; Milford, MA, USA) and HybridSPE® 30 mg/1 cc (Sigma–Aldrich; Steinheim, Germany) cartridges were assessed for the purification of extracts and the isolation of the target THs. Polypropylene (PP) tubes and amber glass vials (1.5 mL) were obtained from VWR International AS (Oslo, Norway). A 12-port Visiprep™ DL (Disposable Liners) SPE vacuum manifold was obtained from Supelco (PA, USA). A TurboVap® LV automated evaporation system (Biotech AB, Uppsala, Sweden) was used for solvent evaporation. The used LC column was an Atlantis C18 T3 (150 mm \times 2.1 mm, 3 μ m) column that was purchased from Waters (Oslo, Norway) and it was connected to a C18 guard column (4.0 mm \times 2.0 mm, 5 μ m) that was purchased from Phenomenex (Værløse, Denmark). ^{125}I radioimmuno assay (RIA) kits were purchased from MP Biomedicals (LCC, NY, USA) for total T_4 (Catalogue No. 06B-254011) and T_3 (Catalogue No. 06-254215) concentrations. A γ -scintillation counter (Cobra Auto-Gamma, model 5003, Packard Instrument Co., Dowers Grove, IL, USA) was used to measure reactivity from the RIA test kits.

2.2. Sample collection

For the method development and validation, matrix standards were prepared from bovine serum samples (surrogate pool matrix), as previously performed [21]. Baikal seal ($n = 16$) plasma samples were collected from the Mishikha region, Russia, which is located at the southern part of Lake Baikal in April 2011 (permission for the sampling was provided by Angara-Baikal Directorate of the Russian Fisheries Agency, ref. 000024). Sampling and handling of the seals were performed in accordance with the Federal Law on fisheries and conservation of aquatic biological resources of the Russian Federation. The sample set consisted of two adult females (8 and 12 years old) and 14 pups. In total, 6 pups were female and the remaining 8 were male (and the age of all pups ranged from 1 to 2 months). The seals were euthanized for sampling of tissues for other research purposes prior to blood sampling from the heart with a 20 mL heparinized syringe. The blood samples were directly transferred into Vacuette® heparinized tubes followed by centrifugation (3000 rpm, 10 min) to collect the plasma. Subsequently, the plasma samples were transferred to cryovials and were immediately snap frozen into liquid nitrogen (-196 °C), and thereafter transferred to -70 °C until analysis. Glaucous gull ($n = 30$) plasma samples were obtained in 2017 and 2018 from Isfjorden, which is near Longyearbyen, Svalbard, Norway. Permissions for the sampling were provided by the Governor of Svalbard (ref. 17/00414-2 and 17/

00414-6). The sample set consisted of 18 female and 12 male adults. Sampling and handling of the sea birds was performed in accordance with the regulations of the Norwegian Animal Welfare Act. Blood samples were collected primarily from the jugular vein, and plasma was obtained and stored according to the procedures specifically indicated for Baikal seals.

2.3. LLE, SPE and HybridSPE®-based sample preparation protocols

Three different sample preparation techniques were assessed for optimal TH extraction: LLE; SPE; and HybridSPE®. Pre- and post-extraction matrix (bovine serum) spikes, fortified with 25 ng/mL of target THs, were prepared (similarly to previous works [20,22,23]) to calculate recoveries and matrix effects for every extraction technique. Method blanks were used for monitoring background contamination.

For the LLE, a generic protocol that involved ethylacetate as the organic solvent was used with minor modifications [24]. Aliquots of 250 μ L of matrix were transferred into 15-mL PP tubes. The samples were fortified with 300 μ L of 1.0 M ammonium acetate. Thereafter, the samples were extracted 3 consecutive times with 3 mL of ethyl acetate each time (3×3 mL). For each extraction, the mixture was shaken in an oscillator shaker for 45 min, and thereafter centrifuged (3500 rpm) for 10 min in room temperature. The supernatants were combined, and 1 mL of Milli-Q water was added. The mixture was centrifuged again, and the supernatant was transferred into another PP tube and concentrated to near dryness under a gentle nitrogen stream (N_2). Finally, 1 mL of (1:1 v/v) MeOH:Milli-Q water was added, vortex mixed, and transferred for LC-MS/MS analysis.

For the SPE, the extraction of the target THs was assessed by both, Oasis® HLB and Strata™-X-CW cartridges. Aliquots of 150 μ L of matrix were transferred into 15-mL PP tubes. The samples were fortified with 300 μ L of 1.0 M ammonium acetate. Both Oasis® HLB and Strata™-X-CW cartridges were conditioned with 3 mL of MeOH and 3 mL of acidified Milli-Q water (adjusted to pH < 3, acidified with HCl solution). Thereafter, the samples were passed through the cartridges, washed with 3 mL of Milli-Q water, and then dried under vacuum for 10 min (8–10 psi). The target THs were eluted with 3 mL of MeOH and 3 mL of MeOH with 2 % (v/v) ammonium hydroxide from the Oasis® HLB and Strata™-X-CW cartridges, respectively. All eluents were concentrated to near dryness under a gentle nitrogen stream (N_2). The solvents were reconstituted with 300 μ L of (1:1 v/v) MeOH:Milli-Q water, vortex-mixed, and transferred for LC-MS/MS analysis.

For the HybridSPE®, two protein precipitation agents, MeOH with 1 % ammonium formate (w/v) and ACN with 1 % formic acid (v/v) were assessed for optimal THs extraction and cartridge functionality. In each case, aliquots of 150 μ L of sample were transferred into 15-mL PP tubes and 450 μ L of the respective precipitation agent were added. The samples were vortex-mixed and centrifuged (3500 rpm) for 10 min in room temperature. The supernatants were collected and passed directly through the pre-washed HybridSPE® cartridges. They were pre-washed with 1 mL MeOH, which was pipetted on the cartridges, while the vacuum on the SPE manifold was kept between 8 and 10 psi. The extracts, ~450 μ L, were collected and transferred directly for LC-MS/MS analysis.

2.4. LC-ESI(+)MS/MS analysis

The chromatographic separation was carried out using an Acquity UPLC I-Class system (Waters, Milford, USA) coupled to a triple quadrupole mass analyser (QqQ; Xevo TQ-S) with a ZSpray ESI ion source (Waters, Milford, USA). The injection volume was 2 μ L and the mobile phase flow rate was 200 μ L/min. The mobile phase comprised Milli-Q

water (0.1 % v/v formic acid) (A) and MeOH (B). The gradient elution started at 50 % (v/v) A, decreased to 20 % A within 4.0 min, then further decreased to 0 % A within 2.0 min (6.0th min), and reverted back to 50 % A at the 6.0th min that was further held for 2.0 min for a total run time of 8.0 min. The specific MS/MS parameters are presented in Table S1. Nitrogen was used as both the desolvation and cone gas, which were set at a flow rate of 800 and 150 L/h, respectively. The electrospray ionization voltage was set at +2.8 kV. The desolvation and source temperatures were set at 500 and 150 °C, respectively. The calibration standard solutions were prepared in (1:1 v/v) MeOH:Milli-Q water.

2.5. HybridSPE®-LC-MS/MS method validation

The calibration of the ESI method was verified by injecting solvent calibration standards at concentrations of 0.25–50.0 ng/mL (0.25, 0.50, 1.00, 2.50, 5.00, 10.0, 25.0 and 50.0 ng/mL). For every target analyte, a specific ¹³C₆-labelled internal standard was used, and quantification was performed with the internal standard method [22,23]. The method limits of detection (LODs) and quantification (LOQs) were estimated for each target analyte (from the respective ¹³C₆-labelled internal standard) as 3 and 10 times the signal from the baseline noise (S/N ratio) in bovine serum matrix, respectively. Precision was assessed through repeatability and reproducibility experiments. For method repeatability (method intra-day precision) experiments, the bovine serum was fortified at two concentrations (10 and 20 ng/mL) and three replicate analyses ($N = 3$) were prepared for each concentration. The precision of the method was also extensively assessed at a low fortified concentration; method repeatability and reproducibility (method intra- and inter-day precision, respectively) were assessed in bovine serum at 1 ng/mL with 5 replicates prepared during the same day ($N = 5 \times 1$) and with 10 replicates prepared in-between two consecutive days ($N = 5 \times 2$), respectively. Since the method intra-day precision at the low fortified concentration (1 ng/mL) was assessed in-between two different days, the results were provided for two days (day 1 and day 2). For instrumental repeatability (instrumental intra-day precision) experiments, solvent matrix was fortified at three concentrations (1, 2 and 5 ng/mL), and three replicate analyses ($N = 3$) were prepared for each fortified concentration. The accuracy (trueness) was evaluated through recovery experiments at the fortified concentrations of 10 and 20 ng/mL of the target THs; absolute and relative recoveries % (as defined in [20]) were calculated in three replicates ($N = 3$) in both concentrations.

2.6. Routine radioimmunoassay analysis of T₄ and T₃

The analysis of T₄ and T₃ was performed according to previous work [21,22,25] with minor modifications. Briefly, 25 and 100 μ L of Baikal seal plasma was added to the test kit tubes of T₄ and T₃, respectively, with the specific radioactive antigen (1 mL of ¹²⁵I Total T₄ and ¹²⁵I Total T₃, respectively), vortex mixed and incubated in a water bath at 37 °C for 2 h. After incubation, the tubes were aspirated, and the reactivity was measured using the γ -scintillation counter. Calibration curves and concentrations of THs in the samples were calculated by the embedded software (SpectraWorks™ Spectrum Analysis Software, Packard Instrument Company, Connecticut, USA) of the γ -scintillation counter. The quality control/quality assurance (QC/QA) of measurements was established by the analysis of the standard reference material, Lyphocheck Immunoassay Plus Control Levels 1, 2 and 3 (BioRad, California, USA). Samples were run in triplicates ($N = 3$) and the repeatability was RSD % (Relative Standard Deviation %) < 10%. LODs were set in accordance to those reported by the kit protocols: 0.07 (T₃) and 2.50 ng/mL (T₄).

2.7. Data analysis and statistical treatment

LC-MS/MS data was acquired with the MassLynx v4.1 software, while quantification processing was performed with TargetLynx (Waters, Milford, USA). Excel (Microsoft, 2018) was used for *t*-test and general descriptive statistics. Principal component analysis (PCA) was performed by the statistical software R (<https://www.R-project.org>; 2018). Data analysis did not include censored data (i.e., non-detects; NDs). Concentrations were reported as ng/mL. The statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. Extraction efficiency of the different techniques

The extraction efficiency of the applied sample preparation protocols that were based on three different sample preparation techniques (SPE, LLE and HybridSPE®) is presented in Fig. S1. In the SPE-based protocols, the recoveries from the Oasis® HLB cartridge were 22.7–63.2 % in Milli-Q water and 53.4–74.2 % in serum, and the recoveries from the Strata™-X-CW cartridge were 29.0–52.7 % in Milli-Q water and 29.2–57.0 % in serum. The two SPE polymeric sorbents (Strata™-X-CW and Oasis® HLB) exhibit hydrophobic, π - π , and hydrophilic intermolecular forces on the target THs. Due to the polar group of *N*-vinylpyrrolidone present in the Oasis® HLB sorbent, there is an increased contribution of hydrogen bonding [26]. The Strata™-X-CW cartridge is designed to increase the retention of basic compounds through a carboxylic acid functional group [26]. The classes with an amine group, such as THs, can be positively charged in an acidic environment, and consequently, promote cation exchange interactions with the cartridge. THs obtain a conjugated molecular system that is prone to be strongly retained by the cartridges through π - π interactions. Overall, the Oasis® HLB sorbent functioned significantly better in the biological matrix, and consequently, it was preferred over the Strata™-X-CW sorbent (Fig. S1); this observation agrees with previous reports [27]. Both SPE-based protocols demonstrated recoveries with higher standard deviations (SDs) when compared to those from the HybridSPE®-based protocols (Fig. S1). The applied LLE-based protocol demonstrated higher recoveries than those from the SPE-based protocols in the Milli-Q water matrix (Fig. S1). However, the serum recoveries from the LLE-based protocol were lower compared to those in Milli-Q water, but were found similar to those from the SPE-based protocols (Fig. S1). The SDs of the T₃ recoveries from the LLE technique were also lower than those from the SPE technique (Fig. S1). The larger variations (SDs) in the recoveries of T₃ in relation to the other target THs were attributed to the concentration step to near dryness.

The HybridSPE® technique has so far been applied in a limited number of sub-trace concentration bioanalytical applications [20,28]. Even though it was previously reported tested for human serum analysis of THs, it was not extensively applied [13]. It can be concluded that this technique is still not commonly reported in literature in contrast to SPE and LLE-based protocols. The HybridSPE® cartridge consists of a zirconium packed-bed, low porosity filter, 0.2 μ m hydrophobic frit assembly [20]. The application of HybridSPE® consists of few, relatively simple and rapid steps: the sample is mixed with a suitable organic solvent, loaded onto the cartridge, filtered on-cartridge (through the cartridge frit) and the sample is ready for analysis. Thus, the samples need less than a minute (<1 min) to pass through the cartridge, avoiding the time exhaustive typical SPE and LLE processes that can compromise the stability of THs, while sample throughput is maximized [13,20]. The optimal results with the application of HybridSPE® were obtained for THs when ACN (1 % v/v formic acid) was used as the precipitation agent. This agent demonstrated absolute recoveries (\pm SD) % of 43.9 (\pm 2.21) % for T₄, 44.3 (\pm 1.02) % for T₃, 42.0 (\pm 1.81) % for *r*T₃ and 45.5 (\pm 1.03) % for T₂ in Milli-Q water matrix, and absolute recoveries (\pm SD) % of 27.6 (\pm 4.50) % for T₄, 26.4 (\pm 1.53) % for T₃, 24.3 (\pm 2.82) % for

*r*T₃ and 25.3 (\pm 2.13) % for T₂ in serum (Fig. S1). The absolute recoveries (%) of the HybridSPE® technique were lower than those from the other two techniques; however, they demonstrated significantly lower uncertainties (lower SDs). The recoveries in the HybridSPE® extraction were not statistically different (*t*-test) when 1, 3 and 5 % v/v formic acid was added to ACN. The second precipitation agent that was assessed for HybridSPE® extraction, MeOH (1 % w/v ammonium formate), provided recoveries < 8% for all THs, but even then, the actual SDs of those showed good precision (maximum recovery SD: \pm 5.61%). Overall, the low uncertainty (low SDs) of the TH absolute recoveries with the HybridSPE® protocol was attributed to the rapid sample preparation that does not impact the stability of THs, but also to the lack of an evaporation and reconstitution step (in contrast to the SPE- and LLE-based protocols) that introduces by-default higher uncertainties [29].

3.2. Matrix effects and ion ratios (%) of THs analysis

The HybridSPE® protocols demonstrated significantly lower matrix effects than the SPE and LLE protocols (Table S2). The HybridSPE® protocol with ACN (1 % v/v formic acid) as the precipitation agent demonstrated matrix effects (%) of -6.10, -6.01, -2.62 and -1.52 % for T₄, T₃, *r*T₃ and T₂, respectively. The matrix effects were found at the same order of magnitude when MeOH (1 % w/v ammonium formate) was used as the precipitation agent. The matrix effects were stronger for T₄ and T₃ in the LLE than the two SPE protocols, while the opposite was observed for *r*T₃ and T₂ (Table S2). Among the two SPE cartridges, only T₄ experienced significantly stronger matrix effects with Strata™-X-CW (-28.7 %) than with Oasis® HLB (-17.0 %). The SRM transition 778.0 > 324.2 *m/z* (T₄), which provided the highest sensitivity (from the two monitored SRMs) for T₄ (Table S1), was used successfully as the quantifier transition for all three matrices. However, the confirmation SRM transition, 778.0 > 732.1 *m/z* (T₄), suffered in the Baikal seal plasma matrix from coeluting matrix interferences (Fig. S2).

The ion ratio (%) for every target TH was calculated from the replicates ($N = 5$) performed in a standard solvent solution (50 ng/mL), as previously described [20]. The calculated ion ratio % (\pm RSD %) for every target TH ranged within the tolerance limits set by the *E.U. Decision 2002/675*: T₄, T₃, *r*T₃ and T₂ demonstrated a ratio of 56.7 (\pm 7.04 %; limits: \pm 20 %), 3.36 (\pm 12.2 %; limits: \pm 30 %), 104 (\pm 7.15 %; limits: \pm 20 %) and 63.1 % (\pm 5.41 %; limits: \pm 20 %), respectively.

3.3. HybridSPE®-LC-MS/MS method performance

The instrumental correlation coefficients for all THs was acceptable in the investigated intervals ($r > 0.998$). T₄, T₃, *r*T₃ and T₂ demonstrated a LOD of 0.17, 0.16, 0.30 and 0.17 ng/mL, respectively, and a LOQ of 0.56, 0.52, 1.00 and 0.56 ng/mL, respectively (Table S3); the values were found in accordance to previous literature [13,30]. The method intra-day precision (method repeatability, RSD %, $N = 3$; when calculated based on the internal standard method) were 2.92–12.0 % and 2.40–11.2 % at the fortified concentration of 10 (medium level) and 20 (high level) ng/mL, respectively (Table S4). The method intra-day precision (method repeatability, RSD %, $N = 5$, $k = 1$ day) and inter-day precision (method reproducibility, RSD %, $N = 10$, $k = 2$ days) were 8.54–15.4 % and 15.4–24.8 %, respectively, when calculated based on the external standard method at the 1 ng/mL concentration of fortification (low level) (Table 1), denoting acceptable chromatographic peak stabilities for all target THs even at traces. The instrumental intra-day precision (instrumental repeatability), when calculated based on the internal standard method, ranged 1.89–8.75 %, 4.82–14.0 % and 3.53–11.3 % at the fortified concentration of 1, 2 and 5 ng/mL, respectively (Table S5). The instrumental and method precision results showed acceptable precision for all THs. The relative recoveries (\pm SD/ \pm RSD %) at the fortified concentration of 10 and 20 ng/mL were 100.0 (\pm 2.91/ \pm 2.91 %) and 110.1 (\pm 8.68/ \pm 7.88 %) % for T₄, 102.2 (\pm 12.2/ \pm 12.0 %) and 99.1 (\pm 7.55/ \pm 7.61 %) % for T₃, 100.5 (\pm 11.6/

Table 1

Method intra-day precision (method repeatability, RSD %, $N = 5$, $k = 1$ day; results were provided for two individual days) and inter-day precision (method reproducibility, RSD %, $N = 10$, $k = 2$ days), calculated based on the external standard method, at the fortified concentration of 1 ng/mL (low level).

Target THs	T_4			T_3		
Precision	Intra-day (day 1; $N=5$)	Intra-day (day 2; $N=5$)	Inter-day (both days; $N=10$)	Intra-day (day 1; $N=5$)	Intra-day (day 2; $N=5$)	Inter-day (both days; $N=10$)
Concentration: 1 ng/mL	13.8	13.3	15.4	15.4	13.1	24.8
Target THs	rT_3			T_2		
Precision	Intra-day (day 1; $N=5$)	Intra-day (day 2; $N=5$)	Inter-day (both days; $N=10$)	Intra-day (day 1; $N=5$)	Intra-day (day 2; $N=5$)	Inter-day (both days; $N=10$)
Concentration: 1 ng/mL	9.53	14.8	18.1	8.54	11.5	20.8

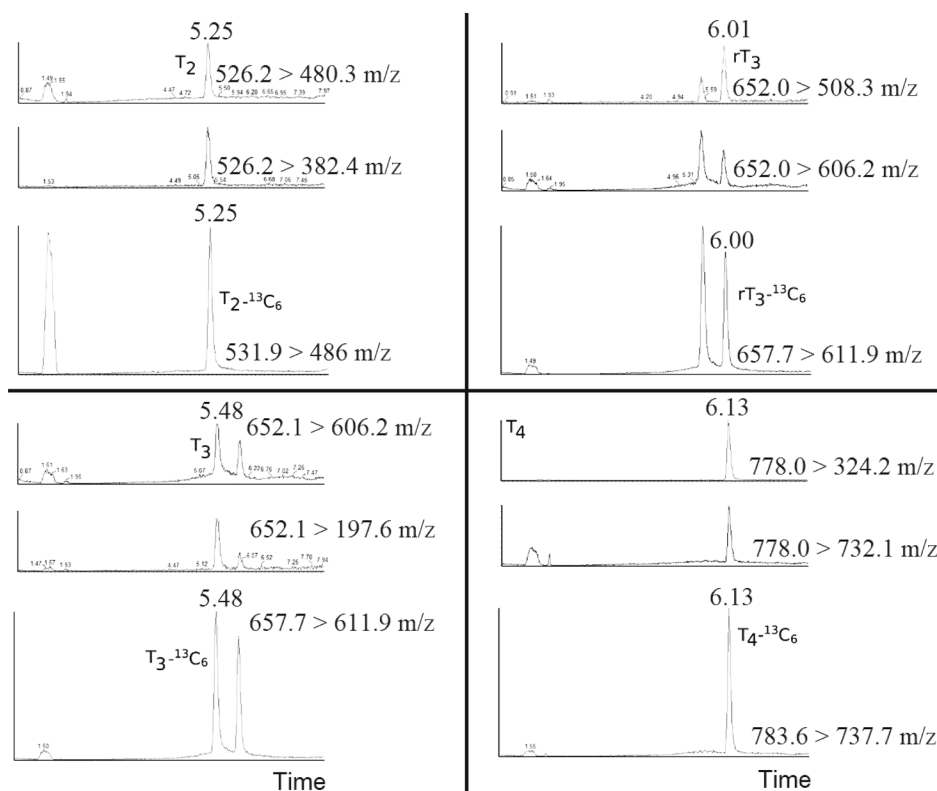


Fig. 1. SRM ion chromatograms of the target THs and internal standards.

± 11.5 %) and $108.0 (\pm 12.1/\pm 11.2)$ % for rT_3 , and $100.5 (\pm 5.69/\pm 5.66)$ % and $104.6 (\pm 2.52/\pm 2.40)$ % for T_2 , respectively. Typical selected reaction monitoring (SRM) ion chromatograms from bovine serum are presented in Fig. 1.

3.4. Analysis of Glaucous gull and Baikal seal plasma samples

A total of 46 plasma samples were analyzed, and the results are presented in Table 2. The highest detection rates were found for T_4 and T_3 followed by rT_3 . T_2 was not detected in any of the two species; previous studies performed also on the same species in blood matrices, did not report measuring or detecting concentrations of T_2 [6,21,31,32]. T_4 and T_3 are previously reported in plasma samples from Glaucous gulls analyzed using RIA, and the results are in good agreement with those found in this study (Table 3). All four studies, including the present study, reported higher T_4 concentrations in females as compared to males (Table 3). Minor differences in THs concentrations among the four

studies can be attributed to physiological differences among the sampled individuals caused by age, reproductive and nutritional status, and the season of sampling. In addition, it is documented that persistent halogenated pollutants (e.g., chlorinated biphenyls) interfere with the THs homeostasis in Glaucous gulls, and differing concentrations of these pollutants among the studies can have influenced the reported TH concentrations [31,32].

For Baikal seals, THs were previously measured in blood serum using LC-MS/MS, and the results for T_3 and rT_3 agreed in general with those measured here in blood plasma (Table 4). Although the mean T_4 concentrations were ~ 2 fold higher in the serum samples of the male Baikal seals [6] than in the plasma samples analyzed here; the variation was large in both matrices. In the females, the T_4 concentrations in the serum and plasma were clearly within the same range (Table 4). The mean T_3 concentrations were higher in the plasma samples than those previously reported in the serum samples. Even though the mean rT_3 concentrations were similar in the females in both studies (Table 4), the mean rT_3

Table 2
Thyroid hormone concentrations (ng/mL) in plasma samples from Glaucous gulls and Baikal seals.

Species	Glaucous gulls (N=30)				Baikal seals (N=16)			
	T ₄	T ₃	rT ₃	T ₂	T ₄	T ₃	rT ₃	T ₂
Descriptive Statistics								
Mean (Whole Population)	23.6	1.70	0.70	n.d.*	16.3	1.11	1.87	n.d.
Median (Whole Population)	21.6	1.30	0.68	n.d.	10.1	0.99	1.29	n.d.
Min. (Whole Population)	5.95	0.37	0.57	n.d.	3.57	0.45	0.66	n.d.
Max. (Whole Population)	44.2	5.61	0.84	n.d.	46.5	2.07	5.91	n.d.
Detection Rate (Whole Population)	30/30	26/30	3/30	0/30	16/16	14/16	9/16	0/16
Mean (Females)	25.7	1.48	0.70	n.d.	20.3	1.08	1.07	n.d.
Median (Females)	22.9	1.20	0.70	n.d.	14.9	1.05	1.12	n.d.
Min. (Females)	17.5	0.37	0.57	n.d.	3.5	0.45	0.66	n.d.
Max. (Females)	44.2	5.61	0.84	n.d.	46.5	1.72	1.40	n.d.
Detection Rate (Females)	18/18	14/18	2/18	0/18	8/8	8/8	4/8	0/8
Mean (Males)	20.3	1.95	0.68	n.d.	12.4	1.14	2.50	n.d.
Median (Males)	18.5	1.43	0.68	n.d.	9.01	0.94	1.62	n.d.
Min. (Males)	5.95	0.56	0.68	n.d.	4.42	0.66	1.15	n.d.
Max. (Males)	39.6	4.36	0.68	n.d.	42.1	2.07	5.91	n.d.
Detection Rate (Males)	12/12	12/12	1/12	0/12	8/8	6/8	5/8	0/8

* n.d.: not detected.

Table 3
Total concentrations of T₄, T₃ and rT₃ reported in plasma samples from Glaucous gulls.

Concentration [ng/mL]	Males			Females			References*
	Mean ± SD	Median	Range	Mean ± SD	Median	Range	
T ₄	19.0 ± 8.90	17.2	10.2-33.9	23.8 ± 7.45	22.8	8.25-39.5	[35]
	17.4 ± 1.20	Not reported	11.7-28.4	17.6 ± 1.06	Not reported	10.8-24.0	[31]
	22.4 ± 9.00	21.8	12.5-48.6	26.6 ± 10.4	29.4	12.5-43.9	[32]
	20.4 ± 8.60	18.5	5.95-39.6	25.7 ± 7.67	22.9	17.6-44.2	This study
T ₃	1.44 ± 0.66	1.27	1.12-2.85	2.38 ± 0.63	1.52	0.53-2.99	[35]
	2.62 ± 0.27	Not reported	1.17-4.29	1.75 ± 0.20	Not reported	0.59-3.32	[31]
	2.51 ± 0.78	2.55	1.17-4.97	1.96 ± 0.66	1.75	0.89-3.42	[32]
	1.95 ± 1.25	1.43	0.56-4.36	1.48 ± 1.33	1.20	0.37-5.61	This study
rT ₃ [#]	0.68	0.68	0.68	0.70±0.19	0.70	0.57-0.84	This study

* Verreault et al. [31]: N = 12 females, 11 males; Verreault et al. [32]: N = 34 females, 32 males; Melnes et al. [35]: N = 24 females, 15 males; This study: N = 18 females, 12 males.

[#] rT₃ detected in only 1 sample from the male population.

concentration in the males was higher in the plasma samples analyzed here than in the previously reported mean concentration in serum. Similarly, to the Glaucous gulls, these minor differences in the THs concentrations between the two studies can be attributed to the differences in the physiological status of the sampled Baikal seals. Sex classification [females (f) and males (m)] based on the sum concentrations of THs and biometric characteristics was visualized through PCA (Figs. S3 and S4). In Glaucous gulls, the classification was more distinct than that observed in Baikal seals. This is due to that the Glaucous gull sample set involved only adults, where sex differences are already

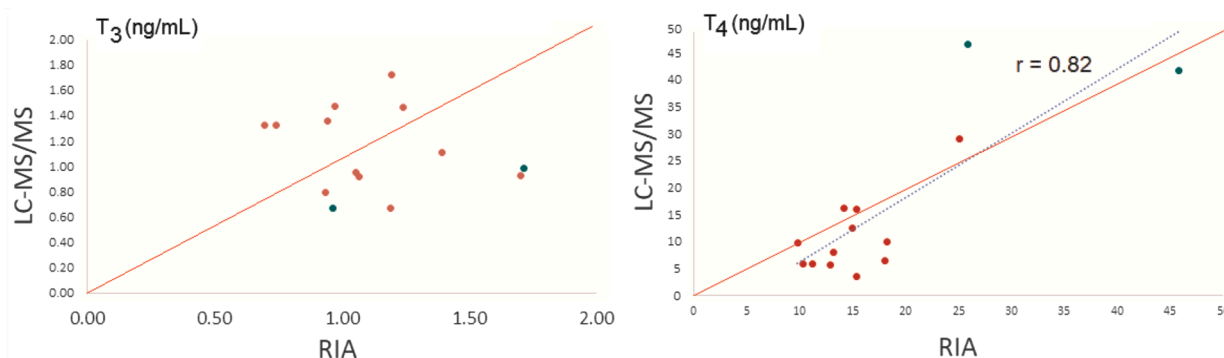
established on the TH profile, while the Baikal seal sample set involved mostly pups, where sex differences among those are not fully pronounced yet. However, the two adult female Baikal seal samples were separated distinctively from all the pups (Fig. S4), which agrees with findings from other seal species, e.g., grey seal pups (*Halichoerus grypus*) [33].

It is noteworthy that T₂ was not detected in any of the samples, whereas rT₃ was only detected in one Glaucous gull sample. Although concentrations of T₂ in healthy humans are reported to be 133 ± 15 [standard error of mean (SEM), N = 29] pg/mL [34], to our knowledge

Table 4Total concentrations of T₄, T₃ and rT₃ reported in Baikal seals.

Concentration [ng/mL]	Males			Females			References*
	Mean ± SD	Median	Range	Mean ± SD	Median	Range	
T ₄	26.0 ± 9.60	27.3	10.5–40.4	24.9 ± 8.4	26.3	11.5–39.6	[6]
	12.5 ± 12.3	9.01	4.42–42.2	20.3 ± 16.8	14.9	3.57–46.5	This study
T ₃	0.76 ± 0.23	0.73	0.42–1.24	0.81 ± 0.33	0.85	0.30–1.43	[6]
	1.14 ± 0.52	0.94	0.66–2.07	1.08 ± 0.42	1.05	0.45–1.72	This study
rT ₃	1.16 ± 0.61	1.23	n.d. [#] –2.30	1.18 ± 0.86	1.23	n.d.–3.99	[6]
	2.50 ± 2.00	1.62	1.15–5.91	1.07 ± 0.31	1.12	0.66–1.40	This study

* Kunisue et al. [6] (analysis in blood serum; LC-MS/MS results presented): N = 24 females, 13 males; This study (analysis in blood plasma): N = 8 females, 8 males.

[#] n.d.: not detected.**Fig. 2.** Cross-array comparison of T₃ and T₄ concentrations between LC-MS/MS and RIA in Baikal seal plasma samples (N = 14); the samples noted in green color are from the two female adults. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

there are no reference concentrations available for seals or birds. With respect to rT₃, there is also a lack of available reference concentrations in seals and birds. Thus, the analytical technique developed in the present study will allow also for rapid and accurate analysis of rT₃ and T₂ in serum and plasma samples, allowing for more detailed studies on the presence and role of these THs in wildlife species.

In the 14 Baikal seal plasma samples, in-which both two THs were detected, cross-array comparison of T₄ and T₃ concentrations was performed between LC-MS/MS and RIA (Fig. 2). The mean concentrations did not demonstrate statistical differences between the two techniques for both T₄ (*t*-test; *p* = 0.61) and T₃ (*t*-test; *p* = 0.93). A significant correlation coefficient (*r* = 0.82; *p* = 0.0004) for T₄ suggests that both RIA and LC-MS/MS technique can be potentially used for the measurement of T₄. However, when focusing on the lower concentration points of T₄, the correlation remains poor (Fig. 2). For T₃, no significant correlation coefficient was identified (*r* = -0.18; *p* = 0.53). The results on T₃ agreed with the previous work in Baikal seal serum samples [6], where the lack of T₃ correlation was attributed to possible immunoglobulin interference in RIA.

4. Conclusions

A rapid HybridSPE® method tailored to LC-MS/MS was developed for the determination of four THs in blood plasma from Glaucous gulls and Baikal seals. Target analyte specific ¹³C internal standards were used for effective compensation of matrix effects and extraction losses during analysis. This allowed for quantification to be performed with the standard solvent calibration curves and alleviated the need to perform it

with matrix match curves. The HybridSPE® protocol demonstrated minor signal suppression (matrix effects: -1.52 to -6.10 %) for all THs. The relative recoveries (%) ranged between 99.1 and 110.1% with excellent precision (RSD: 2.91–12.0 %). These low uncertainties obtained with the HybridSPE® protocol were partially attributed to the rapidity of the specific sample preparation protocol that does not impact the stability of THs. The method inter-day precision at the fortification concentration of 1 ng/mL was < 25 %, when calculated based on the external standard method, denoting acceptable chromatographic peak stabilities for all target THs even at traces. The method was applied successfully in 46 blood plasma samples from Glaucous gulls and Baikal seals, while cross-array comparison between LC-MS/MS and RIA was performed for T₄ and T₃, which were those two THs detected in the majority of samples; all results were found in agreement to previous works demonstrating the highly selective and sensitive valid analysis of THs through the HybridSPE® technique. In the analyzed blood plasma samples, rT₃ demonstrated low detection rate, while T₂ was not detected. Nonetheless, the HybridSPE® technique allows for further in-depth studies on the presence and role of all four THs in wildlife species.

CRediT authorship contribution statement

Kristine Vike-Jonas: Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing - original draft. **Susana Villa Gonzalez:** Methodology, Resources, Software, Writing - review & editing. **Åse-Karen Mortensen:** Resources, Writing - review & editing. **Tomasz Maciej Ciesielski:** Funding acquisition, Investigation, Project administration, Resources, Supervision,

Writing - review & editing. **Julia Farkas**: Investigation, Resources, Writing - review & editing. **Vishwesh Venkatraman**: Investigation, Visualization, Writing - review & editing. **Mikhail V. Pastukhov**: Funding acquisition, Methodology, Resources, Writing - review & editing. **Bjørn Munro Jenssen**: Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing - review & editing. **Alexandros G. Asimakopoulos**: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - review & editing.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jchromb.2020.122447>.

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