Development of an Extensive Analytical Method of Thyroid Hormones in Equine Serum using High-Performance Liquid Chromatography High-Resolution Mass Spectrometry

By
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Abstract

Synthetic thyroid hormones, particularly levothyroxine, are a concern in horse racing due to their performance-enhancing effects. Regulating these substances is essential to prevent malpractice and protect horse safety. However, current analytical methodologies struggle to differentiate between natural and synthetic thyroid hormones. This study developed a sensitive method to simultaneously detect thyroid hormones, their acid metabolites, precursors, and a derivative in equine athlete serum using solid phase extraction using a mixed-mode anion exchange sorbent and liquid chromatography mass spectrometry using parallel ion reaction monitoring based detection and positive and negative mode electrospray ionization. Limits of detection and limits of quantitation, measured in pg/mL, were determined for 3,5,3',5'-tetraiodothyronine (100, 1000), 3,3',5- triiodothyronine (10, 100), 3,3',5'-triiodothyronine (10, 100), 3,5-diiodothyronine (2.5, 5), 3,3'-diiodothyronine (1.25, 2.5), 3-iodothyroacetic acid (7.5, 5), 3,3',5-triiodothyroacetic acid (2.5, 5), 3,3',5,5'-tetraiodothyroacetic acid (5, 50), monoiodotyrosine (2.5, 5), diiodotyrosine (1, 5), and 3-iodothyronamine (1.25, 5). Accuracy, precision, recovery, stability, and matrix effects were determined as validation parameters and were all acceptable. Using this method, reference ranges were derived for serum from a population of pre-race geldings, mares, and stallions. These reference ranges, measured in pg/mL, for geldings, mares, and stallions were 3,5,3',5'-tetraiodothyronine (18100 ± 14200, 18200±15600, 14000±12500), 3,3',5- triiodothyronine (1340 ± 1500, 1180 ± 1230, 1030 ± 1200), 3,3',5'-triiodothyronine (665 ± 521, 722 ± 653, 631 ± 581), 3,5-diiodothyronine (5.19 ± 8.41, 6.40 ± 7.04, 5.82 ± 6.58), 3,3'-diiodothyronine (66.8 ± 52.6, 66.9 ± 62.1, 62.6 ± 57.8), monoiodotyrosine (41.2 ± 96.5, 50.6 ± 125, 42.4 ± 121), diiodotyrosine (70.4 ± 162, 54.3 ± 127, 56.2 ± 157). 3-

iodothyroacetic acid, 3,3’,5-triiodothyroacetic acid, and 3,3’,5,5’-tetraiodothyroacetic acid were below the quantitation range for all sexes and 3-iodothyronamine was not detected.

1.0 Introduction

Thyroid hormones, naturally produced by the thyroid gland via enzymatic pathways, are essential for the regulation of biochemical reactions involved in growth, development, and cellular metabolism [1]. Although hypothyroidism is extremely rare in horses, levothyroxine has often been administered to equine athletes that show nonspecific signs, as it is assumed to increase performance by accelerating metabolism and increasing exercise tolerance [2]. Due to the endogenous nature of thyroid hormones, the relationship between thyroid hormones, their derivatives and their precursors need to be investigated to assess potential effects that levothyroxine may have. Previous research has examined concentrations of 3,5,3',5'-tetraiodothyronine (T4), 3,3',5- triiodothyronine (T3), 3,3',5'-triiodothyronine (rT3), 3,5-diiodothyronine (3,5-T2), 3,3'-diiodothyronine (3,3'-T2), 3-iodothyroacetic acid (TA1), 3,3'5-triiodothyroacetic acid (TA3), 3,3',5,5'-tetraiodothyroacetic acid (TA4), 3-monoiodotyrosine (MIT), 3,5-diiodotyrosine (DIT) and 3-iodothyronamine (T1AM) in human serum [3-10]. However, studies in horses have only been developed to detect T4 and T3 [11-18]. Levels of T4, T3, rT3, 3,5-T2 and 3,3'-T2 in cattle have been examined [8]. Reference ranges for these hormones are summarized in **Table 1.**

Analysis of endogenous hormones and xenobiotics at low concentrations in biological matrices, including serum, usually requires appropriate sample preparation approaches to minimize the impact of matrix components on the detection of targeted compounds. Complicating the analysis for these hormones is their complex relationship and the interplay between them, such as transmutation. To address this issue, antioxidants (dithiothreitol, ascorbic acid and citric acid) have to be added to minimize the deiodination and conversion of thyroid hormones during sample preparation and analysis [3, 19-23].

Table 1. Reference Ranges for Humans, Cattle and Horses for select Thyroid Hormones in serum or plasma. Measured total circulating hormone, free and bound.

Thyroid Hormone	Human	Cattle	Horse
T4 (ng/mL)	43 - 100 [3]	78 - 89 [8]	2.64-3.42[18]
T3 (ng/mL)	0.66 – 1.3 [3]	3.4 - 4.1 [8]	0.814-0.879 [18]
rT3 (ng/mL)	0.15 – 0.64 [3]	<1.4 [8]	Undetermined
3,5-T2 (ng/mL)	<0.0009 [3]	<0.74 [8]	Undetermined
3,3'-T2 (ng/mL)	0.0025 – 0.0077 [3]	<0.92 [8]	Undetermined
TA1 (ng/mL)	< 0.1[10]	Undetermined	Undetermined
TA3 (ng/mL)	0.3 – 0.9 [6]	Undetermined	Undetermined
TA4 (ng/mL)	0.022 – 0.27 [3]	Undetermined	Undetermined
MIT (ng/mL)	0.03 – 0.090[5]	Undetermined	Undetermined
DIT (ng/mL)	0.03 – 0.096[5]	Undetermined	Undetermined
T1AM (ng/mL)	3.55-24.86 [9]	Undetermined	Undetermined

A number of sample extraction approaches including protein precipitation, liquid-liquid extraction, and solid phase extraction (SPE) have previously been evaluated. Protein precipitation has been investigated through the use of organic solvents or acids to remove interfering substances and concentrate the target hormones within the serum [3, 20, 24]. Alternatively, urea has been used for protein denaturation due to its chaotropic nature and ability to maintain basic conditions [22-24]. Finally, Bussy et. al found SPE retained the highest recoveries in plasma and serum matrices for T3, rT3, T4 and 8 other thyroid hormone metabolites [24, 25]. Utilizing SPE, analytes are rapidly isolated through their interaction with a solid stationary phase and extracted from the complex matrix through their suspension or dissolution and cleaned to remove interfering matrices

and eluted prior to analysis. With the removal of salts, proteins, and phospholipids, the matrix's impact is significantly reduced during instrumental analysis which can result in increased sensitivity [25].

3,3',5- Triiodothyronine, T4 and rT3 are the most common THs measured both clinically and in research studies and are assessed as either the free or total compounds. Some common analytical methods include electrochemiluminescent immunoassay (ECLIA), enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA). ECLIA is a time-consuming procedure that only permits a single hormone to be detected per run, with each runtime averaging around 18 minutes. Manufacturer protocol guidelines have warned of a 30% permittable error in accuracy, especially around LOQ levels, which could cause inaccuracies in the estimation of TH concentrations, making interpretation of the results challenging. ELISA has shown limited sensitivity for both T4 and T3 [26]. Although it is more sensitive than ECLIA and ELISA, RIA is easy to perform and provides minimal cross-reactivity between analytes of interest and a high sample throughput. However, it is limited by the use of radioactive materials in sample analysis. Additionally, to achieve these results, RIA requires highly specific radioactive-labeled antibodies to bind to and quantify specific analytes in a sample. Currently, only T4, T3 and rT3 assays are commercially available. Multiplex immunoassays for the other remaining THs and their metabolites remain to be developed [27, 28].

The use of liquid chromatography-tandem mass spectrometry (LC-MS/MS) has allowed for multianalyte detection while resolving some immunoassay limitations [7]. With high selectivity, specificity and reproducibility, LC-MS/MS allows for the determination of multiple compounds in a single analysis with less analytical interference [4]. This methodology, when properly controlled, can allow for good accuracy and precision, rapid analysis, high throughput and is more

reliable than immunoassay techniques when measuring low concentrations of analytes [24, 25, 27]. Gas chromatography-mass spectrometry has also been utilized for TH determination. However due to its laborious derivatization processes, there are fewer methods reported in literature [25]. Developing sensitive and selective analytical approaches to monitor a large number of TH can allow for future evaluation of normal thyroid function and may allow for evaluation of dysregulation of circulating TH from various disease states, metabolic conditions, or modulation of TH from environmental chemicals or deliberate administrations of exogenous TH.

2.0 Background

2.1 Thyroid Hormones

The thyroid gland and its secreted hormones are essential for normal development and growth, acting as the main regulators of metabolism in most tissues through the control of basic metabolic rate, body heat production, oxygen intake and metabolism of carbohydrates and lipids [29]. Thyroid hormones are also essential for normal brain development [9]. The thyroid gland is unique amongst endocrine tissues in its ability to serve as a large reservoir for the hormones through the storage of iodinated tyrosine residues present in thyroglobulin. Thyroglobulin is a large protein produced in follicular cells and stored in the follicular lumen of the thyroid gland where diiodotyrosine (DIT) and monoiodotyrosine (MIT) molecules are formed when iodine attaches to tyrosyl [3]. DIT and MIT act as intermediate metabolites aiding thyroid hormone synthesis of thyroglobulin, forming T4 and T3 as they iodinate [5, 29, 30]. T4 is formed through the oxidation and joining of two DIT molecules and T3 with the coupling of a MIT and a DIT molecule [**Figure 1**, 30].

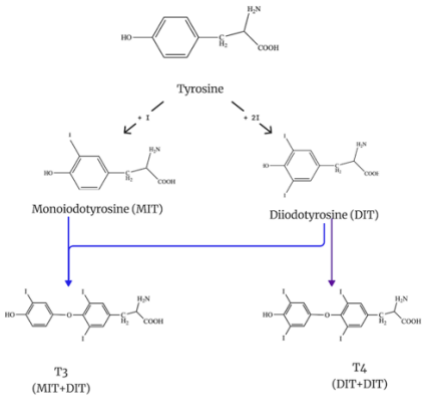

Figure 1. Synthesis of thyroid hormones (created with ChemDraw 21.0.0)

2.2 Hypothalamic-pituitary-thyroid axis

Thyroid hormone synthesis and secretion is primarily regulated by the hypothalamic-pituitary-thyroid (HPT) axis. The signaling of neuroendocrine cells in the hypothalamus stimulates the release of thyrotropin-releasing hormone (TRH), a simple chain containing proline, histamine and glutamine, into systemic circulation TRH binds to specific cells found in the pituitary, which release thyroid-stimulating hormone (TSH) into the bloodstream. Binding of TSH to receptors found in the thyroid gland ultimately results in the production and release of metabolically active T3 and T4, its prohormone, into systemic circulation [4]. These THs can ultimately regulate the production of TRH in the hypothalamus and complete the feedback loop. [**Figure 2**, 11]. The stress reactivity of the HPT axis in female animals is consistently higher than in males [31].

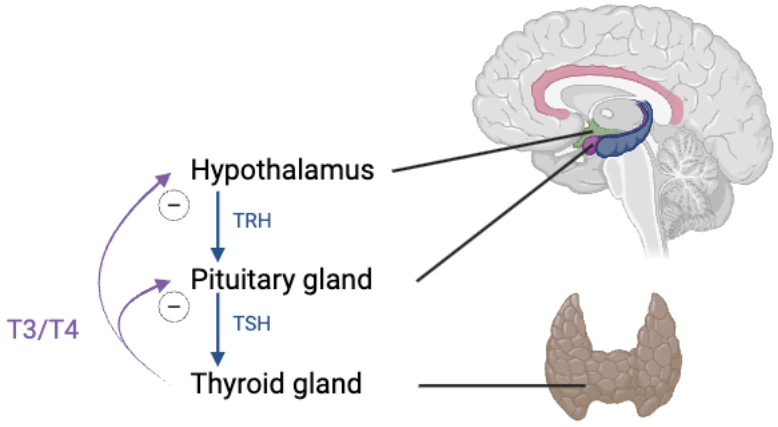

Figure 2. Hypothalamic-pituitary-thyroid (HPT) axis regulation of thyroid gland function

2.3 Thyroid Hormone Regulation

There are two main processes that control the circulating TH concentrations through synthesis, secretion and metabolism allowing for the regulation of TH availability to target cells. The first is the maintenance of stable circulating TH levels through the coordinated action of the HPT axis, controlling TRH and TSH action while simultaneously being negatively controlled by THs already in circulation [32].

The second main process in controlling TH concentrations is via metabolic clearance of THs which reduce the circulating levels of the compounds. Deiodination resulting in the activation and inactivation of the different THs through three iodothyronine deiodinase enzymes (DIO1, DIO2, DIO3), catalyzing the removal of iodine atoms at either the outer phenolic ring (activation pathway) or the inner tyrosyl ring (inactivation pathway) of T4 is one such pathway [**Figure 3**, 34]. DIO1 and DIO2, located in the plasma membrane and endoplasmic reticulum respectively, catalyze the outer ring on T4 contributing to T3 production. DIO1 is responsible for the majority of circulating T3 while DIO2 facilitates the local production of T3 inside cells [35]. DIO3, located

in the plasma membrane, acts as a physiological terminator of TH availability and signaling. DIO3 catalyzes the inner ring deiodination of T4 to form the inactive product rT3 as opposed its metabolically active counterpart, T3 [32]. T3 and rT3 differ in the position of the iodine attached to the tyrosine ring. Due to the competition in receptor binding between the active T3 and inactive rT3, rT3 can bind TH receptors found in targeted tissues which can lead to similar effects as hypothyroidism [36]. T3 converts into 3,5-T2 via DIO1 and 3,3'-T2, a biologically inactive metabolite, via DIO1 and DIO3. rT3 can also be converted into 3,3'-T2 via DIO1 and DIO2 [35].

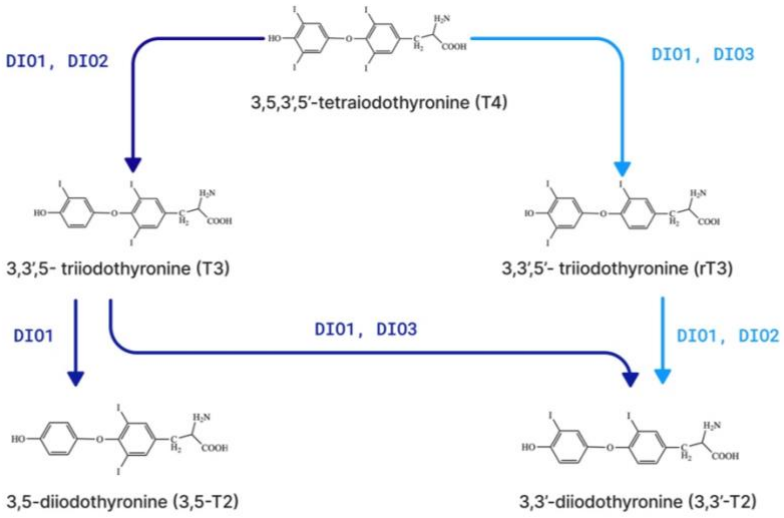

Figure 3. Thyroid hormone metabolism via deiodinase enzymes (created with ChemDraw 21.0.0)

2.4 Phase II Metabolism

Coupled with deiodination reactions, TH reactions are also metabolized through two phase-II reactions, sulfation and glucuronidation, as a means of clearance by decreasing their biological activity, increasing their water-solubility, and increasing excretion into urine or bile [**Figure 4**, 6]. Facilitated by sulfation, T4S is deiodinated to inactive rT3S while the conversion of T4S to T3S is completely blocked [33]. The resulting TH sulfate metabolites suggest their influence on

irreversible inactivation of the thyroid hormone [27, 37]. Sulfated iodothyronines can also act as a T3 reserve from which T3 can be recovered from T3S by tissue sulfatase activity when under low DIO1 activity [6]. TA3 can also be excreted as the main metabolite in bile through sulfation [6]. THs undergo glucuronidation via UDP-glucuronyltransferases (UGT) enzymes and form inactive, non-TR binding TH glucuronides which can be eliminated via urinary and biliary excretion [37]. Increases in excreted T4 glucuronide (T4G) stimulates TSH and thus decreases levels of circulating T4 [27]. Following biliary excretion, T4G can be deconjugated into T4 and may undergo intrahepatic recirculation into the blood, both recycling active TH and regulating their levels [6, 27, 38].

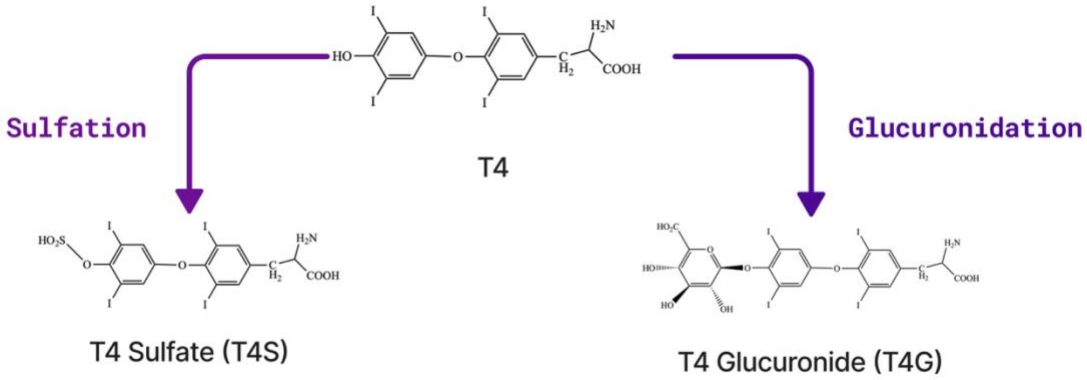

Figure 4. Sulfation and Glucuronidation Mechanisms of T4 (created with ChemDraw 21.0.0)

2.5 Thyroid Hormone Metabolites

2.5.1 Thyronamine

Thyronamines (TAMs) are decarboxylated and deiodinated derivatives of T4 and T3 [**Figure 5**]. Of the thyronamine class, T1AM is an endogenous thyroid hormone [39]. Although its biosynthesis pathway is not fully understood, the expression of thyroid peroxidase (TPO) and

sodium iodine symporter (NIS) outside of thyroid gland can suggest T1AM is formed by extrathyroidal tissues through iodination and the cross-coupling of tyrosine [28, 40]. In rat tissues and human serum, T1AM levels are comparable to total T4 and in rat tissues, greater concentrations than T3. It has been stated that T1AM can be inhibited by antithyroid drugs and decreases under hypothyroidism and also remains low after replacement T4 is administered [28]. Its serum profile, while under T4 withdrawal, indicate either a long T1AM half-life or continuous release into the serum using intracellular hormone deposits [40].

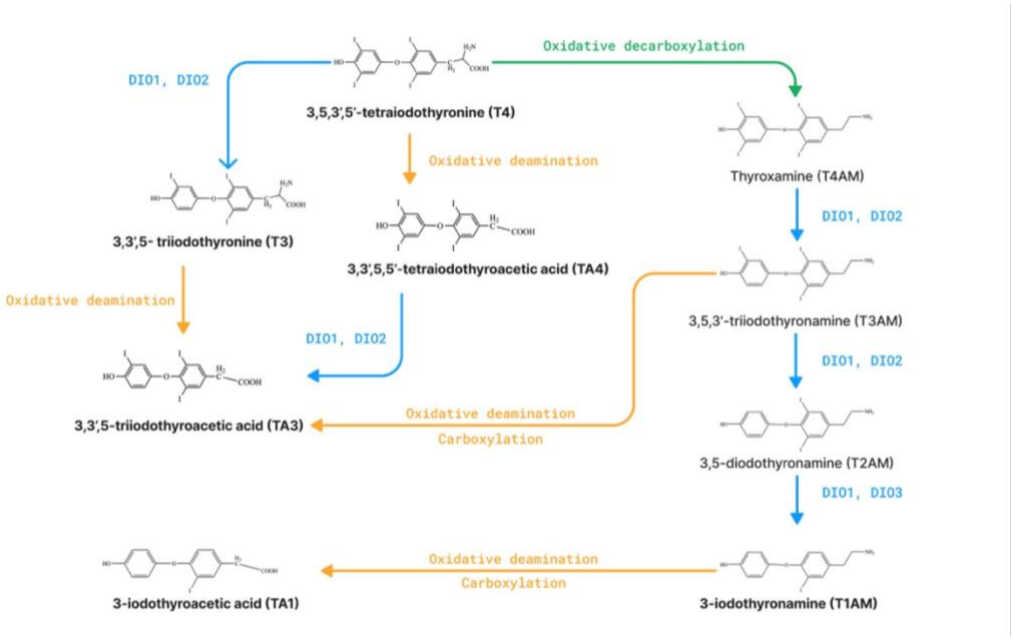

Figure 5. Thyronamine and thyroacetic acid conversion (created with ChemDraw 21.0.0)

2.5.2 Thyroacetic Acids

Iodothyroacetic acids, TA1, TA3 and TA4 are formed from THs and TAMs through an alternative metabolic pathway via oxidative deamination, the removal of an amine group [**Figure 5**, 6]. TA3, the deiodinated metabolite of TA4, can be found in tissues and circulates in blood. TA4 also circulates in blood, but at low nano-molar concentrations [27]. In people, it has been proposed

that TA3 mimics thyroid gland action on TSH, neuronal differentiation, hepatic response, and skeletal metabolic activity, while TA4 inhibits TSH, stimulated aerobic glycolysis and can induce cardiac hypertrophy [4]. While enzymes DIO1 and DO2 direct the deiodination of TA4 to TA3, DIO1 and DIO3 convert TA3 to thyroacetic acid to then be excreted in urine. TA3 can also be excreted as the main metabolite in bile through sulfation [6]. In humans, TA3 and TA4 are glucuronidated faster than their parent compounds, T3 and T4 [33]. TA1 is a major metabolite of T1AM through oxidative deamination and carboxylation and suspected to have a similar pharmacological profile to T1AM and facilitate non-genomic effects of T3 [41]. TA1 was found in human serum at trace amounts, however, were substantially increased in rat serum after being administered T1AM [42].

2.6 Knowledge Gap

The use of exogenous compounds, such as levothyroxine, to improve a horse's metabolic rate, energy balance and overall endurance, can jeopardize horses' safety and well-being, potentially leading to arrhythmias and clinical signs similar to thyrotoxicosis that can have adverse health effects [13, 43]. In horseracing, there is serious concern over the misuse of these agents. Possession of these agents by trainers without a valid prescription for the horse is strictly prohibited. In spite of this prohibition, there have been several trainers sanctioned by the Horseracing Integrity and Welfare Unit for possession of levothyroxine containing products [44]. While the use of these agents is tightly controlled, currently there are no guidelines in place following their detection in biological matrices, such as serum or urine, to allow for sanctions of individuals who misuse the compounds. In order to fill this knowledge gap to identify exogenous levothyroxine in serum samples, it is important to establish the background concentrations of the endogenous hormones

in horses as a reference point. Establishing reference values will provide further understanding of the normal physiological state of equine athletes and aid in detecting any abnormalities in subsequent assessments. This study describes the development and validation of a method for the simultaneous detection of thyroid hormones, their precursors, and their derivatives for clinical testing.

3.0 Materials and Methods

3.1.1 Reference Standards

3,3',5- Triiodothyronine solution, 3,3′,5′-triiodo-L-thyronine solution, L-thyroxine solution, 3,3'5-triiodothyroacetic acid, 3,3',5,5'-tetraiodothyroacetic acid, and 3-iodothyronamine were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). 3,5-Diiodo-L-thyronine and 3,3'-diiodo-L-thyronine were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). 3-Iodo-L-tyrosine and 3,5-diiodo-L-tyrosine were purchased from Toronto Research Chemicals (North York, Ontario, CA). 3-Iodothyroacetic acid was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). The internal standards 3,3',5- triiodo-L-thyronine-$^{13}C_6$ solution, 3,3′,5′-triiodo-L-thyronine-$^{13}C_6$ solution, L-thyroxine-$^{13}C_6$ solution and furosemide-d5 were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) 3,5-diiodo-L-thyronine (4-hydroxyphenyl-$^{13}C_6$) hydrochloride was purchased from Toronto Research Chemicals (North York, Ontario, CA), L-3,3'-diiodothyronine-$^{13}C_6$ solution from Isosciences (Chaska, Minnesota, USA), 3-iodo-tysrosine-$^{13}C_6$ from Santa Cruz Biotechnology (Dallas, Texas, USA), 3,5-diiodotyrosine-$^{13}C_6$ from Acanthus Research (Mississauga, Ontario, CA), and 3-iodothyronamine-d4 Hydrochloride from Muse Chemicals (Paoli, Pennsylvania, USA).

3.1.2 Reagents

Methanol (HPLC grade >99.9%), acetonitrile (HPLC grade >99.9%), water (HPLC grade) and formic acid (~98%) were purchased from Honeywell – Burdick & Jackson (Muskegon, Michigan, USA). Methanol (optima grade) and ammonium hydroxide (ACS reagent grade) were purchased from Fisher Scientific (Pittsburgh, Pennsylvania, USA). Citric acid (ACS reagent >99.5%), L-ascorbic acid (99%) and activated charcoal were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Dithiothreitol (molecular biology grade) and deionized (DI) water from Nanopure water system were purchased from ThermoFisher Scientific (Waltham, Massachusetts, USA).

3.1.3 Glassware

Glass culture tubes (12 x 75mm) and autosampler vials with sealed GL insert (12 x 32mm) were purchased from Duran Wheaton Kimble Life Sciences (Millville, New Jersey, USA). Glass Pasteur pipets (5 ¾") were purchased from Fisher Scientific (Pittsburgh, PA, USA).

3.1.4 Population Samples

Serum samples were acquired 30 to 90 minutes prior to racing from a cohort of healthy equine athletes at racetracks in Southern California. The study encompassed a sample population comprising of 105 geldings, 103 mares, and 103 stallions, ranging in age from 2 to 8 years.

3.2 Preparation of Stock Solutions

3.2.1 Reference and Internal Standard Preparation

Purchased solutions were received in methanol with 0.1N NH_3 at 100 µg/mL. Reference standards for each target analyte were prepared in methanol at 1 mg/mL. Purity correction

calculations were applied to compounds, as needed, to calculate the methanol volume needed for the raw material. Target compounds were mixed into two working solution and diluted with methanol to achieve a final concentration as specified in **Table 2**.

Table 2. Working solution concentrations.

Working Standard Solution	Concentration in solution
1	250 pg/mL T1AM, 3,5-T2, 3,3'-T2, TA3
	500 pg/mL MIT, DIT, TA1
	2.5 ng/mL TA4
	5 ng/mL T3, rT3
	50 ng/mL T4
2	2.5 ng/mL
	5 ng/ml MIT, DIT, TA1
	15 ng/mL TA4
	30 ng/mL T3, rT3
	400 ng/mL T4

An internal standard solution was made by combining internal standards to concentrations equal to used was 2.5 ng/mL for MIT-$^{13}C_6$; 1 ng/mL for DIT-$^{13}C_6$, T1AM-d4 Hydrochloride, 3,5-T2-4-hydroxyphenyl-$^{13}C_6$ hydrochloride L-3,3'-diiodothyronine-$^{13}C_6$; 12.5 ng/mL for T3-$^{13}C_6$ and rT3-$^{13}C_6$; 20 ng/mL for furosemide-d5; and 200 ng/mL for T4-$^{13}C_6$.

3.2.2 Extraction Solution Preparation

An antioxidant solution of citric acid, ascorbic acid and dithiothreitol at equal concentrations of 25 mg/mL was prepared in deionized water. A 5% MeOH 2% ammonia solution was prepared in HPLC grade water. A 20% formic acid solution was prepared in methanol and mixed. A 10% acetonitrile with 0.1% ammonium hydroxide solution was prepared in HPLC grade water.

3.3 Calibrator and Quality Control Preparation

Charcoal stripped horse serum served as a negative control to generate the calibration curve and quality control samples [45]. Pooled horse serum (100 mL), which was prepared in batches as needed, was incubated in an ultrasonic cleaning bath (Branson 5510, Fisher Scientific, Pittsburgh, PA, USA) for 30 minutes at 56°C. Activated charcoal (6.5g) was added and the sample and stirred gently overnight at 4°C with a stir bar. Serum was centrifuged (Sorvall ST 40R, ThermoFisher Scientific, Waltham, Massachusetts, USA) for 2 hours at 4,000 x g and filtered under vacuum through a 0.2 µm Rapid-Flow bottle top filter (ThermoFisher Scientific, Waltham, Massachusetts, USA). It was then transferred into 50 mL centrifuge tubes (Corning Science, Glendale, Arizona, USA) to be stored frozen (-20°C) until use. A calibration curve was made from a serum sample (500 µL) using the working solutions to achieve final concentration specified in **Table 3.**

Quality control samples were prepared at low, mid, and high levels specified in **Table 4.**

Table 3. Volumes and concentrations in spiked calibration curve.

Level	Volume of Working Solution spiked into 0.5 mL serum	Concentration in 0.5 mL serum
1	10 µL of WS #1 25 µL of IS solution[A]	5 pg/mL T1AM, 3,5-T2, 3,3'-T2, TA3 10 pg/mL MIT, DIT, TA1 50 pg/mL TA4 100 pg/mL T3, rT3 1 ng/mL T4
2	25 µL of WS #1 25 µL of IS solution[A]	12.5 pg/mL T1AM, 3,5-T2, 3,3'-T2, TA3 25 pg/mL MIT, DIT, TA1 125 pg/mL TA4 250 pg/mL T3, rT3 2.5 ng/mL T4
3	50 µL of WS #1 25 µL of IS solution[A]	25 pg/mL T1AM, 3,5-T2, 3,3'-T2, TA3 50 pg/mL MIT, DIT, TA1 250 pg/mL TA4 500 pg/mL T3, rT3 5 ng/mL T4
4	10 µL of WS #2 25 µL of IS solution[A]	50 pg/mL T1AM, 3,5-T2, 3,3'-T2, TA3 100 pg/mL MIT, DIT, TA1 300 pg/mL TA4 600 pg/mL T3, rT3 8 ng/mL T4
5	20 µL of WS #2 25 µL of IS solution[A]	100 pg/mL T1AM, 3,5-T2, 3,3'-T2, TA3 200 pg/mL MIT, DIT, TA1 600 pg/mL TA4 1.2 ng/mL T3, rT3 16 ng/mL T4
6	50 µL of WS #2 25 µL of IS solution[A]	150 pg/mL T1AM, 3,5-T2, 3,3'-T2, TA3 300 pg/mL MIT, DIT, TA1 900 pg/mL TA4 1.8 ng/mL T3, rT3 24 ng/mL T4
[A] – Concentration of Internal Standards in 0.5 mL serum - 125pg/mL MIT-$^{13}C_6$, 50pg/mL DIT-$^{13}C_6$, T1AM -d4 Hydrochloride, 3,5-T2-4-hydroxyphenyl-$^{13}C_6$ hydrochloride, L-3,3'-diiodothyronine-$^{13}C_6$; 625 pg/mL T3-$^{13}C_6$, rT3-$^{13}C_6$; 1ng/mL furosemide-d5; 10ng/mL T4-$^{13}C_6$		

Table 4. QC spiking volumes and concentrations.

QC Level	Volume of Working Solution spiked into 0.5 mL serum	Concentration in 0.5 mL serum
Low	40 µL of WS #1	40pg/mL MIT, DIT, TA1; 20pg/mL T1AM, 3,5-T2, 3,3'-T2, TA3; 200pg/mL TA4; 400pg/mL T3, rT3; 4ng/mL T4
Mid	15 µL of WS #2	150pg/mL MIT, DIT, TA1; 75pg/mL T1AM, 3,5-T2, 3,3'-T2, TA3; 450pg/mL TA4; 900pg/mL T3, rT3; 12ng/mL for T4
High	40 µL of WS #2	400pg/mL MIT, DIT, TA1; 200pg/mL T1AM, 3,5-T2, 3,3'-T2, TA3; 1.2ng/mL TA4; 2.4ng/mL T3, rT3; 32ng/mL T4

3.4 Sample Preparation

Prior to preparation, the serum was thawed at room temperature. After thawing, 500 µL of serum was transferred into a 12 x 75 mm glass culture tube. Antioxidant mix (120 µL) and internal standards (25 µL) were added, and samples were incubated for 1 hour. Afterward, 1.9 mL cold acetonitrile was added, the samples were then vortexed (Fisher Scientific, Pittsburgh, PA, USA) and incubated at 4°C for 1 hour. Samples were then then centrifuged at 4000 x g for ten minutes at 4°C. Using glass Pasteur pipets, the supernatant was transferred to new 12 x 75 mm tubes. Samples were evaporated to dryness under nitrogen in a TurboVap LV (Caliper Life Sciences, Hopkinton, Massachusetts, USA) at ~ 40±5°C under a pressure of 10 psi. Dried samples were re-

dissolved with 450 µL of 5% methanol and 2% ammonia in water and vortexed, followed by the addition of 2 mL of deionized water.

3.5 Solid Phase Extraction

Evolute Express strong anion exchange (AX) solid-phase extraction (SPE) cartridges (60 mg, 3 mL, Biotage, San Jose, California, USA) were placed on a Cerex Speedisk positive pressure manifold (Cera Inc., Baldwin Park, California, USA). The SPE columns were conditioned with 3 mL of methanol and rinsed with 3 mL of deionized water. The samples were introduced to the conditioned columns and washed with 3 mL of deionized water and then 3 mL of methanol. Target analytes were eluted from the columns with 20% formic acid in methanol under gravity, followed by low pressure (~1psi) and collected into new 12 x 75 mm tubes. The tubes were evaporated to dryness under nitrogen in a TurboVap LV at ~ 40±5°C under a pressure of 10 psi. After reconstitution with 100 µL of 10% acetonitrile in water, samples were vortexed and centrifuged at 4000 x g for one minute at 4°C.

3.6 LC-MS/MS Analysis

LC-MS analysis used an Agilent 1100 HPLC system coupled with a Q-Exactive High Resolution mass spectrometer (ThermoFisher Scientific, Waltham, Massachusetts, USA). Sample extracts were introduced to the HPLC system with a 75 µL injection volume. Compounds were separated over a 22-minute analytical run with Kinetex 2.6 µm PFP 100Å LC column, 100 x 2.1 mm (Phenomenex, Torrance, CA, USA). Gradient elution was achieved using reversed phase chromatography with mobile phase A: acetonitrile with 0.1% formic acid and mobile phase B: water with 0.1% formic acid. At a 0.4 mL/min flow rate, the initial conditions were 0.00 min, 1%

A held for 2.50 minutes; 2.50 min 50% A; 5.00 min 60% A; 6.00 min 62.0% A; 10.00 min 65% A; 11.00 min 75% A; 13 min 98% A that was held for 5 minutes; then 18.01 min 1% A for 4 minutes. The compounds were then introduced to the mass spectrometer using positive and negative mode heated electrospray ionization. The HPLC flow was diverted to waste for the first 3.30 min when it was introduced to the MS source and at 8.00 min when the flow was sent back to waste following acquisition. The mass spectrometer operated in parallel reaction monitoring (PRM) mode for the parent and product ions and their corresponding collision energies for the compounds shown in **Table 5.** Using Xcaliber Tune software, neat standards (10ng/µL) of each compound were directly infused with 50% mobile phase A and 50% mobile phase B at 0.2 mL/min for compound optimization. Source parameters were: spray voltage 3500V, sheath gas 50 arbitrary units, auxiliary gas 20 arbitrary units, sweep gas 1 arbitrary unit, S-lens RF level 50 arbitrary units, capillary temperature at 320°C, and auxiliary gas heater temperature at 200°C. Xcaliber software (version 4.0.27.19) from Thermo Scientific (Waltham, Massachusetts, USA) was used to control the LC-MS system and for data review.

Table 5. LC-MS method settings. Retention time (min), electrospray ionization mode (+/-), Precursor ion (m/z), Product ion (m/z), Quantifying ion **bolded**, and normalized collision energy (NCE) of targeted compounds.

Analyte	Retention Time (min)	ESI Mode	Precursor ion (m/z)	Product ion (m/z)	NCE
T4	7.01	+	777.6945	**731.6885** 604.7841 350.9749	30
L-T4 - $^{13}C_6$	7.01	+	783.7150	**737.7082** 610.8042 356.9951	30
T3	6.59	+	651.7976	**605.7912** 478.8875 592.7595 507.8665	35
T3 - $^{13}C_6$	6.59	+	657.8188	**611.8123** 484.9074 598.7793 513.8864	30
rT3	7.11	+	651.7976	**605.7912** 478.8875 592.7595 507.8665	35
rT3 - $^{13}C_6$	7.11	+	657.8188	**611.8123** 484.9074 598.7793 513.8864	30
3, 5-T2	6.13	+	525.9011	**479.8953** 381.9697 352.9914	30
3, 5-T2- 4-hydroxyphenyl-$^{13}C_6$	6.13	+	531.9216	**327.1337** 485.9149 299.1389	30
3, 3'-T2	6.47	+	525.9011	**479.8953** 381.9697 352.9914	30
L-3, 3'-T2 - $^{13}C_6$	6.47	+	531.9216	**391.2425** 485.9156 472.8843	30
3-T1AM	6.91	+	356.0144	**338.9870** 309.2424 212.0831	20
3-T1AM- d4 Hydrochloride	6.91	+	362.0343	**345.0070** 218.1033 189.1125	20
TA4	7.47	-	702.6635	**126.9035**	20
TA3	7.05	-	576.7659	**126.9035**	20
TA1	6.39	-	368.9620	**126.9035**	10
Furosemide-d5	6.12	-	334.03156	**290.0425** 315.1965	15
MIT	5.19	+	307.9778	**290.9512** 261.9723	15

MIT - $^{13}C_6$	5.17	+	313.9976	**297.1903** 313.2049 268.1471	15
DIT	5.54	+	433.8749	**387.8689** 416.8479 263.0429	20
DIT - $^{13}C_6$	5.54	+	439.8947	**393.8888** 422.8677	20

3.7 Method Validation

Linearity, accuracy, precision, LOD, LOQ, matrix effect, recovery, dilutional linearity, carryover and stability were all assessed as validation parameters. Linearity was assessed from a 6-point calibration curve using spiked negative control serum samples. Accuracy (%) and precision (relative standard deviation) were assessed at low, mid, and high levels (n=6 per level) over 3 days. Limit of detection was assessed by spiking negative control serum with concentrations 75%, 50%, 25% and 10% below the lowest calibration point and evaluated by the lowest concentration detectable with a signal to noise ratio over 3. Limit of quantitation was determined by the lowest detectable concentration with a signal to noise ration greater than 10 and an accuracy within 20% of the theoretical value. Matrix effect was assessed comparing neat standards at each QC level (n=6 per level) to extracted negative control samples spiked at equivalent concentrations. Carryover was assessed by running a blank sample (10% acetonitrile in water) after the highest calibration point. Dilutional linearity was assessed by spiking 1 mL of negative control serum 8x the highest calibration point and diluting the sample to 15 mL, then aliquoting 0.5 mL of serum (n=6) for analysis. The impact of serum separator tubes was evaluated by spiking 5 mL of negative control serum at the mid QC level, aliquoting half into a serum separator tube, and comparing the recovery. Stability samples were prepared at the mid QC level, without internal standards, and assessed (n=3 per time point) at t=0 and 24hr at room temperature, t= 24hr, 5 days and 2 weeks at 4°C, t= 5 days, 2 and 4 weeks at -20°C. A freeze-thaw cycle was also evaluated, samples (n=3)

were stored in -20°C until being thawed to room temperature and repeated the freeze-thaw cycle for a total of 3 times. Internal standards were added on the day of analysis. Stability was also assessed by comparing extracted negative control samples at each QC level (n=2 per level) that were stored at room temperature for two weeks to samples measured the day of extraction.

4.0 Results

As shown in **Figure 6**, the charcoal-stripping method effectively removed significant quantities of naturally occurring thyroid hormones from pooled serum obtained from horses pre-race.

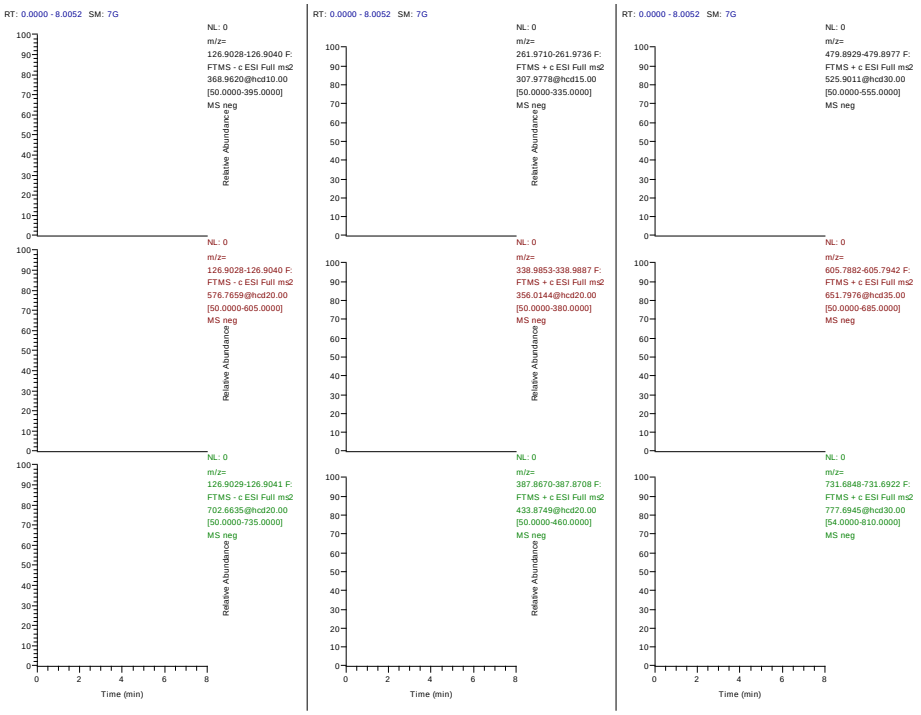

Figure 6. Charcoal-stripped horse serum as a negative control medium.

The outcomes of efforts to achieve chromatographic separation, refine retention times, secure an adequate number of scans, and optimize product ions through normalized collision energy are illustrated in **Figure 7**.

The linearity, LOD, LOQ, and dilutional accuracy were determined with the regression correlation coefficient, R^2, greater than 0.98 and with an applied 1/X weight for all compounds (**Table 6**).

After monitoring a blank sample over the course of 10 days, following the highest calibration point, carryover was observed by 2% MIT-$^{13}C_6$, 0.2% T4, and 0.08% T4-$^{13}C_6$. In response, two blank samples were run following the analysis of the highest calibration point and the QC high. This led to the second blank being free from carryover residue or any lingering analytes of interest. The inter-and intra-day accuracy and precision (%CV) was evaluated by quality control samples at each QC level (n=6 per level) (**Table 7**). Accuracy was calculated from the average of each concentration level divided by the theoretical concentration. Precision was calculated by the dividing the standard deviation of each QC level by the mean. As show in **Table 8**, recovery was calculated by the ratio of the average peak areas of each QC level (n=6 per level) of extracted samples to the post-extracted spike samples. Matrix effects were determined by calculating the ratio of the peak area from samples spiked post-extract to the peak area from neat standards at equivalent concentrations.

Stability assessments were conducted at the mid QC level, results are shown in **Table 9**. For most compounds, there was no significant degradation observed over a two-weeks, regardless of storage conditions at 2-8°C, -20°C, or room temperature post-extraction, or after three freeze-thaw cycles. Exceptions to this trend were 3-T1AM, TA4, TA3, and TA1, which consistently exhibited

instability throughout the stability evaluation, except for post-extraction phase. The serum separator tube had minimal to no impact on the quantitation of analytes, except for T1AM.

Summarized in **Table 10**, the population study encompassed 105 geldings, 103 mares, and 103 stallions, with each target analyte quantified. The data includes: the range, determined by the mean +/- 2SD ; the median; and the count of positive identifications for each analyte. 3-T1AM was not detected in any of the samples.

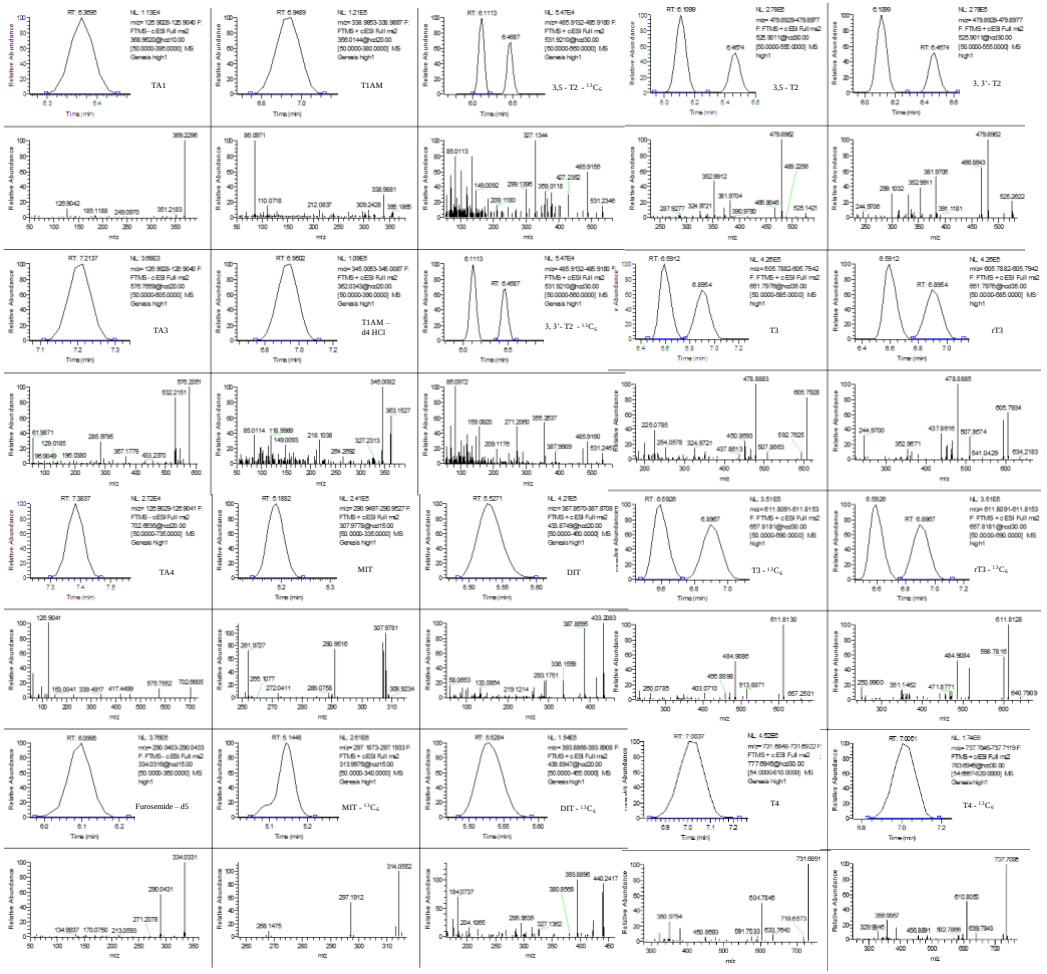

Figure 7: Extracted ion chromatogram and spectra for all compounds at QC high level

Table 6. Linearity, LOD, LOQ, and dilution accuracy of each compound of interest.

Compound	Linearity		LOD (pg/mL)	LOQ (pg/mL)	Dilutional Linearity (% Accuracy)
	Equation	R^2			
T4	Y = 0.106721+0.000242667*X	0.9928	100	1000	97.92
T3	Y = 0.0122933+0.000832475*X	0.9968	10	100	98.42
rT3	Y = 0.0107279+0.000830159*X	0.9966	10	100	97.97
3, 5 – T2	Y = -0.0235316+0.0366236*X	0.9973	2.5	5	98.00
3, 3’ – T2	Y = -0.0253679+0.0366569*X	0.9973	1.25	2.5	98.00
3 – T1AM	Y = -0.00380701+0.0149755*X	0.9927	1.25	5	97.22
TA4	Y = 0.000600429+0.00072274*X	0.9909	5	50	96.58
TA3	Y = -0.00641057+0.00168133*X	0.9887	2.5	5	98.08
TA1	Y = -0.00176073+0.000541916*X	0.9939	7.5	25	98.58
MIT	Y =-0.00256505+0.0139371*X	0.9993	2.5	5	93.84
DIT	Y = 0.0172484+0.0115009*X	0.9996	1	5	99.78

Table 7. Precision and accuracy measured at each QC level (n=6 per level, per day) over a 3-day period.

Compound	Day 1 - Precision			Day 1 - Accuracy			Day 2 - Precision			Day 2 - Accuracy			Day 3 - Precision			Day 3 - Accuracy			Inter-day Precision			Inter-day Acc.		
	low	mid	high	low	mid	high	low	mid	high	low	mid	high	low	mid	high	low	mid	high	low	mid	high	low	mid	high
T4	13.3	10.3	9.27	92.2	83.7	91.8	1.45	12.1	4.12	111	85.9	89.5	3.09	4.39	2.25	103	107	99.8	10.4	14.6	7.35	102	92.3	93.7
T3	8.57	11.1	9.85	95.8	90.4	93.5	3.60	12.5	2.29	107	88.4	91.3	3.31	4.41	2.67	89.0	98.2	102	9.66	10.3	7.51	97.4	92.3	95.6
rT3	13.6	14.4	10.9	94.9	86.6	94.2	3.84	15.0	1.75	110	87.3	92.9	6.99	6.54	2.04	107	104	102	10.1	14.4	7.41	104	92.7	96.5
3, 5 – T2	11.6	15.9	9.09	105	86.6	96.0	8.81	13.6	4.30	111	88.0	97.9	6.02	4.54	5.61	95.8	107	99.4	10.7	14.8	6.38	104	93.7	97.8
3, 3’ – T2	6.98	5.69	12.9	96.9	88.5	90.4	4.10	12.7	7.39	104	90.4	96.3	7.51	9.18	7.11	113	111	99.5	8.90	14.1	9.65	105	96.6	95.4
3 – T1AM	19.9	8.89	11.8	83.1	89.9	99.2	5.17	9.34	3.67	85.1	90.7	104	4.28	5.40	2.24	105	106	103	15.5	11.0	6.98	91.2	95.7	102
TA4	12.7	15.1	11.9	104	94.9	81.3	17.2	16.3	18.7	103	79.2	145	16.9	13.9	21.0	98.1	82.0	72.2	15.1	16.4	37.8	101	85.4	99.4
TA3	14.8	18.6	11.5	113	99.1	83.5	11.8	16.1	26.5	132	88.3	82.2	6.01	15.2	12.8	93.6	114	87.8	18.4	19.1	17.1	113	101	84.5
TA1	39.8	9.49	12.0	64.9	92.4	98.3	17.7	18.8	28.5	136	86.9	92.7	16.4	11.8	16.6	110	120	119	28.5	19.7	16.5	110	101	104
MIT	8.35	10.2	10.6	85.8	87.4	86.6	5.23	13.1	8.99	102	88.4	83.1	4.36	11.2	6.63	111	102	94.6	8.35	10.2	10.6	85.8	87.4	86.6
DIT	17.7	14.9	13.2	93.5	95.2	101	7.83	19.0	6.41	103	86.8	96.6	7.34	3.09	2.20	112	104	101	13.0	14.6	8.41	103	95.3	99.6

Table 8. Recovery and matrix effect of each compound of interest.

Compound	Recovery (%)			Matrix Effect (%)		
	QC low	QC mid	QC high	QC low	QC mid	QC high
T4	61	56	69	62	45	60
T3	53	54	68	94	81	81
rT3	59	57	70	107	84	91
3, 5 – T2	59	57	71	154	141	135
3, 3’ – T2	62	59	71	89	83	82
3 – T1AM	53	65	67	189	154	146
TA4	108	63	92	13	23	23
TA3	60	56	66	108	38	52
TA1	57	67	78	82	59	61
MIT	61	59	68	140	104	104
DIT	64	57	68	160	126	96

Table 9. Stability of compounds at the QC mid level and various temperature conditions, stability of samples at room temperature 2 weeks post-extraction, effects of gel in serum separator tube.

Compound	Room Temperature (% Accuracy)					2-8°C (% Accuracy)			-20°C (% Accuracy)				Serum Separator (% Accuracy)
	t = 0hr	t = 24hr	t = 2week post-extraction			t = 24hr	t = 5day	t = 2week	t = 5day	t = 2week	t =4week	Freeze/thaw Cycle	
			low	mid	high								
T4	92.2	97.4	120	77.4	90.7	97.5	101	103	104	98.1	85.9	83.4	123
T3	108	95.6	113	79.5	94.9	93.6	95.4	96.1	92.8	99.8	100	90.1	117
rT3	98.8	88.6	90.1	86.9	90.4	85.0	85.2	95.1	94.9	86.0	91.5	98.9	112
3, 5 – T2	91.2	93.1	112	80.2	90.3	82.4	97.7	92.5	98.5	96.0	92.6	98.5	124
3, 3’ – T2	87.8	95.0	127	73.4	87.2	93.9	97.7	97.2	99.0	89.3	98.9	90.8	134
3 – T1AM	92.0	62.8	96.2	86.0	95.7	67.8	75.3	60.7	93.9	91.6	87.4	69.0	NF
TA4	96.0	66.2	97.8	99.5	99.5	75.3	68.9	81.9	70.8	70.8	61.4	64.1	96.8
TA3	76.9	57.3	94.5	83.8	91.6	79.0	56.9	94.6	48.5	48.5	22.7	31.2	94.6
TA1	98.4	88.9	125	84.5	110	77.4	65.3	76.7	88.8	49.2	63.3	56.0	105
MIT	98.1	69.5	105	71.3	82.0	76.0	96.9	78.1	99.5	64.4	60.0	47.9	87.9
DIT	95.0	80.9	124	84.3	101	80.1	98.3	99.6	99.4	99.4	91.6	71.4	98.9

Table 10. The range, mean ± 2 standard deviations, the minimum to maximum values, and the median of detected thyroid hormone values of 311 serum samples, categorized by gender. NF = not found. * = single outlier omitted from calculation.

Compound	Gelding (105)			Mare (103)			Stallion (103)		
	Range (pg/mL)	Min. – Max. (pg/mL)	Median	Range (pg/mL)	Min. – Max. (pg/mL)	Median	Range (pg/mL)	Min. – Max. (pg/mL)	Median
T4	18100 ± 14200 (105)	7,290 – 41,600	17,400	18200 ± 15600 (103)	2,130 – 44,300	17,200	14000 ± 12500 (103)	2,360 – 40,800	13,200
T3	1340 ± 1500 (105)	573 – 6,540	1,140	1180 ± 1230 (103)	54.8 – 3,120	1,030	1030 ± 1200 (103)	312 – 3,560	890
rT3	665 ± 521(105)	139 – 1,740	619	722 ± 653 (103)	160 – 1,830	644	631 ± 581 (103)	139 – 1,740	550
3, 5 – T2	5.19 ± 8.41 (38)	<LOQ – 21.2	<LOQ	6.40 ± 7.04 (23)	<LOQ – 13.6	6.78	5.82 ± 6.58 (26)	<LOQ – 15.8	5.31
3, 3’ – T2	66.8 ± 52.6 (105)	25.8 – 151	62.3	66.9 ± 62.1 (103)	2.35 – 175	63.0	62.6 ± 57.8 (103)	11.5 – 153	58.2
3 – T1AM	NF	NF	NF	NF	NF	NF	NF	NF	NF
TA4	<LOQ (56)	<LOQ – 341	<LOQ	<LOQ (77)	<LOQ – 364	<LOQ	<LOQ (70)	<LOQ – 510	<LOQ
TA3	<LOQ (5)	<LOQ – 8.85	<LOQ	<LOQ (11)	<LOQ	<LOQ	<LOQ (7)	<LOQ	< LOQ
TA1	<LOQ (7)	<LOQ – 50.4	<LOQ	<LOQ (5)	<LOQ	<LOQ	<LOQ (3)	<LOQ	<LOQ
MIT	41.2 ± 96.5 (87)	4.51 – 308	25.8	50.6 ± 125 (90)*	<LOQ – 370	29.8	42.4 ± 121 (91)	2.97 – 1,160	20.6
DIT	70.4 ± 162 (92)*	<LOQ – 1,360	51.4	54.3 ± 127 (90)*	<LOQ – 363	37.3	56.2 ± 157 (94)	<LOQ – 2,180	30.3

5.0 Discussion

The development of reference intervals for thyroid hormones in equine athletes is crucial for ensuring the health and safety of the horses. Achieving this requires a sensitive, selective, and highly reproducible analytical method. In this study, our choice to measure total thyroid hormones, instead of free, unbound hormones was driven by the aim of conducting population-level screening. This approach allowed for a comprehensive assessment of the overall concentration of thyroid hormones present in the bloodstream while considering factors such as cost-effectiveness and the framework for assessing health.

The selection of mobile phases comprising 0.1% formic acid in water and acetonitrile for this experiment aligns with established practices [4, 7, 20, 26]. Martínez Brito et. al noted better responses with formic acid for T4, 3,5-T2, 3,3'-T2, T3, rT3, and T4 when compared to ammonium formate, ammonium fluoride and acetic acid. While another paper compared results of unfavorable intensities and ion suppression for the acid metabolites and poor T1AM response [26].

Precursor and product ion masses and collision energies were optimized for each compound via T-infusion of neat standards (10 ng/µL) at 5µL/min with a 50/50 composition of mobile phase A/B at 0.4 mL/min into the Q-Exactive. All compounds were infused using positive mode analysis, however due to their acidic properties, TA1, TA3, and TA4 were examined using negative mode. This allowed for the formation of stable and easily detection anions. In our study, precursor ions for TA4 and TA3 were assessed through a carboxylic acid α-elimination reaction and consistently observed their ionization spectra [26]. However, for TA1, the ion resulting from the elimination reaction was non-specific and appeared inconsistently during direct infusion. Following direct infusion, neat standards were injected into the LC-MS to determine relative retention times for each compound in full scan mode. Subsequently, compounds, along with their respective

normalized collision energies and retention times, were added to the PRM inclusion list.

Achieving chromatographic separation between isomers was a challenge. In this method, there are two pairs of molecules with identical formulas but differing atomic arrangements. Due to their similar properties, isomers tend to elute under comparable conditions, such as the percentage of the organic phase, and at approximately the same elution times. Initially, a Kinetex EVO C18 Core-shell 2.6 µm HPLC column (Phenomenex, Torrance, CA, USA) was assessed. However, when compared to the pentafluorophenyl column, elution separation was improved because of its high degree of steric selectivity in separating halogen-containing analytes and structural isomers. Considering that the majority of compounds exhibited elution within the 6-8 minute timeframe using a 62-65% organic phase, a more gradual organic phase increase was evaluated, 62-65% from 6-10 minutes. Valley detection was enabled in the processing method with an expected width of 1.0 second. To adhere to the HPLC pressure limit of 400 bar while maintaining a flow rate of 0.400 mL/min, column was raised to 30°C to prevent exceeding this limit. During method development, a Thermo Finnigan TSQ Quantum Ultra (Thermo Scientific, Waltham, MA, USA) was used. While the Quantum Ultra exhibited high selectivity for targeted quantitation due to being triple quadrupole instrument, the Q Exactive is a high-resolution mass spectrometer with higher resolving power and sensitivity, accurate mass measurement and ability to provide more detailed structural information. However, due to the Q Exactive's polarity switching time of approximately 500 ms, compounded by the shared elution times of some compounds, polarity switching was insufficient with not enough scans were being collected over a chromatographic peak. To address this issue, retention times were carefully monitored after each run during method development so that the negative PRM runtime could be constricted. To mitigate the impact of polarity switching and encourage the instrument to group the scans together, loop counts were added to both positive

and negative PRM scan groups. This adjustment was aimed to minimize the overlap between the positive and negative PRM scan groups and increase the acquired scans.

The observed carryover values were deemed insignificant, showing no detectable amounts within the relevant concentration ranges of interest, and lower than values reported in existing literature [4]. This may be attributed to the prolonged gradient time, both with organic, followed by aqueous after ionization. To account for matrix effects, compounds were spiked into negative control serum and extracted using the Express AX cartridge. Selected ions for peak identification were determined after comparing the peak area and responses from infused neat standards, injected neat standards, and extracted samples.

The data points for the calibration curve were initially derived from reference ranges shown in **Table 1**. However, adjustments were made during method development after analyzing a subset of horse samples. Detection limits were determined by analyzing concentrations that were 75% to 10% below the lowest point on the calibration curve (**Table 6**). For most compounds, limits of detection were found to be at or below 10 pg/mL, with the exception of T4 which was 100 pg/mL. It is worth nothing that stopping at 10% below the lowest calibrator is a limitation of this approach. Nevertheless, these detection limits remained below the majority of currently published thresholds, as shown in **Table 11**. All limits of quantitation for compounds are at or below the first calibrator level and below.

To compensate for losses incurred during sample preparation and extraction and closely mimic target analyte behavior, specific internal standards were employed. Stable isotope labeled internal standards used included: 3,3',5- triiodo-L-thyronine-$^{13}C_6$, 3,3′,5′-triiodo-L-thyronine-$^{13}C_6$, L-thyroxine-$^{13}C_6$, 3,5-diiodo-L-thyronine (4-hydroxyphenyl-$^{13}C_6$) hydrochloride, L-3,3'-diiodothyronine-$^{13}C_6$, 3-iodo-tysrosine-$^{13}C_6$, 3,5-diiodotyrosine-$^{13}C_6$, 3-iodothyronamine-d4

Hydrochloride, and furosemide-d5. Due to the unavailability of ready-made stock for the acidic analytes, furosemide-d5 was employed as an internal standard. Furosemide-d5 exhibited no interference with the target analytes in the extracted samples and was chosen for its compatibility with negative mode ionization. This ensured that both internal standards and target analytes shared the same ionization polarity, enhancing the data's comparability.

Recovery and matrix effects for each QC level (n = 6 per level) are shown in **Table 8**. Recovery of extracted analytes were calculated by comparing the average area counts between samples spiked pre-extraction to those spiked post-extraction at equal concentrations. Recoveries for T3, rT3, and T4 showed modest improvements when compared to published data that utilized protein precipitation for sample clean-up, yielded recovery rates within the 40-60% [24]. In contrast, another study, similar to this one, incorporated both protein precipitation and SPE, reporting comparable recovery values for 3,5-T2 and T3, while revealing slight enhancements, in this method, for the recovery of T4, rT3, 3,3'-T2, TA4 and TA3 [4]. In most cases, matrix effects were highest at the QC low level. At the QC mid level, T4, T3, rT3, 3,3'-T2, TA4, TA3, and TA1 displayed values above 100, signifying ion enhancement within the sample matrix. Conversely, 3,5-T2, 3-T1AM, MIT, and DIT were below 100, indicating ion suppression. This aligns with findings by Carcelén et. al, who also observed ion suppression in urine samples for MIT and DIT [10]. In a previous study, authors found no matrix effects for T4, T3, rT3, and a T2 isomer using a HyperSep C18 SPE and low-resolution LC-MS [7].

Table 11. Limits of Detection found in literature for analytes of interest.
[a] LOQ, [b]Instrument LOD in standard solution, [c]LOD spiked into gill matrix extract, LLE = liquid-liquid extraction, SPE = solid phase extraction, PPT = protein precipitation

Compound	Sample Pretreatment Approach	Extraction Technique	Detection Method	LOD (ng/mL)	Citation
T4	Chemical Hydrolysis: E. coli, H. pomatia Acetonitrile Ethyl Acetate --- 0.1% Formic Acid in Water 6M HCl, heated 50°C Enzymatic digestion with urea, heated 37°C --- Urea, heated 50°C	LLE SPE HybridSPE RIA SPE SPE PPT --- SPE	LC-MS/MS LC-MS/MS LC-MS/MS γ-scintillation counter LC-MS/MS LC-MS/MS LC-MS/MS LC-MS/MS LC-MS/MS	0.130-0.170 0.08-0.130[a] 0.170 2.50 0.0388 < 0.42 < 0.001[b] 0.2 [c] 0.07	(26) (4) (25) (25) (7) (20) (24) (24) (23)
T3	Chemical Hydrolysis: E. coli, H. pomatia Acetonitrile Ethyl Acetate --- 0.1% Formic Acid in Water 6M HCl, heated 50°C Enzymatic digestion with urea, heated 37°C --- Urea, heated 50°C	LLE SPE HybridSPE RIA SPE SPE PPT --- SPE	LC-MS/MS LC-MS/MS LC-MS/MS γ-scintillation counter LC-MS/MS LC-MS/MS LC-MS/MS LC-MS/MS LC-MS/MS	0.130-0.170 0.029-0.467[a] 0.16 0.07 0.00325 < 0.24 < 0.001[b] 0.2 [c] 0.029	(26) (4) (25) (25) (7) (20) (24) (24) (23)
rT3	Chemical Hydrolysis: E. coli, H. pomatia Acetonitrile Ethyl Acetate Enzymatic digestion with urea, heated 37°C --- Urea, heated 50°C	LLE SPE HybridSPE PPT --- SPE	LC-MS/MS LC-MS/MS LC-MS/MS LC-MS/MS LC-MS/MS LC-MS/MS	0.130-0.170 0.020-0.333[a] 0.30 < 0.001[b] 0.2 [c] 0.057	(26) (4) (25) (24) (24) (23)
3, 5 – T2	Chemical Hydrolysis: E. coli, H. pomatia Acetonitrile Urea, heated 50°C	LLE SPE SPE	LC-MS/MS LC-MS/MS LC-MS/MS	0.130 - 0.170 0.00065-0.011 [a] 0.046	(26) (4) (23)
3, 3' – T2	Chemical Hydrolysis: E. coli, H. pomatia Acetonitrile Ethyl Acetate Urea, heated 50°C	LLE SPE HybridSPE SPE	LC-MS/MS LC-MS/MS LC-MS/MS LC-MS/MS	0.130-0.170 0.00049-0.0079 [a] 0.17 0.034	(26) (4) (25) (23)
3 – T1AM	Urea, heated 50°C ProteinaseK, heated 37C β -glucuronidase, heated 37°C	SPE SPE ---	LC-MS/MS LC-MS/MS LC-MS/MS	0.037 0.0888 0.0355	(23) (46) (28)
TA4	Chemical Hydrolysis: E. coli, H. pomatia Acetonitrile Urea, heated 50°C	LLE SPE SPE	LC-MS/MS LC-MS/MS LC-MS/MS	0.5 0.082-1.308 [a] 0.0067	(26) (4) (23)
TA3	Chemical Hydrolysis: E. coli, H. pomatia Acetonitrile Urea, heated 50°C	LLE SPE SPE	LC-MS/MS LC-MS/MS LC-MS/MS	0.5 0.016-0.25 [a] 0.17	(26) (4) (23)
TA1	β -glucuronidase, heated 37°C	---	LC-MS/MS	88.83 [a]	(28)
MIT	MTBE, Acetonitrile/Acetic Acid Butanol-HCl, heated 70°C	SPE ---	LC-MS/MS IDMS/MS	0.06 [a] 0.018	(5) (10)
DIT	MTBE, Acetonitrile/Acetic Acid Butanol-HCl, heated 70°C	SPE ---	LC-MS/MS IDMS/MS	0.06 [a] 0.046	(5) (10)

For this study, negative control samples require the absence of targeted analytes. Serum, sourced from a supplier for thyroidectomized calf serum, was evaluated and found to not originate from thyroidectomized calves as initially indicated. Ideally, thyroidectomized equine serum would be the preferred choice; however, due to the intricate challenge of removing a thyroid gland without administering exogenous thyroxine supplementation, such a source was not feasible. Additionally, there were no commercially available sources where thyroid hormones were sufficiently depleted or readily available for purchase. To address this limitation while maintaining the matrix's similarities, an alternative approach was explored. Charcoal-stripped horse serum was investigated as a suitable substitute. In an evaluation study that compared thyroidectomized calf serum, charcoal stripped serum, and serum stripped through anion exchange resin, authors found that the values for T4 and T3 to be comparable, thus validating its utility as an appropriate medium for negative control samples [47].

In previous thyroid hormone studies, a reducing solution made up of ascorbic acid, citric acid, and dithiothreitol was added to serum to prevent deiodination and the formation of other target analytes of interest during incubation [4, 9]. The collective action of these reducing agents can reduce active iodine atoms in iodinated compounds back to their inactive forms, thereby stabilizing the molecules and preventing further reactions that might induce deiodination. The use of the antioxidant mixture reduces losses and overall improves the accuracy and reliability of the analytical results.

Serum is a complex biological matrix characterized by high levels of naturally occurring protein and phospholipids poses challenges in terms of analyte ionization and chromatographic separation [25]. These challenges become especially significant when in

contrast to trace concentrations of endogenous thyroid hormones. In a previous study, authors had used horse serum as a blank matrix due to its significantly lower concentrations in comparison to human serum [7]. Consequently, these complexities can impact the accuracy and precision of analytical results and sample clean-up procedures are necessary to minimize these effects. Previously, protein denaturation approaches including chemical hydrolysis, enzymatic digestion and protein precipitation have been examined in combination with different extraction techniques (**Table 11**). In their study, Bussy et. al explored various combinations of sample preparation and extraction techniques. They found that enzymatic digestion followed by protein precipitation offered the most effective recovery, and in terms of individual steps, that enzymatic digestion outperformed protein precipitation for sample preparation [24]. It is worth noting that when enzymatic digestion, via urea treatment and heat, was coupled with SPE, interference in chromatography, specifically for MIT and DIT, was observed [23]. Many other protein precipitation methods and solvents have been tested, including ethyl acetate, 10% trichloroacetic acid, and proteinase K hydrolysis [12, 25, 46]. In this project, precipitation by acetonitrile and hydrochloric acid were both evaluated during initial method development and similar to another study, protein precipitation using acetonitrile was found as the best choice given the highly sensitive LC-MS/MS method and efficiency [24]. However, a drawback inherent to protein precipitation is the requirement for multiple sample transferring steps. Despite the incorporation of additional measures, such as Pasteur pipette transfers aimed at minimizing these steps, there still exists the potential for hindrance to analyte recovery.

In the course of neat sample preparation, a recurring issue emerged as large quantities of analytes consistently adhered to the glass surface of the test tube during the evaporation

process. To address this challenge, adjustments were made: the temperature, initially set at 45°C, was reduced to 40°C. Additionally, the initial pressure, set at 10 psi and later increased to 15 psi after 30 minutes, was subsequently maintained at a constant 10 psi throughout both evaporation steps. Due to these considerations, adjustments were made to measure matrix effects. Neat standards were diluted to match the corresponding concentrations of each QC level in the redissolve, as an alternative to the conventional procedure of evaporating and subsequently resolving the spiked samples.

Investigations into both SPE and LLE techniques were conducted using samples spiked with equal concentrations. For our LLE experiment, spiked samples were deproteinized with acetonitrile, followed by the addition of methyl tert-butyl ether to the supernatant. The mixture underwent agitation on a rotator (Glas-Col™ Apparatus Co., Terre Haute, Indiana, USA) for 30 minutes on a speed setting of 25. The organic layer was transferred, evaporated to dryness, and reconstituted with water: methanol in a ratio of 75:25 (V:V). Three types of SPE columns were assessed for their performance: the UCT Clean Screen DAU, Biotage Isolute HAX, and Waters Oasis MAX. The DAU columns, characterized by their mixed-mode properties, were employed with a validated procedure for screening the acid/neutral fraction from blood extracts. Both HAX and MAX columns, designed for extracting acidic compounds with anion-exchange functionality, were evaluated under identical extraction conditions. In contrast, the LLE experiment yielded the least favorable results, particularly for MIT and DIT. The acid/neutral fraction experiment exhibited suboptimal signal quality and elevated background noise across all target analytes. Notably, HAX cartridge displayed minimal background noise and clear results, but analyte recoveries were below 20%. Consistent with an earlier study, the MAX cartridge showed improved signal quality, absence

of background noise, and notably superior recovery rates [4]. As a result, the MAX cartridge was initially chosen and subsequently, a transition was made to the Evolute Express AX, Biotage's equivalent to the MAX cartridge, which yielded comparable results. The Express AX columns provided several advantages, such as the inclusion of highly wettable sorbents that enable the smooth flow of samples under gravity. This guaranteed a consistent flow rate during conditioning and washing steps for all samples. The non-ionizable hydroxyl groups present in the sorbent ensure their non-reactivity with other substances. This design effectively prevents secondary interactions with the extracted compounds, ensuring a consistent retention and elution process during extraction. As authentic pre-race samples were introduced, both the eluents and dried-down extracts were clean, devoid of particulate matter. Therefore, Express AX cartridges were used for all validation experiments and population study.

A prior LLE experiment that assessed optimal pH conditions for extracted analytes showed better recoveries under basic conditions for T3, TA3, and a T2 isomer, but ultimately opted for a neutral pH to balance extraction conditions [26]. In light of this, neutral to slightly alkaline conditions were upheld throughout the extraction process until reaching the elution phase, which incorporated a formic acid component, as recommended by the manufacturer [48]. Given the high methanol content in the elution solution, the potential for losses in target analytes during the 100% methanol wash was evaluated. Data indicated minor losses were observed. In the initial phase, the eluent solution contained 5% formic acid in methanol. However, upon comprehensive evaluation that entailed incremental adjustments of formic acid concentrations of 5% increments, it was determined that a 20% formic acid solution yielded the optimal results.

Assessment of compound stability in the biological matrix at commonly utilized conditions is essential for evaluating the overall accuracy and reliability of the data. Encouragingly, the thyroid compounds in this method exhibited good stability after a 2-week period in extract at room temperature, as shown in **Table 9**. While the majority of samples remained fixed throughout a 2-week timeframe stored in the refrigerator and freezer, similar to published findings, 3-T1AM, TA4, TA3, and MIT exhibited pronounced degradation as early as 24 hours at room temperature [4]. The thyroacetic acids consistently displayed instability, with over 20% losses following 5 days in 2-8°C. Although the losses were comparatively less severe at -20°C after the same duration, they were also notably poor. By the fourth week at -20°C, most compounds began to exhibit considerable degradation. Samples subjected to three freeze-thaw cycles produced consistent results, characterized by deviations below 20%, for all compounds except for 3-T1AM, TA4, TA3, TA1, and MIT. Although limited information exists regarding the stability of thyroacetic acids, Jongejan et. al also encountered stability issues with TA4, leading them to recommend limiting freeze-thaw cycles to no more than two to preserve its integrity. Similarly, in both this study and their research, T3 and rT3 exhibited stability with variations not exceeding 15%. Nevertheless, the study had also noted that rT3 exhibited lower stability when compared to T3 [4]. Another study, notable for pioneering investigation into the stability of MIT and DIT, yielded results within the range of 93-104% over a 6-month duration at a constant 4°C shielded from light [10]. Despite this study's limited evaluation period of only two weeks at 2-8°C, in contrast to the 6-month duration of the other study, comparable stability recoveries were observed at both the five-day and two-week intervals. This phenomenon might be attributed to samples in this study being stored in appliances equipped with light activation. During initial phases of method development, the suitability of amber

autosampler vials was assessed. This evaluation was prompted by the consideration of factors, such as light, heat, and chemicals on the stability of thyroid hormones and report of rT3 potentially transforming into 3,3'-T2 and 3-T1 under fluorescent light [23, 25]. Ultimately, it was determined the use of amber vials was negligible in analytical outcomes. However, this choice may be reconsidered if stability concerns emerge after extraction. The assessment of the impacts of storage of serum within a serum separator tube containing a gel plug, comprised of a double polymer gel, silica clot activator and silicone-coated interior, was evaluated to confirm analytes of interest were not being retained in the gel. As shown in **Table 9**, minimal losses for all compounds are observed, with the exception of T1AM which was undetected. Notably, while T1AM was effectively extracted from negative control samples, and its spiked internal standard was successfully extracted from population samples, none of the population samples yielded any detection of T1AM. This discrepancy may stem from extremely low endogenous levels; however, results also suggest the analyte could have become bound to the gel plug and subsequently lost during a pre-analytical process. Therefore, further investigation is needed to gain a better understanding of this cause.

Initial data from the population samples suggest a broad concentration range and that, for most compounds, there are no major differences observed among geldings, mares, and stallions for T4, rT3, 3, 5-T2 and 3,3'-T2 (**Table 10**). The T3 measurements in geldings demonstrated notably high values, with both the range and median surpassing those observed in mare and stallion samples. Additionally, stallions displayed higher concentrations of T3 compared to mares. Concentrations of TA4 appeared notably higher in stallions compared to other groups. While there were no large variations in types for TA3 and geldings exhibited

wider TA1 concentration ranges than stallions and mares; it's important to note there were not enough positive identifications for both compounds to establish their reliability. 3-T1AM was not detected in any of the samples, and the absence of existing data for 3-T1AM in horses makes it challenging to corroborate these findings. Shown in **Figure 8**, a structural isomer for TA3, at 7.36 minutes, was consistently present in both negative control samples and equine samples.

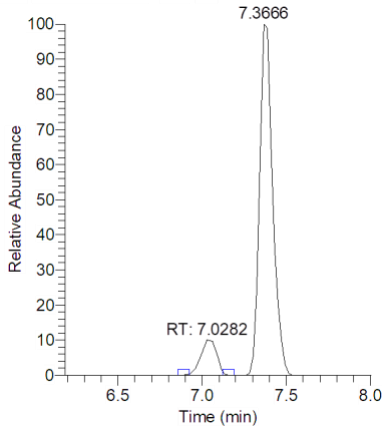

Figure 8. Structural isomer of TA3 at 7.36 min.

Furthermore, it is worth highlighting a single outlier for MIT and DIT extended the maximum range values from 370 pg/mL and 413 pg/mL, respectively, to 1156 and 2179. While the reasons for this outlier remain unknown, prior research suggested that increased iodine intake may lead to elevated DIT:MIT ratios, indicating a potential adaptive response to regulate the over-secretion of T3 [5].

In a previous study, T4 and T3 concentrations were assessed in ten actively racing horses. Among them, two were receiving oral L-thyroxine supplements throughout the study, and the analysis revealed higher T3, T4 and FT4 values in the dosed samples compared to the non-dosed

samples [49]. While it remains uncertain whether any of the participants in the population study received levothyroxine administration, it is noteworthy that two individuals exhibited T4 and T3 values within the top 3% of the study, despite the varying T4:T3 ratios observed throughout the study. Another study also proposed that when humans are administered substantial amounts of L-thyroxine, DIT's already low levels are further decreased, possibly reflecting the heightened secretory activity of the thyroid gland [33].

6.0 Conclusion

A comprehensive method for the simultaneous detection and quantification of 11 thyroid hormones and their metabolites was developed and validated, using high performance liquid chromatography high-resolution mass spectrometry (HPLC-HRMS). Samples were prepared through protein precipitation to eliminate any interfering substances while simultaneously concentrating the target hormones. Evolute Express AX solid phase extraction columns were selected for their precision in facilitating the thorough clean-up of acidic analytes. Compound stability was assessed indicating adequate stability of targeted compounds in serum samples stored at 2-8°C or -20°C for no more than two weeks. Sex-dependent reference ranges were determined for T4, T3, rT3, 3, 5-T2 and 3,3'-T2, MIT, and DIT. While TA4, TA3, and TA1 were successfully detected, I could not establish reliable reference ranges because their concentrations fell below the LOQ. T1AM was not detected in population samples, begging further investigation into its potential binding to the gel plug or the possibility that endogenously produced values are lower than the LOD observed in this study. Significant opportunities for method expansion lie ahead, including the utilization of emerging internal standards for thyroacetic acids, heightened sensitivity for monitoring MIT and DIT, a thorough

assessment of phase 2 metabolites resulting from glucuronidation and sulfation, the investigation of unknown peaks identified in the population study, and the application of this method to evaluate potential changes in thyroid hormone profiles following levothyroxine administration.

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