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# Detection of thyroid hormones T3 and T4 in urine and serum after levothyroxine administration: A pilot study relevant to doping analysis

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ABSTRACT

Thyroid hormones T3 and T4 are clinically relevant hormones that are currently not included on the World Anti-Doping Agency Prohibited List. Pending their inclusion, the possibility of detecting T4 doping was investigated in a T4 administration study involving six healthy volunteers. This pilot study entailed the administration of 1 mg Levothyroxine and the collection of urine and serum samples pre- and post-administration. T3 and T4 quantification methods employing liquid-liquid extraction and liquid chromatography mass spectrometry were developed and validated to analyse all collected samples. The urine and serum steroid profiles were also determined for all samples. All volunteers showed significantly elevated T4 serum concentrations, at least up to 24 h post-administration. From all investigated detection methods, tracking serum T4 concentrations through an individual passport program, employing a 99.7% confidence interval as thresholds, provided the highest test sensitivity, correctly identifying 75% of all post-administration samples as positive. Tracking the T4/T3 ratio in serum provided an identical test sensitivity but requires the additional quantification of T3. No evidence was found indicating the need to include T4 as a confounding factor for the urine or serum steroid profile. Future research should focus on optimizing the extraction protocol and performing a study where T3 is administered in multiple doses and serum samples are collected for a longer period post-administration.

## 1. Introduction

Thyroxine (T4) and triiodothyronine (T3) are tyrosine-based hormones produced by the thyroid gland under influence of thyroid stimulating hormone (TSH). Currently, thyroid hormones are not included on the World Anti-Doping Agency (WADA) Prohibited List [1] as they are deemed to meet the required minimum criteria to be classified as a doping agent [2]. However, there are clear indications of thyroid hormone use in certain athletic niches [3]. In the Netherlands, efforts are ongoing to include these compounds onto the WADA Prohibited List as there are concerns regarding their use among elite skating athletes [4,5]. In the USA, similar concerns have been raised among elite track and field athletes [6]. In the untested environment of fitness and strength athletes thyroid hormones are a common addition to doping regiments [7,8]. Additionally, their potential harmful effect on health has been demonstrated since there is sufficient scientific evidence strongly indicating that excess T3 and T4 are harmful towards bone [9] and cardiovascular health [10]. Contrarily, a clear performance-enhancing effect is thus far undetermined due to lack of concurring evidence [2]. It has been shown that thyroid hormones influence lipid metabolism [11] and skeletal muscle physiology [12]. However, studies demonstrating a clear performance-enhancing effect or targeted fat loss are currently unavailable. Furthermore, reanalysis of Australian doping control samples showed no significant difference in the frequency of suspicious thyroid hormone levels compared to general population prescription rates for these drugs [2,3]. Nevertheless, doping control laboratories should be aware of these trends and can anticipate by investigating their detectability and effects on other physiological parameters. Therefore, this project will investigate the detection of T4 administration.

In the clinical field, these compounds are commonly quantified using immunoassays to assess thyroid gland function [13]. Predominantly free T3 and free T4 fractions are quantified, as their protein-bound fractions

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show no bioavailability [14]. In a routine doping control setting, where the purpose is detecting illicit supplementation, bioavailability is irrelevant, thus quantification of the total fraction (both free and protein-bound fractions) will be performed. Liquid-chromatography mass spectrometry (LC-MS) is the preferred technique for the detection of these compounds in the field of doping [2,3,15–18]. Also in the clinical field LC-MS methods have been developed for these compounds [19–26].

For the first time, urine and serum samples, collected in the context of a T4 administration study involving four male and two female healthy volunteers, are analysed using inhouse developed methods. Additionally, the influence of T4 administration on the endogenous steroid profile of the athlete biological passport (ABP) is investigated, as previous research demonstrated an impact after T3 administration, albeit in only one healthy volunteer [27].

## 2. Methods

### 2.1. Chemicals and reagents

L-thyroxine (T4), 3,3′,5-triiodo-L-thyronine (T3), L-thyroxine-13C6 (T4 internal standard), 3,3′,5-triiodo-L-thyronine-13C6 (T3 internal standard), 3-iodo-L-tyrosine (MIT), phosphate-buffered saline (PBS) tablets, bovine serum albumin (BSA), sodium chloride (NaCl), glacial acetic acid (HOAc), and creatine were purchased from Sigma Aldrich (Bornem, Belgium). Potassium chloride (KCl) was purchased from J.T. Baker (Deventer, Netherlands). LC-MS grade water, methanol (MeOH), acetonitrile (ACN), methyl tert-butyl ether (MTBE), and isopropanol were purchased from Chem-Lab (Zedelgem, Belgium). n-Hexane was purchased from Biosolve (Valkenswaard, Netherlands). LC-MS grade formic acid (HCOOH) and ethyl acetate (EtOAc) were purchased from Fisher Chemical (Madrid, Spain). Urea was purchased from GE Healthcare (Uppsala, Sweden). Sodium acetate was purchased from VWR Chemicals (Leuven, Belgium). RELA 2022 external quality assessment scheme (eQAS) serum samples were kindly provided by the Ghent University Laboratory of Toxicology (Ghent, Belgium).

Synthetic serum was prepared by adding BSA to PBS buffer (1 PBS tablet in 1 L water) to a concentration of 40 mg/mL. Synthetic urine was prepared by adding 14 g NaCl, 3 g KCl, 18.3 g urea, and 2 g creatine to 1 L H2O. Acetate buffer pH 5.2 was prepared by adding 136 g sodium acetate to 800 mL H2O, adjusting the pH to 5.2 with HOAc, and diluting to 1 L with H2O. 75:25 H2O/MeOH containing 0.02% HCOOH was used as reconstitution solution.

### 2.2. Stock and working solutions

T4, T3, and internal standard (ISTD) stock solutions were prepared at 100 μg/mL in MeOH and were stored at −20 °C. Working solutions were prepared at varying concentrations in MeOH and stored at −20 °C. A 10 mg/mL MIT in MeOH stock solution was stored at −20 °C and was diluted to a 100 μg/mL working solution that was stored at 4 °C.

### 2.3. Sample preparation

For serum samples, T3 and T4 were extracted and quantified separately. This means that one sample was aliquoted, extracted, and analysed twice. All tubes and vials were precoated by adding 100 μL 100 μg/mL MIT in MeOH. Vials were rolled for 20 min, tapped dry, and stored in a fume hood overnight to let the remaining MeOH evaporate. To precoated glass screwcap vials, 50 μL 20 ng/mL T3 ISTD or 50 μL 400 ng/mL T4 ISTD was added. Calibrators were spiked by adding following volumes of 4 ng/mL T3 or 400 ng/mL T4: C1 = 25 μL; C2 = 50 μL; C3 = 75 μL; C4 = 100 μL; C5 = 125 μL; C6 = 200 μL. All vials were evaporated to dryness under a stream of nitrogen gas in a Caliper TurboVap LV at 40 °C. 200 μL serum was added to each vial. Synthetic serum was used for the calibrators. 1 mL pH 5.2 acetate buffer was added, followed by brief vortexing. Liquid-liquid extraction (LLE) was performed by adding 4 mL EtOAc and rolling the samples for 15 min. Before transferring the organic phase to a new precoated glass screwcap vial, samples were centrifuged for 5 min at 3200*g* to improve phase separation. The organic phase was evaporated to dryness under a stream of nitrogen gas at 40 °C and reconstituted using 100 μL reconstitution solution. Reconstituted samples were transferred to precoated LC vials and injected onto the LC system.

This serum protocol was adapted to urine with few changes. Most importantly, T3 and T4 are extracted and analysed simultaneously in the same sample. Instead of precoating all vials, 200 μL 100 μg/mL MIT in MeOH was added just before spiking the ISTD. 100 μL 20 ng/mL T3 T4 ISTD spike mix was added to all vials. Calibrators were spiked by adding following volumes of 16 ng/mL T3 T4 spike mix: C1 = 25 μL; C2 = 100 μL; C3 = 200 μL; C4 = 300 μL; C5 = 400 μL; C6 = 500 μL. From this point on, the protocol is identical to the serum protocol, only differing in sample volume (4 mL urine instead of 200 μL serum) and using synthetic urine for the calibrators.

### 2.4. LC-HRMS/MS

Chromatographic separation was performed using a Dionex Ultimate 3000 UHPLC system (Thermo Scientific, Bremen, Germany) equipped with a degasser, an Ultimate dual-gradient rapid separation pump, and an autosampler and column compartment kept at 10 °C and 30 °C, respectively. The setup involved an Acquity UPLC Peptide BEH C18 column (300 Å, 1.7 μm, 2.1 × 50 mm, Waters, Zellik, Belgium), an Acquity Protein BEH C18 precolumn (Waters, Zellik, Belgium), and 95:5 H2O/MeOH and 100% MeOH as aqueous and organic mobile phase, respectively, both containing 0.02% HCOOH. The gradient started at 35% B (organic) for 1.5 min before gradually increasing to 99% B over the course of 4.5 min. 99% B was held for 3 min after which it instantly decreased back to 35%, held for the remaining 3 min. Flowrate was set to a constant 250 μL/min. The injection volume was set to 20 μL and the total runtime was 12 min.

The LC system was coupled to a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany), equipped with an electrospray ionization (ESI) source. The ESI source parameters were the following: spray voltage, 3.2 kV; capillary temperature, 320 °C; auxiliary gas heater, 300 °C; sheath gas flow rate, 25; auxiliary gas flow rate, 10; S-lens, 50. Both positive full-scan and parallel reaction monitoring (PRM, $MS^2$) were used during analysis. The full-scan parameters were set to 70,000 resolution, 300–1200 scan range, 1e6 automatic gain control (AGC) target, and 200 ms maximum ion injection time. The PRM parameters were set to 17,500 resolution, 300–1075 scan range, 1e5 AGC target, 55 ms maximum injection time, and 1.2 *m/z* isolation window. Mass extraction was set to 10 ppm. Table 1 shows the transition ions used for MS analysis.

### 2.5. Validation

Both methods were validated according to Eurachem guidelines [28]. For the serum method, ten different previously collected serum samples, stored at −80 °C, were thawed. For the urine method, ten

**Table 1**
Target analyte precursor ions and their product ions selected for quantification (ion 1) and identification (ion 2). CE = collision energy.

| Target | Elemental composition | Precursor m/z | Product ion 1 m/z | Product ion 2 m/z | CE (eV) |
|---|---|---|---|---|---|
| T3 | C15H12I3NO4 | 651.7973 | 605.7918 | 507.8663 | 32 |
| T4 | C15H11I4NO4 | 777.6940 | 731.6885 | 633.7629 | 32 |
| $^{13}C_6$ T3 | C9$^{13}$C6H12I3NO4 | 657.8174 | 611.8100 | #N/A | 32 |
| $^{13}C_6$ T4 | C9$^{13}$C6H12I4NO4 | 783.7141 | 737.7088 | #N/A | 32 |

different fresh urine samples were collected covering a pH range of 5.0–7.5 and density of 1.002–1.027 g/cm$^3$.

*Linearity* was evaluated by calculating the goodness-of-fit and $r^2$ values of the calibration curves in triplicate. A weighted (1/x) linear regression model was used. *Repeatability* was determined by analysing the lowest, the middle, and the highest calibrator fivefold in addition to the calibration curve, for a total of six replicates. To evaluate *intermediate precision*, the repeatability experiment was performed two more times, once by the same analyst on a different day and once by a different analyst. *Bias* was calculated at the same levels as repeatability and intermediate precision. *Carry-over* was assessed by injecting three blank samples after a sample spiked at three times the concentration of the highest calibrator. *Recovery* was calculated by comparing peak areas of extracted highest calibrators, one spiked as normal, the other spiked after extraction of the calibrator matrix. This experiment was performed in triplicate. *Matrix effect* was evaluated in ten different urine and serum samples using following formula: *Matrix effect (%) = [(peak area urine or serum/peak area blank matrix) – 1] * 100*. *External bias* could only be determined for serum as there were no urine eQAS samples available. It was assessed by analysing the RELA 2022 eQAS serum samples and comparing the results to the mean of values reported by JCTLM listed reference measurement service probing laboratories [29,30].

### 2.6. T4 administration study

The T4 administration study was conducted after approval by the Ghent University Ethical Committee (approval number: B6702022000229). Six healthy volunteers, four male and two female, consumed five 200 μg Levothyroxine tablets simultaneously, for a total dose of 1 mg. The tablets were taken with water in the morning. All volunteers had access to water ad libitum. To assess baseline thyroid hormone levels, urine and serum samples were collected 7 days, 6 days, 3 days, 2 days, 1 day, and 30 min prior to T4 administration. To investigate the effect of the administration on these levels, serum samples were collected 30 min, 1 h, 2 h, 4 h, 6 h, and 1 day post-administration. Serum samples were collected via single phlebotomies, except for the six samples collected on the day of thyroxine administration, where a catheter was used. Urine samples were collected 2 h, 4 h, 6 h, 1 day, 2 days, and 3 days post-administration. An extra urine sample was collected from volunteer 2, 30 min after T4 administration. No +2 days and + 3 days urine samples were collected from volunteer 6. All samples except the first few post-administration samples were collected in the morning and all were stored at −20 °C until analysis. All measured values for urine samples were corrected for specific gravity (SG), measured with an Atago UG-1 digital refractometer, according to following formula: *Corrected value = [(1.02–1)/(SG – 1)] * measured value*.

### 2.7. Steroid profile

All urine and serum samples collected during the T4 administration study had their relevant endogenous steroids quantified and the relevant ratios calculated, as specified by WADA [31]. The urine samples were analysed using a previously published gas chromatography quadrupole time-of-flight mass spectrometry method [32] for testosterone (T), epitestosterone (E), androsterone (A), etiocholanolone (Etio), 5α-androstane-3α 17β-diol (5αAdiol), and 5β-androstane-3α 17β-diol (5βAdiol). The serum samples were analysed for testosterone and androstenedione using a method based on previous research from our group [33].

## 3. Results

### 3.1. Method development

Since T3 and T4 are endogenous hormones, alternative matrices were considered as blank matrix for preparing quality control and calibrator samples. Due to the conserved nature of the thyroid system in mammals, bovine serum or rat serum could not be used as they showed significant interferences. Also charcoal-stripped foetal bovine serum, as well as experimenting with in-house thyroid hormone stripping of serum, likewise resulted in interfering signals. Eventually, a solution of 40 mg/mL BSA in PBS as synthetic serum was opted for. Synthetic urine was prepared as described in *2.1 chemicals and reagents*.

Serum samples were extracted with LLE using EtOAc as the organic solvent. This protocol was chosen for its simplicity and the moderate and good recoveries for T4 and T3 respectively, as observed by Brito and colleagues [16]. The moderate recovery of T4 was less important given its high concentration in serum. Protein precipitation, a common serum clean-up step, was not considered since plasma proteins tend not to dissolve substantially in EtOAc. An alternative protocol employing Oasis MAX and MCX solid-phase extraction (SPE) columns can also be applied [34].

Initially, the idea was to have both T3 and T4 extracted from a single aliquot. Due to repeated incorrect T3 yet correct T4 quantification of eQAS serum samples, it was discovered that the T4 reference standard stock solution contained small amounts of T3. More specifically, T3 was present at 1/100th the T4 concentration. However, since T4 is spiked at a concentration 100 times higher than T3 in serum calibrators, this significantly influenced T3 quantification. To circumvent this issue, T4 and T3 analyses were separated. Consequently, the required sample volume, reagents and consumables, labour time, and instrumental analysis time were doubled. In urine, no such issue was observed since urinary T3 and T4 concentrations are similar, resulting in identical detection ranges. This allowed their simultaneous quantification in a single aliquot.

Analysing the extracted ion chromatogram for T3, an interfering peak is seen, caused by the T3 isomer reverse T3 (rT3). Our method can separate both compounds, with rT3 eluting ±0.4 min after T3. An

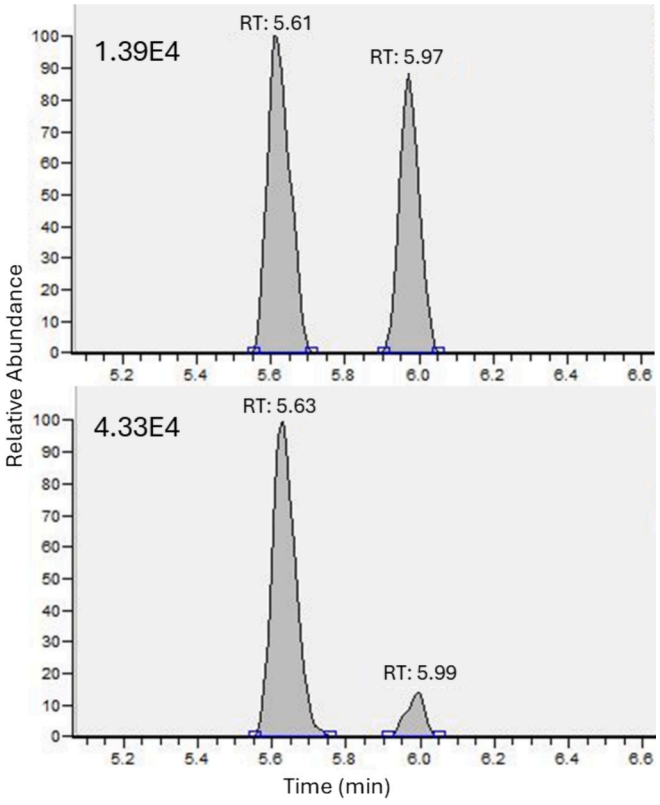


**Fig. 1.** Extracted chromatogram for calibrator 1 (upper chromatogram) and calibrator 4 (lower chromatogram) showing a peak for T3 at RT = 5.61 and for rT3 at RT = 5.97.

example of such a chromatogram is shown in Fig. 1.

A pilot test demonstrated the feasibility of using the validated serum method for urine samples as well. The major hurdle during method development in urine was the use of MIT as a precoating agent. While MIT proved to be a valuable tool in preventing analyte loss through adsorption in the serum extraction protocol, it heavily interfered with the extraction of urine samples. Large variances between replicate samples were found to be caused by the precoating of tubes with MIT. Extracted blanks showed significant yet inconsistent signals for both T3 and T4. Furthermore, an even greater signal for rT3 appeared to be the result of the MIT precoating. Omitting the MIT precoating step from the protocol would have solved this issue, but resulted in unsatisfactory sensitivity for the lowest calibrators, especially for T4. Further experimentation revealed that only precoated vials that were left to dry over time showed these interfering signals. The addition of a fresh MIT solution at the start of the extraction protocol significantly lessened the interference while retaining the desired effect of preventing analyte loss through adsorption.

Due to the non-volumetric transfer of the organic phase, inherent to an LLE protocol, as well as the adsorptive behaviour of T3 and T4, inconsistent losses of either target analyte are inevitable. To correct for this, it is crucial to select an appropriate ISTD. For this reason, isotope-labelled T3 and T4 were opted for, as was the case in published methods from other research groups [3,19,21,35,36].

### 3.2. Validation

Both the serum and urine method were successfully validated for T3 and T4. *Linearity* was compliant with $r^2 > 0.98$ (0.9912; 0.9987; 0.9961; 0.9964) and a goodness of fit below 10% (8.5%; 2.1%; 3.9%; 3.63%) for serum T3 (range: 0.5–4 ng/mL), serum T4 (range: 50–400 ng/mL), urine T3 (range: 0.1–2 ng/mL), and urine T4 (range: 0.1–2 ng/mL) respectively. *Limit of detection* and *limit of quantification* are defined as the lowest calibrator for each individual analyte and matrix, given that no samples at lower concentrations were included during validation. *Repeatability*, *intermediate precision*, and *bias* were compliant for all methods being below 2/3*RSDmax, RSDmax, and 10% (absolute value), respectively. RSDmax was calculated using the Horwitz equation with a 16% lower and 22% upper cutoff value [37]. Results for these parameters are shown in Table 2. *Carry-over* never exceeded 1% and a *recovery* of 44%, 85%, 33%, and 40% was noted for serum T3, serum T4, urine T3, and urine T4, respectively. *Matrix effect* varied significantly between different serum samples (from −36% to +17% for T3 and from −39% to +26% for T4) and even more so between different urine samples (from −10% to +137% for T3 and from −49% to +142% for T4), but this was compensated by our isotopic labelled ISTD. *External bias* was calculated only in serum and measured −1.6% for T3 and 5.3% for T4. Two RELA 2022 eQAS samples were analysed with T4 = 192.0 ng/mL and 143.8 ng/mL and T3 = 1.40 ng/mL and 2.42 ng/mL as mean values determined by JCTLM listed reference measurement service probing laboratories. During validation they were measured at T4 = 207.7 ng/mL and 147.2 ng/mL and T3 = 1.28 ng/mL and 2.55 ng/mL, respectively.

### 3.3. T4 administration study

All serum and urine samples were analysed using the previously described validated methods. During analysis, T3 values in one aliquot of three urine samples and in both aliquots of two urine samples fell below the lowest calibrator. Only seven excretion urines showed T4 values exceeding the highest calibrator, requiring dilution of 2 mL sample with 2 mL water before analysis. All collected data was visualized using scatterplots. Fig. 2 shows the scatterplots for T4, T3, and T4/T3 ratio values for both matrices in just one volunteer for visual clarity. All data from the T3, T4, and T4/T3 ratio analyses in urine and serum can be found in the supplementary material.

To classify a value as significantly different from baseline, two cutoff values were considered. The 95% confidence interval (CI) upper limit and lower limit were calculated using the mean and standard deviation (SD) of pre-administration samples according to following formulae: *95% CI upper limit = mean + 2 * SD*; *95% CI lower limit = mean – 2 * SD*. Similarly, the 99.7% CI upper and lower limits were calculated according to following formulae: *99.7% CI upper limit = mean + 3 * SD*; *99.7% CI lower limit = mean – 3 * SD*. Furthermore, a distinction can be made between calculating these limits using pre-administration values from all volunteers grouped together, or for each volunteer individually. The former results in population thresholds, whereas the latter results in a passport-like system (longitudinal monitoring using thresholds based on previous samples from the same individual), like the ABP used in doping control [38].

Looking at the pre-administration samples, a group-based 95% CI generated two urine T4, two urine T3, two serum T3, and two serum T4/T3 false positives. Using an individual 95% CI, one urine T4, one serum T4, and two serum T3 false positives were generated. Whereas neither 99.7% CI (individual or population) generated any false positives. Due to the generation of false positives, the 95% CI was disregarded in further analyses.

Figs. 3 and 4 give an overview of all post-administration samples and whether they exceed the 99.7% CI upper limit. No samples fall below the 99.7% lower limit. Each row contains a parameter (T4, T3, or T4/T3) and is further subdivided into six rows representing the six volunteers (not labelled for clarity). A green cell marked with an 'x' indicates a value that exceeds the 99.7% CI upper limit, calculated individually (left column) or as a group (right column). Grey cells indicate unavailable values. From Fig. 3, a significant serum T4 elevation is seen in all volunteers up to at least 24 h. For volunteers 2 and 3, the elevation is seen already 30 min after administration, whereas it takes 2 h for the other four volunteers. While both methods detect a significant elevation for all

**Table 2**
Validation data for repeatability, intermediate precision, and bias.

| Target | Level (ng/mL) | Repeatability ($n = 6$) | | | | Intermediate precision ($n = 18$) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean (ng/mL) | Bias (%) | RSD (%) | 2/3*RSDmax (%) | Mean (ng/mL) | Bias (%) | RSD (%) | RSDmax (%) |
| T3 serum | 0.5 | 0.47 | −5.7% | 9.4% | 15 | 0.49 | −2.6% | 17.2% | 22 |
| | 1.5 | 1.44 | −4.2% | 6.1% | 15 | 1.48 | −1.6% | 5.0% | 22 |
| | 4.0 | 4.05 | 1.3% | 3.4% | 15 | 4.14 | 3.5% | 4.9% | 22 |
| T4 serum | 50 | 54 | 7.5% | 4.5% | 15 | 51 | 1.8% | 5.8% | 22 |
| | 150 | 150 | −0.2% | 3.2% | 14 | 150 | 0.0% | 2.7% | 21 |
| | 400 | 408 | 2.0% | 9.5% | 12 | 401 | 0.3% | 6.3% | 18 |
| T3 urine | 0.1 | 0.10 | 1.9% | 2.0% | 15 | 0.10 | 4.5% | 6.7% | 22 |
| | 0.8 | 0.79 | −1.1% | 2.4% | 15 | 0.78 | −2.2% | 2.7% | 22 |
| | 2.0 | 2.09 | 4.6% | 3.3% | 15 | 2.06 | 2.8% | 3.8% | 22 |
| T4 urine | 0.1 | 0.10 | −3.9% | 5.6% | 15 | 0.10 | −0.8% | 5.5% | 22 |
| | 0.8 | 0.80 | 0.5% | 1.2% | 15 | 0.80 | 0.1% | 1.9% | 22 |
| | 2.0 | 2.08 | 3.8% | 1.6% | 15 | 2.06 | 3.1% | 3.0% | 22 |

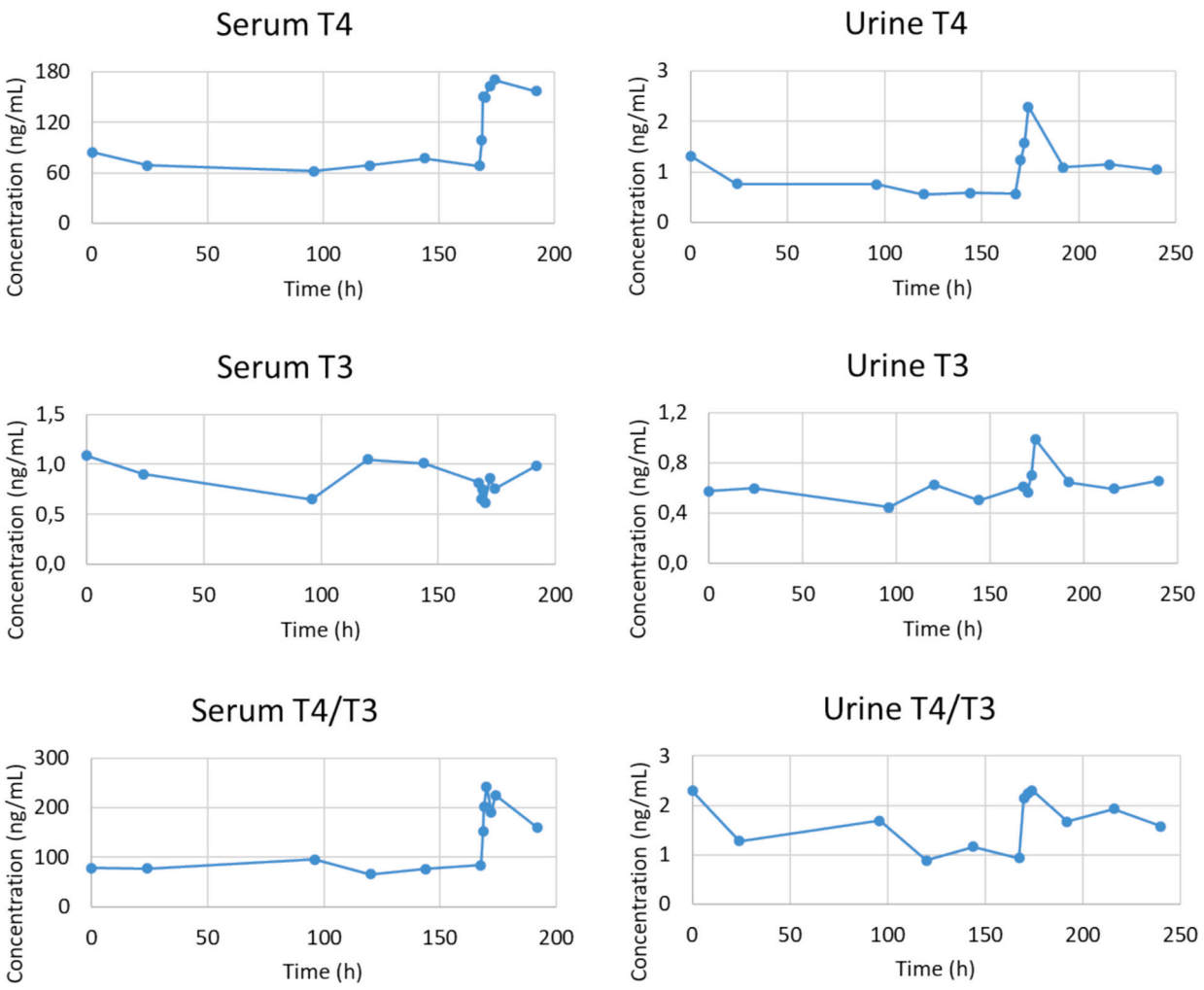


**Fig. 2.** Scatterplots for T4, T3, and T4/T3 ratio in both urine and serum for volunteer 3. Timing started when the first pre-administration sample was collected. Thyroxine was administered at 168 h.

| | Individual CI (passport) | | | | | | Group CI (population threshold) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 30 min | 1 hour | 2 hours | 4 hours | 6 hours | 1 day | 30 min | 1 hour | 2 hours | 4 hours | 6 hours | 1 day |
| T4 | | | x | x | x | x | | | | x | x | x |
| | x | x | x | x | x | x | x | x | x | x | | |
| | x | x | x | x | x | x | | x | x | x | x | x |
| | | | x | x | x | x | | | x | x | | x |
| | | | x | x | x | x | | | x | x | x | x |
| | | | x | x | | x | | x | x | x | x | x |
| T3 | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| T4/T3 | | | | x | | x | | | | x | x | x |
| | x | x | x | x | x | x | x | x | x | x | x | x |
| | x | x | x | x | x | x | x | x | x | x | x | x |
| | | | x | x | x | x | | | x | x | x | x |
| | | | x | x | x | x | | | x | x | x | x |
| | | x | x | x | x | x | | | x | x | | |

**Fig. 3.** Overview of all post-administration serum samples. Those exceeding the 99.7% confidence interval (CI) upper limit are shown in green with an 'x'. Each row containing a parameter (T4, T3, or T4/T3) is further subdivided into six rows representing the six volunteers (not labelled for clarity). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

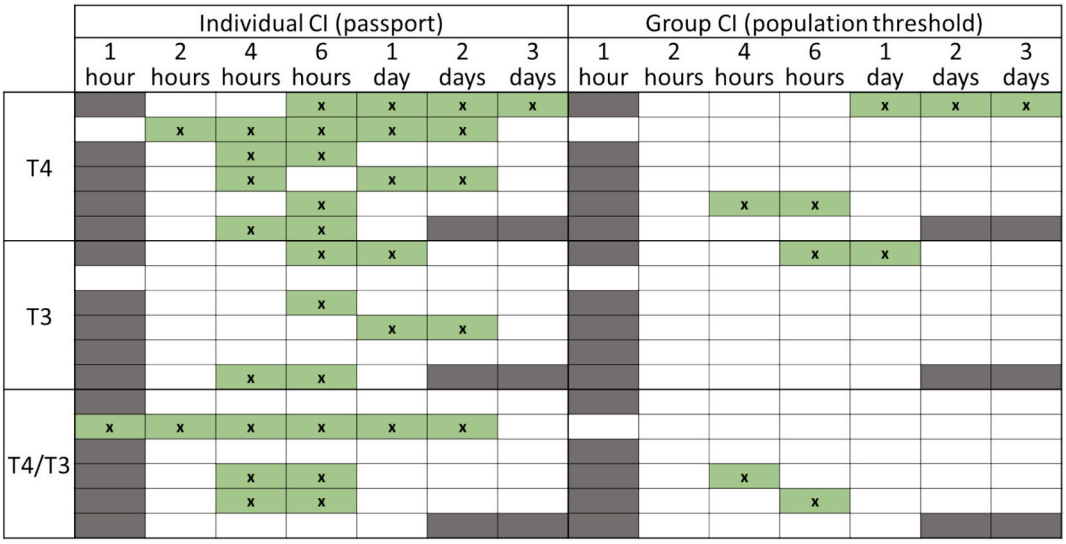


**Fig. 4.** Overview of all post-administration urine samples. Those exceeding the 99.7% confidence interval (CI) upper limit are shown in green with an 'x'. Grey boxes = data not available. Each row containing a parameter (T4, T3, or T4/T3) is further subdivided into six rows representing the six volunteers (not labelled for clarity). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

volunteers, the individually calculated CI performs slightly better, flagging 75% (27/36) of samples as positive, compared to 67% (24/36) using a population threshold. Serum T3 remains constant throughout the duration of the administration study. The serum T4/T3 ratio shows a significant elevation in 75% (27/36) or 69% (25/36) of samples calculated using the individual or group methods, respectively. Unsurprisingly due to the steady T3 values, T4 and the T4/T3 ratio perform almost identically in identifying true positives. Compared to serum (Fig. 3), results in urine (Fig. 4) seem to be much less consistent and impressive for all parameters. Even the best performing method, T4 using an individual CI, only flags 49% (17/35) of samples as positive. For volunteer 5, only one sample is correctly flagged (6 h). Using a population threshold, only 14% (5/35) of samples are flagged. Unlike serum T3, there are some urinary samples showing elevated T3 values, although only in 20% (7/35) of all samples and even in none for two volunteers specifically (2 and 5).

### 3.4. Steroid profile

Similarly to the results collected from T3 and T4 quantification of all urine and serum samples, steroid profile data was visualized using scatterplots and changes in values were assessed using previously discussed 99.7% CI upper and lower limits. Six steroids; T, E, A, Etio, 5αAdiol, and 5βAdiol were quantified in urine. Using these values, five ratios; T/E, A/Etio, 5αAdiol/5βAdiol, 5αAdiol/E, and A/T were calculated. In serum only two steroids; T and androstenedione (Adion) were quantified and their ratio; T/Adion, was calculated. All data from the urine and serum steroid profile analyses can be found in the supplementary material.

Looking at the urinary steroid profiles, there are no markers or ratios that show clear deviations from pre-administration values consistently for all six volunteers (considering a supra-physiological dose is taken). However, volunteer 1 (male) does show some significant (outside of individual 99.7% CI) changes in markers and ratios post-administration. A, Etio, 5αAdiol, T, and E all show decreased values up to 2 (for Etio) or 4 (for the others) hours after thyroxine administration. Additionally, their 5αAdiol/E ratio and A/T ratio are significantly elevated in the samples collected 4 (for both) and 6 (for 5αAdiol/E) hours post administration. Also for volunteer 2 (male) decreased T values 2 and 4 h after administration are seen. Besides these, no other distinct changes can be seen in any of the volunteers, except for a few decreased or elevated values, which can be considered as outliers. To highlight the inter-individual differences in effect (or lack thereof) of thyroxine administration on the urinary steroid profile, scatterplots of all measured markers and calculated ratios are shown for one male (volunteer 1) and one female (volunteer 5) volunteer in Fig. 5.

Similarly to the urinary steroid profile, analysis of the serum steroid profile revealed minimal changes after thyroxine administration. Classifying singular elevated or decreased values as outliers, only two notable digressions are seen. One is the decrease in Adion values for volunteers 1 and 2 (−34% on average for the first 6 h after administration). The other being the increase in T/adion ratio for three out of four male volunteers (+55% on average for the first 6 h after administration). Fig. 6 highlights these findings.

## 4. Discussion

A T4 administration study was performed where participants ingested 1 mg of Levothyroxine at a single point in time. Both pre- and post-administration samples were collected, allowing the measurement of relative changes in T3, T4, and T4/T3-ratio values compared to each participant their baseline levels. From the collected data, it is clear that monitoring individual T4 serum levels over time is the optimal method for detecting T4 doping. Monitoring individual T4/T3-ratio values in serum over time provided an identical test sensitivity (75%) yet entails the quantification of T3 which doubles the number of samples as T3 and T4 are analysed separately in serum. However, if T4 were to be included on the WADA prohibited list, there is a high likelihood that T3 would also be included, motivating the quantification of both analytes. Furthermore, although test sensitivity was identical, not every sample flagged as positive by the T4/T3-ratio was also flagged by solely looking at T4 and vice versa, highlighting the benefit of tracking both. Since thresholds for detecting doping abuse in a passport program are influenced by the variance in values of previous samples from the same individual, stable values result in more narrow thresholds, making it more likely to detect deviations caused by doping. The relative standard deviation (RSD%) of individual T4 serum baseline values ranged from 8.1% to 15.1% in this study. Comparing these values to urine, where the RSD% ranged from 14.5% to 34.9%, at least partially explains why even in a passport-like method, T4 administration is much less likely to be detected in urine than in serum. Further comparing these values to the RSD% of baseline values of all six volunteers combined, 15.9% in serum

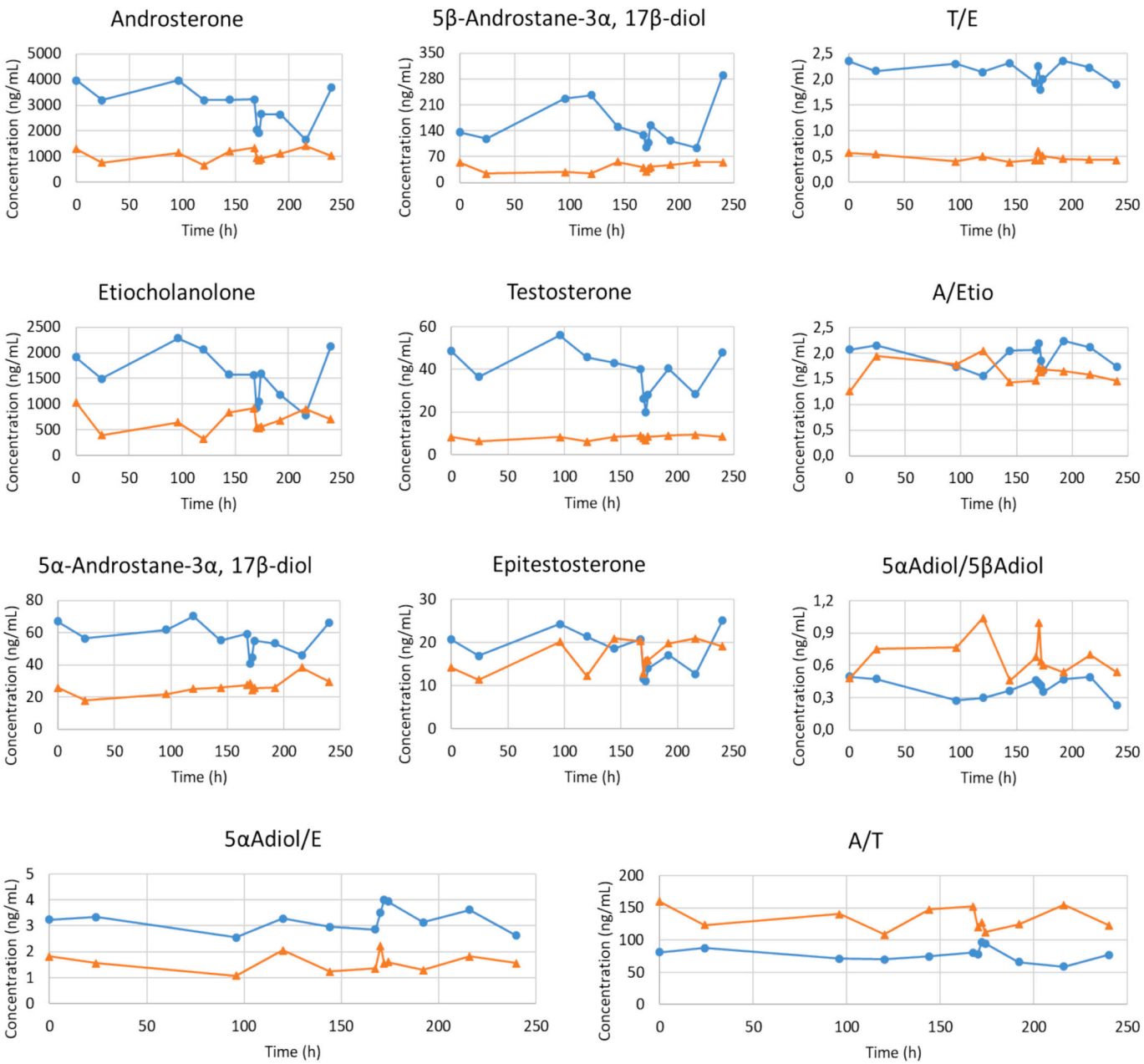


**Fig. 5.** Scatterplots for all measured and calculated urinary steroid profile parameters for volunteers 1 (blue dots) and 5 (orange triangles). Timing started when the first pre-administration sample was collected. Thyroxine was administered at 168 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and 44% in urine, highlights the benefit of a passport program as inter-individual variability becomes more negligible as more samples are collected.

A consideration regarding the developed methods involves the use of BSA in synthetic serum. During development of the serum method, T4 was detected in blank samples at a concentration of 2.5 ng/mL, or 20 times lower than the lowest calibrator (50 ng/mL). This contamination was unsurprisingly caused by using BSA in our blank matrix, since some fraction of T4 is transported in the blood stream bound to albumin [39]. However, the external bias of 5.3% demonstrates the insignificance of this contamination. Other forms of hormone stripped serum can be considered, as other research groups have described [3,16].

Furthermore, although the methods fulfil all validation requirements including quantification of the RELA eQAS samples, moderate recoveries are noted for serum T3, urine T3, and urine T4 analyses. Contradictory to our findings, Brito and colleagues reported a recovery of 86% for serum T3 [16]. No direct explanation could be found, however; the amphoteric character of T3 and T4 and their ability to form zwitterion structures can influence extraction recovery using LLE. Additionally, they exhibit strong adsorption characteristics, demonstrated by a dramatic drop in signal when MIT is omitted from the extraction protocol (data not shown). It is possible some adsorption still takes place despite the use of MIT. This hypothesis is further corroborated by the remarkably higher recovery for serum T4. As this analysis involves significantly higher concentrations compared to the other three analyses, analyte loss through adsorption would have less impact on overall recovery. While BSA was added to our synthetic serum, thyroxine-binding globulin (TBG) and transthyretin (TTR) were not. Their high binding affinity for T4 and T3 [40,41] could potentially cause much lower recoveries in real serum samples than those achieved in our

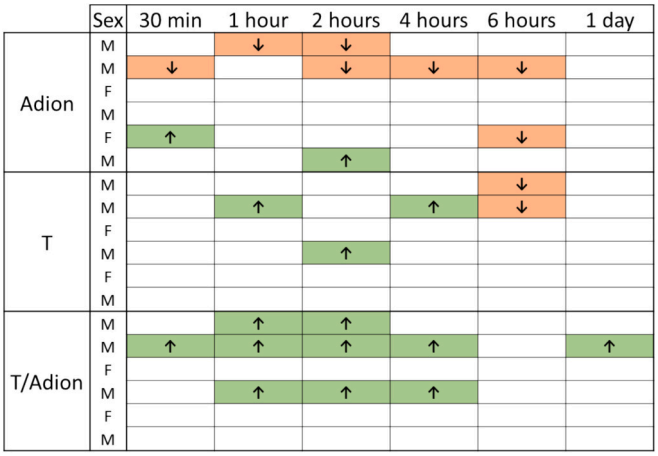


**Fig. 6.** Overview of which blood steroid profile parameters saw a significant increase (green squares with up arrow) or decrease (orange squares with down arrow) for each separate sample compared to baseline values. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

synthetic serum. However, this does not influence quantification as the isotopically-labelled ISTD demonstrated complete equilibration with the (protein-bound) target analytes, as determined in a separate experiment (data not shown).

For this project, a single supraphysiological dose of T4 was administered to healthy volunteers. Future research on this topic should involve a T3 administration study with multiple doses spread out over a longer period, more alike to a doping protocol that an athlete would follow. Additionally, sample collection should be extended in time to observe complete wash-out. We were unable to determine the detection window beyond 24 h, as no later serum samples were collected. Previous research demonstrated a 1–2 day half-life for T3 and a longer 6–7 day, or even 9+ day half-life for T4 [42,43]. To setup an efficient sampling methodology in real-life doping control, it is important to investigate how this translates into the serum T3 and T4 concentrations of a doping athlete.

Previous research from Brito and colleagues demonstrated the effect of thyroid hormone administration on the urinary endogenous steroid profile [27]. They hypothesized that since thyroid hormones are known to upregulate sex hormone-binding globulin (SHBG) [44,45], their administration should be considered as a confounding factor when analysing the steroidal module of the ABP. They found a significant difference in concentration for multiple steroid markers between (especially female) athletes declaring and athletes not declaring thyroid hormone consumption on their doping control form. These findings were further corroborated after administering T3 to a female volunteer. Based on these findings [27], the possibility of seeing a similar effect after T4 administration was investigated. Our results show that despite administration of a supraphysiological dose (1 mg) of T4, of which a part was converted peripherally to T3 as indicated by the significant increase in urinary T3 concentrations in some samples, the steroid profiles were barely affected. Although it is possible that unlike the single high dose in this study, a repeated T4 administration could influence the steroid profile, there is currently little convincing evidence that T4 is a confounding factor. To confirm the findings of Brito and colleagues, a T3 administration study should be performed, as suggested previously. Additionally, an investigation into the steroid profile should involve a balanced cohort of male and female volunteers, as well as consider female contraceptive use and menstrual cycle phase. Given that the T4 administration study had concluded before Brito and colleagues published their steroid profile findings [27], on which our steroid profile investigation is based, no information on contraceptive use or menstrual cycle had been gathered.

Although one volunteer did show significant changes in their urinary steroid profile, this trend was not continued in the five other participants. However, other (un)known confounding factors can also lead to changes in the urinary steroid ABP of an individual. As the observed changes here were limited to one volunteer out of six, no universal change to the steroid profile can be attributed to the thyroxine administration. The serum steroid profile T/adion ratio does show a minimal elevation after thyroxine administration in three out of four male participants. However, this ratio shows no signs of even a slight elevation in the fourth male participant, as is the case for both female participants. Contrary to the findings published by Brito and colleagues [27], any changes in the steroid profile found in this study, were found only in male participants. All measured and calculated parameters in both female volunteers showed no signs of being influenced by thyroxine administration. Furthermore, considering the difficulty in detecting thyroxine doping and the little evidence of its effect on the steroid profile, currently it does not seem worthwhile to consider it as a confounding factor. However, continuous efforts should be made in detecting T4 doping given its potential as a potent and dangerous doping agent.

## 5. Conclusion

The detection of thyroid hormone doping is challenging, as is described extensively by Gild and colleagues [2]. Nonetheless, if, or when, more conclusive evidence is published supporting their prohibition in sports (e.g., clear performance-enhancing effects or malicious use among athletes), the herein developed methods can be readily implemented in doping control laboratories.

Analysis of serum and urine samples collected in a T4 administration study demonstrated that tracking serum T4 levels over time (a biological passport-like system) is the optimal method for detecting T4 doping. Tracking the T4/T3-ratio in serum over time demonstrated similar performance yet requires the additional quantification of T3. T4 administration showed no consistent effect on urine and serum steroid profiles, refuting the need to consider it as a confounding factor.

## CRediT authorship contribution statement

**T. Colpaert:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **K. Van Uytfanghe:** Writing – review & editing, Resources. **G. T'Sjoen:** Writing – review & editing, Project administration. **N. Van Haecke:** Writing – review & editing, Formal analysis. **W. Van Gansbeke:** Writing – review & editing, Formal analysis. **P. Van Renterghem:** Writing – review & editing, Data curation. **M. Olijhoek:** Writing – review & editing, Funding acquisition. **O. de Hon:** Writing – review & editing, Funding acquisition. **G. Coppieters:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization. **P. Van Eenoo:** Writing – review & editing, Supervision, Resources. **K. Deventer:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization.

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## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Thibo Colpaert reports financial support was provided by Doping Authority Netherlands. If there are other authors, they 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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## Data availability

Data will be made available on request.

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