**Determination of thyreostats in bovine urine and thyroid glands by HPLC-MS/MS**

**Abstract:**
The use of thyreostats in livestock is strictly forbidden by European legislation since 1981. The investigation of thyreostats is commonly performed by their detection as derivatives with 3-iodobenzylbromide. Although it leads advantages, the derivatisation procedure can generally cause a decrease in analyte concentrations. With the aim of simplifying the analysis of five thyreostats in both bovine urine and in thyroid glands, two methods were developed without the derivatisation step. Salting-out assisted liquid-liquid extraction was carried out for both matrices, followed by high-performance liquid chromatography coupled with triple-quadrupole mass spectrometry analysis. The methods were validated in agreement with the guidelines of Commission Decision 2002/657/EC. For all the thyreostats evaluated, satisfactory results were achieved; the recovery was within 96% to 104% for both the matrices, while precision (coefficient of variation) was less than 20% for urine and 21% for thyroid glands. The limits of decision and capacities of detection for all the compounds were lower than the recommended values of 10 μg L⁻¹ and 10 μg kg⁻¹, respectively. In urine, the limits of decision ranged from 6.9 to 7.3 μg L⁻¹, and the capacities of detection ranged from 8.5 to 9.7 μg L⁻¹, while in thyroid glands these values varied from 6.6 μg kg⁻¹ to 7.4 μg kg⁻¹ and from 8.0 μg g⁻¹ to 9.7 μg kg⁻¹, respectively. The results obtained show that the methods described are suitable for the direct detection of thyreostats in bovine urine and thyroid glands.
Determination of thyreostats in bovine urine and thyroid glands by HPLC-MS/MS

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Introduction

Thyreostats are drugs that interfere with the mechanism involved in the synthesis of thyroid hormones and cause a condition of deficiency of circulating thyroxine (T4) and triiodothyronine (T3) [1, 2], whose production and release are controlled by the hypothalamus–anterior pituitary axis. The hypothalamus secretes thyrotropin-releasing hormone (TRH), which in turn stimulates the anterior pituitary gland to release thyroid-stimulating hormone (TSH) that induces the production of T3 and T4 by the thyroid, which releases them into the bloodstream. These hormones activate the nuclear transcription of a large number of genes, thus causing the synthesis of enzymes, as well as structural and transport proteins. This leads to an increase in metabolism and maintains the physical and psychological development of the organism. The administration of thyreostats causes an improvement in bodyweight gain mainly due to increased absorption and extracellular retention of water in the edible tissues and in the gastrointestinal tract [3]. Thyreostats are polar amphoteric thionamides with a heterocyclic tautomeric structure, and are mostly derived from thiouracil and mercapto-imidazole. The sequence consisting of nitrogen–carbon–sulphur, known as thioamide, is considered responsible for the thyroid-inhibiting activity (Fig. 1). The best known thyreostatic drugs include the very potent thyroid-inhibiting compounds 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU) and 1-methyl-2-mercaptop-imidazole (tapazole, TAP) [4-6]. The chemical structures of these substances are shown in Figure 1.

The fraudulent use of thyreostats produces low quality meat. Moreover, the edible tissues derived from treated animals might represent a potential risk to the consumer’s health due to the presence of residues and their teratogenic and carcinogenic effects [7-11].

In 1981, the European Union banned their use in animal production both as growth promoters and therapeutic agents [12] and classified them as “substances having anabolic effects and unauthorized substances” belonging to the group A2 as described by the Council Directive 96/23/CE [13]. However, a relationship between the presence of Brassicaceae in feed and thiouracil in urine has been demonstrated by Pinel et al. [9], Vanden Bussche et al. [14] and Kiebooms et al. [15, 16]. The Community Reference Laboratories (CRLs) in 2007 proposed a recommended concentration of 10 μg L⁻¹ in urine and 10 μg kg⁻¹ in thyroid tissue for the purpose of control, as “low concentrations of thiouracil have been detected in bovine animals fed with cruciferous plants, however there is scientific evidence showing that levels above 10 ppb in urine cannot be linked to natural origin due to this contamination” [17]. Recently, Wauters et al. reported concentrations of up to 18.2 μg L⁻¹ in the 99% percentile from 3894 bovines and they suggested that the recommended concentration should be increased to 30 μg L⁻¹ [18]. In fact, the 2015 Italian National Residue Plan already provides this concentration as the limit of detection for thyreostats in urine [19].
Thyreostats analyses typically consist of separation methods based on gas or liquid chromatography associated with a mass spectrometry system of detection. Normally, the extraction of the substances is carried out by using polar solvents more suitable to the chemical characteristics of the thyreostats, such as methanol, acetonitrile or ethyl acetate. Further steps of purification or clean-up with different kinds of solid-phase extraction (SPE) have been reported. Due to the low molecular mass and high polarity of the thyreostats, several authors have proposed a derivatisation step before or after the clean-up, mainly by using 3-iodobenzylbromide (3-IBBr) in the case of HPLC-MS/MS analysis [6]. In the case of GC methods, derivatisation is an unavoidable step in order to convert the analytes into volatile compounds. When HPLC is applied as the separation technique, analytes may be derivatised and, in the analysis of thyreostats, this procedure induces the stabilisation of the chemical structure of the molecule in a specific and single tautomeric form, the reduction of the molecular polarity in order to increase the separation characteristics on the reversed-phase column in the case of HPLC-MS detection, and an increase in the molecular mass [20]. The low molecular mass, particularly, could be disturbed by the chemical noise. In term of sensitivity, the derivatisation leads to an improvement of the signal to noise ratio, and subsequently of the detection capabilities [21]. Despite these advantages, the derivatisation procedure can generally cause a loss in analyte concentrations. Furthermore, removing derivatisation step simplifies, shortens and makes cheaper the whole analysis procedure [22, 23]. Based on these observations, we developed the extraction without derivatisation of the five above-mentioned thyreostats in bovine urine and thyroid glands followed by a sensitive, specific and reproducible HPLC-MS/MS analysis. For the full identification and quantification of the analytes, the criteria established in the 2002/657/EC Commission Decision were followed and the decision limit (CC\(\alpha\)) and the detection capability (CC\(\beta\)) were calculated according to the matrix calibration curve procedure as clarified in the document SANCO/2004/2726 rev. 4 [24, 25].

Materials and Methods
Reagents and chemicals
All solvents were of HPLC-MS grade quality and purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic acid (98–100\%) was from Riedel-de Haën (Sigma-Aldrich). Ultrapure water was obtained through a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). KH\(_2\)PO\(_4\) and NaCl were from Sigma-Aldrich. The analytes 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU), 2-mercaptobenzimidazole or tapazole (TAP) were acquired from Sigma-Aldrich, as well as 5,6-dimethyl-2-thiouracil (DMTU), used as internal standard (I.S.). A stock solution of 1 mg mL\(^{-1}\) was prepared by dissolving the compounds in methanol. Serial dilutions were prepared by diluting the stock solution in the mobile phase, which were then stored at \(-40^\circ\)C.
Phosphate buffer, prepared by dissolving 0.25 M \( \text{KH}_2\text{PO}_4 \) in ultrapure water, was adjusted to pH 7 and then saturated with 0.1% DL- dithiothreitol (DTT; Sigma- Aldrich) as in Vanden Bussche et al. [11].

Sample collection

Urine and thyroid gland samples from Friesian Cows aged 32 to 63 months were collected in a Lombard abattoir after slaughtering, immediately frozen and taken to the laboratory for storage at -40°C until analysis.

Sample extraction

Urine

One millilitre of bovine urine was transferred to a 15-mL glass tube and spiked with 10 ng of internal standard (DMTU) in order to give a final concentration of 10 \( \mu \text{g L}^{-1} \), then vortexed and left for 5 minutes to equilibrate. The samples then underwent denaturation conditions at 65°C for 30 min, after the addition of 1 mL of PBS buffer with 0.1% DTT at pH 7. NaCl (2 g) was added to the solution to mixture as a salting-out reagent.

The extraction was performed by twice repeating these steps: addition of 5 mL tert-butyl methyl ether, centrifugation at 2000 x g for 5 min at 4°C, and collection and transfer of the upper organic layer to a 10-mL polypropylene tube. The extract was dried under vacuum in a rotary evaporator apparatus (Heidolph Instruments GmbH & Co., Schwabach, Germany) at a temperature of 40°C. The residue was dissolved in 200 \( \mu \text{L} \) of the mobile phase (methanol: 0.1% aqueous formic acid, v/v 50:50) and transferred to vials for HPLC. The injection volume was 10 \( \mu \text{L} \).

Thyroid gland

The thyroid gland samples were minced with surgical scissors and homogenised. The sample (1 g) was weighed in a polypropylene tube and 10 ng of internal standard (DMTU) were added, and then the sample was vortexed and left for 5 minutes to equilibrate, then 5 mL of methanol was added. The samples were vortexed, placed in an ultrasonic bath for 10 min and then centrifuged at 2000 x g at 4°C for 10 min. The organic liquid supernatant was then filtrated and transferred to a 15-mL glass tube and 5 mL of PBS buffer with 0.1% DTT at pH 7 were added. The samples underwent denaturation conditions at 65°C for 30 min. To carry out the extraction of the analytes, 2 x 10 mL of Tert-butyl methyl ether and 4 g of NaCl (used as a salting-out reagent) were added to the solution. The sample was centrifuged at 2000 x g for 5 min at 4°C. The upper organic layer was collected and transferred to a 50-mL glass evaporating flask. Lastly, the extracts were combined and dried under vacuum in a rotary evaporator apparatus at 40°C. The residue was dissolved in 200 \( \mu \text{L} \) of the mobile phase and transferred to vials for the autosampler. The injection volume was 10 \( \mu \text{L} \).

HPLC-MS/MS analysis
A Synergi Hydro RP reverse-phase HPLC column C18 (150 x 2.0 mm, i.d. 4 µm) with a C18 4 x 3.0 mm guard column (Phenomenex, Torrance, CA, USA) at a column oven temperature of 30°C was used for the separation, which was performed by an HPLC system that included a Surveyor MS quaternary pump with a degasser, a Surveyor AS autosampler with a column oven, and a Rheodyne valve with a 20-µl sample loop (Thermo Fisher Scientific, San Jose, CA, USA). The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and methanol (solvent B), and the flow rate was set at 200 µL/min. The gradient program is shown in Table 1. The overall run time was 30 minutes. The HPLC system was connected to a TSQ Quantum (Thermo Fisher Scientific, San Jose, CA, USA) triple-quadrupole mass spectrometer with an electrospray interface (ESI) set in the positive (ESI+) ionization mode. The acquisition was made in the multiple reaction-monitoring (MRM) mode. The specific acquisition parameters of all the analytes were optimised by means of direct infusion of standard solutions of the analytes at a concentration of 1 µg mL⁻¹, a flow rate of 50 µL min⁻¹ and a flow rate of the MS pump of 100 µL min⁻¹. The capillary voltage was 3.2 kV; the capillary temperature was 340°C; nitrogen was used as the sheath and auxiliary gas at 30 and 10 arbitrary units, respectively, and argon as the collision gas at 1.5 mTorr; peak resolution was 0.70 Da FWHM. The parent ions, product ions, and collision energy values for each analyte are shown in Table 2. The scan time for each monitored transition was 0.1 s and the scan width was 0.5 amu. The mass spectrometer data acquisition and processing were carried out using Xcalibur™ 2.0.7 SP1 software from Thermo Fisher Scientific Inc.

Method validation

The HPLC-MS/MS method was validated according to the guidelines of Commission Decision 2002/657/EC [24]. MS identification criteria were verified throughout the validation study by monitoring relative retention times, signal-to-noise ratios (S/N) and ion ratios. The instrumental linearity was evaluated through calibration curves in solvent at six levels, (1.0, 5.0, 10, 20, 50, 80, 100 µg L⁻¹) and 10 µg L⁻¹ of DMTU as I.S.

The method validation parameters were determined with fortified blank urine and thyroid gland samples at three concentration levels (5.0, 10, 15 µg L⁻¹ and µg kg⁻¹) in six replicates on three different days (6 samples x 3 concentration levels x 3 series = 54 analyses). Method recovery and precision were evaluated using the matrix curves; recovery is calculated as ratio between the measured concentration to fortified concentration, corrected by internal standard and expressed in percentage; precision is calculated in terms of intra- and inter-day repeatability expressed as the coefficient of variability (CV). The same data from the matrix calibration curves were used to calculate the decision limit (CCα) and the detection capability (CCβ) according to the matrix validation curve procedure described in the Commission Decision 2002/657/EC and clarified in the document SANCO/2004/2726-rev. 4 [24, 25].
Results and Discussion

Sample preparation

Despite the diversity of the matrices analysed, we carried out two similar methods to prepare urine and thyroid glands in order to have the same steps for each matrix.

A preliminary denaturation step of matrix proteins was carried out to disrupt the protein–thyrostat interaction, as reported by Vanden Bussche et al. [11], through the cleavage of the disulfide bonds of the proteins by the addition of a reducing agent, such as DTT. Differently from the above mentioned study, which considered only urine, we adopted this step for both urine and thyroid glands, with a ten-time-lower concentration of DTT.

The polarity of the thyrostats requires the use of an organic polar solvent to extract them from the matrices: we evaluated the applicability of different solvents by several tests using ethyl acetate, chloroform and tert-butyl methyl ether. Comparing the signal intensity of the analytes extracted with the three different solvents, tert-butyl methyl ether was chosen as the best solvent for the extraction. The poorest results were obtained by the extraction performed with ethyl acetate by which we could not extract most of the thyrostats.

In order to facilitate the phase separation and to reduce the miscibility of the analytes in the aqueous phase, this protocol adopted the approach of salting-out-assisted liquid–liquid extraction (SALLE), adding salt (NaCl) prior to the liquid–liquid extraction to favour the transfer of the analytes into the organic solvent [26-28].

Method validation

The analytical procedures developed were subjected to the validation process according to the Commission Decision 2002/657/EC and clarified in the document SANCO/2004/2726-rev. 4 [24, 25].

The HPLC–MS/MS-reconstructed chromatograms of the thyrostats in urine and thyroid glands are shown in Figure 2. DMTU as the internal standard (10 μg L$^{-1}$) is also reported. The analytes were detected and confirmed based on their proper relative retention times and their ion ratios. The relative retention times were within a tolerance limit of 2.5% and the relative ion intensities were within the maximum permitted tolerances [24]. The chromatograms in Figure 3 show the absence of interference peaks at the expected retention times of the thyrostats, hence illustrating a good specificity and selectivity of the method.

For the HPLC-MS/MS confirmation of substances listed in Group A of Annex I of Directive 96/23/EC [13], a minimum of four identification points (IPs) is required [24]. In the present work, we monitored five products ions with the highest intensity. Each one of the five product ions is equal to 1.5 IPs, making a total of 7.5 IPs. The ion giving the highest signal-to-noise ratio was selected for the quantification. The MRM transition intensities were compliant with the maximum tolerances permitted. The parameters obtained for the method validations are given in Tables 3, 4 and 5.
Linearity was verified by using squared correlation coefficients ($r^2$). The regression coefficients of the curves that were built to check the instrumental linearity were higher than 0.982, which indicates a satisfactory linearity for all the analytes. Good linearities were also achieved in urine and in thyroid glands and showed values higher than 0.978 and 0.973, respectively, thus demonstrating a suitable and adequate correlation between the concentration and the acquired response in the sample for both matrices. The precision of the method, which was calculated by applying one-way analysis of variance (ANOVA), was evaluated in terms of intra- and inter-day repeatability, and is expressed as the coefficients of variation (CV) from the replicate samples. Their values were lower than 23%, as proposed by Thompson [29], demonstrating an acceptable precision for the method. The recoveries showed good values ranging from 96% to 104% in urine and from 96% to 104% in thyroid glands. The results regarding the precision, even if similar, are not comparable with the results obtained by Abuin et al [22, 30], who developed methods for the detection of underivatised thyreostats in thyroid, because of the lower concentrations used in this paper. The decision limit (CC$_\alpha$) and detection capability (CC$_\beta$) are very important, debated and decisive points to evaluate. For the estimation of these values, the document of the Commission Decision 2002/657/EC [24] explains both the definition and procedure. However, the approach proposed in the document to evaluate these limits – based on the extrapolation of the calibration curve procedure according to ISO 11843 – may lead to an underestimation of the parameters, as already explained by Galarini et al. [31] and other authors [32-33].

Therefore, CC$_\alpha$ (and, consequently, CC$_\beta$) was determined using a parallel extrapolation to the x-axis at the lowest experimental concentration as clarified in the document SANCO/2004/2726-rev. 4 [25]. Decision limits achieved with this approach were thus experimentally determined, and therefore not underestimated. A comparison with previously published data concerning the detection of non-derivatised thyreostats should consider the differences in the method of CC$_\alpha$ determination. Table 5 shows the obtained CC$_\alpha$ and CC$_\beta$ values, which are lower than the minimum required performance limits (MRPLs) proposed in the CRL guidance document of 2007 in urine and in thyroid glands [17]. Moreover, the TAP analytical limits are lower than those reported in literature for the two matrices, such as MTU in the thyroid gland [11, 22, 30, 34]. Finally, it is worth noting that the validation parameters obtained with our method are comparable between the two different matrices.

**Conclusion**

The methods for the simultaneous direct identification and quantification of five thyreostats without derivatisation in both urine and thyroid gland samples were specific and sensitive. Moreover, the validated methods guarantee a better performance for TAP in both matrices than those reported in the literature. The choice to develop a method without derivatisation and clean-up steps was made due to the advantages in terms of costs and the time of analysis. The
simultaneous determination of five thyreostats in two matrices using similar methods could be useful to make comparative analyses more reliable, because the process variables are the same for urine and thyroid glands.

Furthermore, the measurement of the endogenous TU in urine and thyroid is possible as the analytical limits are all below 10 μg L⁻¹ and 10 μg kg⁻¹, and particularly considering that the CCα – which was determined as clarified by the document SANCO/2004/2726-rev. 4 [25] – is not an estimate, but an experimentally verified concentration with all the characteristics required by the Commission Decision 2002/657/EC [24] for a substance to be quantified.

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Compliance with Ethical Standards

Conflict of Interest Author Luca Maria Chiesa declares that he has no conflict of interest. Author Giuseppe Federico Labella declares that he has no conflict of interest. Author Elisa Pasquale declares that she has no conflict of interest. Author Sara Panseri declares that she has no conflict of interest. Author Radmila Pavlovic declares that she has no conflict of interest. Author Francesco Arioli declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with animals performed by any of the authors.

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Table 1. Gradient table for HPLC method

| Time (min) | Eluent A (%) | Eluent B (%) | Flow rate (µL min⁻¹) |
|-----------|--------------|--------------|-----------------------|
| 0         | 90           | 10           | 200                   |
| 2         | 90           | 10           | 200                   |
| 20        | 30           | 70           | 200                   |
| 24        | 10           | 90           | 200                   |
| 27        | 90           | 10           | 200                   |
| 30        | 90           | 10           | 200                   |

A: 0.1% aqueous formic acid; B: methanol
Table 2. MS/MS conditions for the MRM acquisitions of analytes and the internal standard. Ions for quantification are in bold.

| Analyte     | Precursor ion [M-H]^+ (m/z) | Product ions^{CE} (m/z) | ESI |
|-------------|-----------------------------|--------------------------|-----|
| TAP         | 115                         | 56, 57, 74, 83, 88       | (+) |
| TU          | 128                         | 57, 60, 70, 83, 111      | (+) |
| MTU         | 143                         | 60, 72, 84, 126         | (+) |
| PTU         | 171                         | 60, 67, 86, 112, 154   | (+) |
| PhTU        | 205                         | 77, 86, 103, 146       | (+) |
| DMTU (I.S.) | 157                         | 60, 72, 86, 98, 140    | (+) |

I.S.: internal standard

CE (eV): collision energy
Table 3. Analytical performance (method trueness and precision) data for thyreostat determination in urine.

| Analyte | Concentration level (μg/L) | Recovery % (n = 18) | intra-day (CV; n = 6) | inter-day (CV; n = 18) |
|---------|-----------------------------|---------------------|---------------------|---------------------|
| TAP     | 5                           | 99                  | 8                   | 20                  |
|         | 10                          | 101                 | 5                   | 19                  |
|         | 15                          | 100                 | 5                   | 8                   |
|         | 5                           | 104                 | 15                  | 20                  |
| TU      | 10                          | 98                  | 10                  | 11                  |
|         | 15                          | 101                 | 5                   | 5                   |
| MTU     | 5                           | 104                 | 6                   | 19                  |
|         | 10                          | 96                  | 9                   | 20                  |
|         | 15                          | 101                 | 7                   | 9                   |
| PTU     | 5                           | 104                 | 12                  | 19                  |
|         | 10                          | 96                  | 7                   | 16                  |
|         | 15                          | 101                 | 5                   | 7                   |
| PhTU    | 5                           | 100                 | 11                  | 16                  |
|         | 10                          | 100                 | 5                   | 13                  |
|         | 15                          | 100                 | 3                   | 5                   |

CV: coefficient of variation
Table 4. Analytical performance (method trueness and precision) data for thyreostat determination in thyroid glands.

| Analyte | Concentration level (μg/kg) | Recovery % (n = 18) | intra-day repeatability (CV; n = 6) | inter-day repeatability (CV; n = 18) |
|---------|-----------------------------|---------------------|-----------------------------------|------------------------------------|
| TAP     | 5                           | 104                 | 7                                 | 19                                 |
|         | 10                          | 96                  | 10                                | 20                                 |
|         | 15                          | 101                 | 8                                 | 10                                 |
|         | 5                           | 101                 | 15                                | 21                                 |
| TU      | 10                          | 99                  | 9                                 | 17                                 |
|         | 15                          | 100                 | 7                                 | 9                                  |
|         | 5                           | 99                  | 14                                | 17                                 |
|         | 10                          | 103                 | 9                                 | 10                                 |
|         | 15                          | 103                 | 9                                 | 18                                 |
| MTU     | 5                           | 102                 | 12                                | 20                                 |
|         | 10                          | 98                  | 6                                 | 17                                 |
|         | 15                          | 101                 | 8                                 | 9                                  |
| PTU     | 5                           | 100                 | 12                                | 14                                 |
|         | 10                          | 100                 | 9                                 | 12                                 |
|         | 15                          | 100                 | 9                                 | 9                                  |

CV: coefficient of variation
Table 5. Decision limits (CCα) and detection capabilities (CCβ) calculated for thyreostats in urine and in thyroid glands.

| Analyte | CCα (μg L⁻¹ and μg kg⁻¹) | CCβ (μg L⁻¹ and μg kg⁻¹) |
|---------|---------------------------|---------------------------|
|         | Urine        | Thyroid gland | Urine        | Thyroid gland |
| TAP     | 7.3          | 7.3           | 9.7          | 9.7           |
| TU      | 7.3          | 7.4           | 9.2          | 9.7           |
| MTU     | 7.2          | 7.0           | 9.5          | 8.7           |
| PTU     | 7.2          | 7.4           | 9.2          | 9.6           |
| PhTU    | 6.9          | 6.6           | 8.5          | 8.0           |
Fig. 1. Chemical structure of thyreostats. TU (2-thiouracil), MTU (6-methyl-2-thiouracil), PTU (6-propyl-2-thiouracil), PhTU (6-phenyl-2-thiouracil), TAP (1-methyl-2-mercapto-imidazole; tapazole), DMTU (5,6-dimethyl-2-thiouracil; internal standard).

Fig. 2. HPLC-MS/MS chromatograms and ion spectra of a blank urine (A) and a thyroid gland (B) sample spiked with thyreostats at a final concentration of 5 μg L\(^{-1}\) or μg kg\(^{-1}\), respectively. TU (2-thiouracil), MTU (6-methyl-2-thiouracil), PTU (6-propyl-2-thiouracil), PhTU (6-phenyl-2-thiouracil), TAP (1-methyl-2-mercapto-imidazole; tapazole). The concentration of DMTU (5,6-dimethyl-2-thiouracil; internal standard) is 10 μg L\(^{-1}\) or μg kg\(^{-1}\), respectively.

Fig. 3. HPLC-MS/MS chromatograms of a blank urine (A) and a thyroid gland (B) sample, showing the absence of interfering compounds. TU (2-thiouracil), MTU (6-methyl-2-thiouracil), PTU (6-propyl-2-thiouracil), PhTU (6-phenyl-2-thiouracil), TAP (1-methyl-2-mercapto-imidazole; tapazole).
Figure 1

TU

MTU

PTU

TAP

PhTU

DMTU (I.S.)
Figure 2

(A) TAP

TAP

MTU

PTU

PhTU

PTU

DMTU

(B) TAP

TAP

MTU

TU

PhTU

PTU

DMTU
