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

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Abstract:	<p>Abstract</p> <p>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 $10 \mu\text{g L}^{-1}$ and $10 \mu\text{g kg}^{-1}$, respectively. In urine, the limits of decision ranged from 6.9 to $7.3 \mu\text{g L}^{-1}$, and the capacities of detection ranged from 8.5 to $9.7 \mu\text{g L}^{-1}$, while in thyroid glands these values varied from $6.6 \mu\text{g kg}^{-1}$ to $7.4 \mu\text{g kg}^{-1}$ and from $8.0 \mu\text{g g}^{-1}$ to $9.7 \mu\text{g kg}^{-1}$, respectively. The results obtained show that the methods described are suitable for the direct detection of thyreostats in bovine urine and thyroid glands.</p>

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

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8

9 **Introduction**

10 Thyreostats are drugs that interfere with the mechanism involved in the synthesis of thyroid hormones and cause a
11 condition of deficiency of circulating thyroxine (T4) and triiodothyronine (T3) [1, 2], whose production and release are
12 controlled by the hypothalamus–anterior pituitary axis. The hypothalamus secretes thyrotropin-releasing hormone (TRH),
13 which in turn stimulates the anterior pituitary gland to release thyroid-stimulating hormone (TSH) that induces the
14 production of T3 and T4 by the thyroid, which releases them into the bloodstream. These hormones activate the nuclear
15 transcription of a large number of genes, thus causing the synthesis of enzymes, as well as structural and transport proteins.
16 This leads to an increase in metabolism and maintains the physical and psychological development of the organism. The
17 administration of thyreostats causes an improvement in bodyweight gain mainly due to increased absorption and
18 extracellular retention of water in the edible tissues and in the gastrointestinal tract [3]. Thyreostats are polar amphoteric
19 thionamides with a heterocyclic tautomeric structure, and are mostly derived from thiouracil and mercapto-imidazole.
20 The sequence consisting of nitrogen–carbon–sulphur, known as thioamide, is considered responsible for the thyroid-
21 inhibiting activity (Fig. 1). The best known thyreostatic drugs include the very potent thyroid-inhibiting compounds 2-
22 thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU) and 1-methyl-
23 2-mercapto-imidazole (tapazole, TAP) [4-6]. The chemical structures of these substances are shown in Figure 1.

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

27 In 1981, the European Union banned their use in animal production both as growth promoters and therapeutic
28 agents [12] and classified them as “substances having anabolic effects and unauthorized substances” belonging to the
29 group A2 as described by the Council Directive 96/23/CE [13]. However, a relationship between the presence of
30 Brassicaceae in feed and thiouracil in urine has been demonstrated by Pinel et al. [9], Vanden Bussche et al. [14] and
31 Kiebooms et al. [15, 16]. The Community Reference Laboratories (CRLs) in 2007 proposed a recommended
32 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
33 thiouracil have been detected in bovine animals fed with cruciferous plants, however there is scientific evidence showing
34 that levels above 10 ppb in urine cannot be linked to natural origin due to this contamination” [17]. Recently, Wauters et
35 al. reported concentrations of up to 18.2 µg L⁻¹ in the 99% percentile from 3894 bovines and they suggested that the
36 recommended concentration should be increased to 30 µg L⁻¹ [18]. In fact, the 2015 Italian National Residue Plan already
37 provides this concentration as the limit of detection for thyreostats in urine [19].

38 Thyreostats analyses typically consist of separation methods based on gas or liquid chromatography associated
39 with a mass spectrometry system of detection. Normally, the extraction of the substances is carried out by using polar
40 solvents more suitable to the chemical characteristics of the thyreostats, such as methanol, acetonitrile or ethyl acetate.
41 Further steps of purification or clean-up with different kinds of solid-phase extraction (SPE) have been reported. Due to
42 the low molecular mass and high polarity of the thyreostats, several authors have proposed a derivatisation step before or
43 after the clean-up, mainly by using 3-iodobenzylbromide (3-IBBr) in the case of HPLC-MS/MS analysis [6]. In the case
44 of GC methods, derivatisation is an unavoidable step in order to convert the analytes into volatile compounds. When
45 HPLC is applied as the separation technique, analytes may be derivatised and, in the analysis of thyreostats, this procedure
46 induces the stabilisation of the chemical structure of the molecule in a specific and single tautomeric form, the reduction
47 of the molecular polarity in order to increase the separation characteristics on the reversed-phase column in the case of
48 HPLC-MS detection, and an increase in the molecular mass [20]. The low molecular mass, particularly, could be disturbed
49 by the chemical noise. In term of sensitivity, the derivatisation leads to an improvement of the signal to noise ratio, and
50 subsequently of the detection capabilities [21]. Despite these advantages, the derivatisation procedure can generally cause
51 a loss in analyte concentrations. Furthermore, removing derivatisation step simplifies, shortens and makes cheaper the
52 whole analysis procedure [22, 23]. Based on these observations, we developed the extraction without derivatisation of the
53 five above-mentioned thyreostats in bovine urine and thyroid glands followed by a sensitive, specific and reproducible
54 HPLC-MS/MS analysis. For the full identification and quantification of the analytes, the criteria established in the
55 2002/657/EC Commission Decision were followed and the decision limit ($CC\alpha$) and the detection capability ($CC\beta$) were
56 calculated according to the matrix calibration curve procedure as clarified in the document SANCO/2004/2726 rev. 4 [24,
57 25].

58

59 **Materials and Methods**

60 **Reagents and chemicals**

61 All solvents were of HPLC-MS grade quality and purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic
62 acid (98–100%) was from Riedel-de Haën (Sigma-Aldrich). Ultrapure water was obtained through a Milli-Q system
63 (Millipore, Merck KGaA, Darmstadt, Germany). KH_2PO_4 and NaCl were from Sigma-Aldrich. The analytes 2-thiouracil
64 (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU), 2-
65 mercaptobenzimidazole or tapazole (TAP) were acquired from Sigma-Aldrich, as well as 5,6-dimethyl-2-thiouracil
66 (DMTU), used as internal standard (I.S.). A stock solution of 1 mg mL^{-1} was prepared by dissolving the compounds in
67 methanol. Serial dilutions were prepared by diluting the stock solution in the mobile phase, which were then stored at
68 -40°C .

69 Phosphate buffer, prepared by dissolving 0.25 M KH_2PO_4 in ultrapure water, was adjusted to pH 7 and then
70 saturated with 0.1% DL- dithiothreitol (DTT; Sigma- Aldrich) as in Vanden Bussche et al. [11].

71

72 **Sample collection**

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

75 **Sample extraction**

76 *Urine*

77 One millilitre of bovine urine was transferred to a 15-mL glass tube and spiked with 10 ng of internal standard (DMTU)
78 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
79 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.
80 NaCl (2 g) was added to the solution to mixture as a salting-out reagent.

81 The extraction was performed by twice repeating these steps: addition of 5 mL *tert*-butyl methyl ether,
82 centrifugation at $2000\ \times\ \text{g}$ for 5 min at 4°C , and collection and transfer of the upper organic layer to a 10-mL polypropylene
83 tube. The extract was dried under vacuum in a rotary evaporator apparatus (Heidolph Instruments GmbH & Co.,
84 Schwabach, Germany) at a temperature of 40°C . The residue was dissolved in 200 μL of the mobile phase (methanol:
85 0.1% aqueous formic acid, v/v 50:50) and transferred to vials for HPLC. The injection volume was 10 μL .

86 *Thyroid gland*

87 The thyroid gland samples were minced with surgical scissors and homogenised. The sample (1 g) was weighed in a
88 polypropylene tube and 10 ng of internal standard (DMTU) were added, and then the sample was vortexed and left for 5
89 minutes to equilibrate, then 5 mL of methanol was added. The samples were vortexed, placed in an ultrasonic bath for 10
90 min and then centrifuged at $2000\ \times\ \text{g}$ at 4°C for 10 min. The organic liquid supernatant was then filtrated and transferred
91 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
92 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
93 of NaCl (used as a salting-out reagent) were added to the solution. The sample was centrifuged at $2000\ \times\ \text{g}$ for 5 min at
94 4°C . The upper organic layer was collected and transferred to a 50-mL glass evaporating flask. Lastly, the extracts were
95 combined and dried under vacuum in a rotary evaporator apparatus at 40°C . The residue was dissolved in 200 μL of the
96 mobile phase and transferred to vials for the autosampler. The injection volume was 10 μL .

97

98 **HPLC-MS/MS analysis**

99 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
100 (Phenomenex, Torrance, CA, USA) at a column oven temperature of 30°C was used for the separation, which was
101 performed by an HPLC system that included a Surveyor MS quaternary pump with a degasser, a Surveyor AS autosampler
102 with a column oven, and a Rheodyne valve with a 20- μl sample loop (Thermo Fisher Scientific, San Jose, CA, USA).
103 The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and methanol (solvent B), and the flow rate was set
104 at 200 $\mu\text{L}/\text{min}$. The gradient program is shown in Table 1. The overall run time was 30 minutes. The HPLC system was
105 connected to a TSQ Quantum (Thermo Fisher Scientific, San Jose, CA, USA) triple-quadrupole mass spectrometer with
106 an electrospray interface (ESI) set in the positive (ESI+) ionization mode. The acquisition was made in the multiple
107 reaction-monitoring (MRM) mode. The specific acquisition parameters of all the analytes were optimised by means of
108 direct infusion of standard solutions of the analytes at a concentration of 1 $\mu\text{g mL}^{-1}$, a flow rate of 50 $\mu\text{L min}^{-1}$ and a flow
109 rate of the MS pump of 100 $\mu\text{L min}^{-1}$. The capillary voltage was 3.2 kV; the capillary temperature was 340°C; nitrogen
110 was used as the sheath and auxiliary gas at 30 and 10 arbitrary units, respectively, and argon as the collision gas at 1.5
111 mTorr; peak resolution was 0.70 Da FWHM. The parent ions, product ions, and collision energy values for each analyte
112 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
113 spectrometer data acquisition and processing were carried out using Xcalibur™ 2.0.7 SP1 software from Thermo Fisher
114 Scientific Inc.

115

116 **Method validation**

117 The HPLC-MS/MS method was validated according to the guidelines of Commission Decision 2002/657/EC [24]. MS
118 identification criteria were verified throughout the validation study by monitoring relative retention times, signal-to-noise
119 ratios (S/N) and ion ratios. The instrumental linearity was evaluated through calibration curves in solvent at six levels,
120 (1.0, 5.0, 10, 20, 50, 80, 100 $\mu\text{g L}^{-1}$) and 10 $\mu\text{g L}^{-1}$ of DMTU as I.S.

121 The method validation parameters were determined with fortified blank urine and thyroid gland samples at three
122 concentration levels (5.0, 10, 15 $\mu\text{g L}^{-1}$ and $\mu\text{g kg}^{-1}$) in six replicates on three different days (6 samples \times 3 concentration
123 levels \times 3 series = 54 analyses). Method recovery and precision were evaluated using the matrix curves; recovery is
124 calculated as ratio between the measured concentration to fortified concentration, corrected by internal standard and
125 expressed in percentage; precision is calculated in terms of intra- and inter-day repeatability expressed as the coefficient
126 of variability (CV). The same data from the matrix calibration curves were used to calculate the decision limit ($CC\alpha$) and
127 the detection capability ($CC\beta$) according to the matrix validation curve procedure described in the Commission Decision
128 2002/657/EC and clarified in the document SANCO/2004/2726-rev. 4 [24, 25].

129

130 **Results and Discussion**

131 **Sample preparation**

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

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

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

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

146

147 **Method validation**

148 The analytical procedures developed were subjected to the validation process according to the Commission Decision
149 2002/657/EC and clarified in the document SANCO/2004/2726-rev. 4 [24, 25].

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

156 For the HPLC-MS/MS confirmation of substances listed in Group A of Annex I of Directive 96/23/EC [13], a
157 minimum of four identification points (IPs) is required [24]. In the present work, we monitored five products ions with
158 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
159 highest signal-to-noise ratio was selected for the quantification. The MRM transition intensities were compliant with the
160 maximum tolerances permitted. The parameters obtained for the method validations are given in Tables 3, 4 and 5.

161 Linearity was verified by using squared correlation coefficients (r^2): The regression coefficients of the curves that were
162 built to check the instrumental linearity were higher than 0.982, which indicates a satisfactory linearity for all the analytes.
163 Good linearities were also achieved in urine and in thyroid glands and showed values higher than 0.978 and 0.973,
164 respectively, thus demonstrating a suitable and adequate correlation between the concentration and the acquired response
165 in the sample for both matrices. The precision of the method, which was calculated by applying one-way analysis of
166 variance (ANOVA), was evaluated in terms of intra- and inter-day repeatability, and is expressed as the coefficients of
167 variation (CV) from the replicate samples. Their values were lower than 23%, as proposed by Thompson [29],
168 demonstrating an acceptable precision for the method. The recoveries showed good values ranging from 96% to 104% in
169 urine and from 96% to 104% in thyroid glands. The results regarding the precision, even if similar, are not comparable
170 with the results obtained by Abuìn et al [22, 30], who developed methods for the detection of underivatized thyreostats in
171 thyroid, because of the lower concentrations used in this paper. The decision limit ($CC\alpha$) and detection capability ($CC\beta$)
172 are very important, debated and decisive points to evaluate. For the estimation of these values, the document of the
173 Commission Decision 2002/657/EC [24] explains both the definition and procedure. However, the approach proposed in
174 the document to evaluate these limits – based on the extrapolation of the calibration curve procedure according to ISO
175 11843 – may lead to an underestimation of the parameters, as already explained by Galarini et al. [31] and other authors
176 [32-33].

177 Therefore, $CC\alpha$ (and, consequently, $CC\beta$) was determined using a parallel extrapolation to the x-axis at the
178 lowest experimental concentration as clarified in the document SANCO/2004/2726-rev. 4 [25]. Decision limits achieved
179 with this approach were thus experimentally determined, and therefore not underestimated. A comparison with previously
180 published data concerning the detection of non-derivatized thyreostats should consider the differences in the method of
181 $CC\alpha$ determination. Table 5 shows the obtained $CC\alpha$ and $CC\beta$ values, which are lower than the minimum required
182 performance limits (MRPLs) proposed in the CRL guidance document of 2007 in urine and in thyroid glands [17].
183 Moreover, the TAP analytical limits are lower than those reported in literature for the two matrices, such as MTU in the
184 thyroid gland [11, 22, 30, 34]. Finally, it is worth noting that the validation parameters obtained with our method are
185 comparable between the two different matrices.

186

187 **Conclusion**

188 The methods for the simultaneous direct identification and quantification of five thyreostats without derivatisation in both
189 urine and thyroid gland samples were specific and sensitive. Moreover, the validated methods guarantee a better
190 performance for TAP in both matrices than those reported in the literature. The choice to develop a method without
191 derivatisation and clean-up steps was made due to the advantages in terms of costs and the time of analysis. The

192 simultaneous determination of five thyreostats in two matrices using similar methods could be useful to make comparative
193 analyses more reliable, because the process variables are the same for urine and thyroid glands.

194 Furthermore, the measurement of the endogenous TU in urine and thyroid is possible as the analytical limits are
195 all below $10 \mu\text{g L}^{-1}$ and $10 \mu\text{g kg}^{-1}$, and particularly considering that the $\text{CC}\alpha$ – which was determined as clarified by the
196 document SANCO/2004/2726-rev. 4 [25] – is not an estimate, but an experimentally verified concentration with all the
197 characteristics required by the Commission Decision 2002/657/EC [24] for a substance to be quantified.

198

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204

205 **Compliance with Ethical Standards**

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

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

211 **Informed Consent** Not applicable.

212

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312

313 Table 1. Gradient table for HPLC method

314

Time (min)	Eluent A (%)	Eluent B (%)	Flow rate ($\mu\text{L min}^{-1}$)
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

315

316

317 Table 2. MS/MS conditions for the MRM acquisitions of analytes and the internal standard. Ions for quantification are
318 in bold.

319

Analyte	Precursor ion [M-H] ⁺ (m/z)	Product ions ^{CE} (m/z)	ESI
TAP	115	56 ₂₂ , 57 ₂₀ , 74 ₁₇ , 83 ₁₇ , 88 ₁₆	(+)
TU	128	57 ₃₅ , 60 ₃₄ , 70 ₁₇ , 83 ₂₇ , 111 ₁₆	(+)
MTU	143	60 ₃₂ , 72 ₃₄ , 84 ₁₇ , 86 ₂₃ , 126 ₁₆	(+)
PTU	171	60 ₃₅ , 67 ₂₆ , 86 ₂₇ , 112 ₁₉ , 154 ₁₇	(+)
PhTU	205	77 ₄₁ , 86 ₂₇ , 103 ₂₆ , 105 ₂₅ , 146 ₁₉	(+)
DMTU (I.S.)	157	60 ₃₅ , 72 ₂₉ , 86 ₂₂ , 98 ₁₈ , 140 ₁₆	(+)

I.S.: internal standard

CE (eV): collision energy

320

321

322

323 Table 3. Analytical performance (method trueness and precision) data for thyrostat determination in urine.

324

Analyte	Concentration level ($\mu\text{g/L}$)	Recovery % (n = 18)	Repeatability	
			intra-day (CV; n = 6)	inter-day (CV; n = 18)
TAP	5	99	8	20
	10	101	5	19
	15	100	5	8
TU	5	104	15	20
	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

325

326

327 Table 4. Analytical performance (method trueness and precision) data for thyreostat determination in thyroid glands.

328

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

CV: coefficient of variation

329

330

331

332 Table 5. Decision limits ($CC\alpha$) and detection capabilities ($CC\beta$) calculated for thyreostats in urine and in thyroid glands.
333

Analyte	$CC\alpha$ ($\mu\text{g L}^{-1}$ and $\mu\text{g kg}^{-1}$)		$CC\beta$ ($\mu\text{g L}^{-1}$ and $\mu\text{g kg}^{-1}$)	
	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

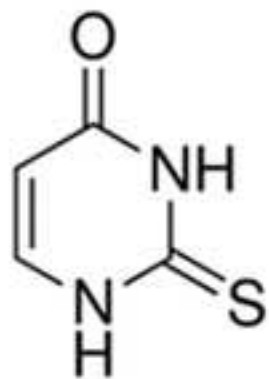
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335 **Fig. 1.** Chemical structure of thyreostats. TU (2-thiouracil), MTU (6-methyl-2-thiouracil), PTU (6-propyl-2-thiouracil),
336 PhTU (6-phenyl-2-thiouracil), TAP (1-methyl-2-mercapto-imidazole; tapazole), DMTU (5,6-dimethyl-2-thiouracil;
337 internal standard).

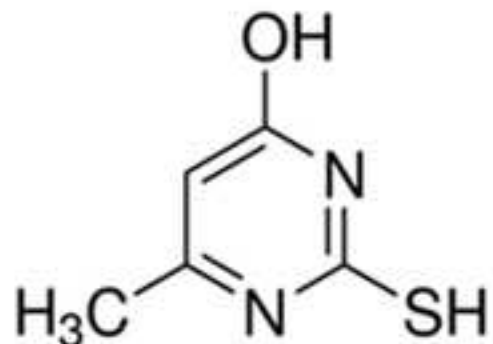
338 **Fig. 2.** HPLC-MS/MS chromatograms and ion spectra of a blank urine (A) and a thyroid gland (B) sample spiked with
339 thyreostats at a final concentration of $5 \mu\text{g L}^{-1}$ or $\mu\text{g kg}^{-1}$, respectively. TU (2-thiouracil), MTU (6-methyl-2-thiouracil),
340 PTU (6-propyl-2-thiouracil),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

342 **Fig. 3.** HPLC-MS/MS chromatograms of a blank urine (A) and a thyroid gland (B) sample, showing the absence of
343 interfering compounds. TU (2-thiouracil), MTU (6-methyl-2-thiouracil), PTU (6-propyl-2-thiouracil), PhTU (6-phenyl-
344 2-thiouracil), TAP (1-methyl-2-mercapto-imidazole; tapazole).

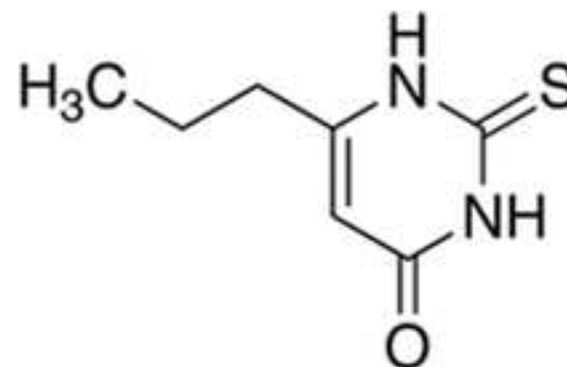
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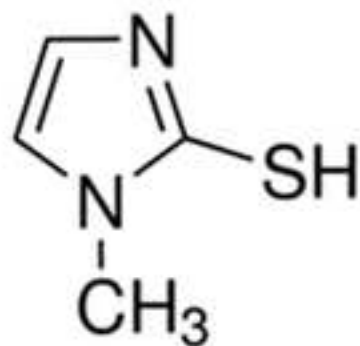
TU



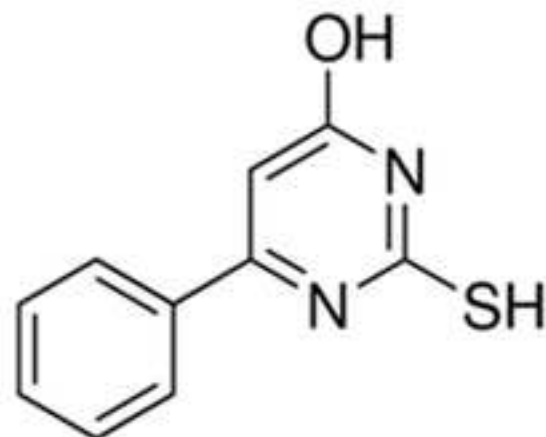
MTU



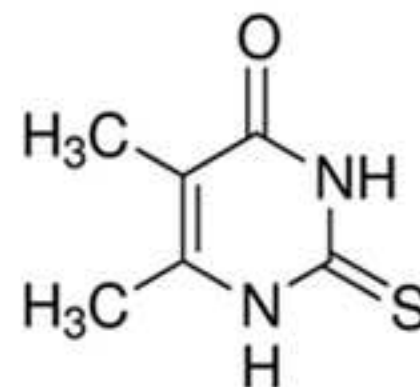
PTU



TAP



PhTU



DMTU (I.S.)

