The occurrence of thiouracil in pig and bovine urine collected from Croatian farms

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Abstract

Thyrostats are a group of forbidden substances in food producing animals that increase water absorption in muscle tissue and the gastrointestinal tract of animals. These substances can be abused as illegal growth promoters as oral drugs on farms before slaughtering. The consequences of their illegal use on farm animals include higher yield though inferior meat quality, while these substances also pose a potential risk to human health, and so their application is banned in European Union Member States. Thiouracil (2-thiouracil) (TU) is the representative, and belongs to the group of thyrostats but also can be naturally present in the urine of farm animals fed with feed containing *Brassicaceae* species. The aim of this study was to monitor TU concentrations in pig and bovine urine in the period from 2015 to 2023. For this purpose, 391 urine samples were collected as a part of the National Residue Control Plan (NRCP) and TU concentrations were determined by liquid chromatography tandem mass spectrometry. TU was detected in 89 of 391 samples, or 22.76%. TU concentration ranged from 1.66 to 28.30 µg/L, and a statistically significant difference ($P < 0.05$) was determined in urine TU concentrations between pigs and bovines. Mean concentrations of TU varied by year, and ranged from 1.66 to 8.06 µg/L in pig urine and from 5.92 to 13.68 µg/L in bovine urine. None of the analysed urine samples contained TU concentration in excess of 30 µg/L, which is the cut-off value to distinguish potentially natural origin resulting from a cruciferous diet (*Brassicaceae* species). The results exclude the possibility of abuse of this substances in the livestock industry in the Republic of Croatia.

Key words: *thyrostatics; thiouracil; urine; pigs; bovines*

Introduction

Thiouracil or 2-thiouracil (TU) belongs to the group of thyrostats, orally active drugs that disturb the normal function of the thyroid gland by inhibiting synthesis of the thyroid hormones triidothyronine (T3) and thyroxine (T4) (Courthey et al., 2002; Vanden Bussche et al., 2011a; Woźniak et al., 2014), and by increasing water absorption and retention within edible tissue and the gastrointestinal tract (Pinel et al., 2006a). Thyrostats are usually easy to use, they are active per os and effective

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after administration with feed (Woźniak et al., 2018). Because of these properties, they can be abused to enhance growth in farm animals. An animal receiving TU in the diet will retain more water and increase carcases weight. Meat produced from those animals will be of inferior quality, due to the high water content (Abuín et al., 2008; Vanden Bussche et al., 2012; Blokland et al., 2021).

However, the possible presence of thyrostat residues in meat and other products derived from treated animals pose a threat to human health (Pinel et al., 2006a, 2006b; Woźniak et al., 2014; Samardžija et al., 2020). The International Agency for Research on Cancer (IARC, 2001) classified these compounds in group 2B (possibly carcinogenic to humans), and they have been banned in Europe for animal fattening purposes since 1981 (EC 81/602, 1981). After oral administration, they cause cardiovascular disorders (accelerated heart rate, dyspnoea and apnoea) and hypertrophy of the thyroid gland (Woźniak et al., 2018). Research indicates that if they are administered to animals at ~ 5 g per day for weight gain, the expected concentration in urine could be over 100 μg/L (Pinel et al., 2006b; Sterk et al., 2014).

Research has shown that the presence of TU in urine can be the result of abuse, but also of a diet rich in cruciferous plants (*Brassicaceae*) (Woźniak et al., 2014). Pinel et al. (2006b) proved that there is a link between genus *Brassicaceae* feeding and detected TU in urine animals, though these concentrations do not exceed 10 μg/L. *Brassicaceae* species plays a significant role in human nutrition (cauliflower, broccoli, cabbage, rapeseed oil) and in animal diet (rapeseed flakes and cakes) (Vanden Bussche, 2011). These plant species contain a goitrogenic compound responsible for the inhibition of conversion of T3 and T4 in a way that reduces the absorption of iodine in the thyroid gland (Arrizabalaga-Larrañaga et al., 2022).

By applying modern analytical techniques (gas chromatography or liquid chromatography tandem mass spectrometry (GC-MS/MS or LC-MS/MS) for determination of thyrostats, it was determined that the threshold of 10 μg/L for TU is too low and resulted in a large number of false positive samples (Vanden Bussche et al., 2009). Accordingly, recent research has indicated and the European Union Reference Laboratory (EURL) has proposed an increase in the threshold level to 30 μg/L (Blokland et al., 2021; Arrizabalaga-Larrañaga et al., 2022). They stated that the differences in the origin of endogenously formed or exogenously administrated TU might result in the differences of formed metabolites. Furthermore, according to Arrizabalaga-Larrañaga et al. (2022), the markers 4-thiouracil and 6-methyl thiouracil should be analysed to distinguish between exogenous and endogenous TU. In the absence of 6-methyl-thiouracil and in the presence of 4-thiouracil, it can be concluded that TU is not originating from illegal treatment (Blokland et al., 2021) and rather can occur endogenously as a consequence of a diet containing cruciferous plants (rapeseed, rapeseed meal, broccoli, cauliflower) (Woźniak et al., 2012; Stark et al., 2014).

The aim of this study was to determine and monitor the TU levels in pig and bovine urine samples collected on Croatian farms over a nine-year period (2015 to 2023) by implementation of the validated analytical method of liquid chromatography tandem mass spectrometry (LC-MS/MS).

Materials and methods

Sampling and sample stabilisation

Samples of pig and bovine urine were collected during the period from

2015 to 2023 on different farms in Croatia as a part of National Residue Control Plan of the Ministry of Agriculture. In total, 391 urine samples (100 mL urine per animal) were taken from bovines (*n*=247) and pigs (*n*=144). Immediately after receiving urine samples in the laboratory, 40 mL of each urine sample was stabilised by adding 1.6 mL 37% hydrochloric acid and 1.6 mL 0.25 M ethylenediaminetetraacetic acid (EDTA). After stabilisation, urine samples were stored in the freezer at -20°C until analysis.

Sample preparation

In 1 mL stabilized urine, 100 μL internal standard concentration (100 μg/L) and 4 mL 1 M phosphate buffer pH=8.0 was added, followed by vortexing. pH was adjusted to 8.0±0.5 by adding 0.1 M NaOH. Further, 100 μL methanol 3-iodobenzyl bromide solution was added and vortexed. This was followed by incubation for 60 minutes at 40°C in a water bath. After cooling to room temperature, 5 mL ethyl acetate was added to samples and vortexed for 60 s at maximum speed. After centrifugation for 5 min at 5000 rpm and 18°C, the ethyl acetate layer was transferred to a glass tube and evaporated under a stream of nitrogen at 55°C. The residues were dissolved in 500 μL acetonitrile/water solution (30/70) and filtered into vials through regenerated cellulose filters (pore size 0.45 μm).

For analysis, HPLC grade solvents, i.e., methanol, acetonitrile and ethyl acetate, were obtained from Sigma Aldrich (St. Louis, MO, USA). Analytical standard of 2-thiouracil was obtained from Sigma Aldrich (St. Louis, MO, USA) and the internal standard Thiouracil $^{13}C^{15}N_2$ (deutereted form) was purchased from the European Reference Laboratory (WFSR, Wageningen, Netherlands). All other chemicals were of analytical grade.

Chromatographic and mass spectrometry conditions

The HPLC equipment consisted of a degasser, a binary pump, column compartment, autosampler (Infinity 1260, Santa Clara, CA, USA) and was coupled with a QQQ 6410 mass spectrometer provided by Agilent Technologies (Santa Clara, CA, USA). Chromatographic separation was performed on a Kinetex C18 HPLC column (3 x 100 mm, and particle size 2.6 μm) (Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.1% acetic acid (A) and acetonitrile (B). Gradient elution was employed, as follows: 0–1 min 60% A, 1–4.5 min 15% A, 4.5–10 min 60% A, with a flow rate of 0.5 mL and column temperature of 40°C. Ionisation was performed in positive ion mode (ESI+), with a source temperature set at 350°C, gas flow rate set at 12 L/min, nebuliser set at 45 psi, and capillary voltage set at 4000 V (+) and 1000 V (-). For TU, one precursor and two product ions, and for the internal standard one precursor and one product ion were monitored.

Method validation was performed using an in-house matrix validation and factorial design by InterVal Plus software version 3.4.0.4. (quo data, Gesellschaft für Qualitätsmanagement und Statistik GmbH, Drestden, Germany).

Data analysis

Statistical analysis was performed using the SPSS Statistics Software 22.0 (SPSS Statistics, NY IBM, 2013, Sankt Ingbert, Germany). Differences between pig and bovine urine were established using analysis of variance (ANOVA), with statistical significance set at 95% ($P = 0.05$).

Results and discussion

Table 1 shows the diagnostic ions, fragmentation voltage, collision energy and retention time for TU and Thiouracil $^{13}C^{15}N_2$ (TU-$^{13}C^{15}N_2$) as the internal standard. Figure 1 shows total ion current (TIC) and multiple reaction monitoring (MRM) chromatograms of the analytes obtained by use of the LC-MS/MS method. The TU calibration curve (Figure 2) consists of six points of the following concentrations: 2.0, 6.0, 8.0, 10.0, 15.0 and 30.0 μg/L.

Method validation gave the values of 1.40 μg/L for the decision limit (CCα) and 1.71 μg/L for detection capability (CCβ). The obtained validation results met all set criteria given by legislation ((EU) 2021/808, 2021), and therefore it can be considered that the implemented LC-MS/MS method is suitable for TU determination.

Literature data has shown that TU can be present in animal tissues as a result of the illegal administration of thyrostats, or can occur endogenously as a consequence of a diet containing cruciferous (*Brassicaceae*) plants (Woźniak et al., 2012, 2014). Plants from the *Brassicaceae* species

Table 1. Diagnostic parameters for thiouracil (TU) and TU $^{13}C^{15}N_2$

Analyte	Precursor ion	Product ions	Fragmentation Voltage (V)	Collision energy (eV)	Retention time (min)	Internal standard
Thiouracil $^{13}C^{15}N_2$	348.0	216.8	110	16	2.782	-
2-thiouracil	345.0	216.8 89.8	110	14 40	2.785	TU-$^{13}C^{15}N_2$

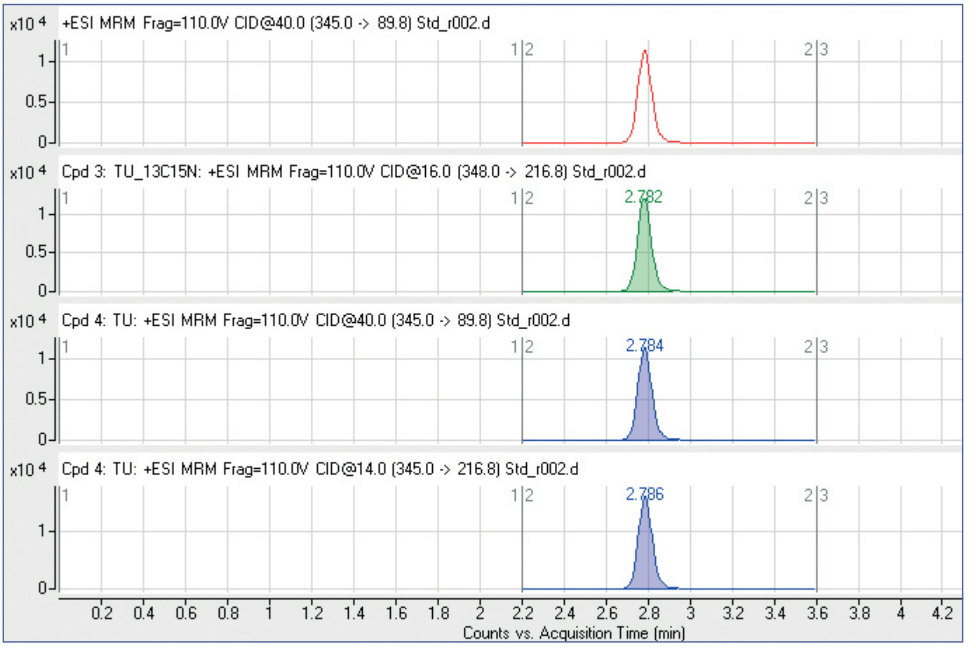

Figure 1. TIC and MRM chromatograms of thiouracil (TU) and TU $^{13}C^{15}N_2$ standard solution with a concentration of 100 μg/L

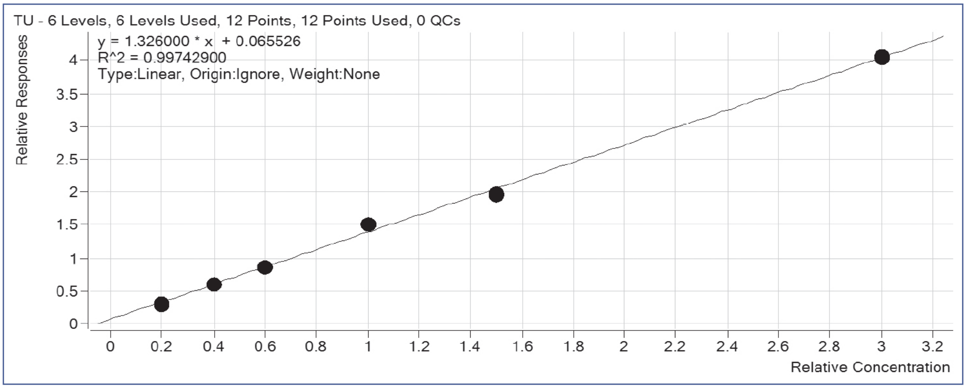

Figure 2. Matrix match calibration curve of thiouracil (TU)

contain glycosinolates that can be an indirect source of TU in a way that these endogenous plant compounds can be converted by myrosinase hydrolysis into TU (Vanden Bussche et al., 2011b; Arrizabalaga-Larrañaga et al., 2022), which can be the source of the TU urine contamination (Tölgyesi et al., 2018). By plant disruption (chewing), glucosinolates located in the plant idioblast cells are releasing from vacuoles together with the plant-derived enzyme myrosinase (ß-thioglucosidase; EC 3.2.1.147) and depending on the pH value, form a different goitrogen that affects thyroid function (Kiebooms et al., 2014). Those authors also stated that the endogenous formation of TU is *Brassicaceae*-induced and occurs under colonic conditions, most likely through myrosinase-like enzyme activity expressed by different common intestinal bacterial species.

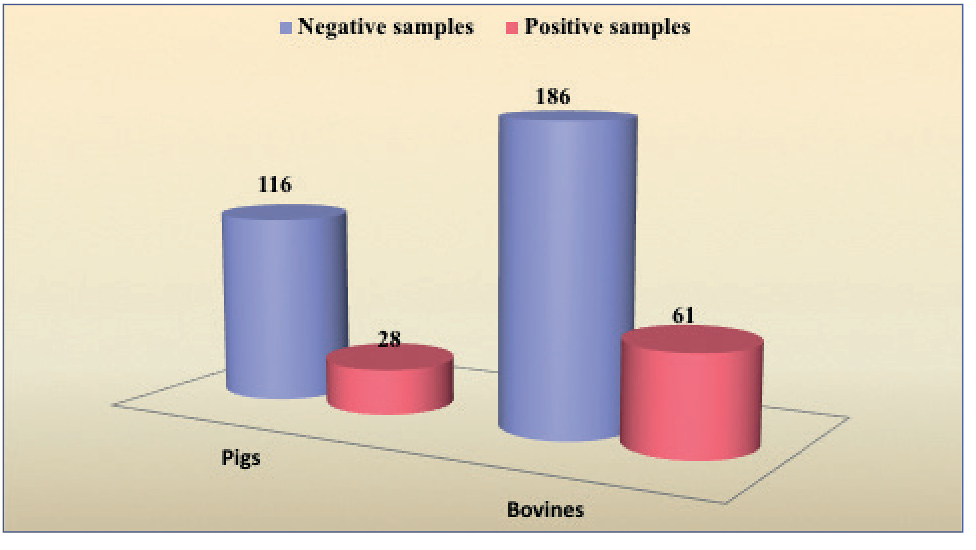

Figure 3. Total number of analysed positive (TU > ccα) and negative (TU < ccα) samples by animal (TU – thiouracil; ccα – decision limit = 1.40 μg/L)

Table 2. Summary overview of determined thiouracil (TU) in urine samples collected from Croatia farms during the period from 2015 to 2023

	Year of sampling								
	2015	2016	2017	2018	2019	2020	2021	2022	2023
Number of samples	43	42	49	44	45	48	47	41	32
Number of positive samples	12	16	10	7	10	11	11	8	5
% positive samples	27.91	30.09	20.41	15.91	22.22	22.92	23.40	19.51	15.63
Min value of TU (μg/L)	3.28	2.31	3.26	5.31	3.66	2.04	1.69	2.07	1.66
Max value of TU (μg/L)	22.75	12.62	28.30	22.80	15.43	17.24	25.16	11.01	1.95

Due to the above, Blokland et al. (2021) claimed that the set TU threshold of 10 μg/L is too low and results in high number of false positive results, and they advised the threshold be raised to 30 μg/L. In this study, performed in the period 2015–2023 on 391 urine samples, TU was detected in 89 urine samples (TU > ccα) (Figure 3), i.e., in 24.14% of pig samples and 32.80% of bovine samples. Table 2 shows a summary overview of the analysed urine samples. The highest percentage of positive samples (30.09%) was obtained in 2016, and the lowest (15.63%) in 2023. Also, the determined concentration of TU varied from 1.66 to 28.30 μg/L, and a statistically significant difference ($P<0.05$) was determined in TU concentrations between the pig and bovine analysed urines.

Mean concentrations of TU varied by year from 1.66 to 8.06 μg/L in pig urine and from 5.92 to 13.68 μg/L in bovine urine.

In a similar study carried out on 537 urine samples collected over an 18-month period in Poland, Woźniak et al. (2011) found that 14.3% of analysed samples were positive for the presence of TU. They stated that in eight samples (one pig and seven bovine samples), the concentration of TU was higher than 10 μg/L while in this study, it exceeded this limit in 17 samples (4.35%). Concentrations in excess of 10 μg/L in this study were determined in two pig and 15 bovine urine samples. Vanden Bussche et al. (2011a) stated that 61.3% of bovine samples, and 96.3% and 57.9% ovine urine samples analysed within that study were a part of the European residue control plan in Belgium and Norway had TU levels below 10 μg/L.

Figure 4 shows the mean ± SD concentrations of TU in urine samples determined in this study by species and year of sampling. It is evident that in 2018 and 2021, the mean concentrations of TU in bovine urine were higher than 10 μg/L. This can be considered naturally present, as none of the analysed urine samples over the years had TU concentrations that exceeded 30 μg/L, which could represent abuse of TU in farm animals. It is also evident that, over the years, lower concentrations of TU were determined in pig urine samples compared to bovine urine samples, which can be explained by the different diet of omnivores and ruminants. Furthermore, TU was not detected in any pig urine samples during 2023. Pinel et al. (2006b) found a relationship between a

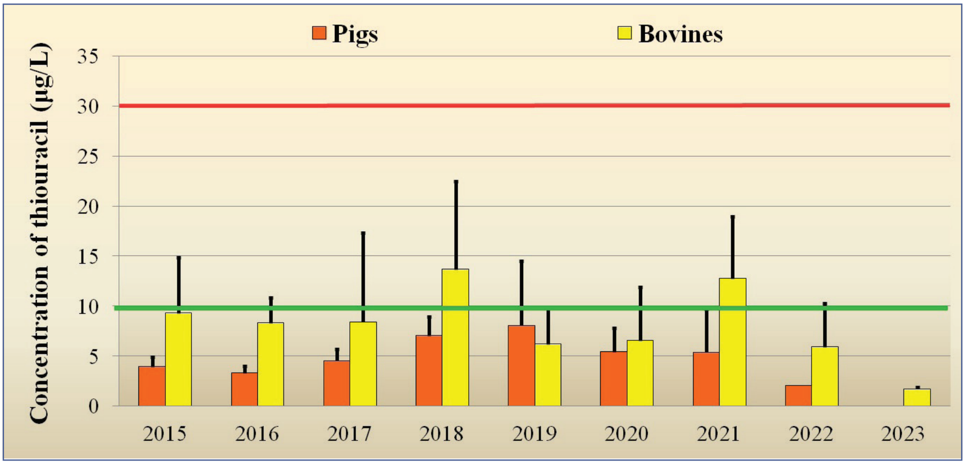

Figure 4. Concentration (mean ± SD) of thiouracil (TU) in urine samples by animal species (pig and bovine) and sampling year (2015-2023) (green line indicates the concentration of 10 µg/L and the red line of 30 µg/L as the threshold for TU)

diet based on cabbage and rapeseed cake in correlation with TU, and determined concentrations in range of 3–7 and 2–9 µg/L TU, respectively. Furthermore, they stated that the polar nature of TU molecule is the reason for its presence in the urine, since polar compounds are easily assimilated and eliminated from urine. Also, the authors showed that a 5-day period was enough for complete elimination of TU from urine.

Conclusions

Over the 9-year period, TU was detected in the both pig and bovine urine samples collected from Croatian farms. TU concentrations in no urine samples exceeded the value of 30 µg/L, considered a threshold value for the suspected abuse of these substances in farm animals in the Republic of Croatia. Therefore, all the detected TU values could be considered endogenous and the consequence of animal feeding containing *Brassicaceae* species. Further research should be focused on the analysis of specific metabolites to determine the origin of TU, i.e., pathways of its occurrence in farm animal urine.

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Pojavnost tiouracila u svinjećim i goveđim urinima prikupljenih na hrvatskim farmama

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Tireostatici predstavljaju skupinu zabranjenih tvari u farmskim životinjama uzgojenim za prehranu ljudi za proizvodnju mesa, koje povećavaju apsorpciju vode u mišićnom tkivu i gastrointestinalnom traktu. Mogu se zlouporabiti kao ilegalni stimulatori rasta u vidu oralnih lijekova na farmama prije klanja. Posljedice njihove primjene u hranidbi farmskih životinja su: veći prirast (randman) i meso lošije kvalitete. Ove tvari predstavljaju potencijalni rizik za zdravlje ljudi pa je njihova primjena u zemljama Europske unije zabranjena. Tiouracil (2-tiouracil) (TU) kao glavni predstavnik skupine tireostatika može biti prirodno prisutan i u urinu domaćih životinja koje se hrane hranom biljnog podrijetla, a koja sadrži vrste *Brassicaceae*. Cilj je ovog istraživanja bio pratiti koncentraciju TU u svinjskom i govedskom urinu u razdoblju od 2015. do 2023. godine. U tu svrhu prikupljen je 391 uzorak urina u sklopu Državnog programa monitoringa rezidua (DPMR), a koncentracije TU određene su metodom tekućinske kromatografije s masenom spektrometrijom (LC-MS/MS). Od ukupnog broja analiziranih uzoraka TU je utvrđen u 89 uzoraka, odnosno 22,76% analiziranih uzoraka sadržavalo je TU. Koncentracije TU kretale su se od 1,66 do 28,30 μg/L, a utvrđena je i statistički značajna razlika ($P < 0,05$) u koncentracijama TU u urinu svinja i goveda. Srednje koncentracije TU varirale su po godinama te su se u svinjskom urinu kretale od 1,66 μg/L do 8,06 μg/L, a u goveđem od 5,92 μg/L do 13,68 μg/L. Niti u jednom od analiziranih uzoraka urina koncentracija TU nije bila viša od 30 μg/L, što predstavlja vrijednost koja se smatra graničnom za razlikovanje TU prirodnog podrijetla (rezultat prehrane biljkama rodova *Brassicacea*) u odnosu na zlouporabu u anaboličke svrhe. Dobiveni rezultati isključuju mogućnost zlouporabe ovih tvari u stočarstvu u Republici Hrvatskoj.

Ključne riječi: *tireostatici, tiouracil, urin, svinje, goveda*