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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · JANUARY 2015

Impact Factor: 2.91 · DOI: 10.1021/jf504475f · Source: PubMed

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JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Perspective

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Toward a New European Threshold to Discriminate Illegally Administered from Naturally Occurring Thiouracil in Livestock

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Supporting Information

ABSTRACT: Thiouracil is a thyrostat inhibiting the thyroid function, resulting in fraudulent weight gain if applied in the fattening of livestock. The latter abuse is strictly forbidden and monitored in the European Union. Recently, endogenous sources of thiouracil were identified after frequently monitoring low-level thiouracil positive urine samples and a "recommend concentration" (RC) of 10 μg/L was suggested by the EURL to facilitate decision-making. However, the systematic occurrence of urine samples exceeding the RC led to demands for international surveys defining an epidemiologic threshold. Therefore, six European member states (France, Poland, The Netherlands, United Kingdom, Norway, and Belgium) have shared their official thiouracil data (2010−2012) collected from bovines, porcines, and small livestock with 95 and 99% percentiles of 8.1 and 18.2 μg/L for bovines (n = 3894); 7.4 and 13.5 μg/L for porcines (n = 654); and 7.4 μg/L (95% only) for small livestock (n = 85), respectively. Bovine percentiles decreased with the animal age (nonadults had significantly higher levels for bovines), and higher levels were observed in male bovines compared to female bovines.

KEYWORDS: *European, threshold, thiouracil, LC-MS/MS, endogenous, urine*

INTRODUCTION

Thiouracil, **1** (Figure 1), is the most studied member of a complex group of substances, the thyrostats, which exert an inhibitory effect on the thyroid function.[1,2] Originally, thyrostats were synthetically designed for the pharmacological treatment of patients with hyperthyroidism or thyrotoxicosis.[1,3−5] Shortly after, this drug found its way into the food chain, particularly for weight gain purposes in livestock.[2,6] Since the drug was inexpensive, easy to obtain, and suitable for oral administration, its popularity was gained rapidly.[7] The increase in the body weight of treated animals is rather spectacular, but due to the decreased production of the thyroid hormones, this treatment eventually only results in water absorption and retention in the soft tissues and the gastrointestinal tract.[7,8]

Thyrostatic residues in meat were later on considered as a potential health risk due to their teratogenic and carcinogenic properties.[9] There was enough compromising evidence to enable the Belgian government to forbid any use of thyrostats in meat producing animals from 1974.[10] This sanction was subsequently adopted by the European Union, prohibiting the use of these drugs for animal fattening purposes in 1981.[11] The zero-tolerance on the European level consequently encouraged the fast development of new detection protocols, which were in accordance with the draft minimum required performance limit (MRPL) of 100 μg/L. The fast evolution and refinement of these techniques resulted, however, in the detection of thyrostats, in particular thiouracil, below the level of 10 μg/L.[12−14] As a result, nowadays, thiouracil positive urine samples are frequently reported in Europe, although low-level abuse at

Received: September 16, 2014
Revised: January 21, 2015
Accepted: January 22, 2015

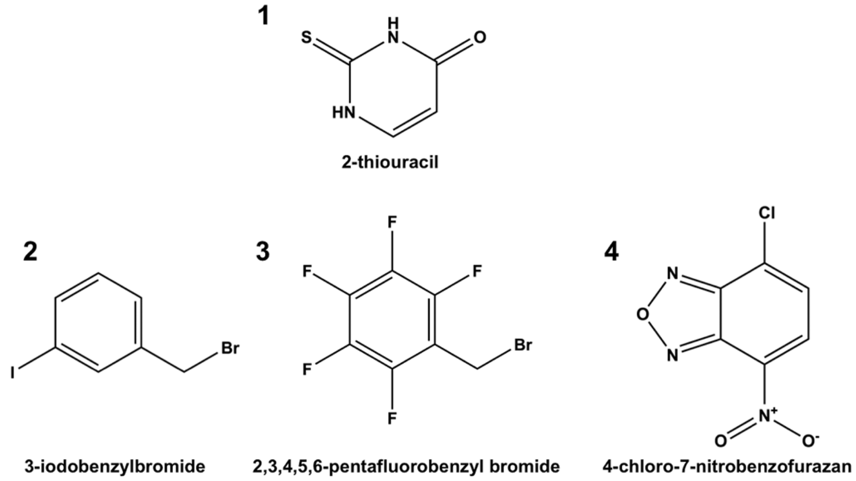

Figure 1. Molecular structure of 2-thouracil, 3-iodobenzyl bromide, 2,3,4,5,6-pentafluorobenzyl bromide, and 4-chloro-7-nitrobenzofurazan.

farms is very unlikely.[15−17] To explain this widely occurring phenomenon, thiouracil was no longer accepted to originate solely from synthetic products. In contrast, the semiendogenous status of thiouracil had to be unraveled.

Pinel et al.[18] performed a first study suggesting species belonging to the family Brassicaceae, commonly used as substantial feed ingredients, as a source of thiouracil. Together with the studies of Vanden Bussche et al.[19,20] and Kiebooms et al.,[21] evidence was gathered on the feed-derived origin of thiouracil. Species in the family Brassicaceae are known to contain glucosinolates, which may be metabolized to thiouracil by the enzymatic interaction with myrosinase at the time of chewing, ingestion, and digestion. A correlation between this particular diet and thiouracil in the urine of livestock was indeed definitely demonstrated.[19−21] However, a relevant concentration of thiouracil was not found in the unprocessed feed itself.[18,19] Additionally, the same correlation could not be demonstrated in a small sample study with humans, despite elevated thiouracil levels in their urine samples, suggesting the existence of additional natural thiouracil sources.[19] Because of these new insights and because of the increasing performance of the newly developed analytical techniques, the zero-tolerance approach of the EU for thiouracil needed revision. As a first step, a guidance paper was published in 2007 by the European Union of Reference Laboratories (EURLs),[22] suggesting a recommended concentration (RC) of 10 μg/L for thiouracil in urine. This RC has no legal value, but it is agreed to be a generally accepted target limit, which should be implemented, wherever possible, by any EU laboratory performing analysis in the light of the thiouracil monitoring programs.

More recently, two member states (France and Poland) suggested new thresholds based on their National Residue Monitoring Plan.[15,17] These studies were performed independently but reported similar outcomes for both countries, suggesting the current RC might be deficient, particularly for certain subgroups of cattle.

To encourage a revision of the existing European guidelines, including the definition of a consensual European threshold for endogenous thiouracil, in this study, a large-scale retrospective epidemiologic study (six member states, 2010−2012) was performed on naturally occurring thiouracil detected by LC-MS/MS in urine samples of bovines, small livestock, and porcines.

Epidemiologic Study Approach: Data Generation, Collection, and Analysis. Retrospective data on urinary thiouracil levels from bovines, small livestock (e.g., sheep, goat), with inclusion of reindeer and porcines, sampled between January 2010 and December 2012, were collected.

Qualified laboratories supplied data from six European countries (i.e., The Netherlands, France, United Kingdom (Great-Britain and Northern-Ireland), Norway, Poland, and Belgium). The contributions of each member state to the total data set of 3894 bovine, 85 small livestock, and 654 porcine records are listed in Table 1. A total of eight laboratories were involved in the chemical analysis (two laboratories for Belgium and the United Kingdom; the Norwegian samples were outsourced to a third Belgian laboratory). These laboratories participated successfully in the 2013 proficiency test for the detection and quantification of thiouracil in bovine urine organized by the EURL (RIKILT, Wageningen).[23] Equal performances in urinary samples of other species, particularly small livestock and porcines, are assumed.

Table 1. Overview of Urinary Thiouracil Data Collection

	bovines	small livestock	porcines
The Netherlands	273	0	139
Belgium	244	0	4
France	1718	0	0
United Kingdom	871	5	2
Norway	432	79	124
Poland	356	1	385
total	3894	85	654

As previously mentioned, the urine samples arrived at the respective laboratories between January 2010 and December 2012 and were each briefly stored at −20 °C prior to analysis. In one laboratory, urine samples were stabilized by adding HCl (37%) and ethylenediaminetetraacetic acid (EDTA) (0.25 M) to prevent degrading of the compounds while extracting at room temperature. At the start of the extraction protocol, an internal standard was added to 1 mL urine. In most cases (7/8 laboratories), the thyrostatic compounds were derivatized with either 3-iodobenzyl bromide, **2** (5 laboratories), 2,3,4,5,6-pentafluorobenzyl bromide, **3** (1 laboratory), or 4-chloro-7-nitrobenzofurazan, **4** (1 laboratory) (Figure 1), followed by solid phase extraction (SPE), (multiple) liquid liquid extraction

DOI: 10.1021/jf504475f
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 2. Population Parameters for the Total (Six Member States) Dataset of Bovine Urinary Thiouracil

	All Bovines				
	total	<6 months	6–12 months	12–24 months	>24 months
n	3894	426	301	575	1079
mean (μg/L)	1.7	3.4	4.1	1.5	1.5
median (μg/L)	0.0	2.0	2.6	0.0	0.4
SD (μg/L)	4.6	4.5	6.1	4	3.6
min (μg/L)	CC_α	CC_α	CC_α	CC_α	CC_α
max (μg/L)	145.0	25.0	64.5	70.0	77.0
$\leq CC_\alpha$ (%)	87.6				
$\leq$RC[a] (%)	96.4				
95% percentile	8.1	14.3	13.3	7.5	6.6
[95% CI[b]] (μg/L)	[7.2–8.9]	[11.0–17.6]	[11.2–14.8]	[5.8–9.4]	[5.0–7.9]
99% percentile	18.2	21.5	28.6	14.8	13.9
[95% CI] (μg/L)	[15.5–20.0]	[18.4–23.2]	[18.2–63.9]	[12.1–17.5]	[11.8–15.3]
	Female Bovines				
	total	<6 months	6–12 months	12–24 months	>24 months
n	1653	136	54	119	947
mean (μg/L)	1.4	2.7	1.3	1.5	1.3
median (μg/L)	0.3	1.6	0.0	0.0	0.4
SD (μg/L)	3.0	4.3	2.3	2.7	2.4
min (μg/L)	CC_α	CC_α	CC_α	CC_α	CC_α
max (μg/L)	34.0	22.1	10.2	15.5	23.1
$\leq CC_\alpha$ (%)	90.1				
$\leq$RC (%)	97.4				
95% percentile	6.5	14.5	5.9	8.2	5.8
[95% CI] (μg/L)	[5.1–7.3]	[5.7–19.9]	[4.4–10.2]	[4.7–9.6]	[4.7–6.9]
99% percentile	15.1	22.1	14.3	15.1	12.5
[95% CI] (μg/L)	[13.0–20.0]	[17.6–22.1]	[n.a.]	[9.1–15.5]	[10.9–14.7]
	Male Bovines				
	total	<6 months	6–12 months	12–24 months	>24 months
n	1168	273	205	449	98
mean (μg/L)	3.0	3.9	4.9	1.5	3.67
median (μg/L)	1.1	2.4	3.13	0.0	0.9
SD (μg/L)	5.8	4.6	6.9	4.3	9.1
min (μg/L)	CC_α	CC_α	CC_α	CC_α	CC_α
max (μg/L)	77.0	25.0	64.5	70.0	77.0
$\leq CC_\alpha$ (%)	76.0				
$\leq$RC (%)	92.6				
95% percentile	12.3	15.0	14.7	7.6	14.5
[95% CI] (μg/L)	[11.1–14.4]	[11.6–18.2]	[11.9–23.0]	[5.5–9.8]	[7.8–37.0]
99% percentile	25	21.0	35.9	14.8	37.0
[95% CI] (μg/L)	[18.4–32.7]	[18.4–25.0]	[22.3–64.5]	[11.8–19.0]	[n.a.]

[a]RC = recommended concentration. [b]CI = confidence interval.

(LLE), a combination of LLE and SPE, or of LLE with duplicate SPE, adapted from previous reports.[12,14] One participating laboratory did not perform any derivatization but added 1% dithiothreitol (DTT; 1,4-bis(sulfanyl)butane-2,3-diol) to the urine aliquot prior to LLE.[13] Thiouracil (and other thyrostats) was finally detected and identified by triple quadrupole (QqQ-MS/MS) mass analyzers after chromatographic separation on a reversed-phase C18 U(H)PLC column.

For each species (cattle, small livestock, and pigs), statistical analysis was performed on the respective data set. Subpopulations (within one species) were created and compared based on gender (male versus female), age (<6 months, 6–12 months, 12–24 months, and >24 months), the combination of gender and age, or the geographical region (member state). Since these subpopulations clearly deviate from a normal distribution, statistical differences between groups were assessed by either Kruskal–Wallis (multiple comparisons) with a posthoc Dunn's test or by the Mann–Whitney rank sum test (comparison of 2 groups), which is significant if $p < 0.05$. The descriptive statistics (mean, median, standard deviation (SD), minimum, maximum, and percentiles) of each subpopulation were acquired, with special interests for specific population parameters such as the 95 and 99% percentiles. For the latter parameters, a 95% confidence interval (CI) was calculated by nonparametric bootstrapping.[24,25] This confidence interval estimates the uncertainty associated with the population's percentile estimates.

Concentration values below the $CC\alpha$ (decision limit) of the respective (sub)populations were set equal to zero. If analysis was performed on a combination of subpopulations with different $CC\alpha$ values, the $CC\alpha$ of the subpopulation with the highest $CC\alpha$ was used for the entire batch. Clearly, this

DOI: 10.1021/jf504475f
J. Agric. Food Chem. XXXX, XXX, XXX–XXX

Table 3. Member State Specific Population Parameters for Bovine Urinary Thiouracil

	member state					
	The Netherlands	Belgium	France	United Kingdom	Norway	Poland
n	273	244	1718	871	432	356
mean (μg/L)	5.8	2.4	1.6	0.8	1.4	0.9
median (μg/L)	4.3	0.0	0.6	0.0	0.0	0.0
SD (μg/L)	5.4	12.0	2.7	4.4	2.5	2.8
min (μg/L)	CC_α	CC_α	CC_α	CC_α	CC_α	CC_α
max (μg/L)	36.4	145	25	77	29.7	23.1
$\leq CC_\alpha$ (%)	46.2	89.8	37.1	94.7	65.7	81.2
$\leq$RC[a] (%)	81.3	93.0	97.8	97.2	99.5	97.2
95% percentile	15.5	14.8	6.2	4.5	5.57	5.24
[95% CI[b]] (μg/L)	[13.5–18.3]	[7.6–29.7]	[4.5–7.1]	[0–7.1]	[4.5–6.2]	[3.9–8.9]
99% percentile	26.4	67.5	14.3	21.56	9.2	15.5
[95% CI] (μg/L)	[18.8–36.4]	[29.0–145.0]	[12.0–16.6]	[13.7–31.0]	[6.9–9.4]	[11.9–20.3]

	Gender					
	France		Norway		Poland	
	male	female	male	female	male	female
n	625	1015	166	218	122	231
mean (μg/L)	2.0	1.4	1.2	1.5	0.9	0.9
median (μg/L)	0.6	0.6	0.0	0.0	0.0	0.0
SD (μg/L)	3.2	2.4	2.0	2.1	2.6	3.0
min (μg/L)	CC_α	CC_α	CC_α	CC_α	CC_α	CC_α
max (μg/L)	25.0	22.1	9.4	8.8	18.2	23.1
$\leq CC_\alpha$ (%)	42.2	33.1	71.1	60.1	79.5	82.3
$\leq$RC (%)	97.3	98.0	100	100	97.5	97.0
95% percentile	7.7	5.0	5.5	5.1	5.0	5.9
[95% CI] (μg/L)	[5.8–9.5]	[4.4–6.7]	[4.4–6.7]	[4.4–6.9]	[3.5–10.4]	[3.5–11.7]
99% percentile	18.0	13.5	9.4	7.9	16.9	15.5
[95% CI] (μg/L)	[13.6–20.3]	[10.7–14.8]	[6.3–9.4]	[6.9–8.8]	[8.8–18.1]	[12.3–23.1]

	Age			
	France			
	<6 months	6–12 months	12–24 months	>24 months
95% percentile	8.8	5.7	7.9	6.2
[95% CI] (μg/L)	[6.1–18.0]	[4.5–8.9]	[5.1–9.9]	[4.7–7.9]
99% percentile	22.3	9.4	12.4	13.5
[95% CI] (μg/L)	[19.8–25.0]	[7.3–9.5]	[10.0–14.3]	[10.8–14.7]

[a]RC = recommended concentration. [b]CI = confidence interval.

approach does not influence the percentiles, but particularly, parametric values such as "mean" and "standard deviation" may be slightly influenced. Statistical analysis was performed with SPSS version 22.0 (IBM, Brussels, Belgium).

Occurrence and Evaluation of Thiouracil in Urine. *Total Bovine Data Set.* A detailed overview of the most important population parameters can be seen in Table 2. Considering the highest CCα (3.9 μg/L) throughout the participating laboratories as a cutoff, the presence of thiouracil was identified in 12.4% (n = 483) of the samples (>CCα = 3.9 μg/L), with 3.6% of the data set (n = 140) containing higher thiouracil levels than the RC (=10 μg/L). Consequently, the 95% percentile is characterized by a concentration below the RC (8.1 [7.2–8.9] μg/L) while the 99% percentile is substantially higher (18.2 [15.5–20] μg/L). A maximum value of 145 μg/L (from Belgium), which could not be explained, was observed within this population (Figure 1).

The animals' gender was known in 72.4% of the sample pool, while their age was defined in 61.1% of the samples. Higher thiouracil concentrations were observed for male bovines compared to female bovines, with significantly higher 95% (12.3 [11.1–14.4] μg/L versus 6.5 [5.1–7.3] μg/L, respectively) and 99% (25.0 [18.4–32.7] μg/L versus 15.1 [13.0–20.0] μg/L, respectively) percentiles, both exceeding the RC (p < 0.01).

Young animals ≤12 months were revealed to be another subpopulation with increased urinary thiouracil levels and 95 and 99% percentiles above the RC (Table 2) (p < 0.001). However, if solely female animals were observed, the definition of "young animals" needed to be adapted to "not older than 6 months", while in male animals both the young (≤12 months) and the older (>24 months) bulls had significantly higher thiouracil levels (p < 0.01).

Member State Specific Bovine Data Set. The descriptive statistics of the six participating member states are presented in Table 3. Frequency plots of these member states are illustrated in Figure 2, and more specific population numbers are added in Table 4.

Overall, the data sets of the two smallest member states, Belgium and The Netherlands, were evaluated as statistically different compared to the remaining four data sets (p < 0.01). Both the 95% (14.8 [7.6–29.7] μg/L and 15.5 [13.5–18.3] μg/L, respectively) and 99% (26.4 [18.8–36.4] μg/L and 67.5 [29.0–145.0] μg/L, respectively) percentiles exceeded the RC

DOI: 10.1021/jf504475f
J. Agric. Food Chem. XXXX, XXX, XXX–XXX

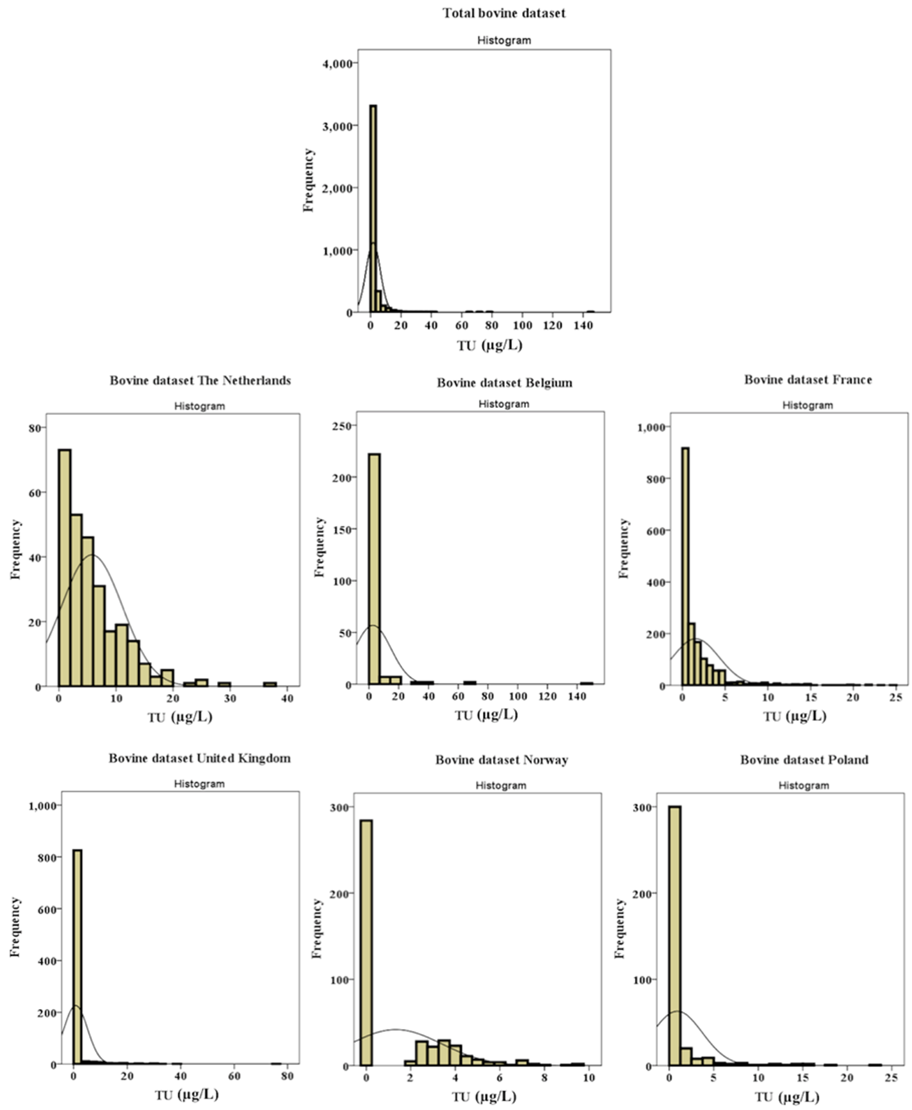

Figure 2. Frequency histograms of the total bovine and member state specific data set indicating the number of samples between given concentrations of urinary thiouracil (μg/L).

Table 4. Member State (Bovine) Population Distributions Based on Age and Gender

	age					gender		
	<6 months	6–12 months	12–24 months	>24 months	unknown	male	female	unknown
The Netherlands	130	143	0	0	0	218	19	36
Belgium	1	1	5	8	229	15	11	218
France	282	121	299	700	316	625	1015	78
United Kingdom	0	0	3	12	856	22	159	690
Norway	4	15	134	178	101	166	218	48
Poland	9	21	134	181	11	122	231	3

substantially, which was not the case for the 95% percentiles of any other member state (max 6.2 μg/L) (nor for the Norwegian 99% percentile (9.2 [6.9–9.4] μg/L)). The Dutch and Belgian data sets are indeed defined by a large sample fraction containing more than 10 μg/L thiouracil (up to 18.7%), while this fraction was limited to 2.8% for the remaining four member states. Due to limited numbers (in one or more subpopulation) and/or lack in detailed information, influences of gender (France, Norway and Poland) and age (France) could only be statistically evaluated in three member states (Table 3). Males and young animals (<6 months) were confirmed to be responsible for higher urinary thiouracil levels, but this could only be statistically confirmed from the largest (French) data set ($p < 0.01$ and $p < 0.01$).

Small Livestock Data Set. Samples (n = 85) collected from 63 sheep, 16 goats, and 6 reindeer originating from Norway (n = 79), United Kingdom (n = 5), and Poland (n = 1) were included in this data set (Figure 2). Due to the limited sample group (n = 85) with only two samples (2.4%) above the RC, only the 95% percentile (7.3 [4.3–17.0] μg/L) could be defined (Table 5).

DOI: 10.1021/jf504475f
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Table 5. Population Parameters for the Datasets of Small Livestock and Porcine Urinary Thiouracil

	small livestock	porcines			
	total	total	The Netherlands	Poland	Norway
n	85	654	139	385	124
mean (μg/L)	1.6	1.5	4.4	0.8	0.1
median (μg/L)	0.0	0.0	3.1	0.0	0.0
SD (μg/L)	3.9	2.83	4.0	1.7	0.6
min (μg/L)	CC_α	CC_α	CC_α	CC_α	CC_α
max (μg/L)	27.0	26.4	26.4	16.6	4.5
95% percentile	7.3	7.4	11.3	3.4	0.0
[95% CI[a]] (μg/L)	[4.3−17.0]	[6.4−8.4]	[10.1−14.0]	[3.0−4.7]	[0.0−2.8]
99% percentile	na	13.5	23.8	8.0	4.1
[95% CI] (μg/L)		[10.5−17.0]	[13.4−26.4]	[5.2−10.5]	[2.2−4.5]

[a]CI = confidence interval.

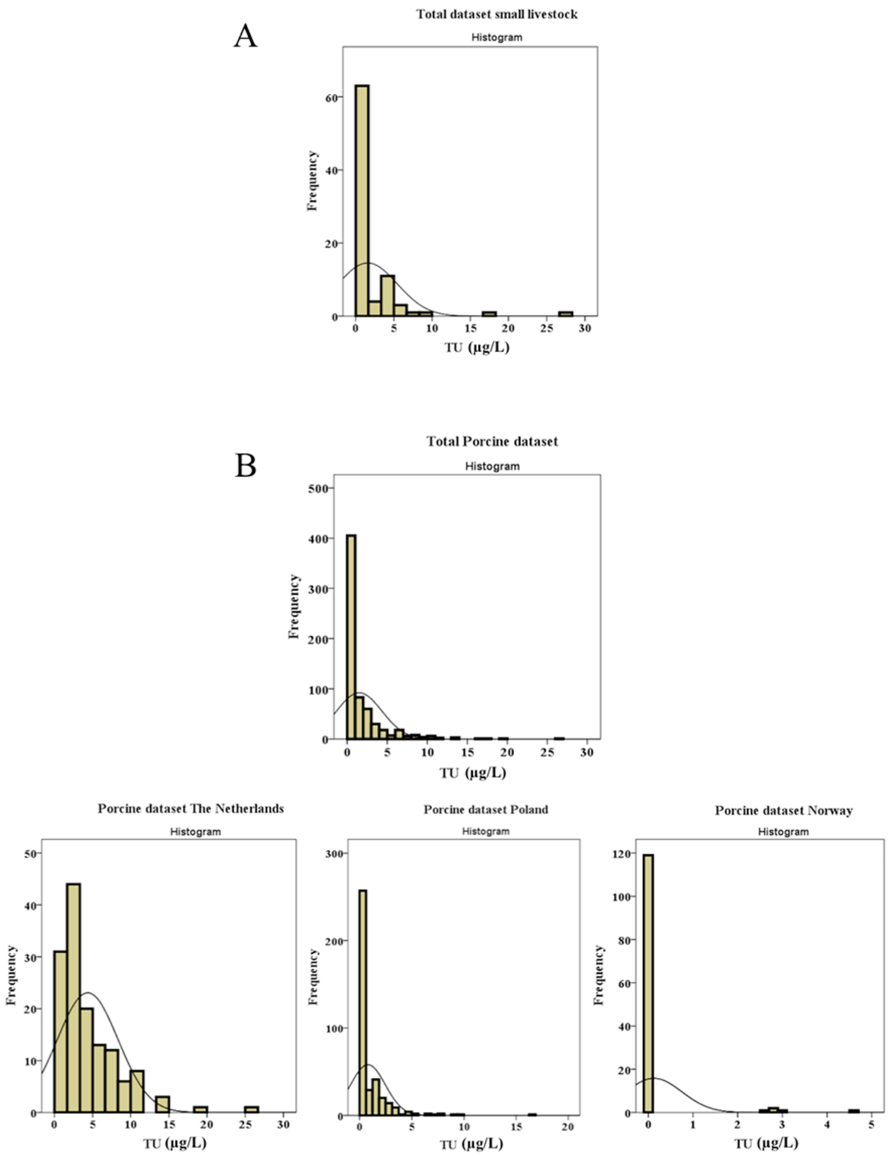

Figure 3. Frequency histograms of (A) the total small livestock population and (B) the total porcine and member state specific data set, indicating the number of samples between given concentrations of urinary thiouracil (μg/L).

Porcine Data Set. The majority of thiouracil data in porcine urine samples was provided by Poland (n = 385), The Netherlands (n = 139), and Norway (n = 124). Limited contributions from Belgium (n = 4) and United Kingdom (n = 2) were included as well.

In 97.7% of the samples, the urinary thiouracil level was below the RC of 10 μg/L, while in 77.1% of the samples,

DOI: 10.1021/jf504475f
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thiouracil could not be detected (<CC_α). The 95% and 99% percentiles for this database were at 7.4 [6.4−8.4] μg/L and 13.5 [10.5−17.0] μg/L, respectively. These thresholds were not influenced by gender or age. However, data sets differed geographically (exclusion of Belgian and British data set), with significantly higher percentiles for The Netherlands (11.3 [10.1−14.0] μg/L and 23.8 [13.4−26.4] μg/L, for the 95% and 99% percentiles, respectively) compared to Norway (<CC_α [0.0−2.8] μg/L and 4.1 [2.2−4.5] μg/L for the 95% and 99% percentiles, respectively) (Table 5; Figure 3).

■ DISCUSSION

With this retrospective study, we aimed to evaluate the applicability of the recommended concentration (RC) for monitoring thiouracil in urine samples of bovines, small livestock, and pigs, advised by the EURL,[22] by statistically approaching sample outcomes gained in several European member states from January 2010 to December 2012. A similar epidemiologic threshold-definition has been previously performed on the national level by two participating member states.[15,17] However, combining up to six member states with a rather scattered distribution throughout Europe allows assessing a broader and larger (thus statistically more powerful) sample size and may identify geographical peculiarities.

Due to the obvious lack of homoscedasticity of the data set, analysis was performed integrally by nonparametric analysis. The population "extremes" were hereby described by 95% and 99% percentiles, covered by their bootstrapped 95% confidence intervals (CI), which give an estimation of the uncertainty of the numeric concentration value associated with the percentiles.[24,25] The inclusion of the CI in this study allows comparing results of similar studies, such as the earlier mentioned French and Polish study. The (bovine and porcine) percentiles reported in these studies fit the respective national CI values of the current study, with one exception for the 99% percentiles for French male bovines and the 95% percentiles for Polish porcines, both below their respective CI. The results of these and the current study are therefore (largely) in agreement, leading toward similar reflections.

It depends on the kind of threshold and its application-scope whether the 95% or 99% percentile, or its upper CI limits, should be taken into account for decision-making.[26,27] Although recommendations on this final choice are out of the scope of this study, the current obtained results, and earlier reports, strongly indicate the need to increase (if 99% percentiles are taken into consideration), or at least refine (if 95% percentiles are considered), the RC that is currently suggested.

Indeed, when selecting the 95% percentiles (with CI) as most stringent thresholds, the present RC is inadequate in very young and/or male bovines. The latter might explain the highest percentiles for the Dutch data set, since 80% of the data involved male animals younger than 1 year (no population details are available on the Belgian data set with similar percentiles). However, high thiouracil levels were also observed in urine samples of Dutch pigs with no statistical interference of age and gender. Another feature, unrelated with any population characteristic, might explain these high thiouracil levels in both bovine and porcine urine. The Dutch protocol seems to contrast with the seven other laboratory protocols by an additional thyrostat-stabilizing step. The stability of thyrostatic drugs in bovine and porcine urine has been extensively studied.[28] Urinary thiouracil concentrations were found to decrease rather drastically if inappropriately stored (e.g., short time at room temperature or prolonged at −70 °C), if the pH exceeded a value greater than 3 and if copper was present. Vanden Bussche et al.[28] suggested therefore a successful pretreatment by which the pH was adapted to 1 and EDTA was added to chelated copper. This treatment turned out to delay thyrostat-decay for 24 h at room temperature, in effect controlling degradation while performing the extraction and at least for three months at −70 °C. This is likely to be responsible for the more elevated thiouracil levels in the Dutch data set. Similarly, lower thiouracil levels (with low percentiles) in Norwegian bovine and porcine urine samples may be due to the applied protocol, which discriminates itself by the lack of derivatization. However, this protocol[13] was successfully validated in accordance with Commission Decision 2002/657/EC and fulfilled the performance criteria of the 2013 proficiency test.[23,29] It is therefore more likely that other factors, related to the Norwegian farming style (e.g., feed, pasture type, housing, etc.), have been influencing the data.

The definition of a European threshold for urinary thiouracil to distinct endogenous from administered thiouracil is not straightforward. Two independent national surveys have previously expressed their concerns and have suggested a revision of the existent RC. Considering these and current study results, the urge for a revision of the recommended concentration and/or the definition of the reference points for action for thiouracil residues in urine is obviously demonstrated, with special emphasis on the identified influencing factors, for example, the applied laboratory protocol (all species), gender (cattle), and age (cattle). Consequently, our findings are in agreement with the latest reflection paper of the EURL in which an increase of the RC from 10 to 30 μg/L is suggested.[30]

However, since the endogenous thiouracil levels are clearly multifactorially influenced and the uncertainty related to the threshold approach may still result in incorrect decision-making with major impact on the farmers' activities, future research should not only aim to identify other endogenous sources of thiouracil and related pathways but also investigate potential biomarkers specific for exogenous thiouracil administration or endogenous thiouracil formation. The discovery of a discriminating biomarker might unequivocally alleviate any shortcomings related to the threshold-approach.

■ ASSOCIATED CONTENT

Supporting Information

Supplementary Table 1: details on the sample extraction and LC-MS/MS detection of urinary thiouracil for the participating laboratories (MeOH = methanol; MeCN = acetonitrile). This material is free of charge via the Internet at http://pubs.acs.org.

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Funding

This research was funded by the Belgian Federal Public Service for Health, Food Chain Safety and Environment with reference number RF12/6260 (THYREOMERK).

Notes

The authors declare no competing financial interest.

DOI: 10.1021/jf504475f
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ACKNOWLEDGMENTS

The authors thank J. Goedgebuer for her technical assistance and M. Andjelkovic, J. Van Loco, and H. van Rhijn for data contributions.

ABBREVIATIONS USED

CI, confidence interval; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate acid; EURLs, European Union reference laboratories; LLE, liquid−liquid extraction; MRLP, minimum required performance limit; QqQ, triple quadrupole; RC, recommended concentration; SPE, solid phase extraction

REFERENCES

(1) Astwood, E. B. Treatment of hyperthyroidism with thiourea and thiouracil. *J. Am. Med. Assoc.* **1943**, *122*, 78−81.
(2) Beeson, W. M.; Andrews, F. N.; Brown, P. T. The effect of thiouracil on the growth and fattening of yearling steers. *J. Anim. Sci.* **1947**, *6*, 16−23.
(3) Shirer, J. W.; Cohen, M. The effects of thiouracil on the thyroid gland. *Ann. Int. Med.* **1945**, *23*, 790−799.
(4) Williams, R. H.; Bissell, G. W. Thiouracil in the treatment of thyrotoxicosis. *Science* **1943**, *98*, 156−158.
(5) Williams, R. H.; Clute, H. M. Thiouracil in the treatment of thyrotoxicosis. *Med. Press Egypt* **1946**, *38*, 169−175.
(6) Braude, R.; Cotchin, E. Thiourea and methylthiouracil as supplements in rations of fattening pigs. *Br. J. Nutr.* **1949**, *3*, 171−186.
(7) Courtheyn, D.; Le Bizec, B.; Brambilla, G.; De Brabander, H. F.; Cobbaert, E.; Van de Wiele, M.; Vercammen, J.; De Wasch, K. Recent developments in the use and abuse of growth promotors. *Anal. Chim. Acta* **2002**, *473*, 71−82.
(8) Vanden Bussche, J.; Noppe, H.; Verheyden, K.; Wille, K.; Pinel, G.; Le Bizec, B.; De Brabander, H. F. Analysis of thyreostats: A history of 35 years. *Anal. Chim. Acta* **2009**, *637*, 2−12.
(9) International Agency for Research on Cancer. Some Thyrotropic Agents. *Monographs on the Evaluation of Carcinogenic Risk to Humans* **2001**, *79*, 53−144.
(10) Belgian Government. Koninklijk Besluit betreffende sommige verrichtingen in verband met stoffen met hormonale, anti-hormonale, anabole, *β*-adrenergische, anti-infectieuze, anti-parasitaire, en anti-inflammatoire werking. *Belgisch Staatsblad* **1974**, 6592.
(11) European Union. Council Directive 81/602/EC concerning the prohibition of certain substances having a hormonal action and of any substances having a thyrostatic action. *Off. J. Eur. Communities: Legis.* **1981**, *222*, 32−33.
(12) Pinel, G.; Bichon, E.; Pouponneau, K.; Maume, D.; Andre, F.; Le Bizec, B. Multi-residue method for the determination of thyreostats in urine samples using liquid chromatography coupled to tandem mass spectrometry after derivatisation with 3-iodobenzylbromide. *J. Chromatogr. A* **2005**, *1085*, 247−252.
(13) Vanden Bussche, J.; Vanhaecke, L.; Deceuninck, Y.; Verheyden, K.; Wille, K.; Bekaert, K.; Le Bizec, B.; De Brabander, H. F. Development and validation of an ultra-high performance liquid chromatography tandem mass spectrometry method for quantifying thyreostats in urine without derivatisation. *J. Chromatogr. A* **2010**, *1217*, 4285−4293.
(14) Wozniak, B.; Zuchowska, I. M.; Zmudzki, J.; Jedziniak, P.; Korycinska, B.; Sielska, K.; Witek, S.; Klopot, A. Liquid chromatography tandem mass spectrometry with ion trap and triple quadrupole analyzers for determination of thyreostatic drugs in urine and muscle tissue. *Anal. Chim. Acta* **2011**, *700*, 155−166.
(15) Le Bizec, B.; Bichon, E.; Deceuninck, Y.; Prevost, S.; Monteau, F.; Antignac, J. P.; Dervilly-Pinel, G. Toward a criterion for suspect thiouracil administration in animal husbandry. *Food Addit. Contam., Part A* **2011**, 1−8.
(16) Pinel, G.; Maume, D.; Deceuninck, Y.; Andre, F.; Le Bizec, B. Unambiguous identification of thiouracil residue in urine collected in non-treated bovine by tandem and high-resolution mass spectrometry. *Rapid Commun. Mass Spectrom.* **2006**, *20*, 3183−3187.
(17) Wozniak, B.; Witek, S.; Zmudzki, J.; Klopot, A. Natural occurrence of thiouracil in urine of livestock in Poland. *Bull. Vet. Inst. Pulawy* **2012**, *56*, 611−615.
(18) Pinel, G.; Mathieu, S.; Cesbron, N.; Maume, D.; De Brabander, H. F.; Andre, F.; Le Bizec, B. Evidence that urinary excretion of thiouracil in adult bovine submitted to a cruciferous diet can give erroneous indications of the possible illegal use of thyrostats in meat production. *Food Addit. Contam.* **2006**, *23*, 974−980.
(19) Vanden Bussche, J.; Kiebooms, J. A.; De Clercq, N.; Deceuninck, Y.; Le Bizec, B.; De Brabander, H. F.; Vanhaecke, L. Feed or food responsible for the presence of low-level thiouracil in urine of livestock and humans? *J. Agric. Food Chem.* **2011**, *59*, 5786−5792.
(20) Vanden Bussche, J.; Vanhaecke, L.; Deceuninck, Y.; Wille, K.; Bekaert, K.; Le Bizec, B.; De Brabander, H. F. Ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry detection of naturally occurring thiouracil in urine of untreated livestock, domesticated animals and humans. *Food Addit. Contam., Part A* **2011**, *28*, 166−172.
(21) Kiebooms, J. A.; Vanden Bussche, J.; Hemeryck, L. Y.; Fievez, V.; Vanhaecke, L. Intestinal microbiota contribute to the endogenous formation of thiouracil in livestock. *J. Agric. Food Chem.* **2012**, *60*, 7769−7776.
(22) CRL. CRLs view on state of the art analytical methods for national residue control plans. *CRL guidance paper*, 2007. http://www.bvl.bund.de/SharedDocs/Downloads/09_Untersuchungen/EURL_Empfehlungen_Konzentrationsauswahl_Methodenvalierungen_EN.pdf?__blob=publicationFile&v=2 (accessed Jan. 16, 2015).
(23) Elbers, I. J. W.; Sterk, S. S.; van Ginkel, L. A. Proficiency test for thiouracil in bovine urine. *Confidential Rikilt Report* **2013**, *2013* (520), 1−37.
(24) Carpenter, J.; Bithell, J. Bootstrap confidence intervals: When, which, what? A practical guide for medical statisticians. *Stat. Med.* **2000**, *19*, 1141−1164.
(25) Wood, M. Statistical inference using bootstrap confidence intervals. *Significance* **2004**, *1*, 180−182.
(26) MacLachlan, D. J.; Hamilton, D. Estimation methods for maximum residue limits for pesticides. *Regul. Toxicol. Pharmacol.* **2010**, *58*, 208−218.
(27) Sanquer, A.; Wackowiez, G.; Havrileck, B. Critical review on the withdrawal period calculation for injection site residues. *J. Vet. Pharmacol. Ther.* **2006**, *29*, 355−364.
(28) Vanden Bussche, J.; Sterk, S. S.; De Brabander, H. F.; Blokland, M. H.; Deceuninck, Y.; Le Bizec, B.; Vanhaecke, L. Thyreostatic drugs, stability in bovine and porcine urine. *Anal. Bioanal. Chem.* **2012**, *403*, 2973−2982.
(29) European Union. European Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Communities: Legis.* **2002**, *221*, 8−36.
(30) EURL. *EURL Reflection Paper: Natural Growth Promoting Substances in Biological Samples*; RIKILT Wageningen UR: Wageningen, 2014; pp 15−21.

DOI: 10.1021/jf504475f
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