Toward the definition of a criterion to suspect thiouracil administration in animal husbandry

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Toward the definition of a criterion to suspect thiouracil administration in animal husbandry

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ABSTRACT

Thyreostats are growth promoters banned in Europe since 1981. The identification of thiouracil (TU) in animal biological matrices is however not anymore systematically interpreted as the consequence of an illegal administration. Indeed, some experimental results pointed out a causality link between cruciferous-based diet and the presence of TU in urine of bovines. The present study aims at investigating, on a large scale ($n > 1300$), the natural occurrence of thiouracil in urine samples collected in different animal species. TU was identified in main breeding animal species: bovine, porcine and ovine. The natural distribution of TU allowed proposing threshold values to differentiate compliant from suspect urine samples. Suggested values are $5.7 \mu\text{g.L}^{-1}$ and $9.1 \mu\text{g.L}^{-1}$ in male adult bovine (6-24 months), $3.1 \mu\text{g.L}^{-1}$ and $8.1 \mu\text{g.L}^{-1}$ in female adult bovine (6-24 months), $7.3 \mu\text{g.L}^{-1}$ and $17.7 \mu\text{g.L}^{-1}$ in calves (< 6 months), $3.9 \mu\text{g.L}^{-1}$ and $8.8 \mu\text{g.L}^{-1}$ in female bovine (> 24 months), $2.9 \mu\text{g.L}^{-1}$ and $4.1 \mu\text{g.L}^{-1}$ in porcine, at 95% and 99% confidence level respectively.

Key words: thiouracil, urine, mass spectrometry, ESI-, breeding animals, endogenous distribution

INTRODUCTION

Thyrostatic compounds are orally active drugs which may be used to increase the weight of cattle prior to slaughter. This weight gain is mainly due to increased water absorption and retention within edible tissue as well as filling of the gastro-intestinal tract by inhibiting thyroid hormones production [Kotter *et al.*, 1959; Derblom *et al.*, 1963]. In particular, 2-thiouracil (TU) is a very strong thyroid-inhibiting compound. The consequence of such abuse is not only the production of inferior meat quality, but overall the potential risk of drug residues to human health, since these compounds have indeed been classified by IARC as belonging to group 2B that represents carcinogenic and teratogenic compounds. For these particular reasons, thyrostats are banned within the EU since 1981 (E.C. Directive 81/602) for animal production. In consequence, reliable, sensitive and specific methods, mandatory based on mass spectrometry, have been set up to ensure efficient control of their illegal use [Le Bizec *et al.*, 1997; De Wasch *et al.*, 2001; Pinel *et al.*, 2005; Zhang *et al.*, 2005]. The latest developed protocols [Pinel *et al.*, 2005; Pinel *et al.*, 2006a; Le Bizec *et al.*, 2009; Vanden Bussche *et al.*, 2010] exhibit performances allowing detection and identification of the thyrostat compounds in biological fluids and edible tissues in the $\mu\text{g.kg}^{-1}$ or $\mu\text{g.L}^{-1}$ range which is in accordance with the requirements of the European Union regarding the provisional Minimum Required Performance Limit (MRPL) provisionally fixed at $10 \mu\text{g.L}^{-1}$. These methods allow unambiguous detection of thyrostats abuse since the concentrations that have to be administrated (5 g / day) in order to observe the expected animal weight gain generate high urine concentrations ($>> 100 \mu\text{g.L}^{-1}$).

Occasionally however, the occurrence of thiouracil in bovine urines from the national monitoring plan with levels in the range $1\text{-}10 \mu\text{g.L}^{-1}$ raised the question of the origin of the substance [Vanden Bussche *et al.*, 2009]. The hypothesis of a contribution from animal feed was recently considered [Pinel *et al.*, 2006b] since some vegetables from the Cruciferous (Brassicaceous) family [Kennedy & Purves, 1941] are known to contain substances, called goitrogens, which impair iodine uptake by the thyroid and subsequently the conversion of T4

(thyroxine) to T3 (triiodothyronine). The experimental study of Pinel et al. (2006b) allowed drawing a correlation between a cruciferous-based animal feeding and the identification of TU in urine of animals; concentrations of TU eliminated in urine during the cruciferous diet were rather low and never exceeded 10 µg.L⁻¹. Even though the relation between feeding and thiouracil occurrence in urine has been established, this observation was the result of a single animal experiment which did not correspond to usual good farming practices, *i.e.* it was performed under *ad libidum* feeding on rapeseed cakes. To complete the global risk analysis, an epidemiological study was necessary to establish the distribution of TU in urine collected in breeding animal. A national monitoring plan has been used to provide urine samples and therefore integrate the potential variability associated to meat animals breeding conditions (species, age, gender, feed...). In this context, more than 1300 urine samples collected in different species (bovine, ovine, porcine) have been analysed for thiouracil according to Pinel et al. (2005). The objective was to investigate the natural distribution of TU and finally, when possible, to propose threshold values authorizing robust classification of samples toward compliant or suspect.

EXPERIMENTAL

Urine samples

The urinary sampling gathered 1322 specimens, involving bovine (n = 1098, including 362 calves), porcine (n = 201) and ovine (n = 26) species. Samples have been collected all over the year 2006 and equally distributed, both over this period of time and on the French territory. Details of the sampling are summarized in Table 1.

Data collection and analysis

Three field laboratories, accredited for thyrostats according to ISO 17025:2005 and running the official French reference method (derivatisation with 3-iodobenzylbromide, SPE C18, detection and identification by LC-ESI(-)-MS/MS) (adapted from Pinel et al., 2005), have been involved in the data production. These laboratories have been submitted to proficiency

tests organised by the French National Reference Laboratory (NRL) which shows $CC\alpha < 0.7 \mu\text{g.L}^{-1}$ for TU: they proved similar performances and homogeneous quantitative data (Z-score in the range ± 1). Data produced in the laboratories, which consisted in TU urinary concentrations, have been analysed in the present study using descriptive statistics.

RESULTS

It shall first be noted that the observed concentrations through the study did not follow a normal distribution whatever the species considered. Therefore, emphasis has been given to the determination of 95 and 99 centiles of the distributions. Nevertheless and owing to the high number of animals included in the study which improves the associated statistical confidence, mean values as well as p-values have also been investigated.

Bovines

Thiouracil concentrations levels determined in the 1098 analysed bovine urine samples (all gender and age considered together) are represented in Figure 1. While TU was not detected in 32% of the samples (observations included in the $[0 - CC\alpha]$ range), it could be identified and quantified in the majority of urines with concentrations ranging from decision limit ($CC\alpha$) to $22.5 \mu\text{g.L}^{-1}$; mean and median values were equal to $1.4 \mu\text{g.L}^{-1}$ and $0.3 \mu\text{g.L}^{-1}$, respectively (Table 1). Several atypically elevated values were noticed (12 samples over the 1098 were found between 11.9 and $22.5 \mu\text{g.L}^{-1}$), explaining this significant difference between the mean and median values. From this urinary distribution, thresholds at 95% and 99% could be established for bovines at $6.2 \mu\text{g.L}^{-1}$ and $12.1 \mu\text{g.L}^{-1}$, respectively. Bovine samples have then been further investigated by age group in order to refine this preliminary result.

The particular case of calves which represented 362 animals out of the 1098 bovine samples was thus studied. The corresponding TU concentration levels are shown in Figure 2 and Table 1. TU could be identified and quantified in more than 80% of the urine samples and

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3 1 concentrations ranged from decision limit ($CC\alpha$) to $22.5 \mu\text{g.L}^{-1}$. Mean and median
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5 2 concentrations, which were equal to $2.2 \mu\text{g.L}^{-1}$ and $1.1 \mu\text{g.L}^{-1}$, respectively, were significantly
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7 3 ($p < 0.0001$) higher than the corresponding values for the bovines older than 6 months-old
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9 4 ($n = 736$) reported at $1.0 \mu\text{g.L}^{-1}$ and $0.1 \mu\text{g.L}^{-1}$, respectively (Table 1). The observed
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11 5 distribution of TU concentrations within the calves' population allowed classifying 95% and
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13 6 99% of the samples below $7.3 \mu\text{g.L}^{-1}$ and $17.7 \mu\text{g.L}^{-1}$, respectively. These thresholds were
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15 7 found significantly higher than those proposed for bovines > 6 months-old, reported at
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17 8 $5.0 \mu\text{g.L}^{-1}$ and $8.8 \mu\text{g.L}^{-1}$, respectively (Table 1). These significant differences between calves
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19 9 and older bovines therefore support the fact that these two populations have to be
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21 10 considered apart in the context of the present issue. The reasons for such differences
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23 11 remains however still unknown and may lie in different metabolisms, breeding or feeding
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25 12 conditions between calves and adult animals. The hereby study has furthermore evidenced
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27 13 that calves exhibit different TU patterns according to their age: it is obvious from Figure 3 that
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29 14 mean TU concentrations increase from very young animals (less than 1 month) to older ones
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31 15 (aged between 5 and 6 months) in the range $1.5 - 4.6 \mu\text{g.L}^{-1}$. Once again, it can be
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33 16 hypothesised that feeding conditions may contribute to this observation, and in particular for
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35 17 calves, the increase in the diet of the vegetable proportion may be considered. Any inter-sex
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37 18 difference was investigated for calves (Figure 4 and Table 1) but did not lead to any
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39 19 significant statistical difference ($p\text{-value} = 0.4035$) between mean TU concentrations for
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41 20 males ($2.1 \mu\text{g.L}^{-1}$) and females ($2.4 \mu\text{g.L}^{-1}$), which does not support to define any gender-
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43 21 dependant thresholds for calves.
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51 23 Adult bovines (> 6 months) were further investigated and the corresponding group therefore
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53 24 divided into two groups, i.e. animals aged between 6 and 24 months and animals older than
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55 25 24 months (Table 1). The reason for this additional categorisation lies in the fact that animals
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57 26 aged more than 24 months only consist in females, which therefore support their study apart.
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59 27 In this context, a close study of the animals aged between 6 and 24 months was performed.
60
28 Thiouracil concentrations levels determined in the 540 analysed bovine urine samples are

represented in Figure 5. Thiouracil was identified and quantified in the majority of urines with concentrations ranging from decision limit ($CC\alpha$) to $20.8 \mu\text{g.L}^{-1}$. Mean and median values were equal to $1.1 \mu\text{g.L}^{-1}$ and $0.1 \mu\text{g.L}^{-1}$, respectively (Table 1); several atypically elevated values were noticed explaining this significant difference between the mean and median values. From this urinary distribution, thresholds at 95% and 99% could be established for bovines aged between 6 and 24 months at $5.2 \mu\text{g.L}^{-1}$ and $8.8 \mu\text{g.L}^{-1}$, respectively. The inter-sex difference was also assessed as presented in Figure 6. Mean TU concentrations were found equal to 1.4 and $0.8 \mu\text{g.L}^{-1}$ in male (M) and female (F) urine samples, respectively. On average, female animals present lower TU concentrations in their urine compared to males, even if the highest values have been observed in female urines. The difference between males and females was found significant, as attested by the low corresponding p-value ($p = 0.00095$), which justifies the proposition of sex-dependant thresholds as follows: $5.7 \mu\text{g.L}^{-1}$ (M) and $3.1 \mu\text{g.L}^{-1}$ (F) at 95% and $9.1 \mu\text{g.L}^{-1}$ (M) and $8.1 \mu\text{g.L}^{-1}$ (F) at 99%. One hypothesis supporting this finding would lie in feeding conditions which are known to be different between female animals, mainly consisting in lactating cows, and male animals which represent meat producing animals.

Finally, and as presented in Table 1, it could be demonstrated that 95% and 99% of the urine samples collected on cows older than 24 months present thiouracil concentrations, respectively below 3.9 and $8.8 \mu\text{g.L}^{-1}$.

From these results it appears obvious that when considering bovine animals, three categories have to be defined, namely the calves (aged < 6 months), the females aged more than 24 months and animals with an age range comprised between 6 and 24 months.

Porcines

Two hundred twenty one porcine urine samples have also been investigated for TU concentrations, which are reported in Figure 7 and Table 1. TU could be identified in more

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3 1 than 80% of the samples. Concentrations ranging from decision limit ($CC\alpha$) to $7.0 \mu\text{g.L}^{-1}$
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5 2 have been obtained while mean and median values were equal to $0.9 \mu\text{g.L}^{-1}$ and $0.5 \mu\text{g.L}^{-1}$,
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7 3 respectively (Table 1). Thresholds at 95% and 99% could be proposed at $2.9 \mu\text{g.L}^{-1}$ and 4.1
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9 4 $\mu\text{g.L}^{-1}$, respectively. A very low standard deviation ($1.0 \mu\text{g.L}^{-1}$) was obtained for porcine which
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11 5 certainly reflects the homogeneous sampling within this specie: all animals involved in the
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13 6 study were indeed from a narrow age group in the range 1-8 months, and furthermore, much
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15 7 standardised breeding practices are observed for porcine. No significant differences between
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17 8 males and females could be observed ($p = 0.2527$), which does not support the proposition
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19 9 of gender-dependant thresholds. As compared to the bovine specie, the urinary TU
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21 10 concentrations in porcine were found significantly lower ($p = 0.0020$, **), this may be linked to
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23 11 the feeding conditions which in this case consist in an omnivore diet including probably not
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25 12 that much cruciferous.
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32 14 *Ovines*
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34 15 Twenty six urine samples collected on ovines have also been investigated in the present
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36 16 study. The TU urinary concentrations were in the range $CC\alpha - 14.3 \mu\text{g.L}^{-1}$; mean and median
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38 17 values were $3.3 \mu\text{g.L}^{-1}$ and $2.2 \mu\text{g.L}^{-1}$, respectively. No significant differences between males
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40 18 and females could be observed ($p = 0.2189$) and thresholds at 95% and 99% could be
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42 19 proposed for this specie at $12.9 \mu\text{g.L}^{-1}$ and $14.3 \mu\text{g.L}^{-1}$, respectively (Table 1). As compared
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44 20 with other species, these values can be considered as rather high, however, because of the
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46 21 reduced number of samples involved, the statistical confidence associated to the resulting
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48 22 values should only be considered as indicative trends for the specie and would need further
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50 23 confirmation through an extended study on a large number of animals.
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55 25 **CONCLUSION**
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57 26 The present study gives a comprehensive overview of the natural occurrence of thiouracil in
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59 27 a large number ($n > 1300$) of urine samples collected in different animal species over a year
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28 on the French territory. TU was identified in main breeding animal species (bovine, porcine

1 and ovine) and in a large proportion of the samples involved (>70%). The natural distribution
2 of TU allowed proposing threshold values to differentiate compliant from suspect urine
3 samples. Suggested values are 5.7 $\mu\text{g.L}^{-1}$ and 9.1 $\mu\text{g.L}^{-1}$ in male adult bovine (6-24 months),
4 3.1 $\mu\text{g.L}^{-1}$ and 8.1 $\mu\text{g.L}^{-1}$ in female adult bovine (6-24 months), 3.9 $\mu\text{g.L}^{-1}$ and 8.8 $\mu\text{g.L}^{-1}$ in
5 female bovine (> 24 months), 7.3 $\mu\text{g.L}^{-1}$ and 17.7 $\mu\text{g.L}^{-1}$ in calves (< 6 months), 3.9 $\mu\text{g.L}^{-1}$ and
6 8.8 $\mu\text{g.L}^{-1}$ in female bovine (> 24 months), 2.9 $\mu\text{g.L}^{-1}$ and 4.1 $\mu\text{g.L}^{-1}$ in porcine, at 95% and
7 99% confidence level respectively.

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9 According to the CRL guidance paper (2007), a provisional MRPL at 10 $\mu\text{g.L}^{-1}$ has been
10 proposed for thyrostatic compounds. This level of performance is undoubtedly too high if a
11 Reference Point for Action (RPA) is decided around 10 $\mu\text{g.L}^{-1}$. It should also be emphasised
12 that robustness of the method regarding TU quantitation and control of the instability of such
13 residue are two issues of prime interest which are genuine pre-requisite before efficient and
14 non ambiguous implementation of the RPA.

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16 This work overall highlights the urgent need for a confirmatory criterion, should it be based
17 on the discovery of a discriminant biomarker or isotope ratio measurement. Two strategies
18 should be considered regarding the biomarker discovery which would either sign for the
19 feeding or the abuse origin of the TU signal; in these cases, biomarkers of interest would
20 either, and respectively, consist in vegetable precursors of the target molecule or direct
21 metabolites (phase I or II) of the exogenous administered thiouracil.

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Table 1: Detailed results of the data analysis for thiouracil urinary concentrations in the different animal species considered

Specie Sex	Total Bovine			Bovine (> 6 months)			Calves (< 6 months)			Porcine			Ovine			
	Male+Female	Male	Female	Male+Female	Male	Female	Male+Female	Male	Female	Male+Female	Male	Female	Male+Female	Male	Female	
n	1098	474	553	736	279	409	362	195	144	201	56	19	26	13	6	
mean	1,4	1,7	1,2	1,0	1,4	0,8	2,2	2,1	2,4	0,9	0,7	0,9	3,3	3,5	1,3	
median	0,3	0,6	0,1	0,1	0,2	0,0	1,1	1,0	1,1	0,5	0,5	0,6	2,2	1,7	1,0	
min	CCα	CCα	CCα	CCα	CCα	CCα	CCα	CCα	CCα	CCα	CCα	CCα	CCα	CCα	CCα	
max	22,5	22,5	20,8	20,8	11,9	20,8	22,5	22,5	18,7	7,0	3,4	5,3	14,3	14,3	3,0	
95% Centile	6,2	6,1	5,9	5,0	5,7	3,3	7,3	6,7	9,0	2,9	1,8	5,3	12,9	14,3	3,0	
99% Centile	12,1	11,2	13,3	8,8	9,1	8,4	17,7	21,4	14,1	4,1	3,4	5,3	14,3	14,3	3,0	
s.d.	2,5	2,6	2,5	2,0	2,1	1,9	3,2	3,1	3,3	1,0	0,7	1,2	3,7	4,1	1,1	
p-value (M vs F)					0,00010			0,40357			0,25277			0,21899		

Bovine Sex	6-24 months			> 24 months
	Male+Female	Male	Female	Female
n	540	279	213	196
mean	1,1	1,4	0,8	0,8
median	0,1	0,2	0,0	0,1
min	CC α	CC α	CC α	CC α
max	20,8	11,9	20,8	15,7
95% Centile	5,2	5,7	3,1	3,9
99% Centile	8,8	9,1	8,1	8,8
s.d.	2,1	2,1	2,0	1,9
p-value (M vs F)		0,00095		

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Figure Captions

Figure 1: Distribution frequency observed for thiouracil concentration levels measured in bovine urine samples (n = 1098)

Figure 2: Distribution frequency observed for thiouracil concentration levels measured in calve urine samples (n = 362)

Figure 3: Thiouracil concentration levels measured in calves urine samples according to their age when known (n = 296)

Figure 4: Distribution observed for thiouracil concentration levels measured in female (F, n=144) versus male calve urine samples (M, n = 195)

Figure 5: Distribution frequency observed for thiouracil concentration levels measured in bovine (6-24 months old) urine samples (n = 540)

Figure 6: Distribution observed for thiouracil concentration levels measured in bovine (6-24 months) female (F, n=213) and male urine samples (M, n = 279)

Figure 7: Distribution frequency observed for thiouracil concentration levels measured in porcine urine samples (n = 201)

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

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Table 1: Detailed results of the data analysis for thiouracil urinary concentrations in the different animal species considered

Specie	Total Bovine			Bovine (> 6 months)			Calves (< 6 months)			Porcine			Ovine			
Sex	Male+Female	Male	Female	Male+Female	Male	Female	Male+Female	Male	Female	Male+Female	Male	Female	Male+Female	Male	Female	
n	1098	474	553	736	279	409	362	195	144	201	56	19	26	13	6	
mean	1,4	1,7	1,2	1,0	1,4	0,8	2,2	2,1	2,4	0,9	0,7	0,9	3,3	3,5	1,3	
median	0,3	0,6	0,1	0,1	0,2	0,0	1,1	1,0	1,1	0,5	0,5	0,6	2,2	1,7	1,0	
min	CC α	CC α	CC α	CC α	CC α	CC α	CC α	CC α	CC α	CC α	CC α	CC α	CC α	CC α	CC α	
max	22,5	22,5	20,8	20,8	11,9	20,8	22,5	22,5	18,7	7,0	3,4	5,3	14,3	14,3	3,0	
95% Centile	6,2	6,1	5,9	5,0	5,7	3,3	7,3	6,7	9,0	2,9	1,8	5,3	12,9	14,3	3,0	
99% Centile	12,1	11,2	13,3	8,8	9,1	8,4	17,7	21,4	14,1	4,1	3,4	5,3	14,3	14,3	3,0	
s.d.	2,5	2,6	2,5	2,0	2,1	1,9	3,2	3,1	3,3	1,0	0,7	1,2	3,7	4,1	1,1	
p-value (M vs F)					0,00010				0,40357			0,25277			0,21899	

Bovine Sex	6-24 months			> 24 months
	Male+Female	Male	Female	Female
n	540	279	213	196
mean	1,1	1,4	0,8	0,8
median	0,1	0,2	0,0	0,1
min	CC α	CC α	CC α	CC α
max	20,8	11,9	20,8	15,7
95% Centile	5,2	5,7	3,1	3,9
99% Centile	8,8	9,1	8,1	8,8
s.d.	2,1	2,1	2,0	1,9
p-value (M vs F)		0,00095		

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