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► **To cite this version:**

Gaud Pinel, Séverine Mathieu, Nora Cesbron, Hubert de Brabander, Daniel Maume, et al.. Demonstration of thiouracil excretion in bovine urine under cruciferous diet. *Food Additives and Contaminants*, 2006, 23 (10), pp.974-980. 10.1080/02652030600806370 . hal-00577490

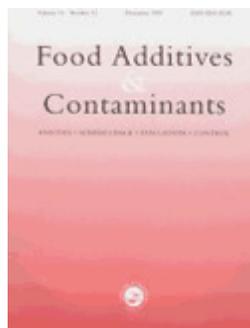
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Demonstration of thiouracil excretion in bovine urine under cruciferous diet

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2006-071.R1
Manuscript Type:	Original Research Paper
Methods/Techniques:	Chromatography - LC/MS, LC/MS
Additives/Contaminants:	Drug residues - hormones, Feeding, Residues, Veterinary drug residues
Food Types:	Animal feed

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Manuscripts

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6 **Evidence that urinary excretion of thiouracil in adult bovine**
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9 **submitted to a cruciferous diet can give erroneous indications of**
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11 **possible illegal use of thyrostats in meat production**
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Abstract

Thyrostats have been banned for use as veterinary drugs in Europe since 1981 because of their carcinogenic and teratogenic properties. Until now, the identification of thiouracil in animal biological matrices is interpreted as the consequence of an illegal administration. The present paper studies the influence of a cruciferous-based feed on the occurrence of thiouracil as a residue in urine. Urine samples collected from two heifers fed on cabbage or rapeseed cakes were analysed for the presence of thiouracil by 3-iodobenzylbromide derivatisation and LC-ESI(-)-MS/MS analysis. Urine collected after cabbage or rapeseed feeding showed thiouracil concentrations in the range 3–7 $\mu\text{g L}^{-1}$ and 2–9 $\mu\text{g L}^{-1}$, respectively, demonstrating a relationship between a diet based on cruciferous vegetables and the occurrence of thiouracil in urine. Thiouracil was excreted in urine in the hours following cruciferous intake. Complete elimination ($< 0.8 \mu\text{g L}^{-1}$) of the compound occurred within 5 days. The precursors in cruciferous vegetables responsible for the thiouracil excretion in urine was proved not to be thiouracil itself.

Key words : thyrostats, thiouracil, urine, rapeseed, cabbage, cruciferous plants, LC-ESI(-)-MS/MS

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 hormone production (Kotter et al. 1959 ; Derblom et al. 1963). In particular, 2-thiouracil (TU) (Figure 1a), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU) and 1-methyl-2-mercapto-imidazole (tapazole, TAP) are very strong thyroid-inhibiting compounds which are inexpensive and easy to obtain. 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. 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 according to the regulation (E.C. Decision 2002/657), 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 newly developed protocols (Pinel et al. 2005) 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) fixed at $100 \mu\text{g L}^{-1}$. 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 ($\gg 100 \mu\text{g L}^{-1}$).

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3 Occasionally however, the occurrence of thiouracil in bovine urine from the national
4 control plan with quantifications in the range 1-10 $\mu\text{g L}^{-1}$ raises the question of the
5 origin of the molecule, for example arising from low level abuse, end of the kinetic of
6 elimination, endogenous excretion or originating from animal feed? The latter
7 hypothesis could be considered since some vegetables from the Cruciferae
8 (Brassicaceae) family (Kennedy & Purves 1941) are known to contain substances,
9 called goitrogens, which impair iodine uptake by the thyroid or impair its incorporation
10 into thyroxine. These substances are called goitrogens and include thiouracil and
11 thioglucosides. Thioglucosides or glucosinolates are in particular found in cruciferous
12 vegetables (such as cabbage, cauliflower and rapeseed) (Tian et al. 2005).
13 Glucosinolates constitute a well-defined group of secondary plant metabolites in
14 cruciferous plants. Glucosinolates undergo hydrolysis with the endogenous plant
15 enzyme myrosinase (thioglucoside glucohydrolase EC 3.2.3.1) into a range of
16 biological compounds. Enzymatic hydrolysis occurs after cell wall disruption caused by
17 processes such as cutting, cooking or freezing (Verkerk et al. 1997). Very few studies
18 deal with the natural occurrence of thiouracil in vegetables as a free compound or as an
19 aglycone moiety (De Brabander 1984) and the influence of cattle feed on the presence
20 of this compound in biological matrices has never been investigated up to now. To
21 avoid false positive indications of drug abuse, and since these vegetables are
22 susceptible to be present in bovine feed through complementary nitrogen feedingstuffs,
23 it is of major importance to elucidate this possible contribution from feed. Work aimed
24 at identifying the thiouracil in bovine urine through different mass spectrometric
25 evidence has been reported recently (Pinel et al. 2006) and was a necessary preliminary
26 to the present work. The present paper aims at assessing the influence of a cruciferous
27 vegetable feed based on the concentration range for thiouracil in urine. Thiouracil
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3 determination was carried out according to a previous published method based on LC-
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5 (ESI(-))-MS/MS determination after 3-iodobenzylbromide derivatisation (Pinel et al.
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7 2005).
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12 [Insert Figure 1 about here]
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15 16 17 **Experimental**

18 19 *Animal experiment*

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22 Two different experiments were conducted with heifers to evaluate the contribution of
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24 two cruciferous vegetables, cabbage and rapeseed, on the occurrence of thiouracil in
25
26 urine upon consumption of these feedingstuffs.
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29 Cabbages were collected directly from farms in January 2005 and rapeseed cakes were
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31 obtained from a local agricultural cooperative store.
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34 One heifer has been fed with fresh cabbage twice a day, morning and afternoon, over 9
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36 days from D₀ to D₈ (except on D₃). Urine samples were collected during the same
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38 period twice a day, except on D₃.
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41 The second experiment was conducted over 19 days, from D₀ to D₁₈, with a different
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43 heifer from the one used in the first experiment. Feed was kept unchanged with hay for
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45 7 days, then the heifer was fed with rapeseed cakes *ad libitum* for 4 days and finally
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47 returned to hay for 8 days. Urine samples were collected twice a day during this period.
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49 For subsequent analysis of the vegetable matrices, cabbage leaves and stems were
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51 separated, grounded and freeze-dried.
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Chemicals

Standards were obtained from Sigma-Aldrich (St. Quentin Fallavier, France). Stock solution of 2-thiouracil (TU) was prepared in methanol at a concentration of $100 \text{ ng } \mu\text{L}^{-1}$. Working solutions were obtained by 100x and 1000x dilutions in methanol ($1 \text{ ng } \mu\text{L}^{-1}$ and $0.1 \text{ ng } \mu\text{L}^{-1}$, respectively). The internal standard was synthesised in our laboratory, 2-benzyl-6-propyl-2-thiouracil (BPTU). Chemicals for extraction and purification steps were of analytical grade. Solvents for the preparation of the mobile phase were of HPLC-grade. They were all obtained from SDS (Peypin, France). Derivatisation reagent 3-iodobenzylbromide (3IBBr) was obtained from Sigma-Aldrich (St Louis, MO, USA) and prepared extemporaneously (2 mg mL^{-1} in methanol). Phosphate buffer, pH 8, was made up of 94.5 mL Na_2HPO_4 0.2 M and 5.5 mL KH_2PO_4 0.2 M.

Material

Silica gel SPE disposable columns (1 g) were obtained from UCT (Bristol, PA, USA). Separation of thyrostatic compounds was performed on a Nucleosil C_{18} AB column ($5 \mu\text{m}$, $2.5 \times 50 \text{ mm}$) (Interchim, Montluçon, France) with a MeOH (A) and H_2O containing 0.5% acetic acid (B) gradient, run at $300 \mu\text{L}/\text{min}$ on an Alliance 2690 HPLC chromatograph (Waters, USA). The gradient was linear as follows: A/B 30/70 to 70/30 from 0 to 20 min.

MS measurements

A triple quadrupole mass analyser (Quattro-LC, Micromass, UK) was used fitted with an ESI source operated in the negative mode. The analytes were detected using selected reaction monitoring (SRM) acquisition mode. The following transitions have been monitored for BPTU and TU, respectively: $259 > 225$, 58, 42 and $343 > 127$, 95, 58, 42.

Derivatisation, extraction and clean-up

The analytical protocol describing the derivatisation, extraction and purification of thyrostats from urine has been published elsewhere (Pinel et al. 2005). Briefly, thyrostats contained in urine were derivatised with 3-IBBr (pH 8, 1 h, 40 °C) prior to extraction (Figure 1b). After a liquid/liquid extraction of the derivatised compounds (diethyl-ether, pH adjusted to 3 with HCl 35 %), the samples were cleaned-up on silica cartridges (elution with a mixture of hexane/ethyl acetate 40/60) and injected onto the LC column. Analysis of vegetables followed the same method with an additional solid/liquid extraction step at the beginning of the protocol. Indeed, to 2 g of cabbage or rapeseed, 5 mL of methanol have been added. The extraction proceeded for 30 min under agitation and the extract was obtained after centrifugation (3000 g, 20 min). The methanolic extracts were dried under a gentle stream of nitrogen before the derivatisation step occurs.

Results and discussion

Cruciferous vegetables were chosen for this study because cabbage is historically the plant in which a goitrogen property is attributed to the presence of anti-thyroid agents (Kennedy & Purves 1941). Cabbage is furthermore a plant on which cattle traditionally feed on during winter on farms. Rapeseed was chosen as it belongs to the Cruciferae family and is incorporated into animal feed as nitrogen source.

Feeding and consequences on thiouracil concentrations in urine

All the urine samples collected during the different animal feeding experiments were analysed in order to establish the influence of feeding on thiouracil concentrations in

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3 urine. Urine samples arising from the experiment with cabbage feed were analysed for
4 their content of thiouracil. Typical chromatograms are presented on Figure 2. The
5 reference chromatogram (total ion current) shows the signals associated to the internal
6 standard BPTU ($100 \mu\text{g L}^{-1}$) eluted at 15.2 min and to thiouracil ($10 \mu\text{g L}^{-1}$) with a
7 retention time of 12.1 min (Figure 2a). The urine sample collected in the morning of the
8 experiment (D0 morning), before the heifer had been provided with any cabbage, did
9 not contain any free thiouracil as shown on extracted ion chromatograms on Figure 2b.
10 The extracted ion chromatograms of urine sample collected in the evening of day 5
11 (Figure 2c) provided unambiguous identification of thiouracil in the sample, with
12 respect to number of monitored reactions, ion-ratio and retention time criteria,
13 according to the European Decision 2002/657/EC and as shown in previous work (Pinel
14 et al. 2006). The concentration of thiouracil in the sample was found to be $6 \mu\text{g L}^{-1}$. The
15 concentrations calculated in the different urine samples collected during the experiment
16 are shown on Figure 3. These results clearly indicate that thiouracil appears in the urine
17 samples within the first 10 hours of the experiment (D₀ evening). Thiouracil was
18 always present in urine samples collected in the evening during the experiment and
19 seldom in the morning samples. The highest concentration was reached on D₁ in the
20 evening ($7 \mu\text{g L}^{-1}$). According to these results, the correlation with a cabbage-based
21 feeding and the occurrence of thiouracil in urine samples at concentrations between 5
22 and $10 \mu\text{g L}^{-1}$ was established. To complete the results, it would have been of interest to
23 check the absence of thiouracil in urine samples collected some evenings before the
24 experiment with the feeding of cabbage started. A possible explanation for the
25 occurrence of thiouracil in the evening urine samples could be the polar nature of the
26 compounds present in the cabbage which is a water-based vegetable. Polar compounds
27 such as thiouracil would be very easily assimilated and rapidly eliminated in urine.
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3 Indeed, thyrostats, and in particular thiouracil, are known to be rapidly eliminated in
4 urine after oral administration (De Brabander 1984).
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15 Urine samples collected during the rapeseed experiment were analysed for their content
16 of thiouracil. Typical chromatograms are presented in Figure 4. As already shown with
17 the cabbage experiment, thiouracil, which was not present in the urine samples
18 collected before feeding with rapeseed cakes (extracted ion chromatograms shown on
19 Figure 4b), appeared in the samples collected during the experiment (extracted ion
20 chromatograms shown on Figure 4c). The calculated thiouracil concentrations in urine
21 samples collected during the whole animal experiment are presented on Figure 5. In
22 this experiment, no difference could be found between samples collected in the
23 morning or in the evening. A possible explanation could be that rapeseed cakes are
24 anhydrous stuffs and polar compounds are not released so rapidly as they are from
25 aqueous stuffs such as cabbage. Three main periods can be distinguished: (a) Urine
26 collected during the seven days before feeding with rapeseed did not contain any
27 thiouracil; (b) Urine collected during the feeding with rapeseed cakes in which
28 thiouracil was identified in concentrations in the range $1.5\text{--}7.5\ \mu\text{g L}^{-1}$, and (c) Urine
29 collected once the feeding with rapeseed cakes has been stopped and in which
30 thiouracil was identified and quantified at concentrations as high as $9\ \mu\text{g L}^{-1}$. This latter
31 part showed a typical elimination kinetic behaviour since TU concentrations decreased
32 with time to be undetectable ($< 0.8\ \mu\text{g L}^{-1}$) 6 days after the end of the rapeseed feeding.
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[Insert Figures 4 and 5 about here]

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6 This second animal experiment confirmed the conclusions already drawn after the first
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8 one as described above: a cruciferous-based feeding regime leads to the excretion and
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10 detection of thiouracil in urine of animals during the time of the feeding.
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12 Concentrations were observed to be below $10 \mu\text{g L}^{-1}$ and are in accordance with values
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14 sometimes reported for urine within the French national control plan. According to
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16 these results, which have never been described before, the identification of thiouracil in
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18 urine samples at concentrations up to $10 \mu\text{g L}^{-1}$ should henceforth not lead
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20 automatically to the conclusion of illegal abuse of thiouracil. Results will have to be
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22 interpreted keeping in mind that thiouracil may possibly originate from feed. These
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24 recent observations of thiouracil at trace level have certainly to be linked with the use
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26 of new generation of analytical instruments which have led to decreased detection
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28 limits and allowed measurement of concentration levels that were undetectable with
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30 older and less sensitive instruments.
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39 To understand the origin of the urinary excretion of thiouracil, we investigated the
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41 composition of the feedingstuff used in this study, particularly focusing onto similar
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43 chemical structures to that of thiouracil. Analysis of the different samples, cabbage
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45 leaves, stems and rapeseed cakes did not show evidence of the presence of free TU.
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48 These results raise the question of the origin of thiouracil in urine. The certain
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50 identification of thiouracil was a necessary preliminary, which has been achieved
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52 recently (Pinel et al. 2006) through different and independent mass spectrometric
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54 determinations (LC-MS/MS, GC-MS/MS, HRMS) in bovine urine samples from
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56 animals never treated with any thyrostatic compounds. Even though thiouracil was
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3 identified unambiguously, the question of its origin remains unanswered. Further work
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5 is needed to exclude any analytical pitfall and precursor(s) identity has to be elucidated.
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10 **Conclusion**

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12 The present study allowed a correlation to be seen between the use of a cruciferous-
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14 based animal feed and the identification of thiouracil in the urine of animals through
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16 animal experiments and LC-MS/MS analysis of the collected urine. This study, which
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18 is the first of its kind, will lead in future to a more careful interpretation of samples
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20 suspected of being “non compliant” ones with regard to thiouracil abuse.
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22 Concentrations of thiouracil eliminated in urine during the cruciferous diet were rather
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24 low and up-to 10 $\mu\text{g L}^{-1}$. Even though the relation between components in animal feed
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26 and thiouracil occurrence in urine has been established, neither free thiouracil nor its
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28 precursors have yet been identified in cabbage or rapeseed. Further work should be
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30 conducted in order to investigate on the potential thiouracil -releasing compounds in
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32 vegetables. The discrimination between urines containing thiouracil of a natural origin
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34 or as a consequence of drug abuse should rely for example on potential and specific
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36 phase I or phase II metabolites that will be searched in the future. The use of isotopic
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38 methods such as GC-C-IRMS could also help in such discrimination.
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48 **Acknowledgements**

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50 We thank the clinical staff from *Maladie des Animaux d'Élevage* (National Veterinary
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52 School – Nantes, France) for providing the heifers and their help for conducting the
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54 animal experiments.
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Figures

Figure 1: Tautomeric forms of 2-thiouracil (a) and chemical structure of TU derivatised with 3-IBBr (b).

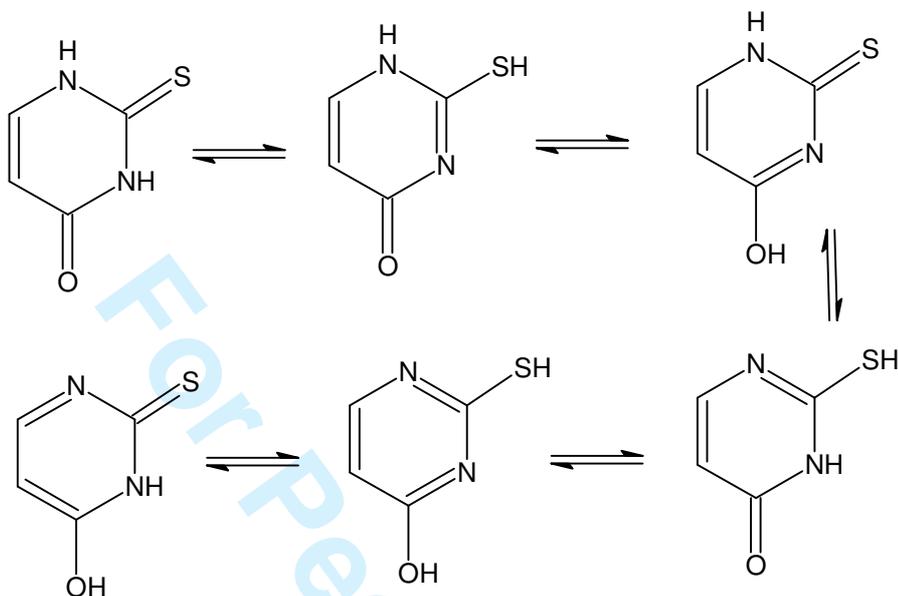
Figure 2: Cabbage Experiment. Ion chromatograms of (a) reference sample fortified with TU ($10 \mu\text{g L}^{-1}$) and BPTU as internal standard ($100 \mu\text{g L}^{-1}$), (b) urine sample collected the first morning of the experiment and (c) urine sample collected on D₅ evening. LC-ESI(-)-MS/MS analysis and SRM acquisition mode.

Figure 3: Thiouracil elimination ($\mu\text{g L}^{-1}$) in urine samples collected twice a day on a heifer fed on cabbage during 9 days.

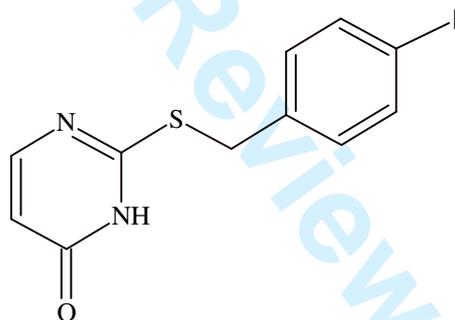
Figure 4: Rapeseed Experiment. Ion chromatograms of (a) reference sample fortified with TU ($10 \mu\text{g L}^{-1}$) and BPTU as internal standard ($100 \mu\text{g L}^{-1}$), (b) urine sample collected the first morning of the experiment and (c) urine sample collected on D₁₀ evening. LC-ESI(-)-MS/MS analysis and SRM acquisition mode.

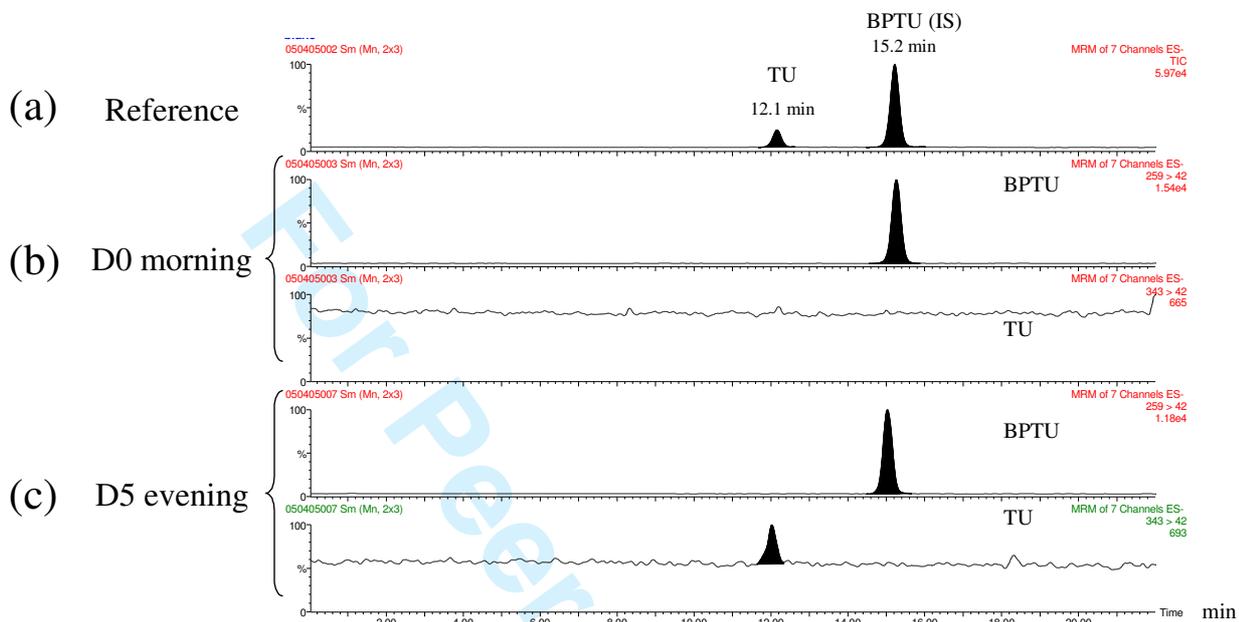
Figure 5: Thiouracil elimination ($\mu\text{g L}^{-1}$) in urine samples collected twice a day (morning and evening) on a heifer fed on rapeseed cakes during 19 days.

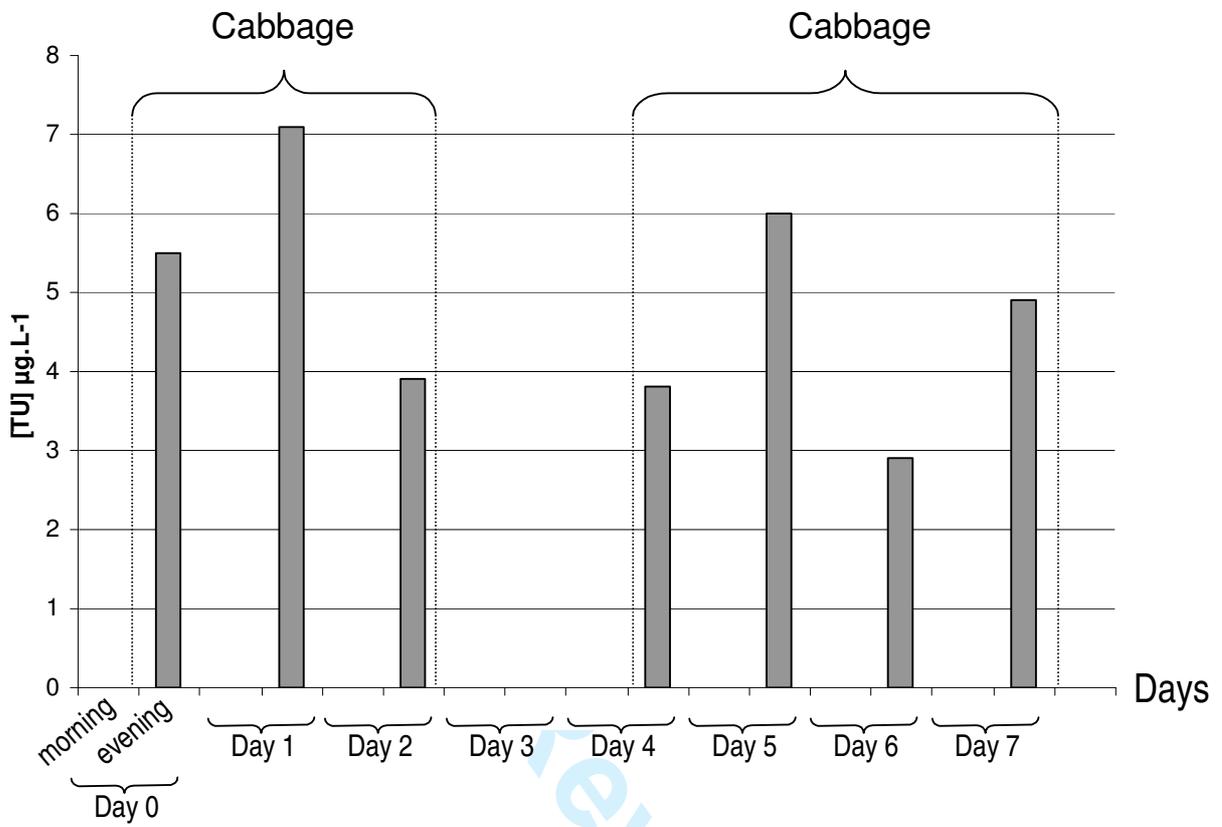
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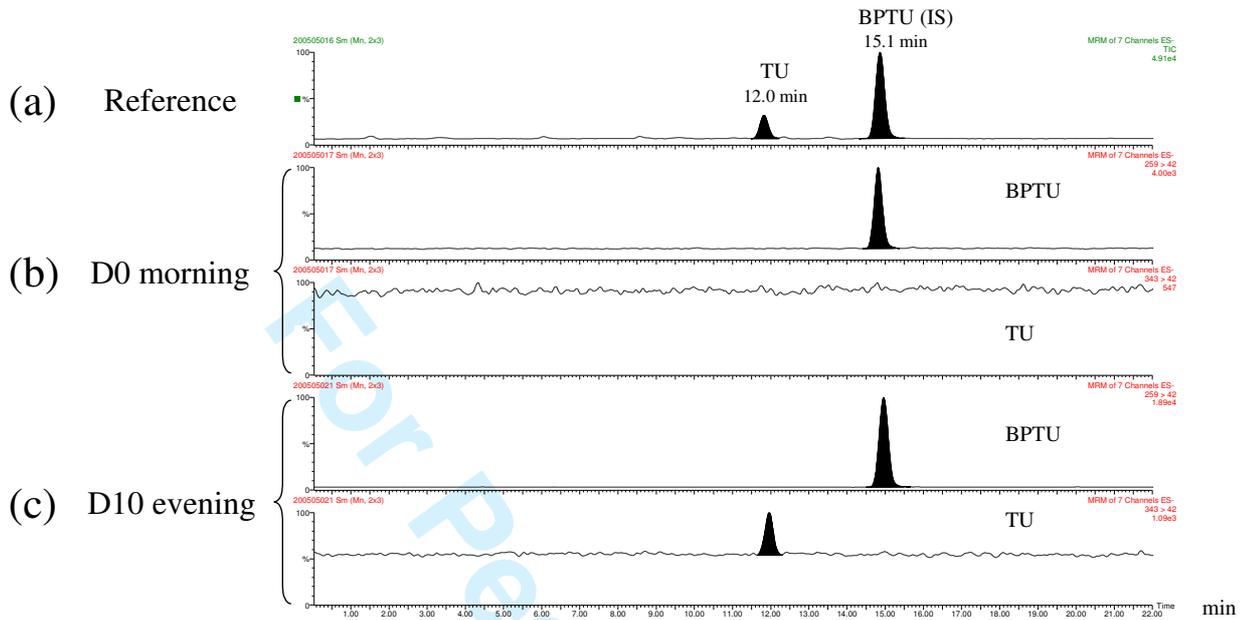




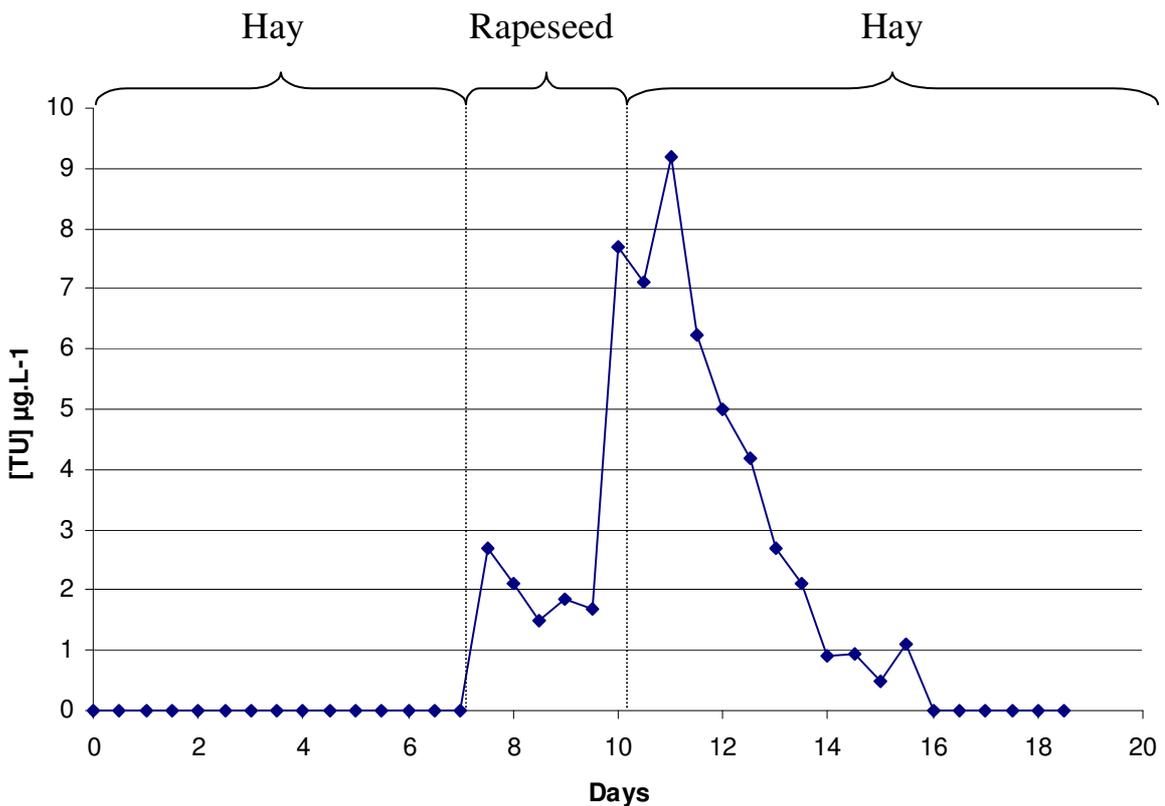


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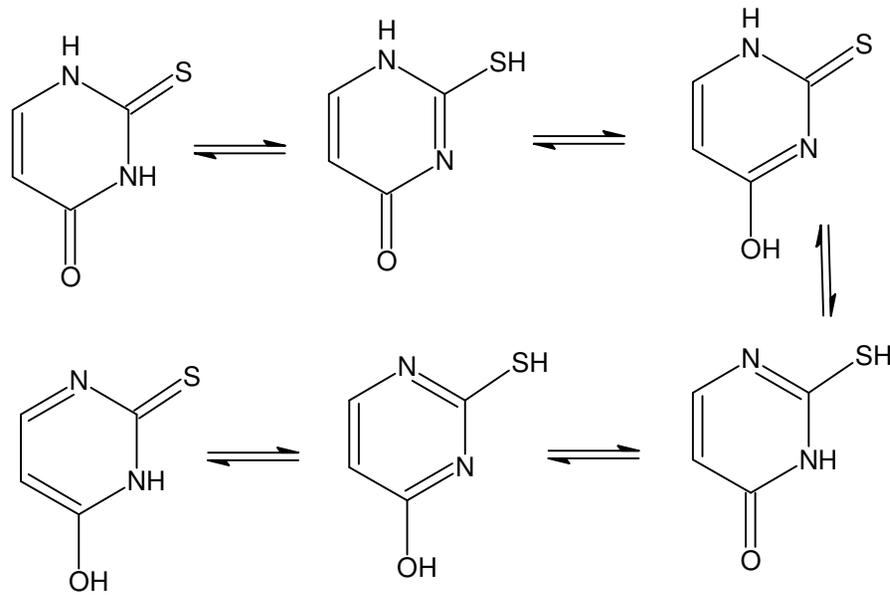


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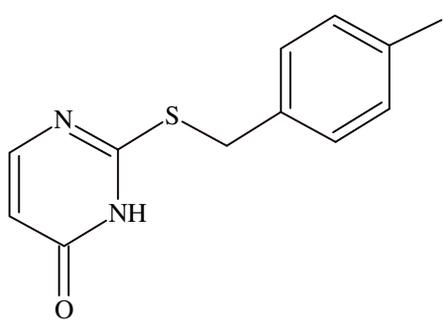


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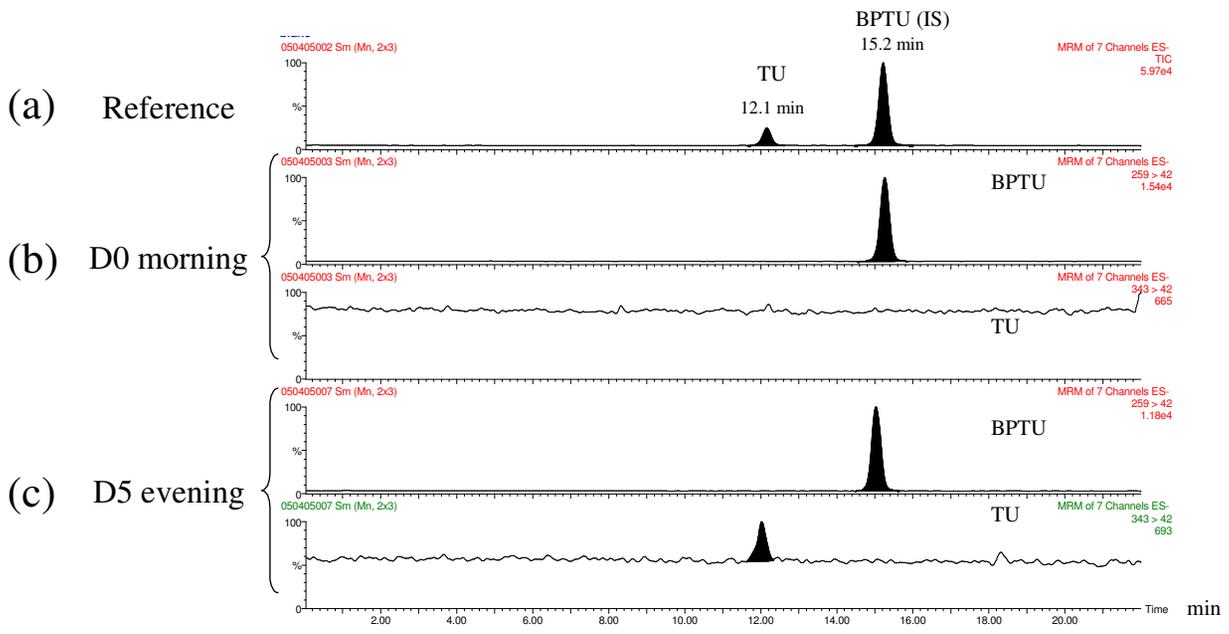
(a)

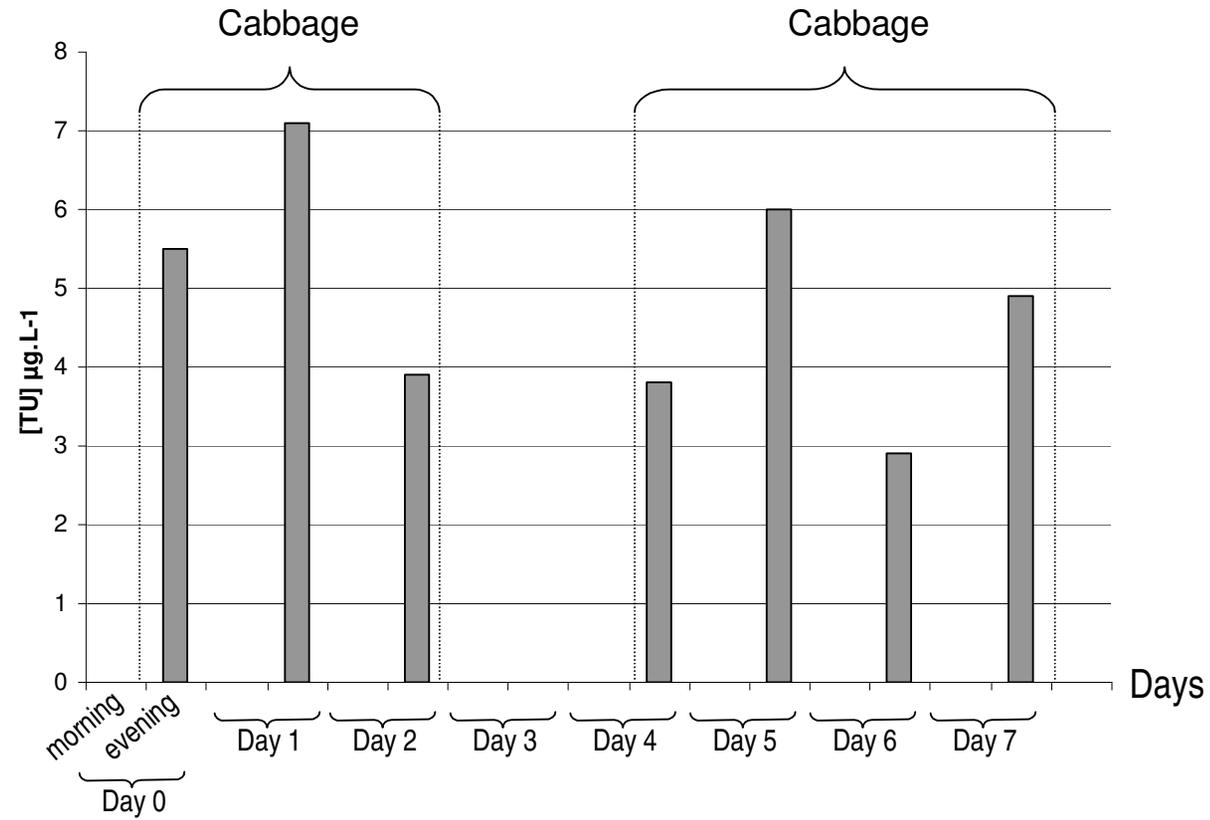


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