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Feed or Food Responsible for the Presence of Low-Level Thiouracil in Urine of Livestock and Humans?

Julie Vanden Bussche,*[,†] Julie Kiebooms,[†] Nathalie De Clercq,[†] Yoann Deceuninck,[§] Bruno Le Bizec,[§] Hubert F. De Brabander,[†] and Lynn Vanhaecke[†]

[†]Laboratory of Chemical Analysis, Research Group of Veterinary Public Health and Zoonoses, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium

[§]ONIRIS, LABERCA, F-44307 Nantes, France

ABSTRACT: In recent years, questions have been raised on the possible semiendogenous status of the alleged xenobiotic thyreostatic drug thiouracil; thiouracil has been detected in the urine of various animals (livestock and domesticated) at concentrations between 1 and 10 μg L^{-1} and also in human urine. Although several studies suggest Brassicaceae-derived feed as potential origin, no traces of thiouracil have been detected in feed so far. Therefore, the aim of this study was to elucidate the origin of thiouracil in the urine of livestock and humans. To this purpose various Brassicaceae feed and food sources (e.g., rapeseed, rapeseed coarse meal, cabbage, cauliflower, broccoli) were investigated for the presence of thiouracil. In addition, the impact of the Brassicaceae-related β-thioglucosidase enzyme was evaluated. This myrosinase enzyme appeared to be crucial, because without its catalyzed hydrolysis no thiouracil could be detected in the various Brassicaceae-derived samples. Therefore, a sample pretreatment with incorporated enzymatic hydrolysis was developed after ensuring the quality performance of the extracted myrosinase mixture with a single-point glucose assay. Upon enzymatic hydrolysis and LC-MS^2 analysis, thiouracil was successfully detected in samples of traditional rapeseed, rapeseed-'00' variety coarse meal (values of erucic acid <2% and glucosinolates <25 μmol g^{-1}), and rapeseed cake at 1.5, 1.6, and 0.4 μg kg^{-1}, respectively. As for the food samples, broccoli and cauliflower displayed thiouracil concentrations of 6.0 and <1.0 μg kg^{-1}, respectively. To the best of the authors' knowledge this study is the first to report the presence of naturally occurring thiouracil in feed and food samples. Future research should investigate the pathway of thiouracil formation and identify its possible precursors.

KEYWORDS: enzymatic hydrolysis, myrosinase, mass spectrometry, liquid chromatography, Brassicaceae, naturally occurring thiouracil

1. INTRODUCTION

Thiouracil is categorized as a xenobiotic thyreostat, an orally active drug that upon administration disturbs the normal metabolism of the thyroid gland by inhibiting the production of the hormones triiodothyronine and thyroxine.[1] This goitrogenic activity may be attributed to the presence of a thiocarbamide group.[2] The thyroid-inhibiting activity is in particular displayed by 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 1-methyl-2-mercaptoimidazole (TAP, tapazole), and 2-mercaptobenzimidazole (MBI). In livestock, the administration of thyreostats results in a considerable live weight gain, mainly caused by increased water retention in edible tissue and augmented filling of the gastrointestinal tract.[3,4] Consequently, these growth-promoting agents negatively affect the meat quality of treated animals. In addition, some xenobiotic thyreostats such as thiouracil are listed as compounds with teratogenic and carcinogenic properties and thus pose a possible human health risk (International Agency for Research on Cancer).[5] These arguments led in 1981 to a ban on their use for animal production in the European Union.[6]

Because of the established zero-tolerance levels for the use of thyreostatic drugs in animal production, European legislation demands its member states to develop confirmatory methods to detect and quantify thyreostatic compounds in various matrices (e.g., urine, feces, meat, thyroid gland). As a guideline for these methods, the European Union set out a provisional minimum required performance limit (MRPL) of 100 μg L^{-1} or μg kg^{-1}. To achieve this, new confirmatory methods have been developed and ameliorated over time, as reviewed by Vanden Bussche et al.[7] Nowadays, the most common method used consists of a 3-iodobenzylbromide (3-IBBr) derivatization followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).[8] Recently, this application was transferred to the new high-end technology of ultraperformance liquid chromatography coupled to tandem MS (UPLC-MS/MS).[9] Both methods comply with the provisional MRPL, and even with the recommended concentration (RC) of 10 μg L^{-1} or μg kg^{-1}, as set out by the European Union Reference Laboratories (EURL) in 2007.[10] More recently, a U-HPLC-MS/MS method for thyreostats in urine without derivatization was presented as well, also complying with the RC.[11]

In recent years, however, thiouracil has occasionally been detected in the urine of livestock at concentrations between the CC_α value and 10 μg L^{-1}. The detection of these residues has raised questions on its origin, namely, synthetic or natural. Up to

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now, only a few studies have addressed this issue, which is in the scientific literature referred to as the possible semiendogenous status of thiouracil. The onset was given by Pinel et al., who investigated the hypothesis of Brassicaceae plant or derivative (e.g., cabbage and rapeseed cake) consumption being responsible for the presence of TU in bovine urine.[12] Brassicaceae are known to contain glucosinolates, the precursor molecules of the naturally occurring thyroid-inhibiting compounds, such as the oxazolidine-2-thiones and thiocyanates.[13,14] Glucosinolates are well-defined secondary plant metabolites, which upon cell disruption (e.g., grounding, cooking, freezing) are hydrolyzed by the endogenous β-thioglucosidase plant enzyme, myrosinase (EC 3.2.1.147).[15−17] This hydrolysis generates a range of biologically active compounds, which are converted to derivates with a natural thyreostatic action.[13,14] Hydrolysis induced by ingestion has also been reported, with the intestinal microbiota as most probable mediators for this catalysis.[17−22] Pinel et al. investigated the possible correlation of Brassicaceae administration and TU present in bovine urine.[12] Although a correlation was established, no thiouracil could be detected in the feed itself. Another study that explored the status of TU was not confined to bovine urine alone. Urine of livestock (bovine, porcine, ovine, etc.), domesticated animals (canine), and even humans was subjected to U-HPLC-MS/MS analysis.[24] Most animals displayed traces of TU, below 10 μg L^{-1}. As for the humans, for whom the influence of a Brassicaceae diet was investigated, TU was retrieved in 66.7% of the samples. No significant differences could, however, be detected between the period of Brassicaceae restriction and administration. These two independent studies confirm the natural origin of thiouracil detected in the urine of untreated species. So far, however, the origin of this thiouracil signal in urine is still undefined. Because thiouracil is considered not only as a synthetic but also as a naturally occurring compound, it is referred to in general as a "semiendogenous" compound. However, if thiouracil is detected in a natural source of origin, it will be defined by the term "naturally occurring". In case evidence indicates that this thiouracil is released from the same matrix where it has been produced, it will be defined as an "endogenous" compound.

The aim of this study was to investigate and elucidate the origin of thiouracil in the urine of livestock and humans. For this purpose, various Brassicaceae vegetables (food samples, e.g., cauliflower and broccoli) and derivatives (feed samples, e.g., rapeseed, rapeseed coarse meal, and feeding cabbage) were investigated for the presence of thiouracil. Additionally, the influence of hydrolysis catalyzed by the β-thioglucosidase enzyme myrosinase was investigated, due to its well-known presence in plants of the Brassicaceae family.

2. MATERIALS AND METHODS

2.1. Reagents and Chemicals. The glucosinolate sinigrin ((−)-sinigrin hydrate, ≥ 99.0%) was obtained from Sigma-Aldrich (St. Louis, MO), D-(+)-glucose was from Merck (Darmstadt, Germany), and the chemical standard 2-thiouracil (TU) and internal standard 6-ethyl-2-thiouracil (ETU) were also obtained from Sigma-Aldrich. The deuterated internal standard 6-propyl-2-thiouracil-D5 (PTU-D5) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). Stock solutions of the chemical standards were prepared in methanol at a concentration of 200 ng μL^{-1}. Working solutions were prepared by 200× and 2000× dilutions in methanol (1 and 0.1 ng μL^{-1}, respectively). When necessary, sonication was applied to ensure the complete dissolution of the substances. Solutions were stored in dark glass bottles at 7 °C. For the internal standards ETU and PTU-D5, spike levels of 50 μg L^{-1} were used.

Reagents were of analytical grade (VWR International, Merck, Darmstadt, Germany) when used for extraction and purification steps and of Optima MS grade for MS application (Fisher Scientific U.K., Loughborough, U.K.), respectively.

The derivatization reagent, 3-iodobenzylbromide (3-IBBr, Sigma-Aldrich), was prepared extemporaneously (2 mg mL^{-1} methanol). Phosphate buffer, made up of 0.2 M Na_2HPO_4 and 0.2 M KH_2PO_4 in deionized water, was prepared and adjusted to a pH of 8.

Table 1. Monitored Transitions of the LC-MS^2 Method for the Thyreostatic Drug Thiouracil and Its Internal Standards, Ethylthiouracil and Propylthiouracil-D5, Respectively

analyte	$[M - H]^-$	product ions	collision energy (eV)	retention time (min)
thiouracil	343	182, 215, 309	44	10.3
ethylthiouracil	371	210, 243, 337	44	14.3
propylthiouracil-D5	385	127, 262, 356	30	15.4

2.2. Preparation of Myrosinase Solution. For the enzymatic treatment of the feed and food samples, a myrosinase solution was prepared according to the method of Wrede.[25] Five grams of yellow *Sinapsis alba* L. seed (white mustard seed), purchased at a local store, was homogenized with 30 mL of water, stirred for 30 min at room temperature, and centrifuged at 12000*g* for 10 min. The supernatant was mixed with an equal volume of 90% ethanol. The mixture was centrifuged, and the precipitate ws washed with 10 mL of 70% ethanol and centrifuged again. Subsequently, the ethanol fraction was removed and the remainder dissolved in 5 mL of water. This solution contains ca. 20 mg of enzyme mL^{-1} solution.[26] For the hydrolysis, phosphate buffer (pH 7) made up of 0.5 M $Na_2HPO_4 \cdot 2H_2O$ and 0.5 M KH_2PO_4 was preferred, and this was dissolved in deionized water.

Additionally, commercially available myrosinase purified from white mustard seeds (*S. alba* L.) was purchased at Sigma-Aldrich (thioglucosidase, 25 UN) to serve as a control for the enzymatic activity. This was dissolved in 100 μL of water, and 30 μL of this solution was required per sample of 1.5 mL.[27]

2.3. Instrumentation. *2.3.1. LC-MS^2.* Separation of the thyreostatic compounds was achieved at 30 °C on a Symmetry C18 column (5 μm, 150 mm × 2.1 mm, Waters, Milford, MA) with water containing 0.5% acetic acid (A) and methanol (B), run at 0.3 mL min^{-1} on a Finnigan Surveyor LC system (Thermo Electron, San Jose, CA). The linear gradient was as follows: A/B 50:50 for 3 min, increasing the amount of methanol during 17 min to 0:100, and allowing the column to re-equilibrate for 10 min at 50:50. A linear ion trap mass spectrometer (LTQ, Thermo Electron) was used, fitted with a heated electrospray ionization source (HESI) operating in the negative ion mode. The following working conditions were applied: source voltage at 5 kV; vaporizer and capillary temperature at 250 and 275 °C, respectively; sheath and auxiliary gas at 30 and 5 arbitrary units (au), respectively. The transitions monitored are displayed in Table 1.

2.3.2. HPLC-ELSD. For the detection of glucose, the samples were injected on a Prevail Carbohydrate ES column (5 μm, 250 mm × 4.6 mm, Grace Davison Discovery science, Deerfield, IL) coupled to a HP Agilent 1100 HPLC system (Palo Alto, CA). The mobile phase, consisting of acetonitrile and water (60:40), was pumped isocratically at 0.5 mL min^{-1}. The evaporative light scattering detector (Alltech model 3300 ELSD, Grace Davison Discovery Science) was operated at 40 °C, with a N_2 pressure of 4 bar, a nebulizer gas at a flow of 1.5 L min^{-1}, and a gain factor set at 4.

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Table 2. Overview of the Various Pretreatments Applied on the Different Feed and Food Samples, Prior to Myrosinase Hydrolysis, Extraction, Cleanup, and LC-MS^2 Analysis[a]

	pretreatment			
sample	freeze-dried	ground	inactivation[b]	concentration (μg kg^{-1} dry wt)
broccoli	+	+		5.7 ± 0.5
	+	+	+	6.0 ± 1.0
cauliflower	+	+		0.9 ± 0.1
	+	+	+	0.6 ± 0.3
feeding cabbage	+	+		ND
rapeseed	+			0.4 ± 0.2
	+	+	+	0.4 ± 0.3
	+	+	+	1.5 ± 0.3
	+			0.1 ± 0.1
rapeseed-'00' coarse meal	+	+		1.6 ± 0.2
rapeseed cake	+	+		0.4 ± 0.1
feed 30% rapeseed-'00'	+	+		ND

[a] Additionally, the concentration (μg kg^{-1}) of thiouracil and the obtained standard deviation (n = 6) were calculated by means of the standard addition approach and corrected with the obtained recovery yield. ND, not detected. [b] Inactivation by means of heating, that is, 2 h of boiling.

2.3.3. Data Analysis. For both instrumental procedures data processing was performed using Xcalibur 2.0.7 software (LCA; Thermo Fisher Scientific, San Jose, CA).

2.4. Food and Feed Samples. As an animal feed, rapeseed and its extraction products (rapeseed flakes, cake, and coarse meal) generated as side products from the oil industry are of high interest. These are considered as a valuable, cheap, and easily obtainable source of nitrogen. For this purpose, only the '00' varieties defined with low erucic acid (<2%) and glucosinolates (<25 μmol g^{-1}) levels may be used.[23] In our study, animal feed (30% rapeseed-'00'), feeding cabbage, rapeseed coarse meal derived from '00' variety, and traditional rapeseed (*Brassica napus* L. partim Napoleon, Ilvo, Melle, Belgium) with high glucosinolate content were included. Alternatively as food sources, the Brassicaceae vegetables broccoli and cauliflower were incorporated in the study.

Prior to analysis, all samples were freeze-dried. In addition, some samples received additional pretreatments to investigate the influence of the myrosinase enzyme coexisting in the matrix (Table 2). As pretreatment, different combinations of grounding and inactivation through boiling (2 h) were conducted.[27,28]

Because specimens of cabbage, cauliflower, and broccoli contain elevated levels of water (90%), freeze-drying resulted in the concentration of matrix constituents that led to elevated background noise.[29] For this reason, aliquots of 0.25 g were utilized for the analysis of cabbage, cauliflower, and broccoli. For all other samples an amount of 0.5 g of sample was selected. To each sample was added the internal standard PTU-D5 at a concentration of 50 μg kg^{-1}.

2.5. Enzymatic Hydrolysis. The most optimal conditions for myrosinase-catalyzed hydrolysis were pH 7 and incubation at 37 ± 1 °C.[26] Therefore, 9.5 mL of phosphate-buffered saline at pH 7 was added to each sample, with an additional 0.5 mL of myrosinase solution. Next the samples were placed in a heated (37 ± 1 °C) ultrasonic bath for 4 h and subsequently placed overnight in an oven at 37 ± 1 °C.

2.6. Enzymatic Activity Determination. The activity was measured by a myrosinase-coupled enzymatic procedure, during which glucose is formed as a result of the unimolar reaction between myrosinase and the glucosinolate sinigrin. As substrate, sinigrin was selected because this was the only commercially available purified glucosinolate. The first experimental setup was performed to investigate if the extraction procedure of myrosinase from white mustard seeds was successful. For this purpose, five different samples of 1.5 mL at pH 7 (made up from 0.1 M ammonium acetate and 0.025 M ammonium bicarbonate) containing 34 mM sinigrin were composed in duplicate. The first sample type did not contain any myrosinase, and then 0.5 or 1.0 mL of the myrosinase mixture (section 2.2) was added. The fourth sample contained 30 μL of the synthetic purified myrosinase solution. As for the fifth and final sample, 0.5 mL of myrosinase mixture was added with additionally 2 mM ascorbic acid, which has been reported to increase the enzymatic activity of the enzyme.[30,31] The samples subsequently underwent a 4 h of incubation at 37 ± 1 °C, after which the hydrolysis reaction was stopped by boiling the samples for 5 min.[32] A second experiment investigated the kinetics of the enzymatic hydrolysis rate. For this experiment, three samples (5 mL) at pH 7 containing 34 mM sinigrin and 1.67 mL of myrosinase solution were incubated under conditions identical to those of the previous experiment, at 0, 1, 1.5, 2, 2.5, 3, 3.5, and 4 h; 0.5 mL of each batch was sampled, boiled (5 min), and analyzed for glucose. Of each sample 10 μL was injected on the HPLC-ELSD system.

2.7. Sample Extraction and Purification. For analysis of the feed and food samples, the analytical method with incorporated derivatization step was selected because of the more extensive sample cleanup as compared to the method without derivatization. The addition of solid phase extraction (SPE) to the sample cleanup was required for the adequate removal of matrix interferences and proper detection of thiouracil. Before the actual sample cleanup, all samples first underwent the enzymatic hydrolysis as described under section 2.5. The next day prior to the analytical analysis, specimens were cooled, passed over a filter paper, and adjusted to pH 8 by adding 8 mL of phosphatic buffer. Afterward, derivatization, extraction, and sample cleanup were performed, which have already been published elsewhere.[8] Briefly, 400 μL of 3-IBBr (2 mg mL^{-1} MeOH) was added to the sample and placed at 40 ± 1 °C (1 h). Next, the pH was adjusted to 3.6 ± 0.1, followed by liquid/liquid extraction with diethyl ether (3 × 5 mL). Then the samples were purified on cyclohexane-conditioned (15 mL) silica cartridges (SI SPE, 0.5 g), washed with 6 mL of cyclohexane, and eluted with a mixture of *n*-hexane/ethyl acetate (40:60). The collected fraction was evaporated to dryness under a gentle stream of nitrogen at 50 °C. Finally, the dried residue was redissolved in a total volume of 160 μL of mobile phase consisting of 50:50 0.5% acetic acid in water/MeOH.

2.8. Quality Assurance. Prior to sample analysis, a standard mixture of the target compounds was injected to check the operational conditions of the chromatographic device. To every Brassicaceae-related sample, which was analyzed in 6-fold, two procedure internal standards (IS) were added prior to enzymatic hydrolysis at a concentration of 50 μg kg^{-1} (PTU-D5 and ETU). To ensure that the IS did not degrade to our analyte of interest (thiouracil) during hydrolysis, every sample was additionally analyzed in triplicate without addition of the IS. Even more, each sample endured cochromatography (10 μg kg^{-1} of TU) in triplicate to demonstrate the specificity and acknowledge the identity of thiouracil.

Identification of the thyreostats was based on the retention time relative to the IS and on the ion ratios of the product ions, carried out according to criteria described in Commission Decision 2002/657/EC.[33] After identification, the analyte concentrations were calculated by means of the standard addition approach.

2.9. Standard Addition Approach. For calculating the unknown concentration of the analyte in question, the standard addition approach as described in Commission Decision 2002/657/EC was applied.[33] After hydrolysis, extraction, and cleanup, the obtained dried samples were divided into two aliquots of analogous mass (m) and volume (V).

One aliquot, the unknown, was reconstituted in methanol/aqueous acetic acid (50:50) for injection on the LC-MS^2 system. After analysis of the unknown, the concentration of the identified analyte (thiouracil) was estimated by fitting its area ratio, $\chi_{unknown}$, in a calibration curve in water undergoing the extraction procedure. On the basis of this estimation, the other aliquot was spiked with a similar known concentration of the identified analyte (A). Final reconstitution of this aliquot was identical to that of the first aliquot. LC-MS^2 analysis of this aliquot resulted in an area ratio of χ_{known}. Using the following formula, the unknown concentration ($C_{unknown}$) was calculated:

$$C_{unknown} = \chi_{unknown} V_{unknown} \rho_A V_A / (\chi_{known} V_{known} m_{unknown} - \chi_{unknown} V_{unknown} m_{known})$$

$$\text{with } V_{unknown} = V_{known} \text{ and } m_{unknown} = m_{known}$$

$$C_{unknown} = \chi_{unknown} \rho_A V_A / (\chi_{known} - \chi_{unknown})$$

with C = concentration, X = area ratio, V = volume, ρ = concentration, m = mass, and A = identified analyte.

In addition, the recovery yield of the sample pretreatment (hydrolysis, extraction, and cleanup) was taken in account, so analyte losses during this pretreatment were considered, which resulted in more accurate calculations for the concentration of thiouracil in the samples.

3. RESULTS AND DISCUSSION

3.1. Enzymatic Activity of the Myrosinase Enzyme. Before application of the prepared myrosinase solution, a quality performance check was required. Therefore, a small-scale study was set up to determine and acknowledge the hydrolytic activity of the extracted myrosinase solution. In the literature, two alternatives were described for investigating the enzymatic activity. Most commonly used is a spectrophotometric assay, during which the myrosinase-induced decomposition of the substrate, in this case the glucosinolate sinigrin, is monitored.[30,32,34] The second option, a single-point glucose assay, measures the myrosinase activity by determining the release of glucose from sinigrin.[29,32] This study opted for the quantification of glucose released during sinigrin hydrolysis, because an HPLC analysis method coupled to evaporative light scattering detection (ELSD) for the detection of organic sugars including glucose, with a detection limit of 0.1 g L^{-1}, was available in our laboratory.[35]

The results of the first experimental setup, which compared the enzymatic activity from our myrosinase mixture (different volumes and addition of ascorbic acid) to the purchased purified myrosinase, clearly confirmed the hydrolytic activity of our extracted myrosinase enzyme, as displayed in Figure 1. Adding 0.5 mL of myrosinase solution sufficed to obtain similar results as with the synthetic myrosinase (30 μL). As reported in the literature, the addition of L-ascorbic acid (2 mM) stimulated the activity of myrosinase through catalysis of the hydrolysis of sinigrin, resulting in an increased release of glucose.[30,31] However, because of the small quantity added (0.528 mg), these samples displayed greater standard deviations (Figure 1), which may be the result of variation in weighing (8.9%) and the limited sensitivity of our analytical scale below 0.1 mg.

The kinetics of sinigrin hydrolysis reached its optimum after 1.5 h at 37 ± 1 °C. This hydrolysis rate was maintained until the end of the incubation (4 h) (Figure 2). The myrosinase-induced hydrolysis already occurred at room temperature (t_0), which is in line with previous results.[27−29]

These results were able to display the expected biological activity of the extracted enzyme mixture and the incubation time optimum, both valuable and essential pieces of information, before enzyme-catalyzed hydrolysis was applied.

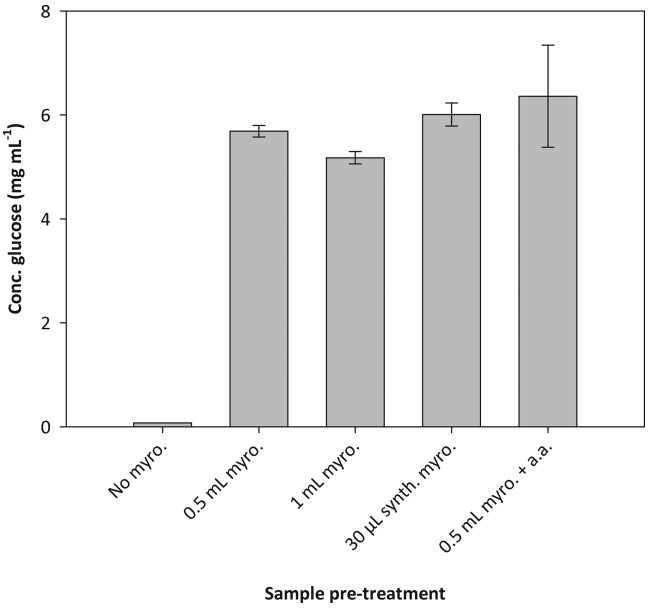

Figure 1. Graphic representation of the detected mean area of glucose by HPLC-ELSD, with error bars representing the standard deviation of the mean detected (n = 4). Glucose was formed as a byproduct of the myrosinase (myro.) catalyzed hydrolysis of sinigrin (34 mM). For no myrosinase treatment, the standard error bar is absent due to too low value (SD = 4.4 E^{-5}). synth., synthetic; a.a., 2 mM ascorbic acid.

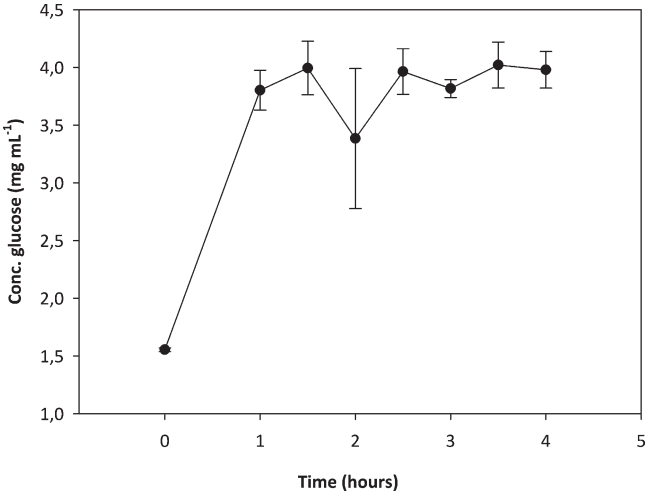

Figure 2. Kinetics of the myrosinase-catalyzed hydrolysis of sinigrin (n = 3), by monitoring the release of glucose by HPLC-ELSD. Error bars correspond to standard deviations on the mean, with n = 3.

3.2. Chemical Analysis of Brassicaceae Vegetables and Derivatives. The scientific literature suggested Brassicaceae-derived feed as origin of the detected low-level thiouracil residues in the urine of various species,[12] which our study used as a starting point. Upon purification, extraction, and analysis of a rapeseed cake according to the method of Pinel et al., no thiouracil could be detected.[8] For this reason during the study, sample pretreatment was successfully altered by incorporating an enzymatic hydrolysis catalyzed by the myrosinase enzyme

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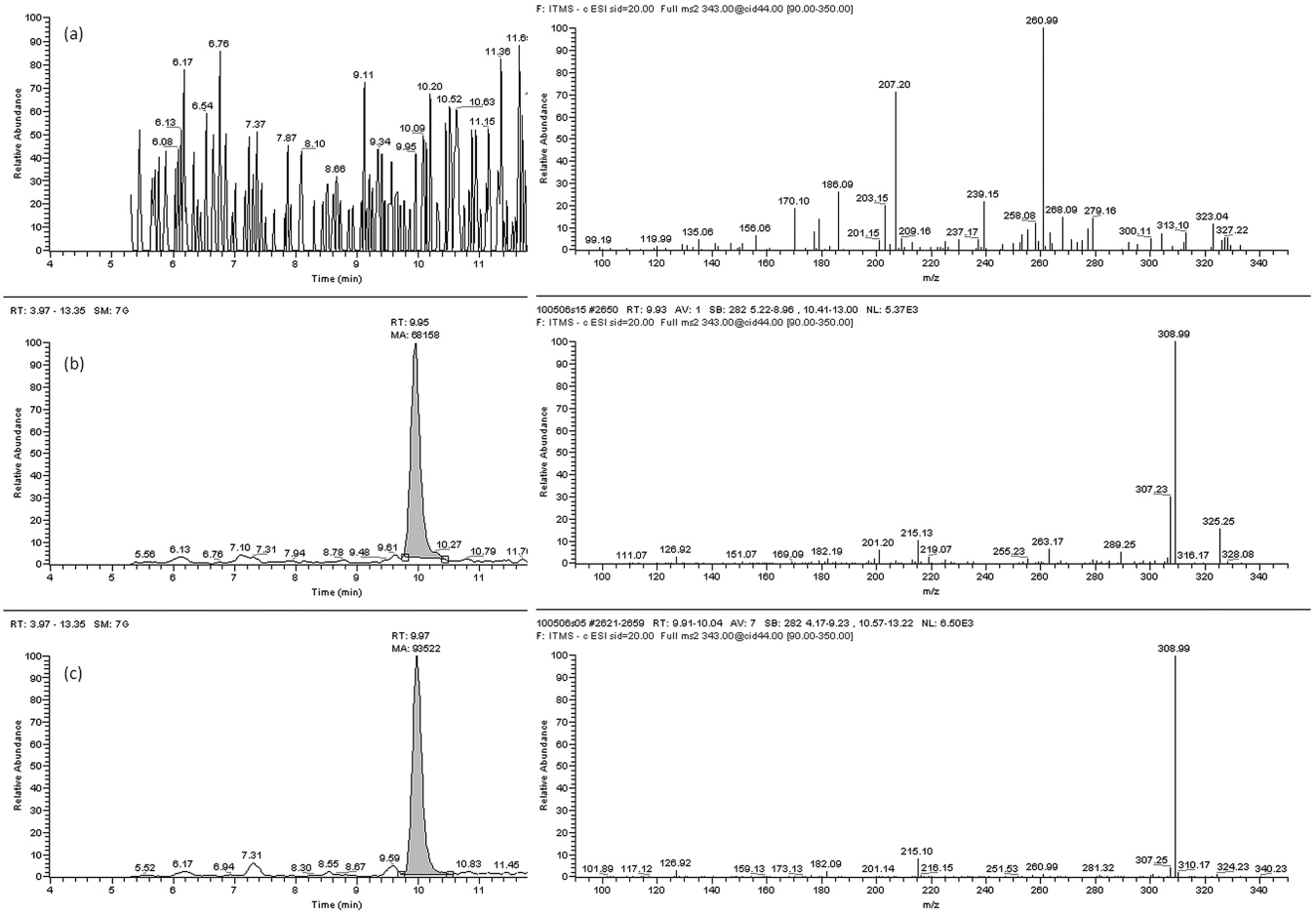

Figure 3. LC-MS^2 chromatogram (and spectra) of identical rapeseed coarse meal samples with (a) no incubation, (b) incubation at 37 ± 1 °C for 4 h, and (c) incubation at 37 ± 1 °C for 4 h with myrosinase addition (0.5 mL).

naturally prevalent in all Brassicaceae members. Reanalysis of the identical rapeseed cake, kindly provided by Pinel et al. (LABERCA, ONIRIS, Nantes, France), with the incorporated enzymatic hydrolysis step led to the detection and identification of thiouracil. Because the enzyme was extracted from *S. alba* L., a member of the Brassicaceae family, the extract was also analyzed for the presence of TU, to exclude possible false-positive results. No TU could indeed be detected in the myrosinase solution, confirming the genus *Brassica* from the Brassicaceae family as its origin. In addition, some experiments indicated that even without the addition of the myrosinase solution, upon incubation at 37 °C, TU could be discovered as well. However, this TU residue was detected to a lesser extent (Figure 3) as compared to rapeseed meal that was exposed to a hydrolysis step with exogenously administered myrosinase prior to extraction and analysis. This background TU level may be explained by the presence of a residual amount of endogenous myrosinase enzyme. For this reason, the involvement of endogenous myrosinase, naturally prevalent in the original feed or food specimens, was further explored. To this purpose, selected Brassicaceae vegetables (cauliflower and broccoli) were cooked to inactivate the myrosinase enzyme,[29,30] and the residual TU concentrations detected were compared to those of raw samples. As for the rapeseed (*Brassica napus* L. partim Napoleon), different combinations of pretreatment were conducted, that is, grinding and inactivation by cooking.[27,28,34]

After the initial optimization of the enzymatic hydrolysis, all pretreated feed and food samples were analyzed by LC-MS^2. Within several samples (traditional rapeseed, rapeseed-'00' coarse meal, rapeseed cake, broccoli, cauliflower) traces of TU were detected, as presented in Table 2. The obtained concentrations were calculated by means of a standard addition approach,[33] with the recovery yield of the entire sample pretreatment taken into account. Of all feed and food samples, broccoli obtained the highest concentration of TU at 6.0 μg kg^{-1} (dry weight). These results show concentration profiles similar to those of the oxazolidine-2-thiones content of rapeseed, broccoli and cauliflower,[36] which provides another indication that TU might follow a pathway of synthesis similar to that of the known naturally occurring thyreostatic drugs. Traditional rapeseed and rapeseed coarse meal also displayed significant concentrations of TU, 1.5 and 1.6 μg kg^{-1}, respectively (Table 2). As for the feeding cabbage and the meal with 30% rapeseed-'00', a very high background noise was generated, to the extent that even spiked samples (10 μg kg^{-1} TU) did not yield a detectable thiouracil signal. Other samples such as cauliflower, rapeseed cake, and most of the pretreated traditional rapeseed demonstrated only low concentrations of TU (<1.0 μg kg^{-1}). It must be mentioned that the levels of detected naturally occurring thiouracil in these samples were in general lower than the retrieved signals in urine. For example, the rapeseed cake, in which 0.4 μg of thiouracil kg^{-1} could be detected, was also administered to a

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heifer by Pinel et al., which in turn resulted in urine samples found positive for thiouracil up to 9 μg L^{-1}.[12]

The data obtained in this study unambiguously proved the necessity of myrosinase-catalyzed hydrolysis for the detection of thiouracil in feed and food matrices of the Brassicaceae family. Therefore, it seems that thiouracil might follow a pathway of synthesis similar to that of the known naturally occurring thyreostats, that is, oxazolidine-2-thiones and thiocyanates, that is, hydrolysis of a precursor molecule present in Brassicaceae plants or vegetables.

The different pretreatments (grinding and inactivation) of the different feed and food specimens rapeseed, broccoli, and cauliflower led to the detection of small differences in TU concentrations, but a real trend was difficult to establish. Noticeable, however, was that inactivated samples tended to generate higher TU signals. In light of the hypothesis that TU follows a pathway of synthesis similar to that of the known naturally occurring thyreostats, the following explanation may be formulated for this observation. For the naturally occurring thyreostatic drugs the pH level is generally accepted to influence the type of byproduct formed.[13] In the case of inactivated samples, only exogenous myrosinase is present, under the proper circumstances (pH 8), to generate the highest yield of thiouracil formation. The normal noninactivated samples, however, contain residual endogenous myrosinase, which might prior to the sample pretreatment already catalyze the hydrolysis of some of the precursor molecules. However, due to improper and uncontrolled circumstances at that time, this may lead to the formation of other byproducts, resulting in a lower yield of thiouracil.

The aim of the present study was to investigate the possible natural origin of low-level thiouracil residues detected in the urine of various animals (livestock and domesticated) and humans. To the best of our knowledge this study is the first to report the presence of naturally occurring thiouracil in feed and food samples, hereby elucidating and acknowledging a natural origin for the low-level thiouracil residues detected in the urine of various species.[24] These combined results give an indication that Brassicaceae members are most likely not the sole source of contamination and that other, yet unknown, factors might contribute to the presence of naturally occurring thiouracil in urine samples.

For future experiments, the addition of ascorbic acid might prove useful, because it is known to positively influence the hydrolysis rate of the myrosinase enzyme.[30,31] Even more, future research should be performed to investigate the pathway of thiouracil formation and identify its precursors, possibly belonging to the glucosinolates.

AUTHOR INFORMATION

Corresponding Author

*Phone: +32 9 264 73 40. Fax: +32 9 264 74 92. E-mail: Julie.Vandenbussche@ugent.be.

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