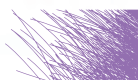


Analytical Approaches To Unravel The Semi-Endogenous Status Of Thiouracil

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Analytical approaches to unravel the semi-endogenous status of thiouracil

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ANF	anti-nutritional factor
APCI	atmospheric pressure chemical ionisation
API	atmospheric pressure ionisation
APPI	atmospheric pressure photoionisation
a.u.	arbitrary units
B.	Bifidobacterium
BENELUX	Belgium, the Netherlands, and Luxembourg
3-BrBBr	3- bromobenzylbromide
BTU	6-benzyl-2-thiouracil
Ca ²⁺	calcium
cAMP	cyclic adenosine monophosphate
Canola	Canadian oil, low acid
CC _α	decision limit
CC _β	detection capability
CE	collision energy
CID	collision-induced dissociation
ClO ₄ ⁻	perchlorate
CN ⁻	cyanide
Cu ²⁺	copper
CV	coefficient of variation (also known as relative standard deviation)
Da	Dalton or atomic mass unit (u)
DAD	diode array detection
DTT	DL-dithiothreitol
DIT	di-iodinated tyrosine
DMTU	6-dimethyl-thiouracil
EC	Enzyme Commission number
ECD	electron capture detection
EDTA	ethylenediaminetetraacetate
ELISA	enzyme-linked immunosorbent assay
ELSD	evaporative light scattering detection
(H)ESI	(heated) electrospray ionisation
ETU	6-ethyl-thiouracil
EU	European Union
EURL	European Union Reference Laboratories

FID	flame ionisation detection
FPD	flame photometric detection
FFT	fast fourier transform
FWHM	full width at half maximum
GC	gas chromatography
GC-C-IRMS	gas chromatography-combustion-isotope ratio mass spectrometry
GC-MS	gas chromatography mass spectrometry
Gls	glucosinolates
ha	hectare
HCD	high energy collisional dissociation
HCl	hydrochloric acid
HEPT	height equivalent of theoretical plate
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
HRMS	high-resolution mass spectrometry
HSS	high strength silica particles
IARC	International Agency for Research on Cancer
3-IBBr	3-iodobenzylbromide
IC	ion chromatography
IC-MS/MS	ion chromatography coupled to tandem mass spectrometry
IP	identification point
IS	internal standard
LC-MS	liquid chromatography mass spectrometry
LC-MS/MS	liquid chromatography tandem mass spectrometry
Li ⁺	lithium
LOD	limit of detection
LTQ	linear trap quadrupole
MBI	2-mercaptobenzimidazole
MEKC	micellar electrokinetic chromatography
MeOH	methanol
[M+H] ⁺	protonated molecular ion
Mn ²⁺	manganese
MSTFA	<i>N</i> -methyl- <i>N</i> -trimethylsilyl-trifluoroacetamide
min	minute

MIT	mono-iodinated tyrosine
MRL	maximum residue limit
MRPL	minimum required performance limit
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MS ⁿ	multiple stage mass spectrometry
MSPD	matrix solid-phase dispersion
MTU	6-methyl-2-thiouracil
<i>m/z</i>	mass-to-charge ratio
NaCl	sodium chloride
NBD-Cl	7-chloro-4-nitrobenzo-2-oxa-1,3-diazol
ND	not detected
OZT	oxazolidine-2-thione
PFBBBr	pentafluorobenzylbromide
PTU	6-propyl-2-thiouracil
PTU-D5	6-propyl-d5-2-thiouracil
PhTU	6-phenyl-thiouracil
Q	quadrupole
QqQ	triple quadrupole mass analyser
R	variable side chain
R ²	correlation coefficient
RC	recommended concentration
REM	rapeseed extraction meal
RIA	radio immuno assay
RPA	reference points for action
RSD	relative standard deviation (also known as coefficient of variation)
SCN ⁻	thiocyanate
SD	standard deviation
SDS	sodium dodecyl sulphate
SIM	single ion monitoring
S/N	signal-to-noise ratio
SPE	solid phase extraction
SRM	selected reaction monitoring
5-VOT	5-vinyl-1,3-oxazolidine-2-thione or goitrin

NOTATION INDEX

T3	triiodothyronine
T4	thyroxine
TAP	1-methyl-2-mercaptoimidazole or tapazole
Tg	thyroglobulin protein
TSP	thermionic specific detection
TU	4(6)-R-2-thiouracil
U-HPLC	ultra-high performance liquid chromatography
UPLC	ultra performance liquid chromatography
TLC	thin layer chromatography
TOF	Time-of-Flight
TPO	thyroid peroxidase enzyme
TRH	thyrotropin-releasing hormone
TS	thyreostatic drugs
TSH	thyroid-stimulating hormone
UK	United Kingdom
USA	United States of America
UV	ultraviolet

CHAPTER I

GENERAL INTRODUCTION TO THYREOSTATIC DRUGS

Adapted from:

Vanden Bussche J, Noppe H, Verheyden K, Wille K, Pinel G, Le Bizec B, De Brabander HF. 2009. Analysis of thyreostats: A history of 35 years. *Analytica Chimica Acta* 637: 2-12.

CHAPTER I

GENERAL INTRODUCTION TO THYREOSTATIC DRUGS

1. Thyreostatic drugs

Nowadays the term 'thyreostats' or 'thyreostatic drugs' (TS) is used to refer to a complex group of substances that interfere with the thyroid function, resulting in a decreased production of the thyroid hormones triiodothyronine and thyroxine [1, 2]. In the past also 'anti-hormones' was used to refer to thyreostats, although this nomenclature is not correct, since anti-hormones counteract the action of a hormone, not its production as is the case for thyreostats [3].

1.1 Xenobiotic thyreostats

Thyreostatic drugs comprise three structurally different types of compounds. The first type are the sulphated (position 2) heterocyclic aromatic compounds containing two nitrogen atoms at positions 1 and 3 (pyrimidine), referred to as thiouracil (TU) and analogues. For the latter, substitution (R) takes place on position 6 with either an ethyl, dimethyl, propyl or phenyl group (Figure I.1). As for the second and third structural type, they only comprise of 2-mercaptobenzimidazole (MBI) and tapazole (TAP), respectively. MBI is a heterocyclic aromatic compound, consisting of a benzene and a sulphated imidazole molecule. The structure of tapazole or 1-methyl-2-mercaptobenzimidazole consists of a methylated and sulphated imidazole molecule (Figure. I.1).

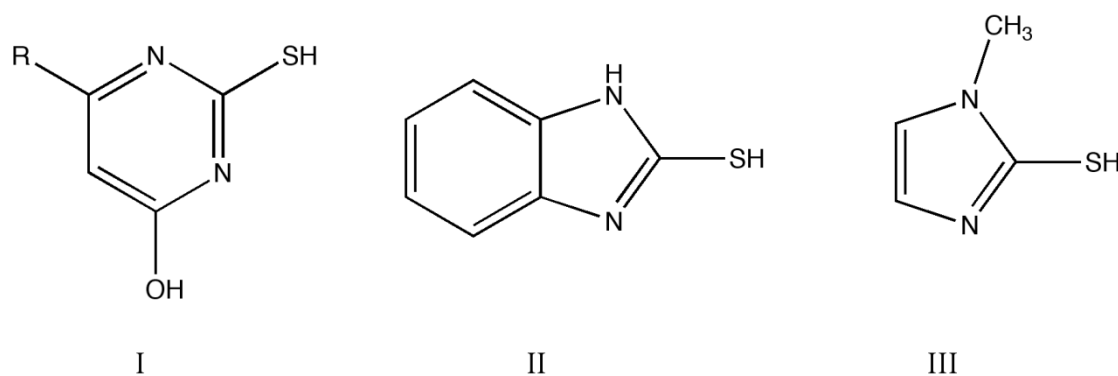


Figure I.1: Structural formulae of thyreostatic drugs. I: 4(6)-R-2-thiouracil (TU); II: 2-mercaptobenzimidazole (MBI); III: 1-methyl-2-mercaptoimidazole (TAP).

All of the xenobiotic thyreostats are polar and amphoteric molecules, characterized with a low molecular weight (114-204 Da) [2]. Within their formulae a common element is displayed, namely the nitrogen-carbon-sulphur sequence (thioamide), presumed responsible for the thyroid-inhibiting activity [4, 5].

Besides the therapeutic use of some of these compounds in human and feline medicine, the xenobiotic thyreostats have also been illegally exploited for fattening purposes in animal husbandry [1, 2]. The use of the most powerful thyreostatic agents results in a weight gain caused by the increased filling of the gastro-intestinal tract as well as the retention of water in edible tissues, by inhibiting the thyroid hormone production [6, 7]. This leads to the production of meat of lower quality and is considered as an abuse, because water is sold for the price of meat [8, 9]. Besides that, it has been proposed that residues of thyreostatic drugs may be teratogenic and carcinogenic [10-13]. For example in Spain, the consumption of meat contaminated with thyreostats has been related with an increased incidence of aplasia cutis, a characteristic scalp defect [10]. For these reasons, the use of thyreostatic drugs for animal fattening purposes has been prohibited in Belgium since 1974 [14]. In the European Union (EU), there is a general agreement on the ban of these substances since 1981 [15]. Within the framework of residue control of xenobiotic thyreostats, 2-thiouracil, 6-methyl-2-thiouracil, 6-propyl-2-thiouracil, 1-methyl-2-mercaptobenzimidazole and 2-mercaptobenzimidazole are of most interest [2].

1.2 Naturally occurring thyreostats

A second main group within the class of thyreostatic compounds are the natural occurring thyreostats, which comprise the oxazolidine-2-thiones (OZTs) and thiocyanates [1]. The latter has a dual origin, since this can also be chemically synthesised. In line with the xenobiotic thyreostats, it is the thionamide group of these naturally occurring thyreostats that is responsible for its thyroid inhibiting activities.

The natural thyreostats originate from precursors (glucosinolates), present in plants of Brassicaceae (syn. Cruciferae) and related families [16-18]. These glucosinolates (GIs) are secondary plant metabolites consisting of a β -thioglucose moiety, a sulphonated oxime moiety, and a variable side chain derived from an amino acid [19-22]. More than 120 different glucosinolates have already been identified and widely described in literature [23-25]. These glucosinolates co-exist in the plant, however are physically separated from the endogenous β -thioglucosidase or myrosinase enzyme (EC

3.2.1.147). Cellular disruption of the plant tissue during processing or ingestion facilitates the contact between glucosinolates and the myrosinase enzyme, which results in the hydrolysis of these secondary plant metabolites [16, 22-23]. However, hydrolysis induced by ingestion has also been reported. In this case, the bacterial microbiota of the gastro-intestinal tract have been shown responsible for the hydrolysis [22, 26-27]. With humans different digestive strains were found capable of metabolising glucosinolates, e.g. *Bacteroides thetaiotaomicron*, *Bacteroides vulgates*, *Bifidobacterium (B.) adolescentis*, *B. longum*, *B. pseudocatenulatum*, *Escherichia coli* and *Lactobacillus agilis* strain R16 [28-31].

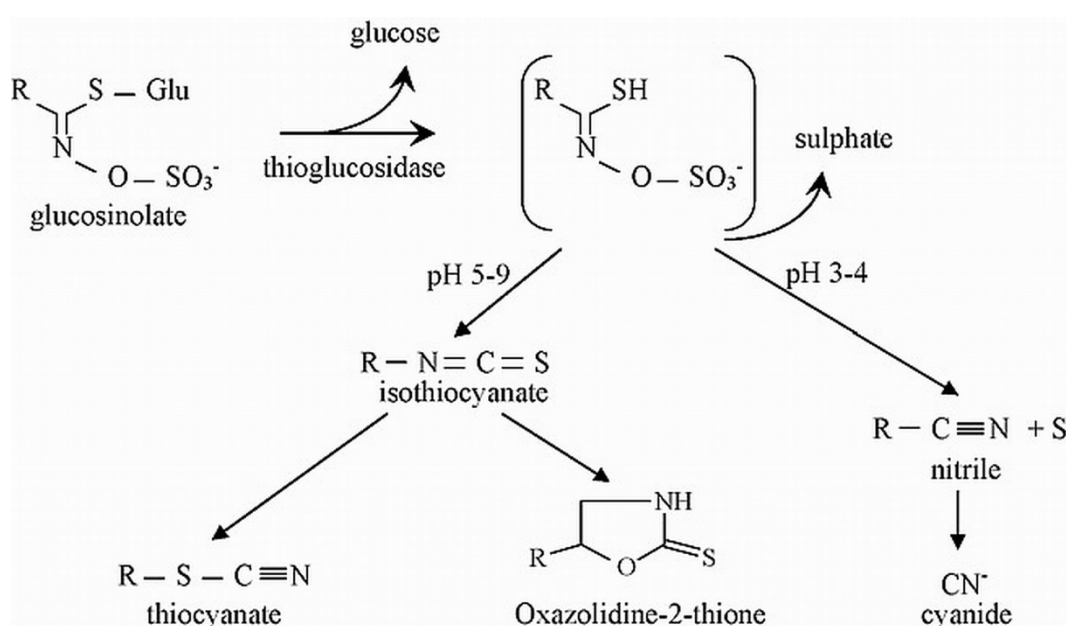


Figure I.2: Schematic representation of glucosinolate hydrolysis and factors that may influence the outcome of the different metabolites (R = variable side chain).

After hydrolysis, a glucose molecule and an unstable aglucone moiety are formed. The latter rapidly undergoes spontaneous rearrangement, eliminating sulphur, to yield a variety of several biological active compounds, e.g. isothiocyanates, thiocyanates, oxazolidine-2-thiones, and nitriles (Figure I.2). The specific metabolite formation depends entirely upon the pH and the nature of the glucosinolates side chain (R) [16, 19, 32]. At acidic pH (3-4) sulphur is split off and a nitrile is produced, which can split further to form cyanide (CN^-). At pH 5 to 9 isothiocyanates are formed, from which the naturally occurring TS, thiocyanates (SCN^-) and oxazolidine-2-thiones can be generated. This formation depends

upon the structure of the variable side chain (R). In case of the OZTs, an appropriate located β -hydroxyl substituent is required for the spontaneous cyclisation to take place.

Of the OZTs, 5-vinyl-1,3-oxazolidine-2-thione (5-VOT, goitrin) is the most commonly screened and detected analyte within the framework of residue analysis for the naturally occurring thyreostats [18, 22, 33-38]. Its goitrogenic activity is well-known, i.e. 133 % if compared to the activity of propylthiouracil [1, 36]. 5-VOT is formed from the glucosinolate progoitrin or 2-hydroxybut-3-enylglucosinolate, as a result of a myrosinase catalysed hydrolysis reaction [22, 38].

1.3 Other thyreostatic agents

Beside the xenobiotic and natural occurring thyreostats, a large number of other molecules like inorganic ions such as lithium (Li^+) [39-44], perchlorate (ClO_4^-) [45-48] and thiocyanate (SCN^-) [45, 49-50], but also veterinary drugs including sulphonamides [4, 51-54] may exert a thyreostatic action [55-56]. Moreover iodide, which is an essential element for the synthesis of the thyroid hormones, at high concentrations can also modulate on the function of the thyroid gland by producing a mild and transient inhibition of organic binding of iodide and hence of the hormone synthesis. This inhibition is known as the Wolff–Chaikoff effect [50, 57].

1.4 Legislation

When thyreostatic drugs are administrated for livestock breeding, residues may occur in edible matrices derived from these treated animals. Due to the potential human health risks, the EU issued certain regulations concerning the use of substances with thyreostatic action. Subsequently, guidelines and criteria were set for the detection of thyreostatic drugs abuse. In this paragraph, the most relevant legislation concerning thyreostatic drug residues in matrices from animal origin is presented.

In Belgium, substances with thyreostatic action have been prohibited since 1974 [14]. The EU adopted this legislation in 1981, as Council Directive 81/602/EC [15]. Later on, Council Directive 85/358/EC [58] amended Council Directive 81/602/EC [15], to guarantee a uniform application for detection and monitoring of thyreostatic drugs in all member states. Council Directive 96/22/EC [59], the revision of Council Directive 81/602/EC [15] described the prohibition on the use of certain substances with

hormonal or thyreostatic action in stock farming. Additionally, it promulgates that member states have to prohibit the import of meat from treated animals from third countries.

The measures to monitor the residue control of certain substances (listed in Annex I), e.g. thyreostats in live animals and animal products are described by Council Directive 96/23/EC [60]. Two groups of substances are included in Directive 96/23/EC [60] listed in Annex I, based on Commission Regulation No. 2377/90 [61]. Group A comprises substances having hormonal or thyreostatic action, β -agonists (Directive 96/22/EC) [59] and veterinary drugs that have been banned (included in Annex IV of Council Regulation (EC) No 2377/90) [61]. Group B comprises other veterinary drugs and contaminants.

For good implementation of directive 96/23/EC [60], it is necessary to determine common criteria for the interpretation of test results in official control laboratories. Also important, in particular for substances not authorized or prohibited by the EU, is the progressive establishment of a minimum required performance limit (MRPL) for analytical methods. For thyreostats, a suggested MRPL has been fixed at $100 \mu\text{g L}^{-1}$ or $\mu\text{g kg}^{-1}$. Additionally, in December 2007 a recommended concentration (RC) for TU, MTU, PTU and TAP of $10 \mu\text{g L}^{-1}$ or $\mu\text{g kg}^{-1}$ in urine or thyroid has been proposed by the European Union Reference Laboratories (EURLs) in the EURL guidance paper [62]. However, this document has no legal force and serves only as a technical guidance for the laboratories performing analytical thyreostatic drugs detection for the residue control plan. This RC is considered as a more up-to-date value for setting the sensitivity level of the analytical detection method for thyreostatic drugs. Nevertheless, an EU supported legislation concerning the sensitivity level for TS detection is required. Therefore Council Regulation (EC) No 470/2009, which repeals Council Regulation (EC) No 2377/90 introduces the concept of reference points for action (RPA) [61, 63]. This is the level of a residue for which no maximum residue limit has been set, and which will be detected and confirmed by official control laboratories in accordance with Community requirements. So far no RPA has been established for TS, most likely the RPA will take the recommended concentration into account.

To ensure a harmonized implementation of Directive 96/23/EC [60], Commission Decision 2002/657/EC [64] lays down the technical guidelines and performance criteria for residue control. Within this Commission decision (2002/657/EC) [64], a system of identification points (IPs) is introduced in order to interpret the obtained data (chromatograms, spectra) when detection methods are used other than full-scan mass spectrometer (MS) techniques. This system is based on the number and the ratio of the ions in the obtained MS spectrum. For the confirmation of the banned substances, listed as group A

(e.g. thyreostats), a minimum of four IPs is required. Since the implementation of the 2002/657/EC criteria [64], few studies describe the applicability of these guidelines for determination of thyreostats in urine and thyroid [65-67]. Parameters that need to be evaluated during the validation procedure are selectivity, specificity, linearity, trueness, recovery, applicability, ruggedness, stability, repeatability, reproducibility, decision limit (CC_{α}) and detection capability (CC_{β}).

2. Thyroid gland

2.1 Anatomy and histology of the thyroid gland

The butterfly-shaped thyroid belongs to the endocrine glands and is with all vertebrates located in the anterior neck just inferior to the larynx (Figure I.3). This gland maintains the level of metabolism in the tissues that is optimal for their normal function, and also regulates the calcium (Ca^{2+}) balance. The thyroid tissue is made up of hollow spherical follicles separated by connective tissue rich in capillaries. Each follicle is surrounded by a layer of epithelial cells, which comprises two types: the follicular and parafollicular cells, with a central lumen called colloid that contains the thyroglobulin protein (Tg).

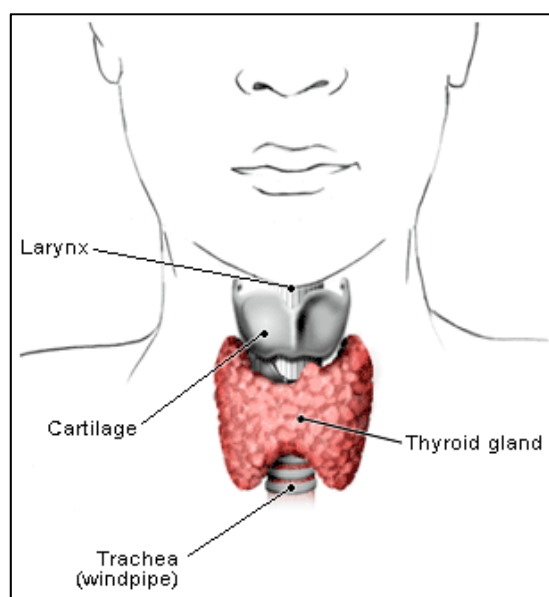


Figure I.3: Anterior view of the thyroid gland.

Most of the thyroid tissue consists of follicular cells, which secrete the iodine-containing thyroid hormones triiodothyronine (T3) and thyroxine (T4). The parafollicular cells secrete the hormone calcitonin, which depresses excessive levels of Ca^{2+} in the blood. The produced thyroid hormones

stimulate the O_2 consumption of most of the cells in the body, help to regulate lipid and carbohydrate metabolism, and are necessary for normal growth and maturation [68-70].

2.2 Production thyroid hormones

In order to produce these hormones, the thyroid gland needs iodine and tyrosine (an amino acid). First off iodine, a vital trace mineral found in food, is ingested, converted to iodide, and actively transported from the blood circulation to the colloid of the gland. This transport mechanism is called the sodium-iodide transporter, a membrane protein, also referred to as the iodide trapping mechanism or pump [71]. In the colloid of the follicular cell, the iodide is oxidized by the thyroid peroxidase enzyme (TPO) and bound in a matter of seconds to tyrosine molecules attached to the thyroglobulin protein (Figure 1.4) [72]. This reaction results in either mono-iodinated tyrosine (MIT) or di-iodinated tyrosine (DIT), which are the precursors of the hormonally active iodothyronines T_4 and T_3 . If two DIT molecules couple together, the result is the formation of T_4 , in case of DIT and MIT coupling, T_3 is formed. Both of these coupling reactions are catalyzed by the TPO.

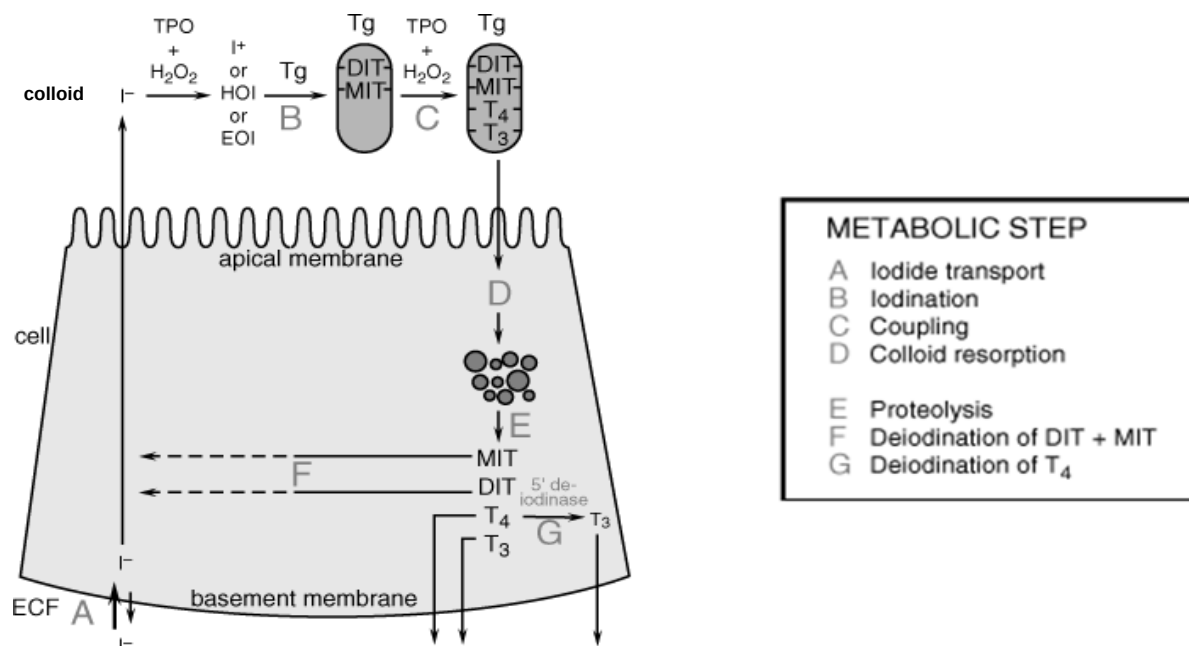


Figure 1.4: Schematic illustration of a follicular cell showing the key aspects of thyroid iodine transport and thyroid hormone synthesis. (MIT, mono-iodinated tyrosine; DIT, di-iodinated tyrosine; T_3 , triiodothyronine; T_4 , thyroxine; Tg, thyroglobulin; TPO, thyroid peroxidase; HOI, hypiodous acid; EOI, enzyme-linked species; ECF, extracellular fluid)

Finally, the thyroid hormone release is mediated by endocytosis of the colloid: or macropinocytosis by pseudopods or micropinocytosis by small coated vesicles, both processes stimulated by thyroid-stimulating hormone. The relative importance of the two pathways varies among species, with micropinocytosis thought to be predominating in humans. Following endocytosis, endocytotic vesicles fuse with lysosomes, and proteolysis occurs. The iodotyrosines released from Tg are rapidly deiodinated, where the released iodine is recycled. Thyroid hormones are released from Tg in the lysosome, and diffuse out of the follicle cell into the capillaries surrounding the cell, thereby entering the circulation [68-70].

2.3 Regulation hormone production

To initiate secretion of the stored thyroid hormones into the blood circulation, the anterior pituitary gland releases the thyroid-stimulating hormone (TSH), which signals the follicle cells to reclaim Tg by endocytosis. This TSH is in turn down regulated by a direct inhibitory feedback of high circulating thyroid hormone levels. The release of TSH, and thus of thyroid hormones can also be initiated by thyrotropin-releasing hormone (TRH) produced by the hypothalamus. Therefore, changes in the level of circulating thyroid hormones or environmental changes like temperature will directly influence the thyroid hormone synthesis and secretion by TSH and TRH, respectively [68-70].

2.4 Thyroid disorders

Disfunction of the thyroid comprises several different diseases all involving problems with the thyroid gland and the synthesis of hormones. The disorders are commonly separated into two major categories, hypothyroidism (or myxedema) and hyperthyroidism (or thyrotoxicosis), depending on whether serum thyroid hormone levels (T3 and T4) are decreased or increased, respectively.

Hypothyroidism displays a decreased synthesis of thyroid hormones and low levels of circulating thyroid hormones. Patients present complaints like fatigue, weakness, lethargy, abnormal weight gain, tiredness, baldness, temperature intolerance, constipation, menstrual disturbances, and an enlarged thyroid (goiter) [68, 70, 73-74]. For hyperthyroidism other factors beside over-activity of the thyroid gland like inflammatory thyroid diseases can cause this disorder. Complaints comprise a goiter, rapid heartbeat, palpitations, excess sweating, diarrhea, weight loss, muscle weakness, and unusual sensitivity to heat [5, 68, 70, 73, 75].

Effective treatment of most thyroid disorders is readily available. Treatment of the hypothyroid patient is straightforward and consists of hormone replacement therapy. For a hyperthyroid patient more options for treatment are available, one of these utilises anti-thyroid or thyreostatic drugs to decrease hormone synthesis and secretion [73, 76]. In the USA, propylthiouracil and methimazole are the two thyreostatic drugs used, whereas in the UK and the rest of Europe mainly methimazole or carbimazole, which is metabolised to methimazole [5, 77-79] are prescribed.

2.5 Inhibition pathway of thyreostatic drugs on the thyroid gland

Thyreostatic drugs are a complex group of different types of compounds that inhibit the function of the thyroid gland, resulting in a lower level of circulating thyroid hormones. The mechanism of action of these compounds on the thyroid gland differs.

Xenobiotic thyreostats and OZTs inhibit the formation of thyroid hormones by interfering with the incorporation of iodine on tyrosine molecules anchored to Tg. They also inhibit the coupling of the iodotyrosines (MIT and DIT) to form iodothyronines (T3 and T4). This implies that they interfere with the oxidation of the iodide ion and iodotyrosine groups. Taurog (2000) proposed that the drugs bind to and inactivate the peroxidase enzyme, thereby preventing oxidation of iodide or iodotyrosine groups to the required active state [80]. Over a period of time, the inhibition of hormone synthesis results in the depletion of stores of iodinated Tg as the protein is hydrolyzed and the hormones are released into the circulation. Only when the preformed hormone is depleted and the concentrations of circulating thyroid hormones begin to decline, clinical effects become noticeable. There is some evidence that the coupling reaction is more sensitive to the action of propylthiouracil, than the iodination reaction [80]. Inorganic anions, with thiocyanate and perchlorate as most important, interfere with the active transportation of the iodide from circulation to the follicle cells of the thyroid gland. These anions are as iodide monovalent and therefore competitive inhibitors for the iodide uptake at the sodium-iodide transporter [81].

Lithium, the most important cationic thyreostatic agent, increases renal secretion of iodide and inhibits the coupling reaction of iodotyrosines to form iodothyronines. In addition, it inactivates the adenylycyclase enzyme that catalyzes the formation of cyclic adenosine monophosphate (cAMP), necessary for the stimulation of the thyroid gland by TSH [81].

Veterinary drugs, like sulphonamides and dexamethasone, inhibit the thyroid peroxidase activity and thus interfere with iodination and coupling of tyrosine residues or inhibits the glandular secretion of hormone, respectively [54, 82-83].

3. Brassicaceae

The genus *Brassica* of the Brassicaceae plant family is of most importance for horti- and agriculture. This genus comprises all sorts of cabbages and rapeseed. For human consumption a large variety, e.g. cauliflower, broccoli, white cabbage, green cabbage, savoy cabbage, Chinese cabbage, watercress, mustard, and rapeseed oil is available and included in the normal diet. For animal feed, rapeseed and its extraction products (rapeseed flakes, cake, and coarse meal) generated as by-products from the oil industry and the biodiesel fuel production are of high interest. In this section the focus will be set on rapeseed and its by-products. Before its use in human and animal consumption, rapeseed however had to undergo manipulation due to the anti-nutritional factors (ANFs) of rapeseed.

3.1 Anti-nutritional factors of rapeseed

Rapeseed can be classified according to the fatty acid content of the extracted oil, or according to the glucosinolate content of the rapeseed extraction meal (REM). Rapeseed contains 40-42 % oil, a mixture of glycerol and fatty acids of which 5 % saturated and 95 % unsaturated, with 35 % of the fatty acids consisting of erucic acid [24, 84-86]. After the oil extraction, a rapeseed degradation product is delivered that contains 35 % proteins, a possible valuable and cheap nitrogen source for animal feed [84-86].

However, the presence of erucic acid and GIs are unwanted, as they are considered as anti-nutritional factors [22, 24]. Human consumption of rapeseed oil with a normal erucic acid level (35 %) is linked to possible heart and digestion problems [87]. As for the glucosinolates, a substantial intake has proven to be deleterious to animal health [18, 88-90], not because of the glucosinolates themselves but due to the formation of the toxic breakdown products, e.g. OZTs, nitriles, and thiocyanates [56]. In light of nutritional concerns, the industry turned their attention in 1970 to developing seeds with little erucic acid present (< 2 %), the 'single low' or '0' varieties of rapeseed [91-93]. A major market constraint remained however, namely the utilisation of the high-protein meal generated from the oil extraction

process. The presence of the glucosinolates in REM limits its utilisation. As a result, genetic manipulation strategies were applied capable of lowering the average glucosinolates content of 166 $\mu\text{mol g}^{-1}$ to 38 $\mu\text{mol g}^{-1}$, which are referred to as the 'double-low' or '00' or 'Canola' (Canadian oil, low acid) varieties of rapeseed [22, 94]. Modern '00'-rapeseed varieties show a remarkable low glucosinolates content of 8-15 $\mu\text{mol g}^{-1}$ seed that meets the criteria of 25 $\mu\text{mol g}^{-1}$ as set by Commission regulation No 1035/2003 [95]. This Regulation also demands an erucic acid level below 2 %. Nowadays, for all application the 'double-low' or '00' varieties are considered as standard, for biofuel however 'traditional' rapeseed may be used with unaltered levels of erucic acid and glucosinolates [84, 86].

3.2 Human consumption

Rapeseed oil gained new interest because of the availability of the '00' rapeseed varieties with improved fatty acid composition. This oil, besides the traditional olive oil becomes more and more employed in the kitchen. Currently, rapeseed production in the EU member communities occupies 6.5 million ha, making it the predominant oil crop in Europe [86]. In addition, it displays a good ratio, e.g. 4, between the polyunsaturated fatty acids omega-6 and omega-3 [96]. These are essential cholesterol-lowering fatty acids, which also decrease cardiovascular risk.

3.3 Animal feed

Oil extraction from rapeseed produces a protein-rich material, e.g. REM of potential high value for animal feed. For animal and human consumption, only rapeseed of the '00' variety may be used, characterized by a low erucic acid level (< 2 %) and a maximum level of 25 μmol glucosinolates per g of seeds [95]. The REM can be produced in three different products, rapeseed flakes, cake, or coarse meal. The type of extraction product formed depends on the oil extraction process. If cold compression (50-60 °C) occurs, rapeseed cake with 11-20 % of fat is formed. Hot compression (80-140 °C) results in rapeseed flakes with a fat percentage of 6-10 %. For the coarse colza meal, hot compression is followed by etherification and toasting with a fat percentage of 1-4 % [84].

Table I.1: Biological effects of glucosinolate intake on different animal species.

Animal species	Total glucosinolates (mmol kg ⁻¹ diet)	Effect on animal
<u>Monogastrics</u>		
Fish	2.2	Tolerated level
	19.3	Severe growth depression & thyroid disturbances
Pigs	0.8	Tolerated level
	1 - 3	Reduced feed intake & growth
	7	Severe growth depression
	9 - 10	Liver and thyroid hypertrophy
Poultry	5.5	Tolerated level
	8 - 15	Severe growth depression
Rabbits	7.9	Tolerated level
	17 - 25	Severe growth depression & increased mortality
<u>Ruminants</u>		
Cow	< 11	Tolerated level
	11	Iodine deficiency
	12 - 24	Depressed feed intake & milk production
Sheep	1 - 2	Weight loss during lactation
	< 4	Tolerated level
	> 4	Iodine deficiency & thyroid hypertrophy

The total dietary intake of rapeseed extraction products remains restricted, even with the lowered glucosinolates content toxic degradation products such as OZTs, nitriles, and thiocyanates are still formed. The level of tolerance appears to be species dependant (Table I.1) [22]. Ruminants are less affected than monogastric animals because the microbiota of their digestive system induces transformation of glucosinolates and of their metabolites [97].

4. Methodology

Thyreostatic drugs are considered as a possible human health risk and concerns have been raised about the presence of their residues in edible matrices of animal origin. To uphold the established 'zero-tolerance' level of thyreostatic drugs, analytical approaches for detecting residues of these compounds in edible or biological matrices are a necessity. Residue analysis like all other fields underwent a continuous and tremendous evolution, going hand in hand with the progress in instrumental development. In this section a brief overview is provided of the evolution in analysis of thyreostatic drugs in various matrices of animal origin. This overview displays the broad variety of extraction and clean-up techniques necessary for eliminating matrix interferences, but also the different analytical detection approaches. Additionally, the latest technological improvements in analytical approaches based on liquid chromatography coupled to mass spectrometry will be discussed more in detail.

4.1 Analysis of thyreostatic drugs over time

In former years, the symptoms of the thyroid disorder hypothyroidism, caused by the administration of thyreostatic drugs, were used as an indication. Later on, the determination of thyreostatic drugs in matrices of animal origin was dominated by chromatographic separation methods (GC or LC) coupled to sensitive and specific detection techniques such as MS. More recently, LC coupled to MS, and more specific coupled to multiple or tandem MS, gained in popularity. The conventional residue control plan of thyreostatic drugs nowadays focuses on the analysis of (suspected) samples for the xenobiotic thyreostats.

4.1.1 Monitoring the physiological parameters of hypothyroidism

Hypothyroidism is a disorder of the thyroid gland [74, 98] that can originate from the administration of TS. Subsequently, the thyroid hormone production (T3 and T4) is inhibited and decreased levels of T3 and T4 in the blood may be detected. As such, the alteration in the levels of the circulating T3 and T4 hormones, which can be easily measured by immunological techniques like radio immuno assay (RIA) or enzyme-linked immunosorbent assay (ELISA), can form an indication of TS abuse [2, 99].

Also a morphological investigation can indicate the improper use of TS. The treated animal displays a mucous inflated skin, caused by the deposition of mucopolysaccharides and is in a sedated state (weak

muscles and retarded tendon reflexes). Also, in many cases the histological image of the thyroid gland will be altered, which can be determined through a microscopic investigation [100-101].

Beside these, the enlargement of the thyroid gland, also called a goiter, is the only semi-quantitative parameter that can be applied for the detection of TS abuse and is therefore easy to determine at slaughterhouse level [2, 8].

4.1.2 Chemical analysis

It must be pointed out that prior to the analysis a sample pre-treatment is an absolute requirement for eliminating interfering substances. To extract thyreostats from matrices (e.g. animal tissue, excreta, plasma, and organs) most often methanol [65-66, 102-103] has been used as a solvent, but also acetonitrile [104-105] and ethyl acetate [106-108] have been reported. In the beginning, a mercurated affinity column was used for the clean-up of extracts [1, 3, 102, 109], while nowadays solid phase extraction (SPE) has gained more interest. In most cases, Silica SPE cartridges [65, 106-107, 110] has been mentioned, but anion exchange [104], amino-propyl and alumina cartridges [108] have also been reported. Some even resort to the technology of matrix solid-phase dispersion (MSPD), because homogenisation, disruption, extraction, and clean-up are combined in one procedure [111-112].

In the beginning of the 1970s Van Waes introduced the first chemical detection approach based on a colorimetric reaction [113]. This so called “Van Waes” method consisted of a very complex clean-up with aluminium oxide, followed by a colorimetric reaction of methyl-thiouracil with 2,6-dichloroquinonimide. The result of this reaction was a distinctive yellow colour [113]. The limit of detection (LOD) was in the order of 1-10 mg kg⁻¹. As such, only very high concentrations of thyreostatic drugs could be detected with this method. For this, the method was only found suited for the thyroid matrix and not for other edible matrices of animal origin, like meat.

Later on a more specific analysis method, namely thin layer chromatography (TLC) was described by Gissel and Schaal (1974) [11]. Thiouracil and analogues, such as MTU and PTU were detected at 0.5-1.0 mg kg⁻¹ in animal tissue and urine. De Brabander and Verbeke (1975) [109] developed a TLC method for TU, MTU, PTU, TAP, and PhTU with a detection level in the order of 10 µg kg⁻¹. As sample pre-treatment a selective extraction procedure, based on the specific complex formation between

thyreostats and mercury ions, was performed [1, 109]. This TLC method was based on the reaction of these compounds with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol (NBD-Cl). As for the visualization of these non-fluorescent TS-NBD adducts, different agents, e.g. glycine, ethanolamine, 2-mercaptoethanol, thioglycolic acid, 3-mercaptopropionic acid, 2-aminoethanethiol, and cysteine were described. The most powerful ones contained the $\text{SHC}_2\text{H}_4\text{NH}_2$ sequence (e.g. cysteine and 2-aminoethanethiol). Finally, cysteine was chosen as spraying agent, due to the stability of the formed fluorescent adduct (Cys-NBD). As for confirmatory purposes, two-dimensional thin layer chromatography (2D TLC) was reported by De Brabander and Verbeke (1975) [109]. In this approach, the drugs were distributed over a two-dimensional space instead of over a linear space, resulting in an improved separation. Later on, this method was adopted by the BENELUX (Belgium, Netherlands and Luxemburg) and the EU for qualitative analysis of thyreostatic drugs [114-116]. High performance thin layer chromatography (HPTLC) [1, 109, 117] succeeded TLC, with later on the development of the 4x4 HPTLC, where 4 samples and 4 standards could be developed simultaneously on 1 HPTLC plate [118-119].

Since the 1960s, separation methods based on gas and liquid chromatography gained in popularity, due to the achievement of higher specificity and selectivity. With the introduction of gas chromatography (GC) in thyreostatic drug residue analysis, it became clear that coupling of GC with MS was a requirement to achieve adequate quality control criteria [120-123]. Using derivatisation agents such as benzylchloride [120], pentafluorobenzylbromide (PFBBBr) [121] or methylation prior to GC-MS analysis [104, 122-124] resulted in the achievement of lower LODs. Unfortunately, the recovery of certain thyreostatic compounds, like tapazole remained low. Therefore, De Brabander et al. proposed in 1992 an efficient method combining HPTLC and GC-MS analysis [117]. In this case, suspected spots appearing on the TLC plate were scraped off. These were then eluted (diethyl ether), evaporated and derivatised with MSTFA (*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide) to form silyl-derivatives that were subsequently analysed by GC-MS (Figure I.5). Later on, this derivatisation (MSTFA) prior to the GC-MS analysis is combined with a second derivatisation, executed before the purification of the sample. This supplementary derivatisation increased the recovery and repeatability of the analysis, and increased the number of active site adsorptions. For this, NBD-Cl [102], PFBBBr [110-112] or 3-BrBBBr [125] have been described as derivatisation agents. These methods were found suitable for confirmation of thyreostatic drug abuse in biological matrices (e.g. animal tissue, milk, thyroid, and urine).

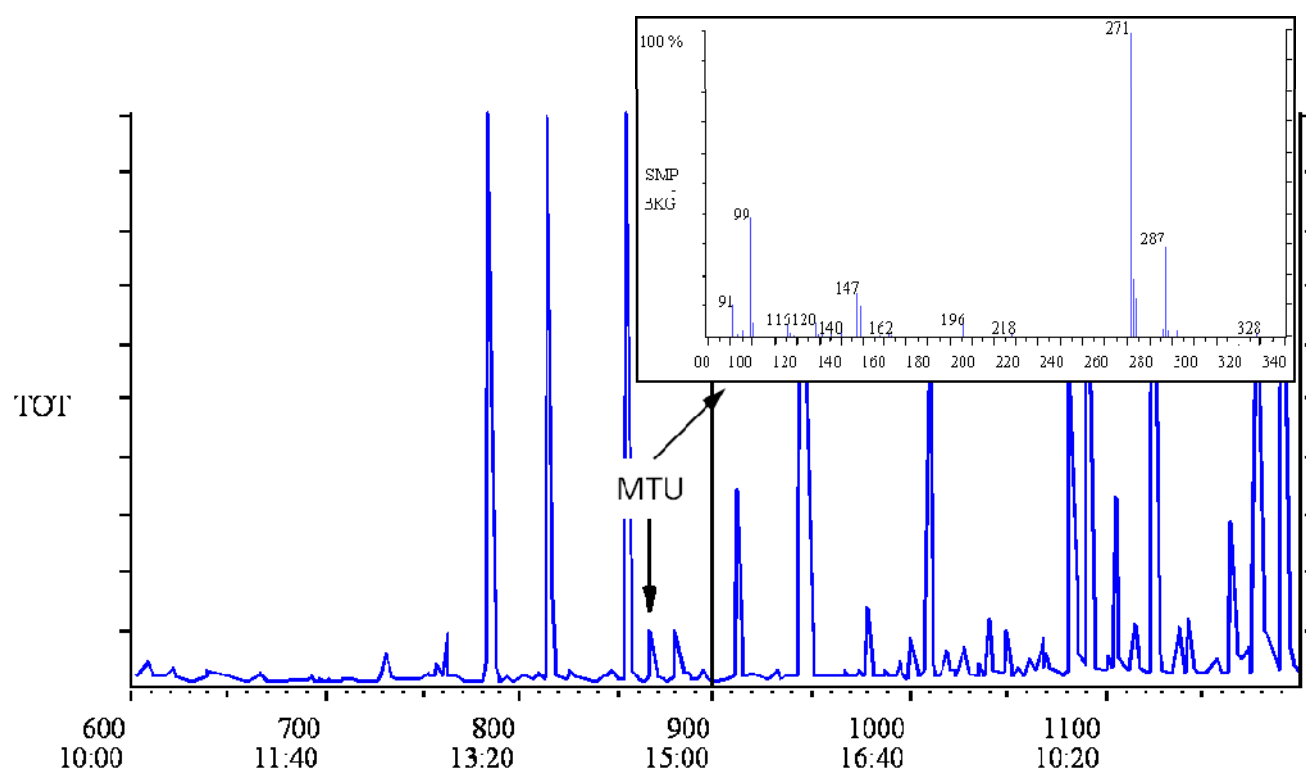


Figure 1.5: Gas Chromatogram of MTU, derived from a suspected spot on a HPTLC plate, with corresponding mass spectrum.

Additionally, high-performance liquid chromatography (HPLC) methods were described for matrices such as urine, meat, serum, plasma and thyroid, all in the range of mg kg^{-1} levels. Only Buick et al. (1998) was able to reach a lower LOD, in the range of $10 \mu\text{g kg}^{-1}$ [107]. As for the detection UV (ultraviolet) [126-130], electrochemical [123, 129], chemiluminescence [131] and diode array detection (DAD) [107] were reported. With the introduction of atmospheric pressure interfaces like ESI (electrospray ionisation) and APCI (atmospheric pressure chemical ionisation), liquid chromatography coupled to mass spectrometry (LC-MS) became the method of choice for (thyreostatic) residue control [65, 103, 106]. The first LC-MS method was introduced by Blanchflower et al. (1997) [106]. Without derivatisation 5 TS (TU, MTU, PTU, PhTU and TAP) were detected in urine and thyroid tissue at concentration levels of $25 \mu\text{g kg}^{-1}$, based on APCI ionisation and SIM-MS (single ion monitoring). Later on, LC-MSⁿ and LC-MS/MS methods in combination with ESI were developed and applied on matrices such as urine, faeces, muscle, liver, animal feed and hair, which obtained higher sensitivity and specificity by derivatisation with NBD-Cl [103] or 3-iodobenzylbromide (3-IBBr) [65]. Within the advantages of ion trap mass spectrometry, multiple stage mass spectrometry (MSⁿ) originated. De Wasch et al. (1998) combined HPTLC with an ion trap mass spectrometer in MSⁿ mode for confirmatory

purposes [132]. Thus, in case of HPTLC suspected samples, the remainder of the extract was subjected directly into the ion trap mass spectrometer (Finnigan MAT LCQ), operating in MS^n . Ionisation was performed by ESI and fragment ions were acquired up to MS^3 [132]. It must be pointed out that nowadays nearly all detection methods of thyreostatic drugs use GC or LC coupled to multiple or tandem MS [65-66, 102-103, 132], in the attempt of improving the analytical accuracy as well as the sample throughput. One method resorted to micellar electrokinetic chromatography (MEKC) for the detection of thyreostats in animal feed by DAD, at the level of 20 mg kg^{-1} [133].

4.1.3 Monitoring natural thyreostatic drugs: oxazolidine-2-thiones

OZTs, originating from the glucosinolates present in the Brassicaceae plants, are naturally occurring sulphur compounds. Of the Brassicaceae family some plants like rapeseed may be used as cheap and readily available protein source for animal feed. However, an important limiting factor for the commercial exploitation of rapeseed-derived feedstuff for livestock, is the reported goitrogenicity and anti-nutritional effects. For this reason, '00'-rapeseed with lower erucic acid and glucosinolate content has been developed. The derived by-products of this '00'-rapeseed contain lower ANF-levels and are being commercialized as protein supplement for the feed industry. Nevertheless, even with the lowered glucosinolate content, OZTs are still present. Therefore, the derived by-products of the '00'-rapeseed needs to be analysed for OZTs, especially for 5-VOT, which requires sensitive and specific methods for investigating of various biological matrices (e.g. plant, milk, egg, and tissue) [37].

Before the determination of 5-VOT in biological matrices, sample pre-treatment is required. For the selective extraction steps, liquid chromatography [134], liquid/ liquid extraction [135-136] and also the specific complex formation of OZTs with mercury ions [1, 36-37, 137] have been reported. Initially, 5-VOT was isolated by two-dimensional paper chromatography, from plant material and milk and then quantified by UV spectrometry [16, 138]. Madesjki (1974) [139] described a TLC method in combination with UV detection for the measurement in eggs. Later on separation techniques such as GC and LC were reported. For GC, various detectors have been described, e.g. ECD (electron capture detection) [3, 36, 134, 137], FID (flame ionisation detection) [134, 140], FPD (flame photometric detection) [134] and TSP (thermionic specific detection) [134]. As for LC only UV detection has been reported [37-38, 135-136]. The first gas chromatographic methods provided insufficient accuracy for the determination in a physiological medium [140-141], but later on adequate sensitive GC analysis

was reported for the determination in milk [134], plasma, urine and muscle tissue [3, 36, 137]. To ameliorate the sensitivity of the analysis, derivatisation with butylheptafluoro [134] or pentafluorobenzoyl chloride [3, 36, 137] derivatives has been described. As for HPLC, normal phase [135-136] as well as reversed phase [37-38] has been reported. Only Mabon et al. (1999) [38] applied HPLC for other matrices than milk, such as animal feed and biological matrices (e.g. organs, muscle tissue, and plasma). It needs to be stated however, that chromatographic methods coupled to MS have only been reported for the determination of the purity grade of synthesized oxazolidine-2-thiones and not for the analysis of biological matrices [142].

In literature, the adverse effects (e.g. goitrogenicity and anti-nutritional aspects) of 5-VOT that originate from the ingestion of progoitrin, have been extensively studied. Most of the reports dealt with ruminants and poultry, but also products derived thereof, such as milk and eggs. Cows, put on a diet containing 6 g of goitrin a day, showed varying levels of this compound in biological matrices and tissues: 15-200 $\mu\text{g kg}^{-1}$ in plasma, 80-250 $\mu\text{g kg}^{-1}$ in urine and 70 $\mu\text{g kg}^{-1}$ in [36, 137]. Additionally 5-VOT appeared in the milk of lactating cows [143]. As a result, concerns were expressed of this 'goitrogenic milk', especially for children, due to the reports of endemic goiter in Tasmania [144] and Finland [145-147], presumably attributed to Brassica containing diet of cows. In poultry, high intake of progoitrin, resulted in reduced synthesis of thyroid hormones [148], translated to a reduced basal metabolism and growth retardation [149]. Other adverse effects like lower fertility and eggs with fishy odour ('egg taint') were also reported [150-151]. According to Madejski, besides milk, eggs as well could be a potential source of 5-VOT for the consumer [139]. It must be pointed out that past concerns of animal derived products containing OZTs, such as 'goitrogenic milk' or the 'crabby eggs', remain.

4.1.4 Monitoring of inorganic thyreostats

Data on inorganic thyreostats, comprising both anions (e.g. SCN^- and ClO_4^-) and cations (e.g. Li^+), originated mainly from East-European countries. For the regulatory control of inorganic thyreostats abuse, analytical approaches for the determination of these compounds needed to be developed. Afterwards, these methods were applied to obtain more information on excretion profiles, residue levels and zootechnical effects [47, 152-155]. This information was helpful to inform the analysts and inspection services on the nature and magnitude of the expected concentrations of drug residues after

illegal treatment. In this section a brief overview of the available literature of the most important members of this group, e.g. SCN^- (natural and chemical origin), ClO_4^- , and Li^+ is given.

For the residue control of SCN^- caution is required, because of the natural and chemical nature of this compound. De Brabander et al. (1977) [1, 156] described a quantitative method for the detection in urine, based on the method of Nota and Palombari (1973) [157]. This method measured the concentration of cyanogen bromide that resulted from the reaction of SCN^- and bromine by gas solid chromatography. Later on, a GC-MS method was reported for measuring thiocyanate levels in biological fluids [158]. Even more recently, an ion chromatography coupled to tandem mass spectrometry (IC-MS/MS) method was described for amniotic fluid samples [159].

Nowadays, most detection methods for perchlorate are based on ion chromatography (IC) [160-163] or IC coupled to electrospray ionisation mass spectrometric detection (IC-ESI-MS) [161], but also HPLC (UV) [164] is still employed.

For the detection of lithium an ion exchange chromatography method was developed by Batjoens et al. (1993) [160].

4.2 Current trends in chromatographic separation: from HPLC to U-HPLC

Since the development of proper interface systems, high performance liquid chromatography has become the predominant technology in residue analysis when coupled to a specific and sensitive detection system like mass spectrometry. An interface and ionisation system converts analytes from liquid phase to gas phase, reduces pressure for mass analysis and finally converts the analytes into ions. One of the primary drivers for evolution of HPLC has been the constant development in packing materials used to effect separations. Throughout the last decennia, there has been a trend to use packing material with smaller particles size (Figure I.6). However, in conventional HPLC the choice of particle size must be a compromise, since the smaller the particle size, the higher the generated column back-pressure. The generated pressure is considered as the major limitation of the conventional chromatographic separation system.

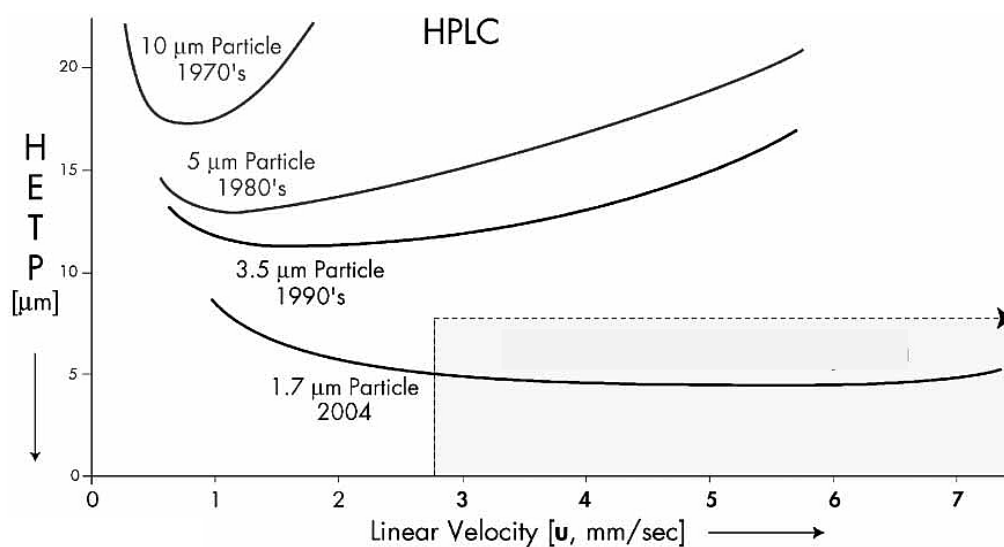


Figure I.6: van Deemter plot, illustrating the evolution of particle sizes over the last three decades.

In modern residue analysis the observed trends towards decreasing the laboratory costs and increasing the number of analytes to be detected, emphasised even more the 'need for speed', which led to the development of ultra-high performance liquid chromatography (U-HPLC). This new development in analytical separation retains the practicality and principles of HPLC while increasing the overall interlaced attributes of speed, sensitivity, and resolution by working with sub-2 micron particles [165-168]. The underlying principles of this evolution are governed by the van Deemter equation that describes the relationship between linear velocity (flow rate) and plate height (HETP, or column efficiency) [169]. As indicated by the van Deemter equation (Figure I.6), the efficiency of the chromatographic process is proportional to the decrease in particle size. As for the mobile phase flow rate, increases do not negatively affect the efficiency, as observed for the 10 and 5 μm particles. However, sub-2 micron columns require much higher operating pressures, and a system properly designed to capitalize this is required. Therefore, systems that can reliably deliver the requisite pressures and maintain the separation efficiency of small particles with tightly managed volumes have been developed [165-166, 168]. The analytical pumps capable of operating in both gradient and isocratic separation modes, while delivering solvents smoothly and reproducibly at these pressures, became commercially available. The newly on the market available ultra-high or very high pressure pump systems embodied all of these requirements [165, 168]. For these reasons, the innovative U-HPLC technology could play a significant role in the future of liquid chromatography [170].

4.3 Current detection technologies hyphenated with liquid chromatography

In residue analysis, mass spectrometry has become the detector of choice for chromatographic separation [170-171]. The power of this technique lies in the fact that the mass spectral data generated provides valuable information about the molecular weight, structure, identity, and quantity with a high degree of confidence. Moreover, the selectivity obtained with this instrument cannot be compared to a commonly used UV detector.

The MS principle consists of ionising chemical compounds to generate charged molecules or molecule fragments, sorting and identifying the ions according to their mass-to-charge (m/z) ratio and measuring the abundance of the ions. As such the MS comprises an ion source, a mass analyser, and a detector. In context of my doctoral thesis only those techniques used will be discussed, only briefly however since all of the techniques are common values in the world of residue analysis.

4.3.1 Ionisation source

Earlier LC-MS systems were only successful for a limited number of compounds due to the limitations of the interface technique. In this case, the mobile phase molecules are not separated from the wanted compounds, and ionisation was performed under vacuum condition. With the introduction of atmospheric pressure ionisation (API) techniques, the number of compounds successfully analysed by LC-MS greatly expanded. In API, the analyte molecules are ionised first at atmospheric pressure, next the ions are mechanically and electrostatically separated from neutral molecules. Common atmospheric pressure ionisation techniques are: electrospray ionisation (ESI), atmospheric pressure chemical ionisation, and atmospheric pressure photoionisation (APPI).

ESI is a soft ionisation technique that generates gas phase ions from a typical liquid-phase. The LC eluent is nebulized into a chamber at atmospheric pressure, after passing the capillary needle on which a strong electrostatic field is applied. The heated drying gas evaporates the solvent into droplets. As the droplets shrink, the charge concentration in the droplets increases. Eventually, the repulsive force between ions with like charges exceeds the cohesive forces and non-fragmented ions are ejected into the gasphase. These ions are attracted to and thus proceed to the mass analyser. The heated

electrospray ionisation (ESI) interface is even better capable of mobile phase desolvation, due to thermal desolvation assistance in the capillary needle [172].

During APCI, the analyte solution is introduced into a pneumatic nebulizer and desolvated in a heated quartz tube before interacting with the corona discharge creating ions. The LC eluent is sprayed through a heated (typically 250 °C - 400 °C) vaporizer at atmospheric pressure that vaporizes the liquid. The resulting gas phase solvent molecules are subsequently ionised by electrons discharged from a corona needle and transfer their charge to the analyte molecules through chemical reactions. Positive ions are formed through proton transfer, while negative ions are formed through electron transfer or proton loss [172].

4.3.2 Mass analyser

During this study, three types of mass analysers hyphenated with liquid chromatography were utilised, i.e. an ion trap (LTQ, Thermo Fisher Scientific), a triple quadrupole (QqQ, TSQ Vantage, Thermo Fisher Scientific), and an orbitrap (Exactive, Thermo Fisher Scientific) mass spectrometer. The latter two are coupled to the latest U-HPLC technology that requires fast switching mass spectrometers. The QqQ and Orbitrap achieve these fast acquisition rates and obtain sufficient data points across the narrow U-HPLC generated chromatographic peaks.

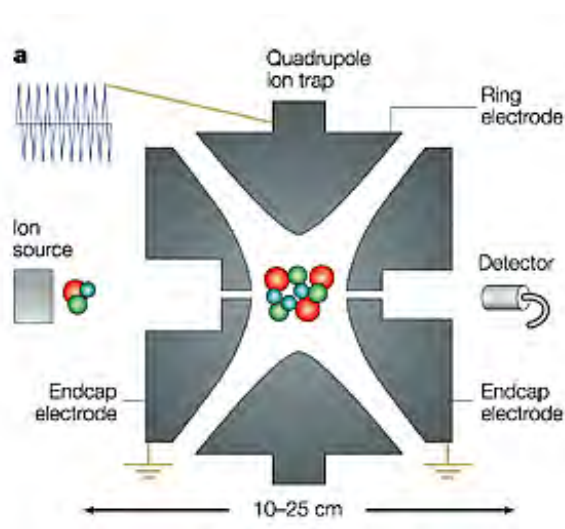


Figure I.7: Schematic of an ion trap mass analyser

The ion trap mass analyser, also referred to as the quadrupole ion trap consists of three hyperbolic electrodes: the ring electrode and the entrance and exit end-cap electrodes (Figure I.7). By applying voltages, these electrodes form a cavity which traps the ions in a stable oscillating trajectory. The exact motion of the ions is dependent on the voltages applied and their individual mass-to-charge (m/z) ratios. Ion traps have the advantage of being able to perform multiple stages of mass spectrometry (MS^n) without additional mass analysers. After trapping the precursor ions, collision-induced dissociation (CID) is applied to generate structural information. For detecting the ions, the potentials are altered to destabilise the ion motions resulting in ejection of the ions through the exit end-cap [172-173].

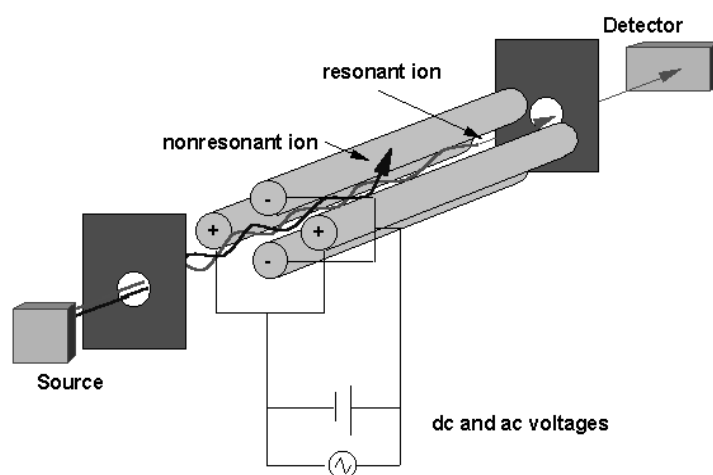


Figure I.8: Schematic of a quadrupole mass analyser

A quadrupole mass analyser consists of four parallel rods arranged equally spaced around a central axis (Figure I.8). By applying precisely controlled voltages to opposing sets of poles, a 'mass filter' is created. Only ions with a particular mass-to-charge ratio will pass through the filter to be detected at a particular applied voltage. A triple quadrupole allows fragmentation of ions in quadrupole 2, the CID collision cell. For quadrupole mass analysers different types of MS/MS experiments are possible. The three most common types used are displayed in Figure I.9, namely product ion scans, which is the most common employed with ESI ionisation, precursor ions scans, and neutral loss scans. Selected reaction monitoring (SRM) is depicted as the most sensitive operating mode for MS/MS [172].

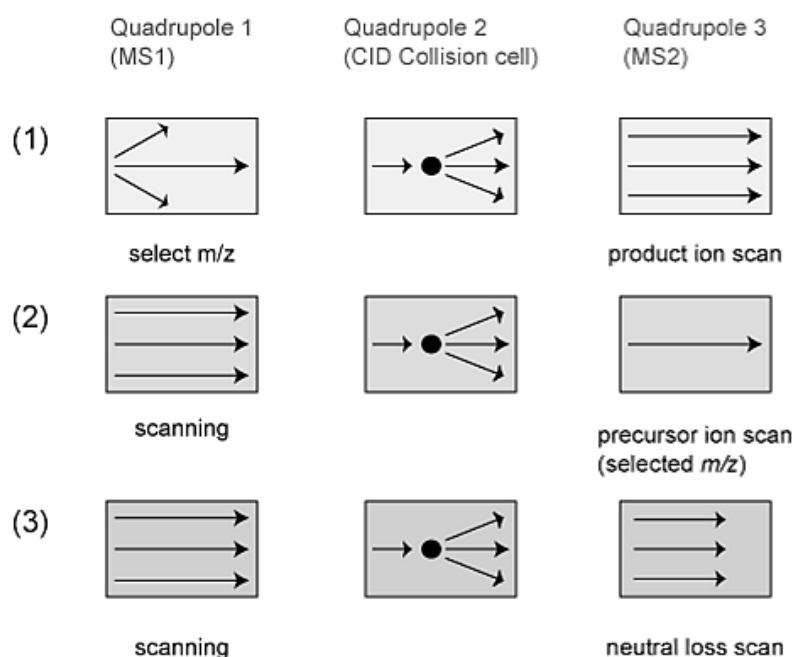


Figure I.9: : Schematic representation of the three common types of MS/MS experiments, i.e. product ion scan (1), precursor ion scan (2), neutral loss scan (3).

The Orbitrap bench top mass analyser is a high resolution, accurate mass detection system. The success of this system is highly dependent on the ability to trap ions and inject packages of ions into the orbitrap. Therefore it is preceded by an external injection device based on trapping ions in RF-only gas-filled curved quadrupole (the C-trap, Figure I.10). Inside the orbitrap, ions are electrostatically trapped while rotating around the central electrode and performing axial oscillation. These oscillating ions induce an image current, which can be detected using a differential amplifier. This is followed by a Fast Fourier Transformation (FFT), employed to convert the recorded time-domain signal for ions with different masses into a frequency, resulting in an accurate reading of their m/z and high mass resolution. This system is capable of generating fragmentation information in a non-selective manner, 'all ions MS/MS' by using a High Energy Collisional Dissociation (HCD) cell without precursor ion selection. In this way structural information can be obtained on compounds of interest and fragment ions can be used for confirmation in targeted analyses [174].

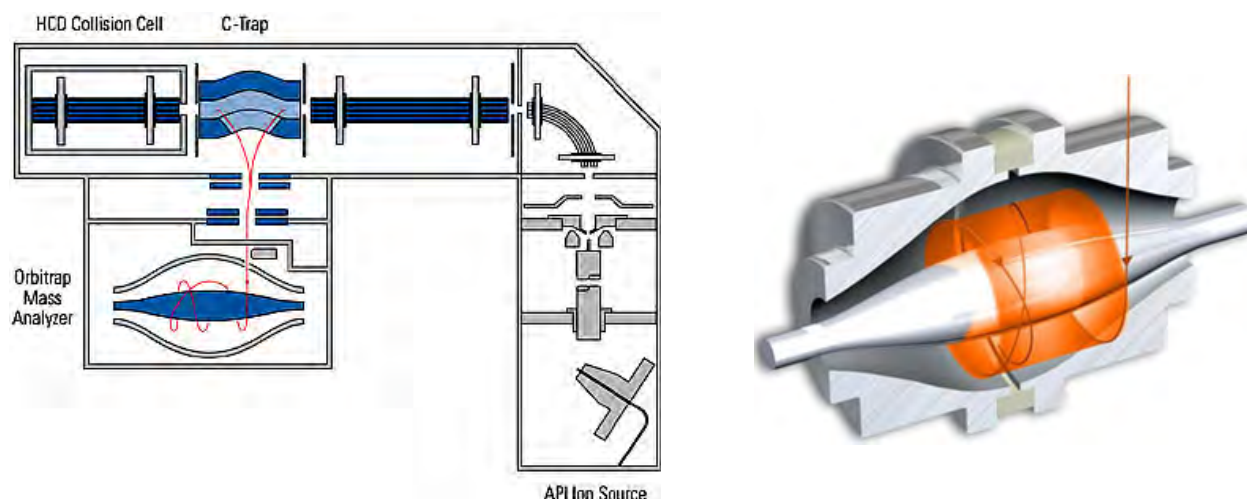


Figure I.10: Schematic overview of a bench-top Orbitrap mass spectrometer, the Exactive (Thermo Fisher Scientific) and the Orbitrap mass analyser.

4.3.3 Detector

The detection of the ion trap and QqQ mass spectrometer is achieved by an electron multiplier, used to detect the presence of ion signals emerging from the mass analyser. The basic physical process that allows an electron multiplier to operate is called secondary electron emission. When ions hit a surface, it causes the electrons in the outermost area of the atom to be released, which are known as secondary electrons. These electrons are emitted and then accelerated through an electric field, which is generated by applying the proper voltage to the surface of the tube. The electric field forces the emitted electrons to hit the following wall, and these electrons, like the ion, also cause electrons to be emitted. This cascade continues until enough electrons are emitted to create a measurable current.

For the Orbitrap mass spectrometer, mass analysing and detecting functions are performed by one and the same, the orbitrap. The oscillating ions inside the trap induce an image current into the two outer halves of the trap, which can be detected using a differential amplifier. To convert this current from the time towards the frequency domain Fast Fourier Transformation is used. Because the frequency of oscillation and hence the induced current is specific to any given m/z , it can be converted into a spectrum.

5. Conceptual framework of this study

Thiouracil, belonging to the group of the xenobiotic thyreostats, is known to be illegally administrated to livestock for growth-promoting purposes, recent years however, questions have been raised concerning the status of this exogenous drug. Therefore, the main goal of the present study was to investigate the possible endogenous prevalence of thiouracil. The specific goals and scope of this study were as follows:

- ✓ To develop a new analytical approach to specifically and accurately analyse all xenobiotic thyreostats in urine without derivatisation
- ✓ To provide data prior to analysis concerning the stability of the xenobiotic thyreostats, with respect to sample storage and conservation
- ✓ To investigate the possible endogenous prevalence of thiouracil, this within a wide variety of species
- ✓ To elucidate the origin of the alleged endogenously produced or prevalent thiouracil

The work presented in this dissertation comprises 4 research chapters, preceded by a general introduction to the subject (**CHAPTER I**).

CHAPTER II describes the development and validation of a new analytical method, this without derivatisation for the detection and quantification of xenobiotic thyreostats in urine. To this purpose ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (U-HPLC-MS/MS) is used. The challenge of this analytical approach lays in the elimination of the commonly used derivatisation step.

CHAPTER III investigates the stability of the xenobiotic thyreostats in urine of livestock. The effects of preservation at room temperature, preservation duration and number of freeze-thaw cycles will be evaluated. In addition the benefit of different conservation approaches is investigated. The most optimal pre-treatment is subsequently applied on incurred urine samples, which is of relevance for the national residue control plan.

CHAPTER IV aimed at endorsing the possible endogenous prevalence of thiouracil. Urine samples of animals (livestock and domesticated) with known and unknown clinical backgrounds were analysed for thiouracil with the newly developed analysis method described in **CHAPTER II**. In addition, a small-scale 9-day human experiment with Brassicaceae vegetables was performed to investigate if this endogenous prevalence could be extrapolated to the human population.

CHAPTER V investigates the origin of the naturally occurring thiouracil in urine of livestock and domesticated animals and humans. For this purpose various feed and food matrices, Brassicaceae related were investigated for the presence of naturally occurring thiouracil. In addition, the mechanism of formation within the concerned Brassicaceae species, with a special emphasis on the myrosinase enzyme.

CHAPTER VI presents a general conclusion of the different research chapters. Additionally, some future recommendations are formulated.

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CHAPTER II

DEVELOPMENT OF AN ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY METHOD FOR QUANTIFYING THYREOSTATS IN URINE WITHOUT DERIVATISATION

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CHAPTER II

DEVELOPMENT OF AN ULTRA-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY METHOD FOR QUANTIFYING THYREOSTATS IN URINE WITHOUT DERIVATISATION

Abstract:

Thyreostatic drugs, illegally administrated to livestock for fattening purposes, are banned in the European Union since 1981 (Council Directive 81/602/EC). For monitoring their illegal use, sensitive and specific analytical methods are required. In this study an U-HPLC-MS/MS method was described for quantitative analysis of eight thyreostatic drugs in urine, this without a derivatisation step. The sample pre-treatment involved a reduction step with dithiothreitol under denaturing conditions at 65 °C, followed by liquid/liquid extraction with ethyl acetate.

This analytical procedure was subsequently validated according to the EU criteria (2002/657/EC Decision), resulting in decision limits and detection capabilities ranging between 1.1 and 5.5 $\mu\text{g L}^{-1}$ and 1.7 and 7.5 $\mu\text{g L}^{-1}$, respectively. The method obtained for all xenobiotic thyreostats, a precision (relative standard deviation) lower than 15.5 %, and the linearity ranged between 0.982 and 0.999. The performance characteristics fulfil not only the requirements of the EU regarding the provisional minimum required performance limit (100 $\mu\text{g L}^{-1}$), but also the recommended concentration fixed at 10 $\mu\text{g L}^{-1}$ in urine set by the European Union of Reference Laboratories. Future experiments applying this method should provide the answer to the alleged endogenous status of thiouracil.

1. Introduction

Thyreostats are orally active drugs, which upon administration disturb the normal metabolism of the thyroid gland by inhibiting the production of the hormones triiodothyronine and thyroxine [1, 2]. This goitrogenic activity may be attributed to the presence of a thiocarbamide group [3]. 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 gastro-intestinal tract [4, 5]. Consequently, these growth-promoting agents negatively affect the meat quality of treated animals. In addition, xenobiotic thyreostats are listed as compounds with teratogenic and carcinogenic properties and thus pose a possible human health risk (International Agency for Research on Cancer) [6]. These arguments led in 1981 to a ban on their use for animal production in the European Union [7].

In light of the residue control plan, which must ensure the elimination of thyreostat abuse, European legislation demands its member states to develop confirmatory methods to detect and quantify thyreostatic compounds in various biological matrices (e.g. urine, faeces, meat, thyroid gland, etc.). As a guideline for these methods, the European Union set out a provisional minimum required performance limit (MRPL) of $100\text{ }\mu\text{g L}^{-1}$ or $\mu\text{g kg}^{-1}$. In December 2007, the European Union of Reference Laboratories (EURLs) posted a guidance paper containing their view on 'state of the art' analytical methods for the national residue control plan [8]. This document, which has no legal force (serves only as a technical guidance), comprises recommended concentrations of substances for which no maximum residue limits (MRLs) have been established [9]. The recommended concentration for analysing thiouracil, methyl-thiouracil, propyl-thiouracil, and tapazole in urine and in the thyroid gland was set at a concentration of $10\text{ }\mu\text{g L}^{-1}$ or $\mu\text{g kg}^{-1}$.

The development of analytical methods, fulfilling the provisional MRPL or more recent the recommended concentration is challenging due to the noteworthy chemical properties of thyreostatic drugs. The polar, amphoteric character of these drugs, and their ability to adopt different tautomeric forms, negatively affects the extraction yield from biological samples, but also their chromatographic separation. Additionally, in case of mass spectrometric detection (MS) their small molecular weight (110-210 Da) limits the sensitivity. The majority of the currently available analytical methods circumvents these difficulties by applying derivatisation before analysis, which increases the molecular weight, lowers the polarity, and stabilizes the molecule in a single tautomeric form. For thin layer

chromatography the use of derivatisation agent 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol (NBD-Cl) has been reported [1, 10-13], whereas for gas chromatography coupled to MS, benzylchloride [14], methylating agents [15-17] or MSTFA (N-methyl-N-trimethylsilyl-trifluoroacetamide) have been used. The extraction yield prior to GC-MS analysis could be even more increased when combined with other derivatisation agents, specifically used for stabilising the compounds in one tautomeric form, e.g. pentafluorobenzylbromide (PFBBBr) [18-21] and 3-bromobenzylbromide (3-BrBBR) [21-22]. For LC-MS analysis only the use of NBD-Cl [De Wasch 2001] and more recently 3-iodobenzylbromide [24-25] have been described. A more detailed overview of the substantial evolution in thyreostatic analysis has been reviewed earlier [26].

For routine analysis, the method of Pinel et al. [24], exploiting a 3-iodobenzylbromide derivatisation is generally accepted as the most optimal procedure [26-27]. This method generated decision limits (CC_α) and detection capabilities (CC_β) in the range of 0.1-5.2 $\mu\text{g L}^{-1}$ and 2.6-23.2 $\mu\text{g L}^{-1}$, respectively. Recently Löhmus et al. [25] transferred this method to the new ultra performance liquid chromatographic (UPLC) technique coupled to tandem MS and obtained for all thyreostats CC_α and CC_β values in agreement with the EURL guidance paper ($< 10 \mu\text{g L}^{-1}$). Application of these highly sensitive analytical procedures as opposed to earlier methods for urine of cattle, gave rise to the detection of thiouracil (TU) in the concentration range of 0-10 $\mu\text{g L}^{-1}$. The origin of this signal, which is assumed to be illegal administration, is however still a matter of debate since this signal could be retrieved in urine of untreated cattle as well [21, 28]. Identification was carried out by 3 independent mass spectrometric approaches (i.e. LC-MS/MS, GC-MS/MS and HRMS), each using a different derivatisation procedure [21]. These approaches acknowledged the presence of TU in urine of untreated cattle. For this reason Pinel et al. [28] proposed the possible endogenous formation of the xenobiotic thyreostat, thiouracil. However, the liability of a derivatisation step, which may result in possible false-positive identification of TU should be considered. For investigation of this suggested endogenous status, the development of a highly sensitive method for the detection of underivatised thyreostats, more specific for thiouracil in urine could offer a conclusive answer. In addition, the avoidance of a derivatisation step simplifies the sample pre-treatment and reduces laboratory costs. Recently Abuin et al. [29-30] published such a simplified method, in thyroid tissue. Nevertheless, this method for underivatised thyreostats obtained too high values for the decision limit and detection capability.

Therefore, the aim of the present study was to develop a confirmatory quantitative method for the determination of underivatised thiouracil in urine aliquots, which for future reference could be employed to conclude on the paradox surrounding the semi-endogenous status of TU. To this purpose ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (U-HPLC-MS/MS) was used. Subsequently, the method was validated according to the criteria set in Commission Decision 2002/657/EC [31]. All xenobiotic thyreostats, monitored by the European control plans were included in the validation.

2. Materials and Methods

2.1 Reagents and chemicals

Standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). The deuterated internal standard (IS), 6-propyl-d5-2-thiouracil (PTU-D5) was provided by Toronto Research Chemicals Inc. (Toronto, Canada). Stock solutions of the thyreostatic drugs: 2-thiouracil (TU), 6-dimethyl-thiouracil (DMTU), 6-ethyl-thiouracil (ETU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-thiouracil (PhTU), 1-methyl-2-mercaptoimidazole (tapazole, TAP) and 2-mercaptobenzimidazole (MBI) were prepared in methanol at a concentration of 200 ng μL^{-1} . Working solution were prepared by 200 x and 2000 x dilution in methanol (1 ng μL^{-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 in the refrigerator.

Reagents where of analytical grade when used for extraction and purification steps, and of HPLC-grade for (U)-HPLC-MS/MS application. They were obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific UK (Loughborough, UK), respectively. Phosphate buffer, dissolved in deionised water, was controlled and adjusted to a pH of 7. For extraction purposes, the required amount of phosphate buffer, pH 7, was saturated with 1 % of DL-dithiothreitol (DTT)(purity 99 %, Sigma-Aldrich, St. Louis, MO, USA).

2.2 Instrumentation

The LC system consisted of a Thermo Electron (San José, USA) Accela U-HPLC pumping system, coupled with an Accela Autosampler and Degasser. Chromatographic separation was achieved by reversed

phase chromatography and gradient elution. Separation of the thyreostatic compounds was carried out on a Acquity UPLC HSS T3 column (High Strength Silica particles) (1.8 μm , 100 mm x 2.1 mm, Waters, Milford, MA, USA), kept at 35 °C. An Acquity UPLC in-line filter (2.1 mm, 0.2 μm , Waters) was used to improve analytical column lifetime. The mobile phase constituted of 0.1 % aqueous formic acid and 0.1 % formic acid in methanol, and was pumped at a flow rate of 0.3 mL min⁻¹. Optimized separation of all analytes was obtained using a linear gradient starting with a mixture of 95 % aqueous formic acid and 5 % formic acid in methanol. After 1.65 min the amount of acidified methanol was increased to 100 % in 5.2 minutes and kept there for 0.5 min. Finally, the column was allowed to re-equilibrate for 2 minutes at initial conditions, this before each run. All analytes could be separated in a total runtime of only 9.4 minutes. Analysis was performed on a triple quadrupole mass analyser (TSQ Vantage, Thermo Electron, San José, USA), fitted with a heated electrospray ionisation (HESI II) source operating in positive ion mode.

The following working conditions were applied: spray voltage at 3.5 kV; vaporizer and capillary temperature at 370 °C and 300 °C, respectively; sheath and auxiliary gas at 40 and 20 arbitrary units (a.u.), respectively; cycle time of 0.8 s. Argon pressure in the collision cell (Q2) was set at 1.5 mTorr and the mass resolution at the first (Q1) and third (Q3) quadrupole was set at 0.7 Da at full width at half maximum (FWHM). Precursor ion, S-lens RF amplitude, and collision energy (CE) in Q2 were optimized individually per compound (Table II.1). Quantification and confirmation data were acquired in selected reaction monitoring (SRM) mode, the transitions followed are displayed in Table II.1. Instrument control and data processing were carried out with Xcalibur Software (Thermo Electron, San José, USA).

Table II.1: Collected SRM transitions and compound specific MS parameters (product ions in bold were used for quantification purposes).

Analyte	tR (min)	Precursor ion (<i>m/z</i>)	Product ions (<i>m/z</i>)	S-lens (RF Amplitude) (V)	Collision Energy (eV)
TU	1.6	129.0	112.1	49	15
			84.1		27
			60.1		34
			57.1		37
TAP	2.3	115.0	81.1	54	32
			74.1		17
			57.2		19
MTU	2.9	143.0	126.1	53	15
			86.1		23
			84.1		17
			60.1		36
DMTU	4.6	157.1	140.1	67	17
			98.2		19
			72.1		34
			60.1		37
ETU	4.8	157.1	78.2	48	23
			72.1		36
			60.1		36
PTU	5.4	171.1	154.1	60	16
			122.2		18
			86.1		25
			60.1		36
PTU-D5 ^a	5.4	176.1	159.2	62	17
			117.2		19
			86.1		28
			60.1		34
MBI	5.3	151.0	118.1	67	25
			93.1		23
			91.1		34
			65.2		36
PhTU	6.0	205.0	188.1	102	18
			146.1		19
			103.1		27
			77.1		38

^a: Internal standard (IS)

2.3 Samples

Urine samples from bovine, ovine, and porcine origin were obtained from veterinary sampling in light of the European residue control plan. Upon arrival at our laboratory, samples were stored at -20 °C, and thawed before analysis. The thawed samples were centrifuged for 10 min at 4000 x g, and aliquots of 1 ml were used for analysis. To each sample 50 ng of internal standard (PTU-D5) was added, to obtain a final concentration of 50 $\mu\text{g L}^{-1}$. As for the spiked samples, a standard solution (0.1 or 1.0 ng μL^{-1}) containing all 8 thyreostats was added, vortexed vigorously for one minute and left to equilibrate for at least 15 min before extraction.

2.4 Sample extraction and purification

One mL of DTT-enriched (1 %) phosphate buffered saline at pH 7 was added to each sample, followed by vortexing vigorously for one minute. Subsequently, the samples were placed under denaturing conditions, at 65 °C for 30 min, next the denaturated, reduced extracts were purified with 2 x 5 mL ethyl acetate. Finally, the combined extracts were evaporated to dryness under a gentle stream of nitrogen at 60 °C and the dried residue redissolved in a total volume of 200 μL of mobile phase consisting of 10/90 MeOH/0.1 % aqueous formic acid. This ratio is slightly different than the initial conditions of the U-HPLC-MS/MS procedure, but the elevated methanol concentration was necessary to ensure good dissolution of the analytes. Of the obtained extract, 10 μL was injected on column.

2.5 Quality assurance

Prior to the sample analysis, a standard mixture of the targeted compounds was injected to check the operational conditions of the chromatographic devices. To every sample, a procedure internal standard (IS) was added at a concentration of 50 $\mu\text{g L}^{-1}$, prior to the extraction. The identification of the thyreostats were based on their retention time relative to the IS and on the ion ratios of the product ions, carried out according to the criteria described in Commission Decision 2002/657/EC [31].

After identification, the analytes concentration was calculated by fitting its area ratio in a eight-point calibration curve, established by blank urine samples spiked with 8 thyreostats in the range of 2.5-100 $\mu\text{g L}^{-1}$ and the IS at 50 $\mu\text{g L}^{-1}$. Area ratios were determined by integration of the area of an analyte under the specific SRM chromatograms in reference to the integrated area of the internal standard.

3. Results and Discussion

3.1 U-HPLC and MS parameters

Initially, our analytical procedure was developed on a HPLC system (Finnigan Surveyor, Thermo Electron, San Jose, CA, USA) coupled to a LTQ linear ion trap mass analyser (Thermo Electron, San Jose, CA, USA). To determine optimal MS conditions, the default parameters for the HESI-probe, capillary temperature, vaporizing temperature, sheath gas pressure, and auxiliary gas pressure were further optimized by individually infusing the analytical thyreostatic standards. During this tuning step acetic acid and formic acid were evaluated as candidate mobile phase additives, to enhance ionisation. Addition of formic acid at 0.1 % in the mobile phase provided the most optimal compromise between ionisation and peak geometry.

For chromatographic separation different columns, with a special emphasis on columns with a higher affinity for polar compounds were tested, i.e. normal reversed phase Symmetry C18 (5 μ m, 150 mm x 2.1 mm, Waters), Cogent Bidentate C18 (4 μ m, 75 mm x 2.1 mm, Micro Solv), Gemini C6-phenyl (3 μ m, 50 mm x 2.0 mm, Phenomenex), Nucleodur Sphinx (5 μ m, 250 mm x 4.0 mm, Machery-Nagel), Atlantis T3 (3 μ m, 150 mm x 2.1 mm, Waters), and Hypercarb (5 μ m, 100 mm x 2.1 mm, Thermo Scientific). The choice of column was mainly determined by the achieved retention time, the base peak separation, and the peak efficiency, measured as peak width at the baseline. Evaluation of the retention was based on the earliest eluting analyte thiouracil. Reasonable retention times were obtained with the Nucleodur Sphinx, Atlantis T3, and Hypercarb column and these were subjected to further investigations. Subsequently, base peak separation of structurally related thyreostats (ETU & DMTU) was compared, for which all three columns obtained good results. With regard to the peak efficiency, the Hypercarb displayed relatively broad peaks, whereas the Atlantis T3 provided the narrowest peaks. This could be explained by the relative small particle size of the column, 3 instead of 5 μ m. Therefore, the Atlantis T3 column was chosen for chromatographic separation of thyreostatic drugs. Additionally, retention times, optimal separation, and good resolution were ameliorated by careful selection of the gradient program. Moreover the methanol gradient was chosen in such a manner that reasonable retention times and a good base peak separation of structurally related thyreostats occurred. The organic solvent methanol was preferred, as the higher elution strength of acetonitrile proved to be disadvantageous for retention of the relatively polar thyreostats.

According to Commission Decision 2002/657/EC [31], LC-MS/MS analysis of banned substances requires 4 identification points (IP), which accords to one precursor (1 IP) and two product ions (2 x 1.5 IP). However, upon fragmentation with the linear ion trap mass analyser only 1 product ion could be observed. Therefore the use of a triple quadrupole analyser (QqQ) was preferred. Since this high-end fast-scanning QqQ apparatus allows the combination with ultra-high performance liquid chromatography, a method transfer to U-HPLC was performed at the same time. The equivalent U(H)PLC columns: Nucleodur Sphinx (Machery-Nagel), Acquity HSS T3 (Waters), and Hypersil Gold (Thermo Scientific) of those providing the best retention and base peak separation in classical HPLC were evaluated for their performances. The Acquity HSS T3 (1.8 μ m, 50 mm x 2.1 mm) provided not only the highest signal-to-noise-ratio (S/N) and peak intensity, but most important the best retention of TU. When analysing pre-spiked urine extracts, detection of thiouracil was ambiguous. Subsequently, some of these analysed extracts were post-spiked to investigate the ion suppression phenomenon [32]. Post-spiking resulted in the detection of TU, linking the lack in detection to co-elution of isobaric interferences. This problem was resolved when switching to a longer, 100 mm instead of 50 mm HSS T3 column, as depicted in Figure II.1. Indeed, Wren [33] acknowledges that the use of longer columns results in better resolution because of greater retention and selectivity.

The protonated molecular ion $[M+H]^+$ was selected as precursor ion for all compounds, four transitions per analyte were acquired, except for TAP and ETU for which only 3 transitions could be obtained (Table II.1). For each compound, based on peak intensity and signal-to-noise-ratio, the two most intense transitions were selected for validation purposes.

RT: 0.00 - 7.19

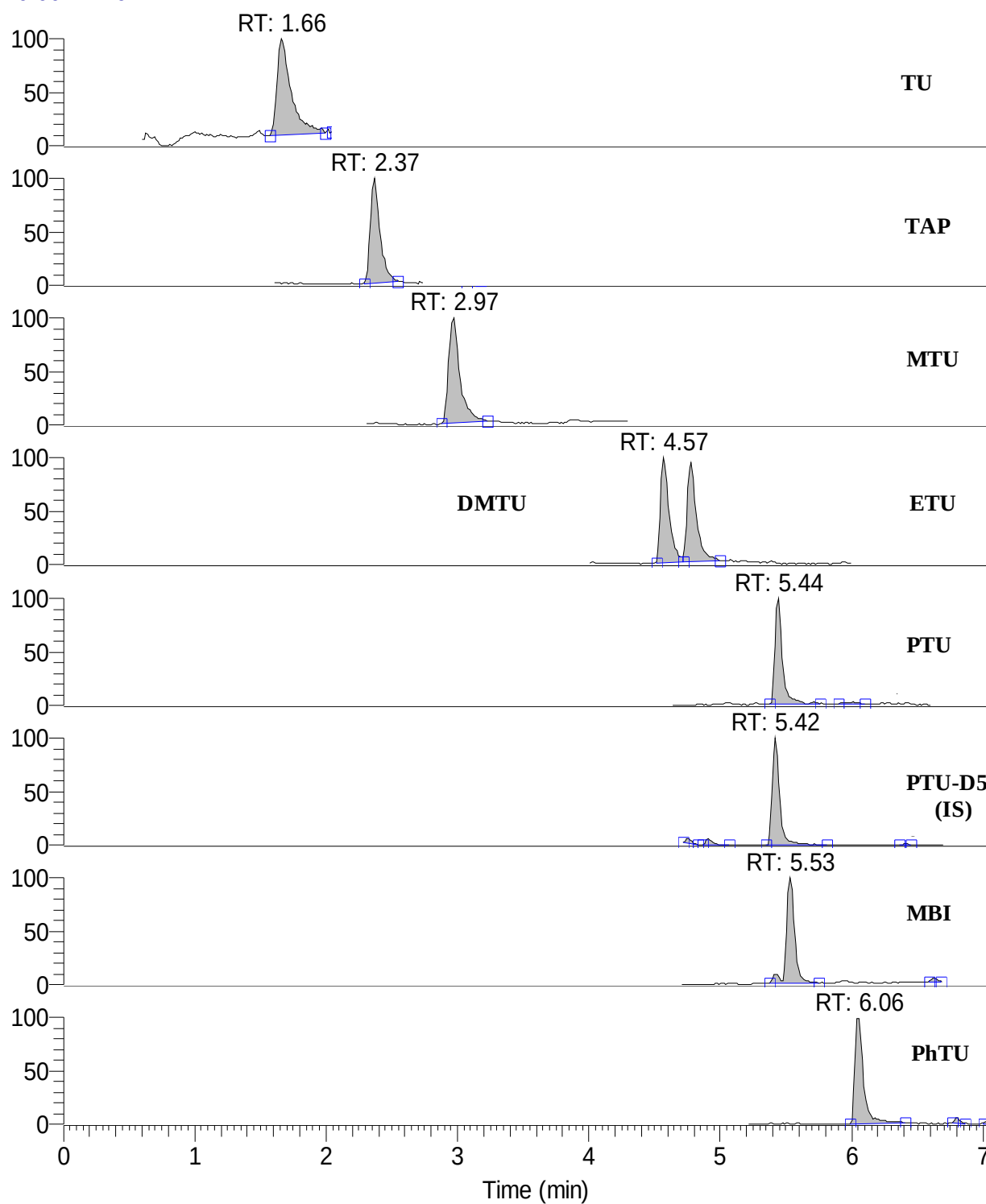


Figure II.1: SRM chromatogram of an extracted urine, spiked with 8 thyreostats at $10 \mu\text{g L}^{-1}$ and the internal standard, PTU-D5 at $50 \mu\text{g L}^{-1}$.

3.2 Development of sample pre-treatment

Preliminary experiments were performed to evaluate the performance of different solvents in extracting thyreostats from urine. Therefore three organic solvents, immiscible with water but polar enough to extract thyreostats, were selected i.e. chloroform, dichloromethane, and ethyl acetate. Before extracting fortified urine samples, experiments with fortified water samples were conducted during which ethyl acetate obtained the highest extraction yield. However when applied to urine samples, even with optimization of the pH (pH 2-10) no thyreostatic drugs could be detected. As a result of these observations, the influence of a clean-up step prior to the analysis of underivatised thyreostats was investigated. In literature the use of Silica SPE (solid phase extraction) cartridges has been described for thyreostats [23, 29, 34]. This SPE sorbent was applied in an indirect procedure, LLE prior to Si SPE, as well as in a direct one, redissolving an evaporated urine sample in a less polar, more desired solvent before loading onto the sorbent. Other types of SPE sorbents were also tested, e.g. Oasis HLB/WAX/MAX (Waters), Envi-Carb (Supelclean), and HR-X (Machery-Nagel), but all unsuccessful when applied to urine samples. Some of these samples were post-spiked upon detection to check for ion suppression, but this was not the case. Therefore, the hypothesis was posed that endogenous matrix constituents present in urine interfered with the extraction. Most likely this concerns some kind of protein as indicated by Blanchlower et al. [34].

Therefore, different strategies to eliminate these interferences and allowing the extraction of thyreostats from urine were compared. During a first series of experiments, the known interaction between thyreostats and metal ions was exploited [1, 13, 35-36]. To this purpose metal ions, more specific manganese (Mn^{2+}) was added to the sample in order to interact with the thiol group of thyreostatic drugs and enhance the extraction yield [35]. An alternative strategy was preventing these interactions by adding the chelating agent EDTA (ethylenediaminetetraacetate) [27, 34]. Salting out (NaCl; 2.5-5 g) to aid the partition of the thyreostats into organic solvent was also tested, as was potential hydrolysis with β -glucuronidase (50-100-150-200 μ L; 2h-4h overnight; 50-60-65 $^{\circ}$ C). A second series of experiments focused on the potential disruption of protein-thyreostat interaction, and more specific denaturation of matrix proteins. To this end, the supplementation of different organic solvents, i.e. acetone, methanol, acetonitrile (0.5-1.0-2.0-3.0 mL), and an acidic treatment (HCl; 1-6 M; 50-100-200 μ L) were exploited. Subsequently, the impact of mechanical disruption was evaluated by adding Zirconia beads ($\varnothing$ 0.1-0.5 mm, 0.5-0.75-1.0 g) at elevated temperatures (60-65 $^{\circ}$ C; 2-4 h) [37-38].

Finally, the supplementation of reducing agent DTT or the anionic surfactant SDS (sodium dodecyl sulphate) (1mL of buffer saturated with 1 %), as a means to disrupt disulfide or non-covalent bonds respectively, was examined [39-40].

The two most promising methods were mechanical disruption using zirconia beads and addition of the reducing agent DTT. The beads application has been reported for disrupting bacterial, yeast, and fungal cells to extract DNA [37-38, 41-42], whereas DTT has been for cleaving disulfide bonds [39]. Both protocols were subjected to further experimentation. Parameters such as the size and amount of the beads, pH, temperature, and means of disruption were important for optimization of the mechanical disruption procedure. In the finalized protocol 1 g of zirconia beads ($\varnothing$ 0.5 mm) was supplemented to the urine sample set at pH 5.2, afterwards placed for 2 h in an ultrasonic bath at 65 °C, with additional manual shaking, followed by LLE. For the sample pre-treatment protocol applying DTT, the pH of the sample proved to be of crucial importance, since the reducing power of DTT is limited to pH values above 7. However, the addition of DTT (1mL of buffer at pH 7, 1 % DTT) alone proved to be insufficient, since no extraction of thyreostats occurred. This indicated that the disulfide bonds were buried, and thus not accessible to solvents. Therefore the protocol had to be carried out under denaturing conditions, 65 °C, which resulted in the extraction of thyreostats from urine. The extraction yield was not improved by increasing the duration of the denaturation step and therefore kept at 30 minutes.

When comparing the two finalized protocols for zirconia beads and DTT (n=18), normalized area ratios of respectively, 0.0039 ± 0.006 (CV 15.9 %) and 0.054 ± 0.007 (CV 12.8 %) were obtained. In the end, the DTT procedure proved to be the most sensitive method, as demonstrated in Figure II.2, but also the least liable to differences in urine samples, and therefore selected for validation purposes.

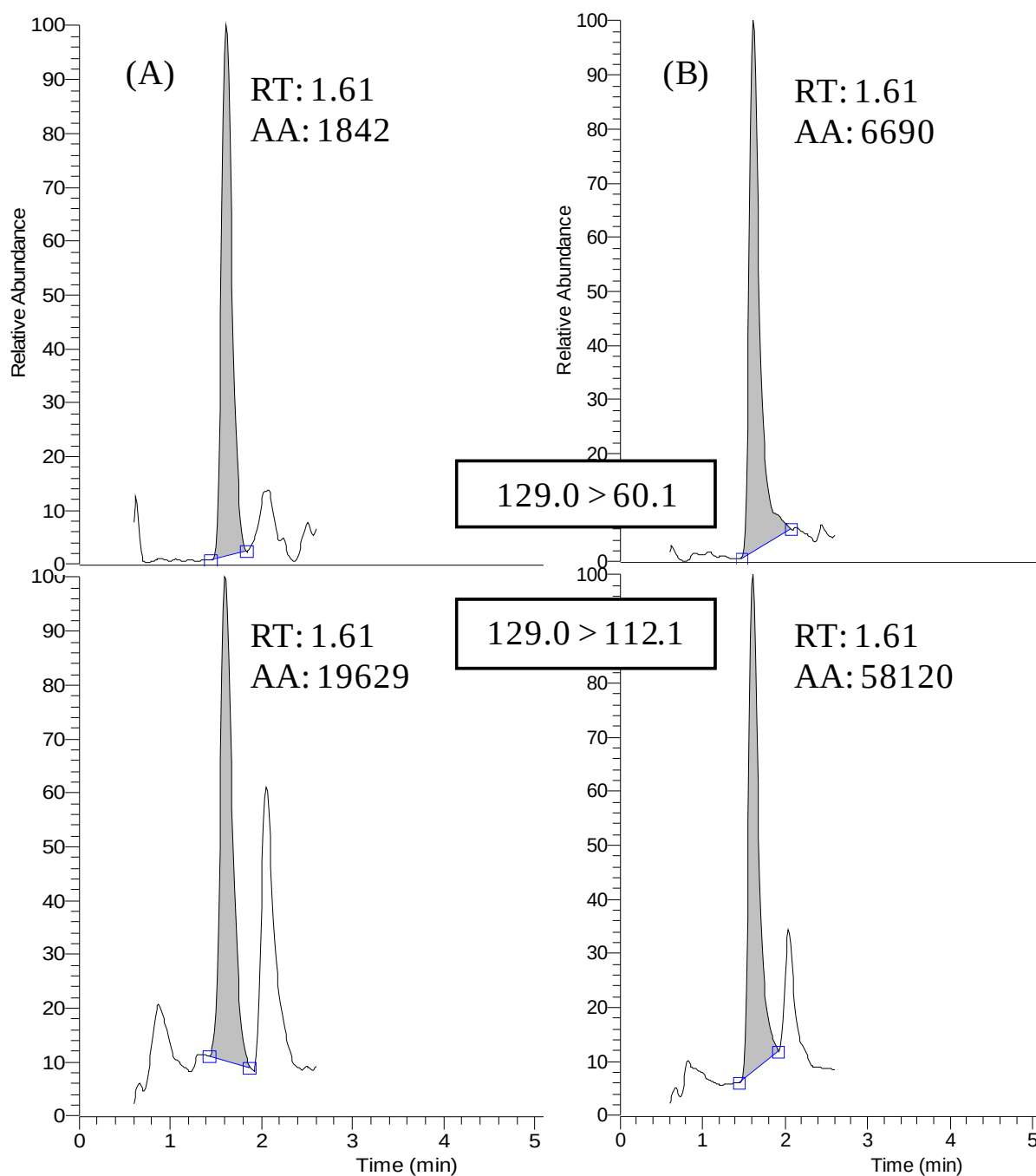


Figure II.2: SRM chromatogram of a blank urine sample (mixture of 6 different urines) fortified at $10 \mu\text{g L}^{-1}$ of thiouracil, where zirconia beads (A) and DTT (B) were used to aid in the extraction.

3.3 Validation

The newly developed analytical method was validated according to the criteria specified in Commission Decision 2002/657/EC for quantitative confirmation [31]. For each compound 4 transitions were monitored, except for TAP and ETU (Table II.1). The primary and secondary product ion were used for quantification purposes.

Firstly, an appropriate internal standard was chosen, capable of anticipating fluctuations in the signal intensity upon extraction of thyreostats from urine samples. The use of an isotopically labelled IS in MS-based chemical analysis has always been recommendable, as well as compounds that are structurally related to the analyte (basic structure identical) [31]. For these reasons the deuterated propyl-thiouracil (PTU-D5), DMTU, and ETU were compared as internal standards. Due to its superior performance PTU-D5 was preferred and used as IS throughout this study.

The validation procedure used, adopted in part a particular protocol proposed by Antignac et al. [43]. This was tailored for validating analytical methods based on MS detection and tried to give a compromise between the 2002/657/EC European Decision requirements and practical aspects and limitations related to laboratory work [31]. The validation protocol was designed as follows:

Analysis of 20 blank urine samples from various origins (bovine, porcine, and ovine) was performed to check for the ruggedness of the method. This set up permitted to determine the specificity by calculating the average (μ_N) and standard deviation (σ_N) of the noise amplitude, expressed relative to the internal standard signal amplitude. The calibration curve was realized on a mixture of 6 previously analysed blank urine samples. Seven fortification levels were included with the previously estimated noise average (μ_N) as a forced intercept. The linearity of this calibration graph was evaluated by calculation of the correlation coefficient (R^2) and the sensitivity i.e. the slope of the fitted curve (a). Based on these data, the decision limit (CC_α) was calculated taking in account the equation of the calibration graph (Eq. (1)), where I is the signal amplitude and C the concentration, and the definition of CC_α (Eq. (2)). This combination led to the expression given by Eq. (3).

$$I_{CC_\alpha} = \mu_N + a \cdot CC_\alpha \quad (1)$$

$$I_{CC_\alpha} = \mu_N + 2.33 \sigma_N \quad (2)$$

$$CC_\alpha = (2.33 \sigma_N) / a \quad (3)$$

For calculating CC_β , 20 blank samples were spiked at the determined CC_α level. This permitted to estimate the repeatability through the standard deviation of the signal amplitude (σ_S). In order to minimize the estimation error, the signal relative standard deviation ($(R.S.D.)_S$) was preferred above the standard deviation (σ_S). Finally with σ_N , a , and $(R.S.D.)_S$, the detection capability (CC_β) could be calculated, taking in account the calibration equation (Eq. (4)), where I is the signal amplitude and C the

concentration, and the definition of CC_{β} (Eq. (5)). The combination of these two formula led to the final expression given by Eq. (6).

$$I_{CC\beta} = \mu_N + 2.33 \sigma_N + 1.64 CC_{\beta} \quad (4)$$

$$I_{CC\beta} = \mu_N + 2.33 \sigma_N + 1.64 (R.S.D.)_S CC_{\beta} \quad (5)$$

$$CC_{\beta} = [2.33 \sigma_N + 1.64 \mu_N (R.S.D.)_S] / a [1 - 1.64 (R.S.D.)_S] \quad (6)$$

For the mean recovery and precision, 18 identical blanks, originating from a mixture of 6 different urines were amended with targeted compounds at 0.5, 1.0, and 1.5 times the recommended concentration of $10 \mu\text{g L}^{-1}$ and the IS, splitted in three sets ($n=6$). This was performed by two operators on three different days.

3.3.1 Specificity

The specificity of the method was demonstrated by analysis of blank urine samples and samples fortified with each analyte separately or with a mixture of all analytes. The fortification level was set at $10 \mu\text{g L}^{-1}$, in accordance with the recommended concentration of the EURL guidance document (2007) [8]. More recently, endogenous production of TU in the $0\text{--}10 \mu\text{g L}^{-1}$ range has been reported [25, 28]. Therefore blank urine samples ($n=20$), containing a background concentration lower than $1 \mu\text{g L}^{-1}$ of TU were selected to validate the method. For each analyte spiked, chromatograms showed a significant increase in peak area and intensity at its specific retention time compared to the blanks, taking a S/N of at least 3 into account (Figure II.1). No other matrix substances interfered at these retention times. Therefore, the newly developed method was found to be specific for all eight thyreostatic drugs (TU, TAP, MTU, DMTU, ETU, PTU, MBI, and PhTU) in the presence of matrix components.

3.3.2 Selectivity

In accordance with the European Criteria 2002/657, analytes were identified on the basis of their relative retention time, i.e. the ratio of the chromatographic retention time of the analyte to that of the internal standard [31]. In addition, a system of identification points was used to interpret the data, based on the ion ratios of the precursor and product ions in the acquired spectrum [31]. For the confirmation of thyreostats, listed in Group A of Annex I of Directive 96/23/EC [44], a minimum of 4

identification points (IPs) is required [31]. Precursor (1 IP) and product ions (1.5 IP/ion) of each analyte are presented in Table II.1. The individual relative retention time ($n=18$) of the extracted thyreostatic compounds showed in every case a standard deviation lower than 0.006, with a coefficient of variation smaller than 0.90 %. This falls well within the stated tolerance level of 2.5 % for liquid chromatography. As a result, the identification of the eight thyreostatic compounds, extracted from urine samples was unambiguously. As for the identification points, a maximum of 7 could be designated to the analytes with 4 monitored transitions. As for TAP and ETU, where only three transitions were monitored, a maximum of 5.5 IP's could be assigned. When analysing urine samples fortified at 5-10-15 $\mu\text{g L}^{-1}$, the minimum required amount of IP's, set at 4, was easily achieved.

3.3.3 Calibration curves

The linearity of the developed method was evaluated for each thyreostatic compound by preparing calibration curves in blank matrix, which consisted of a mixture of 6 different bovine urines. The blank samples were fortified within a range of 2.5-100 $\mu\text{g L}^{-1}$, but no equidistant steps were used. More notice was given to the low concentration range, since this part strongly influences the CC_α - and CC_β -values. Moreover by using these fortification levels, the concentration domain globally used in practice (0-100 $\mu\text{g L}^{-1}$) could be verified. Linear regression analysis was carried out by plotting the peak area ratios of the analyte against the IS versus the analyte concentrations. The estimated noise average of the pool of blanks ($n=20$) was used as a forced intercept [43]. Good linearity was obtained, all correlation coefficients (R^2) were ≥ 0.991 , only TAP was slightly inferior, but still acceptable at 0.982.

3.3.4 Mean recovery

Since no certified reference material was available, trueness of the measurements was assessed by fortifying blank urine samples (pool of 6) with 0.5, 1.0, and 1.5 times the recommended concentration as stated in the EURL guidance document (10 $\mu\text{g L}^{-1}$). This was performed in six replicates. As can be deduced from Table II.2, all calculated mean recoveries fulfil the criteria put forward in the EC/2002/657 stating that a mass fraction between 1 and 10 $\mu\text{g L}^{-1}$ should obtain a mean recovery range of 70-80 %, whereas a mean recovery of 80-110 % should be required for a mass fraction of, or greater than 10 $\mu\text{g L}^{-1}$. Only DMTU, which exceeds this limit by 1 % at 1 times the fortification level, did not fulfil this criterion. The recoveries (98.1-111 %) obtained with this innovative analytical method were

highly satisfactory. However to the best of our knowledge, besides an older study of Blanchflower et al., which obtained inferior recoveries, little has been reported on the recoveries of thyreostatic drugs in urine [34]. More recent reports concerning urine did not mention recoveries [24-25], or handled extraction of other matrix like the thyroid gland with recoveries lower than 75 % [18, 45].

Table II.2: Precision and mean recovery of the developed method for eight thyreostats, analysed in urine.

Analyte	Nominal concentration ($\mu\text{g L}^{-1}$)	Recovery ^a		Repeatability ^b		Within laboratory reproducibility ^c
		mean $\pm$ S.D. (%)	R.S.D. (%)	overall mean $\pm$ S.D. ($\mu\text{g L}^{-1}$)	R.S.D. (%)	R.S.D. (%)
TU	5	91.2 $\pm$ 16.1	17.6	9.8 $\pm$ 1.4	14.0	17.7
	10	89.1 $\pm$ 12.4	14.0			12.3
	15	98.2 $\pm$ 10.4	10.6			7.5
TAP	5	103 $\pm$ 16.2	15.8	11.3 $\pm$ 11.9	11.9	16.4
	10	101 $\pm$ 14.6	14.4			13.5
	15	105 $\pm$ 11.2	10.7			9.9
MTU	5	106 $\pm$ 4.8	4.5	10.4 $\pm$ 0.8	7.4	5.5
	10	103 $\pm$ 2.5	2.5			4.8
	15	91.1 $\pm$ 2.3	2.5			9.2
DMTU	5	92.8 $\pm$ 14.9	16.0	10.8 $\pm$ 1.6	14.5	18.0
	10	111 $\pm$ 13.2	11.9			13.1
	15	110 $\pm$ 10.1	9.2			8.8
ETU	5	91.5 $\pm$ 10.6	11.5	10.3 $\pm$ 1.2	12.1	12.2
	10	107 $\pm$ 13.1	12.2			9.9
	15	109 $\pm$ 11.9	10.9			12.1
PTU	5	95.1 $\pm$ 4.6	4.9	10.5 $\pm$ 0.7	6.2	6.5
	10	100 $\pm$ 1.7	1.7			6.0
	15	104 $\pm$ 3.7	3.5			6.2
MBI	5	99.5 $\pm$ 10.1	10.1	10.5 $\pm$ 0.9	8.9	12.2
	10	105 $\pm$ 13.0	12.3			10.4
	15	107 $\pm$ 10.3	9.6			11.9
PhTU	5	93.3 $\pm$ 11.9	12.8	11.0 $\pm$ 1.8	16.4	16.7
	10	108 $\pm$ 15.4	14.3			12.2
	15	110 $\pm$ 11.9	10.9			15.4

^a: 18 aliquots of identical blank samples, and fortify six aliquots at each of 0.5, 1.0, and 1.5 times the recommended concentration of 10 $\mu\text{g L}^{-1}$.

^b: three series of six replicates of fortified samples of an identical matrix at 0.5, 1.0, and 1.5 times the recommended concentration of 10 $\mu\text{g L}^{-1}$, under identical conditions.

^c: four series of six replicates of fortified samples of an identical matrix at 0.5, 1.0, and 1.5 times the recommended concentration of 10 $\mu\text{g L}^{-1}$, analysed by 2 different operators.

3.3.5 Precision

To evaluate the precision of the method, repeatability and within-laboratory reproducibility were determined. Repeatability was evaluated by calculating the coefficients of variation (CV). To this purpose, data from three series of six replicates of samples of an identical origin fortified at 0.5, 1.0, and 1.5 times the recommended concentration of $10 \mu\text{g L}^{-1}$ were used. These analyses were carried out on different occasions by the same analyst under repeatable conditions. For all thyreostats considered, good repeatability was obtained, since the individual overall calculated CVs for each compound were well below 20 % (Table II.2). This outcome was not evaluated by the Horwitz equation, too high values would be obtained, because of the low concentration range used [31]. However, in accordance with the Commission Decision 2002/657/EC, the CVs obtained for mass fraction lower than $100 \mu\text{g L}^{-1}$ were as low as possible [31].

For evaluation of the reproducibility only the within-laboratory reproducibility was considered. Four series of six replicates of fortified samples at 0.5, 1.0, and 1.5 times the recommended concentration of $10 \mu\text{g L}^{-1}$ were analysed by different operators on different days. The results, summarized in Table II.2 indicate the good precision of the method. The obtained CVs were in accordance with the commission Decision (2002/657/EC) stating that in case of repeated analysis of a sample carried out under within-laboratory reproducibility conditions, the intra-laboratory coefficient of variation of the mean should not exceed 20 % in case of a mass fraction of, or greater than $10 \mu\text{g L}^{-1}$.

3.3.6 Decision limit (CC_α) and detection capability (CC_β)

Different procedures to determine the decision limit (CC_α) and the detection capability (CC_β) are reported in literature [31, 43]. The decision limit CC_α is defined as the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant. During this study, the CC_α and CC_β were determined by analysis of 20 blank urine samples respectively, non-fortified and fortified at CC_α level. The signal associated with CC_α corresponds to the maximal noise amplitude. The detection capability CC_β is defined as the lowest concentration at which a method is able to detect contaminated samples with a statistical certainty of $1-\beta$ (error probability = 5 %). Table II.3 summarizes the calculated CC_α - and CC_β - values for the different thyreostats. Because the concentrations for CC_α , obtained by this

approach concern relatively low values, a preliminary experiment was conducted to check if all compounds were detected when spiked at their CC_{α} -level (Figure II.3).

Table II.3: Decision Limits (CC_{α}) and detection Capabilities (CC_{β}) calculated for the eight thyreostats in urine according to the 2002/657/EC Decision.

Analyte	CC_{α} ($\mu\text{g L}^{-1}$)	CC_{β} ($\mu\text{g L}^{-1}$)
TU	2.2	3.0
DMTU	1.7	2.3
ETU	1.2	2.1
MTU	1.1	1.7
PTU	2.2	3.3
PhTU	1.6	2.4
TAP	5.5	7.5
MBI	1.1	1.7

The determination of CC_{α} and CC_{β} occurred in the context of a confirmatory method for banned substances, as such a minimum of 2 product ions was required. Subsequently, the two most intense transitions were used for the calculations. Decision limits and detection capabilities ranged respectively, between 1.1 and 5.5 $\mu\text{g L}^{-1}$ and between 1.7 and 7.5 $\mu\text{g L}^{-1}$. These results are highly satisfactory since the performances are far below the suggested MRPL of 100 $\mu\text{g L}^{-1}$ in urine samples. Even so, if in the future the recommended concentration (EURL guidance document 2007) [8] of 10 $\mu\text{g L}^{-1}$ for TU, MTU, PTU, and TAP in urine would be legalized, this method would still easily meet the requirements.

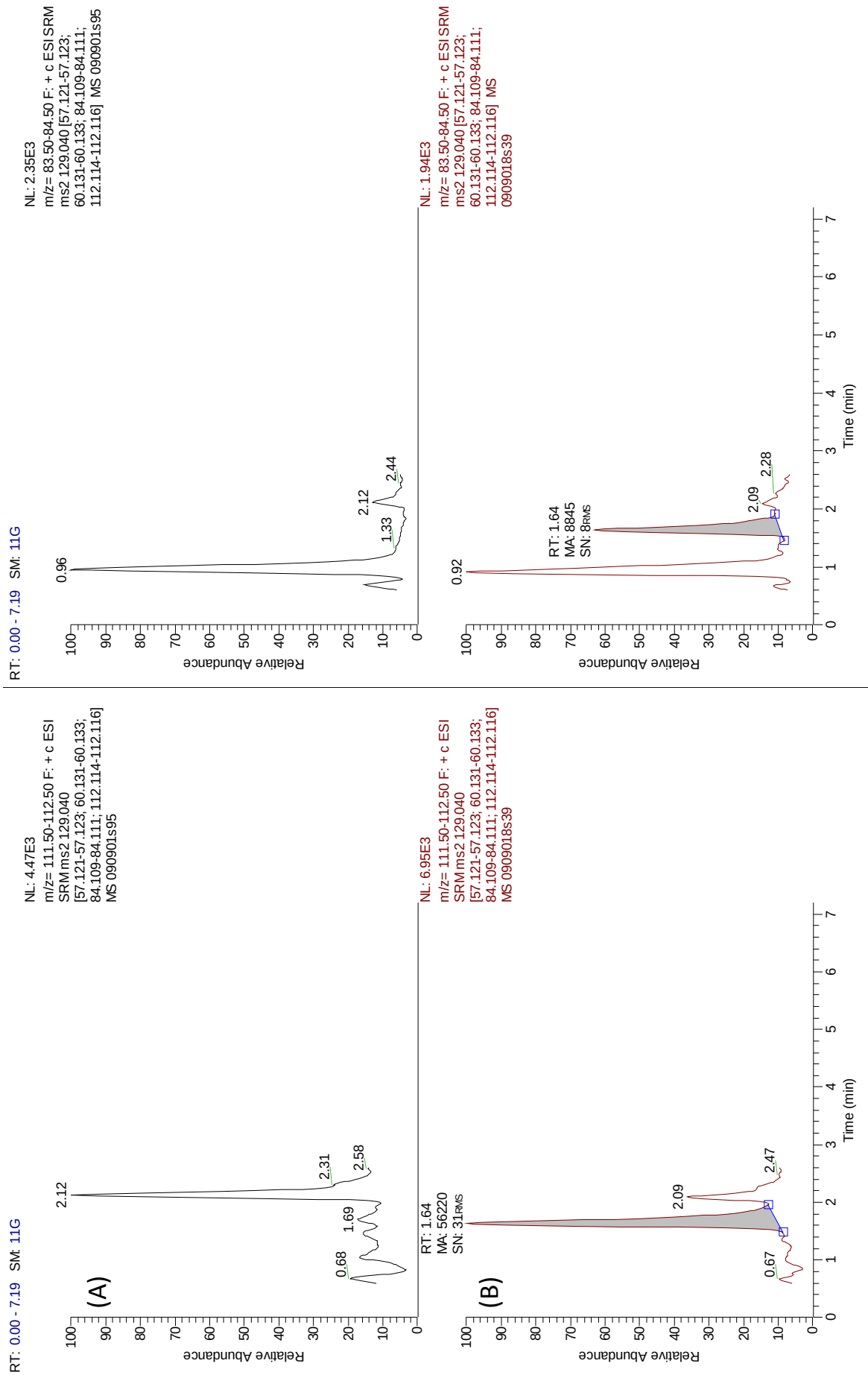


Figure II.3: SRM chromatogram of a blank urine sample reduced by DTT and subsequently extracted with ethyl acetate (A), and a fortified urine at the CC_a level of thiouracil (2.2 $\mu\text{g L}^{-1}$) (B).

4. Conclusion

Ultra-high performance liquid chromatography coupled to tandem mass spectrometry proved to be a suitable technique for the quantification and confirmation of eight thyreostats (TU, TAP, MTU, DMTU, ETU, PTU, MBI, and PhTU) in urine samples without derivatisation. The selected reaction monitoring mode of the triple quadrupole mass analyser easily allowed quantification at the level of the recommended concentration of $10\text{ }\mu\text{g L}^{-1}$ for TU, MTU, PTU and TAP, resulting in low CC_α and CC_β values. Moreover, the newly developed U-HPLC protocol enabled shorter analysis times (10 min) and, consequently, a higher throughput, while maintaining good peak separation and resolution. The concise sample pre-treatment, consisting of a reduction step followed by a simple LLE even further allowed to reduce analysis time and costs. Omitting the derivatisation resulted not only in a decreased analysis time and laboratory costs, but should also reduce the possibility of obtaining possible false-positive results. To that purpose, the application of this method in laboratories involved in the official control of residues of thyreostatic drugs in urine samples could be highly advantageous.

5. Acknowledgements

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CHAPTER III

STABILITY OF THYREOSTATIC DRUGS IN BOVINE AND PORCINE URINE

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CHAPTER III

STABILITY OF THYREOSTATIC DRUGS IN BOVINE AND PORCINE URINE

Abstract:

Thyreostatic drugs, illegally administrated to livestock for fattening purposes, are banned in the European Union since 1981. For monitoring their illegal use, sensitive and specific analytical methods are required. In this context, the knowledge of the stability in a matrix is of primary importance. This study aimed at evaluating the effects of preservation, number of freeze-thaw cycles, and matrix-related variables on the stability of thyreostatic drugs in the urine of livestock. Finally, the developed conservation approach was applied on incurred urine samples, which displayed traces of the thyreostat thiouracil below the recommended concentration of $10 \mu\text{g L}^{-1}$.

The stability study confirmed the negative influence of preservation (8 h) at room temperature and at -70°C , decreases in concentration of more than 78.0 % were observed for all thyreostats, except for 1-methyl-2-mercaptoimidazole and 2-mercaptobenzimidazole. Additionally, investigation of matrix-related variables indicated significant impacts of the presence of copper ($p = 0.001$) and the pH ($p = 0.002$). Next, an optimized pre-treatment (pH 1 and 0.1 M ethylenediaminetetraacetic acid disodium salt dehydrate) significantly differing from the original conservation approach ($p < 0.05$) was developed, which proved capable of delaying the decrease in concentration and improved the detection in time for both spiked as well as incurred urine samples. In the future, it seems highly advisable to apply the developed pre-treatment on incurred urines upon sampling, before thyreostat analysis. Additionally, it is recommendable to limit preservation of urine samples at room temperature, but also in the freezer prior to thyreostat analysis.

1. Introduction

Xenobiotic thyreostats are orally active drugs, which upon administration disturb the normal metabolism of the thyroid gland by inhibiting the production of the hormones triiodothyronine and thyroxine [1, 2]. This goitrogenic activity may be attributed to the presence of a thiocarbamide group [3]. In particular, 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 1-methyl-2-mercaptoimidazole (TAP, tapazole), and 2-mercaptobenzimidazole (MBI) display this strong thyroid-inhibiting action. 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 [4, 5]. Consequently, these growth-promoting agents negatively affect the meat quality of treated animals. In addition, xenobiotic thyreostats are listed as compounds with teratogenic and carcinogenic properties and thus pose a possible human health risk (IARC) [6]. These arguments led in 1981 to a ban on their use for animal production in the European Union [7].

In light of the residue control plan, which must ensure the elimination of thyreostat abuse, European legislation demands its member states to develop confirmatory methods to detect and quantify thyreostatic compounds in various biological matrices (e.g. urine, faeces, meat, thyroid gland, etc.). As a guideline for these methods, the European Union set out a provisional minimum required performance limit (MRPL) of $100\ \mu\text{g L}^{-1}$ or $\mu\text{g kg}^{-1}$. To achieve this, new confirmatory methods have been developed and ameliorated over time, as reviewed by Vanden Bussche et al. (2009) [8]. 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) [9]. Recently this application was transferred to the new high-end technology of ultra-high performance liquid chromatography coupled to tandem MS (UPLC-MS/MS) [10]. Both methods comply with the MRPL, and even with the recommended concentration (RC) of $10\ \mu\text{g L}^{-1}$ or $\mu\text{g kg}^{-1}$, as set out by the European Union Reference Laboratories (EURL) in 2007 [11]. To ensure that detection methods applied in residue control are sufficiently robust and repeatable in time, the Commission Decision 2002/657/EC stated technical guidelines and performance criteria for validation of analytical detection methods [12]. During this procedure parameters like selectivity, specificity, linearity, trueness, recovery, applicability, ruggedness, repeatability, reproducibility, decision limit (CC_α) and detection capability (CC_β) are investigated. Imperative for the ruggedness of samples and their analytical results is also the

knowledge of the stability of an analyte in a matrix. In many cases, this part of the validation study is reduced to a minimum or even omitted.

During routine analysis for residue control, occasionally non-compliant samples of forbidden substances (group A, Annex I of CD 96/23/EC) [13] have been reported, in which case a second analysis, performed at another laboratory can be requested. For thyreostatic drugs however this confirmatory analysis has not always shown to be straightforward. In 2004 and 2006, when re-analysing non-compliant urine samples, the European Union Reference Laboratory (RIKILT, the Netherlands) reported a significant decrease in detected thyreostat concentration. This decrease may be ascribed to the lack of stability of thyreostats in matrix. In this context, the knowledge of the stability of an analyte is essential. Even more, it may support anomalous findings obtained during re-analysis.

Therefore, this study aimed at determining the stability of thyreostatic drugs in urine of livestock. Parameters such as preservation time, amount of freeze-thaw cycles, and stability at room-temperature were investigated. Subsequently, a study to determine the kinetics and possible causes of the decrease in concentration was performed. These observations led to the development of a conservation approach, for which experiments, on a short and long-term basis were set-up on spiked urine samples of bovine and porcine origin by RIKILT (Institute of Food Safety, Wageningen University, the Netherlands). After establishing a pre-treatment (pH = 1 and 0.1 mL of 0.1 M EDTA), the effectiveness of this procedure was tested upon incurred urine samples (bovine and porcine) at the Laboratory of Chemical Analysis (LCA, Ghent University, Belgium). For the incurred samples in particular the stability of thiouracil (TU) was investigated. This analyte has drawn a lot of attention lately because of its possible endogenous status and its presence in urine of untreated livestock animals at concentrations below $10 \mu\text{g L}^{-1}$ [8, 14-15]. Therefore, the investigation of the stability of low-level thiouracil ($< 10 \mu\text{g L}^{-1}$) in incurred urine samples was considered as an important additional aspect within this stability study. To this end, all incurred urines were upon sampling divided into two aliquots, one remained unaltered, the second acidified and supplemented with EDTA. Again, the effect of freeze-thaw cycles and preservation time was investigated.

2. Material and methods

2.1 Reagents and chemicals

Standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of the thyreostatic drugs: 2-thiouracil (TU), 6-benzyl-2-thiouracil (BTU), 6-dimethyl-2-thiouracil (DMTU), 6-ethyl-2-thiouracil (ETU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU), 1-methyl-2-mercaptoimidazole (tapazole, TAP), and 2-mercaptobenzimidazole (MBI) were prepared in methanol at a concentration of 200 ng μL^{-1} . Working solutions were prepared by 200 $\times$ and 2000 $\times$ dilution in methanol (1 ng μL^{-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 DMTU or ETU, spike levels of 5 and 10 $\mu\text{g L}^{-1}$, respectively, were used. Reagents were of analytical grade when used for extraction and purification steps, and of Optima® MS grade for MS application, which were obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific UK (Loughborough, UK), respectively.

The derivatisation reagent, 3-iodobenzylbromide (3-IBBr, Sigma-Aldrich, St-Louis, MO, USA), was prepared extemporaneously (2,5 mg mL^{-1} or 2 mg mL^{-1} in 1 mL methanol for RIKILT and LCA, respectively). As for the chelating agent, a 0.1 M solution of ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA) was prepared in water (Sigma-Aldrich, St-Louis, MO, USA). Phosphate buffer, made up of 0.2 M Na_2HPO_4 and 0.2 M KH_2PO_4 in deionised water, was controlled and adjusted to a pH of 8.

2.2 Instrumentation

The samples were analysed at two different locations, RIKILT (Institute of Food Safety, Wageningen University, The Netherlands) and Laboratory of Chemical Analysis (LCA, Ghent University, Faculty of Veterinary Medicine, Belgium). Both laboratories used liquid chromatography coupled to mass spectrometry, UPLC-MS/MS and LC-MS², respectively.

2.2.1 UPLC-MS/MS

Separation of the thyreostatic compounds was achieved at 60 °C on a Acquity UPLC BEH C18 column (1.7 μm , 100 mm $\times$ 2.1 mm, Waters, Milford, MA, USA) with as mobile phases water containing 0.5 %

acetic acid (A) and acetonitrile (B), run at 0.25 mL min^{-1} on a Waters Acquity Ultra Performance system (Waters, Milford, MA, USA). The linear gradient was as follows: A/B 60/40 for 2 min, increased the amount of acetonitrile during 7 minutes to 15/85, and allowed the column to re-equilibrate for 3 min at 60/40. A Micromass Quattro Ultima Pt. mass spectrometer (Waters, Micromass, Manchester, UK) was used, fitted with an electrospray ionisation source operating in the positive ion mode. The following working conditions were applied: capillary voltage at 3.4 kV and a cone voltage of 40-70 V; source and desolvation temperature at 110 and 350 °C, respectively; cone and desolvation gas flow at 112 and 742 L Hr⁻¹, respectively; cycle time of 0.39 s and a collision cell pressure of 1.90e^{-3} mbar [16]. The transitions monitored are displayed in table III.1.

Table III.1: Monitored transitions per individual thyreostatic drug for the UPLC-MS/MS method (RIKILT, Wageningen University, The Netherlands).

Analyte	[M + H] ⁺	Product ion 1 (Coll. En.)	Product ion 2 (Coll. En.)	Retention time (min)
Tapazole	331	217 (16)	90 (30)	1.7
Thiouracil	345	217 (14)	90 (30)	3.3
Methylthiouracil	359	217 (14)	90 (30)	4.3
Mercaptobenzimidazole	367	217 (14)	90 (30)	4.5
Dimethylthiouracil	373	217 (14)	90 (30)	5.3
Propylthiouracil	387	217 (16)	90 (30)	6.3
Phenylthiouracil	421	217 (16)	90 (30)	6.6
Benzylthiouracil	435	217 (14)	90 (30)	6.8

2.2.2 LC-MS²

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, USA) with as mobile phases water containing 0.5 % acetic acid (A) and methanol (B), run at 0.30 mL min^{-1} on a Finnigan Surveyor LC-system (Thermo Electron, San Jose, USA). The linear gradient was as follows: A/B 50/50 for 3 min, increasing the amount of methanol during 17 minutes to 0/100, and allowed the column to re-equilibrate for 10 min at 50/50. A linear ion trap mass spectrometer (LTQ, Thermo Electron, San Jose, USA) was used, fitted with a heated

electrospray ionisation source operating in the negative ion mode, except for TAP which required the positive ionisation 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 (a.u.), respectively. The transitions monitored are displayed in table III.2.

Table III.2: Monitored transitions per individual thyreostatic drugs for the LC-MS² method (LCA, Ghent University, Belgium).

Analyte	[M + H] ⁺	[M - H] ⁻	Product ions	Collision energy (eV)	Retention time (min)
Tapazole	331		114, 217	33	2.3
Thiouracil		343	182, 215, 309	44	9.4
Methylthiouracil		357	196, 229, 323	43	11.3
Mercaptobenzimidazole		365	148, 237, 332	32	10.2
Ethylthiouracil		371	210, 243, 337	44	13.4
Propylthiouracil		385	257, 351	45	14.7
Phenylthiouracil		419	171, 291, 385	35	15.7

2.3 Samples

For the experiments performed at RIKILT, blank bovine and porcine urine samples from healthy animals were provided by Animal Science Group (Lelystad, The Netherlands).

At LCA incurred urine samples, for investigating the stability and effect of the developed pre-treatment on low-level TU, were obtained from veterinary sampling in light of the European residue control plan. Upon sampling these urines were divided in two aliquots, one remained unaltered, the second one acidified with hydrochloric acid (HCl) (pH 1) and supplemented with 0.1 M EDTA (0.1 mL).

All samples were stored at -20 °C, and thawed before analysis. The thawed samples were then centrifuged for 10 min at 4000 × g, and aliquots of 1 mL were used for analysis. To each sample 5 or 10 ng of internal standard (DMTU or ETU) was added, to obtain a final concentration of 5 or 10 µg L⁻¹, respectively. As for the spiked samples, a standard solution (0.1 or 1 ng µL⁻¹) containing all thyreostats was added.

2.4 Sample extraction and purification

The analytical detection method applied by RIKILT [16] and LCA were both based on the protocol of Pinel et al. (2005) [9], with minor modifications. After the addition of 4 or 5 mL phosphate buffer to 1 mL of urine, the solution was derivatised with 100 μ L of a 3-IBBr solution (at a concentration of 5 or 4 mg per 2 mL of methanol for RIKILT and LCA, respectively) and placed at 40 °C during 1 hour. Before the liquid/ liquid extraction with either ethyl acetate (3 $\times$ 5 mL) or diethylether (3 $\times$ 3 mL), the solution was adjusted to a pH-value between 2 and 4. The combined extracts were finally evaporated under a gentle stream of nitrogen and redissolved in either 500 μ L of 30/70 acetonitrile/water (RIKILT) or 120 μ L 50/50 methanol/water (LCA). In both cases, 20 μ L was injected onto the column.

2.5 Quality assurance

Prior to the sample analysis, a standard mixture of the target compounds was injected to check the operational conditions of the chromatographic devices. To every sample, a procedure internal standard (IS) was added at a concentration of 5 or 10 μ g L⁻¹ (DMTU or ETU, respectively), prior to extraction. 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 the criteria described in Commission Decision 2002/657/EC [12].

After identification, the analytes concentration was calculated, for the quantitative LC-MS/MS method this was performed by means of a calibration curve (0-1-2-5-10-20-50-100 μ g L⁻¹). The LC-MS² detection method however was a semi-quantitative method, therefore concentrations were assessed based on a blank urine spiked with a known concentration of the thyreostatic drugs (TU at 5 μ g L⁻¹, TAP 25 μ g L⁻¹, others at 2 μ g L⁻¹). Area ratios were determined by integration of the area of an analyte under the specific chromatograms in reference to the integrated area of the internal standard.

2.6 Data analysis

Data processing was performed using Masslynx, Quanlynx V4.1 (RIKILT; Waters, Milford, MA, USA) and Xcalibur™ 2.0.7 software (LCA; Thermo Fisher Scientific, San José, USA). All data were statistically interpreted using 2-way ANOVA, using the statistical software package S-PLUS 8.0 (Seattle, WA, USA).

The level of significance was set at 5 %, where the null hypothesis will be rejected if the obtained p-value is below the significance level of 0.05.

3. Results and discussion

3.1 Spiked urines samples

To determine the stability of thyreostatic drugs in urine and establish the matrix-related variables, analyses of spiked urine samples of bovine and porcine origin were performed by RIKILT. The thyreostatic drugs investigated, comprised TAP, TU, BTU, MTU, MBI, PTU, and PhTU and were spiked as a mixture. Different spike levels were used, individually chosen per experiment between the range of the provisional MRPL ($100 \mu\text{g L}^{-1}$) and the RC ($10 \mu\text{g L}^{-1}$), to display the most beneficial kinetics.

3.1.1 *Stability of thyreostats in urine*

During a first experiment the stability of thyreostatic drugs was investigated in urine during preservation in the freezer (-70°C). Aliquots of 1 mL of bovine and porcine urine, but also of water ($n = 4$) were spiked at $20 \mu\text{g L}^{-1}$ and stored at -70°C . Each sample underwent 1, 2, 3 or 4 freeze-thaw cycles with a defrosting period of 3 hours during 4 consecutive days. A significant decrease in thyreostat concentration could be noted, which showed a correlation with the increase of number of freeze-thaw cycles, for TAP this effect was less pronounced (Figure III.1). Additionally, a significant matrix effect of urine could be observed [17]. Indeed, it appeared that the urine matrix was responsible for the decrease in thyreostat concentration, since the concentration of analytes remained stable in water. Even more, the observed losses were more pronounced in bovine than in porcine urine (Figure III.1). Noticeable however was that upon addition of 3-IBBr derivatised thyreostats to blank urine, limited losses were observed during preservation. Moreover, with the incorporation of 4 freeze-thaw cycles, the losses of all analytes were below 10 %, except for BTU and TAP, with 36.3 and 39.2 % respectively. Whereas without derivatisation, losses between 54.8 and 97.4 % were obtained, These results stress the stability of the derivatised thyreostats as opposed to the original underderivatised compounds.

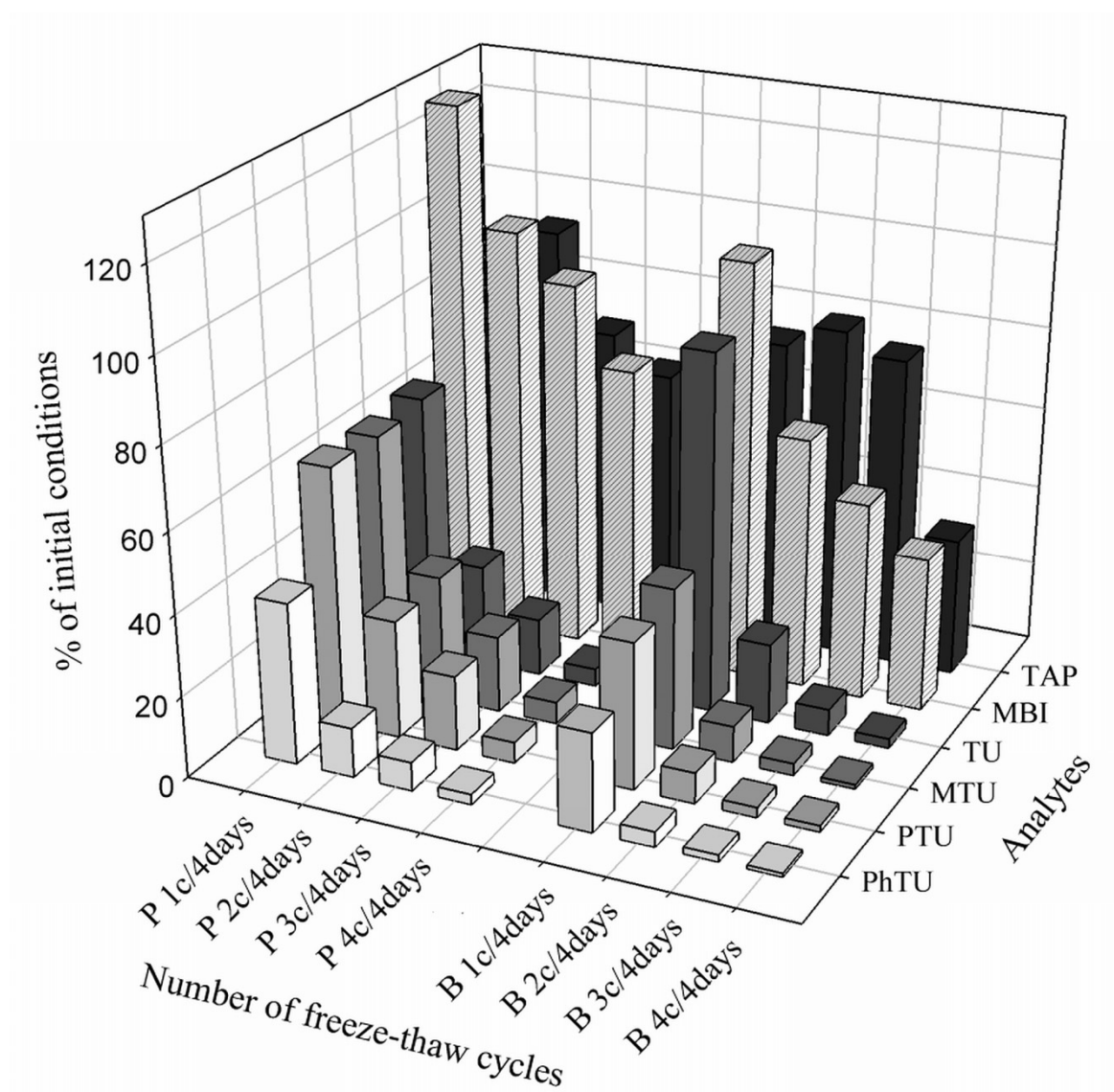


Figure III.1: The effect of the number of freeze-thaw cycles (1, 2, 3, or 4) in bovine (B) and porcine (P) urine on the thyreostat concentration (% of initial conditions), spiked at $20 \mu\text{g L}^{-1}$, performed during 4 consecutive days.

3.1.2 Study of the matrix-related variables

Besides the insight in the rate of decrease of thyreostat concentration in urine, the mechanism behind this noticeable decrease was of interest as well. Possible causes including pH, daylight, enzyme activity, salt, and metal ion concentration (Cu^{2+}) were kept under consideration [18,19]. The above described experiment (section 3.1.1) indicated a higher loss in bovine than in porcine urine. For this reason, subsequent experiments were conducted with bovine urine to simulate the 'worst-case scenario'.

To monitor the decrease in thyreostat concentration, bovine urine samples (10 mL) were spiked with all thyreostats at $20 \mu\text{g L}^{-1}$ and kept at room temperature for 8 hours. Every hour one mL aliquot was taken, prepared, derivatised, and analysed. This experiment indicated a linear decrease in time of the thyreostatic drugs in bovine urine. A correlation coefficient (R^2), which represented the area ratio of the analyte plotted against its concentration level, higher than 0.91 was obtained for all analytes except for TAP, for which a R^2 of 0.82 was calculated. All analytes displayed a concentration decrease per hour of 10.0 to 12.2 %, while for MBI this only amounted to 6.0 %.

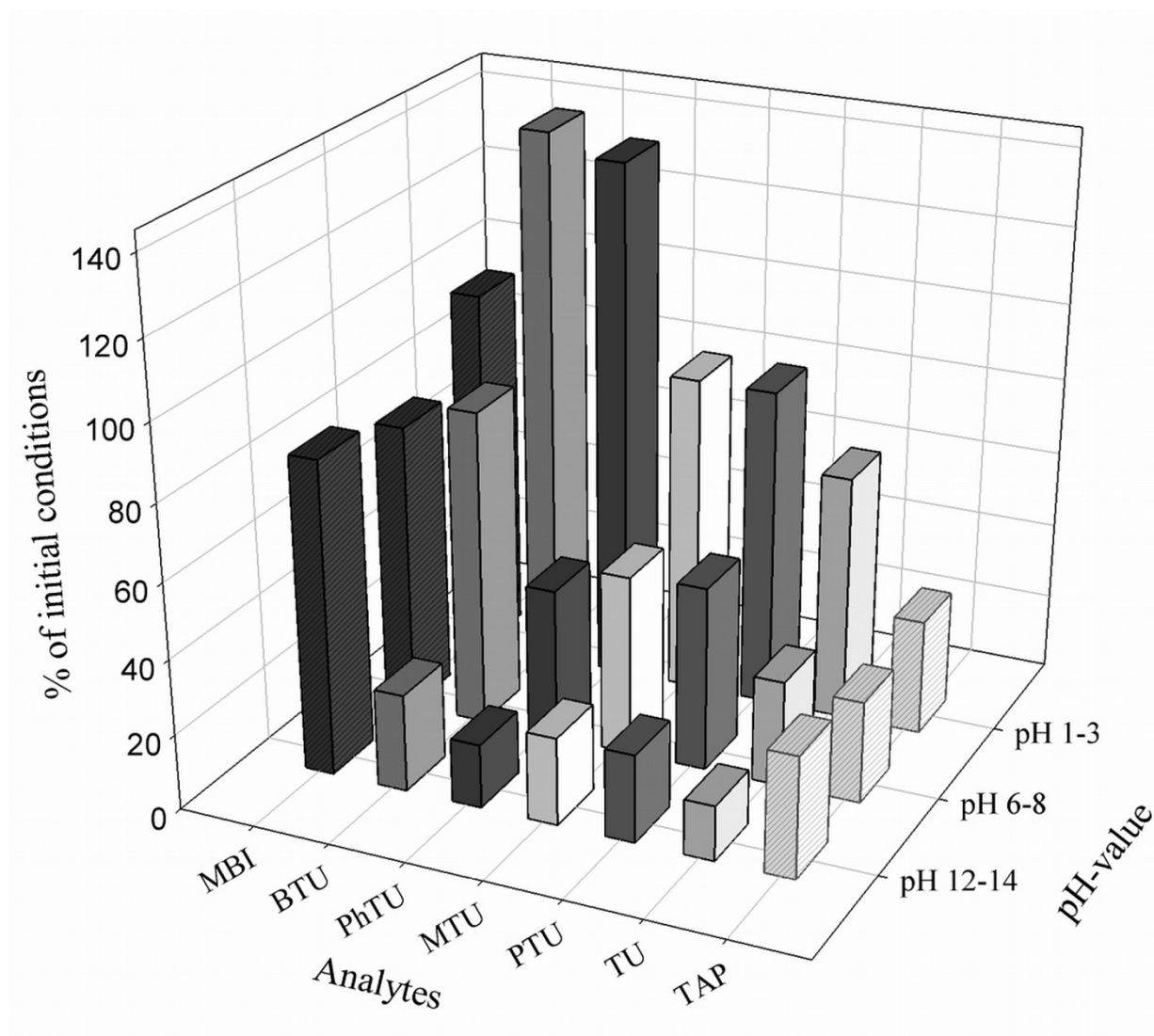


Figure III.2: The effect of different pH-values on the thyreostat concentration ($20 \mu\text{g L}^{-1}$) in bovine urine when left 8 hours at room temperature before clean-up, 3-IBBr derivatisation, and UPLC-MS/MS analysis.

For investigating the matrix-related variables behind the decrease in thyreostat concentration, spiked ($20 \mu\text{g L}^{-1}$) urine aliquots of 1 mL were placed 8 hours at room temperature, before conducting sample

clean-up and UPLC-MS/MS analysis. Several variables were evaluated to this end, namely daylight vs. darkness, pH 1-3 vs. pH 7-8 vs. pH 12-14, boiling (8 min) to inhibit enzyme activity, 1 M NaCl and a blank. The effect of daylight, boiling, and salt addition proved not to be significant ($p > 0.050$). The impact of the pH-value however was significant ($p = 0.002$), in particular for thiouracil and its analogues (Figure III.2). At lower pH-values, the highest concentration was retrieved for all analytes. In general, the analytes remained stable at pH 1-3, only thiouracil still displayed losses up to 35 %. The effects of 1 M CuSO_4 and 1 M Na_2SO_4 were investigated in water samples to exclude the interfering effect of ions inherent to urine. The addition of copper (II) caused a significant decrease ($p = 0.001$) in analyte concentration, for thiouracil and analogues a loss of more than 80 % was noted, for TAP and MBI this amounted around 50 %. The interaction between the thiol group of the thyreostats and copper ions or other metal ions is generally well known [1, 19-21].

3.1.3 *Increasing stability of thyreostatic drugs in urine*

Subsequent experiments were intended to elucidate of the parameters that would maintain or prolong the stability of thyreostats in urine during preservation and also to investigate the effectiveness of the possible pre-treatment steps. The above described experiments (section 3.1.2) demonstrated that a pH-value greater than 3 and the presence of copper significantly influenced the loss of thyreostatic drugs in urine. For this reason, the pre-treatment consisted of a fixed pH-value of 1 and the addition of the chelating agent ethylenediaminetetraacetate acid (EDTA), which inhibits the interaction between copper ions and thyreostats by scavenging these ions.

To investigate the effectiveness of this pre-treatment, additional experiments with freeze-thaw cycles were conducted. Four blank bovine urines (5 mL) were set at pH 1, 7, 13 or supplemented with 0.1 M EDTA (0.1 mL). Each pre-treated sample was divided in 4 aliquots of 1 mL, and subsequently spiked at $100 \mu\text{g L}^{-1}$ with a mixture of 7 thyreostats and internal standard. These samples were frozen at -70°C and underwent 1, 2, 3, or 4 freeze-thaw cycles (defrosted during 3 hours) during 4 consecutive days. This experiment demonstrated that the thyreostatic concentration was more stable at pH 1 and that the addition of EDTA also assisted in the process (Figure III.3) [18].

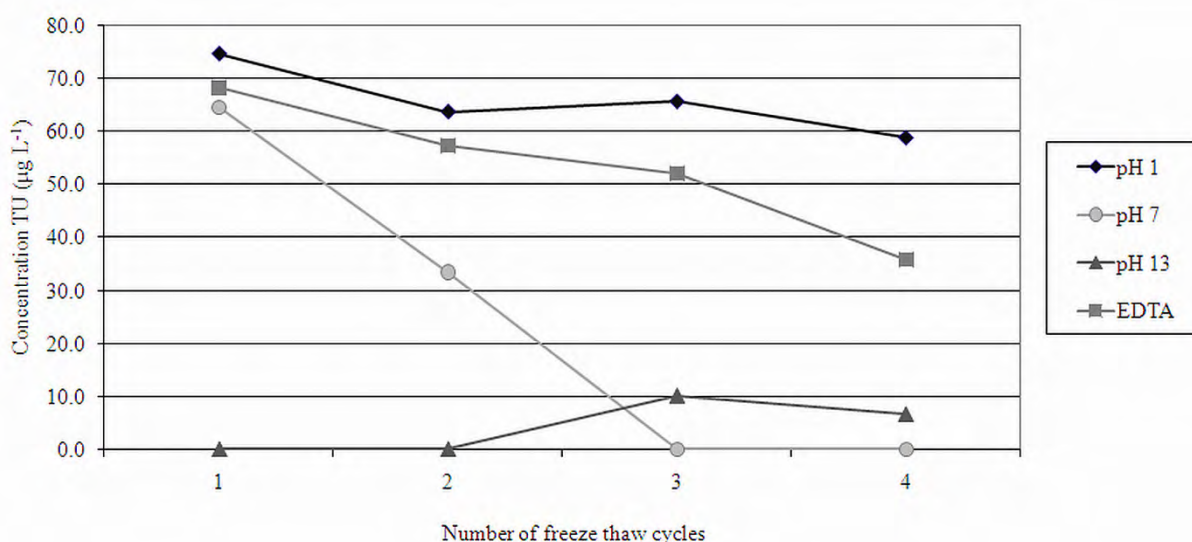


Figure III.3: The effect of different pre-treatments on the concentration ($\mu\text{g L}^{-1}$) of the thyreostatic drug, thiouracil in bovine urine upon storage at $-70\text{ }^{\circ}\text{C}$ and upon exposure to a number of freeze-thaw cycles, before UPLC-MS/MS analysis.

Next, the stability of thyreostats in pre-treated urine (pH 1 and 0.1 mL of 0.1 M EDTA) was compared to untreated urine, this on a short and long-term basis. For the short-term stability, the losses in thyreostatic concentrations at room temperature were followed up during 24 hours. To this extent, ten different bovine urines were selected (20 mL each), and divided in two aliquots of 10 mL, one portion unaltered, the other set at pH 1 and supplemented with 0.1 mL of 0.1 M EDTA. Spike levels were set at $50\text{ }\mu\text{g L}^{-1}$ and 1 mL aliquots were taken, prepared, derivatised, and analysed at 0, 2, 4, 6, 8, and 24 h. The results demonstrated that the pre-treatment of pH 1 and addition of EDTA stopped or delayed the decrease of thyreostats for 24 h at room temperature, with the exception of TAP for which a significant loss was observed between 8 and 24 h (Table III.3).

To evaluate the long-term stability, bovine urine samples were frozen and analysed once every month, this during 3 months. Again untreated urine was compared to pre-treated urine (pH 1 and EDTA), but also storage time versus the number of freeze-thaw cycles were included as variables in the set-up. To this end, bovine urine (untreated and pre-treated) was either frozen in bulk and underwent the freeze-thaw cycles in bulk, or was frozen in separate aliquots of 1 mL that only endured 1 freeze-thaw cycle. During this long-term stability experiment samples were prepared, derivatised, and analysed after 0, 1, 2, and 3 months. No significant difference ($p > 0.050$) was observed between the behaviour of bulk urines or separate aliquots (Table III.4). The number of freeze-thaw cycles on a long-term basis proved

to be of lesser significance than the time of preservation. As for the effect of pre-treatment, setting the bovine urine at pH 1 and adding 0.1 mL of 0.1 M EDTA clearly delayed the losses in thyreostat concentrations for 3 months at -70 °C. TAP again formed an exception, although its decrease in concentration was delayed in common with the other thyreostats, after 3 months the concentration of the pre-treated urine was similar to that of the untreated sample.

Table III.3: Short-term stability of thyreostatic drug concentration ($\mu\text{g L}^{-1} \pm \text{S.D.}$) in bovine urine ($n = 10$) compared to identical urines with a pre-treatment (pH 1 and 0.1 mL of 0.1 M EDTA), both spiked at $50 \mu\text{g L}^{-1}$ and kept for 0, 2, 4, 8 and 24 hours at room temperature before sample preparation and UPLC-MS/MS analysis.

Analyte	Untreated						Pre-treated					
	t_0	t_2	t_4	t_6	t_8	t_{24}	t_0	t_2	t_4	t_6	t_8	t_{24}
TAP	22.1 ± 6.5^a	23.0 ± 3.2^a	16.4 ± 2.5^{abc}	17.0 ± 5.0^{ab}	12.8 ± 3.9^{bc}	9.1 ± 8.5^c	20.2 ± 6.1^d	22.9 ± 9.5^d	22.7 ± 8.6^d	19.8 ± 8.1^d	21.0 ± 8.1^d	15.5 ± 16.3^c
TU	15.4 ± 5.4^a	9.3 ± 4.1^b	4.4 ± 3.7^c	2.5 ± 2.6^c	1.3 ± 1.5^c	0.3 ± 0.4^c	12.2 ± 5.8^d	12.3 ± 4.5^d	14.5 ± 5.4^d	13.0 ± 5.9^d	13.7 ± 6.5^d	14.8 ± 6.8^d
BTU	20.1 ± 5.8^a	10.5 ± 4.9^{bc}	7.2 ± 4.9^c	3.6 ± 3.9^{cd}	1.7 ± 1.7^{cd}	0.8 ± 0.9^d	23.0 ± 7.2^e	25.4 ± 10.4^e	20.4 ± 10.6^e	24.6 ± 9.1^e	22.2 ± 11.7^e	23.7 ± 6.0^e
MTU	19.3 ± 5.0^a	14.7 ± 2.8^b	8.3 ± 3.7^c	5.2 ± 3.6^{cd}	2.9 ± 2.4^{de}	0.7 ± 1.1^e	14.8 ± 5.4^f	14.3 ± 4.2^f	16.8 ± 4.6^f	16.2 ± 5.5^f	16.8 ± 6.3^f	17.1 ± 6.5^f
MBI	28.5 ± 7.1^a	27.5 ± 4.6^{ab}	19.5 ± 5.7^{bc}	15.6 ± 6.1^{cd}	11.6 ± 6.0^{cd}	7.9 ± 8.8^d	35.2 ± 5.7^e	37.0 ± 6.9^e	38.7 ± 8.7^e	36.1 ± 8.8^e	30.6 ± 9.1^e	26.9 ± 16.3^f
PTU	20.7 ± 4.1^a	15.1 ± 6.1^b	9.6 ± 5.7^c	5.5 ± 4.5^{cd}	2.9 ± 2.8^d	0.8 ± 1.1^d	16.9 ± 4.8^e	18.7 ± 5.6^e	18.5 ± 5.0^e	19.2 ± 5.2^e	17.4 ± 4.7^e	18.9 ± 4.2^e
PhTU	19.9 ± 6.4^a	7.9 ± 3.9^b	5.2 ± 4.2^{bc}	2.6 ± 2.8^c	1.3 ± 1.2^c	0.7 ± 0.7^c	26.8 ± 9.2^d	29.3 ± 13.4^d	23.9 ± 15.1^d	30.3 ± 12.1^d	27.7 ± 16.3^d	26.6 ± 6.2^d

a, b, c, d, e, f: Means within a row with a different superscript differ ($p\text{-value} < 0.05$)

Table III. 4: Long term stability of thyreostatic drug concentration ($\mu\text{g L}^{-1}$) in bovine urine, with individual portions compared to bulk additional to the untreated versus pre-treated portions (pH 1 and 0.1 mL of 0.1 M EDTA), with a spike level of $100 \mu\text{g L}^{-1}$ and kept during 3 months at -70°C . At 0, 1, 2 and 3 months samples were prepared for UPLC-MS/MS analysis.

Analyte	Untreated ($\mu\text{g L}^{-1}$)								Pre-treated ($\mu\text{g L}^{-1}$)							
	Portion				Bulk				Portion				Bulk			
	t_0	t_1	t_2	t_3	t_0	t_1	t_2	t_3	t_0	t_1	t_2	t_3	t_0	t_1	t_2	t_3
TAP	147.8	25.3	6.8	34.4	175.8	11.0	7.5	11.9	120.9	80.1	37.7	52.7	118.3	92.9	49.5	41.9
TU	93.6	15.9	0.8	9.4	102.0	3.3	0.8	0.0	76.7	71.3	46.9	48.9	68.0	87.6	58.6	72.5
BTU	90.7	26.6	4.2	34.1	92.5	13.3	4.8	6.0	114.1	99.3	55.7	56.1	113.1	105.5	86.1	84.8
MTU	89.4	52.7	9.0	33.5	88.6	30.3	11.9	6.4	74.5	71.9	50.1	48.8	66.1	73.4	60.4	63.5
MBI	65.2	1.8	0.4	4.4	71.3	0.5	0.5	1.6	83.4	82.6	27.2	63.2	74.5	61.5	41.8	48.3
PTU	77.2	48.0	9.2	43.4	78.6	28.6	12.8	9.1	64.3	60.8	59.4	59.3	66.3	70.7	73.5	74.7
PhTU	90.7	26.6	4.2	38.9	92.5	13.3	4.8	6.4	114.1	99.3	55.7	76.0	113.1	105.5	86.1	102.0

3.2 Incurred samples

In light of the national control plan, LCA (Ghent University, Belgium) performed LC-MS² analyses of incurred urine samples for thyreostatic drugs. In the event of non-fraudulent action, residue analysis of these samples should lead to compliant results. In case residue traces are detected, their concentrations should be below the RC of 10 µg L⁻¹. Nevertheless, these compliant samples could still be considered as positive, if the detected residue concentration is higher than the decision limit (CC_α) and when all criteria are fulfilled as stated in EC/2002/657 [12]. In recent years, many compliant urines samples were found positive for thiouracil at concentration levels below the RC [8,10,14], which in turn has raised the question of a semi-endogenous status of thiouracil [14,15]. For this reason, only results of thiouracil could be presented in this section, since all other thyreostats monitored were absent. For residue analysis, it is also of importance to observe the behaviour and stability of low-level thiouracil (< 10 µg L⁻¹) [11] in urine and investigate the benefit and necessity of conducting a pre-treatment (pH = 1 and EDTA).

In this section, the behaviour of thiouracil in bovine and porcine urine was not compared, because the number of urines was too limited. The urine samples, bovine (n = 13) as well as porcine (n = 10) were supplied in two aliquots to our laboratory, one untreated and one pre-treated (pH 1 and 0.1 mL of 0.1 M EDTA). Immediately upon arrival, 1 mL of each aliquot of all samples was prepared, derivatised, and analysed by LC-MS². The remainder of the aliquots was divided in a smaller portion (2 mL), which was subsequently stored at -20 °C and re-analysed after 5 months of preservation, and a larger portion (10 mL). The latter was also stored at -20 °C, but reanalysed every month during 5 months to monitor the effect of the number of freeze-thaw cycles.

Table III.5: LC-MS² analysis upon arrival (t = 0 months) of the incurred urine samples for low-level thiouracil concentration, comparing untreated to pre-treated (pH 1 and 0.1 mL of 0.1 M EDTA) urine samples.

Thiouracil (3-IBBr)	Bovine (n = 13)		Porcine (n = 10)	
	Untreated	Pre-treated	Untreated	Pre-treated
N° of samples positive for TU	3	5	7	9
Mean concentration (µg L ⁻¹)	1.1 ± 0.1	5.1 ± 3.0	2.4 ± 0.8	2.5 ± 1.4

The analysis upon arrival, already indicated a beneficial effect of the pre-treatment (pH 1 and 0.1 mL of 0.1 M EDTA), as depicted in Table III.5. The number of samples in which TU was detected was higher in case of pre-treatment. To investigate the effect of the preservation time, all aliquots (10 mL) from bovine and porcine origin, untreated and pre-treated were re-analysed 5 additional times at 1, 2, 3, 4, and 5 months. A large decrease in TU concentration, both for untreated as well as for pre-treated urine, was noted after 1 month of preservation. Nevertheless, a significant difference ($p < 0.0001$) could be observed in TU concentration between the untreated and pre-treated samples. Even more, the pre-treatment of urine (pH 1 and EDTA) prolonged the detection of TU in time (Figure III.4). Additional analyses were performed to investigate the effect of the number of freeze-thaw cycles, this on the aliquots, who were preserved during 5 months ($-20\text{ }^{\circ}\text{C}$) without any freeze-thaw cycles. In this case however, significant differences ($p > 0.050$) could be detected in TU concentration as compared to an identical aliquot that underwent 6 cycles during 5 months at $-20\text{ }^{\circ}\text{C}$. Even more, the results displayed a pernicious effect of long-term storage for TU detection in urine, independent of the number of freeze-thaw cycles. This effect however was reduced when handling pre-treated samples (pH 1 and EDTA), nevertheless one month of preservation led to a 50 % reduction of the original detected concentration.

Addition of HCl (pH 1) and EDTA proved to be most beneficial for the stability of low-level TU in urine of bovine and porcine origin. It resulted in a higher detection rate, translated not only in a higher number of samples positive for TU, but also in an increased concentration. Even more, a prolonged detection in time was observed. As for the storage conditions, the time spent at storage appeared responsible for the decrease in thiouracil concentration, rather than the number of freeze-thaw cycles, which is valuable information for transportation means (i.e. from sampling location to the analytical laboratory, etc.). These results imply that storage time should be kept to a minimum. For future purposes, it might be of interest to investigate the elimination kinetics of thyreostatic drugs in non-compliant urine samples, to establish a maximum duration on the storage time in case of confirmatory analysis.

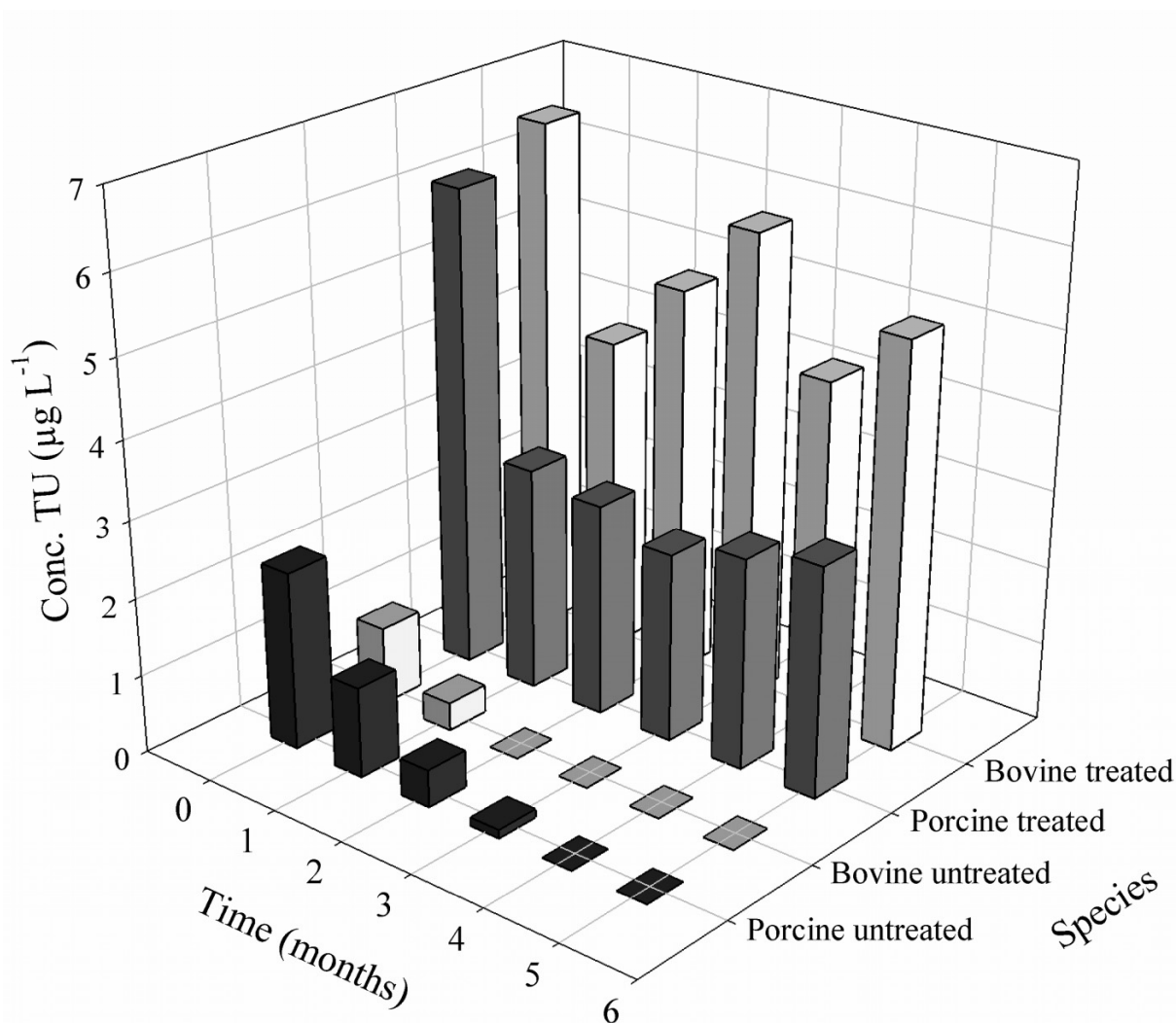


Figure III.4: The effect of pre-treatment (pH 1 and 0.1 mL of 0.1 M EDTA) on the stability of low-level thiouracil concentrations of incurred bovine ($n = 1$) and porcine ($n = 1$) urine samples during a 5 month preservation at $-20\text{ }^{\circ}\text{C}$, with a monthly LC-MS² analysis

4. Conclusion

The present study investigated the stability of thyreostatic drugs, comprising TAP, TU, BTU, MTU, MBI, PTU, and PhTU in bovine as well as porcine urine. At room temperature a linear decrease was observed in time for all analytes. When preserved at $-70\text{ }^{\circ}\text{C}$ on a short-term basis (4 days) this decrease was inversely related to the increase in number of freeze-thaw cycles. On a long-term basis (5 months) however, the number of freeze-thaw cycles proved irrelevant for the decrease in thyreostats concentration. In this case, the time of preservation was correlated with the concentration decrease. Additionally, an effective pre-treatment was developed by adjusting the pH-value to 1 upon sampling

and supplementing 0.1 mL of 0.1 M of EDTA. This treatment delayed the decrease in thyreostat concentration in urine, and even prolonged its detection over time. However for the incurred urine samples, this decrease or disappearance in signal for the low-level thyreostat ($< 10 \mu\text{g L}^{-1}$), thiouracil was unavoidable with long-term preservation at $-20\text{ }^{\circ}\text{C}$, even with pre-treatment. This is important knowledge for conducting residue analysis for legislative as well as research purposes.

In the future, when analysing thyreostatic drugs in urine in light of national residue control plans, it is highly recommendable to conduct the suggested pre-treatment of pH 1 and EDTA upon sampling. Furthermore, it is advisable to limit the storage time of urine at room temperature, but also in freezer (-20 or $-70\text{ }^{\circ}\text{C}$) to a maximum of 3 months, which should be a feasible period for the executing laboratories.

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CHAPTER IV

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

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CHAPTER IV

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

Abstract:

Thiouracil (TU) belonged to the xenobiotic thyreostats, which are growth-promoting agents, illegally used in animal production. Recently, it has been reported that thiouracil is suspected to have a natural origin. The EURL guidance paper (2007) acknowledged this, by stating that thiouracil concentrations below $10 \mu\text{g L}^{-1}$ might have a natural origin derived from Brassicaceae consumption. The present research aimed at endorsing this possible natural occurrence. Urine samples of animals (livestock and domesticated) with known and unknown clinical backgrounds were analysed for thiouracil with a newly developed ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry analysis method without derivatisation. In addition, a small-scale 9-day human experiment with Brassicaceae vegetables was performed to investigate if this natural prevalence could be extrapolated to the human population.

The untreated animals displayed thiouracil concentrations below $10 \mu\text{g L}^{-1}$ acknowledging the alleged natural occurrence of thiouracil. As for the humans, in 66.7% of the urine samples thiouracil was found above the CC_α of $2.2 \mu\text{g L}^{-1}$. However the correlation with the Brassicaceae diet proved not significant ($p = 0.095$). Nevertheless, these results demonstrate the natural occurrence of thiouracil in urine of animals and humans. The exact origin of this natural thiouracil trace still needs to be identified.

1. Introduction

Thiouracil (TU) belonged to the group of xenobiotic thyreostats. These are orally active drugs, which upon administration disturb the normal metabolism of the thyroid gland by inhibiting the production of the hormones triiodothyronine and thyroxine [1, 2]. This goitrogenic activity may be attributed to the presence of a thiocarbamide group [3]. 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 gastro-intestinal tract [4, 5]. Consequently, these growth-promoting agents negatively affect the meat quality of treated animals. In addition, some xenobiotic thyreostats such as thiouracil (TU) are listed as compounds with teratogenic and carcinogenic properties and thus pose a possible human health risk (IARC) [6]. These arguments led in 1981 to a ban on the use of thyreostats for animal production in the European Union [7].

In recent years, questions have been raised with regard to the status of thiouracil. The onset of this was given in 2006 by Pinel et al., who reported a correlation between the supplementation of a Brassicaceae diet to cattle and the presence of TU in bovine urine [8]. This was considered as a first indication that thiouracil might have a natural origin. In December 2007, the European union of Reference Laboratories (EURLs) acknowledged this possibility by posting a guidance paper stating a recommended concentration (RC) of $10\text{ }\mu\text{g L}^{-1}$ for thiouracil in urine [9]. According to their opinion, all values below this RC could be linked to a natural origin. In general, for detecting thiouracil in urine at these low-level concentrations, LC-MS analysis based on the protocol of Pinel et al. (2005) was performed [10]. Prior to the analysis of TU, which is a small, amphoteric, and relatively polar molecule, derivatisation with 3-iodobenzylbromide (3-IBBr) was conducted to aid in extraction and detection [10-12]. However, the use of a derivatisation step may lead to possible false-positive results. To this purpose, an U-HPLC-MS/MS analysis procedure has recently been developed, allowing the detection of thiouracil in urine without derivatisation. This method proved able to detect TU well below the RC of $10\text{ }\mu\text{g L}^{-1}$, with a decision limit (CC_{α}) and detection capability (CC_{β}) of $2.2\text{ }\mu\text{g L}^{-1}$ and $3.0\text{ }\mu\text{g L}^{-1}$, respectively [13]. In addition, this newly developed method, without derivatisation, significantly reduced the likelihood of false-positive results, and was designed to assist in investigating the natural occurrence of TU.

The aim of the present study was to acknowledge the presence of thiouracil in urine of untreated livestock, this in the low-level range ($< 10 \mu\text{g L}^{-1}$). Therefore, field samples of various species were analysed using our newly developed method, to exclude possible false-positive results. If thiouracil is detected in these field samples, it concerns most likely, as stated by the EURL guidance paper, a contamination of natural origin derived from feed [9]. This poses yet another question, can this natural prevalence be extrapolated to the human population, and if so, is this then correlated to any dietary habits, possibly elucidating the natural origin? Therefore, this study also aimed at unravelling the presence of thiouracil in human urine and investigated a possible correlation with the administration of a Brassicaceae-containing diet. The generated data may also be of value for indirect risk assessment, regarding the body burden of TU. To this purpose, a small-scale experiment with six healthy volunteers was conducted, during which the volunteers were asked to consume Brassicaceae vegetables. The sampled urines were subsequently analysed for the presence of thiouracil by means of ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry (U-HPLC-MS/MS) [13].

2. Experimental

2.1 Reagents and chemicals

The chemical standard 2-thiouracil (TU) and deuterated internal standard 6-propyl-2-thiouracil-D5 (PTU-D5) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Toronto Research Chemicals Inc. (Toronto, Canada), respectively. Stock solutions were prepared in methanol at a concentration of $200 \text{ ng } \mu\text{L}^{-1}$. Working solutions were prepared by $200\times$ and $2000\times$ dilutions in methanol ($1 \text{ ng } \mu\text{L}^{-1}$ and $0.1 \text{ ng } \mu\text{L}^{-1}$, respectively). When necessary, sonication was applied to ensure the complete dissolution of the substances. Solutions were stored in dark glass bottles at 7°C .

Reagents were of analytical grade when used for extraction, and of Optima® LC-MS grade when used for U-HPLC-MS/MS analysis. They were obtained from VWR International (Merck, Darmstadt, Germany) and Fisher Scientific (Loughborough, UK), respectively. Phosphate buffer, dissolved in deionised water, was made up from $0.5 \text{ M Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ and $0.5 \text{ M KH}_2\text{PO}_4$, adjusted to a pH of 7. For extraction purposes, the required amount of phosphate buffer, pH 7, was saturated with 1% of DL-dithiothreitol (DTT, purity 99%, Sigma-Aldrich, St. Louis, MO, USA).

2.2 Instrumentation

Separation of thyreostatic compounds was carried out at 35 °C on an Acquity HSS T3 column (High Strength Silica particles) (1.8 μm , 100 mm $\times$ 2.1 mm, Waters, Milford, MA, USA), coupled to an Accela U-HPLC pumping system (Thermo Fisher Scientific, San Jose, USA). In addition, an Acquity UPLC in-line filter (2.1 mm, 0.2 μm , Waters) was used to improve analytical column lifetime. The mobile phase constituted of 0.1 % aqueous formic acid and 0.1 % formic acid in methanol, and was pumped at a flow rate of 0.3 mL min⁻¹. Optimized separation of the analytes was obtained using a linear gradient starting with a mixture of 95 % aqueous formic acid and 5 % formic acid in methanol. After 1.65 min the amount of acidified methanol was increased to 100 % in 5.2 min and kept there for 0.5 min. Finally, the column was allowed to re-equilibrate for 2 min at initial conditions, this before each run.

Analysis was performed on a triple quadrupole mass analyser (TSQ Vantage, Thermo Fisher Scientific, San Jose, USA), fitted with a heated electrospray ionisation (HESI II) source operating in positive ion mode. The following working conditions were applied: spray voltage at 3.5 kV; vaporizer and capillary temperature at 370 and 300 °C, respectively; sheath and auxiliary gas at 40 and 20 arbitrary units (a.u.), respectively; cycle time of 0.8 s. Argon pressure in the collision cell (Q2) was set at 1.5 mTorr and the mass resolution at the first (Q1) and third (Q3) quadrupole was set at 0.7 Da at full width at half maximum (FWHM). Precursor ion, S-lens RF amplitude, and collision energy (CE) in Q2 were optimized individually per compound (Table IV.1). Quantification and confirmation data for thiouracil were acquired in the selected reaction monitoring (SRM) acquisition mode. The transitions followed for TU and PTU-D5 are also displayed in Table IV.1.

Instrument control and data processing were carried out by means of Xcalibur Software (2.0.7, Thermo Fisher Scientific, San José, USA). Additionally, data was statistically interpreted using ANOVA (S-PLUS, Seattle, WA, USA), level of significance was 5%.

Table IV.1: Collected SRM transitions and compound specific MS parameters (product ions in bold were used for quantification purposes).

Analyte	tR (min)	Precursor ion (<i>m/z</i>)	Product ion (<i>m/z</i>)	S-lens (RF amplitude) (V)	Collision energy (eV)
Thiouracil	1.6	129	112.1	49	15
			84.1		27
			60.1		34
			57.1		37
			159.2		17
Propylthiouracil- D5 ^a	5.4	176.1	117.2	62	19
			86.1		28
			60.1		34

^a Internal standard.

2.3 Samples livestock and domesticated animals

The urine samples were divided into two groups. The first group comprised bovine ($n = 222$), porcine ($n = 63$) and ovine urine samples ($n = 19$) obtained from veterinary sampling in light of the European residue control plan of Belgium and Norway. Upon arrival at laboratory, samples were stored at $-20\text{ }^{\circ}\text{C}$, and thawed before analysis. The second group consisted of animals (seven porcine, one bovine, one equine, and one canine urine sample) housed at the Faculty of Veterinary Medicine (Ghent, Belgium), with a known clinical background. All samples, taken in a non-invasive manner by a veterinarian, were stored at $-20\text{ }^{\circ}\text{C}$.

Prior to analysis, the samples were thawed and subsequently centrifuged for 10 min at $4000 \times g$, aliquots of 1 mL were used for the analytical procedure. To each sample, 50 ng of internal standard (PTU-D5) was added, to obtain a final concentration of $50\text{ }\mu\text{g L}^{-1}$. As for the spiked samples, a standard solution (0.1 or $1.0\text{ ng }\mu\text{L}^{-1}$) of thiouracil was added.

2.4 Human experiment

A small-scale 9-day study (D₀-D₈), which consisted of a control and test period that every volunteer had to endure, was performed with healthy female (n = 3) and male volunteers (n = 3), aged 27-33 years old. No pre-selection was conducted because a control period (D₀-D₂) was included. During this period, all volunteers were asked to refrain from any Brassicaceae vegetables or derivatives (e.g. mustard and rapeseed oil) consumption to foresee a natural baseline level and obtain a certain degree of volunteer screening. No other restrictions were imposed on the diet. This experimental design was based on two different studies both dealing with the conversion of glucosinolates after ingestion of a Brassicaceae vegetables [14-15]. During the test period (D₃-D₅), processed Brassicaceae vegetables (e.g. cauliflower, broccoli, and Savoy cabbage) were provided to the volunteers and they were asked to consume a minimum of 150 g at dinner. This was followed by a 3-day control period (D₆-D₈), during which the volunteers were asked for a second time to refrain from any Brassicaceae consumption as a follow-up to monitor the residual concentration of thiouracil [14].

Urine samples were collected twice a day by the volunteers, this during the whole experiment, and immediately frozen (-20 °C) upon sampling. This pilot study supplied commercially available food products in normal physiological quantities, and urine collection occurred in a non-invasive manner. Therefore, no authorization from the medical ethical committee was required [16].

2.5 Sample cleanup and U-HPLC-MS/MS analysis

The analytical protocol describing the sample clean-up and U-HPLC-MS/MS analysis has been published earlier [13]. Briefly, thiouracil contained in urine was reduced by 1% DTT at pH 7, this under denaturing conditions (30 min, 65 °C). Afterwards, a liquid/ liquid extraction was performed with ethyl acetate. This was followed by evaporation under nitrogen and dissolution of the dried extract (A/B, 90/10), with subsequent injection onto the U-HPLC system.

3. Results and discussion

3.1 Thiouracil in animal urine

In the framework of the national control plan of Belgium and Norway, our Laboratory (Ghent University, Belgium) frequently received urines of livestock for the routine analysis of thyreostats. These obtained urines, subsequently analysed never exceeded the recommended concentration for TU of $10 \mu\text{g L}^{-1}$. Nevertheless, 61.3% of the bovine urines obtained levels of TU below the RC, for porcine urine this was 96.3%, and for ovine urine 57.9% of the samples. The clinical background of these animals was unknown. Illegal administration for growth-promoting purposes however, seemed highly unlikely at these low concentrations [1, 17] and the possibility of a natural origin more plausible.

Next, urine sampled from different species with a thoroughly annotated clinical background, and no history of thyreostatic drug administration, was analysed. The experiment comprised seven porcine, one bovine, one equine, and one canine urine sample. All samples, besides the urine originating from the mare, displayed traces of thiouracil below $5 \mu\text{g L}^{-1}$. Thiouracil was identified according to the criteria of retention time, monitored transitions, and ion ratios as set by the EC/2002/657 [18]. Additionally, a co-chromatographic experiment was conducted to ascertain the presence of TU. Therefore, all untreated urines underwent a second analyses upon addition of TU at $5 \mu\text{g L}^{-1}$ (Figure IV.1). When TU was added an increase in signal was observed at the retention time corresponding to TU, confirming the identity of the analyte. This implies that thiouracil, i.e. below $10 \mu\text{g L}^{-1}$, was detected in urine of different animal species, who never received any thyreostatic treatment. Moreover, the collected urine samples were analysed with U-HPLC-MS/MS, significantly reducing the likelihood of possible false-positive results. Therefore, it may be concluded that thiouracil has a natural origin, most likely originating from the feed source [8]. These data, because of the incompleteness of possible influencing parameters, such as age and sex of the animal, type of feed, were not investigated for significant differences within the different species. Further research should however be performed to this purpose.

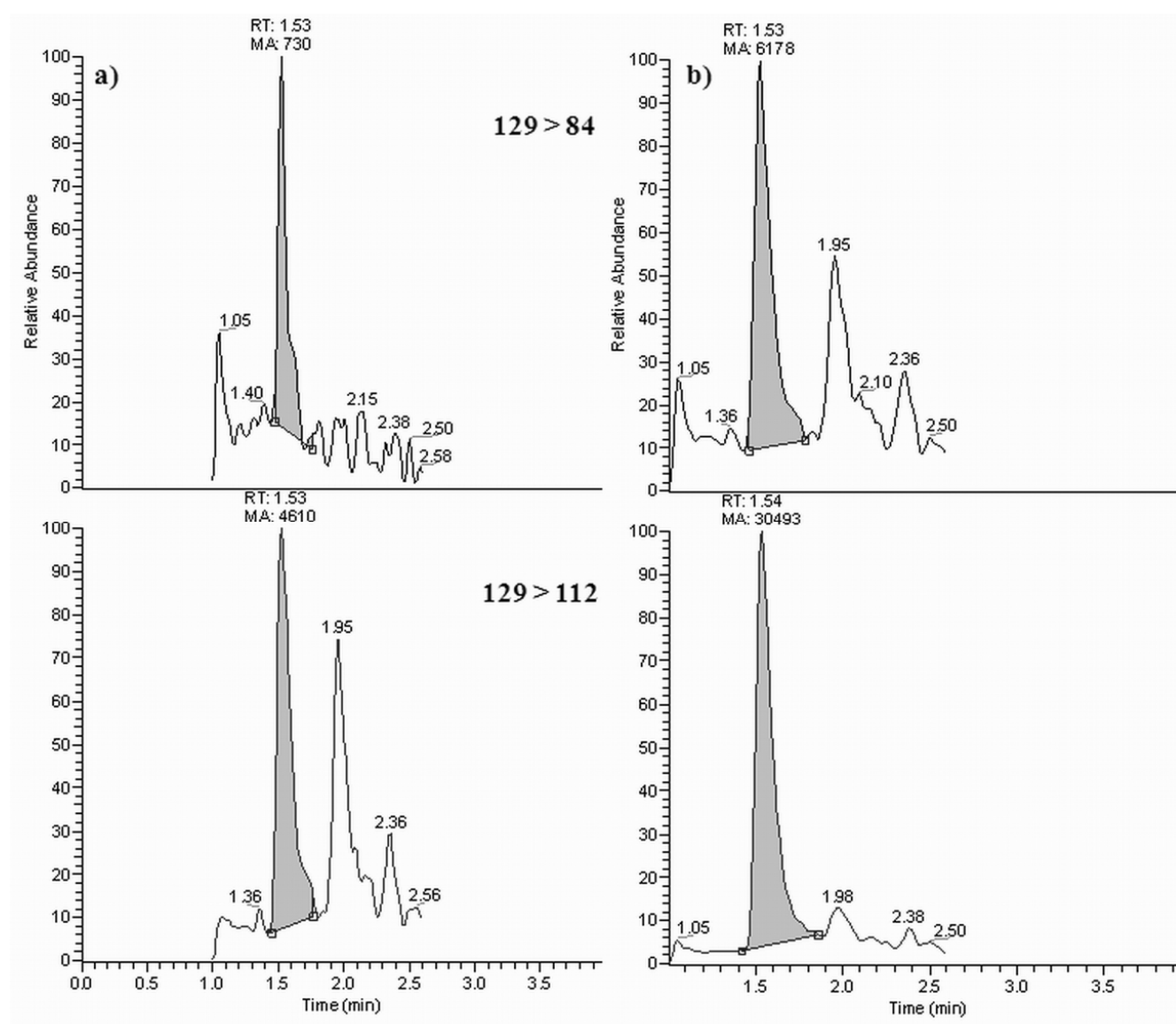


Figure IV.1: SRM chromatogram of (a) a bovine urine, and (b) the same urine spiked with $5 \mu\text{g L}^{-1}$ of thiouracil, after U-HPLC-MS/MS analysis.

3.2 Thiouracil in human urine

After submitting all obtained urine samples to U-HPLC-MS/MS analysis [13], it became clear that thiouracil was excreted by all healthy volunteers (Figure IV.2). In 66.7 % of the samples, concentrations higher than the CC_α -value ($2.2 \mu\text{g L}^{-1}$) were reached. By including two blank periods in the beginning and at the end of the treatment period, during which volunteers were asked to refrain from Brassicaceae vegetables and derivatives consumption, it was intended to display the elimination kinetics of thiouracil in human urine, as demonstrated by Pinel et al. (2006) in bovine urine [8].

However, a correlation between the presence of thiouracil in the urine and the Brassicaceae-rich diet could not be observed. Moreover, no significant differences in detected TU concentrations ($p > 0.05$) were demonstrated between the blank periods and the Brassicaceae period, although a p -value of 0.095 could be obtained. Additionally, it needs to be highlighted that the measured concentrations of TU for two male volunteers occasionally exceeded the recommended concentration of $10 \mu\text{g L}^{-1}$.

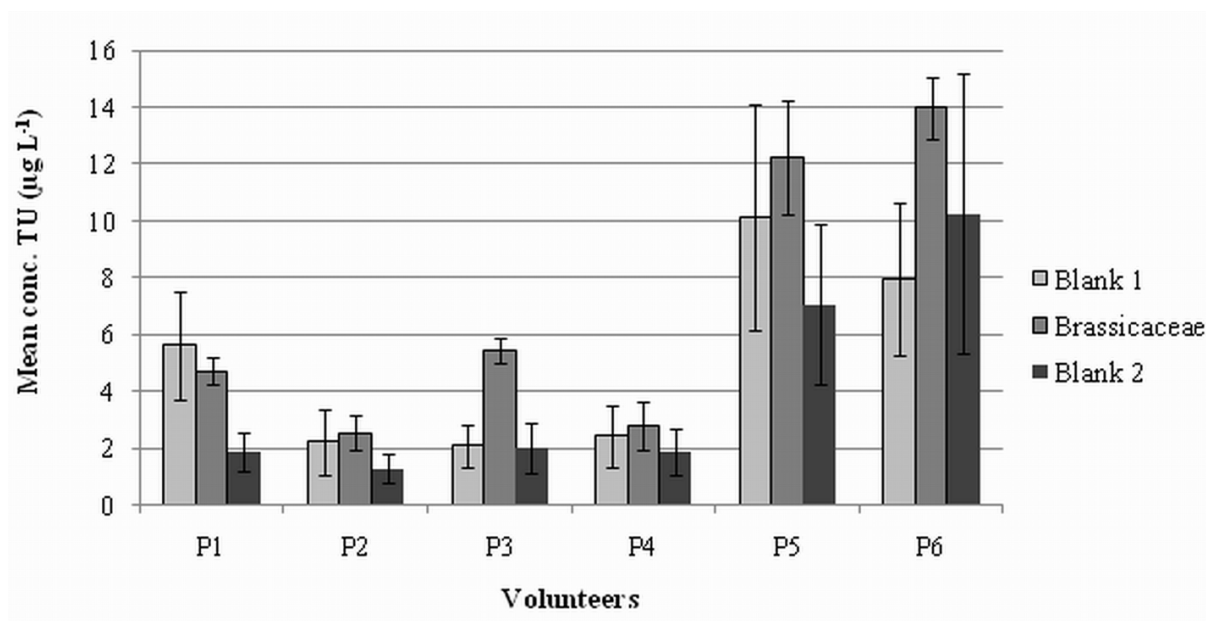


Figure IV.2: Graphic representation of the mean concentration of thiouracil ($\mu\text{g L}^{-1}$), with error bars representing the standard error of the mean detected, in feminine (P1-3) and masculine (P4-6) human urine, during a 9-day experiment that included two blank periods and a Brassicaceae-rich diet period.

Because of these unexpected results, co-chromatography was conducted on some samples, with spike levels of $5 \mu\text{g L}^{-1}$ of TU to confirm its presence (Figure IV.3). Moreover, six samples were re-analysed by LC-MS with the 3-IBBr derivatisation protocol [10], by two different laboratories (LCA, Ghent university, Belgium and LABERCA, ONIRIS, Nantes, France), which confirmed the obtained results and concentration levels. To the best of our knowledge this study is the first to report the presence of thiouracil in human urine.

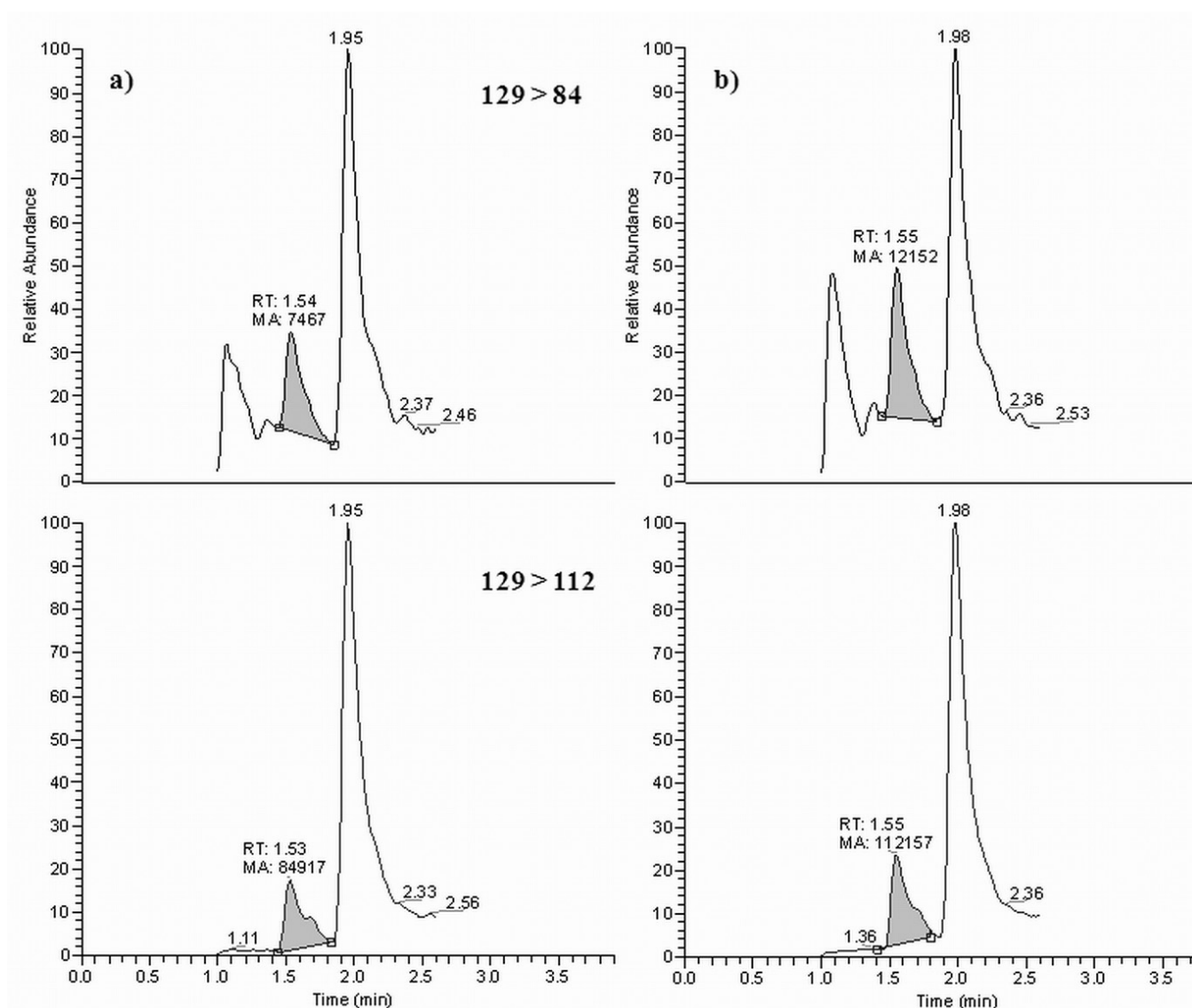


Figure IV.3: SRM chromatogram of (a) a human urine, and (b) the same urine spiked at 5 µg L⁻¹ of thiouracil by U-HPLC-MS/MS analysis.

All six volunteers were healthy subjects, with no record of a thyroid disease or thyreostatic treatment. Because of the high prevalence (66.7 %) of TU in the analysed urines, the presence appears to be intake-related. However the contamination source comprised more than only Brassicaceae vegetables and derivatives, which was enforced by the presence of TU in urine derived from the blank period (D₀-D₂). The additional sources of the contamination could however not be identified. The above mentioned results indicate the natural origin of thiouracil, that was detected in human urine.

4. Conclusion

In this work the thyreostatic compound, thiouracil was detected in the urine of untreated animal species, livestock as well as a domesticated animals, this below the recommended concentration of 10 $\mu\text{g L}^{-1}$. Traces of thiouracil below the recommended concentration are accepted by the European Union of Reference Laboratories as contaminations of natural origin. In our study the alimentation between the animals differed and was not controlled, therefore the contamination source of these traces could not be identified.

Even more, the obtained results were transferable to the human population. A small-scale experiment indicated the presence of TU in human urine, this in 66.7 % of the samples analysed. Noteworthy was that the values for two male volunteers sometimes even exceeded the recommended concentration of 10 $\mu\text{g L}^{-1}$. As for the contamination source, the Brassicaceae-rich diet did enforce the presence of the analyte, however in a non-significant way ($p = 0.095$). This provided a clear indication that the Brassicaceae diet was not the sole source of contamination for the naturally occurring thiouracil detected in human urine.

Finally, from these results it can be concluded that the alleged xenobiotic thyreostat, thiouracil can occur naturally. Up till now, its exact origin remains unknown, but evidence points towards a nutritional origin. Future work will focus on the elucidation of this exact source. In light of this manuscript, another question that requires proper investigation has surfaced with respect to: the impact of this low-level naturally occurring thiouracil trace on the functionality of the thyroid gland. Does it affect the thyroid hormone profile and as such may impose to a possible health risk? Or on the other hand, could thiouracil possibly be a biomarker indicating a perturbed function of the thyroid gland? At this point these questions remain unanswered and further investigation regarding the body burden of naturally occurring TU is required.

5. Acknowledgements

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CHAPTER V

FEED OR FOOD RESPONSIBLE FOR THE PRESENCE OF LOW-LEVEL THIOURACIL IN URINE OF LIVESTOCK AND HUMANS?

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CHAPTER V

FEED OR FOOD RESPONSIBLE FOR THE PRESENCE OF LOW-LEVEL THIOURACIL IN URINE OF LIVESTOCK AND HUMANS?

Abstract:

In recent years, questions have been raised on the possible semi-endogenous status of the alleged xenobiotic thyreostatic drug thiouracil. This, because thiouracil has been detected in urine of various animals (livestock and domesticated) at concentrations between 1 and 10 $\mu\text{g L}^{-1}$, and in humans. 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 urine of livestock and humans. To this purpose various Brassicaceae feed and food sources (e.g. rapeseed and 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, since without its catalyzed hydrolysis no thiouracil could be detected in the various Brassicaceae-derived samples. Therefore, a sample pre-treatment with incorporated enzymatic hydrolysis was developed after assuring the quality performance of the extracted myrosinase mixture with a single-point glucose assay.

Upon enzymatic hydrolysis and LC-MS² analysis, thiouracil was successfully detected in samples of traditional rapeseed, rapeseed '00'-variety coarse meal (values erucic acid < 2 % and glucosinolates < 25 $\mu\text{mol g}^{-1}$), and rapeseed cake at 1.5, 1.6, 0.4 $\mu\text{g kg}^{-1}$, respectively. As for the food samples, both broccoli as cauliflower displayed thiouracil concentrations of 6.0 $\mu\text{g kg}^{-1}$ and < 1.0 $\mu\text{g kg}^{-1}$, respectively. To the best of our 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 TU formation and identify its possible precursors.

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 (IARC) [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, faeces, meat, thyroid gland, etc.). As a guideline for these methods, the European Union set out a provisional minimum required performance limit (MRPL) of $100 \mu\text{g L}^{-1}$ or $\mu\text{g kg}^{-1}$. To achieve this, new confirmatory methods have been developed and ameliorated over time, as reviewed by Vanden Bussche et al. (2009) [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 ultra performance 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 $10 \mu\text{g L}^{-1}$ or $\mu\text{g kg}^{-1}$, as set out by the European Union of Reference Laboratories (EURL) in 2007 [10]. More recently, a U-HPLC-MS/MS method for thyreostats in urine without derivatisation was presented as well, also complying with the RC [11].

In recent years, however, thiouracil has occasionally been detected in urine of livestock at concentrations between the CC_α -value and $10 \mu\text{g L}^{-1}$. The detection of these residues has raised questions on its origin, namely synthetic or natural. Up to now, only few studies addressed this issue,

which is in scientific literature referred to as the possible semi-endogenous status of thiouracil. The onset was given by Pinel et al. (2006), 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 hydrolysed by the endogenous β -thioglucosidase plant enzyme, myrosinase (EC 3.2.1.147) [15-17]. This hydrolysis generates a range of biological active compounds, which are converted to derivatives 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. (2006) 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 did not confine to bovine urine alone. Urine of livestock (bovine, ovine, etc.), domesticated animals (canine) and even humans was subjected to U-HPLC-MS/MS analysis [23]. Most animals displayed traces of TU, this below the RC of $10 \mu\text{g L}^{-1}$. 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 urine of untreated species. So far however, the origin of this thiouracil signal in urine is still undefined.

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

2. Material and methods

2.1 Reagents and chemicals

The glucosinolate sinigrin ((-)-sinigrin hydrate, $\geq 99.0\%$; Sigma Aldrich, St-Louis, MO, USA), D(+)-glucose (Merck, Darmstadt, Germany), the chemical standard 2-thiouracil (TU) and internal standard 6-ethyl-2-thiouracil (ETU) were obtained from Sigma-Aldrich (St. Louis, MO, USA). 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 $200\text{ ng }\mu\text{L}^{-1}$. Working solutions were prepared by $200\times$ and $2000\times$ dilutions in methanol ($1\text{ ng }\mu\text{L}^{-1}$ and $0.1\text{ ng }\mu\text{L}^{-1}$, 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 $50\text{ }\mu\text{g L}^{-1}$ 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 UK, Loughborough, UK), respectively. The derivatisation reagent, 3-iodobenzylbromide (3-IBBr, Sigma-Aldrich, St-Louis, MO, USA), was prepared extemporaneously (2 mg per mL methanol). Phosphate buffer, made up of $0.2\text{ M Na}_2\text{HPO}_4$ and $0.2\text{ M KH}_2\text{PO}_4$ in deionised water, was prepared and adjusted to a pH of 8.

2.2 Preparation of myrosinase solution

For the enzymatic treatment of the feed and food samples, a myrosinase solution was prepared according to Wrede [24]. Five gram of yellow *Sinapsis alba* L. seed (white mustard seed), purchased at a local store was homogenised with 30 mL of water, stirred for 30 min at room temperature and centrifuged at $12\,000\times g$ for 10 min . The supernatant was mixed with an equal volume of 90% ethanol. The mixture was centrifuged, the precipitate 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 enzyme per mL solution [25]. For the hydrolysis, phosphate buffer pH 7 made up of $0.5\text{ M Na}_2\text{HPO}_4\cdot 2\text{H}_2\text{O}$ and $0.5\text{ M KH}_2\text{PO}_4$ was preferred, this was dissolved in deionised water.

Additionally, commercially available myrosinase purified from white mustard seeds (*Sinapsis alba* L.) was purchased at Sigma-Aldrich (thioglucosidase, 25 UN, St. Louis, MO, USA) 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 [26].

2.3 Instrumentation

2.3.1 LC-MS²

Separation of the thyreostatic compounds was achieved at 30 °C on a Symmetry C18 column (5 μm , 150 mm $\times$ 2.1 mm, Waters, Milford, MA, USA) 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, USA). 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, San Jose, USA) was used, fitted with a heated electrospray ionisation 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 (a.u.), respectively. The transitions monitored are displayed in table V.1.

Table V.1: Monitored transitions of the LC-MS² 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

2.3.2 HPLC-ELSD

For the detection of glucose, the samples were injected on a Prevail Carbohydrate ES column (5 μm , 250 mm x 4.6 mm, Grace Davison Discovery science, Deerfield, IL, USA) coupled to a HP Agilent 1100 HPLC system (California, USA). 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, Deerfield, IL, USA) operated at 40 °C, with a N₂-pressure of 4 bar, a nebulizer gas at a flow of 1.5 L min⁻¹, and a gain factor set at 4.

2.3.3 Data analysis

For both instrumental procedures data processing was performed using Xcalibur™ 2.0.7 software (LCA; Thermo Fisher Scientific, San José, USA).

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 easy obtainable source of nitrogen. For this purpose, only the '00'-varieties defined with low erucic acid (< 2 %) and glucosinolates (< 25 $\mu\text{mol g}^{-1}$) levels may be used [27]. 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 pre-treatments to investigate the influence of the myrosinase enzyme co-existing in the matrix (Table V.2). As pre-treatment, different combinations of grounding and inactivation through boiling (2 hours) were conducted [26, 28]. Since specimens of cabbage, cauliflower, and broccoli contain elevated levels of water (90 %), freeze-drying resulted in the concentration of matrix constituents, which 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 the internal standard PTU-D5 was added at a concentration of $50 \mu\text{g kg}^{-1}$.

2.5 Enzymatic hydrolysis

The most optimal conditions for myrosinase-catalysed hydrolysis were pH 7 and incubation at $37 \pm 1^\circ\text{C}$ [25]. 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 ($37 \pm 1^\circ\text{C}$) ultrasonic bath for 4 hours, and subsequently placed overnight in a oven at $37 \pm 1^\circ\text{C}$.

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 since this was the only commercially available purified glucosinolate. The first experimental set-up was performed to investigate if the extraction procedure of myrosinase from white mustard seeds was successful. For this purpose, 5 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, then 0.5 mL 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 of ascorbic acid, which has been reported to increase the enzymatic activity of the enzyme [30-31]. The samples subsequently underwent a 4 h incubation at $37 \pm 1^\circ\text{C}$, 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 of sinigrin and 1.67 mL of myrosinase solution, were incubated under identical conditions as during 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 analysed 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, samples first underwent the enzymatic hydrolysis as described under section 2.5. The next day prior to the analytical analysis, specimens were cooled down, passed over a filter paper and adjusted to pH 8 by adding 8 mL of phosphatic buffer. Afterwards derivatisation, extraction, and sample clean-up were performed which has already been published elsewhere [8]. Briefly, 400 μL of 3-IBBr (2 mg mL^{-1} MeOH) was added to the sample and placed at $40 \pm 1^\circ\text{C}$ (1 h). Next, the pH was adjusted to 3.6 ± 0.1 , followed by liquid/ liquid extraction with diethylether (3 x 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 analysed in six-fold, two procedure internal standards (IS) were added prior to enzymatic hydrolysis at a concentration of $50 \mu\text{g kg}^{-1}$ (PTU-D5 and ETU). To ensure that the IS did not degrade to our analyte of interest (thiouracil) during hydrolysis, every sample was additionally analysed in triplicate without adding the IS. Even more, each sample endured co-chromatography ($10 \mu\text{g kg}^{-1}$ 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 analytes concentration was 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 clean-up, 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-MS² system. After analysis of the unknown, the concentration of the identified analyte (thiouracil) was estimated by fitting its area ratio, χ_{unknown} in a calibration curve in water undergoing the extraction procedure. Based on this estimation, the other aliquot was spiked with a similar known concentration of the identified analyte (A). Final reconstitution of this aliquot was identical as that of the first aliquot. LC-MS² analysis of this aliquot resulted an area ratio of χ_{known} . Using the following formula, the unknown concentration (C_{unknown}) was calculated:

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

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

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

With:

C concentration

X area ratio

V volume

ρ concentration

m mass

A identified analyte

In addition, the recovery yield of the sample pre-treatment (hydrolysis, extraction, and clean-up) was taken in account, so analyte losses during this pre-treatment 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 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, this with a detection limit of 0.1 g L^{-1} was available in our laboratory [35].

The results of the first experimental set-up, 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 V.1. Adding 0.5 mL of myrosinase solution sufficed to obtain similar results as with the synthetic myrosinase (30 μL). As reported in 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 V.1), which may be the result of variation in weighing (8.9 %) and the limited sensitivity of our analytical scale below 0.1 mg.

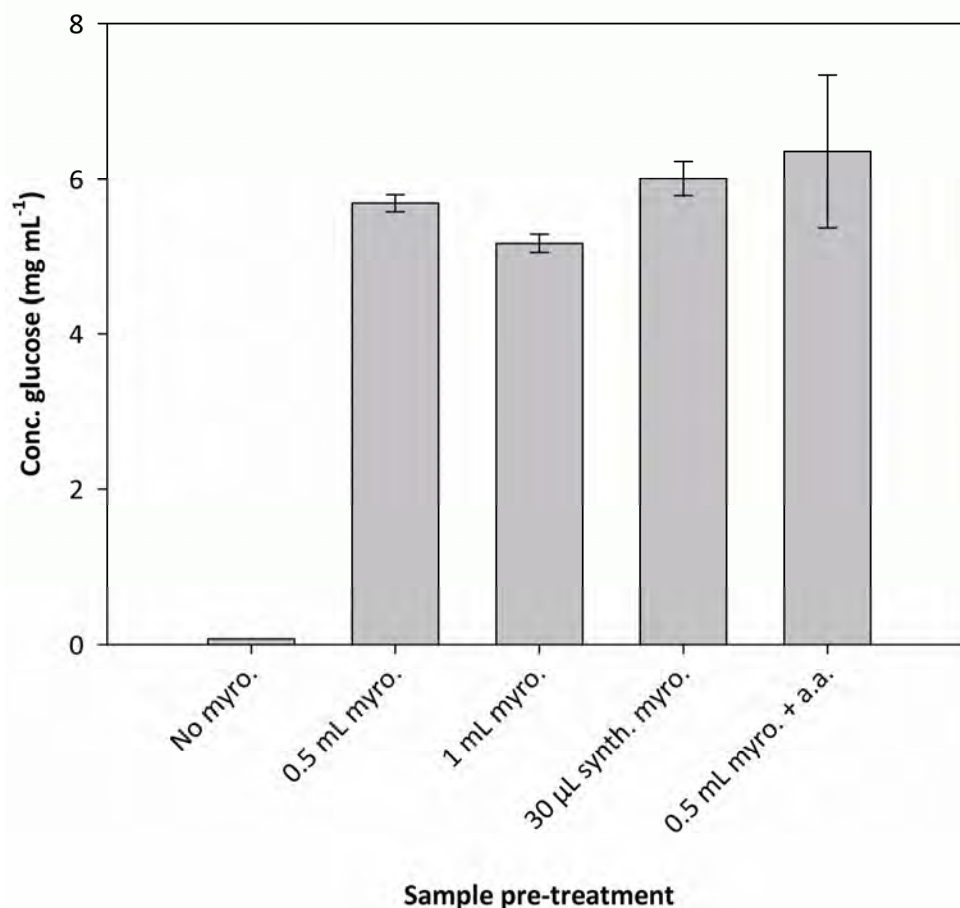


Figure V.1: Graphic representation of the calculated concentration of glucose by HPLC-ELSD, with error bars representing the standard deviation of the mean detected ($n = 4$). Glucose was formed as a by-product of the myrosinase (myro.) catalysed hydrolysis of sinigrin (34 mM). For no myrosinase treatment, the standard error bar is absent due to a too low value ($SD: 4.4 \times 10^{-5}$). (synth.: synthetic; a.a.: 2 mM ascorbic acid).

The second experiment investigated the kinetics of sinigrin hydrolysis, which reached its optimum after 1.5 h at 37 ± 1 °C. This hydrolysis rate was maintained till the end of the incubation (4 h) (Figure V.2). Noticeable was that the myrosinase-induced hydrolysis already occurred at room temperature (t_0), which is in line with previous reports [26, 29].

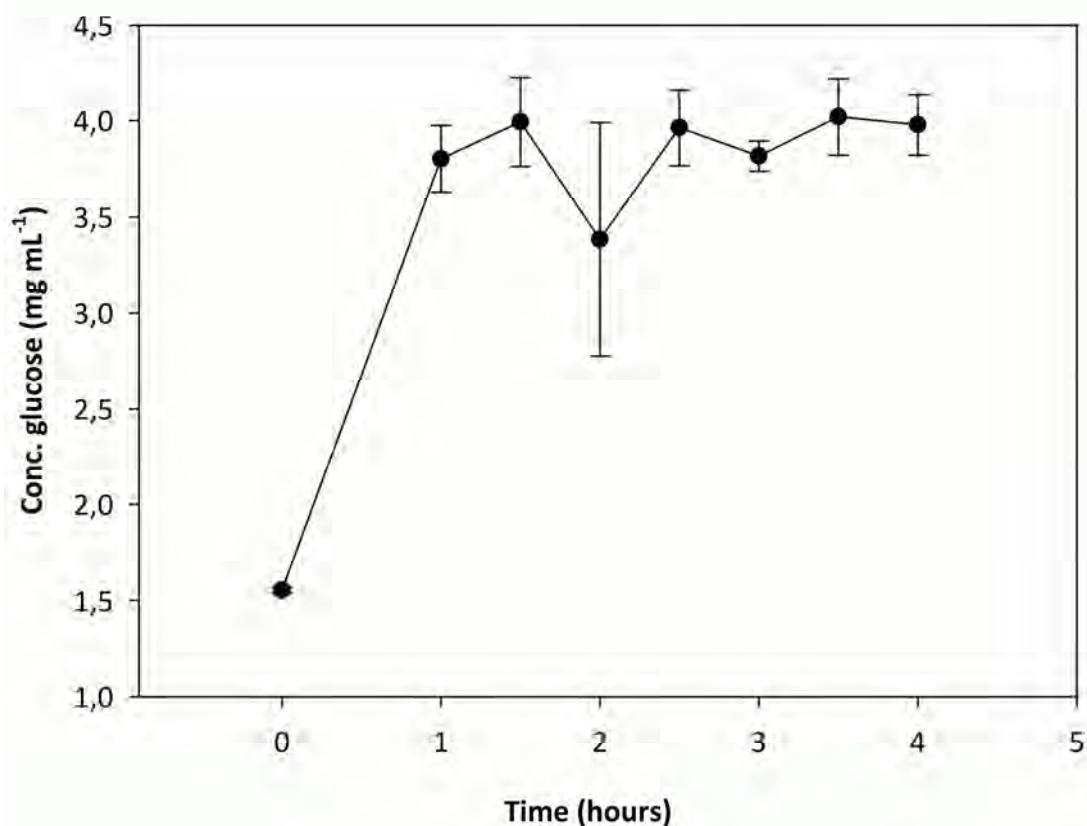


Figure V.2: Kinetics of the myrosinase-catalysed hydrolysis of sinigrin, by monitoring the release of glucose by HPLC-ELSD. Error bars correspond to standard errors on the mean, with $n = 3$.

3.2 Chemical analysis of Brassicaceae vegetables and derivatives

Upon purification, extraction, and analysis of a rapeseed cake according to Pinel et al. (2005) no thiouracil could be detected [8]. For this reason during the study, sample pre-treatment was successfully altered by incorporating an enzymatic hydrolysis catalysed by the myrosinase enzyme naturally prevalent in all Brassicaceae members. Re-analysis 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 TU. Because the enzyme was extracted from *Sinapsis alba* L., member of the Brassicaceae family, the extract was also analysed 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, though upon incubation at 37 °C,

TU could be discovered as well. This TU-residue was however detected to a lesser extent (Figure V.3), as compared to rapeseed meal that was exposed to a hydrolysis step with exogenously administrated 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 [26, 28] and the residual TU concentrations detected compared to those of raw samples. As for the rapeseed (*Brassica napus* L. partim Napoleon), different combinations of pre-treatment were conducted, i.e. grounding and inactivation by cooking. [26, 28, 34].

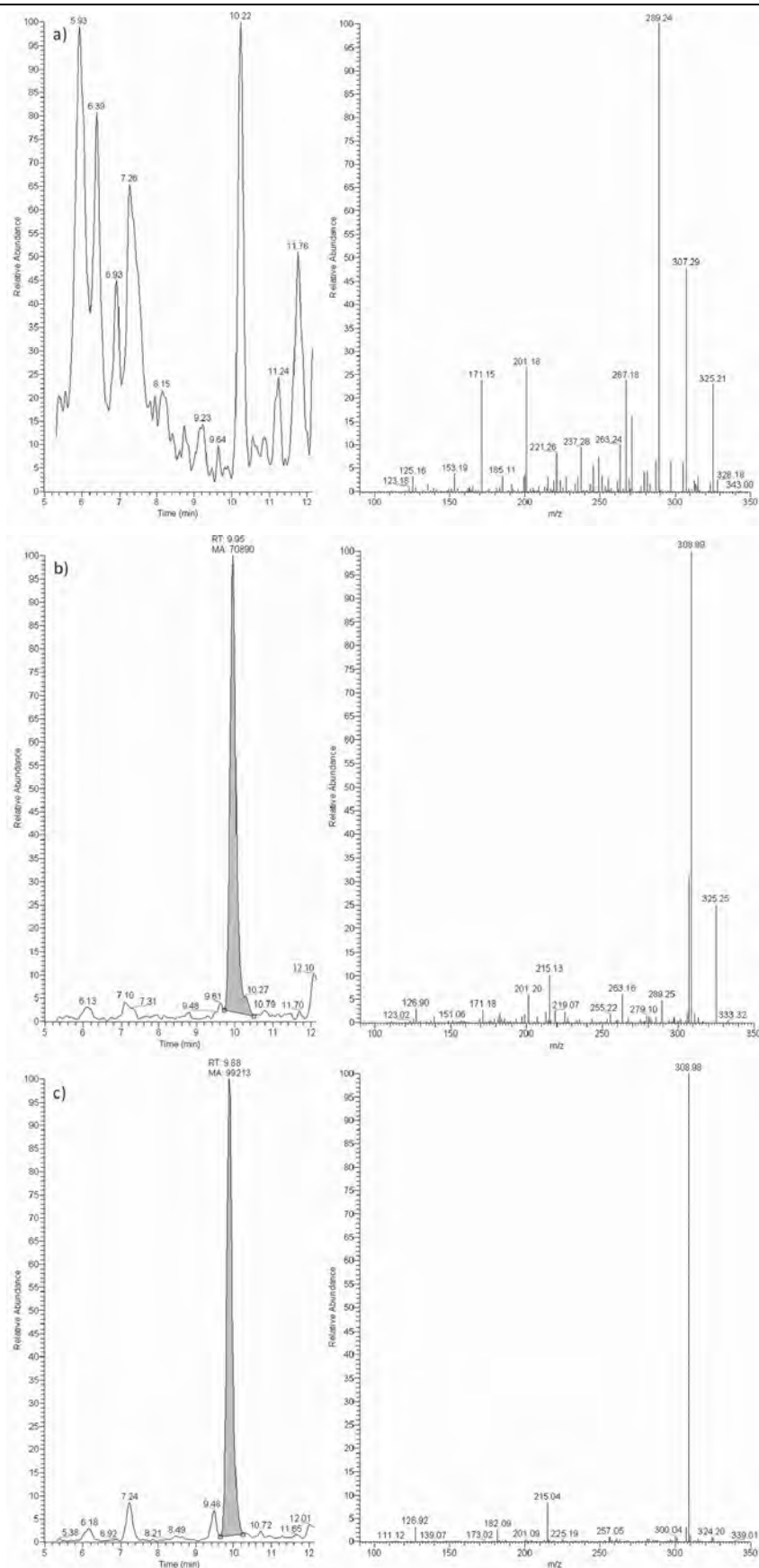


Figure V.3: LC-MS² chromatogram (and spectra) of identical rapeseed-'00' coarse meal samples with (a) no incubation, (b) incubation at 37 ± 1 °C for 4 hours, and (c) incubation at 37 ± 1 °C for 4 hours with myrosinase addition (0.5 mL).

After the initial optimisation of the enzymatic hydrolysis, all pre-treated feed and food samples were analysed by LC-MS². Within several samples traces of TU were detected, as presented in Table V.2. The obtained concentrations were calculated by means of a standard addition approach [33], with the recovery yield of the entire sample pre-treatment taken into account. Of all feed and food samples, broccoli obtained the highest concentration of TU at 6.0 $\mu\text{g kg}^{-1}$ (dry weight). These results show similar relative concentration differences in comparison to the oxazolidine-2-thiones content of rapeseed, broccoli and cauliflower [36], which provides another indication that TU follows a similar pathway of synthesis as the known naturally occurring thyreostatic drugs. Traditional rapeseed and rapeseed-‘00’ coarse meal also displayed significant concentrations of TU (Table V.2). As for the feeding cabbage and the meal with 30 % rapeseed-‘00’, a very high background noise was generated, to this extent that even spiked samples (10 $\mu\text{g kg}^{-1}$ TU) did not yield a detectable thiouracil signal. Other samples like cauliflower, rapeseed cake and most of the pre-treated traditional rapeseed demonstrated only low concentrations of TU (< 1.0 $\mu\text{g kg}^{-1}$). It must be mentioned that the levels of detected natural occurring thiouracil in these samples were in general lower than the retrieved signals in urine. For example, the rapeseed cake in which 0.4 $\mu\text{g thiouracil kg}^{-1}$ could be detected, was also administrated to a heifer by Pinel et al. (2006), which in turn resulted in urine samples found positive for thiouracil up to 9 $\mu\text{g L}^{-1}$ [12].

The obtained data in this study unambiguously prove the necessity of myrosinase catalysed hydrolysis for the detection of thiouracil in feed and food matrices of the Brassicaceae family. Therefore, it seems that thiouracil might follow a similar pathway of synthesis as the known naturally occurring thyreostats, i.e. oxazolidine-2-thiones, thiocyanates, i.e. hydrolysis of a precursor molecule present in Brassicaceae plants or vegetables. As for the different pre-treatments (grounding 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 was however that inactivated samples tended to generate higher TU signals. In light of the hypothesis that TU follows a similar pathway of synthesis as 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 by-product formed [13]. In 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 non-inactivated samples, however, contain residual endogenous myrosinase, which might prior to the sample pre-treatment already catalyse 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 by-products, resulting in a lower yield of thiouracil.

Table V.2: An overview of the various pre-treatments applied on the different feed and food samples, prior to extraction, clean-up, and LC-MS² analysis. Additionally, the concentration of thiouracil was calculated by mean of the standard addition approach, and corrected with the obtained recovery yield. (ND: not detected)

Sample	Pre-treatment			Concentration ($\mu\text{g kg}^{-1}$ dry weight)
	Freeze-Dry	Ground	Inactivation ^a	
Broccoli	+	+		5.7
	+	+	+	6.0
Cauliflower	+	+		0.9
	+	+	+	0.6
Feeding cabbage	+	+		ND
Rapeseed	+			0.4
	+	+		0.4
	+		+	1.5
	+	+	+	0.1
Rapeseed-‘00’ coarse meal	+	+		1.6
Rapeseed cake	+	+		0.4
Feed 30 % Rapeseed-‘00’	+	+		ND

^a: Inactivation by means of heating, i.e. 2 hours of boiling.

4. Conclusion

The present study investigated the possible origin of low-level thiouracil residues in the urine of various animals (livestock and domesticated) and humans. Scientific literature suggested Brassicaceae-derived feed as origin [12], which our study used as a starting-point. The impact of the Brassicaceae myrosinase enzyme appeared to be crucial, since without its catalyzed hydrolysis no thiouracil was detected in the various Brassicaceae-derived samples. Upon enzymatic hydrolysis, thiouracil could be detected in samples of traditional rapeseed, rapeseed-‘00’ coarse meal, and rapeseed cake at 1.5, 1.6, 0.4 $\mu\text{g kg}^{-1}$, respectively. As for the food samples, both broccoli as cauliflower displayed signals for thiouracil of 6.0 $\mu\text{g kg}^{-1}$ and < 1.0 $\mu\text{g kg}^{-1}$, respectively. These data unambiguously prove the necessity of the myrosinase catalysed hydrolysis, which may indicate that thiouracil follows a similar pathway of synthesis as the known naturally occurring thyreostats, i.e. oxazolidine-2-thiones and thiocyanates.

To the best of our knowledge this study is the first to report the presence of endogenous thiouracil in feed and food samples, hereby elucidating a possible natural origin for the low-level thiouracil in urine of various species [23]. For future experiments, the addition of ascorbic acid might prove useful, since 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 TU formation and identify its precursors, possibly belonging to the glucosinolates.

5. References

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CHAPTER VI

GENERAL DISCUSSION AND FUTURE RESEARCH PERSPECTIVES

CHAPTER VI

GENERAL DISCUSSION AND FUTURE RESEARCH PERSPECTIVES

1. General research outcome

1.1 Positioning of this research

Thiouracil belongs to the group of xenobiotic thyreostatic drugs, which displays growth-promoting properties when orally administered to livestock [1-2]. In agricultural practices these drugs may be illegally applied to obtain a considerable live weight gain, mainly caused by increased water retention in edible tissue and augmented filling of the gastrointestinal tract [3-4]. Beside the fact that thyreostatic drugs negatively affect the meat quality of treated animals, they are also listed as compounds with teratogenic and carcinogenic properties (International Agency for Research on Cancer) [5]. Therefore, consumption of thyreostatic drug residues may pose a possible human health risk. The above mentioned arguments led in 1981 to a ban on their use for animal production in the European Union [6].

To maintain the zero-tolerance level for thyreostatic drugs, screening approaches for different biological matrices such as meat, thyroid, urine etc. had to be developed to ensure the absence of residues in the derived food products intended for the consumer market. Initially, macroscopic (presence of an enlarged thyroid or goiter), microscopic (alteration of the histological image of the thyroid gland), and clinical-biological (change in levels of free circulating thyroid hormones) methods were used as screening tools to detect thyreostatic drug abuse, but eventually an evolution towards robust chemical analytical methods became inevitable [7]. The evolution in chemical screening and confirmatory techniques has been ongoing with continuous developments and improvements. The introduction of analytical instruments such as ultra-HPLC, high-end fast switching triple quadrupole MS, high-resolution, accurate mass, and full scan MS (e.g. Time-of-Flight and Fourier transform MS) in particular have contributed to this unforeseen speed (Figure VI.1). Additionally, the use of these analytical instruments resulted in detection methods with higher sensitivity and specificity, accompanied by decreasing decision limits (CC_α).

It is exactly this technical evolution, enabling constantly lower detection limits, which led to the discovery of thiouracil in urine samples of various livestock species below the recommended concentration of $10 \mu\text{g L}^{-1}$ [8-9]. In light of the national residue control plans, this was contemplated as a considerable problem, since thyreostatic drugs are issued as banned substances. However, bearing the knowledge of the well-described elimination kinetics of thyreostatic drugs in mind [1, 10], it may be deduced that these low values do not sustain any growth-promoting effects. This finding, in combination with the report of Pinel et al. (2006) indicating a correlation between the administered Brassicaceae diet and the presence of trace-levels of thiouracil in bovine urine [8], raised questions on the origin of the detected thiouracil. In this context, the term 'semi-endogenous' status of thiouracil was born, yet to be proven.

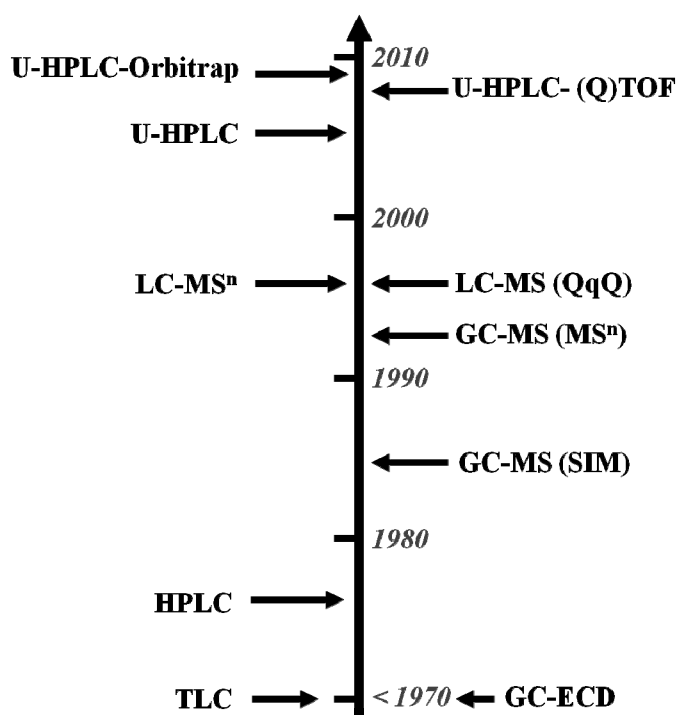


Figure VI.1: Evolution of methods used in residue analysis (adapted from De Brabander et al. 2009 [11])

Therefore the aim of this doctoral thesis was to:

- ✓ Ascertain the identity of the detected analyte thiouracil in urine by the development and validation of a quantitative ultra-high performance liquid chromatography tandem mass spectrometry method (U-HPLC-MS/MS) (CHAPTER II)

- ✓ Investigate the potential influence of the stability of thyreostatic drugs on the analytical results, with a focus on thiouracil in urine during storage on a short- (4 days) and long-term basis (3-5 months) (CHAPTER III)
- ✓ Confirm the potential status of thiouracil as 'naturally occurring' by screening urine samples of a large population of animals, comprising a smaller group with known medical backgrounds (CHAPTER IV)
- ✓ Investigate the possible sources of origin of the naturally occurring thiouracil by analysing Brassicaceae-derived feed and food samples (CHAPTER V)

1.2 Main research findings and scientific contributions

The main accomplishments of this work are, in a nutshell (Figure VI.2):

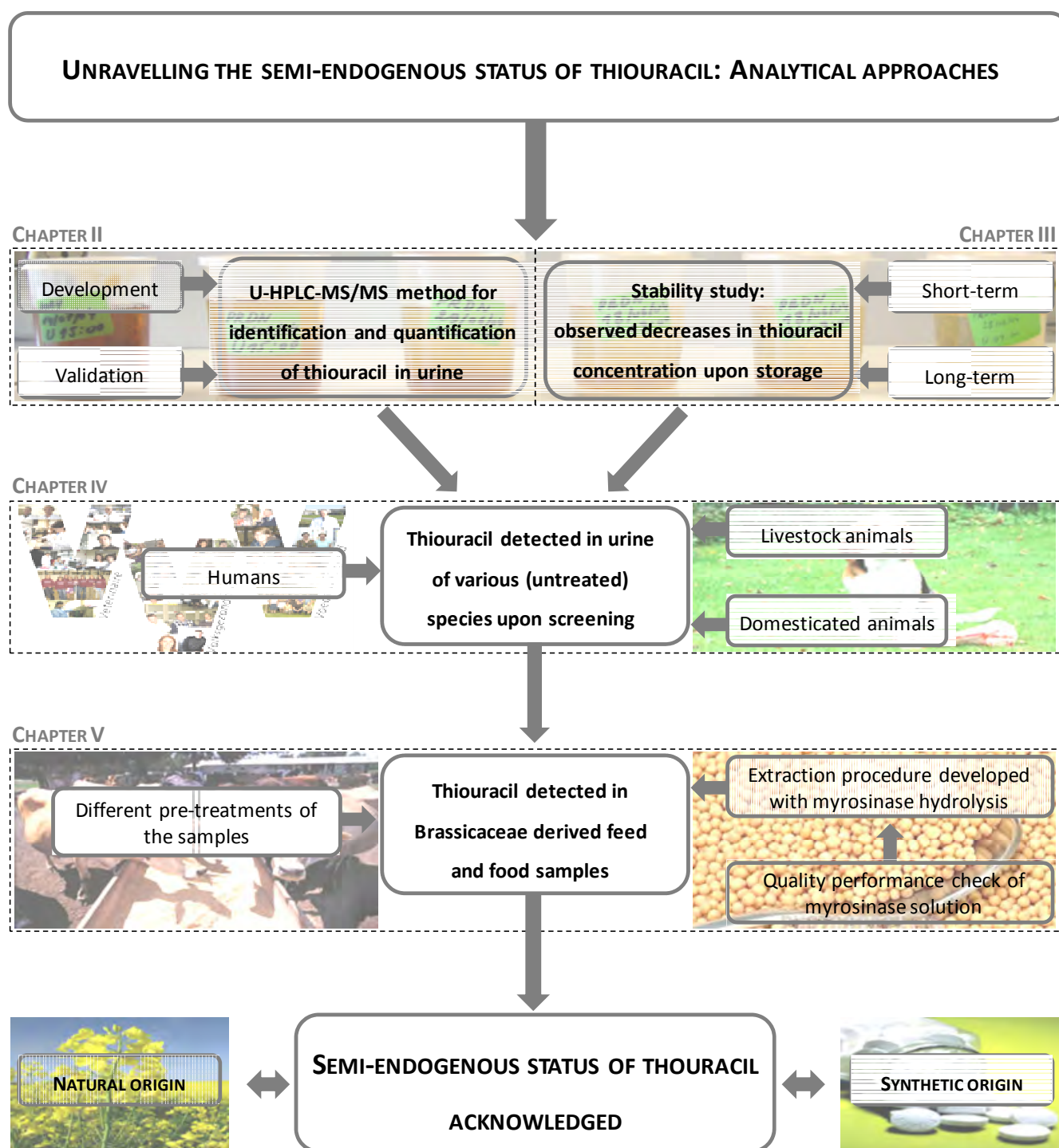


Figure VI.2: Schematic overview of the main research accomplishments of this work.

1.2.1 *An U-HPLC-MS/MS analysis method for detecting thiouracil in urine*

The development of a confirmatory analytical procedure for thyreostatic drugs is challenging due to the noteworthy chemical properties of these compounds. The polar, amphoteric character of these drugs, and their ability to adopt different tautomeric forms by delocalizing the π -electrons in the ring structure, negatively affects the extraction yield from biological samples, but also their chromatographic separation [12-13]. Additionally, in case of mass spectrometric detection the small molecular weight of thyreostats (110-210 Da) limits the selectivity and as a result the sensitivity of the detection. For the above mentioned reasons and to obtain decision limits (CC_{α}) and detection capabilities (CC_{β}) below $10 \mu\text{g L}^{-1}$ (recommended concentration) [14], the majority of the currently available analytical methods circumvents these difficulties by applying derivatisation before analysis [9, 13, 15-16]. However, it needs to be highlighted that the use of a derivatisation step may increase the likelihood of false-positive results. In order to adequately investigate the semi-endogenous status of thiouracil, an analytical method enabling the detection below the recommended concentration of $10 \mu\text{g L}^{-1}$ in urine without derivatisation is required. For this reason, the development and validation, according to the criteria specified in Commission Decision 2002/657/EC [17], of a new U-HPLC-MS/MS analysis method for detecting thyreostatic drugs, including thiouracil in urine ($< RC$ of $10 \mu\text{g L}^{-1}$) is described in **CHAPTER II**.

This new procedure omitted the derivatisation step to reduce this likelihood of possible false-positive results. With this validated method CC_{α} - and CC_{β} -values were obtained that easily reached below the recommended concentration of $10 \mu\text{g L}^{-1}$, this for all thyreostatic compounds in urine. In addition, 60 urine samples obtained from veterinary sampling in light of the European residue control plan were analysed with this newly developed method as well as with the 3-IBBr derivatisation method. Even though these methods were run on different instrumentation, U-HPLC-MS/MS (TSQ Vantage, Thermo Fisher Scientific) and LC-MS² (LTQ, Thermo Electron) instruments respectively, a correlation factor of 0.74 was obtained (**UNPUBLISHED DATA**). This nicely demonstrates that the newly developed method is adequate, specific, sensitive, and robust. Additionally, the combination of the concise sample pre-treatment with an U-HPLC-MS/MS runtime of not even 10 min resulted in lower analysis time and costs and as result a significant higher sample throughput. Beside these facts, the actual novelty of this detection method lays with the omission of the derivatisation step in combination with the low CC_{α} - and CC_{β} -values ($< 10 \mu\text{g L}^{-1}$). The only recent study reporting the detection of underivatised thyreostats

was by Abuin et al. (2008) [18], but suffered from unacceptable high CC_{α} - and CC_{β} -values. Other detection methods did obtain low CC_{α} and CC_{β} -values ($< 10 \mu\text{g L}^{-1}$), this however only when including a derivatisation step [9, 13].

The analytical procedure for the detection of underivatised thyreostatic drugs in urine, described in **CHAPTER II** was devised not only to obtain a novel highly advantageous routine analysis method, but also to assist later on in the investigation of the natural occurrence of thiouracil in urine of various species (**CHAPTER IV**).

1.2.2 *Stability of thyreostatic drugs in urine*

Imperative for the ruggedness and the analytical results of routinely acquired samples, is the knowledge of the stability of the analytes of interest in a matrix. For thyreostatic drugs however, this has not always shown to be as straightforward [19]. In this context, the stability of thyreostatic drugs in urine was investigated in **CHAPTER III**.

During the first part of the study analyses on spiked urine samples of bovine and porcine origin were performed to determine the stability of the analytes in a urine matrix and establish the possible matrix-related variables. The results indicated that thyreostatic drugs were unstable (decreases up to 78 %, with MBI as exception) upon storage at room temperature as well as upon freezing (-20 or -70 °C). As for the matrix-related influences, the pH and the presence of copper proved to be significant (p-value < 0.05). Therefore, a pre-treatment consisting of a pH-adjustment (pH 1) and the addition of a chelating agent (0.1 M EDTA) was developed. This treatment displayed a positive impact on the stability of the thyreostatic drugs in urine. Subsequently, field samples (bovine and porcine) obtained in light of the European residue control plan were screened for the presence of thyreostatic drugs. Only traces of thiouracil below the recommended concentration were detected, referred to as 'semi-endogenous' thiouracil. These low-level residues of thiouracil in urine appeared unstable, and even though the applied pre-treatment of pH 1 and 0.1 M EDTA postponed this decline, it was not completely eliminated during a storage period of 5 months. The results of this study proposed to limit the storage time of urine at room temperature, but also in a freezer (-20 or -70 °C) to a maximum of 3 months, which should be a feasible period for the executing laboratories. Furthermore, the developed pre-

treatment of pH 1 and EDTA upon sampling was proven as highly advisable for analysis of thyreostatic drugs in urine.

1.2.3 *The natural occurrence of thiouracil detected in urine of various species*

Two independent research groups described the presence of thiouracil below $10 \mu\text{g L}^{-1}$ (RC) in livestock urine [8-9]. Our newly developed and validated U-HPLC-MS/MS method without derivatisation (CHAPTER II) was specifically designed to aid in unravelling this 'semi-endogenous' status of thiouracil. With this new analytical method 304 urines of bovine, porcine, and ovine origin were analysed (CHAPTER IV). In total 71.8 % was found positive for thiouracil ($> \text{CC}_\alpha$ and $< 10 \mu\text{g L}^{-1}$), as such acknowledging the previously described low-level residues of thiouracil [8-9]. To exclude the limited, but still existing possibility of illegal thiouracil administration, a limited group of various animal species with known medical backgrounds (i.e. no thyreostatic drug treatment or thyroid disorder) were screened for thiouracil in urine. Since thiouracil was indeed detected in the urine of these animals, the natural origin of thiouracil could as such be acknowledged (CHAPTER IV).

In addition, the final part of CHAPTER IV also included an extrapolation of this study to humans, which is to the best of our knowledge the first study in its kind. The investigated human urines displayed a similar trend as described in the first part of this chapter, 66.7 % of the samples contained thiouracil residues higher than the CC_α ($2.2 \mu\text{g L}^{-1}$). Besides, a correlation between the presence of these residues and the supplemented Brassicaceae diet was investigated as potential origin of the naturally occurring thiouracil. This proved however to be non-significant ($p = 0.095$), and indicated that the contamination source comprised more than only Brassicaceae vegetables and derivatives. Nevertheless taken all of this into account, it indicates that thiouracil detected in urine of livestock, domesticated animals, and humans has a natural origin. Evidence points towards a nutritional origin, most likely still Brassicaceae related. In light of these findings, further investigations on the involvement of Brassicaceae-derived feed and food samples were carried out in CHAPTER VI.

1.2.4 *Thiouracil originates from Brassicaceae derived feed and food samples*

The main goal of CHAPTER VI was to investigate and elucidate more in depth the origin of the semi-endogenous thiouracil residues retrieved in urine of animals and humans. As a starting point,

Brassicaceae seed, feed derivatives and vegetables were analysed on the presence of thiouracil [8]. The extraction procedure described by Pinel et al. (2005) [13] did not enable the detection of thiouracil in these matrices [8]. For this reason, a myrosinase enzyme solution, extracted from yellow *Sinapsis alba* L. seed (mustard seed) was added to aid in the release of thiouracil from the Brassicaceae derived matrix. This experimental adaptation to the extraction procedure of Pinel et al. (2005) [13] was based on the well-studied formation of the naturally occurring thyreostats, i.e. oxazolidine-2-thiones and thiocyanates. These originate from a myrosinase catalysed hydrolysis reaction of a precursor (glucosinolate) present in Brassicaceae plants [20-21].

Prior to the use of the extracted enzyme mixture, quality performance of the hydrolysis activity and kinetics were conducted by means of a single-point glucose assay [22-23], with an HPLC-ELSD analysis method [24]. After assessing the activity of the extracted myrosinase mixture, the hydrolysis was successfully incorporated into the sample clean-up. When applying this pre-treatment and the sample clean-up prior to LC-MS² analysis, thiouracil was retrieved in various types of Brassicaceae feed and food samples.

To the best of our knowledge this is the first study able to retrieve thiouracil in animal feed matrices derived from Brassicaceae. In addition, the analysed vegetables, broccoli and cauliflower (*Brassica oleracea*) displayed traces of thiouracil. This finding can at least partially explain the presence of thiouracil in the analysed human urine aliquots (**CHAPTER IV**), however the additional contamination sources are still unidentified. The existence of additional sources is enforced by the presence of thiouracil in urine collected from the period before Brassicaceae consumption, also referred to as the blank period. The findings reported in **CHAPTER V** have a considerable impact on the interpretation of the obtained analytical results of thyreostatic drugs analysis, this in light of the national residue control plans.

2. Future perspectives

Against the background of this dissertation - unravelling the semi-endogenous status of thiouracil - some future research topics may be identified that are directly or indirectly relevant for residue analysis in light of the European control plan for thyreostatic drugs. Further research may aim at the identification of the possible precursors, the elucidation of the formation pathway, and retrieval of the

influence of the metabolic activity of the digestive system on the 'semi-endogenous' thiouracil formation. This may ultimately lead to new biomarkers providing the key to substantiate the naturally occurring thiouracil from its exogenous analogue.

2.1 Other analytical approaches

2.1.1 *Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry*

This dissertation has described the research undertaken to enable the confirmation of the semi-endogenous status of thiouracil. However, another problem remains to be tackled: how to distinguish unequivocally between the naturally occurring (*de novo* formed) and synthetic administrated thiouracil. For the different European member states involved in residue analysis, this is an important matter in order to uphold the zero-tolerance policy [25].

In agricultural practices, but also in sports the abuse of for example steroids for growth-promoting and performance-enhancing effects has already led to an analytical approach that distinguishes endogenous from exogenous steroids [26-29]. For this, gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) has been used for discriminating between steroids originating from synthetic precursors and from their chemically identical natural analogues. The GC-C-IRMS instrument enables the precise measurement of differences in stable isotope ratios ($^{13}\text{C}/^{12}\text{C}$ ratio) that results from fractionation patterns inherent to the source of steroids [27-28]. The reliability of this analytical instrument for measuring isotope variations has already been shown in a large number of applications fields, including the rigorously demanding regulatory control field of residue analysis [26, 28-29].

Investigating the possibility of retrieving an isotope variation between the naturally occurring thiouracil and its synthetic analogue would provide an advantage for the residue control plan of the different member states. In this way, a reliable analytical method would be offered to uphold the zero-tolerance policy concerning thyreostatic drugs in biological matrices.

2.1.2 *Accurate mass high-resolution mass spectrometry*

The goal of **CHAPTER II** was to develop a method without derivatisation able to detect and quantify the thyreostatic drugs thiouracil in urine. This method reduced the likelihood of false-positive results due to omitting the derivatisation step. Nevertheless, a remote possibility of false-positive results may still exist, even with this newly developed method. To obtain a 100 % certainty of the identity of thiouracil in urine, the technology of accurate mass high-resolution mass spectrometry (HRMS) should offer the solution. Mass spectrometric devices such as Time-of-Flight (TOF), Fourier transform ion cyclotron resonance (FT-ICR) or Fourier transform Orbitrap possess the capacity to provide reliable identification of thiouracil in a complex matrix like urine with the necessary resolving power, mass accuracy, sensitivity, and dynamic range [28, 30]. To the best of our knowledge, the only report made so far on thiouracil and HRMS analysis includes derivatisation [31]. The performance of a HRMS analysis without derivatisation would be highly advisable to ascertain once and for all the identity of low-level thiouracil in urine ($< 10 \mu\text{g L}^{-1}$).

2.2 Retrieving the origin of naturally occurring thiouracil

Brassicaceae derived-feed samples and vegetables have been acknowledged in this work as source of origin for naturally occurring thiouracil (**CHAPTER V**). Evidence from this dissertation also points towards a similar synthesis pathway for this ‘semi-endogenous’ thiouracil as for the known natural thyreostats, i.e. oxazolidine-2-thiones and thiocyanates, formed by hydrolysis of glucosinolates, secondary plant metabolites present in Brassicaceae [20-21].

Further research is however warranted to investigate the identity of the possible natural precursors of thiouracil. To that end, feed or food samples tested positive for thiouracil should be extracted with a general extraction protocol as described by Pinel et al. (2005) [13]. Subsequently, preparative separation techniques (preparative HPLC) may be applied to obtain a fractionation of the extracted feed, during which the suspected precursors could be collected. Through myrosinase catalysed hydrolysis (**CHAPTER V**) and LC-MS² analysis, thiouracil may be detected and as such result in the identification of the fraction comprising the precursor. Afterwards, the precursor-containing fraction could be analysed by high-resolution mass spectrometry or NMR spectroscopy to elucidate its chemical configuration. The careful interpretation of these obtained data could lead to the identification of this

unknown precursor molecule, potentially followed by the elucidation of the mechanism of thiouracil formation.

In case a glucosinolate is identified as precursor, Brassicaceae plants, vegetables, and derived-feed samples may indeed be considered as the sole vegetable origin of the naturally occurring thiouracil. If structure elucidation indicates a different sort of molecule as precursor, it needs to be kept in mind that the origin of this precursor may not be limited to the Brassicaceae family. To this end, additional research could be undertaken to look for other sources of origin.

Besides, another possible origin needs to be highlighted: 'Endogenous thiouracil originating from within the body of the host (animal and human)'. In scientific literature thiouracil is acknowledged as a minor component of transfer RNA (tRNA) [32-33]. However, no information could be retrieved concerning the frequency of this occurrence. Nevertheless, this information indicates the possibility of endogenous presence of thiouracil. To this purpose, it might be of interest to transfer the U-HPLC-MS/MS analysis method for thiouracil in urine (**CHAPTER II**) to plasma and apply this method on plasma samples derived from animals with known medical backgrounds (no thyreostatic treatment) on a controlled diet (omitting Brassicaceae-derived feedstuff). By this approach, it could also be assessed if thiouracil might have an additional 'endogenous' origin.

2.3 Influence of the digestive system

The significant role of the β -thioglucosidase enzyme myrosinase in the formation of naturally occurring thiouracil from Brassicaceae-derived feed and food matrices has been proven in this dissertation (**CHAPTER V**). In addition, literature also reports that intestinal microbiota are probable mediators for this hydrolysis [34-39]. This of course raises the question: 'Do the different gastro-intestinal processes that Brassicaceae derived-feed undergoes upon consumption, have a possible influence on the formation of thiouracil?'.

Recommendable in light of the national residue control plan would be to investigate this possibility through *in vitro* incubation experiments (Figure VI.3). For this, two model organisms should be used, i.e. pigs on the one hand and cattle on the other hand. With respect to the monogastric model (pig), the processes in the stomach, small intestine and large intestine are of main importance [40]. Stomach digestions should evaluate the potential influence of an acidic pH, the enzyme pepsin, and the

presence of other food constituents. Small intestinal digestions will simulate the impact of a more basic environment and the action of bile salts and enzymatic secretions, besides the presence of a first microbial community. Finally, a large intestinal digestion will be performed to investigate the influence of micro-aerophilic and obligate anaerobic intestinal bacteria on the formation of thyreostats from feed. The intestinal inocula will be derived from fresh centrifuged intestinal content of a porcine or bovine. The most important aspect, which distinguishes the gastro-intestinal tract of ruminants, in this case cattle from that of pigs, is the presence of the rumen. Therefore, an additional digestion experiment should be performed to evaluate the effect of the rumen microbial community on the formation of thiouracil from Brassicaceae derived-feed. For this purpose, *in vitro* batch incubations with rumen content as inoculum [41] should investigate the degradation of feed components and the formation of metabolites taking place when bacteria are exposed to the substrate during incubation.

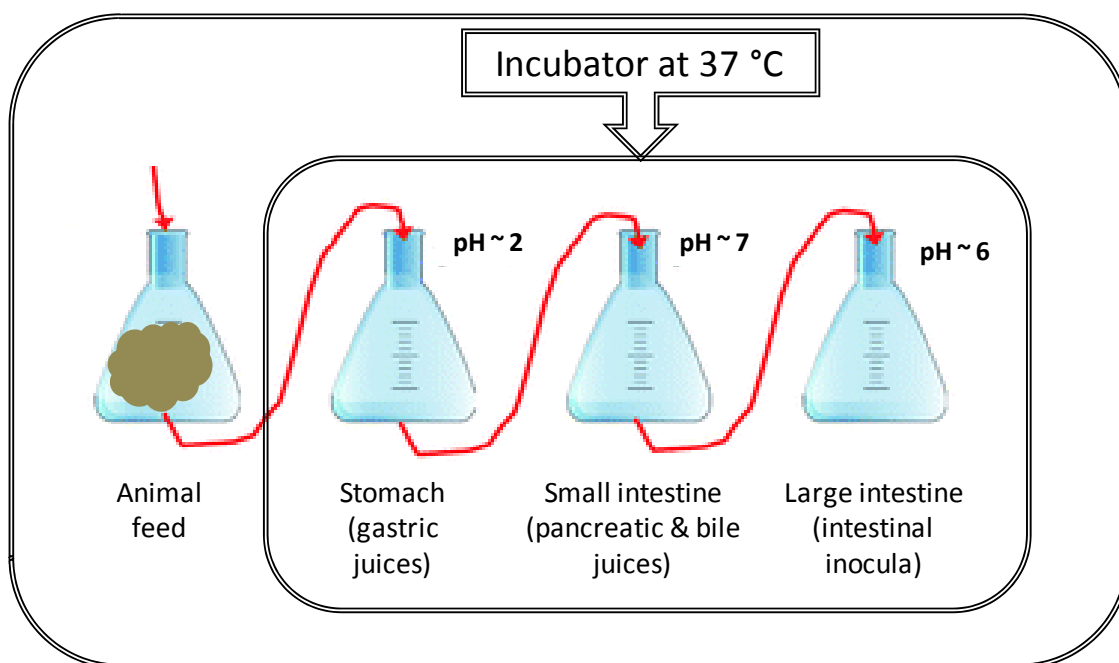


Figure VI.3: Schematic representation of a static *in vitro* incubation model.

Additionally, the digestive samples obtained from the *in vitro* batch incubation experiments could be employed to search for a selective and objective biomarker, which should ultimately enable the distinction between synthetic (exogenous) and naturally present thiouracil. To this end, mass spectrometric techniques based on accurate mass determination, such as time-of-flight (TOF) and

Fourier transform (Orbitrap), are indispensable for the identification of these unknown reaction intermediates [42].

Furthermore, an *in vivo* experiment with a representative amount of animals (bovine and/or porcine) could be designed to investigate the potential of these selected biomarkers. Three different treatments could be considered: a control diet, a diet supplemented with the synthetic thyreostat thiouracil and a diet exclusively composed of rapeseed (Brassicaceae), taking into account that between the different treatment periods sufficiently long adaptation periods should be included to avoid possible carry-over effects. The collected digests and urine samples could subsequently be screened for the selected biomarkers, by using accurate mass determination and NMR spectroscopy, or what may be referred to as a metabolomic approach [42].

2.4 Impact on the general health conditions

Thyreostatic drugs such as thiouracil are known to interfere with the function of the thyroid gland. For growth-promoting effects a daily dosage of 5 g needs to be administered [2], which in turn generates, when analysed with sensitive detection techniques, signals in urine considerably higher than $100 \mu\text{g L}^{-1}$ [9, 13]. The influence of semi-endogenous thiouracil, retrieved in urine at concentrations between the CC_α and $10 \mu\text{g L}^{-1}$, on the thyroid metabolism and its implication on the general health of the animal or human is however unknown. Most likely the effect will be less pronounced than in case of administration for weight-gain, because of the small amounts of thiouracil naturally present in the feed samples.

To investigate this, a feed sample found positive for thiouracil ($\text{CC}_\alpha <$ and $< 10 \mu\text{g L}^{-1}$) should be administered to for example a heifer, which should only receive this thiouracil-positive feed and water supply *ad libitum*. On a regular basis, urine and plasma samples should be taken and analysed for the presence of thiouracil and the concentration of the free circulating thyroid hormones T3 and T4 [43], respectively. Additionally, some urine and plasma samples of the heifer should be collected before the diet of thiouracil-positive feed, to foresee a natural baseline level for the analytical experiment. Interesting would also be to include a bovine calf and a monogastric animal to the study, because these have been described as more susceptible to the effects of compounds with anti-thyroid action [1, 34].

As such these results may give an indication of the effect of the continuous and long-term exposure to these low-level thiouracil residues.

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SUMMARY

SUMMARY

In recent years, scientific concern and debate has risen on the presence and the possible origin of thiouracil, below the recommended concentration of $10\ \mu\text{g L}^{-1}$ in urine of livestock. The analyte thiouracil finds itself in a grey-zone, on the one hand a known synthetic analyte, on the other hand possessing a possible natural origin. As a result, the term semi-endogenous was born, however scientifically constructed proof was still required. The research of this doctoral thesis was situated within this context and performed in close collaboration with the Laboratoire d'Etude des Résidus et Contaminants (ONIRIS, Nantes, France).

In the introduction, **CHAPTER I**, a concise overview is given of the different groups of existing thyreostatic drugs and their influence on the pathway of hormone synthesis of the thyroid gland. In this regard, the legislative framework dedicated to the analytical detection of thyreostatic residues in biological matrices has been discussed. The Brassicaceae plant family is also briefly mentioned, to point out its relevance in the diet of animals and humans. Additionally, a summary of the evolution in analytical approaches to detect thyreostatic drugs in various matrices is given, with the analytical instruments used in this doctoral thesis discussed separately. Finally, the conceptual framework and the different research phases (**CHAPTER II-V**) of this doctoral thesis dealing with the possible semi-endogenous status of thiouracil have been formulated.

In **CHAPTER II** the development and validation of an ultra-high performance liquid chromatography coupled to triple quadrupole mass spectrometry method that enables the detection of all xenobiotic thyreostats in urine, without derivatisation and with a sensitivity well below the recommended concentration of $10\ \mu\text{g L}^{-1}$, is presented. This analytical procedure was validated according to the EU criteria (2002/657/EC Decision), resulting in decision limits and detection capabilities in urine of $1.1 - 5.5\ \mu\text{g L}^{-1}$ and $1.7 - 7.5\ \mu\text{g L}^{-1}$, respectively. This U-HPLC-MS/MS method obtained an overall precision of 15.5 % (relative standard deviation). Other method performance characteristics e.g. calibration, recovery, selectivity, and specificity are also presented individually for each targeted compound.

The stability of thyreostatic drugs in urine of livestock during storage was investigated in **CHAPTER III**. The first part used spiked urine samples to examine the effects of preservation, number of freeze-thaw

cycles, and matrix-related variables. The significant impact ($p < 0.05$) of copper ions and the pH-value on the stability of thyreostatic drugs in urine, led to the development of a sample pre-treatment. This pre-treatment, consisting of an acidification to pH 1 and the addition of 0.1 M ethylenediaminetetraacetic acid disodium salt dehydrate, proved capable of delaying or even completely inhibiting the decrease in thyreostatic concentration and improved its detection in time. In the second part of **CHAPTER III** this newly developed sample pre-treatment was applied on multiple collected field samples, containing traces of thiouracil below $10 \mu\text{g L}^{-1}$. These untreated urine samples and their pre-treated duplicates were simultaneously analysed by LC-MS². Application of the sample pre-treatment displayed a significant improvement in stability as compared to their untreated duplicates. The obtained results clearly indicated the necessity of limiting the preservation time of urine samples at room temperature, but also in the freezer prior to thyreostat analysis. Additionally, the implementation of the developed pre-treatment (pH 1 and 0.1 M EDTA) upon sampling appeared to be highly beneficial for the stability of thyreostatic drugs in urine. For future purposes when analysing for thyreostatic drugs in light of the European national control plans, it seems highly recommendable to conduct this suggested pre-treatment.

The aim of **CHAPTER IV** was to confirm the possible natural occurrence of thiouracil. Firstly, a large group of urine samples derived from livestock animals (bovine, porcine, and ovine) was analysed with the newly developed U-HPLC-MS/MS analysis method without derivatisation (**CHAPTER II**). This approach acknowledged the frequent presence of thiouracil in urine below the RC of $10 \mu\text{g L}^{-1}$. Next, this natural occurrence of thiouracil was confirmed by analysing urine samples of animals (livestock and domesticated) with known clinical backgrounds. Secondly, it was examined if this natural prevalence could be extrapolated to the human population. To this end, a small-scale 9-day human *in vivo* experiment with Brassicaceae vegetables was performed, to investigate the presence of thiouracil in human urine and evaluate a possible correlation with the administered Brassicaceae diet. In 66.7 % of the collected human urine samples, traces of thiouracil ($> \text{CC}_\alpha$ of $2.2 \mu\text{g L}^{-1}$) were found, however no significant correlation could be measured between the consumed Brassicaceae diet and the presence of thiouracil.

As a final step in elucidating the semi-endogenous status of thiouracil detected in urine of animals and humans, a search was conducted in **CHAPTER V** to identify possible natural sources of origin. To this purpose, various Brassicaceae derived feed and food sources (rapeseed, rapeseed coarse meal,

cabbage, cauliflower, broccoli, etc.) were investigated for the presence of thiouracil. After optimisation of the enzymatic hydrolysis protocol and the required quality performances of the extracted enzyme solution, the different feed and food samples were analysed for thiouracil. Upon enzymatic hydrolysis, sample clean-up, and LC-MS² analysis, thiouracil was successfully detected in traditional rapeseed, rapeseed '00'-variety coarse meal (values erucic acid < 2 % and glucosinolates < 25 $\mu\text{mol g}^{-1}$), and rapeseed cake at 1.5, 1.6, 0.4 $\mu\text{g kg}^{-1}$, respectively. For broccoli and cauliflower thiouracil concentrations of 6.0 $\mu\text{g kg}^{-1}$ and < 1.0 $\mu\text{g kg}^{-1}$, respectively were found. These findings acknowledge the natural origin of thiouracil.

In **CHAPTER VI** general conclusions and future research perspectives were formulated. In short, this doctoral study was able to provide scientific evidence that defines thouracil as a semi-endogenous compound, with a natural as well as a synthetic origin. This semi-endogenous thiouracil was retrieved not only in urine of livestock, but could be extrapolated to urines of domesticated animals (canine) and humans. Finally, a source of origin for the naturally occurring thiouracil was detected and identified as Brassiceceae-derived feed and food.

SAMENVATTING

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De laatste jaren is er een toenemende wetenschappelijke bezorgdheid en discussie ontstaan omtrent het voorkomen en de mogelijke herkomst van thiouracil in nutsdieren onder de ‘aangeraden concentratie’ van $10 \mu\text{g L}^{-1}$. Thiouracil bevindt zich in een grijze zone, enerzijds erkend als synthetisch component, anderzijds is er sprake van een natuurlijke herkomst. Binnen deze context is de term semi-endogeen ontstaan, wetenschappelijk bewijs hiervoor moet echter nog aangeleverd worden. Binnen dit onderwerp situeert zich het onderzoek uit dit doctoraat.

In de inleiding, **HOOFDSTUK I**, wordt een overzicht gegeven van de verschillende chemische verbindingen met thyreostatische werking en hun storende invloed op de hormoon synthese van de schildklier. De wettelijke omkadering zoals die geldt voor het illegaal misbruik van thyreostatica bij nutsdieren en hun chemische analyse in diverse biologische matrices wordt aangekaart. Tevens wordt ook de relevantie van het voorkomen van planten en/of plantproducten, afkomstig van de Brassicaceae familie, in het dierlijk en menselijk dieet aangehaald. Bovendien wordt een overzicht gegeven van de evolutie in analytische methoden voor de detectie van thyreostatica in diverse matrices. Tot slot wordt ook het conceptueel kader van dit doctoraatsonderzoek geschetst, waar de problematiek van een mogelijke semi-endogene status van thiouracil wordt behandeld en de opbouw van de verschillende onderzoeksfasen (**HOOFDSTUK II-V**) van dit doctoraat wordt geschetst.

In **HOOFDSTUK II** werd de ontwikkeling en validatie beschreven van een U-HPLC-MS/MS methode, die in staat is om thyreostatica te detecteren in urine, dit zonder derivatisatie stap en met een gevoeligheid lager dan de ‘aangeraden concentratie’ van $10 \mu\text{g L}^{-1}$. Deze analytische methode werd gevalideerd volgens de Europese criteria (2002/657/EC beslissing) met beslissingsgrenzen en detectievermogens voor alle thyreostatica in urine tussen $1.1 - 5.5 \mu\text{g L}^{-1}$ en $1.7 - 7.5 \mu\text{g L}^{-1}$, respectievelijk. Deze U-HPLC-MS/MS methode behaalde een precisie van 15.5 % (relatieve standaard afwijking). Overige prestatiecriteria zoals calibratie, selectiviteit, specificiteit en terugvinding werden voor elk van de beschreven componenten ook individueel gerapporteerd.

De stabiliteit van thyreostatica in urine in functie van bewaring werd onderzocht in **HOOFDSTUK III**. In een eerste deel werd de stabiliteit van de componenten onderzocht aan de hand van aangerijkte stalen. Dit om effecten van bewaring, aantal vries-dooi cyclussen en matrix-gerelateerde variabelen na

te gaan. Als matrix-variabele bleek de aanwezigheid van koperionen en de pH-waarde een significante impact ($p < 0.05$) te hebben op de stabiliteit van de thyreostatische componenten in urine. Hiertoe werd een voorbehandeling ontwikkeld waarbij een pH-waarde van 1 en een toevoeging van 0.1 M ethyleendiaminetetra-azijnzuur als meest optimaal werden beschouwd. Toepassing van deze voorbehandeling resulteerde in een vertraging of zelfs het stoppen van de concentratiedaling, met een verlengde detectie tot gevolg. In het tweede deel van **HOOFDSTUK III** werd deze behandeling toegepast op verzamelde urinemonsters, die spoorgehalten aan thiouracil ($< 10 \mu\text{g L}^{-1}$) bevatten. Aan de hand van LC-MS² analyse werden deze stalen parallel geanalyseerd met hun behandelde duplicaat, wat een significante verbetering in stabiliteit aantoonde ten opzichte van de onbehandelde stalen. Uit deze resultaten bleek het aangeraden om de bewaringstermijn van urinestalen voorafgaand aan de analyse op kamertemperatuur, alsook in de diepvries te beperken. Bovendien werd aangetoond dat het uitvoeren van een staalvoorbehandeling (pH 1 en EDTA) bij staalafname enkel maar een positieve uitwerking heeft op de stabiliteit van thyreostatica in matrix. Deze methode kan dus aanbevolen worden voor toekomstige stalen, bemonsterd in de context van het nationale controleplan.

Het doel van **HOOFDSTUK IV** bestond erin om de natuurlijke oorsprong van thiouracil te bevestigen. Hiervoor werden met de U-HPLC-MS/MS methode zonder derivatisatie, ontwikkeld in **HOOFDSTUK II**, een grote hoeveelheid urinestalen geanalyseerd. Deze aanpak bevestigde het herhaaldelijk voorkomen van thiouracil in urinemonsters onder de grens van $10 \mu\text{g L}^{-1}$. Om de natuurlijke herkomst van de gedetecteerde thiouracil residuen te achterhalen, werden ook urine stalen van nuts- en huisdieren met gekende medische achtergrond geanalyseerd, waarbij opnieuw thiouracil werd teruggevonden ($< 10 \mu\text{g L}^{-1}$). In een volgende stap werd nagegaan of deze natuurlijke prevalentie ook kon uitgebreid worden naar de menselijke populatie. Aan de hand van een kleinschalige *in vivo* studie (9 dagen) werd enerzijds onderzocht of thiouracil voorkwam in geanalyseerde menselijke urinestalen en anderzijds of er een mogelijke correlatie bestond met het Brassicaceae-dieet dat aan de betreffende proefpersonen werd toegediend. In 66.7 % van de humane urinestalen werd thiouracil teruggevonden ($> \text{CC}_\alpha$ van $2.2 \mu\text{g L}^{-1}$), maar een significante correlatie tussen het Brassicaceae-dieet enerzijds en het voorkomen van thiouracil in de urine anderzijds, kon niet bevestigd worden.

Als laatste stap in het ophelderen van de semi-endogene status van thiouracil, werd in **HOOFDSTUK V** gezocht naar mogelijke bronnen van herkomst. Hiertoe werden verschillende van Brassicaceae afgeleide voeder- en voedingsbronnen (koolzaad, koolzaadschroot, voederkool, broccoli, bloemkool,

etc.) geanalyseerd op de aanwezigheid van thiouracil. Deze analyses vonden pas plaats na optimalisatie van de enzymatische hydrolyse en de kwaliteitstesten van de bereide enzymoplossing. Na enzymatische hydrolyse, staalopzuivering en LC-MS² analyse kon thiouracil gedetecteerd worden in traditioneel koolzaad, koolzaadschroot-'00' variëteit (erucazuur < 2 % en glucosinolaten < 25 µmol g⁻¹) en koolzaadkoek aan respectievelijk 1.5, 1.6, 0.4 µg kg⁻¹. Voor broccoli en bloemkool bedroeg deze concentratie respectievelijk 6.0 µg kg⁻¹ en < 1.0 µg thiouracil per kg. Deze bevindingen tonen de natuurlijke oorsprong van thiouracil aan.

In **HOOFDSTUK VI** worden tenslotte de algemene conclusies en toekomstperspectieven van dit doctoraatsonderzoek geformuleerd. Samenvattend kan gesteld worden dat voldoende wetenschappelijk bewijs geleverd is om thiouracil als een semi-endogene component te bestempelen, gekenmerkt door een natuurlijke en een synthetische herkomst. Meer nog, deze semi-endogene thiouracil is niet enkel aangetroffen bij nutsdieren, maar ook bij huisdieren (hond) en mensen. Finaal is ook de bron van herkomst teruggevonden en geïdentificeerd als Brassicaceae gerelateerde voeder- en voedingsproducten.

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Personalia

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Education

1994-2000: Sint Lodewijkscollege, Bruges
ASO: Latin / Mathematics

2000-2003: Ghent University
Bachelor in Biomedical Science, Graduated with Honours

2003-2005: Ghent University
Master in Biomedical Science, Growth and Cell differentiation
Graduated with Honours

Oct. 2008: School for Advanced Residue Analysis in Food (SARAF),
2 weeks training at Laberca, ONIRIS, Nantes, France.

2008-2009: Advanced Academic English: writing skills (course)

Professional activities

2005- 2007: *Innogenetics, Zwijnaarde*

Part of the team dedicated to the HLA-Antigen project, designed for the new technology platform "4-MATTM".

My main task within the team was to design, test, and improve a new protocol for the purification of HLA Antibodies and perform the necessary Quality Control routines.

The research, mainly based on a trial and error improvement cycle, consisted out of a wide range of analytical techniques.

2007-2011: *Doctoral fellowship*

PhD research granted by Ghent University (Dehousse), at the department of Veterinary Public Health & Food Safety, Laboratory of Chemical analysis on the project: 'Analytical approaches to unravel the semi-endogenous status of thiouracil'.

2008-2009: Close collaboration with and performed research (5 weeks) at the Laboratoire d'Etude des Résidus et Contaminants dans les Aliments of l'Ecole Nationale Vétérinaire, Agroalimentaire et de l'Alimentation Nantes-Atlantique (LABERCA, ONIRIS), Nantes, France.

2009-2010: Tutor of 4 Master students in Veterinary Medicine and Pharmaceutical Science.

2009-2010: Tutor assistant for the practical theory and exercise session on the primary components of the course 'Food and Environmental Chemistry' at the Faculty of Veterinary Science (Ghent University).

Sept. 2010: Achievement of 'outstanding Poster Award for Feed' at the Exchange conference, held on 28th of September 2010, Ghent, Belgium.

2010-2011: Department head 'at interim' at the department of Veterinary Public Health & Food Safety, Laboratory of Chemical analysis (6 months).

Scientific publications

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Conference, workshops and seminars

⇒ 10th International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analysers/ 10th International Symposium on Advances in Extraction Techniques (HTC/ExTech-10), Bruges, Belgium, 2008.

(POSTER PRESENTATION)

⇒ User meeting Thermo Fisher Scientific, Elewijt-Zemst, Belgium, 2008.

⇒ Conference on Residues of Veterinary Drugs in Food (EuroResidue VI), Egmond aan Zee, The Netherlands, 2008.

(POSTER PRESENTATION)

⇒ Trends in Food Analysis VI Symposium, Ghent, Belgium, 2009.

(POSTER PRESENTATION)

⇒ Waters Application Seminar-UPCL, Ghent, Belgium, 2009.

⇒ EuroAnalysis-The Impact of Analytical Chemistry on Quality of Life, Innsbruck, Austria, 2009.

(POSTER PRESENTATION)

⇒ Workshop AOAC International, Paris, France, 2009.

(POSTER PRESENTATION)

⇒ 11th International Symposium on Hyphenated Techniques in Chromatography and Hyphenated Chromatographic Analysers/11th International Symposium on Advances in Extraction Techniques (HTC/ExTech-10), Bruges, Belgium, 2010.

(POSTER PRESENTATION)

⇒ 6th International Symposium on Hormone and Veterinary Drug Residue Analysis (VDRA), Ghent, Belgium, 2010.

(ORAL AND POSTER PRESENTATION)

⇒ Exchange Conference-Open Innovation for Food, Feed and Health, Ghent, Belgium, 2010.

(POSTER PRESENTATION)

⇒ Workshop FOD: Food Safety, Security and Environment, Brussels, Belgium, 2010.

(ORAL PRESENTATION)

