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Review

Analysis of thyreostats: A history of 35 years

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ABSTRACT

Thyreostatic drugs (TS), illegally administrated to livestock for fattening purposes, are banned in the European Union since 1981 (Council Directive 81/602/EC). This paper reviews the trends in the analytical approaches for the determination of TS drugs in biological matrices. After a brief introduction on the different groups of compounds with a thyreostatic action, the most relevant legislation regarding the residue control of these compounds is presented. An overview of the analytical possibilities for the determination of TS in animal matrices, covering sample extraction, purification, separation techniques and detection methods is provided. Additionally, a brief outline of animal experiments is described that illustrates the excretion and distribution profiles of TS residues. Finally, the novel developments in TS analysis are highlighted. Also the possible semi-endogenous status of thiouracil is discussed.

Contents

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1. Thyreostats

Nowadays the term 'thyreostats' or 'thyreostatic drugs' (TS) is used to refer to a complex group of substances that inhibit the thyroid function, resulting in a decreased production of thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) [1,2]. In the past also 'anti-hormones' was used, although this nomenclature was not correct. Anti-hormones counteract the action of a hormone, not its production [3].

The use of thyreostatic drugs for animal fatting purposes is prohibited in Belgium since 1974 (KB 12.04.1974) [4]. In the European Union, there is a general agreement on the ban of these substances since 1981 [5].

The use of this group of compounds for animal fattening purposes 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 TS may be teratogenic and carcinogenic [10,11]. For example in Spain, the consumption of meat contaminated with thyreostats has caused an increased incidence of aplasia cutis, a characteristic scalp defect [10]. Consequently, these particular reasons induced the need for the development of sensitive and specific analytical methods for the determination of thyreostatic drugs in matrices of animal origin.

Thyreostats can be divided into two main groups, respectively the xenobiotic and the natural occurring sulfur compounds [1]. These are polar and amphoteretic thioamides, characterized with a low molecular weight. Within their formulae a common element is displayed, the nitrogen–carbon–sulfur sequence (thioamide) (see Fig. 1), presumed responsible for the thyroid-inhibiting activity.

Additionally, a large number of other molecules like inorganic ions such as lithium (Li^+) [12–18], perchlorate (ClO_4^-) [19–23] and thiocyanate (SCN) [19,24,25], but also veterinary drugs like sulfonamides [26–32] may have a thyreostatic action [33,34].

Within the framework of residue control of xenobiotic TS, 4(6)-R-2-thiouracil (R = hydrogen, methyl, propyl, phenyl), tapazole (TAP) and 2-mercaptobenzimidazole (MBI) are of most interest [1]. These synthetic drugs are the most powerful TS agents. Above this, they were cheap and readily available on the black market.

The group of natural occurring thyreostats comprises thiocyanates and oxazolidine-2-thiones (OZT's). Thiocyanates may be classified within the group of inorganic ions because besides their natural origin, they can be chemically synthesized. The group of natural TS originates from precursors (glucosinolates), present in plants of *Brassicaceae* (syn. *Cruciferae*) and related families [35–37]. Generally, only in case of cellular disruption of the plant tissue, the hydrolysis of the glucosinolates occurs [35,38,39]. This reaction is catalyzed by a β-thioglucosidase enzyme, also called myrosinase (EC

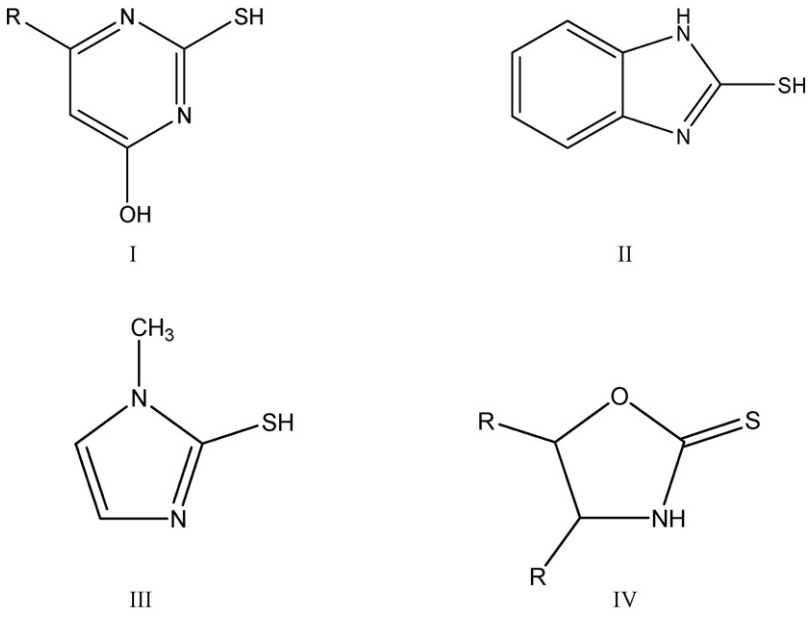

Fig. 1 – Structural formulae of thyreostats. I: 4(6)-R-2-thiouracil (R = hydrogen, methyl, propyl, phenyl); II: 2-mercaptobenzimidazole (MBI); III: 1-methyl-2-mercaptoimidazole (TAP); IV: 1,3-oxazolidine-2-thione (OZT).

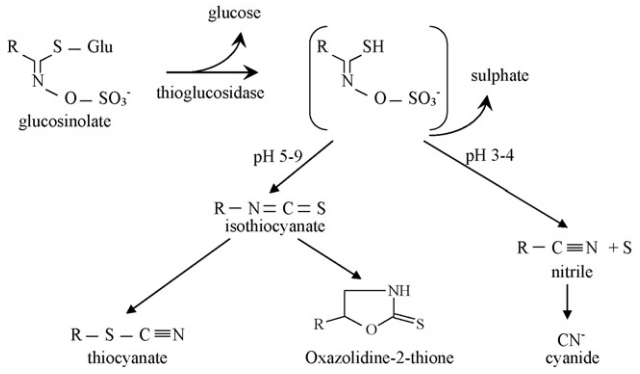

Fig. 2 – Schematic representation of glucosinolate hydrolysis and factors that may influence the outcome of the different metabolites (R = variable side chain).

3.2.1.147), which co-exists with the glucosinolates. However, hydrolysis induced by ingestion has also been reported. The myrosinase is then produced by the bacterial microflora of the gastro-intestinal tract [39–41]. After the hydrolysis, the remainder of the glucosinolate can be transformed into several biological active compounds. This depends entirely upon the pH and the structure of the side chain (R) (see Fig. 2) [35]. At low pH (3–4) sulfur is split off and a nitrile is produced, which can split further forming cyanide (CN^-). At pH 5–9 isothiocyanates are formed, from which the natural occurring TS, thiocyanates (SCN^-) and oxazolidine-2-thiones (OZT's) can be generated. This formation depends entirely upon the structure of the variable side chain (R). In case of the OZT's, an appropriate located β-hydroxyl substituent is required for the spontaneous cyclization. Within the framework of residue analysis of natural occurring thyreostats, especially the detection of 5-vinyl-1,3-oxazolidine-2-thione (5-VOT, goitrin) has been highlighted [37,42–46].

2. Legislation

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

In Belgium, substances with thyreostatic action were prohibited since 1974 (KB 12.04.1974) [4]. The EU adopted this legislation in 1981, as Council Directive 81/602/EEC [5]. Later on, Council Directive 85/358/EEC [47] amended Council Directive 81/602/EEC [5], to guarantee a uniform application for detection and monitoring of TS in all Member States.

Council Directive 96/22/EC [48], the revision of Council Directive 81/602/EEC [5] described the prohibition on the use of certain substances with hormonal or thyreostatic action in stockfarming. 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 the Council Directive 96/23/EC [49]. Two groups of substances are included in Directive 96/23/EC [49] listed in Annex I, based on Commission Regulation No. 2377/90 [50]. Group A comprises substances having hormonal or thyreostatic action, β-agonists (Directive 96/22/EC) [48] and veterinary drugs that now have been banned (included in Annex IV of Council Regulation (EEC) No 2377/90) [50]. Group B comprises other veterinary drugs and contaminants.

For good implementation of directive 96/23/EC [49], it is necessary to determine common criteria for the interpretation of test results of 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) of analytical methods. For thyreostats a suggested MRPL is fixed at 100 $\mu g\,L^{-1}$ or $\mu g\,kg^{-1}$. Additionally, in December 2007 an MRPL of 10 $\mu g\,L^{-1}$ or $\mu g\,kg^{-1}$ is proposed by the Community Reference Laboratories (CRLs) in the CRL guidance paper. However, this document has no legal force and serves only as a technical guidance for the analytical methods of TS residue control.

To ensure a harmonized implementation of Directive 96/23/EC [49], Commission Decision 2002/657/EC [51] lays down the technical guidelines and performance criteria for residue control. Within this Commission decision (2002/657/EC) [51], 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 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 [51], few studies describe the applicability of these guidelines for determination of thyreostats in urine and thyroid [52,53]. Parameters that need to be evaluated during the validation procedure are selectivity, specificity, linearity, trueness, recovery, applicability, ruggedness, stability, repeatability, reproducibility and decision (CC_α) and detection limits (CC_β).

3. Determination of xenobiotic thyreostatic drugs

Because of their possible effects on human health, there have been concerns about the presence of these compounds in edible matrices of animal origin. Consequently, there was a need for the development of analytical approaches for the detection of these TS. Conventionally, the residue control plan of thyreostatic drugs only screened for xenobiotic TS in (suspected) samples. In former years, the symptoms of the thyroid disorder hypothyroidism, caused by the administration of TS, were used as an indication. Over the last years, the determination of TS in matrices of animal origin has been dominated by chromatographic separation methods (GC or LC) coupled to sensitive and specific detection techniques such as MS. Recently, LC coupled to MS, and more in spe-

cific coupled to multiple MS, has gained in popularity. In this section, the most relevant detection methods for TS abuse are presented. Additionally, novel analytical approaches are discussed.

3.1. *Monitoring the physiological parameters of hypothyroidism*

Hypothyroidism is a disorder of the thyroid gland [54–56] that can originate from the administration of TS. Subsequently, the thyroid hormone production (triiodothyronine, T_3 and thyroxine, T_4) is inhibited and decreasing levels of T_3 and T_4 in the blood may be detected. As such, the alteration in the levels of the circulating T_3 and T_4 hormones, which can be easily measured by immunological techniques like radio immuno assay (RIA) or enzyme linked immuno sorbent assay (ELISA), can be an indication of TS abuse [2,57].

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 [58,59].

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].

3.2. *Chemical analysis*

3.2.1. *Extraction*

It must be pointed out that sample pretreatment prior to the analysis is an absolute requirement for eliminating interfering substances. To extract thyreostats from matrices (e.g. animal tissue, excreta, plasma and organs) most often methanol [52,53,60,61] is used as a solvent, but also acetonitrile [62,63] and ethyl acetate [64–66] have been reported. In the beginning, a mercurated affinity column was used for the clean-up of extracts [2,3,60,67], nowadays solid phase extraction (SPE) gained in interest. In most cased Silica SPE cartridges [52,64,65,68] are mentioned, but anion exchange [62], amino-propyl and alumina cartridges [66] have also been reported. Some even resort to the technology of matrix solid-phase dispersion (MSPD), because homogenization, disruption, extraction and clean-up are combined in one procedure [69,70].

3.2.2. *Detection*

In the beginning of 1970s a colorimetric method, the so called "Van Waes" method was introduced [71]. This method consisted of a very complex clean up with aluminium oxide, followed by a colorimetric reaction of methyl-thiouracil (MTU) with 2,6-dichloroquinonimide. The result of this reaction was a distinctive yellow color [71]. The limit of detection (LOD) was in the order of 1–10 $mg\,kg^{-1}$. As such, only very high concentrations of TS 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, 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) [67] developed a TLC method for TU, MTU, PTU, TAP and PhTU with a detection level in the order of 10 $\mu g\,kg^{-1}$ (ppb). As sample pretreatment a selective extraction procedure, based on the specific complex formation between TS and mercury ions, was performed [2,67]. 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 contain the $SHC_2H_4NH_2$ 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 purpose, two-dimensional thin layer chromatography (2D TLC) was reported by De Brabander and Vebeke (1975) [2,67]. Here, 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 (European Union) for qualitative analysis of TS [72–74]. High performance thin layer chromatography (HPTLC) [2,67,75] succeeded TLC, with later on the development of the 4×4 HPTLC, where 4 samples and 4 standards are developed simultaneously on 1 HPTLC plate [76,77].

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 TS residue analysis [78], it became clear that coupling of GC with mass spectrometry (MS) was a requirement to achieve quality control criteria [79–82]. Using derivatization agents like benzylchloride [79], pentafluorobenzylbromide [80] or methylation prior to GC–MS analysis [62,81–83] lower limits of detection (LOD) could be reached. Unfortunately, the recovery of certain TS compounds, like tapazole remained low. Therefore, De Brabander et al. proposed in 1992 an efficient method combining HPTLC and GC–MS analysis [75]. In this case, a suspected spot originated on the TLC plate, it was scraped off. This was then eluted (diethyl ether), evaporated and derivatized with MSTFA (*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide) to form silyl-derivatives that was subsequently analyzed by GC–MS (see Fig. 3). More recently, the derivatization (MSTFA) prior to the GC–MS analysis is combined with a second derivatization, executed before the purification of the sample. This supplementary derivatization solved problems concerning, recovery, repeatability and active site adsorptions, for this NBD-Cl [60], PFBBr [68–70] or 3-BrBBr [84] have been described. These methods were found suitable for confirmation of TS abuse in biological matrices (e.g. animal tissue, milk, thyroid and urine).

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,

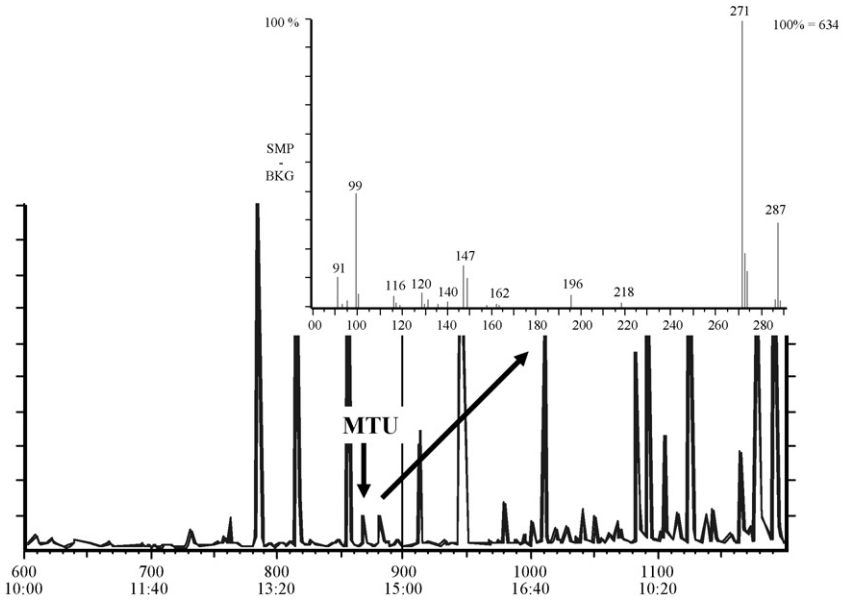

Fig. 3 – Gas chromatogram of MTU, derived from a suspected spot on a HPTLC plate, with corresponding mass spectrum.

in the range of 10 $\mu g\,kg^{-1}$ [65]. As for the detection UV (ultraviolet) [85–90], electrochemical [88,89], chemiluminescence [91] and diode array detection (DAD) [65] were reported. With the introduction of atmospheric pressure interfaces like ESI (electrospray ionization) and APCI (atmospheric pressure chemical ionization), liquid chromatography coupled to mass spectrometry (LC–MS) became the method of choice for (thyreostatic) residue control [52,61,64]. The first LC–MS method was introduced by Blanchflower et al. (1997) [64]. Without derivatization 5 TS (TU, MTU, PTU, PhTU and TAP) were detected in urine and thyroid tissue at concentration levels of 25 $\mu g\,kg^{-1}$, based on APCI ionization and SIM-MS (single ion monitoring). Later on, LC–MS^n 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 derivatization with NBD-Cl [61] or 3-iodobenzylbromide (3-IBBr) [52]. Within the advantages of ion trap mass spectrometry, multiple stage mass spectrometry (MS^n) originated. De Wasch et al. (1998) combined HPTLC with an ion trap mass spectrometer in MS^n mode for confirmatory purposes [92]. 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. Ionization was performed by ESI and fragment ions were acquired up to MS^3 [92]. It must be pointed out that nowadays nearly all detection methods of TS use GC or LC coupled to multiple or tandem MS [52,53,60,61,92], 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 $mg\,kg^{-1}$ [93].

3.3. *Animal experiments with MTU*

In addition to methods for the determination of TS residues, there is an important need for knowledge of the fate and excretion of TS in cattle. Because data on distribution and excretion is rather scarce, animal experiments were conducted by De Brabander (1986) [2]. MTU was used in these experiments, since it was in those days the most important synthetic TS drug.

For obtaining knowledge of the distribution of TS, De Brabander (1986) [2] measured the MTU concentration in different matrices using HPTLC. Thyroid, kidney and various muscle tissues were collected from 5 regulatory control animals (bovines). As can be seen in Table 1 concentrations of MTU ($mg\,kg^{-1}$) in the thyroid were 10–100 times higher than those measured in the muscle tissues [2,94]. Additionally, concentration of MTU measured in the different muscle tissues were of the same magnitude. Although for meat sampling the M. Diaphragma is recommended for reducing the sampling cost, based on the described results sampling muscle tissue for residue analysis of TS was found to be irrelevant.

For obtaining data about the excretion of MTU in bovines, different experiments were described by De Brabander (1986) [2,94]. In case of the ingestion of a single dosage (4 g), the MTU levels were measured in urine, plasma and milk. The

Table 1 – Detected concentrations of MTU ($mg\,kg^{-1}$) in different matrices of animal origin ($n = 5$)

Sample tissue	Mean concentration ($mg\,kg^{-1}$)	Stdev ($mg\,kg^{-1}$)
Thyroid	42.36	8.82
Kidney	1.58	0.96
M. Long Dorsi	1.55	0.97
M. Psoas	1.23	0.83
Cervical muscle	1.00	0.66
M. Gastrocnemius	1.03	0.67
M. Trapezius	0.92	0.59
M. Solius	1.31	1.12
M. Diaphragma	1.16	0.76

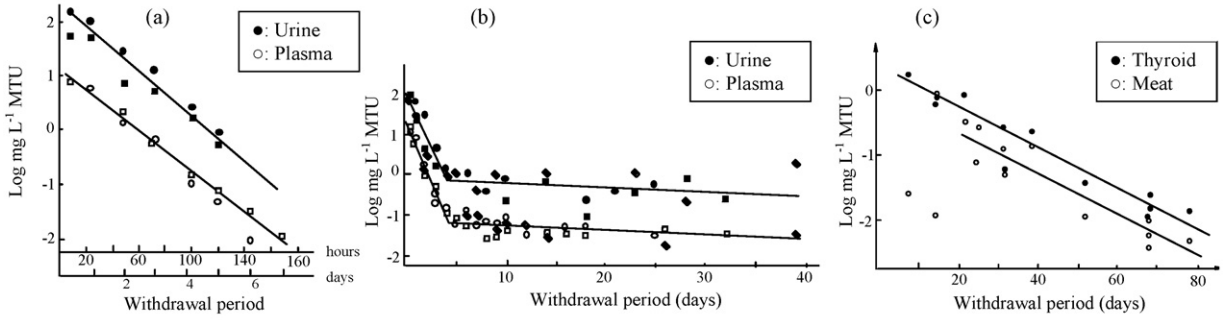

Fig. 4 – Residue levels of MTU in bovines: (a) in plasma and urine, after 5 g of MTU day^{-1} for 14 consecutive days; (b) in plasma and urine, after 5 g of MTU day^{-1} for 28 consecutive days; (c) in thyroid and meat (n = 13), after 5 g of MTU day^{-1} for 28 consecutive days.

highest concentrations of MTU residues were found in urine, the lowest in milk. After the treatment a rapid decline was seen. Detection was possible until 2–3 days after the administration. Two other experiments measured the MTU levels in plasma and urine after multiple administration, a dosage of 5 g of MTU day^{-1}, respectively for 14 (Fig. 4a) and 28 consecutive days (Fig. 4b). As presented in Fig. 4a, the MTU levels in urine and plasma, expressed as Log μg mL^{-1} MTU, in function of time, decreased linearly and the concentration dropped below the limit of detection within 6–8 days. For an administration period of 28 days, higher MTU concentrations and biphasic elimination appeared (Fig. 4b). Thus, in the first 4 days an initial rapid decline was observed, followed by a slower decreasing rate, resulting in a time detection of 40 days. The final experiment detected MTU in the thyroid and meat of 13 different animals, after various withdrawal periods. The concentration present in the thyroid was 3 times higher then in meat, but a parallel decrease was shown (Fig. 4c). Within this setup a withdrawal period of approximately 80 days was calculated.

Meat processing experiments were also conducted, to observe if cooking diminished the MTU content of processed meat [2,75]. 100 g of meat, containing 1.25 mg kg^{-1} of MTU, was heated during 1 h at 90 °C. This resulted in 65 g cooked meat with a concentration of 1.44 mg kg^{-1}, or nearly the same as the original meat and 35 g drip (=fluid lost during cooking), with a very low concentration of MTU (0.88 mg kg^{-1}). 75% of the residues of MTU remained in the cooked meat and MTU was not destroyed by the heating process.

From these experiments it was concluded that thyreostats, and more in specific MTU accumulate in the thyroid. Nevertheless residues may be detected in urine, plasma and meat. The measurement results obtained after the administration of a single dosage of MTU to cattle, confirm the initial rapid disappearance of MTU in urine, plasma and milk found also described by other authors [95–97]. In case of prolonged administration of MTU (28 days), a slower elimination than with a single dosage was seen. As such, appreciable residues levels were found in the thyroid, muscular tissue, plasma and urine, even after a withdrawal period of 1 month. Additionally, when edible matrices were processed for consumption (cooked), thyreostatic residues (MTU) remained present in meat, resulting in a potential risk for consumer's health.

4. Monitoring natural thyreostatic drugs: oxazolidine-2-thiones

Originating from the *Brassicaceae*, OZT's are natural occurring sulfur compounds. Species from this family (e.g. rape seed) may be used as cheap and readily available protein supplements for animal feed. However, the reported goitrogenicity and anti-nutritional effects of these compounds, especially of 5-vinyl-1,3-oxazolidine-2-thione (5-VOT, goitrin), are an important limiting factor for the commercial exploitation of brassica derived feedstuff to domestic livestock. Nowadays newer varieties with lower glucosinolate content are being commercialized as protein supplement. However, even when lowering the glucosinolate content, the amount of 5-VOT in feed needs to be controlled, which requires sensitive and specific methods for investigation of various biological matrices (plant, milk, egg, tissue, etc.).

Before the determination of 5-VOT in biological matrices, sample pretreatment is required. For the selective extraction steps, liquid chromatography [98], liquid liquid extraction [99,100] and also the specific complex formation of oxazoldine-2-thiones with mercury ions [2,46,101–103] were reported.

Initially 5-VOT was isolated by two-dimensional paper chromatography, from plant material and milk and then quantified by UV sprectrometry [35,104]. Madesjki (1974) [105] 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)

Table 2 – Detected thiocyanate concentrations in tissue and biological fluid, after daily dose of 6 mg KSCN (SCN$^-$)

Sample tissue	Control rat (mg kg^{-1})	SCN$^-$ treated rat (mg kg^{-1})
Meat	4.0	7.0
Liver	2.4	7.0
Kidney	4.3	10.3
Plasma	6.7	16.7
Urine	5.8	154.0

[3,101,102,98], FID (flame ionization detection) [98,106], FPD (flame photometric detection) [98] and TSP (thermionic specific detection) [98]. As for LC only UV detection [46,99,100,103] was reported. The first gas chromatographic (GC) methods provided insufficient accuracy limits for the determination in a physiological medium [106,107], but later on adequate GC analysis originated for the determination in milk [98], plasma, urine and muscle tissue [3,101,102]. To ameliorate the sensitivity of the analysis, reports of butylheptafluoro [98] or pentalfluorobenzoyl chloride [3,101,102] derivatives have been made. As for high pressure liquid chromatography (HPLC), normal phase [99,100] as well as reversed phase [46,103] has been reported. Only Mabon et al. (1999) [103] 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 that chromatographic methods coupled by MS only have been reported for the determination of the purity grade of synthesized oxazolidine-2-thiones, not for the analysis of biological matrices [108].

In literature the adverse effects (e.g. goitrogenicity and anti-nutritional aspects) of 5-VOT that originate from the ingestion of progroitrin, has been extensively studied. Most of the reports concerned ruminants and poultry, but also their derived products, respectively 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 $\mu g\,kg^{-1}$ in plasma, 80–250 $\mu g\,kg^{-1}$ in urine and 70 $\mu g\,kg^{-1}$ in [101,102]. Additionally 5-VOT appeared in the milk of lactating cows [109]. As such, concerns were expressed of this 'goitrogenic milk', especially for children, due to the reports of endemic goiter in Tasmania [110] and Finland [111–113], presumed attributed to Brassica containing diet of cows. In poultry high intake of progoitrin, resulted in reduced synthesis of thyroid hormones [114], which is translated to a reduced basal metabolism and to growth retardation [115]. Other adverse effects like lower fertility and eggs with fishy odor ('egg taint') were also reported [116,117]. According to Madejski, beside milk, eggs could be a potential source of 5-VOT for the consumer [105]. Recently, a series of studies were reported on the influence of rapeseed meal on lamb physiology and performance [118–121]. Especially the alteration of the thyroid histology, correlated with the goitrogenicity of 5-VOT was reported and little influence of the antinutritional effects were seen. This study concluded that low-gluosinolates rapeseed meal could be used as a sole protein supplement. However studies of the long-term effects of rapeseed meal on the reproductive processes are still required. It is perhaps an indication that adult ruminants are better capable in detoxification of the glucosinolate degradation products due to their different digestion process, compared to monogastric animals [118]?

Finally, it must be pointed out that the concern of the animal derived products, for example the 'goitrogenic milk' or the 'crabby eggs' remains.

5. Monitoring of inorganic thyreostats

Based on the available literature, the thyreostatic action of certain inorganic ions [12–25,122] has been reported quite a lot. This group comprises both anions (e.g. SCN^- and ClO_4^-) and cations (e.g. Li^+) are comprised. Both groups interfere with the thyroid function, but in a different manner. The anions interfere with the iodide (I^-) concentration in the thyroid through competitive inhibition. The cations inhibit the transfer of iodotyrosines to iodothyronines, block the secretion of thyroid hormones (T_3 and T_4) and increase also the renal secretion of iodide [2].

The data on inorganic thyreostats originated mainly from East-European countries. Their investigations concerned mostly the zootechnical effects of these compounds [21,23,123–125]. However, little work has been done on the possible transfer of the TS residues to biological matrices and on the excretion profile.

For the regulatory control of the abuse of inorganic thyreostats, analytical approaches for the determination of these compounds needed to be developed. The methods were then applied to obtain more information on excretion profiles and residue levels. This was helpful to inform the analysts and inspection services on the nature and magnitude of the expected concentrations of drug residues after illegal treatment. Therefore, in this section a brief overview of the available literature is given of the most important members of this group, e.g. thiocyanate (natural and chemical origin), perchlorate and lithium.

5.1. *Thiocyanate (SCN^-)*

Caution is required for the residue control of SCN^-, because of the natural and chemical nature of this compound.

De Brabander et al. (1977) [2,126] described a quantitative method for the detection in urine, based on the method of Nota and Palombari (1973) [127]. This method measured the concentration of cyanogen bromide (BrCN) that is the result of the reaction of SCN^- and bromine by gas solid chromatography (GSC). Later on, a GC–MS method was reported for measuring the thiocyanate levels in biological fluids [128]. Even more recently, an ion chromatography coupled to tandem mass spectrometry (IC–MS/MS) method was described for amniotic fluid samples [129].

Studies, conducted with Wistar rats (see Table 2) by De Brabander and Verbeke (1977) [126] were set up for better understanding the excretion profile and the occurrence of SCN^- residues in biological fluids (plasma, urine) and tissues (liver, meat, and kidney) [2]. As can be seen in Table 2, a daily treatment of 6 mg of KSCN for 3 consecutive days, resulted in a 2–3-fold increase in the levels measured in plasma, kidney and liver as compared to the control group. Due to this administration, levels of SCN^- in urine increased 50–100 times in comparison with the normal concentration. Nonetheless after ceasing the administration, the SCN^- concentrations in urine dropped to their normal values after 3–5 days. In meat, the SCN^- levels underwent an increase of 11% after long term exposure (49 days), this consisted of a low but significant increase.

These experiments indicate that SCN^- residues can be transferred to animal tissues. However, even with a prolonged administration period, after ceasing the treatment the residues are rapidly excreted.

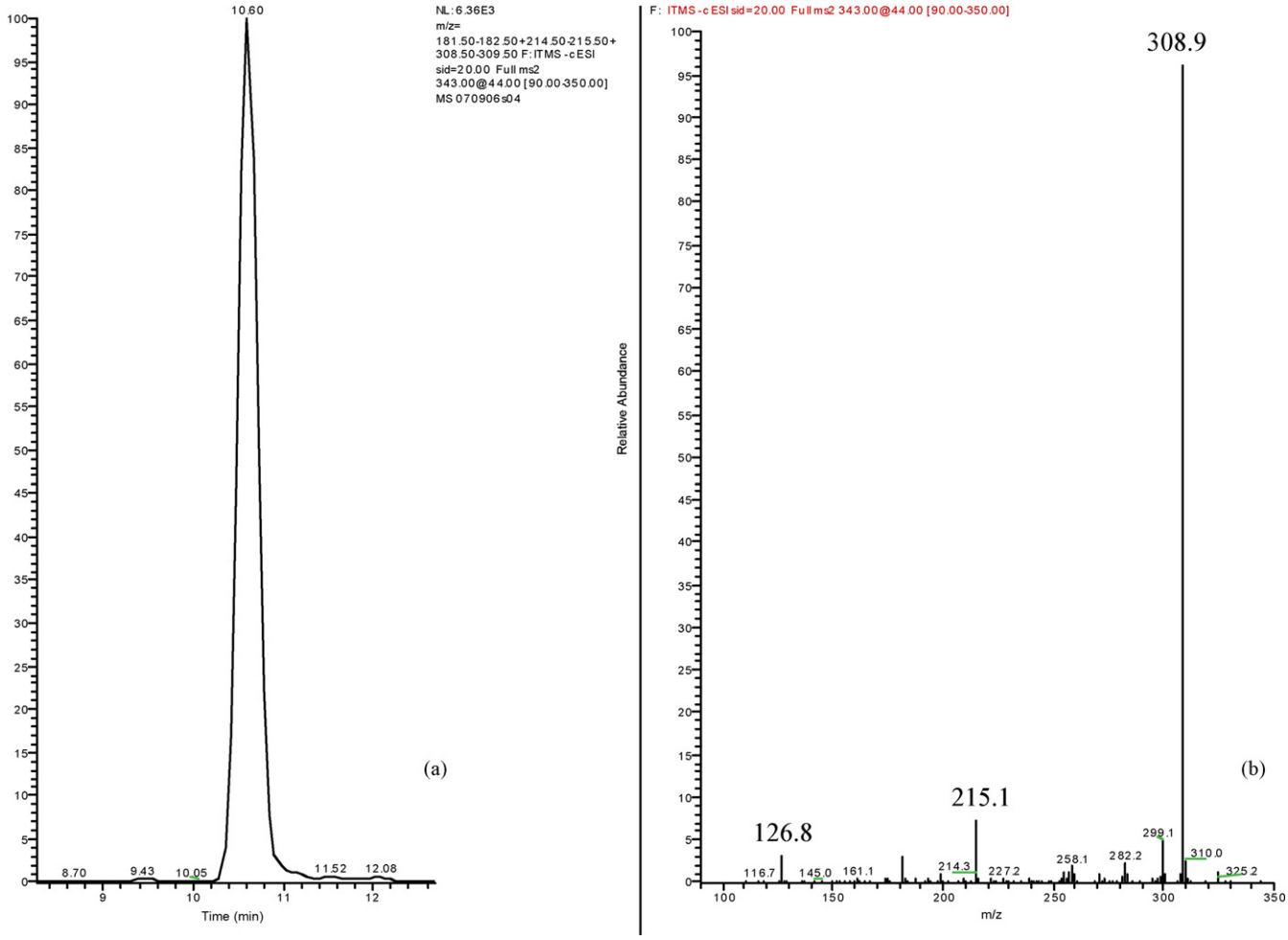

Fig. 5 – (a) Chromatogram (S/N = 1900) and (b) MS^2 spectrum of urine (1 mL) fortified with thiouracil at 5 μg L^{-1}.

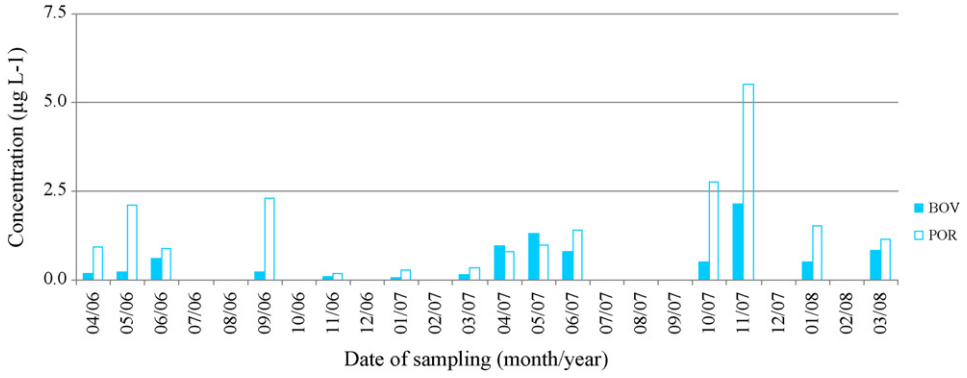

Fig. 6 – Overview of data of the occurrence of TU in bovine (BOV) and porcine (POR) urine sample from a European country (2006–2008).

5.2. *Perchlorate (ClO_4^-)*

Nowadays most detection methods for perchlorate are based on ion chromatography (IC) [130–133] or IC coupled to electrospray ionization mass spectrometric detection (IC–ESI-MS) [131], but also HPLC (UV) [134] is mentioned.

Batjoens et al. (1993) also studied the excretion profile of perchlorate residues in bovine urine, after a single time dosage and after a long-term administration [130]. With a single administration dosage of 2, 4 or 6 g, the ClO_4^- residues dropped below the LOD of 0.1 mg kg^{-1} for the 2 and 4 g dosage, this after 48 h. For the 6 g dosage, up to 72 h was needed for complete removal of the ClO_4^- residues. Additionally, at the moment that the administration period of 4 g day^{-1} for 10 consecutive days was ceased a similar profile was seen in comparison with the single administration.

Unfortunately, even with a prolonged administration a rapid decline of perchlorate residues is seen in cattle urine. This made it very difficult for the analyst to detect and control its abuse in cattle fattening.

5.3. *Lithium (Li^+)*

For the detection of lithium an ion exchange chromatography method was developed by Batjoens et al. (1993) [135].

A similar study as for perchlorate [130] was set up to study the excretion profile of Li^+ in urine of bovines [135]. The first study consisted of a single oral dosage of 3 or 9 g. A biphasic elimination profile was seen, with a rapid decline in the first 36 h, while the remaining Li^+ ions were released slowly during the following 6–7 days. As such, it was possible to detect residues of Li^+ in urine up to 144 h after administration of 3 g and up to 168 h for 9 g dosage. Secondly, a daily dosage of 6 g day^{-1} (10 consecutive days) is administrated. The residues were detected for a period between 192 and 288 h, after ceasing the treatment. When reviewing the excretion profile, a rapid decline in Li^+ level is monitored during the first 2 days. However, the rate of decline takes up much more time compared to the single dosage.

In residue control, the abuse of Li^+ for fattening purposes has the advantage of displaying a long withdrawal period, even when concerning a single dosage. The higher the amount of the single dosage, the longer small traces of residues could be found in urine. In case of a long administration profile even longer withdrawal periods were seen, 8–12 days.

6. Recent developments

Former detection methods of TS (TU and analogues) in matrices of animal origin are time consuming, demanding a lot of organic reagents and are labour intensive. In this section new developments in the framework of TS detection in animal matrices are highlighted. New developments are mainly focused on a derivatization prior to GC– or LC–MS/MS analysis.

Because of the chemical properties of TS drug, the purification is a true analytical challenge. Firstly, these compounds have the capacity in delocalizing the π-electrons in the ring structure, which results in various tautomeric forms. Thiouracil and analogues can have up to 6 tautomeric forms [136]. As such derivatization is performed before the extraction to lock the TS into a single tautomeric form, which will benefit the efficiency and repeatability of the extraction procedure. Secondly, the low molecular weight of TS results in a low sensitivity (signal to noise ratio) on the mass spectrometer. Through derivatization a significant increase of the molecular weight is provided, which is beneficiary for the signal specificity. In case of detection on the LC–MS, the high polar behaviour of TS interferes with the retention and separation on the stationary phase in reversed phase liquid chromatography. As such, a decline in polarity is seen with the application of derivatization, resulting in an improved separation [52].

For GC–MS/MS, derivatization is performed before the extraction, respectively with 3-bromobenzylbromide (3-BrBBr) and *N*-methyl-*N*-(trimethylsilyl)-trifluoro-acetamide (MSTFA) [84]. As for LC–MS/MS analysis, 3-iodobenzylbromide (3-IBBr) has been selected as the most efficient derivatization reagent, executed before the purification [52].

Fig. 5 displays a fortified (5 $\mu g L^{-1}$) urine sample, derivatized with 3-IBBr that is analyzed by LC–MS/MS. Using this technique, considerably lower LOD values of 0.2–0.4 $\mu g L^{-1}$ are detected, in comparison with 50–100 $\mu g L^{-1}$ before.

Recently, Abuín et al. (2008) described a validation procedure, according to the European Criteria 2002/657/EC, of an ultra-performance liquid chromatography (UPLC) method coupled to tandem mass spectrometry, for the detection of thyreostatic drugs in thyroid tissue [53]. Due to the difficult nature of thyreostatic compounds, it is surprising that here no derivatization is performed in this method.

7. TU... status of semi-endogenous substance?

Based on experimental studies, a correlation between a cruciferous-based diet and the occurrence of thiouracil in urine of cattle is proposed [137]. Because a clear TU concentration (up to 9 $\mu g L^{-1}$) in urine could be detected after feeding of rapeseed cakes, the erroneous indication of possible illegal use of TU may be supposed.

As seen in Fig. 6, similar results were obtained when urine samples (bovine and porcine) were processed within the framework of a control plan for a European country. Occasionally TU was detected, at concentrations within the range of 1–6 $\mu g L^{-1}$. These results indeed raised the question of the possible semi-endogenous status of TU.

As such, the use of an improved detection method [52] has revealed a possible 'natural' presence of TU in urine of non-treated animals. However, further research is needed for the discrimination between low level abuse and natural presence of TU, and maybe other TS.

At this moment, a TS level of 10 $\mu g L^{-1}$ or $\mu g kg^{-1}$ (CRL guidance paper, 2007) instead of 100 $\mu g L^{-1}$ is proposed as a provisional MRPL of TS.

8. Conclusions

Due the increasing production of livestock, the illegal use of growth promoting agents remains an important problem. As such, an adequate residue control plan is an absolute requirement to monitor the illegal treatment of animal livestock. Thyreostatic drugs are banned, resulting in a zero-tolerance level that is established by national and international legislation. Therefore, analytical approaches have to be developed for the detection of TS residues in biological matrices.

Throughout the years, the increasing scientific knowledge and technical improvements resulted in analysis methods with increasing sensitivity and specificity. Techniques such as GC and LC coupled to mass spectrometry achieved LODs in the range of 0.1 $\mu g kg^{-1}$ or $\mu g L^{-1}$. In the future, UPLC coupled to MS^n, will most likely gain in popularity due to the reduced analysis time and solvents costs.

Within the framework of TS residue control, the possible natural occurrence of TU is of most importance. This indicates that in the future a more careful interpretation is needed

of samples suspected of being 'non-compliant' with regard to thiouracil abuse.

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