

Determination of the thyreostats in animal feeding stuffs using liquid chromatography-tandem mass spectrometry

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Abstract

A rapid liquid chromatography tandem mass spectrometry method was developed and validated to detect and confirm five thyreostatic drugs: tapazole, thiouracil, methylthiouracil, propylthiouracil, and phenylthiouracil in animal feeding stuff samples. Thyreostats were extracted from feed with methanol, and then degreasing of the extract with petroleum ether was performed, followed by the derivatisation of the compounds with 3-iodobenzylbromide in basic medium (pH 8.0). The derivatives were extracted with diethyl ether and analysed by gradient elution on a Poroshell 120-EC C18 column with triple quadrupole MS detection with turbo spray source in positive ionisation mode. The method was validated in accordance with the Commission Decision 2002/657/EC. For validation level of $10 \mu\text{g kg}^{-1}$, the recovery ranged from 82% to 97.5% for all examined compounds. The repeatability and reproducibility did not exceed the limit of 20% for all analytes. The linearity was good for all thyreostats in the whole range of tested concentrations, as proved by the correlation coefficients greater than 0.99. The decision limits ($CC\alpha$) ranged from $1.63 \mu\text{g kg}^{-1}$ to $3.95 \mu\text{g kg}^{-1}$, whereas the detection capabilities ($CC\beta$) ranged from $2.74 \mu\text{g kg}^{-1}$ to $6.73 \mu\text{g kg}^{-1}$. The developed analysis is sensitive and robust, and therefore useful for quantification and confirmation of thyreostats in residue control programme.

Keywords: feeding stuff, thyreostats, liquid chromatography, mass spectrometry.

Introduction

Thyreostats are a group of compounds known as thionamides, which inhibit the synthesis of thyroid hormones: triiodothyronine (T3) and thyroxine (T4) (10). This property favours the processes of fattening animals; therefore these compounds could be applied illegally to animals for anabolic purposes. The weight gain of animals is mainly due to water retention in edible tissue and augmented filling of the gastrointestinal tract (6). However, due to the cancerogenic and teratogenic properties of thyreostatic drugs and possible harmful effects on public health (18), their use in food producing animals has been prohibited in Europe by Council Directive 81/602/EC (15). Monitoring for illicit use of these substances is conducted in accordance with EU Directive 96/23/EC (16), which is implemented in each member state according to the National Residue Control Plans (16).

Antithyroid drugs are mostly studied in urine, muscles, and thyroid gland (5, 22). According to the guidelines of Directive 96/23, samples for the study of prohibited substances (Group A) must also be taken from live animals on the holding. Therefore, besides biological samples of animal origin, it is recommended to take samples of drinking water and feeding stuffs.

In addition, in the cases where the prohibited compounds in animal tissues are detected, additional sampling must be conducted, including samples of the feed from the place of origin of the animals. Because in recent years, one of thyreostatic drug - thiouracil (TU) was detected in bovine and porcine urine samples (19, 30), development of the method for determining this group of compounds in feeding stuffs seems to be necessary. The presence of thiouracil in the urine of animals may result from its illegal use or from diet rich in cruciferous plants. For several years, it has been reported that thiouracil is of a natural origin derived

from *Brassicaceae* consumption (24). According to latest reports presented by Vanden Bussche *et al.* (27), thiouracil may naturally occur in feed and food samples. Thiouracil was successfully detected in samples of traditional rapeseed, rapeseed-'00' variety coarse meal, and rapeseed cake at 1.5, 1.6, and 0.4 $\mu\text{g kg}^{-1}$, while broccoli and cauliflower displayed thiouracil concentrations of 6.0 and $<1.0 \mu\text{g kg}^{-1}$ respectively. A crucial element in the preparation of feed samples was enzymatic hydrolysis using myrosinase, because no thiouracil could be detected in the various *Brassicaceae*-derived samples without the enzyme. Therefore, the finding of TU in feed samples without the use of enzymatic hydrolysis step may indicate the illegal use of this compound. Various methods were developed for the determination of thyrostatic drugs in animal tissues (21) and urine (7), but only few studies concerned feed analysis. The low molecular weight, high polarity, and the existence of tautomeric forms of thyrostats caused difficulties associated with the analytical determination of this group of compounds for many years. These inconveniences are reduced by application of derivatisation, and 3-iodo-benzylbromide has been tested and selected as the most efficient derivatisation agent when detection is performed by LC-MS/MS (23). When analysis is performed by GC method, derivatisation converts the analytes into volatile compounds, suitable for this technique (31). Recently, the LC-MS/MS methods have been published for the determination of thyrostats in urine and thyroid gland without derivatisation (2, 28), and detection limits of these methods were even higher.

For extraction of thyrostats from matrix, polar solvents such as acetonitrile (9), methanol (12), and ethyl acetate (8) are usually used. The extracts are then cleaned-up by liquid-liquid extraction (LLE) with diethyl (23) ether or dichloromethane (20) or combination of LLE with solid phase extraction (SPE), mainly with silica cartridges, but also with C18 columns (1, 11, 25). The use of gel permeation chromatography (GPC) as an alternative clean-up strategy was also described (2).

As emphasised previously, there is a limited number of methods for the analysis of thyrostats in feed. A method for the identification of eight thyrostatic drugs in animal feeding stuff samples was published in 2005 (23). Thyrostats were extracted with methanol, and derivatisation with 3-iodobenzylbromide in a strong basic condition was conducted. Then, clean-up step on reversed-phase C18 was performed followed by silica SPE cartridge. The compounds were analysed by LC-MS/MS and the method was able to reach limits of 150 $\mu\text{g kg}^{-1}$.

The aim of this study was to develop a multiresidue, rapid, and sensitive LC-MS/MS confirmatory method for the detection of five thyrostatic drugs in feed samples.

Material and Methods

Reagents and chemicals. Acetonitrile (LC-MS reagent), methanol (HPLC isocratic grade), n-hexane (ultra-resi analysed), and sodium hydroxide were purchased from Mall Baker (Deventer, the Netherlands). Analytical grade reagents such: diethyl ether, acetic acid (99.5%), ortho-phosphoric acid (85% purity), boric acid, and concentrated hydrochloric acid were obtained from POCH (Poland). Water was purified using a Milli-Q system (Millipore, USA). Derivatisation reagent 3-iodobenzyl bromide (3IBBr) was provided by Sigma-Aldrich (Germany). Britton-Robinson buffer (0.4 M), pH 8.0 was prepared by dissolving boric acid (24.73 g) in warm water (800 mL), then adding ortho-phosphoric acid (26.7 mL), acetic acid (23 mL), 50% sodium hydroxide solution, and diluting with water to 1000 mL. A 50% sodium hydroxide solution in water was prepared by dissolving the solid substance (500 g) in water (500 mL). A 0.1% acetic acid solution was prepared by mixing acetic acid (0.25 mL) with water (249.75 mL). Solution of 3IBBr was drawn up by dissolving reagent (10 mg) in methanol (4 mL). The injection solvent comprised of acetonitrile and 0.1% acetic acid solution (25:75, v/v).

Standards of thyrostats: 1-methyl-2-mercaptoimidazole (TAP), 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU), and internal standard (IS) 5,6-dimethyl-2-thiouracil (DMTU) were obtained from Sigma-Aldrich (Germany). The standards were stored in accordance with the manufacturer's recommendation. Primary standard stock solutions of each compound were prepared in methanol at a concentration of 1 mg mL⁻¹. Intermediate standards solutions were prepared by further dilution of stock solution with methanol to obtain final concentrations of 100 $\mu\text{g mL}^{-1}$ and 10 $\mu\text{g mL}^{-1}$. For fortification purposes, a mixed standard solution containing each analyte (except IS) at a concentration of 1 $\mu\text{g mL}^{-1}$ was prepared. Working standard solutions were stored at 2-8°C for not longer than three months.

LC-MS/MS measurement. The chromatographic separation of six thyrostatic drugs was achieved using a 1100 HPLC binary pump system (Agilent Technologies, Germany) on a Poroshell 120 EC-C18 analytical column (150 mm × 2.1 mm, 2.7 μm) (Agilent Technologies, Germany) with an octadecyl guard cartridge (4 mm × 2 mm) (Phenomenex, USA) and the column temperature was maintained at 40°C. A gradient LC system using 0.1% acetic acid in water

(mobile phase A) and acetonitrile (mobile phase B) at a flow of 0.2 mL min⁻¹ was operated; the injection volume was 25 μL . The gradient increased from 25% B at 0 min to 65% B at 4 min, 70% at 15 min, then returned to 25% at 18 min and equilibrated for 7 min. The total run time of the method was 25 min.

For integration and data acquisition Analyst 1.4.2 software (ABSCIEX, Canada) was used.

All LC-MS/MS analyses were carried out using the API 4000 triple quadrupole (QqQ) instrument with a Turbo Ion-Spray source (ABSCIEX, Canada) operating in positive Electrospray Ionisation mode. Nitrogen was used as nebulisation and desolvation gas. Source and desolvation temperatures were set at 500°C and 120°C, respectively, the capillary voltage was set at 5500V. MS was operated in multiple reactions monitoring (MRM) mode. For thyreostats, at least two transitions per analyte were monitored, using optimised ion source (capillary and cone potentials) and MS/MS (collision energy) parameters. The precursor product transitions for each analyte, as well as their corresponding collision energies are presented in Table 1.

Sample extraction and purification. Samples of feed were grounded and mixed before preparation. Two grams of feed were weighed to centrifuge tube and 10 mL of methanol were added followed by 20 µL of internal standard (1 µg mL⁻¹ DMTU) to obtain final concentration of 10 µg kg⁻¹. The sample was vortexed and left for 10 min, then 5 mL of methanol phase (equivalent of 1 g of feed) were transferred to a new tube and degreasing step of extract was performed using liquid-liquid extraction with petroleum ether (3 × 3 mL). The extract was evaporated to dryness under a stream of nitrogen at 60°C (±2°C). The residues were dissolved in 5 mL of Britton-Robinson buffer, pH 8.0,

next 100 µL of methanol 3-iodobenzyl bromide solution was added and the whole sample was mixed. The derivatisation was conducted in the dark at 40 °C (±2°C) over 1 h. After derivatisation, the reaction mixture was cooled to room temperature and the pH of the solution was adjusted to 4.5 by adding a few drops of concentrated hydrochloric acid. Then, the tube was centrifuged (10 min, 8000 rpm) and supernatant was collected to another tube. Afterwards, the mixture was extracted two times with 3 mL of diethyl ether. The combined extracts were passed through anhydrous sodium sulphate layer and evaporated to dryness under a gentle stream of nitrogen at 40°C (±2°C). The residue was reconstituted in 600 µL of the mobile phase consisting of acetonitrile and 0.1% acetic acid aqueous solution (25:75, v/v). Twenty-five microlitres were finally injected onto the LC-MS system.

Matrix-matched calibration. Matrix matched calibration curves were prepared and used for quantification. Samples were fortified at levels corresponding to 0, 1, 5, 10, 30, and 50 µg kg⁻¹ by adding 0, 20, and 100 µL aliquots of a 0.1 µg mL⁻¹ standard solution, and 20, 60, and 100 µL of a 1 µg mL⁻¹ standard solution respectively. After fortification, the samples were held for 10 min prior to extraction as described above. Calibration curves were prepared by plotting the response factor (peak area analyte/internal standard peak area) as a function of analyte concentration.

Table 1. LC-MS/MS ion acquisition parameters (MRM) mode used for identification and confirmation of thyreostats

Compound	MRM transition (m/z)	Collision energy (eV)	Declustering Potential (V)
TAP	331 > 217*	31	100
	331 > 114	35	
	331 > 90	58	
TU	345 > 217*	26	89
	345 > 90	61	
MTU	359 > 217*	26	95
	359 > 90	65	
PTU	387 > 217*	29	95
	387 > 90	70	
PhTU	421 > 217*	35	106
	421 > 90	70	
DMTU (IS)	373 > 217*	27	105
	373 > 90	65	

* Transitions used for quantification

Method validation. The samples of feed for validation did not contain the residues of the analytes of interest. The method developed was submitted to a validation process in accordance with Commission Decision 2002/657/EC general requirements (14). The experiments were conducted by the software “ResVal” (v 2.0) (CRL Laboratory, the Netherlands) (4), which was intended for the validation of analytical methods. For estimation of linearity, precision (repeatability and within-laboratory reproducibility), recovery, decision limit ($CC\alpha$), and detection capability ($CC\beta$) of the method, blank feed samples were fortified with each analyte at 5, 10 and 15 $\mu\text{g kg}^{-1}$. Six replicate test portions, at each of the three fortification levels, were analysed. In addition, one blank sample and once on level 20, also on level 50 $\mu\text{g kg}^{-1}$ were performed. This 21 replicate research set was conducted three times on three separate days. The linearity was studied with six calibration levels between 0 and 50 $\mu\text{g kg}^{-1}$. The $CC\alpha$ and $CC\beta$ of the method were calculated according to the calibration curve by ResVal software. $CC\alpha$ is the concentration corresponding to the intercept + 2.33 times the standard error of the intercept. $CC\beta$ is the concentration corresponding to the signal at $CC\alpha$ + 1.64 times the standard error of the intercept.

For checking signal specificity, 10 different feed samples from a variety of sources were tested. The samples were analysed in duplicate, initially only fortified with internal standard, and then fortified with both internal standard and the analytes of interest at a concentration equivalent to 10 $\mu\text{g kg}^{-1}$.

For evaluation of matrix effect, the difference between the mass spectrometric response for the

analytes in standard solution and the response for the same analytes at the same concentration in feed matrix was performed.

The uncertainty for each compound for validation level of 10 $\mu\text{g kg}^{-1}$ was evaluated using ResVal software. According to this validation software, the uncertainty was calculated as the sum of variances of reproducibility on levels 5-15 $\mu\text{g kg}^{-1}$ and variance of the matrix effects. The combined uncertainty was multiplied by a coverage factor of 2 to estimate expanded uncertainty.

Results

The summary of the validation results of thyreostats in feeding stuff samples is presented in the Tables 2 and 3.

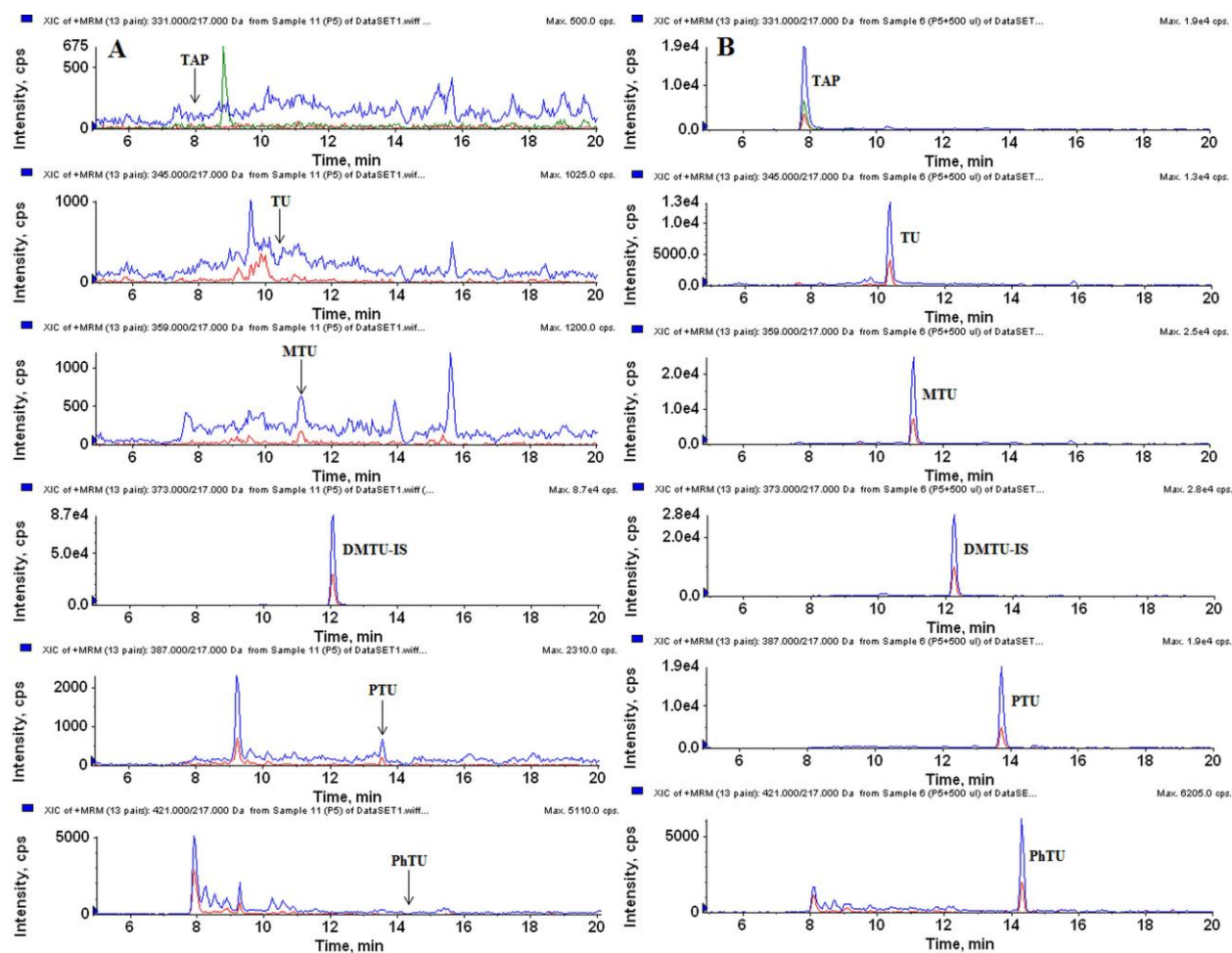
The recovery and precision of the method were determined using spiked feed samples at three levels analysed on three different days. The method was characterised by a good recovery, above 81%, and correct precision, within-laboratory reproducibility did not exceed 30% CV. The calculated concentrations of $CC\alpha$ and $CC\beta$ of the thyreostats were much less than 10 $\mu\text{g kg}^{-1}$, and demonstrated high sensitivity of the method. For tapazole and propylthiouracil a significant matrix effect was observed. The linearity was good for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients (r^2) exceeding 0.99 value for all curves. The specificity studies showed no interferences in the range of the retention times of analytes as shown in Fig. 1.

Table 2. Recovery and precision of the method

Analyte	Spike level ($\mu\text{g kg}^{-1}$)	Recovery (%)	Repeatability (RSD,%)	Within- laboratory reproducibility (RSD,%)
Tapazole	5	99.2	20.8.6	30.4
	10	97.5	18.3	18.3
	15	95.8	14.3	17.5
Thiouracil	5	86.9	7.4	19.5
	10	82.1	16.2	16.9
	15	77.7	19.6	19.6
Methylthiouracil	5	92.8	3.8	22.2
	10	87.8	12.1	19.8
	15	86.4	9.6	18.8
Propylthiouracil	5	98.9	15.3	15.3
	10	89.4	12.9	18.1
	15	81.8	12.2	17.6
Phenylthiouracil	5	93.3	19.1	20.1
	10	84.1	15.5	19.9
	15	82.4	17.7	17.7

Table 3. $CC\alpha$, $CC\beta$, measurement of uncertainty (%), and matrix effect (%) values of each of the analytes

Analyte	$CC\alpha$ ($\mu\text{g kg}^{-1}$)	$CC\beta$ ($\mu\text{g kg}^{-1}$)	M.U. (%)	ME (%)
Tapazole	1.92	3.28	36	70
Thiouracil	2.39	4.07	28	27
Methylthiouracil	1.63	2.74	35	13
Propylthiouracil	1,73	3.0	33	-11
Phenylthiouracil	3.95	6.73	34	-59

**Fig. 1.** LC-MS/MS chromatograms of: (A) blank feed sample; (B) samples of feed fortified with thyreostats at $10 \mu\text{g kg}^{-1}$

Discussion

The previously published method for urine and muscle samples was used as a starting point for the development of this procedure (29). The positive ionisation was used in the method, as only this ionisation mode is suitable for all investigated compounds. Negative ionisation, used mainly for thiouracil and its derivatives, provides unsatisfactory results for tapazole (23). In this method, for each analyte a precursor ion (parent mass) and two fragments (corresponding to strong and weak ion) are

monitored (Table 1). This yields four identification points, required for a confirmatory method. Throughout the validation study, LC-MS/MS identification criteria were verified by monitoring relative retention times and ion ratios. In all instances, these were within the tolerances set out in Commission Decision 2002/657/EC (14).

For chromatographic separation of the thyreostats, three columns were tested: Nucleosil® 100-5 C18 AB (125 mm \times 2 mm, 5 μm) column, Inertsil® ODS-3 analytical column (150 mm \times 2.1 mm, 3 μm), and the Poroshell 120-EC C18 column (150 \times 2.1 mm,

2.7 μm). All columns revealed similar selectivity for compounds tested and the correct chromatographic separation of the thyreostats was obtained, however with different gradient conditions. Nevertheless, better results were achieved on Poroshell 120-EC C18 column. Especially, peak originating from tapazole has gained in intensity and symmetry and, therefore, this column was chosen for further study instead of the previously proposed Nucleosil[®] 100-5 C18 AB.

To separate analytes from the matrix components, a liquid-liquid extraction (LLE) with organic solvents is very often used. To verify the efficiency of extraction of thyreostats from feed samples enriched to a concentration of 50 $\mu\text{g kg}^{-1}$, in addition to methanol, other reagents such as t-butyl methyl ether and mixture of methanol and Britton-Robinson buffer at pH 8.0 (50:50,v/v) were tested. The best results were obtained for methanol (97% recovery), which was previously applied in the method for muscle samples. For remaining reagents, recovery was slightly lower and amounted to above 84% for t-butyl methyl ether and 86% for mixture. Since some feed samples contain large amounts of fat and are rich in protein, the stage of degreasing and removal of protein from sample extract seems to be necessary to avoid large matrix effect. Degreasing of the extract was performed by using a triple extraction with petroleum ether, while denaturation of protein was performed by heating at 60°C. Irrespective of the type of feed, precipitated protein was not observed, and that is why this step was omitted. The derivatisation of thyreostats with 3IBBr, the most effective derivatisation reagent, was applied. After derivatisation, the compounds were extracted from basic aqua phase with diethyl ether after acidification of the derivative mixture to pH 4.5 instead of previously proposed pH 2.5, which greatly improved the reproducibility for tapazole. Taking into account the variety of the feed samples, it can be concluded that the treatment of the samples was appropriate for the purpose, and chromatograms for feed extracts showed the absence of matrix peaks, as presented in Fig. 1.

The method was validated according to the criteria specified in Commission Decision 2002/657/EC (14) for a quantitative confirmatory method, covering specificity, calibration curve linearity, recovery (accuracy), repeatability, $CC\alpha$ and $CC\beta$, measurement of matrix effect, and uncertainty.

The specificity of the method was demonstrated by analysis of 10 different feed samples and parallel samples fortified with the mixture of the analytes at 10 $\mu\text{g kg}^{-1}$. No interference for any of the transitions was observed around the thyreostats retention times (Figure 1), therefore, the method was found to be specific for the thyreostatic drugs investigated.

The linearity of the chromatographic response was tested with matrix matched curves for each compound using blank samples spiked from 0 to 50 $\mu\text{g kg}^{-1}$. The linearity was good for all analytes in the whole range of

tested concentrations, as proved by the correlation coefficients (r^2) exceeding 0.99 value for all curves.

Because no certified reference materials are available, instead of trueness, recovery rate was assessed. Recovery was expressed in terms of percentage of measured concentration vs fortified concentration. The recovery and precision of the method were determined using spiked feed samples at three levels analysed on three different days. Because the method was performed utilising internal standard, recovery of each sample was individually corrected. As shown in Table 2, the correct recovery for all compounds tested was obtained. The application of internal standard improved, together with the precision of the method expressed as within-laboratory reproducibility, which did not exceed 30% CV. These results are in agreement with the 2002/657/EC accepted ranges for these parameters.

The values of $CC\alpha$ and $CC\beta$ were calculated according to the calibration curve prepared for five calibration levels. Blank feed samples were fortified at 0.5, 1, 1.5, 2, and 5 times validation level of 10 $\mu\text{g kg}^{-1}$ for each analyte and were analysed on three different days. Because there is no permitted limit for thyreostats in animal feed and no MRPL level was established, the level of 10 $\mu\text{g kg}^{-1}$ has been used for the validation as this level is suggested by the Community Reference Laboratory for these analytes in other matrices (17). The calculated values of $CC\alpha$ and $CC\beta$ presented in Table 3 are all below 4 $\mu\text{g kg}^{-1}$ and 7 $\mu\text{g kg}^{-1}$ respectively. Compared to values reported in the literature for thyreostatic compounds (23), the $CC\alpha$ and $CC\beta$ values obtained in this study are much lower indicating high sensitivity of the presented methodology.

Matrix effect is a common problem for LC-MS/MS with ESI mode, which can lead to signal suppression or enhancement and is often difficult to eliminate through cleanup procedures (3, 26). In order to estimate the matrix effect, a comparison of the chromatographic response of each analyte in standard solution and in feed matrix was conducted. As the data in Table 2 presents, a significant effect on signal responses of tapazole and phenylthiouracil was observed; for the first compound enhancement of signal was noted; whereas, for PhTU ions suppression was detected. Therefore, for quantification of thyreostats, matrix matched calibration curves were used. The expanded uncertainty was calculated applying a coverage factor of 2, which gives a level of confidence of approximately 95% (13). The expanded uncertainty evaluated for validation level of 10 $\mu\text{g kg}^{-1}$ ranged between 28% and 36% for five analytes (Table 2).

The proposed method was applied for the detection of the thyreostats in feed samples collected on farms and submitted to the laboratory in the frame of the National Residue Control Programme. The samples

of feed were from different species such as calves, cattle, swine, and sheep, and differed in composition. Some of the samples derived from farm where thiouracil was detected in cattle's urine above $10 \mu\text{g L}^{-1}$, revealed the presence of rapeseed meal (one in at about 40%). Out of the 15 analysed feed samples, no thiouracil and other compounds were detected above decision limits of the method. The elaborated method is characterised by high sensitivity and is a useful tool for official control of feeding stuff for thyreostatic drugs.

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