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Multi-residue method for the determination of thyreostats in urine samples using liquid chromatography coupled to tandem mass spectrometry after derivatisation with 3-iodobenzylbromide

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Abstract

Thyreostats are banned compounds in Europe since 1981 (directive 81/602/EC) because of their carcinogenic and teratogenic properties. The control of their illegal use in breeding animals is amongst the most difficult because of their low molecular weight, high polarity and the existence of tautomeric forms. The analytical procedure described in this paper involves, after a derivatisation step with 3-iodobenzylbromide, the detection and identification at low level (μg/L) by LC-ESI(−)-MS/MS of eight thyreostats in various biological matrices (urine, muscle, liver, thyroid, animal feed, faeces and hair). The urine method has been validated according to the EU criteria (2002/657/EC decision): CC_α and CC_β were found in the range (0.1–5.2 μg/L) and (2.6–23.2 μg/L), respectively. The performances fulfil the requirements of the EU regarding the provisional minimum required performance limit (MRPL) fixed at 100 μg/L in urine.

Keywords: Thyreostatic drugs; Urine; Thiouracil; Mercaptobenzimidazole; Derivatisation; LC-ESI-MS/MS; MRPL

1. Introduction

Thyreostatic compounds are orally active drugs which may be used to increase the weight of cattle prior to slaughter. This weight gain is mainly due to increased water absorption and retention within edible tissue as well as filling of the gastro-intestinal tract [1,2]. The consequence of such abuse is not only the production of inferior meat quality, but overall the potential risk to human health of drug residues. For these particular reasons, thyreostats are banned within the EU since 1981 (directive 81/602/EC) [3] for animal production.

Thyreostats are polar and amphoteretic thionamides, which are small molecules derived from thiouracil or mercapto-imidazole (Fig. 1). Extraction and analysis of these molecules is a real analytical challenge. The first difficulty concerns the extraction from biological matrices in which they occur through different tautomeric forms. The second difficulty, when separated onto reversed-phase liquid chromatography, is due to their high polarity which prevents a good retention and separation of these compounds on the stationary phase. Finally, the analysis by mass spectrometry of these small molecular weight analytes (100–200 u) is not satisfactory in term of sensitivity (signal to noise ratio) since the signals are usually disturbed by the chemical noise, as illustrated by Blanchflower et al. [4]. Thyreostats were first studied with colorimetric methods based on the reactivity of thiol or thione groups, with for example 2,6-dichloroquinone-chloroimide or 7-chloro-4-nitrobenzo-2-oxal-1,3-diazole (NBD-Cl) [5,6]. Various techniques have been described in the literature for the detection of administration of thyreostats to animals. These range from weight measurement of the thyroid gland [7] to various methods based on thin layer chromatography [8–10], gas chromatography using

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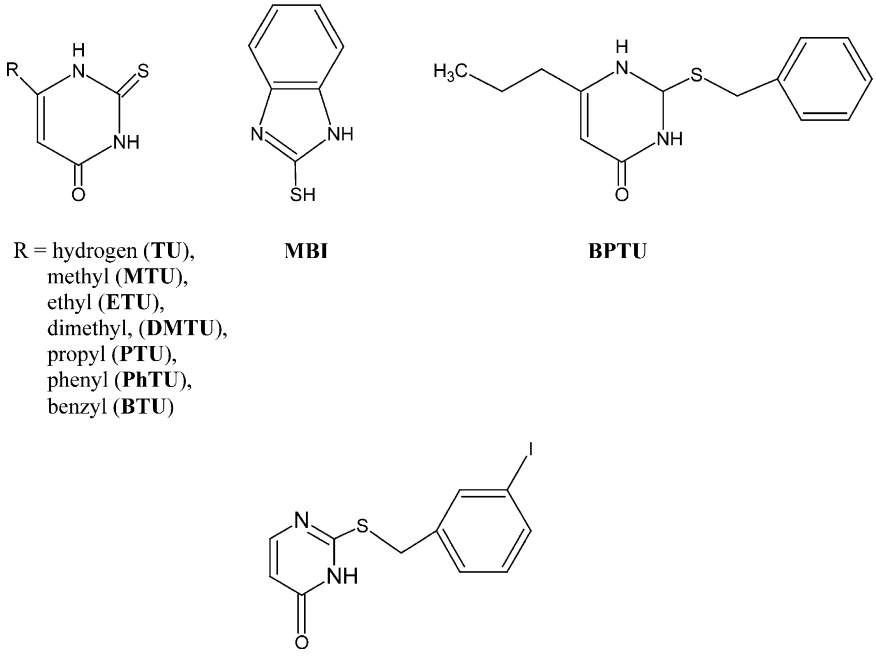

R = hydrogen (**TU**), methyl (**MTU**), ethyl (**ETU**), dimethyl, (**DMTU**), propyl (**PTU**), phenyl (**PhTU**), benzyl (**BTU**)

MBI

BPTU

TU-3IBBr

Fig. 1. Elemental composition and chemical structures of thiouracil (TU), methyl-thiouracil (MTU), ethyl-thiouracil (ETU), dimethyl-thiouracil (DMTU), propyl-thiouracil (PTU), phenyl-thiouracil (PhTU), benzyl-thiouracil (BTU), mercaptobenzimidazole (MBI) and benzylpropylthiouracil (BPTU). Chemical structure of TU derivatized with 3-iodobenzylbromide.

nitrogen-phosphorus detection, flame photometric detection, or mass spectrometry detection [11,12] and high performance liquid chromatography using either UV [13] or electrochemical detection [14]. More recently, methods based on liquid chromatography coupled to mass spectrometry have been published: Blanchflower et al. [4] reported an LC-MS method using an atmospheric pressure chemical ionization (APCI) interface and selected ion monitoring (SIM). A method based on LC-ESI-MS/MS-full scan measurements on ion trap detector was recently developed [15]. Detection performances of the different published methods were 50 μg/L in urine using GC-MS [11,12], 20 μg/kg, for six different thyreostats, in thyroid samples using LC-MS/MS [15] and 25 μg/kg in muscle with a GC-MS/MS detection [16].

The present work aimed at increasing the performances of detection of thyreostats in various biological matrices, extending the monitoring to a wide range of compounds. For this purpose, different derivatives have been studied and compared for thyreostats in terms of extraction, specificity, quality of the chromatographic separation, ionization efficiency and specificity of the fragmentation.

2. Experimental

2.1. Chemicals

Standards were obtained from Sigma–Aldrich (St. Quentin Fallavier, France). Stock solutions of the thyreostatic drugs (Fig. 1): 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-ethyl-2-thiouracil (ETU), 5,6-dimethyl-2-thiouracil (DMTU), 6-propyl-2-thiouracil (PTU), 6-benzyl-2-thiouracil (BTU), 6-phenyl-2-thiouracil (PhTU), 2-mercaptobenzimidazol (MBI) were prepared in methanol at a concentration of 100 ng/μL. Working solutions were obtained by 100× and 1000× dilutions in methanol (1 and 0.1 ng/μL, respectively).

The internal standard was synthesised in the laboratory, 2-benzyl-6-propyl-2-thiouracil (BPTU) (Fig. 1).

Chemicals for extraction and purification steps were of analytical grade. Solvents for the preparation of the mobile phase were of HPLC-grade. They were all obtained from SDS (Peypin, France).

Derivatisation reagents 3-iodobenzylbromide (3IBBr), 4-iodobenzylbromide (4IBBr), penta-fluorobenzylbromide (PFBBr), 3-bromobenzylbromide (3BrBBr), 3-bromobenzylchloride (3BrBCl), 2-iodobenzylchloride (2IBCl) were obtained from Sigma–Aldrich (St Louis, MO, USA) and prepared extemporaneously (2 mg/mL in methanol).

Phosphate buffer, pH 8, was made up of 94.5 mL 0.2 M Na_2HPO_4 and 5.5 mL 0.2 M KH_2PO_4.

2.2. Materials

Silica gel SPE disposable columns (1 g) and reversed-phase C18 SPE cartridges (2 g) were obtained from UCT (Bristol, PA, USA).

Separation of thyreostats compounds was performed on a Nucleosil C18 AB column (5 μm, 2.5 mm × 50 mm) (Interchim, Montluçon, France) with a MeOH (A) and H_2O containing 0.5% acetic acid (B) gradient, run at 300 μL/min on an Alliance 2690 HPLC chromatograph (Waters, USA). The gradient was linear as follows: A/B 30/70 to 100/0 in 20 min. A triple quadrupole mass analyser (Quattro-LC, Micromass, UK) was used fitted with an ESI source operated in the negative mode. Mass spectrometric analysis were done in the following working conditions: capillary was set at 3 kV, cone voltage at 40 V, acquisistion from *m*/*z* 2–1000 (time scan 4 s, inter-scan delay 0.03 s, dwell time 0.1 s, centroïd mode), source temperature at 120 °C and desolvatation temperature at 300 °C. Nitrogen was used as nebulizing and auxiliary gas at flow rates of 90 and 600 L/h, respectively. Argon was the collision gas and collision energy was set at 40 eV. The analytes were detected using selected reaction monitoring (SRM) acquisition mode. The data were collected and analysed with the MassLynx software.

2.3. Extraction and derivatisation

2.3.1. Urine

Urine samples (bovine and porcine) were centrifuged for 10 min at 3000 × *g*. Five millilitres of phosphate buffer, pH 8, were added to 1 mL of urine. To the sample, 100 ng of internal standard (BPTU) was added. A methanolic derivative solution (100 μL) was added, tubes were placed in an ultrasonic bath for 10 min and the derivatisation was allowed to proceed in the dark at 40 °C over 1 h. Thereafter, the reaction mixture was adjusted to pH between 2 and 3 by adding 100 μL of HCl 35%. The derivatized extract was then purified successively with 3, 2 and 2 mL of diethyl ether. The combined extracts were dried over sodium sulphate and evaporated to dryness under a gentle stream of nitrogen.

2.3.2. Tissues, animal feed, faeces, hair

Tissues (muscle, liver, thyroid) and faeces were freeze dried. All the different matrices were grounded. To 1 g of animal feed, 100 mg of hair or 250 mg of tissue (corresponding to 1 g fresh tissue) 5 mL of methanol were added. To 2.5 g of dried faeces (corresponding to 20 g fresh faeces), 25 mL of methanol were added. Samples were vortexed, placed in an ultrasonic bath for 10 min and agitated for 1 h at room temperature to allow the analytes to extract from the matrix. Samples were centrifuged (15 min, 3000 rpm) and the supernatants were filtered over a 0.45 μm membrane (Whatman, Springfield Mill, UK). To the filtrate, 100 ng of internal standard (BPTU) was added. Samples were evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 5 mL of phosphate buffer, pH 8, and the derivatisation was performed as described for urine samples.

2.4. Clean-up

The residue was dissolved in 100 μL CH_2Cl_2 and vortexed. Prior to the application on the column 300 μL cyclohexane was added and the mixture was vortexed. For the clean-up procedure of the sample extracts, the silica cartridges were conditioned with 15 mL of cyclohexane. The extract was applied onto the column. The column was washed with 6 mL of cyclohexane. Thyreostats were eluted with 6 mL of a mixture of hexane/ethyl acetate 40/60 (v/v) which was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 150 μL of mobile phase consisting of 30/70 MeOH/H_2O containing 0.5% acetic acid. Of this extract, 75 μL were injected on the column.

For faeces and hair matrices, the clean-up was exactly the same as for the urine samples. Concerning tissue and animal feed, it was necessary, prior to the silica cartridge clean-up, to run a reversed-phase SPE. After liquid/liquid extraction, the residue was dissolved in 100 μL methanol and 900 μL H_2O, and the mixture was vortexed. The C_{18} cartridges were conditioned with 10 mL MeOH then 10 mL water. The extract was applied onto the column. The column was washed with 5 mL MeOH/H_2O 50/50. Analytes were eluted with 6 mL MeOH/H_2O 90/10 which was evaporated under a stream of nitrogen to give an aqueous residue. HCl (100 μL) 35% was added to obtain a pH between 2 and 3. A liquid/liquid extraction with diethyl ether was performed as described previously and the clean-up on silica cartridge was realised as described for urine samples.

3. Results and discussion

3.1. Derivatisation study

The aims of the derivatisation step were to stabilize the thyreostat drugs under a single tautomeric form, to give them an apolar behaviour and to increase their masses. For this purpose, halogenated derivatisation reagents containing two halogens groups were tested: firstly because it allows a nucleophilic substitution to occur on the thyreostats and secondly because it gives rise to a significant increase in mass [12,15]. To fulfil this latter point, derivatising reagents containing a benzene ring were of real interest. Six different compounds were studied: 3-iodobenzylbromide (3IBBr), 4-iodobenzylbromide (4IBBr), penta-fluorobenzylbromide (PFBBr), 3-bromobenzylbromide (3BrBBr), 3-bromobenzylchloride (3BrBCl) and 2-iodobenzylchloride (2IBCl). Derivatisation occurred before the extraction step because it allows to deal with a single form of the thyreostat drug which is much more convenient for the subsequent extraction. Indeed, thyreostats belonging to the TU group exhibit six different tautomeric forms owing to the delocalisation of the π electrons in the ring structure [12]. The place of the derivatisation in the protocol is the same than in those already published [12,15]. Derivatisation occurred in aqueous solution at pH eight for two main reasons: the substances are soluble in aqueous media in alkaline conditions and the tautomeric forms favourable to nucleophilic substitution are predominant in the pH conditions.

Comparison of the different derivatising reagents was realised using the thiouracil as model compound. All the potential derivatisation reagents allowed the derivatisation to occur; MS/MS fragmentations and diagnostic ions were studied for the six TU-derivatives. A significant increase in mass could be highlighted since it passed from 128 u to values in the range 295–343 u. Iodo-derivatives gave rise to higher masses than bromo- or fluoro-ones. In each case, four to seven diagnostic ions could be monitored. In the case of bromo-derivatives, two series of diagnostic ions could be followed because of the isotopic ratio of the considered halogen (^{79}Br and ^{81}Br). This leads to very specific diagnostic ions, however, the signal is therefore divided by two. The choice of the negative ion mode for the detection of the molecules permitted to decrease the chemical noise and then to increase the signal to noise ratio.

The relatively more apolar behaviour of the derivatives was checked on reversed-phase HPLC: retention time was significantly increased in all cases since before derivatisation, TU eluted almost in the void volume whereas after derivatisation the retention time was around 10 min. Influence of the halogen nature on the retention behaviour could also be evaluated: brominated derivates showed lower retention time than fluorated one and over all than iodinated ones. An influence of the halogen position on the ring could also be noticed since retention was increased with the position on the ring (4 better than 3, better than 2).

Finally, the efficiency of the derivatisation, extraction and ionization steps of the derivatized molecules on the signal intensity was evaluated and is reported in Fig. 2. The relative intensities have been calculated according to the internal standard, indeed, since the BPTU does not support any derivatisation, its signal intensity is therefore independent from the derivatising reagents used. It appeared that the signal intensities are strongly correlated to the reagent, iodobenzylbromide ones giving rise to the more intense signals. Results clearly showed the influence of the halogen group and its position on the intensity of the corresponding signal: intensities were the worst for fluoro-derivatives and the best for brominated ones. When the same halogen group was considered, its position

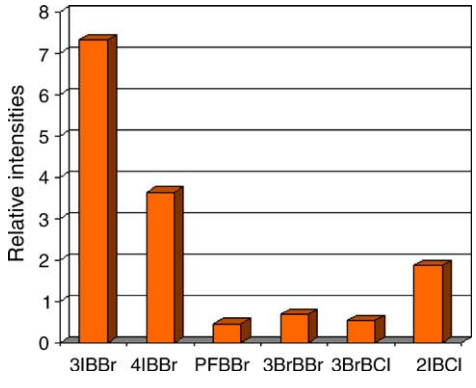

Fig. 2. Relative intensities of the signals observed for the different TU-derivatives. Results are expressed as ratio of peak height in between derivatized TU and internal standard.

was favourable in the position 3 rather than in the position 4 on the aromatic ring. It appeared that for steric reasons, the presence of five fluor atoms on the benzene ring could prevent the derivatisation to occur in the best conditions when compared to derivatisation reagents containing only one halogen atom.

Finally the nature of the halogen group allowing nucleophilic substitution to occur seemed to be of less importance in the signal intensity obtained for the corresponding derivatives, substitution with brome atoms being somewhat more interesting than with chlore.

According to these observations, the derivatisation reagent of choice was the 3-iodobenzylbromide (3IBBr). The eight thyreostats of interest were tested for this reagent regarding the reaction efficiency, signal sensitivity and specificity. The derivatisation with 3IBBr as described in the present work allowed to reach, for each of the eight thyreostat compounds the different goals: conversion of the different tautomeric forms into a single one before the extraction step, significant increase in mass (+215 u) with positive consequence onto signal specificity, enhancement of the sensitivity, and improvement of the derivative stability (over a week instead of a day for NBDCl derivatives [15]), colorless derivatized extract which does not contaminate the ion source of the mass spectrometer (as observed with NBDCl derivatives), decreasing of compound polarity leading to improved separation on RPLC.

3.2. Application to biological samples

Urine samples have been supplemented by 5 μg/L of each of the thyreostat compounds and 100 μg/L of the internal standard BPTU. After derivatisation with the 3IBBr, the compounds of interest were extracted and purified before injection on LC-ESI-MS/MS. A typical ion chromatogram of urine sample spiked at 5 μg/L with each thyreostat is shown in Fig. 3a. All the molecules of interest are easily detected at this concentration level and their identification is non ambiguous. Previous published methods for urine allowed the detection of thyreostat drugs at levels around 50 μg/L [11]. The present method is a real improvement since more thyreostat compounds are monitored and over all because the identification level is 10 times lower.

The method has been validated according to the EU criteria (2002/657/EC decision) [17]. 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. Decision limits were based on the analysis of 20 blank samples. The signal associated to CC_α corresponds to a 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-β (error probability = 5%). Detection capability was based on the analysis of 20 blank urine samples fortified at the level of 15 μg/L (presumed identification performance of the method according to a pre-validation). Calculated CC_α and

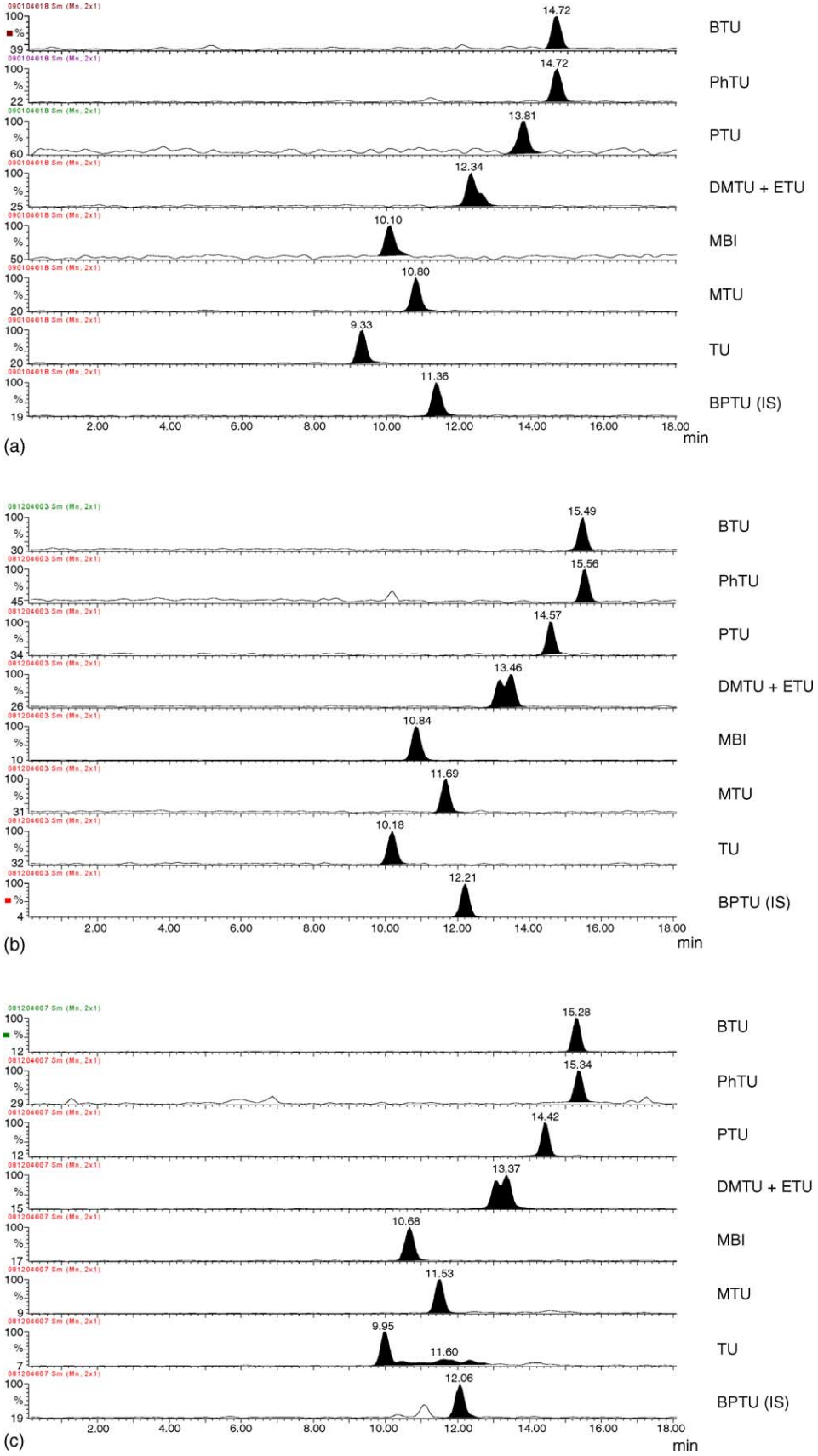

Fig. 3. Mass chromatograms of urine (a), muscle (b) and animal feed (c) samples fortified with thyreostats at 5, 15, and 150 μg/kg, respectively. LC-ESI(−)-MS/MS analysis and SRM acquisition mode.

Table 1
Decision limits (CC_α) and detection capabilities (CC_β) calculated for the eight thyreostats in urine according to the 2002/657/EC decision

Analytes	CC_α (μg/L)	CC_β (μg/L)
TU	0.7	4.8
MTU	1.6	4.2
ETU	0.7	2.5
DMTU	0.8	2.9
PTU	0.5	4.6
PhTU	5.1	18.7
BTU	2.3	20.5
MBI	0.1	23.2

CC_β for the different thyreostats are reported in Table 1. Decision limits (CC_α) obtained in screening (1 transition) were in the range 0.1–5.1 μg/L, and the detection capabilities (CC_β) obtained in confirmation (two transitions, expected ratio) are in the range 2.5–23.2 μg/L. These results are satisfactory since the performances are below the suggested minimum required performance limit (MRPL) fixed at 100 μg/L in urine sample.

The method has successfully been applied to other biological matrices (Muscle, Liver, Thyroid, Faeces, Hair, Animal feed). For tissues and animal feed, in order to obtain clean extracts, it was necessary to add a clean-up step on reversed-phase C_{18} gel before the silica cartridge SPE. Thyreostats could be identified at levels of 15 μg/kg in the different considered tissues, 5 μg/kg in dried feaces and 50 μg/kg in hair (Fig. 3b). In animal feed, the detection of drugs of interest was 150 μg/kg which is in accordance with the levels that would be used for illegal abuse (Fig. 3c). Previous studies on thyroid [15] and muscle samples [16] reached the levels of 20 and 25 μg/kg, respectively. The present method allows to decrease identification limits of thyreostat compounds in complex biological matrices.

4. Conclusion

In order to improve performances of methods dedicated to the detection of thyreostat compounds, the study preliminary focussed onto their derivatisation prior to the first extraction step. The aims of the derivatisation are numerous and include the stability of the compounds, the reduction of their polarity to improve their separation, the increase of their molecular weight to allow lower detection capabilities and limits of decision. The 3-iodobenzylbromide has been selected as the most efficient derivatisation reagent.

The protocol has been successfully applied to biological matrices (urine, tissues, animal feed, faeces and hair) spiked with eight different thyreostats (TU, MTU, ETU, DMTU, PTU, PhTU, BTU, MBI). Performances of identification in all these matrices have been improved in comparison with already published results. Furthermore, the number of monitored compounds has also been extended since eight molecules can be followed with this multi-residue method.

Further work is being carried out on matrices (urine, faeces, hair) collected on treated animal. Kinetics of elimination are under investigation and will be published soon.

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