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Simultaneous determination of thyreostatic residues in animal tissues by matrix solid-phase dispersion and gas chromatography–mass spectrometry

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Abstract

A method for determination of thyreostatic residues in animal tissues by matrix solid-phase dispersion (MSPD) and gas chromatography-mass spectrometry in selected ion detection mode was developed. Thyreostatic compounds in different matrices were extracted and purified by combination of MSPD and subsequent solid-phase extraction. Silica gel was selected as the solid support of both procedures and the conditions of the procedures were optimized. Thyreostats were derivatized with pentafluorobenzylbromide (PFBBr) in strong basic medium and then with *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA), which can improve the yields of derivatization for thyreostats, the repeatability, and therefore the limits of detection (LOD) of thyreostats. The limits of detection reached 10 μg/kg (2-thiouracil, 6-methyl-2-thiouracil and 6-propyl-2-thiouracil), 20 μg/kg (6-phenyl-2-thiouracil) and 50 μg/kg (tapazole) with high recoveries (more than 70% for most of thyreostats) and relative standard deviations between 4.5% and 8.7%.

Keywords: Thyreostats; Matrix solid-phase dispersion; Animal tissues; Derivatization; Gas chromatography–mass spectrometry

1. Introduction

Thyreostatic substances are a series of derivatives of thiocarbamide with different substituents, such as 2-thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU) and 1-methyl-2-mercapto-imidazole (TAP). Fig. 1 shows the structural formulae of these compounds.

The thyreostats can decrease the production of thyroid hormone, inhibit the normal metabolism and lower the gastro-intestinal motility, and the use of thyreostats will increase the weight of animals before slaughter by enhancing the water retention in the subcutaneous and muscular tissues as well as in the gastro-intestinal tract [1–3]. Consequently, the quality of the foodstuff becomes inferior and consumption of the meat containing thyreostat residues may be harmful to human health. Thus, the use of thyreostats has been prohibited in most countries.

Many methods such as TLC [4,5], GC [6,7], and HPLC [8–10] for the analysis of thyreostat residues were developed in order to control the illegal use of these compounds in the early stage, whereas these methods lack the function of confirmation for the structure of thyreostats and have low sensitivities. De Brabander and Verbeke [11] reported an effective purification method in which a mercuric complex of the thyreostats was formed on a mercurated ion exchange column. This method was used for the analysis of thyreostats coupled with different detection methods [12–14] in the following years. But the use of mercurated ion exchange column lead the analysis becomes longer and complicated, and low recovery of PhTU. Furthermore, the mercury-containing reagents will contaminate the environment, and so it would be highly desirable to eliminate their use from analytical procedures.

GC–MS [12,15] and LC–MS [16] have been used for the determination and confirmation of thyreostatic residues

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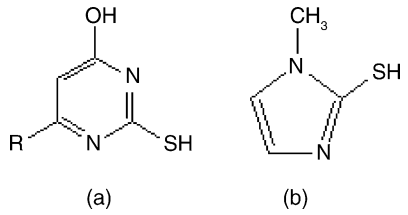

Fig. 1. Structural formulae of thyreostats (R = hydrogen (TU), methyl (MTU), propyl (PTU), phenyl (PhTU)) (a) and TAP (b).

recently years, but the purification step prior to analysis is mainly based on the liquid-liquid extraction and solid-phase extraction. Because of the highly polar and hydrophilic nature of the thyreostats, it is difficult to extract them from tissue efficiently, resulting relative lower recoveries of analytes [15].

Matrix solid-phase dispersion (MSPD) has been widely utilized to the determination of residues for various biological systems [17–24]. It combines the procedures of homogenization, disruption, extraction and purification, and avoids the use of large amounts of solvents and has been proven to be a good alternative to liquid–liquid extraction [25]. It can be used alone or with solid-phase extraction for the purification and enrichment purposes [26–27].

The solid support previously utilized in matrix solid-phase dispersion is mainly non-polar bounded solid-phase (typically C_8 or C_{18}), and there are some reports relating the use of silica gel as solid supports [28–35]. The aim of this report is to develop a procedure for extraction and purification of thyreostats in animal tissues by matrix solid-phase dispersion, in which silica gel was selected as the dispersed sorbent, and a GC–MS method in selected ion mode for determination of thyreostat residues in animal tissues.

2. Experimental

2.1. Apparatus and analysis conditions

The analysis was performed on a Trace-MS spectrometer (ThermoQuest, Finnigan San Jose, CA, USA). Data was collected with a Xcalib software data process system. Separation was carried out on a DB-5 ms fused-silica capillary column (J & W Scientific, Folsom, CA, USA), 30 m × 0.25 mm I.D., 0.25 μm film thickness. High purity helium (99.999%) was used as carrier gas (1.0 ml min^{-1}). The temperature program was: initial 100 °C, hold 2 min, rate 15 °C min^{-1} to 260 °C, and then rate 10 °C min^{-1} to 290 °C, hold 5 min. The split/splitless injector was set to 250 °C and 1 μl was injected without split.

The mass spectrometer was operated in the electron impact (EI) mode using 70 eV ionization voltages. The ion source temperature was 200 °C and the GC–MS interface was set to 250 °C. The analyses were performed by selected ion detection mode.

SPE instrument, 10 port vacuum manifold (Agilent Technologies, P.A., CA, USA).

2.2. Reagents and preparation of solutions

2-Thiouracil (TU), 6-methyl-2-thiouracil (MTU), 6-propyl-2-thiouracil (PTU), 6-phenyl-2-thiouracil (PhTU), 1-methyl-2-mercapto-imidazole (TAP) and internal standard dimethyl-thiouracil (DMTU) were from Sigma (Saint Louis, MO, USA). *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) (97%) and penta-fluorobenzylbromide (PFBBr) (97%) were from Acros Organics (New Jersey, USA). Methanol was HPLC grade (Fisher, USA), and all other solvents were analytical grade and used as supplied. Silica gel was from Phenomenex (California, USA), 40–63 μm; Merck (Darmstadt, Germany), 40–63 μm and YMC (Kyoto, Japan), 50 μm. It was activated prior to use (at 150 °C, after washing with methanol). The silica gel of Phenomenex was used to optimization of conditions.

Accurately weighted 25 mg TAP, TU, MTU, PTU, PhTU and dissolved in 25 ml methanol to obtain the stock solution of the thyreostats at a concentration of 1 mg/ml. The internal standard solution was achieved by dissolving 25 mg of DMTU in 25 ml of methanol. The working solutions were prepared as required by further diluting with methanol.

NaOH/ethanol solution: 1.0 g of sodium hydroxide was dissolved in 50 ml of anhydrous ethanol.

2.3. Matrix solid-phase dispersion

Two hundred to three hundred grams of samples to be analyzed were chopped and homogenized. 0.50 g sample was weighed accurately and placed into a mortar with 2.0 g silica gel. The standard solution of thyreostats and internal standard DMTU were added. Blending them with a pestle for a few minutes until obtained a homogeneous mixture. After 30 min, the mixture was transferred into a 10 ml syringe barrel which was used as MSPD column. Washed them with 10 ml chloroform, and eluted with 5 ml methanol/chloroform. The eluent was collected into a tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen.

2.4. Derivatization with PFBBr

In above residue, 500 μl NaOH/ethanol (0.1 mol/l) and 25 μl PFBBr were added and mixed adequately. The derivatization reaction proceeded about 10 min in water bath of 25 °C. Then, diluted hydrochloric acid (about 0.1 mol/l) was added to adjust the pH of the solution to 3.0 (±0.3). The solution was extracted with 1 ml dichloromethane for three times. The combined organic solvent (about 3 ml) was evaporated to dryness at 40 °C under a gentle stream of nitrogen after dried with a little anhydrous sodium sulfate.

2.5. Solid-phase extraction

The silica SPE cartridge (1.0 g) was conditioned with 5 ml ethyl acetate and 5 ml dichloromethane, respectively, and then 1.0 ml solution of the sample extract in hexane was loaded. After rinsing the column with 5 ml chloroform, elution was performed by 5 ml dichloromethane/ethyl acetate (v/v = 20:80). The eluent was collected into a tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen.

2.6. Derivatization with MSTFA

Fifty micoliters MSTFA and 50 μl dichloromethane was added into the tube and reacted at 60 °C for 30 min. The solution was diluted to 1.0 ml with hexane for GC–MS analysis.

2.7. Effect of the ratio of sample to silica gel

Accurately weighed 0.30, 0.50, 0.70 g samples and placed into a mortar with 2.0 g silica gel, then 30, 50, 70 μl (1.0 μg/ml) standard solution were added, respectively. Other procedures were as described in above sections.

2.8. Effect of the amount of silica gel

Accurately weighed 0.25, 0.50, 0.75 g samples and placed into a mortar with 1.0, 2.0, 3.0 g silica gel, respectively, then 25, 50, 75 μl (1.0 μg/ml) standard solution were added. The volumes of rinsing and eluting solvent were 8 and 4 ml when silica gel was 1.0 g, and 12 ml and 8 ml when silica gel was 3.0 g, respectively. Other procedures were as described in above sections.

3. Results and discussions

3.1. Derivatization and detection of thyreostats

For gas chromatography–mass analysis of thyreostats, derivatization prior to analysis is needed. The most common derivatization reagent is MSTFA and the –OH and –SH of the thyreostats are silylated [12], but the yield of derivatization is low due to the tautomeric forms of thyreostats in solution [36] and high activity of –SH group [37], especially in a complex matrix that not purified completely. So, we attempt to modify the derivatization reaction in order to improve the yield of derivatization and limit of detection. Pentafluorobenzylbromide can react with the compound containing –SH group in strong basic medium forming stable derivatives [38], the –OH of the thiouracil derivative can be subsequently silylated by MSTFA (see Fig. 2). In this report, we attempt to conduct the derivatization reaction in NaOH/ethanol medium because the alcoholate ion can accelerate the reaction between –SH and halohydrocarbon [37]. The concentrations of alkali and derivatization reagent, the time and temperature of derivatization, stability of the derivative were investigated to optimize the conditions of derivatization reaction.

The alkali concentration and reaction temperature are two important factors influencing the derivatization reaction. The responses (peak area) of thyreostatic derivatives increase significantly with the increase of the alkali concentration when the alkali concentration was below 0.1 mol/l. The responses increase slightly above 0.1 mol/l of the alkali concentration, indicating that the reaction was nearly completed. Higher alkali concentration will cause more interference peaks in the chromatograph, so the alkali concentration was selected as 0.1 mol/l.

The reaction proceeds very well below 30 °C and high temperature is not advantageous, the responses of the derivatives decrease significantly above 30 °C. Therefore, the reaction temperature was determined as 25 °C. Generally, the concentration of the derivatization reagent PFBBr is in large excess (10 times over the analytes), there is no remarkable difference among the responses in higher concentration and the amount used can be adjusted according to the concentration of thyreostats. In this report, 25 μl PFBBr is chosen when the volume of NaOH/ethanol is 500 μl. The reaction is very fast and can be completed in 10 min, and the responses have not obvious alterations after 10 min. The derivatives are stable in 24 h, which guarantees the accomplishment of the analysis. Finally, the conditions of the derivatization reaction are: 500 μl 0.1 mol/l NaOH/ethanol, 25 μl PFBBr, 25 °C, 10 min.

After two steps of derivatization, the analytes were detected on a quadruple mass spectrometer under EI conditions

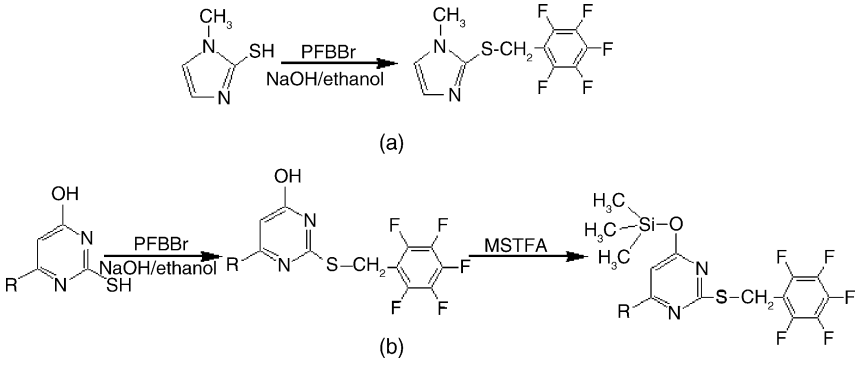

Fig. 2. Derivatization reactions of thyreostats.

Table 1
Selected ions of thyreostatic derivatives

Thyreostats	Selected ions
TAP	241, 261, 275, 294
TU	360, 365, 380, 381
MTU	374, 379, 394, 395
PTU	402, 407, 422, 423
PhTU	436, 441, 456, 457

in selected ion detection mode. The abundant fragment ions can be selected to the quantitative analysis and confirmation of the thyreostats. The selected ions are listed in Table 1 and Fig. 3 shows the selected ion chromatograms of standards. For TU and its 6-position derivatives, the selected ions are M^+, $(M+1)^+$, $(M\text{-}CH_3)^+$ and $(M\text{-}HF)^+$ were. For TAP, and the selected ions of TAP are M^+, $(M\text{-}F)^+$, $(M\text{-}CH_2F)^+$ and $(M\text{-}CH_2F\text{-}HF)^+$.

3.2. Optimization conditions of the purification procedures

3.2.1. The conditions of MSPD

The common solid support used in MSPD procedures is reserved-stationary phase (C_8, C_{18}) [25]. There are some reports [28–35] mentioning the application of normal-stationary phase. Because there are high polar groups (–OH and –SH) in the molecules of thyreostats, which limits the applicability of techniques such as solvent partitioning and the more common SPE phases [39–40], silica gel was selected as the solid support of MSPD procedure.

The rinsing and eluting strengths of solvent were optimized based on the 2.0 g solid support (silica gel) and 0.5 g pork sample. Fifty micoliters (1 μg/ml) solutions of thyreostats and internal standard (DMTU) were added individually, corresponding to 100 μg/kg. The suitable strength of rinsing and eluting solvents were determined by examining the recoveries of thyreostats. Recoveries were obtained by comparing the relative area of analytes to internal standard in the spiked matrix to that of the standard solution at the same concentration. The results are summarized in Table 2.

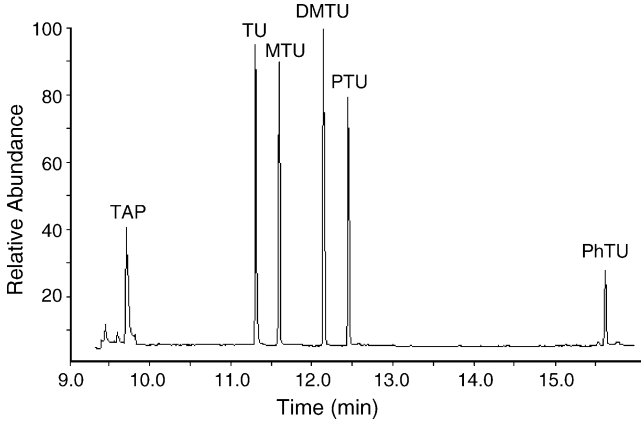

Fig. 3. Selected ion chromatograms of thyreostatic derivatives (50 μg/l).

Table 2
Recoveries of thyreostats obtained with different eluting solvents

Eluting solvent	TAP	TU	MTU	PTU	PhTU
5 ml $CHCl_3$	nd	nd	nd	nd	nd
10 ml $CHCl_3$	nd	nd	nd	nd	nd
5 ml $CH_3OH/CHCl_3$ (v/v = 15:85)	33.0	69.4	79.3	84.8	75.6
5 ml $CH_3OH/CHCl_3$ (v/v = 25:75)	70.5	76.9	86.2	88.1	78.8

nd: not detected; relative standard deviations are between 3.5% and 7.6% ($n = 5$).

Chloroform cannot elute any analytes from the column, so it was a suitable rinsing solvent. The mean recoveries of analytes increased with the strength of eluting solvent, and when the volume ratio of CH_3OH to $CHCl_3$ reached 25/75, the mean recoveries of thyreostats were all above 70%. The interference will become significant when the eluting strength increases. Therefore, the rinsing and eluting conditions were 10 ml CH_3Cl and 5 ml $CH_3OH/CHCl_3$ (v/v = 25/75), respectively.

Because the activities of silica gel are greatly affected by water, experiments in various conditions, ratios of sample to silica gel (w/w), the amount of silica gel used and the silica gel obtained from different manufacturers, have been designed in order to check the adaptability of the method. In these experiments, the spiking level of thyreostats and internal standard were 100 μg/kg in pork samples.

3.2.2. Effect of the ratio of sample to silica gel on recoveries of thyreostats

The pork samples used in the experiments contained about 70% of water, and so different amount of pork sample equals to the variations of water. The most common ratio of sample to solid support ranges from 1:3 to 1:5 (w/w) [21,25] and too high a sample to sorbent ratio might exceed the dispersion ability of sorbent and thus influence MSPD performance. 0.3, 0.5, 0.7 g samples to 2.0 g silica gel were tested individually in this experiment to observe the effect of the ratio of sample to sorbent on the recoveries of thyreostats. The results showed that the mean recoveries of most thyreostats are between 77% and 90% and the relative standard deviations are in the range of 3–8% ($n = 5$) except for TAP whose recoveries are between 62% and 71% with the same RSDs range. It indicated that the variations of water in silica gel did not affect its functions as a solid support for purification and extraction of the thyreostats in pork samples.

3.2.3. Effect of the amount of silica gel used and the silica gel from different manufacturer on recoveries of thyreostats

In order to examine the extensive suitability of silica gel as a sorbent for thyreostats in MSPD procedure, the influence of the amount of solid support and the silica gel from different manufacturers on the recoveries of thyreostats were investigated. The ratios of pork sample to solid support were fixed to 1:4. The strength of rinsing and eluting solvent keep consistent while their volumes were adjusted according to the

amount of solid support used without further optimization (see Section 2.8).

The results showed that the mean recoveries of TAP and TU decreased about 10% due to the loss in rinsing process when 1.0 g of silica gel was employed as the solid support, and the recovery of PhTU decreased about 10% when the amount of solid support increased to 3.0 g probably because of the lack of eluting strength. The mean recoveries of thyreostats in other conditions are between 71% and 90% with RSDs ranged from 2% to 8% ($n = 5$). It can be concluded that the use of different amount of silica gel in the MSPD process had not much influence on the recoveries of thyreostats if the rinsing and eluting conditions were further optimized.

The average recoveries of most thyreostats are between 71% and 90% with RSDs ranged from 2% to 8% ($n = 5$) when the silica gel obtained from Phenomenex, Merck and YMC was used as solid support in optimized conditions (spiking level 100 μg/kg), only the recovery of TAP on the solid support from Merck is slightly lower, only 64.4%. The differences of recovery on the silica gel from various manufacturers are minor for most of the thyreostats.

3.2.4. Conditions of solid-phase extraction

Animal tissues are a complex non-homogeneous mixture of chemical substances that makes it hard to isolate and determine analytes of interest. When the spiking level is above 100 μg/kg, one step of MSPD will give satisfactory results (see Fig. 4), but there are still some interferents in the chromatogram. To improve the limits of detection (LOD) of thyreostats, subsequent purification using solid-phase extraction after MSPD is needed. In order to optimize the SPE conditions, the negative pork samples were purified using MSPD method developed above and then the thyreostats and internal standard were spiked.

There are two options for subsequent SPE procedure. One is that the samples carry out SPE procedure directly and another derivatize with PFBBr prior to the SPE procedure. Table 3 lists the results of the two methods. The difference is significant between the two methods. The recoveries of thyreostats are relative low when the spiked samples were purified directly in the SPE procedure, but satisfactory results were obtained when the spiked samples were derivatized with PFBBr prior to the SPE. TU and its 6-position derivatives have six tautomeric forms because of the delocalization of the π electrons in the ring

Table 3
The average recoveries of thyreostats in solid-phase extraction

Analyte	TAP	TU	MTU	PTU	PhTU
Thyreostats	47.4	43.0	69.8	63.7	49.8
Derivatives of thyreostats	90.5	90.1	92.1	94.2	79.3

The optimized rinsing and eluting solvents are 5 ml chloroform and 5 ml methanol/chloroform (v/v = 15:85), respectively for thyreostats and the conditions for derivatives of thyreostats described in Section 2.5. The standard deviations were between 7.8% and 15.3% for thyreostats, and between 2.0 and 3.9% for PFBBr derivatives of thyreostats ($n = 5$).

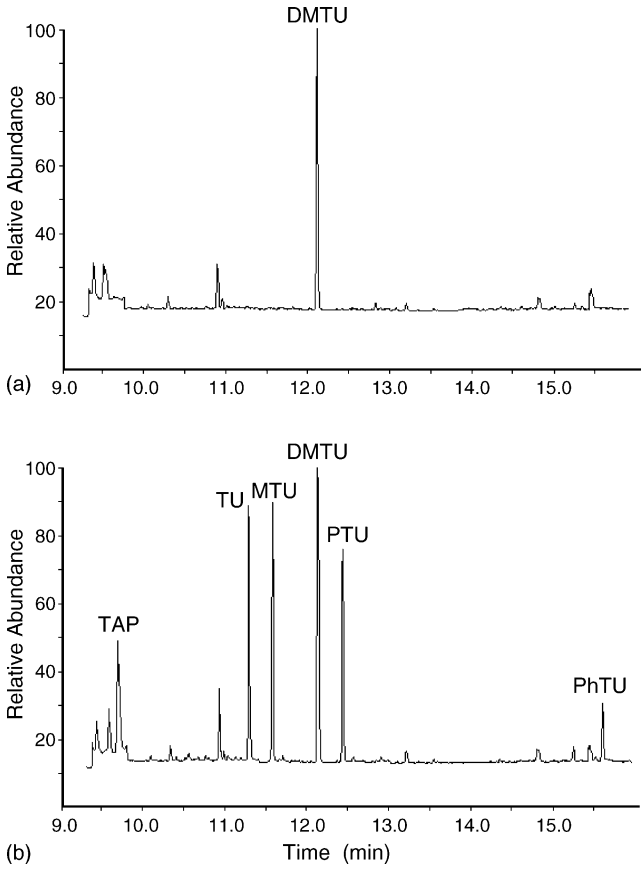

Fig. 4. Selected ion chromatograms for the negative pork sample (a) and spiked pork sample (b, 100 μg/kg).

structure [38]. The derivatization with PFBBr can decrease the polarity of these compounds and lock the molecule in one tautomeric form, which makes the purification become easier.

3.3. Recovery yields at different spiking levels and limit of detection

Five kinds of negative pork sample obtained from different supermarkets were used to examine the optimized procedures developed above (three repeats for each kind of sample at each spiking level). Table 4 summarizes the average recoveries from negative pork samples at five different fortification levels (10, 20, 50, 100, 500 μg/kg). The average recoveries of most thyreostats are above 70% with inter-assay RSDs between 2% and 9%. The recovery of TAP is relative low in most cases due to the loss in pre-treatment process, especially in lower level of fortification.

The limits of quantification (LOQ) for thyreostats were determined corresponding with a signal to noise ratio of 5. The limits of detection were the same with LOQ because in lower signal to noise ratio, the relative intensities of fragment ions selected for detection will change and cause uncertainty for the confirmation of thyreostats. From the data in Table 4, the LOD for TU, MTU and PTU was 10 μg/kg, PHTU 20 μg/kg

Table 4
The recoveries (%) and RSD (%) of thyreostats at different spiking levels

Spiking level (μg/kg)	TAP		TU		MTU		PTU		PhTU	
	R	RSD	*R*	RSD	*R*	RSD	*R*	RSD	*R*	RSD
10	–	–	71.1	8.7	82.7	6.6	82.0	4.5	–	–
20	–	–	73.8	4.7	83.3	5.2	89.0	3.8	78.3	7.1
50	51.1	8.4	75.2	4.2	80.2	4.5	88.5	4.0	73.0	5.7
100	63.8	7.7	78.3	4.0	79.4	3.9	83.0	3.6	76.5	5.2
500	70.0	3.2	82.5	2.6	83.3	1.9	87.0	2.2	77.3	2.3

R: average recoveries, RSD: relative standard deviation.

Table 5
The recoveries (%) of thyreostats in different matrices

Matrix	TAP	TU	MTU	PTU	PhTU
Pork	63.8	78.3	79.4	83.0	76.5
Pig thyroid	50.3	78.4	85.5	79.3	77.2
Pig's liver	47.3	68.9	71.1	63.6	55.6
Beef	60.3	87.2	89.4	97.2	72.7

The relative standard deviations were between 3.3% and 7.7% ($n = 5$).

and TAP 50 μg/kg due to their low response and interference (see Fig. 3).

3.4. Application on different matrices

Different matrices have been tested based on the developed method in the fortification level of 100 μg/kg and the results are listed in Table 5. For pork, Pig thyroid and beef samples, the results are satisfactory. The mean recoveries of most thyreostats ranged from 70% to 97% with acceptable RSDs, 3.3% to 7.7%. The mean recoveries for Pig's liver were lower than 70% probably because of the more complex of the matrix.

Silica gel is a porous and granular form of silica, which is a polymer of silicic acid. As to the internal structure of each small silica gel granule, there are a vast network of inter-connecting microscopic pores, and as to the silica gel surface, the structure terminates in either siloxane group (≡Si–O–Si≡) or silanol groups (≡Si–OH). There are three types of silanol groups, the isolated silanols, the vicinal or bridged silanols, where two hydroxyl groups attached to two different silicon atoms are bridged by hydrogen bond, and the geminal silanols which consists of two hydroxyl groups attached to the same silicon atom [41].

Thyreostats are high polar compounds because of the existence of the thiohydroxy group. Polar molecules usually associate with silanols on and in the silica particle by means of hydrogen bonds [25]. However, there is about 70% of water in the pork sample we used, and water is more polar than thyreostats. So the forming of hydrogen bond between the silica gel and water has precedence over that between the silica gel and thyreostats. On the other hand, the inter-connecting microscopic pores of the silica gel would attract and hold moisture by physical adsorption and capillary condensation [41]. Thus a film of water on the surface of the silica gel came into being. Then the thyreostats would disperse into the water film to an equilibration and be eluted by the polar methanol–chloroform mixture.

4. Conclusions

A method for simultaneous determination and confirmation of thyreostatic residues in animal tissues by gas chromatography-mass spectrometry has been developed. The samples were extracted and purified by combination of MSPD and subsequent solid-phase extraction using silica gel as the solid support. The procedures of pretreatment method were investigated and optimized. Two steps of derivatization were selected, with PFBBr in strong basic medium and then with MSTFA, which can improve the yields of derivatization for thyreostats, the repeatability, and therefore the limits of detection of thyreostats. The method developed has distinct advantages over the methods previously reported.

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