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Gas chromatographic determination of residues of thyreostatic drugs in bovine muscle tissue using combined resin mediated methylation and extraction

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Abstract

A method is described for the assay of residues of the thyreostatic substances 2-thiouracil, 6-methyl-2-thiouracil, 5- and 6-propyl-2-thiouracils and 6-phenyl-2-thiouracil in beef muscle at levels of quantitation down to 25 μg/kg for thiouracil and 15 μg/kg for substituted thiouracils. Analytes are extracted from the muscle matrix with acetonitrile and methylated by absorption onto a macroporous anion exchange resin followed by treatment with methyl iodide in acetonitrile at room temperature. Supercritical carbon dioxide is also a suitable derivatization solvent. Determination is carried out using gas chromatography with mass spectrometric detection. The methyl ester of 2,4,5-trichlorophenoxyacetic acid serves as an internal standard while 5-methyl-2-thiouracil is a suitable surrogate. 5-Ethyl-2-thiouracil is added to minimise losses of the analytes during the analysis. The method exhibits a linear range of 25–625 μg/kg for the substited thiouracils and 50–1250 μg/kg for 2-thiouracil. Recovery of the analytes ranges from 50% for 6-phenyl-2-thiouracil to 90% for 2-thiouracil with coefficients of variation of less than 10%.

Keywords: 2-Thiouracil; 6-Methyl-2-thiouracil; 5(6)-Propyl-2-thiouracil; 6-Phenyl-2-thiouracil

1. Introduction

The thyreostatic drug group comprises substances which inhibit the production of thyroid hormones in both humans and animals. One side effect of their use is the build up of water in muscle tissues and this property has led to their illegal use in some sections of the meat industry to increase the meat yield. The result of such abuse is not only the potential risk to human health of drug residues but also production of inferior meat. The use of thyreostatic drugs is therefore banned in many countries and the Australian National Residue Survey has routinely monitored, for a number of years, the thiouracil class of thyreostatics comprising 2-thiouracil (**1**) and the simple 6-methyl, 6-propyl and 6-phenyl derivatives (**2**–**4**) in Fig. 1.

A number of methods for the detection, determination and confirmation of thyreostatic drugs in animal muscle, urine and plasma have been previ-

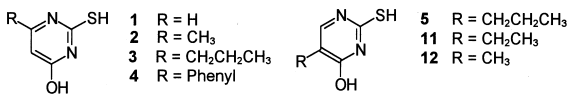

Fig. 1. Structural formulae of thiouracils examined.

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ously reported. These include several methods based on high-performance liquid chromatography using both UV and electrochemical detection [1–5], thin layer chromatography [6,7] and gas chromatography using nitrogen–phosphorus detection, flame photometric detection, electron capture detection or mass selective detection [8–11]. The procedures used to clean up extracts have also been as varied as those used in determination and range from mercury salt affinity chromatography [9] to chromatographic work-up on Sephadex [11] or silica gel [5]. Successful separation of the thiouracils from co-extracted matrix components is hampered by their highly polar, hydrophilic nature which limits the applicability of techniques such as solvent partitioning and the more common SPE phases.

The derivatization of polar analytes in supercritical carbon dioxide has been the subject of much recent interest for extending the applicable range of supercritical fluid extraction. The preparation of less polar derivatives during extraction not only facilitates solubility of the analyte but also yields products readily analysed by GC. We have previously reported a procedure in which anionic analytes trapped on an ion exchange resin were simultaneously methylated and eluted using methyl iodide in either supercritical carbon dioxide or in acetonitrile [12]. In that work the resin mediated methylation of carboxylic acids, phenols and substances containing an acidic –NH– group with methyl iodide either in supercritical carbon dioxide or in acetonitrile was compared. Results indicated that SFE conditions gave the more rapid derivatization of acids and phenols, once minimum conditions of temperature, pressure, reaction times and reagent concentrations had been met. This was extended to a simple gas chromatographic method for the determination of polybasic organic acids in fruit juices after isolation and methylation on ion exchange resins [13]. However, it was found that the use of SFE conditions was not an advantage during methylation of compounds containing an acidic –NH– grouping and for 6-propyl-2-thiouracil, yields of the dimethyl derivative were similar in either solvent.

The purpose of this project was to realise the potential of our previous work on resin mediated methylation by developing an efficient and sensitive analytical method for the in situ derivatization/extraction of highly polar thyreostatic compounds in meat extracts using heterogeneous derivatization with methyl iodide. The main challenges of this work were centred on the ability of the resin-mediated derivatization procedure to trap and methylate, in consistently high yield, low concentrations of the various thiouracils in a matrix of co-extractives.

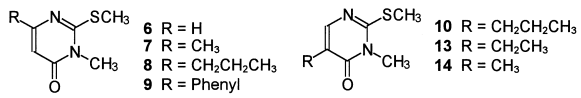

Fig. 2. Structural formulae of thiouracil methyl derivatives.

We now report a new method for the quantitative determination of the thiouracil thyreostatic agents **1**–**4** in bovine muscle by gas chromatography–mass spectrometry (GC–MS) after sample clean-up by trapping onto macroporous ion exchange resin followed by methylation with methyl iodide in either supercritical carbon dioxide or acetonitrile to give the dimethyl derivatives **6**–**9** shown in Fig. 2.

The 5-substituted thiouracils **5**, **11** and **12**, which have not been reported to be used as thyreostats, are employed in the method as surrogates and internal standards giving the derivatives **10**, **13** and **14** on methylation.

2. Experimental

2.1. Reagents

Analytical grade hexane and HPLC grade acetonitrile and methanol were obtained from Mallinckrodt Australia (Melbourne, Australia) and used as supplied. Analytical grade methyl iodide was obtained from Mallinckrodt Speciality Chemical Corp (Paris, KA, USA). Methyl 2,4,5-trichlorophenoxyacetate, 2-thiouracil, 6-methyl 2-thiouracil, 5- and 6-propyl-2-thiouracils and 6-phenyl-2-thiouracil were obtained from the Curator of Standards, Australian Government Analytical Laboratories (Sydney, Australia). 5-Ethyl-2-thiouracil was obtained from the Sigma Chemical Co. (St Louis, MO, USA).

2.2. Resins and support columns

Analytical grade anion exchange resin AG MP-1, 63–150 mm (chloride form) was supplied by Bio-Rad Laboratories (Sydney, Australia). Before use, the resin was washed with 10 bed volumes of water and an equal volume of methanol and dried at room temperature under vacuum. For some experiments, the AG MP-1 resin was converted to the fluoride form by treatment with at least 20 bed volumes of aqueous 1 *M* sodium fluoride followed by thorough rinsing with distilled water then methanol, before drying by vacuum aspiration. Empty 3 ml solid phase extraction cartridges (containing only the bottom frit), to which 10 ml reservoirs could be fitted, were used to prepare ion-exchange columns. AG MP-1 resin (100 mg) was added to each of these cartridges, then washed with acetonitrile–0.1 *M* NaOH (80:20, v/v) (ca. 1 ml). Samples or standard solutions were than applied directly to the reservoir. Preliminary experiments showed that the loading rate was important; approx. 2 ml/min was found to give good results.

2.3. Standards

Stock solutions of each thiouracil, except for 5-ethyl-2-thiouracil, were prepared at concentrations of 0.2 mg/ml in 0.1 *M* aqueous sodium hydroxide. 5-Ethyl-2-thiouracil stock was prepared as a 1 mg/ml solution in 0.1 *M* aqueous sodium hydroxide and methyl 2,4,5-trichlorophenoxyacetate was prepared as a 0.2 mg/ml solution in acetonitrile. These stock solutions were kept at −18°C until required. Thiouracil stock solutions had a storage life of 4 weeks whereas no deterioration of methyl 2,4,5-trichlorophenoxyacetate was noted after 1 year.

It is important to note that thiouracils have only limited stability in very dilute solution, particularly the parent substance **1** and the phenyl analogue **4**. Investigations into analyte stability showed that the standard stock solution of thiouracils in 0.1 *M* NaOH lost between 25 and 35% of analytes after storage for one week at 4°C and were even less stable when prepared and stored in methanol. No significant degradation occurred after storage at −18°C for 4 weeks.

2.4. Isolation of thiouracils from beef muscle

Homogenised beef muscle samples (5 g) were weighed into 50-ml polypropylene centrifuge tubes and the appropriate amount of thiouracil stock solutions added to produce three different spiking levels. These were 25, 50 and 500 ng/g for 2-thiouracil and 12.5, 25 and 250 ng/g for 6-methyl-2-thiouracil, 5- and 6-propyl-2-thiouracil and 6-phenyl-2-thiouracils.

Spiked samples were vortexed for one min and allowed to stand in the dark for 30 min for equilibration to occur. The mixture was then extracted with 10 ml of acetonitrile by inverting the sealed tube at 100 rpm for 30 min then centrifuging at 2500 rpm for 5 min. The solvent layer was decanted into another centrifuge tube and the residual beef muscle was re-extracted in the same way with a further 5 ml of acetonitrile. The fat in the combined acetonitrile extracts was removed by partition with 10 ml hexane followed by centrifuging at 2500 rpm for 5 min. The hexane layer was removed, and the acetonitrile solution partitioned with a further 10 ml hexane. The acetonitrile was then evaporated to ca. 2 ml at 50°C under a slow stream of nitrogen. 5-Ethyl-2-thiouracil stock solution (100 μl) was added to this solution which was then made up to 10 ml with 0.1 *M* aqueous NaOH. This mixture was then added to a column prepared from 100 mg of resin and a slight vacuum applied to aid elution. The resin column was washed with 2–3 ml water followed by 3×1 ml methanol and dried for 15 min by aspiration of the resin bed under vacuum.

2.5. Derivatization in acetonitrile

Methylation was achieved by adding 300 μl of 0.5 *M* methyl iodide in acetonitrile to the resin bed which was then protected from light by covering with aluminium foil and allowed to stand for one hour at room temperature (25°C). The methylated products were eluted into a low volume GC vial with 2 aliquots of 0.5 ml of acetonitrile and methyl 2,4,5-trichlorophenoxy-acetate added as internal standard. Using a stream of nitrogen at 50°C, the volume was reduced almost to dryness and 0.1 ml of acetonitrile added prior to analysis by GC–MSD.

Recovery was determined by comparing the area ratio of analytes/internal standard in the spiked matrix extract to the area ratio of analytes/internal standard for the standard solution at the same concentration.

2.6. Derivatization in supercritical carbon dioxide

The dried resin carrying the trapped analytes was transferred into a 2.5 ml ISCO extraction vessel. Neat methyl iodide (75 μl) was added to the top of the resin prior to pressurization with CO_2 and samples were allowed to react under pressure for 20 min with no flow. This reaction step was performed at a constant pressure of 200 bar and 80°C and was followed by extraction of the products into toluene with 20 ml of pressurised CO_2 under the same conditions. After evaporation of the toluene to near dryness under a stream of nitrogen at 50°C, the residue was taken up in 100 μl of acetonitrile and analysed by GC–MSD.

2.7. Analysis of standards

The linearity of detector response in the concentration range of 50, 125, 500 and 1250 ng/ml for 2-thiouracil and 25, 65, 250 and 650 ng/ml for the other thiouracils was carried out on standards prepared and derivatized by the procedure detailed above.

2.8. Supercritical fluid extraction

An ISCO Model SFX 2-10 extraction unit and an ISCO Model 260D (Lincoln, NE) syringe pump were used for supercritical fluid extraction and derivatization. Extraction conditions were similar to those we described in [12]. Fused-silica restrictor tubing with 50 mm I.D. and approximately 25 cm in length was used to control the flow rate to 0.9–1.5 ml/min of pressurised CO_2. Extractions were performed at a constant pressure of 200 bar. The quantity of pressurised CO_2 consumed was used to monitor the length of the extraction. This was the simplest way to overcome inconsistencies in flow rate. Waste extraction fluid was vented through charcoal traps to minimise the release of methyl iodide and toluene.

2.9. Gas chromatography and data acquisition

Analyses were carried out on a Hewlett–Packard 5890 series II gas chromatograph fitted with a Hewlett–Packard 5971A mass selective detector (MSD) and an HP Ultra 2 (cross linked 5% Ph-Me silicone) column, 25 m×0.2 mm I.D.×0.33 mm film thickness and split/splitless injector operating at 250°C in the splitless mode. Helium pressure in the injector was adjusted to give a carrier gas flow of 1 ml/min. The MSD temperature was maintained at 280°C and a 5 min solvent delay employed. After injections of 1 μl of sample, an initial oven temperature of 70°C was maintained for 0.5 min then ramped to 90°C at 10°C/min then to 280°C at 20°C/min and held for 2.5 min. The analytes were detected in single ion monitoring (SIM) mode using standard autotune calibrations. Ions with m/z 109, 125, 198, 232 and 170 were selected for quantitation of compounds **6**–**10**, respectively, based on considerations of relative abundance and lack of background noise. The surrogate, compound **14** was monitored using its fragment of m/z 125. Methyl 2,4,5-trichlorophenoxyacetate, the internal standard, was monitored as the total ion current for two main ions at m/z 268 and 233.

3. Results and discussion

3.1. Optimisation of methylation conditions with standards

Investigations using a standard mixture of the thyreostatic thiouracils confirmed that, as was the case for 6-propyl-2-thiouracil in the work reported in reference [12], comparable methylation efficiency could be obtained for these compounds in acetonitrile or supercritical carbon dioxide. This is demonstrated in the data in Table 1. Good yields of methylated thiouracils were obtained after reaction in acetonitrile for one hour at room temperature and the simplicity of the procedure favoured its use over the more complex SFE technique in the analytical method developed here. As can be seen in the table, as little as 50 mg of the resin could be used to trap the analytes from standard solution for methylation, but to avoid possible problems with overloading by

Table 1
Methylation of thiouracils relative to internal standard by different procedures

Compound	A	B	C
1	1.28	1.06	1.13
2	2.33	2.25	2.18
5	0.45	0.45	0.43
3	–	0.51	0.54
4	0.16	0.14	0.17

Results are expressed as ratio of peak area of methylated analyte to peak area of internal standard (methyl ester of 2,4,5-T).
For A and B, amount of resin used=0.1 g, amount of ''chaperone'' added=100 μg.
For C, amount of resin used=0.05 g, amount of ''chaperone'' added=50 μg.
A utilised methyl iodide in supercritical carbon dioxide at 90°C. B and C utilised methyl iodide in acetonitrile at room temperature.

sample matrix co-extractives, 100 mg of resin was routinely used for analysis of samples.

It was found in previous work [12] that the use of the fluoride form of the resin was preferable to the hydroxide form. Not only was the fluoride ion readily replaced by other anions but this form of the resin was neutral and therefore did not cause base-catalysed analyte degradation. In this work we discovered that thiouracils have a strong affinity for the resin and will readily replace the chloride ion. Therefore the use of the resin in the chloride form, which is the form supplied commercially, is completely suitable for direct use and the 2-thiouracils **1**–**5** could be converted in good yield to the dimethyl derivatives **6**–**10**.

3.2. Use of 5-ethyl-2-thiouracil as a recovery aid

Preliminary work on the volume of resin required for effective trapping of the thiouracils revealed that yields of the dimethyl derivatives of the analytes were reduced by increasing the quantity of sorbent used. This effect was most marked when using standard solutions of low concentration. By contrast, initial work with fortified meat samples showed little variation in the recovery of analytes across the detectable range. These factors suggested the possibility of a small amount of irreversible binding of the analytes to the resin. Consequently, it was found beneficial to add a moderate amount of a non-target thiouracil to the extract before loading it onto the ion exchange column to act as a ''chaperone'' to low concentrations of analyte. The presence of a relatively high and constant concentration of 5-ethyl-2-thiouracil (**11**) during the trapping process ensured reproducibility of analyte recoveries across the applicable concentration range in both standards and samples. This substance has the advantages of being relatively inexpensive, structurally similar to the target analytes, the source of a convenient surrogate standard (as discussed below) and has no recorded use as a thyreostatic agent. It was found to be much more effective in preventing recovery losses than other compounds including thiourea, 2-mercaptopyrimidine and quinoxaline 2-carboxylic acid, which were also tested as potential ''chaperones''.

Incorporation of 5-ethyl-2-thiouracil as a ''chaperone'' for other thiouracils furnished improved and consistent yields of the target analytes as their dimethyl derivatives. The optimum quantity of **11** required to ensure consistency of results was investigated. When less than 25 μg of **11** was added to thiouracil standard solutions of low concentration prior to the methylation step, yields of dimethyl thiouracils were very poor. Addition of increasing amounts of **11** up to 200 μg increased both the yield of dimethyl-thiouracils and the reproducibility of results when tested on standards. However, the effective increase in yield above 100 μg was not large, and this quantity was chosen for the routine method to ensure that resin saturation by the recovery aid would not occur.

3.3. Use of surrogates and internal standards

In this work, the methyl ester of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was found to be a suitable internal standard with chromatographic properties similar to the dimethyl-thiouracils **6**–**10**. During the development of the use of a ''chaperone'' for the thyreostatic thiouracils, it was found 5-ethyl-2-thiouracil (**11**), as supplied commercially, was contaminated with a small percentage of the impurity 5-methyl-2-thiouracil (**12**). When an excess of **11** was introduced as an aid to the recovery of thyreostatics, it was found that the small amount of the impurity **12** added into the analysis was both constant and present at a similar concentration to the target thyreostats. **12** could be methylated under the

same conditions and in the same yield as other thiouracils and it was therefore a useful surrogate on which to assess the recoveries of target analytes during the methylation process.

3.4. Performance of the method in analysis of bovine muscle

The use of an ion exchange resin in this unified procedure for trapping and derivatizing the thiouracils resulted in an excellent clean-up of meat extracts for mass selective detection. Representative GC–MS traces from the analysis of the methyl derivatives of the thiouracils **1**–**5** recovered from beef muscle at the lowest concentrations evaluated in this study are shown in Fig. 3 and Fig. 4. The recovery of the analytes of interest from spiked meat samples is shown in Table 2. These recoveries are sufficiently high and repeatable to permit the use of the method for multi-residue determination of thiouracil residues in bovine muscle over a linear range of at least 25–625 μg/kg (substituted 2-thiouracils) or 50–1250 μg/kg (2-thiouracil). Linear regression data for the analysis is recorded in Table 3.

The least well recovered analyte proved to be 6-phenyl-2-thiouracil. However, the coefficient of variation for this and the other substituted 2-thiouracils at 12.5 μg/kg remains well below 10%. Therefore, with the use of 5-methyl-2-thiouracil as a surrogate, the method is suitable for the determination of these substituted thiouracils down to at least 12.5 μg/kg and of 2-thiouracil, which is less sensitively detected, at 25 μg/kg. The ultimate limit of detection (LOD) for a method can be defined as three times the standard deviation of the blank response. For chromatographic methods, this is most readily determined by use of the standard deviation

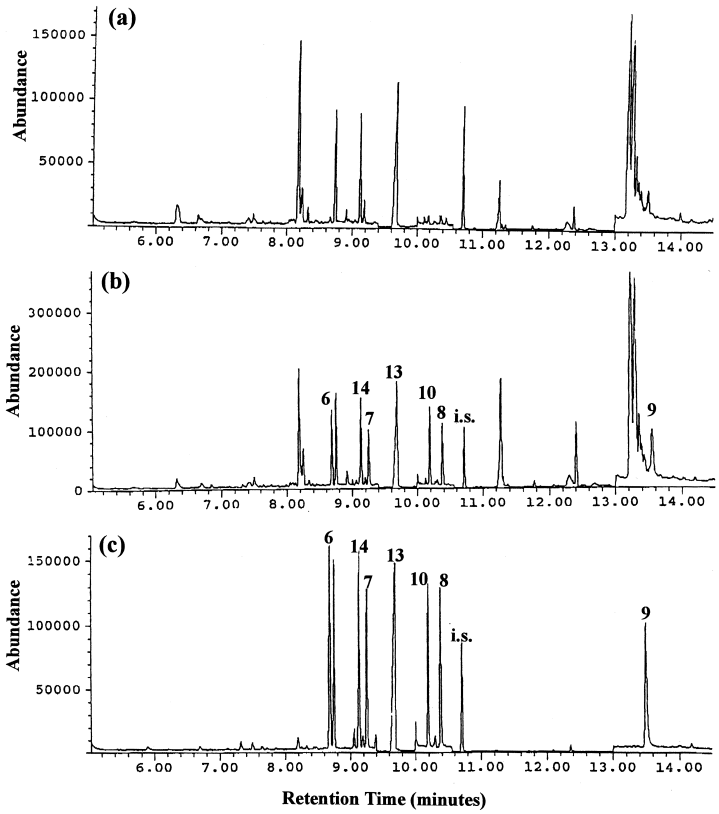

Fig. 3. Total ion chromatograms of (a) bovine muscle, (b) bovine muscle fortified with thyreostats (25 μg/kg **1** and 12.5 μg/kg each of **2**–**5**), analysed using resin mediated methylation in acetonitrile. (c) Standard solution of equivalent concentration.

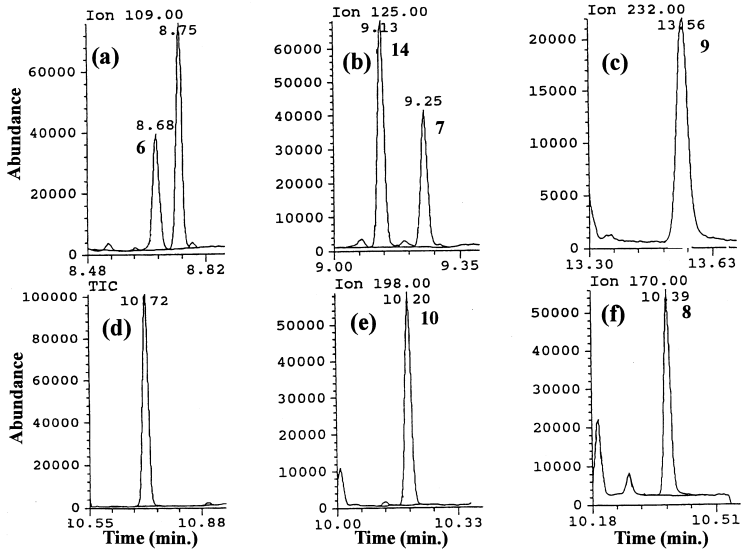

Fig. 4. Selected ion chromatograms from the analysis by resin mediated methylation in acetonitrile of a sample of bovine muscle fortified with thyreostats (25 μg/kg of **1** and 12.5 μg/kg each of **2**–**5**). Dimethyl derivatives of (a) 2-thiouracil (**6**), (b) 5-methyl-2-thiouracil surrogate (**14**) and 6-methyl-2-thiouracil (**7**), (c) 6-phenyl-2-thiouracil (**9**), (e) 5-propyl-2-thiouracil (**10**) and (f) 6-propyl-2-thiouracil (**8**). (d) 2,4,5-T methyl ester internal standard.

Table 2
Mean recovery and precision for beef muscle samples spiked with 2-thiouracil (**1**), 6-methyl-2-thiouracil (**2**), 5-propyl-2-thiouracil (**5**), 6-propyl-2-thiouracil (**3**) and 6-phenyl-2-thiouracil (**4**): (n=8)

Compound	Concentration (μg/kg)		Recovery (%)	Precision (% C.V.)	LOD (μg/kg)
	Added	Found (S.D.)			
1	25.0	22.5 (2.3)	90.0	9.9	8
	50.0	42.3 (2.5)	84.5	6.0	
	500.0	412.5 (14.0)	82.5	3.4	
2	12.5	8.8 (0.7)	70.0	7.6	2.7
	25.0	20.5 (1.1)	82.0	5.6	
	250.0	201.5 (3.8)	80.6	1.9	
5	12.5	9.8 (0.6)	78.4	6.6	2.5
	25.0	20.1 (0.9)	80.5	4.3	
	250.0	194.5 (3.7)	77.8	1.9	
3	12.5	8.4 (0.4)	66.9	4.5	1.7
	25.0	21.3 (1.0)	85.3	4.7	
	250.0	208.8 (8.1)	83.5	3.9	
4	12.5	7.0 (0.4)	55.9	5.9	2.2
	25.0	12.6 (0.5)	50.5	3.9	
	250.0	174.3 (12.4)	69.7	7.1	

Table 3
Regression lines for resin assisted methylation for beef muscle samples spiked with 2-thiouracil (**1**), 6-methyl-2-thiouracil (**2**), 5-propyl-2-thiouracil (**5**), 6-propyl-2-thiouracil (**3**) and 6-phenyl-2-thiouracil (**4**): (n=8)

Compound	Slope	Intercept	Correlation coefficient (r^2)
1	0.704	−0.231	0.999
2	0.707	−0.145	0.999
5	0.720	0.030	1.000
3	0.844	−0.446	0.995
4	0.670	−0.104	1.000

Range: 50–1250 μg/kg in beef muscle for compound **1**; 25–625 μg/kg in beef muscle for the compounds **2**–**5**.

of the results for samples spiked at a level close to the detection limit as an approximation of that of the blank. On that basis the detection limits of this method are of the order of 10 μg/kg for 2-thiouracil and 5 μg/kg for the substituted analogues as shown in Table 2.

4. Conclusion

Resin mediated methylation can be used as the basis of a simple, cost effective procedure for the multi-residue determination of part per billion levels of 2-thiouracil and its analogues in beef muscle. Trapping and washing the analytes on an anion exchange resin prior to their release by derivatization with methyl iodide in acetonitrile solvent, produces sufficiently clean extracts for their reproducible determination by GC–MS. Although supercritical carbon dioxide is also a suitable solvent for the derivatization, the use of acetonitrile permitted a much simpler procedure, with no loss in performance. The procedure described uses small quantities of solvents and reagents, can be readily batched and is brief enough to allow the analysis of 2 batches per day by a single analyst. The nature of the trapping and derivatization steps indicate that the method may also be easily adapted to the methylation and GC analysis of other analytes containing an acidic nitrogen functionality.

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