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# DETECTION OF ANTITHYROID RESIDUES IN MEAT AND SOME ORGANS OF SLAUGHTERED ANIMALS

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## SUMMARY

A simple method is described for the routine detection of antithyroid residues in thyroid, liver, kidney and meat contaminated at levels as low as 10 ppb (10 parts per $10^9$).

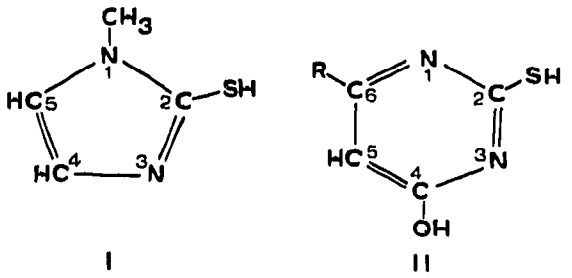
Tissue samples (2 g) are homogenized in methanol, contaminating lipids and amino acids are removed and the antithyroid residues are subjected to reaction with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) in buffer. The NBD derivatives are extracted with diethyl ether and separated by thin-layer chromatography. After spraying with cysteine or mercaptoethylamine, the antithyroid residues appear as fluorescent spots. The detection limit of these compounds is of the order of 200 pg.

## INTRODUCTION

The use of thyroid-inhibiting substances in cattle breeding has gained in popularity in recent years. These substances allow a considerable increase in live weight gain, although it results mainly from increased filling of the gastrointestinal tract and augmented water retention in the slaughter animals[1]. Of the many thyriostatic drugs examined, thiouracil and 2-mercaptoimidazole derivatives (Fig. 1) belong to the most biologically active substances[2].

As the presence of residues of these highly potent antithyroid drugs in meat may constitute a health hazard, regulations in different countries of the EEC prohibit the use of antithyroid drugs (called antihormones: AH) in breeding of cattle. However, the lack of sensitive, specific methods for the detection of AH residues in animal tissues hampered the efficient control of the use of these substances.

Analyses for these AH substances in thyroid and meat involve a chromatographic clean-up of the extract prior to colorimetric development with Grote's reagent[3] or 2,6-dichloroquinonimide[4]. Interfering substances obscure the reactions, and Gissel and Schaal[5] therefore proposed one-dimensional thin-layer chromatography (TLC) and spraying with dichloroquinonimide for the identification of methylthiouracil. The practical detection limit of the AH substances by these methods is about 0.5–1 ppm.



I II

Fig. 1. Structural formulae of thyriostatic drugs: I, 1-methyl-2-mercaptoimidazole (tapazole); II, 4(6)-R-2-thiouracil (R = H, methyl, *n*-propyl or phenyl).

As techniques that involve the formation of fluorescent derivatives afford a simple means of attaining high sensitivity and specificity in the analysis of compounds that have a suitable functional group, their combination with TLC would permit the unequivocal detection of small amounts of these thyroid-inhibiting drugs. In this paper, a convenient, simple and reliable method is presented that permits the routine detection of AH residues in meat and organs from slaughtered animals at the level of 10 ppb*.

## EXPERIMENTAL

### *Apparatus*

The following apparatus was used: extraction tubes of 10, 15 and 50 ml; rotary vacuum evaporator with vacuum source; water-bath; homogenizer (Ultra-turrax); chromatographic tanks; cabinet for chromatography with longwave lamp (366 nm) and contrast filter.

### *Reagents and reference compounds*

Silica gel 60 TLC plates without fluorescence indicator were obtained from Merck, Darmstadt, G.F.R. (Cat. No. 5721), silica gel SIL-G25 HR from Machery, Nagel & Co., Düren, G.F.R., and Dowex 50W-X8, 20–50 mesh, from Fluka, Buchs, Switzerland.

4(6)-Phenyl-2-thiouracil (ØTU) and 4(6)-methyl-2-thiouracil (MTU) were purchased from Fluka, 4(6)-propyl-2-thiouracil (PTU), 2-thiouracil (TU), 2-mercapto-1-methylimidazole (tapazole) (TAP) and NBD-Cl (7-chloro-4-nitrobenzo-2-oxa-1,3-diazole) from Aldrich-Europe, Beerse, Belgium, and 2-[2-$^{14}$C]thiouracil from the Radiochemical Centre, Amersham, Bucks., Great Britain.

Methyl Cellosolve (ethylene glycol monoethyl ether), Sequanal grade was purchased from Pierce, Rockford, Ill., U.S.A., PPO (2,5-diphenyloxazole) and dimethyl-POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene] from Packard Instrument Co., La Grange, Ill., U.S.A., diethyl ether, free from peroxides, from Gifrez & Barbezat, Decines, France, and light petroleum (b.p. 40–60°), p.a. grade, from Merck.

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* Throughout this article the American billion ($10^9$) is meant.

All other reagents used were reagent-grade products from different manufacturers.

*Pre-treatment of ion exchanger.* Dowex 50W-X8 (1 lb) is washed on a Buchner funnel with four volumes of 4 *N* hydrochloric acid, distilled water and four volumes of 2 *N* sodium hydroxide solution. The resin is heated at 70° in two volumes of 2 *N* sodium hydroxide solution for 1 h and then washed with water, four volumes of 4 *N* hydrochloric acid and finally with water to a slightly acidic or neutral reaction.

*Solutions*

Stock solutions of the antithyroid compounds in methanol were prepared at a concentration of 200 μg/ml.

*NBD-Cl solution.* NBD-Cl (5 mg) is dissolved in 2 ml of acetone. This solution is prepared fresh daily and stored in the dark.

*Spray solutions.* I: denaturated alcohol–isopropanol–25% ammonia (50:50:2, v/v/v). II: cysteine (or mercaptoethylamine)–distilled water (3:100, w/v). Just before spraying, 2 ml of solution II are mixed with 100 ml of solution I.

*Extraction and clean-up*

A 2-g amount of tissue (thyroid, meat, liver or kidney) is minced, placed in an extraction tube and, after addition of 10 ml of methanol, is homogenized using an Ultra-turrax for 20 sec. The homogenate is shaken for 20 min and centrifuged at 2000 *g* for 3 min. The supernatant is decanted off and mixed with 1 ml of 1 *N* hydrochloric acid. Fat is removed by extracting the supernatant three times with 4 ml of light petroleum (b.p. 40–60°). A Dowex 50W-X8 column is prepared by filling a 300 × 9 mm column with water and Dowex 50W-X8 to a resin bed height of 150 mm. Prior to use, 15 ml of 75% methanol are passed through the column. The lower phase of the extract is percolated through the column at a rate of *ca.* 2 ml/min. The column is washed with 5 ml of 75% methanol and drained. The percolated extract and column washings are concentrated to a volume of *ca.* 1 ml on a rotary evaporator at 40°. The concentrated extract is mixed with 5 ml of phosphate buffer, pH 8, and 0.5 ml of methanol. The pH is controlled and eventually adjusted to 8. For removal of polar lipids, the buffer is extracted successively with 4, 3 and 3 ml of diethyl ether, the ether phases are removed and the buffer solution is placed under a stream of nitrogen for evaporation of dissolved ether.

*Reaction*

A 0.1-ml volume of a 2.5 mg/ml solution of NBD-Cl in acetone is added to the buffer solution and the reaction vessel is placed in a water-bath at 40° for 1 h. The reaction proceeds in darkness. After cooling, 3 ml of diethyl ether are added, 1 ml of 1 *N* hydrochloric acid is added so as to bring the pH to 3–4 and the mixture is shaken. After separation of the layers, the ether fraction, containing the derivatized thyriostatic drugs, is transferred into a second extraction tube and the buffer phase is again extracted twice with 2 ml of diethyl ether. The combined ether fractions are dried over sodium sulphate and concentrated to 0.2 or 0.5 ml under a stream of nitrogen.

*Preparation of standard solutions*

Standard solutions are prepared by diluting 0.1 ml of a stock solution in 5 ml of phosphate buffer, pH 8. Derivatization and extraction are carried out as mentioned above. The volume of the ether fraction is reduced to 0.5 ml.

*Thin-layer chromatography*

The extracts are analyzed by TLC using pre-coated silica gel 60 plates, activated by heating at 110° for 1 h. Development is normally carried out in standard tanks (not lined with filter-paper).

*One-dimensional screening analyses.* The combined standards (MTU, TAP, TU; 50 ng each) and six to eight samples are spotted on one chromatographic plate. For thyroid tissue, a sample of 50 $\mu$l of the concentrated 0.5-ml ether solution, equivalent to 0.2 g of thyroid tissue, are spotted. When analyzing meat, liver or kidney, 0.2-ml concentrated ether solution is used, and spotting 50 $\mu$l corresponds to 0.5 g of tissue. The plate is developed with chloroform–ethanol (95:5, v/v) and, after drying, the plate is examined under daylight and under UV light (366 nm). Yellow spots, visible by daylight, with $R_F$ values corresponding to those of the standards, indicate the presence of concentrations of thyriostatic drugs greater than 50 ng. UV fluorescent spots are marked on the plate with a pencil. The plate is sprayed (see reagents), and the excess of NBD-Cl and the antithyroid drugs appear as highly fluorescent yellow-green spots.

*Two-dimensional development.* The identities of the spots located in the one-dimensional screening analyses are confirmed by two-dimensional development with chloroform–ethanol (95:5, v/v) in the first dimension and chloroform–propionic acid (95:5, v/v) in the second dimension. Trace amounts of AH substances can be detected by spotting up to 100–150 $\mu$l of the concentrated ether extract.

*Determination of $^{14}$C-labelled 2-thiouracil*

A Packard Tri-Carb 3003 liquid scintillation counter was used, with toluene–methyl Cellosolve (4:1, v/v) containing 1.2% of PPO and 0.004% of dimethyl-POPOP as the scintillation liquid. The homogenized samples were spiked with $10^5$ c.p.m. of 2-[2-$^{14}$C]thiouracil and were analyzed as described above. Volumes of 0.1–0.5 ml of the aqueous or solvent solutions were taken from the different steps of the isolation. The volatile solvents were evaporated under a stream of nitrogen, 20 ml of the scintillation liquid were added and the vials were placed in the liquid scintillation counter. The values were corrected for background activity and quenching. The overall recovery was calculated for each step in the procedure.

## RESULTS AND DISCUSSION

*Reaction of NBD-Cl with antithyroid drugs*

The reaction velocity of NBD-Cl with antithyroid drugs was determined spectrophotometrically. Solutions containing 40 $\mu$g of antithyroid drug and 250 $\mu$g of NBD-Cl, dissolved in 3 ml of buffer, were prepared. The extinction at 420 nm, measured against a blank containing the same amount of NBD-Cl, was recorded at 10-min intervals for 2 h. In this way, the optimum pH and temperature of the reaction were established.

Fig. 2 shows the course of the reaction of MTU with NBD-Cl at 35° and various pH values as a function of time. It is evident that pH 8 is the optimum for the reaction. The reaction was carried out at this pH at 25, 35, 45 and 55°, and the optimum temperature was found to be 40°. At pH values greater than 8 or temperatures greater than 45°, a strong colour was obtained for the blank, caused by decomposition of the NBD-Cl, resulting in an apparent decrease in extinction (see Fig. 2, curve for pH 9).

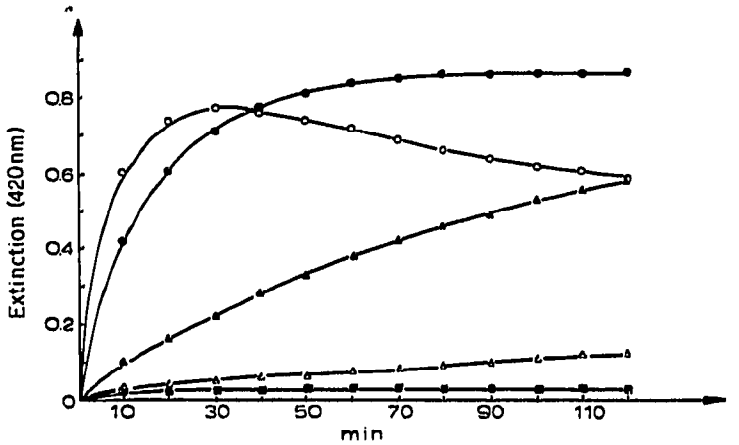


Fig. 2. Course of reaction of MTU with NBD-Cl at 35° and at various pH values. pH: ■, 5; △, 6; ▲, 7; ●, 8; ○, 9.

Fig. 3 illustrates the course of the reaction of the different antithyroid drugs with NBD-Cl under optimum conditions (pH 8, 40°) as a function of time. It can be seen that MTU, TAP and PTU were completely derivatized after 1 h. TU reacts more slowly, being 80% derivatized after 1 h.

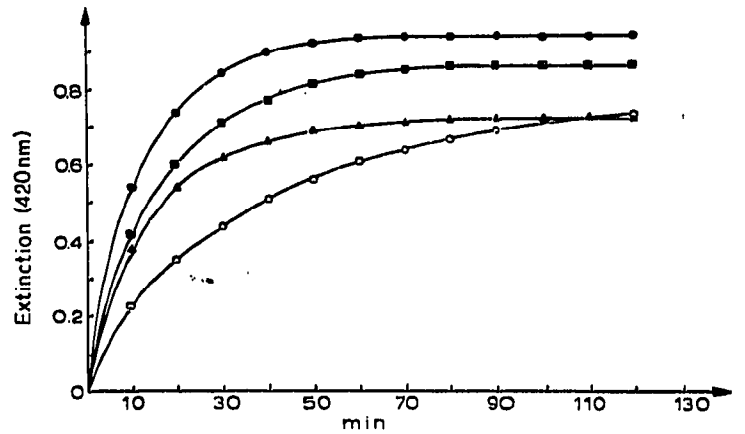


Fig. 3. Course of reaction of thyriostatic drugs with NBD-Cl under optimum conditions (pH 8; 40°). ●, TAP; ■, MTU; ▲, PTU; ○, TU.

*Thin-layer chromatography*

Two types of pre-coated silica gel 60 plates, development in unlined and lined tanks and several solvents were tried. The tests were performed with standard solutions.

The pre-coated plates were Merck 5721 (0.25 mm) and Machery, Nagel & Co. SIL-G25 HR (0.25 mm). From chromatograms run on these plates (Fig. 4), it can be concluded that the spots are less diffuse on the Merck plates, which also have the practical advantage of being harder, so that handling and storage of the plates is easy.

The plates were developed in both lined and unlined tanks. In lined tanks, a solvent vapour-saturated atmosphere was ensured by lining the tank with filter-paper (Whatman No. 1). Fig. 4 shows that the best separation between the antithyroid drugs and the excess of NBD-Cl was obtained with unlined tanks and Merck 5721 plates.

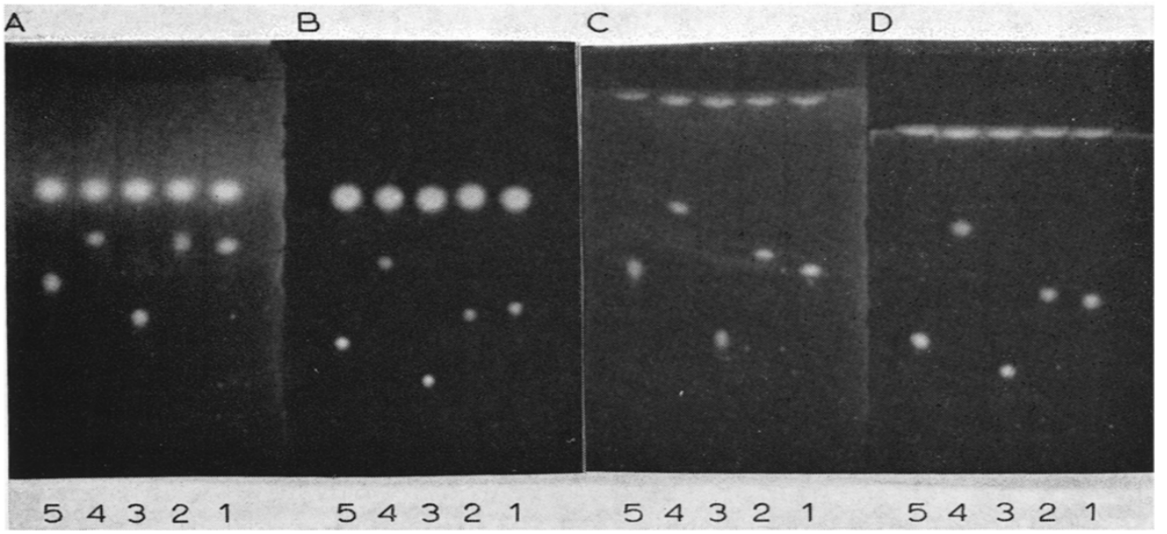


Fig. 4. Chromatograms of standards (A = SIL-G25 HR, lined; B = Merck 5721, lined; C = SIL-G25 HR, unlined; D = Merck 5721, unlined). Solvent systems: A and B, chloroform–ethanol (90:10); C and D, chloroform–ethanol (95:5). 1 = PTU; 2 = ØTU; 3 = TU; 4 = TAP; 5 = MTU.

Tables I and II give the $R_F$ values obtained using different solvents with both lined and unlined tanks and for the two types of plate. In routine analyses, solvents 2 and 4 (Table I) were chosen.

*Spray-induced fluorescence*

Ghosh and Whitehouse[6] stated that NBD-Cl reacts with amino groups to yield a highly fluorescent derivative. Reaction with –SH groups yields a less fluorescent product[7]. Although thyriostatic drugs contain both –NH and –SH functions (Fig. 1), the reaction products with NBD-Cl are not fluorescent. On the contrary, the derivatives absorb UV light at 366 nm so that high concentrations of derivatives and the excess of NBD-Cl can be seen as dark spots on a chromatographic plate.

TABLE I

$R_F \times 100$ VALUES OF SOME SUITABLE SOLVENTS IN UNLINED TANKS

| *Solvent mixture* | *Merck 5721 plate* | | | | | | *SIL-G25 HR plate* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *MTU* | *TAP* | *TU* | *ØTU* | *PTU* | *NBD* | *MTU* | *TAP* | *TU* | *ØTU* | *PTU* | *NBD* |
| Chloroform–ethanol (90:10) | 58 | 81 | 51 | 74 | 76 | 92 | 63 | 91 | 58 | 64 | 79 | 95 |
| Chloroform–ethanol (95:5) | 32 | 67 | 21 | 45 | 42 | 98 | 50 | 67 | 30 | 53 | 50 | 99 |
| Chloroform–propionic acid (90:10) | 46 | 52 | 33 | 82 | 74 | 89 | 31 | 30 | 25 | 62 | 48 | 78 |
| Chloroform–propionic acid (95:5) | 48 | 58 | 34 | 76 | 68 | 89 | 47 | 40 | 38 | 79 | 68 | 87 |
| Chloroform–acetic acid (90:10) | 71 | 68 | 63 | 74 | 74 | 83 | 78 | 50 | 61 | 89 | 89 | 94 |
| Chloroform–acetic acid (95:5) | 52 | 51 | 41 | 59 | 59 | 82 | 51 | 39 | 43 | 63 | 61 | 74 |

TABLE II

$R_F \times 100$ VALUES OF SOME SUITABLE SOLVENTS IN LINED TANKS

| *Solvent mixture* | *Merck 5721 plate* | | | | | | *SIL-G25 HR plate* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *MTU* | *TAP* | *TU* | *ØTU* | *PTU* | *NBD* | *MTU* | *TAP* | *TU* | *ØTU* | *PTU* | *NBD* |
| Chloroform–ethanol (90:10) | 27 | 48 | 16 | 33 | 35 | 65 | 36 | 48 | 29 | 44 | 45 | 67 |
| Chloroform–ethanol (95:5) | 19 | 41 | 13 | 28 | 26 | 63 | 44 | 56 | 34 | 56 | 54 | 69 |
| Chloroform–propionic acid (90:10) | 53 | 60 | 40 | 70 | 66 | 84 | 41 | 38 | 32 | 69 | 59 | 76 |
| Chloroform–propionic acid (95:5) | 29 | 36 | 20 | 46 | 44 | 63 | 23 | 24 | 19 | 44 | 34 | 63 |
| Chloroform–acetic acid (90:10) | 52 | 53 | 48 | 63 | 59 | 76 | 6 | 13 | 5 | 11 | 12 | 56 |
| Ethyl acetate–acetone (95:5) | 23 | 9 | 19 | 47 | 36 | 63 | 16 | 31 | 7 | 17 | 30 | 68 |

We found that the dark spots can be converted into highly fluorescent spots by spraying them with an alkaline cysteine solution. As cysteine forms a fluorescent adduct with NBD-Cl[7], it is probable that the thyriostatic drug, bound to NBD-Cl, is exchanged with cysteine. The induced fluorescence remains stable for several months when the plates are stored in darkness. The principle of exchanging the investigated molecule with a (more) fluorescence-inducing moiety is interesting because by this means various products can be detected in smaller amounts. This principle can probably be applied to other fluorophores also. One can always try to increase the fluorescence by spraying with the product that gives the highest fluorescence with the fluorophore used. These phenomena are not always well understood. In this work, the following compounds were tested as spray reagents: glycine (GLY); ethanolamine (EA); 2-mercaptoethanol (MEL); thioglycolic acid (TGA); 2-mercaptoethylamine (MEA) and cysteine (CYS). Spraying with amines was not successful as the fluorescence induced was weak and occurred only after several hours. The fluorescence induced by some thiols (MEL, TGA) was strong but the reaction time was long (3–4 h) and TAP could not be exchanged. The two successful sprays were CYS and MEA. This suggests that both –SH and $-NH_2$ groups situated in $\beta$-positions relative to each other are needed for a rapid reaction. The proposal of a reaction mechanism would be only speculation; an intramolecular S → N transfer, as proposed by Birkett *et al.*[7], may be a possible explanation.

The activation and fluorescence spectra on TLC plates were measured with a Farrand Mark I spectrofluorimeter. A small silica plate was dipped in NBD-Cl solution and, after drying, the plate was sprayed, incubated for 3 h in darkness and

placed in the cell so as to form an angle of 45° with analyzer and excitator. The maxima derived from these measurements are given in Table III.

A knowledge of the activation and fluorescence maxima is necessary for the determination of optimum conditions for quantitative analyses of AH substances. During some preliminary work, test plates were scanned with a Vitatron TLD-100. A linear relationship between concentration of AH substances and fluorescence was found in the range 2–50 ng. However, the fluorescence measured depended on the solvents used during development. Quantitative determination of the AH substances following two-dimensional TLC will probably involve the anti-diagonal technique described by Beljaars *et al.*[8].

TABLE III

ACTIVATION AND FLUORESCENCE MAXIMA OF NBD DERIVATIVES

| *Spray reagent* | *Activation (nm)* | *Fluorescence (nm)* |
|---|---|---|
| EA, GLY | 340, 470 | 540 |
| TGA, MEL | 340, 450 | 515 |
| CYS, MEA | 340, 470 | 540 |

*Detection limits of thyriostatic drugs*

Detection limits were determined by spotting diluted standard solutions. The plates were developed with chloroform–ethanol (95:5, v/v) and sprayed with cysteine solution. The results are given in Table IV. It can be seen that the yellow colour, visible in daylight, was intensified by spraying. When activated at 366 nm, concentrations approximately 100 times lower can be detected. For thyroid tissue, the values in parts per billion were calculated from the detection limits of the standards, taking into account that 50 $\mu$l (*e.g.*, one tenth of the sample) were spotted and that an overall yield of 50% was obtained. When more than 50 $\mu$l are spotted, detection limits can easily be calculated in a similar manner. When the plate was sprayed with a cysteamine solution, the intensity of fluorescence was 5–10 times greater than with cysteine.

TABLE IV

DETECTION LIMITS OF THYRIOSTATIC DRUGS BEFORE AND AFTER SPRAYING WITH CYSTEINE

| *Drug* | *Standards (ng)* | | | *Thyroid tissue (ppb)* | | |
|---|---|---|---|---|---|---|
| | *Before spraying* | *After spraying* | | *Before spraying* | *After spraying* | |
| | | *Daylight* | *366 nm* | | *Daylight* | *366 nm* |
| MTU | 50 | 10 | 0.2 | 500 | 100 | 2 |
| TAP | 50 | 20 | 0.4 | 500 | 200 | 4 |
| TU | 25 | 10 | 0.4 | 250 | 100 | 4 |
| ØTU | 25 | 10 | 0.6 | 250 | 100 | 6 |
| PTU | 50 | 10 | 0.1 | 500 | 100 | 1 |

*Analyses of extracts from animal tissues*

The overall recovery of [$^{14}$C]thiouracil, added to 2 g of minced tissue, was determined (Table V). No difference in reproducibility was observed between samples derived from thyroid tissue, meat, kidney and liver.

The extraction yield of [$^{14}$C]thiouracil in methanol can be increased to 97 ± 1.5% by re-extracting the centrifuged residue with 5 ml of acidified methanol. This second extraction step was omitted from the procedure.

TABLE V

OVERALL RECOVERY OF [$^{14}$C]THIOURACIL ADDED TO MEAT AND ORGAN SAMPLES FROM SLAUGHTERED ANIMALS

| *Extraction step in procedure* | *Recovery (%)* | *Standard deviation (%)* |
|---|---|---|
| Extraction with methanol | 89.6 | ± 1.3 |
| After extraction with light petroleum | 89.5 | ± 1.3 |
| After column adsorption | 86.0 | ± 2.4 |
| After extraction with diethyl ether | 81.6 | ± 2.8 |
| Extraction of NBD derivative (diethyl ether) | 23.4 | ± 1.4 |
| Extraction of NBD derivative (methyl ethyl ketone) | 43.1 | ± 2.1 |

After passage through the ion-exchange column, evaporation and extraction with diethyl ether, about 80% of the [$^{14}$C]thiouracil could be recovered. However, following derivatization with NBD-Cl and extraction with diethyl ether or methyl ethyl ketone, only 23 and 43%, respectively, of the [$^{14}$C]NBD derivative could be detected in the organic phase. As the extraction yield of [$^{14}$C]thiouracil, after reaction with NBD-Cl in buffer, is 70 and 80% with diethyl ether and methyl ethyl ketone, respectively, these results suggest that extracts from animal tissues still contain substances that slow down the reaction of NBD-Cl with thiouracil.

In a series of experiments, TAP, MTU and TU were added to blank samples of meat at a concentration of 10 ppb (10 ng/g) and analyzed by two-dimensional TLC. By spotting 100 $\mu$l of the 250-$\mu$l concentrated extract, spots of the antithyroid drugs were easily detected. One analyst can carry out about ten analyses per day. Pre-treatment of ten samples, derivatization and extraction take about 4 h and the development of the plates takes about 2.5–3 h; to this must be added the time required for screening analysis (1 h).

*Use of procedure in the control of antithyroid residues in animal tissues*

The method described has been accepted by the Belgian Ministry of Public Health for the control of antithyroid residues in slaughtered animals. The procedure has been in routine use in different laboratories for 2 months. Contrary to the methods hitherto described[3–5], our procedure enables antithyroid residues to be detected in meat as well as in thyroid tissue, kidney and liver.

From experiments with different levels of MTU, Böhnel[3] concluded that only thyroid tissue contained detectable MTU residues. Moreover, the MTU content of thyroid tissue declined rapidly, 50% of its initial content being broken down within

the first 5 days of storage. However, from several analyses performed on different cows and steers, we conclude that detectable levels of MTU residues in thyroid tissue are always associated with the presence of MTU residues in meat, kidney and liver (see Fig. 5).

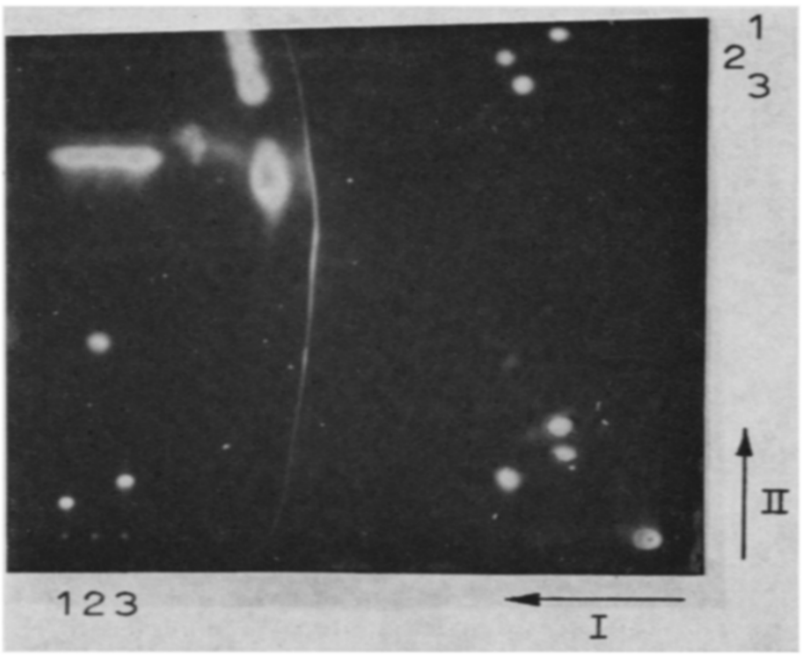


Fig. 5. Two-dimensional chromatogram showing MTU residues in kidney. 1 = TU; 2 = TAP; 3 = MTU.

Tissues taken from five different animals, slaughtered 5 days before sampling and kept at 5° in the slaughterhouse, still contained appreciable amounts of MTU residues. Recently, we analyzed two meat *(longissimus dorsi)* samples that had been frozen at −15° for 1 year. From these samples, taken from cows of which the thyroid contained 1.8 ppm of MTU (as analyzed by the method of Van Waes[4]), an intense spot of MTU could be detected on the thin-layer chromatogram.

It therefore appears that the procedure described here is reliable and sensitive and permits the routine detection of antithyroid residues in a variety of animal tissues even if they have been stored and frozen for long periods.

## CONCLUSIONS

The method described is capable of the detection of antithyroid residues in animal tissues at a level of 10 ppb or lower. In comparison with earlier methods, the procedure described is five times more rapid, simple, one thousand times more sensitive and ten times cheaper.

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