HPTLC Analysis of Residues of Anabolics in Meat and Kidney Fat

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Chromatographic clean-up
Anabolics
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Summary

A column chromatographic method is described for the faster clean-up of anabolic residues in meat and kidney fat prior to HPTLC analysis. After enzymic hydrolysis, to free the conjugated hormones, the anabolics were extracted from the matrix with methanol. The estrogens were separated from the androgens and gestagens by group fractionation through a coupled Polyclar-phenylsilane column. This clean-up procedure was developed to speed up the routine detection of various anabolics in meat and kidney fat samples by HPTLC with fluorescence detection. This faster clean-up may also be coupled to an additional clean-up by HPLC prior to HPTLC analysis.

1 Introduction

The use of the steroids as growth promoters in the fattening of animals is prohibited in all EEC member states. In Belgium checks on the illicit application of anabolics is carried out by the Institute of Veterinary Inspection (IVK-IEV) according to EEC directives [1, 2].

In regulatory control at the farm, urine and/or feces of the animals may be sampled. In urine, substantial amounts of the anabolics and/or of their metabolites are present after treatment with the steroids. For the analysis of residues of anabolics in urine a routine HPTLC method has been described previously [3]. This procedure may be combined with an additional clean-up by HPLC with column switching as described by *F. Smets* [4, 5]. However, the use of an automated HPLC will considerably increase the cost of the analysis.

At the retail level (butchers' shop, supermarket) or in the case of import/export, sampling is restricted to tissue only. At the slaughterhouse, tissue as well as excreta can be sampled. Some anabolics (*e.g.,* the gestagens medroxyprogesterone acetate, megestrol acetate, and chlormadinone acetate) are concentrated in kidney fat, which is to be considered as the target tissue for the tracing of this group of drugs. After illicit administration the identity of the anabolics is not known. From the analysis of injection sites it is known that cocktails of different anabolics are used [6]. In order to protect the consumer, screening of the samples by a multiresidue method should be carried out. HPTLC is a valuable multiresidue method for the determination of residues of steroids [7, 8, 9]. In our laboratory a HPTLC method with fluorescence detection was developped by *R. Verbeke* [10]. Using this method, anabolics could be detected at the 0.5 – 10 ppb level. However, the original clean-up described [10] is time consuming and may lead to bottlenecks in routine analysis. Therefore, modifications of the *Verbeke* method have been described [11] and are used in regulatory control in Belgium [12].

In this paper an improved column chromatographic clean-up procedure for anabolics in meat and kidney fat is presented. This method allows routine HPTLC detection of anabolic residues within a reasonable time interval. During application in regulatory control, the method has been proved to be particularly useful for the detection of residues of gestagens.

This faster clean-up can also be used in addition to a clean-up by HPLC. By fraction collection very "clean" extracts are obtained. The reduction of matrix components considerably improves the interpretation of the two-dimensional HPTLC (2D-HPTLC) analysis. However, the use of HPLC also increases the cost of analysis.

2 Experimental

2.1 Apparatus

The following apparatus was used: homogenizer (*e.g.,* Waring Blender with a reservoir of 250 ml); microwave oven (*e.g.,* Zanker); centrifuge equipped with metal centrifugation tubes of 300 ml (*e.g.,* Sorvall); rotary vacuum evaporator; waterbath; extraction flasks (250 and 500 ml); ten-port vacuum manifold (*e.g.,* Vac-Elut); empty reservoirs of 8 ml and corresponding frits of 20 μm (Analytichem International, Harbor City, CA, USA); nitrogen evaporator (*e.g.,* Techni Dry Block);

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chromatographic columns and tanks; UV-transilluminator (λ = 366 nm) (*e.g.*, c-62, U.V. Products Inc., San Gabriel, Calif., USA); white light trans-illumination source (local supplies shop); homemade single sample applicator [10] or "4 x 4" sample applicator [13, 14, 15]; LKB 1219 Rack *beta* liquid scintillation counter (Bromma, Sweden); and Zeiss KM 3 TLC scanner in the fluorescence mode (Zeiss, Oberkochen, FRG).

The HPLC used was a Perkin-Elmer LC-5 (Norwalk, USA) equipped with an automatic injector and coupled to a Frac 100 fraction collector (Pharmacia, Bromma, Sweden).

2.2 Reagents and Reference Compounds

Glucuronidase-sulfatase enzyme suspension (*Helix pomatia* juice: 100000 Fishman units/ml β-glucuronidase + 1000000 Roy units/ml sulfatase) was obtained from I.B.F. (Clichy, France).

Most reference steroids were obtained from Steraloids (Wilton, NY, USA). Zeranol, zearalenone, and medroxyprogesterone (acetates) were gifts from Upjohn (Puurs, Belgium). Trenbolone, trenbolone acetate, [6,7-^{3}H]-trenbolone, and [6,7-^{3}H]-trenbolone acetate were gifts from Roussel – Uclaf (Paris, France). Other radioactive steroids ([monoethyl-^{3}H]-diethylstilboestrol; [2,4,6,7-^{3}H]- or [4-^{14}C]-estradiol; [4-^{14}C]-testosterone; [4-^{14}C]-progesterone; and [^{3}H]-zeranol) were purchased from Amersham (Bucks, Great Britain).

Silica gel thin layer plates or HPTLC plates without fluorescence indicator, and reversed phase plates (RP-18) were obtained from Merck (Darmstadt, FRG; Cat. No. 5721, 5631, and 5914, respectively). Polyclar AT (a water-insoluble polyvinyl-pyrrolidone for binding phenols) [16] was from Serva (Heidelberg, FRG), and phenylsilane bonded to silica was from Analytichem International (Harbor City, CA, USA).

All other reagents (*n*-hexane, dichloromethane, chloroform, acetone, benzene, cyclohexane, and ethyl acetate) were reagent grade products from Merck. Diethyl ether, free from peroxides, was obtained from Gifrer & Barbezat (Decines, France).

Pretreatment of Polyclar AT: In a glass-stoppered flask (250 ml), 20 g of resin were swelled overnight in distilled water. Small particles were removed by sedimentation for 10 min in a 500 ml graduated cylinder and elimination of the supernatant. This procedure was repeated three times. The resin was washed with 250 ml 0.3 M Na_2CO_3 in 30 % methanol and finally with distilled water to neutral pH. The resin can be stored in water and is ready for use.

Treatment of phenylsilane: The commercial bonded phase was used without pretreatment.

2.3 Solutions

Stock solutions of the hormones in methanol were prepared at a concentration of 100 μg/ml.

Chromatographic solvent systems: (1) = chloroform – acetone (9 + 1, *v/v*); (2) = chloroform – acetone – hexane (25 + 5 + 20, *v/v/v*); (3) = chloroform – benzene – ethanol (9 + 1 + 0.25, *v/v/v*); (4) = *n*-hexane – diethyl ether – dichloromethane (4 + 3 + 2, *v/v/v*); (5) = cyclohexane – ethyl acetate – ethanol (60 + 40 + 2.5, *v/v/v*); and (6) = tetrahydrofuran – *n*-hexane (35 + 65, *v/v*)

2.4 Columns

2.4.1 Polyclar Column

Columns provided with a PTFE stopcock and a female B14 glass joint (upper) and a male B14 glass joint (lower) were used. The columns were filled up to a height of 5 cm (1 x 5 cm) with the stored resin. No further treatment was necessary.

2.4.1 Phenylsilane Column

Phenylsilane (500 mg) was filled into an empty 8 ml reservoir provided with a porous frit of polyethylene (as an alternative, pre-packed columns can be used). Before use, the bonded phase was solvated by percolating the columns with 10 ml of methanol followed by 20 ml of distilled water. The phenylsilane (lower) column was coupled to the Polyclar (upper) column by the lower male B14 joint.

2.5 Extraction Procedure

An overall scheme of the extraction procedure is given in **Figure 1.** In the description of the extraction procedure, the quantities of solvents used for meat samples are different from those for kidney fat. The quantities of solvent for kidney fat are given between brackets.

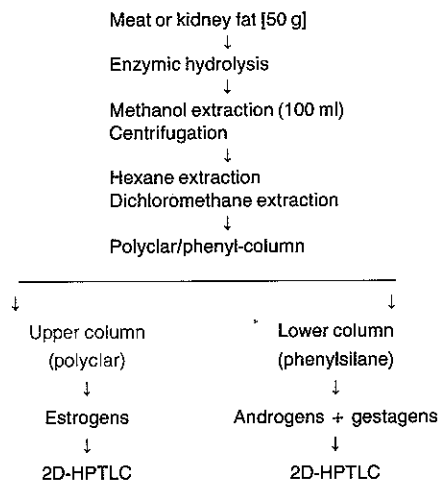

Figure 1

Overall scheme of the extraction procedure.

Meat or (kidney) fat is cut into small pieces. Fifty grams is weighed into a glass flask, and 40 ml distilled water is added. The fat samples are melted in a waterbath at 80 °C (or in a microwave oven). Afterwards, the content is homogenized with a Waring Blender for 1 min (30 s at low and then 30 s at high speed). The homogenate is transferred into the original flask and a drop of chloroform (as anti-bacterial agent) and 10 ml sodium acetate buffer (0.2 M, pH 5.2) are added. The pH is checked and, if necessary, adjusted to pH 5.2 After addition of 400 µl glucuronidase-sulfatase enzyme suspension, the hormone conjugates are hydrolysed during 2 h at 62 °C (or overnight at 37 °C). After hydrolysis the content of the flask is shaken with 180 ml methanol (100 ml for kidney fat) and centrifuged for 10 min at 9000 rpm (13000 g). The supernatant is extracted twice with 50 ml hexane for the removal of the triacylglycerols. Afterwards, the anabolics are extracted into 150, 90, and 90 ml portions of dichloromethane (for the analysis of fat use 90, 75, and 75 ml). The combined dichloromethane phases are washed with 50 ml 0.05 M Na_2CO_3 followed by 50 ml distilled water. (This washing step is omitted in analyses needing optimum yield for zeranol.) The dichloromethane phases are evaporated to near dryness on a rotary evaporator.

2.6 Group Fractionation of Hormones

The evaporated extract is taken up in 1 ml of methanol and then diluted with 10 ml of distilled water. The extract is ready for a one-step passage through the coupled Polyclar/phenylsilane columns to separate estrogens from androgens + gestagens. The meat or fat extract is quantitively transferred to the top of the Polyclar column and allowed to drain into the coupled columns using the Vac-Elut coupled to a vacuum source. The evaporation flask is rinsed once with 1 ml of methanol and then with 10 ml of distilled water. The combined rinsings are also transferred to the top of the Polyclar column. After the rinsings drain into the surface, the columns are uncoupled.

2.6.1 Estrogen Fraction

The estrogens are eluted from the Polyclar column with 15 ml of methanol. The eluate is evaporated to dryness and transferred to a fibrinogen tube with 2, 1, and 1 ml of ethanol. The extract is concentrated to 100 µl under a flow of nitrogen.

2.6.2 Androgen and Gestagen Fraction

The phenylsilane column is washed with 10 ml 30% methanol. The elution of androgens and gestagens from the phenylsilane column is performed with 10 ml methanol. The eluate is evaporated to dryness and the residue is transferred to a fibrinogen tube with 2, 1, and 1 ml of ethanol. The extract is concentrated to 100 µl under a flow of nitrogen.

2.7 HPLC Purification

A spironolactone solution (10 µl; 2 mg/100 ml) is added to the estrogen and androgen fraction. The (relative) retention times of the most important steroids are determined, against spironolactone (internal standard), with standard solutions by UV detection. Spironolactone is a product with a structure similar to an androgen but exempt of anabolic activity. These retention times are given in **Table 1.**

Table 1

Retention times (*RT*) of the most important steroids.

Steroid	*RT* [min]
Estrogens	
17β-Estradiol	5.6
Ethinylestradiol	6.3
Dienestrol	7.6
DES	8.0
Spironolactone	9.2
Androgens	
Trenbolone	5.2
Nortestosterone	6.5
Testosterone	7.8
Spironolactone	9.2
Methyltestosterone	9.6
Gestagens	
Chlormadinone acetate	14.9
Megestrolacetate	15.0
Medroxyprogesterone acetate	16.0

The chromatographic conditions are as follows: column, RPC18 column (5 µ ROSIL 15 cm, Alltech, Eke, Belgium); elution, acetonitrile – water (50 + 50, *v/v*). The different fractions are collected and evaporated to dryness under a stream of nitrogen. The fractions are taken up in 100 µl ethanol. During elution the retention time of spironolactone is recorded to control and adjust the window setting for fraction collection.

2.8 Sample Application

A fraction (5 – 10 µl) of the concentrated extract (50 – 100 µl) of the estrogen or androgen and gestagen or HPLC fraction is applied on a HPTLC plate by the home-designed sample applicator described in [10]. As an alternative, a faster "4 x 4" developing mode and a "4 x 4" sample applicator could be used [13, 14, 15].

2.9 HPTLC

The extracts are analyzed by two-dimensional chromatography on precoated silica gel 60 nanoplates (HPTLC plates, 10 x 10 cm or 5 x 5 cm). TLC on 20 x 20 cm plates could also be used, but the application volumes should be adapted. De-

velopment is carried out in nonsaturated tanks. At the starting point of the nanoplates, up to 10 µl of the extract is spotted. Appropriate concentrations of the reference mixtures are applied in the side lanes. The reference mixtures contain 2 – 20 ng of the steroids presumed to be present in the sample. Chromatographic development is carried out over a distance of 3 – 4 cm using solvent (5) for the androgens and gestagens and solvent (3) for the estrogen fraction. The plate is air dried and the starting point of the sample is overspotted with 5 – 10 ng of the steroids presumed to be present. This procedure is useful for additional R_f comparison between an unknown spot and the reference after the second development. The plate is then turned 90° and run in the second direction, using the appropriate solvents [androgens and gestagens = solvent (1); estrogens = solvent (4)]. As an alternative, a "4 x 4" developing mode can be used. In this mode four samples are developed in two dimensions on one HPTLC plate [13, 14].

2.10 Fluorescence Detection

Before dipping, the nanoplates are dried at 95 °C for 15 min. Fluorescence is induced by dipping in a 5 % sulfuric acid – ethanol solution for 30 s. The plates are viewed under UV light (λ = 366 nm) for fluorescent spots (*e.g.* trenbolone) and then incubated at 95 °C for 10 min. The fluorescence is observed under trans-illumination at λ = 366 nm and with visible (white) light.

The identity of the hormones is evaluated by comparing the R_f values and the fluorescent (and visible) colors of the reference substances (**Table 3**) with those of unknown spots under trans-illumination. Confirmation of the identity of the hormones is obtained by co-chromatography – the sample is overspotted with a known amount of the anabolic on a second plate. After the development and the fluorescence reaction, the anabolics in the sample must coincide exactly with the reference compounds added to the sample. If necessary, an additional confirmation of the identity of the steroids may be obtained by HPTLC on reserved phase plates (RP-18); first dimension, methanol – distilled water – toluene (75 + 20 + 5, *v/v/v*); second dimension, *n*-hexane – dichloromethane – acetonitrile (40 + 10 + 5, *v/v/v*).

3 Results and Discussion

3.1 Extraction

Initially the extraction procedure used was the same as described by *Verbeke* [10]. During our investigations it was found that the use of a microwave oven (instead of a waterbath) for melting the fats may improve the speed of the analysis. The time for melting one fat sample (50 g) in a waterbath (80 °C) was *ca.* 30 min. In a microwave oven the same fat sample was melted within *ca.* 7 min. However, the melting time in a microwave oven increases with the number of samples placed into the oven. So, microwave melting was carried out during the preparation of a second sample. In examining a large number of tests, no degradation of residues of steroids in meat or kidney fat samples has been observed after microwave-melting.

3.2 Group Fractionation

A group fractionation into estrogens and androgens and gestagens was obtained by passing the extract through a coupled polyclar/phenylsilane column. The phenolic anabolics (estrogens) are quantitatively retained by the polyvinylpyrrolidon column (= Polyclar), as described for the analysis of bovine urine [3]. Polyclar is a water insoluble polyvinylpyrrolidone for binding phenols [16]. The 3-keto steroids (androgens and gestagens) pass through the upper column and are selectively bound to the phenylsilane column. The Polyclar adsorbent was packed into a glass column as described in [3]. The phenylsilane column was a commercial solid phase column. A commercial ten-port vacuum manifold (Vac-Elut) was used for the collection of the eluates.

3.2.1 Elution of Polyclar Column

All estrogens were quantitatively eluted from the Polyclar column with methanol (**Table 2**). In comparison with urine samples [3], the interference of matrix components from meat and kidney fat samples leading to "dirty" chromatograms was much smaller. So, a more polar solvent could be used for elution (100 % methanol instead of 0.2 M Na_2CO_3 in 15 % methanol). In comparison with urine the elution volume could also be reduced (15 ml instead of 90 ml). This improved the speed of analysis.

Table 2

Recovery of labelled anabolics in the different clean-up steps (*n* = number of individual experiments).

Substance	Recovery [mean ± *SD* (n)]	
	PC-column	Phenyl-column
[^{3}H]-DES	69 ± 9.0 (6)	–
[^{14}C]-17β-Estradiol	76 ± 3.6 (6)	–
[^{14}C]-Nortestosterone	–	75 ± 8.6 (5)
[^{14}C]-Testosterone	–	82 ± 7.0 (8)
[^{14}C]-Progesterone	–	70 ± 9.3 (4)

3.2.2 Elution of Phenylsilane Column

During a first series of experiments the cyanopropyl column, used for the clean-up of urine [3], was tested for meat and kidney fat extracts. It was found that the androgens and gestagens present in a meat extract could not quantitatively be retained on a cyanopropyl column. After passage of an extract, the bonded phase material was very contaminated (visual observation). Even the use of larger amounts of bonded phase material was unsuccessful. Consequently, a phenylsilane column was tested with more success. After

washing with 30 % methanol, the androgens were quantitatively eluted with methanol. Also, in comparision with urine analysis, a smaller elution volume was obtained (10 ml *versus* 50 ml).

In Table 2 the elution yields of some labelled steroids from polyclar and phenylsilane columns are shown.

3.3 HPLC Purification

This faster clean-up may also be coupled to an additional clean-up by HPLC with fraction collection. The first intention of the use of this extra clean-up procedure was to facilitate the interpretation of the HPTLC plates. Meat and kidney fat extracts obtained by the procedure described above are still "crude" extracts, containing a lot of matrix components. Due to the presence of other fluorescent spots, these HPTLC plates can only be evaluated by experienced analysts. The HPTLC plates obtained after the HPLC purification are much cleaner and interpretation is facilitated.

Moreover, some "close" anabolics (*e.g.*, nortestosterone and testosterone) are collected in different fractions. Therefore, confusion between those two drugs is avoided. This is very important since testosterone is a natural steroid, whereas nortestosterone is xenobiotic (at least for bovine samples).

3.4 HPTLC and Sulfuric Acid Induced Fluorescence

For the optimum two-dimensional chromatography of the androgen and gestagen and estrogen fractions, different solvent combinations had to be tested. With the solvents selected (**Tables 3** and **4**) the best separation between the steroids and the matrix components was obtained. The solvent combinations used for 2D-HPTLC of HPLC fractions are less critical. After dipping the plates in 5 % sulfuric acid in ethanol a detection limit of 1 – 10 ng for individual steroids was obtained, as described by *Verbeke* [10]. The anabolics were identified by comparing the relative R_f values and the different fluorescence colors (and colors under white light trans-illumination). The relative R_f values (for androgens to methyltestosterone, R_f (1) = 0.53 and R_f (5) = 0.46; for estrogens to 17-β-estradiol, R_f (3) = 0.23 and R_f (4) = 0.30) were measured in triplicate for different solvent combinations and are given in Tables 3 and 4.

By combination of solvents (5) and (1) for androgens, an insufficient separation between medroxyprogesterone acetate (MPA) and chlormadinone acetate (CAP) was obtained. This problem was solved by adding *n*-hexane to the chromatographic solvent (instead of (1): (2) = chloroform – acetone – *n*-hexane; 25 + 5 + 20, *v/v/v*) Megestrol acetate (MEGA) and medroxyprogesterone acetate (MPA) could not be separated with the normal solvent systems used. The differentiation between those two drugs is carried out on the basis of the color produced by illumination with white light. MEGA produces a blue color (like CAP) while MPA is "dirty" green. Recently, it was found that smaller amounts of MPA could be

Table 3

Relative R_f values of some estrogens on HPTLC plates after sulfuric acid induced fluorescence.

Substance	Relative R_f* value in solvent+ (3)	366 nm UV (4)	Color** in
Benzestrol	0.77	1.46	GY
Dienestrol	0.82	1.60	RD
trans-Diethylstilboestrol	0.99	1.81	RD
cis-Diethylstilboestrol	0.36	1.02	RD
17β-Estradiol	1.00	1.00	YW
Estriol	0.09	0.02	YW-BN
Estrone	1.77	1.80	YW
Ethinylestradiol	1.27	1.53	YW
Hexestrol	0.86	1.68	GN
Mestranol	2.68	2.30	YW
Zearalenone	1.36	1.52	GN-YW
Zeranol	0.82	0.54	GN-YW

*Relative to 17 β-estradiol ($R_{f(3)}$ = 0.23; $R_{f(4)}$ = 0.30). +Solvent systems: (3) = chloroform – benzene – ethanol (9 + 1 + 0.25, *v/v/v*); (4) = *n*-hexane – diethyl ether – dichloromethane (4 + 3 + 2, *v/v/v*).
**Abbreviations: BE = blue; BN = brown; BT = bright; GN = green; LT = light; OE = orange; PU = purple; RD = red; YW = yellow.

Table 4

Relative R_f values of some anabolics of the "androgen" fraction of HPTLC plates after sulfuric acid induced fluorescence.

Substance	Relative R_f value* in solvent+ (1)	(5)	Color in (366 nm) UV
1-Dehydrotestosterone	0.65	0.67	OE-BN
5α-Dehydrotestosterone	1.15	1.27	BE-PU
Epitestosterone	0.83	0.95	YW-BN
Ethinyltestosterone	1.23	1.35	YW-BN
Medroxyprogesterone	1.14	1.23	BE-PU
Medroxyprogesterone acetate	1.61	1.26	YW
Melengestrolacetate	1.61	1.26	YW-BN
17α-Methyltestosterone	1.00	1.00	YW-GN
4,9,11-Methyl-testosterone	1.02	1.02	YW-GN
19-Nortestosterone	0.80	0.79	YW-LT
Progesterone	1.61	1.36	BE-GN
Testosterone	0.88	0.89	YW
Trenbolone	0.80	0.84	BE-BT
Trenbolone acetate	1.63	1.48	BE-BT
Vinyltestosterone	1.28	1.34	YW-GN
Megestrolacetate	1.66	1.23	YW
Chlormadinone acetate	1.66	1.1	YW

*Relative to 17α-methyltestosteron ($R_{f(1)}$ = 0.53; $R_{f(5)}$ = 0.46). +Solvent systems: (1) = chloroform – acetone (9 + 1, *v/v*); (5) = cyclohexane – ethyl acetate – ethanol (60 + 40 + 5, *v/v/v*). For color abbreviations see Table 3.

masked by larger amounts of MEGA. A separation between the two drugs was achieved by using solvent (2) in the first dimension and (6) in the second dimension.

In routine analysis, sometimes a very unpleasant phenomenon occurs during the induction of fluorescence by dipping with ethanol – sulfuric acid. For some batches of HPTLC plates the thin layer loosens from the glass plates and cracks during heating. Plates showing this phenomenon are very difficult to handle and to evaluate. In most cases the analysis should be restarted. The problem was solved at first by purchasing "good" batches from the manufacturer on basis of the lot number. However, recently it was found that this phenomenon could be avoided in two other ways: 1) by heating the plates before dipping, or 2) by dipping the plates first into an ethanol solution (before the final dip into the ethanol – sulfuric acid solution). The first solution was included in the description of the procedure. The exact reason for this phenomenon is unknown. The occurence of small amounts of water "trapped" between the thin layer and the glass plate could produce an exothermic reaction at contact with sulfuric acid. However, this is only a hypothesis.

Table 5

Overall recovery of hormones added to meat and kidney fat.

Substance (*n*)	Recovery [%] [mean ± *SD*]	Concentration range* added [µg/l]
Estrogens		
Dienestrol (5)	74 ± 9.0	1 – 3
Diethylstilboestrol (6)	80 ± 5.1	0.5 – 2
17β-Estradiol (9)	79 ± 5.0	1 – 2
Ethinylestradiol (8)	76 ± 7.4	2 – 4
Euqilinin (3)	70 ± 1.5	2 – 3
Hexestrol (9)	75 ± 5.1	2 – 10
Zeranol (5)	29 ± 7.7	6 – 10
Androgens and Gestagens		
1-Dehydrotestosterone (3)	78 ± 3.6	2 – 8
5-Dihydrotestosterone (3)	77 ± 5.6	1 – 3
Medroxyprogesterone (4)	74 ± 11.9	2 – 4
Medroxyprogesterone acetate (5)	78 ± 3.5	1 – 4
Melengestrol acetate (4)	75 ± 3.3	2 – 3
17-Methyltestosterone (8)	76 ± 10.4	1 – 3
4,9,11-Methyltestosterone (4)	79 ± 6.3	1 – 2
19-Nortestosterone (7)	73 ± 10.2	1 – 5
Testosterone (5)	81 ± 6.8	1 – 2
Trenbolone (8)	73 ± 9.8	1 – 2
Vinyltestosterone (4)	78 ± 5.9	1 – 2
Chlormadinone acetate (8)	81 ± 3.3	1 – 2
Megestrol acetate (5)	73 ± 8.9	1 – 5
6-Dehydroprogesterone (3)	73 ± 4.0	2 – 10

(*n*) = number of individual experiments. *Concentration range added and determined by TLC scanning.

3.5 Overall Recovery

The recovery of the anabolics through the entire procedure was measured with blank samples of meat and fat spiked with hormones in the concentration range of 0.5 – 10 ppb. The amount of the anabolics in the final extract was determined by TLC scanning in the fluorescence mode using the anti-diagonal technique of *Beljaars et al.* [17].

In **Table 5** the recoveries of the unlabelled steroids are shown. The recoveries obtained for the most important anabolics are high (70 – 90 %). The lower recovery for zeranol is caused by the washing step of the dichloromethane extracts with 0.05 M Na_2CO_3. The sensitivity of the method for zeranol may be improved by omitting that step. However, this increases the interference of the matrix components with the other anabolics.

4 Conclusions

The method described permits the multi-residue analysis of anabolics at residue levels of 0.5 – 10 ppb in meat and kidney fat. The results obtained with this procedure were comparable with those obtained with the original method described by *Verbeke* [10, 18]. However, the present method is considerably faster; one analyst is able to clean-up at least 20 samples a week.

By combination of the faster clean-up with the faster "4 x 4" HPTLC developing mode [13, 14, 15], the delay between sampling and the first results is considerably shortened. This permits the release of "negative" carcasses for the meat trade on a shorter time interval. Moreover, more time could be spent for additional tests (*e.g.*, HPLC clean-up or MS confirmation) on "suspect" samples with the remainder of the extract.

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