Improved Chromatographic Clean-Up of Anabolics in Bovine Urine

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Summary

A rapid and selective chromatographic method is described for the clean-up of anabolics in bovine urine using group fractionation. After enzymic hydrolysis, to free the conjugated hormones, the anabolics were extracted from urine with diethyl ether. The estrogens were separated from the androgens + gestagens by group fractionation through a coupled Polyclar/Cyanopropyl column. This clean-up procedure was developed to permit the routine detection of various anabolics in bovine urine samples by HPTLC with fluorescence detection.

1 Introduction

In the EEC the use of steroids as growth promotors in cattle fattening is prohibited. EEC countries have to follow a sampling plan and analyze a certain number of samples [1, 2].

The illegal use of hormonal compounds in animal production may be detected by analyzing different kinds of matrices such as : injection sites, body fluids (plasma, bile), tissues (meat, kidney fat), or excreta (urine and feces). The choice of matrix is complex and depends on many factors: the sampling circumstances, the type of hormone, the species of animal, *etc*.

In regulatory control on the living animal, the analysis of urine is the method of choice; as this is where relatively high levels of anabolics are found. Moreover, bovine urine is a well known matrix and handling and storage of samples is easy. Sampling of feces is easier for the veterinarian but the analysis is more difficult; hitherto, the clean-up procedure for the analysis of feces has been much more tedious than that for urine.

At the slaughterhouse, tissue, body fluids, or excreta may be sampled. At this stage of the regulatory control urine is important. Urine provides the best evidence of illegal treatment with certain anabolics (*e.g.* 19-nortestosterone). For other anabolics (*e.g.* medroxyprogesterone acetate and chloromadinone acetate) kidney fat is a better indicator.

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With illicit administration, the identity of the anabolics is not known. Screening by several radioimmunoassays (RIA) [3] or a multi-residue method should be used on the selected urine sample. HPTLC with fluorescence detection [4–8] is a valuable multi-residue method for the determination of steroids at the ppb level. However, the original clean-up described [4] is time consuming and may lead to bottlenecks in routine analysis. HPLC with column switching may be used for the separation and purification of anabolics present in bovine urine [9, 10]. However the use of an automated HPLC will considerably increase the cost of analysis.

In this paper an improved column chromatographic clean-up procedure for anabolics in bovine urine is presented. This method permits routine HPTLC detection of anabolic residues in urine at the ppb level within a limited time interval.

2 Experimental

2.1 Apparatus

The following apparatus were used: centrifuge, rotary vacuum evaporator, waterbath, extraction flasks (500 ml), chromatographic columns and tanks, UV transilluminator (366 nm) (c-62; U.V. Products Inc., San Gabriel, CA, USA), home made single sample applicator or "4×4" sample applicator [11, 12], and a LKB 1219 Rack beta liquid scintillation counter (Bromma, Sweden).

2.2 Reagents and Reference Compounds

– Glucuronidase-sulfatase enzyme suspension (*Helix pomatia* juice: 100,000 Fishman units / ml β-glucuronidase + 1,000,000 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 α-nortestosterone were a gift from *Dr. Stephany* (RIVM, Bilthoven, The Netherlands). Medroxyprogesterone acetate was a gift 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 were obtained from E. Merck (Darmstadt, FRG) (Cat. No. 5721 and 5631, respectively), Polyclar AT (a water insoluble polyvinylpyrrolidone for binding phenols) [13] from Serva (Heidelberg, FRG) and Cyanopropylsilane bonded to silica from J.T. Baker Chemicals B.V. (Deventer, Holland).

– All other reagents (*n*-hexane, dichloromethane, chloroform, acetone, benzene, cyclohexane, and ethyl acetate) were reagent-grade products from E. 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 resin was swelled overnight in distilled water. Small particles were removed by sedimentation in a 500 ml graduated cylinder for 10 min and elimination of the supernatant. This process 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.

Pretreatment of Cyanopropylsilane: Low molecular weight materials and fines were removed by allowing sedimentation of the bonded phase in methanol. Cyanopropylsilane (100 g) was suspended in 1000 ml methanol in a graduated cylinder. After sedimentation (2×5 min) the supernatant was decanted. A last sedimentation (5 min) was performed in 500 ml distilled water and the supernatant was siphoned off. The bonded phase was stored in water-methanol (95 : 5, v/v).

2.3 Solutions

Stock solutions of the hormones in methanol were prepared at a concentration of 100 µg/ml.
Chromatographic solvent systems: 1 = *n*-hexane – diethyl ether – dichloromethane (5 : 9 : 6, v/v/v); 2 = chloroform – acetone (9 : 1, 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).

2.4 Columns

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 with the stored resin up to a height of 5 cm (1×5 cm). No further treatment was necessary.

Cyanopropylcolumn: The same glass columns were filled to a height of 4 cm (1×4 cm) with cyanopropyl. Before use the bonded phase was solvated by percolating the columns with 30 ml of a 30% acetic acid solution followed by 30 ml of methanol. The washing was completed with distilled water until the pH was neutral.

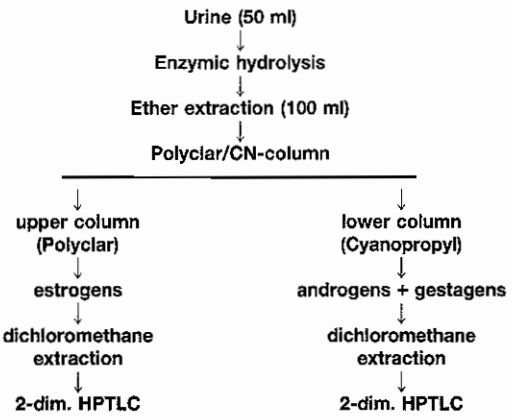

Figure 1

Overall scheme of the extraction procedure.

2.5 Extraction Procedure

An overall scheme of the extraction procedure is given in **Figure 1.**

Fifty ml of urine was filtered on a glass wool filter. A drop of chloroform (as antibacterial agent) and 10 ml sodium acetate buffer (0.2 M, pH 5.2) were added to the filtrate. The pH was checked and adjusted to pH 5.2 if necessary (by using a few drops of 0.1 M HCl). After addition of 50 µl glucuronidase-sulfatase enzyme suspension the hormone conjugates were hydrolyzed for 2 h at 62 °C. Afterwards, the enzyme digest was extracted once with diethyl ether (100 ml). The ether phase was collected in a round-bottomed flask and evaporated to dryness on a rotary evaporator at 40 °C.

2.6 Group Fractionation of Hormones

The evaporated extract was taken up in 1 ml of methanol and then diluted with 10 ml of distilled water. The extract was ready for a one-step passage through the coupled Polyclar/Cyanopropyl columns to separate estrogens from the androgens + gestagens. The urine extract was quantitively transferred to the top of the Polyclar column and allowed to drain into the coupled columns. The evaporation flask was rinsed first with 1 ml of methanol and then with 10 ml of distilled water. The combined rinsings were also transferred to the top of the Polyclar column. After the rinsings had drained into the surface, the columns were uncoupled.

Estrogen Fraction: The Polyclar column was washed with 20 ml of 15% methanol and the estrogens were eluted with 90 ml of 0.2 M Na_2CO_3 in 15% methanol. The pH of the eluate was adjusted to 5 with 6 N HCl. Afterwards, 50 ml methanol was added. The eluate was extracted with 30 ml, 10 ml, and 10 ml of dichloromethane. The CH_2Cl_2 phases were collected in one extraction flask and successively washed with 10 ml 0.2 M Na_2CO_3 and 10 ml distilled water. The extract was evaporated to dryness and finally dissolved in 100 µl of ethanol. This extraction procedure has been described [4].

Androgen + Gestagen Fraction: The elution of androgens + gestagens from the cyanopropyl column was performed with 50 ml of a solution of 10% acetic acid in 20% methanol (acetic acid-methanol-distilled water (1:2:7, v/v/v). After addition of 30 ml methanol the eluate was extracted with 20 ml, 5 ml, and 5 ml dichloromethane. The dichloromethane phases were collected and evaporated to dryness. The residue was dissolved in 100 µl of ethanol.

2.7 Sample Application

A fraction (5–10 µl) of the concentrated extract (50–100 µl) of the estrogen or androgen + gestagen fraction was applied a HPTLC plate by a home designed sample applicator described previously [4]. Alternatively, a faster "4×4" developing mode and a "4×4" sample applicator could be used [11, 12].

2.8 High Performance Thin Layer Chromatography

The extracts were analyzed by two-dimensional chromatography on precoated silica gel 60 nanoplates (HPTLC plates : 10×10 cm or 5×5 cm). TLC on 20×20 cm plates could also be used but the application volumes should be adapted. Development was carried out in nonsaturated tanks. At the starting point of the nanoplates up to 10 µl of the extract was spotted. Appropriate concentrations of the reference mixtures were applied in the side lanes. The reference mixtures contained 2–20 ng of the steroids presumed to be present in the sample. Chromatographic development was carried out over a distance of 3–4 cm using solvent 5 for the androgens + gestagens and solvent 3 for the estrogen fraction. The plate was air dried and the starting point of the sample was 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 was then turned 90° over and run in the second direction using the appropriate solvents (androgens + gestagens = solvent 2; estrogens = solvent 4). Alternatively a "4×4" developing mode could be used. In this mode 4 samples are developed in two dimensions on one HPTLC plate [11, 12].

2.9 Fluorescence Detection

The plates were air-dried and the fluorescence reaction was induced by dipping the nanoplates in a 5% sulfuric acid-ethanol solution for 30 s. The plates were viewed under UV light (366 nm) for fluorescent spots (*e.g.* trenbolone) and then incubated at 95°C for 10 min. The fluorescence was observed under transillumination at 366 nm.

The identity of the hormones was evaluated by comparing the R_f values and the fluorescence colors of the reference substances (**Table 3**) with those of unknown spots under transillumination. Confirmation of the identity of the hormones was obtained by co-chromatography: the sample is overspotted with a known amount of the anabolic on a second plate. After development and the fluorescence reaction, the anabolics in the sample must coincide exactly with the reference compound added to the sample.

3 Results and Discussion

3.1 Ether Extraction

After the enzymic hydrolysis, the anabolics are quantitatively extracted from 50 ml urine by 100 ml of diethyl ether. This quick and simple extraction replaces the time consuming XAD-2 adsorption column described before [4]. In **Table 1** the efficiency of the ether extraction of some labeled anabolics is shown. Since the extraction yields are high, just one extraction with 100 ml diethyl ether is sufficient for the quantitative removal of the anabolics from the urine. In comparison with the XAD-2 eluate this ether extract is more easily evaporated to dryness with a rotary evaporator. This is an additional advantage which also speeds up the analysis.

Table 1

Extraction yields of labeled anabolics from urine with diethyl ether. (*n* = number of individual experiments.)

Substance *(n)*	Extraction Yield (%) (mean ± *SD*)
[^{3}H] DES (5)	94 ± 6.8
[^{14}C] 17β-estradiol (6)	99 ± 0.5
[^{14}C] estriol (4)	82 ± 4.3
[^{14}C] estrone (3)	96 ± 4.2
[^{14}C] 17α- OH-progesterone (3)	99 ± 0.8
[^{14}C] progesterone (3)	99 ± 0.6
[^{14}C] testosterone (20)	97 ± 3.7
[^{3}H] zeranol (5)	88 ± 3.8

3.2 Group Fractionation

Selective adsorption and elution is necessary to perform sufficient group fractionation. Therefore, the extract was passed through a coupled Polyclar/Cyanopropyl column. The phenolic anabolics (estrogens) are quantitatively retained by the polyvinylpyrrolidone column (Polyclar) [13]. The 3-keto steroids (androgens + gestagens) are selectively bound to the cyanopropyl sorbent. The elution pattern of the different hormones from the two columns was studied.

Elution of Polyclar column: By the elution of the Polyclar column with a 75% methanol solution all estrogens were quantitatively retrieved. However, the optimal elution characteristics may not be judged solely on the basis of the elution yield of the hormones, as the possibility of proper identification of the anabolics on the thin layer plates is also very important. This was especially true with highly contaminated urines as the obtained chromatograms were "dirty" and could not be used for adequate differentiation of the spots from the anabolics and the spots originating from the matrix. The methanol concentration used was too high and a great deal

of unknown impurities, retained by the Polyclar column were eluted. The optimal methanol concentration was obtained after an experimental study. In this study an adequate recovery of all bound hormones and a sufficient quantity of the thin layer chromatograms was combined. The best elution solvent hitherto found is 0.2 M Na_2CO_3 in 15% methanol. In **Figure 2** the optimum elution patterns of ^{3}H-DES and ^{3}H-zeranol from a Polyclar column are given.

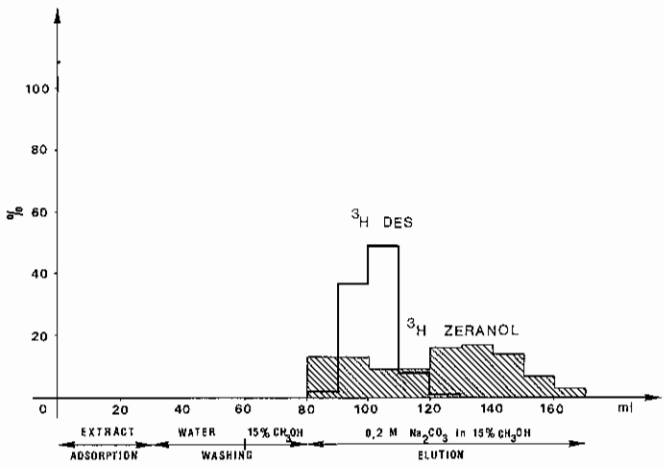

Figure 2

Adsorption and elution pattern of [^{3}H]-DES and [^{3}H] zeranol on a Polyclar column (% of total radioactivity eluted).

Elution of cyanopropyl column: Experiments with different concentrations of methanol showed that a 20% methanol solution was necessary for the sufficient elution of some strongly adsorbed anabolics like progesterone. Elution in a smaller volume was obtained by using 10% acetic acid in 20% methanol. In **Figure 3** the optimum elution pattern of some androgens and gestagens from a cyanopropyl column

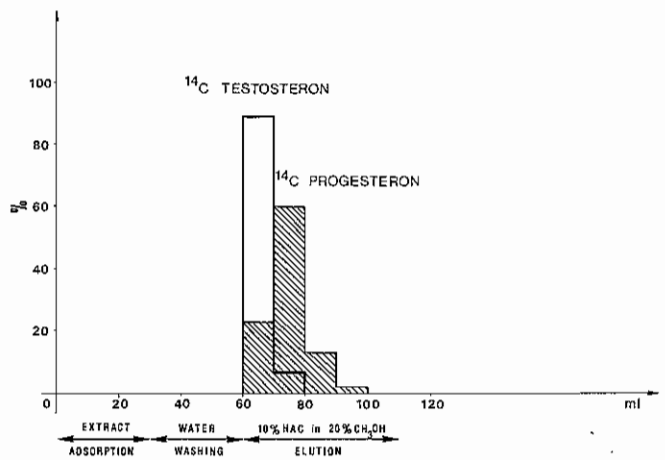

Figure 3

Adsorption and elution pattern of [^{14}C] testosterone and [^{14}C]progesterone on a cyanopropyl column (% of total radioactivity eluted).

is given. In **Table 2** the elution yields of some labeled steroids from Polyclar and cyanopropyl columns are shown.

Table 2

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

Substance	Recovery (%) PC-column*	CN-column*	dichloro-methane(%)*
[^{3}H] DES	82 ± 5.9(6)	–	82 ± 6.0(9)
[^{14}C] 17β-estradiol	97 ± 2 6)	–	96 ± 3.3(6)
[^{14}C] estriol	25 ± 3.5(3)	–	10 ± 1.5(3)
[^{3}H] zeranol	63 ± 2.4(4)	–	62 ± 3.1(4)
[^{14}C] testosterone	–	95 ± 3.8(8)	93 ± 6.5(8)
[^{14}C] progesterone	–	97 ± 2.1(8)	96 ± 3.0(8)

* mean ± *SD (n)*

3.3 Dichloromethane Extraction

Before application to the thin layer plate further purification of the column eluates was necessary. Therefore a ternary extraction of a methanol-water mixture with dichloromethane was performed. For most steroids this extraction, described earlier [4], gave very good yields. In Table 2 the recovery of some labeled steroids after dichloromethane extraction is given.

3.4 HPTLC and Sulfuric Acid Induced Fluorescence

Different solvent combinations were tested for two-dimensional chromatography of the androgen + gestagen and estrogen fractions. An optimal R_f of the spots and a sharp

Table 3

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

Substance	Relative R_f values* in solvent system 3	4	Color in UV (366 nm)
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
Ethynylestradiol	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 17β-estradiol (R_{f3}, 0.23; R_{f4}, 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; and YW = yellow.

separation between the different anabolics and the spots originating from the matrix were obtained. After dipping the plates in 5% sulfuric acid in ethanol the detection limit was 1–10 ng of individual steroids [4]. The anabolics were identified by comparing the relative R_f values and the different fluorescence colors. The relative R_f values (for androgens to methyltestosterone: R_{f1}, 0.40; R_{f2}, 0.53; R_{f5}, 0.46; for estrogens to 17 β-estradiol: R_{f3}, 0.23; R_{f4}, 0.30) were measured in triplicate for different solvent combinations and given in **Table 3** and **Table 4.**

Table 4

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

Substance	Relative R_f value* in solvent system 1	2	5	Color in UV (366 nm)
1-Dehydrotestosterone	0.57	0.65	0.67	OE-BN
5α-Dehydrotestosterone	1.42	1.15	1.27	BE-PU
Epitestosterone	0.85	0.83	0.95	YW-BN
Ethynyltestosterone	1.58	1.23	1.35	YW-BN
Medroxyprogesterone	1.20	1.14	1.23	BE-PU
Medroxyprogesterone acetate	1.71	1.61	1.26	YM
Melengestrol acetate	1.65	1.61	1.26	YW-BN
17α-Methyltestosterone	1.00	1.00	1.00	YW-GN
4,9,11-Methyltestosterone	0.98	1.02	1.02	YW-GN
19-Nortestosterone	0.85	0.80	0.79	YW-LT
Progesterone	1.82	1.61	1.36	BE-GN
Testosterone	0.97	0.88	0.89	YW
Trenbolone	0.78	0.80	0.84	BE-BT
Trenbolone acetate	1.77	1.63	1.48	BE-BT
Vinyltestosterone	1.47	1.28	1.34	YW-GN

* relative to 17α-methyltestosterone (R_{f1}, 0.40; R_{f2}, 0.53; R_{f5}, 0.46). Solvent systems: 1 = *n*-hexane–diethyl ether–dichloromethane (5 : 9 : 6, v/v/v). 2 = chloroform-acetone (9 : 1, v/v). 5 = cyclohexane–ethyl acetate–ethanol (60 : 40 : 2.5, v/v/v). For abbreviations see Table 3.

On combination of solvents 1 and 2 (androgens) sometimes an insufficient separation between 19-nortestosterone and testosterone was found. This problem was solved by using another chromatographic solvent instead of *n*-hexane-diethyl ether-dichloromethane (5 : 9 : 6, v/v/v). With solvent 5 (cyclohexane-ethyl acetate-ethanol (60 : 20 : 2.5, v/v/v)) a good separation between 19-nortestosterone and testosterone was obtained.

3.5 Overall Recovery

The recovery of the anabolics through the entire procedure was measured with blank urines spiked with hormones in concentrations of 0.5–10 ppb. The concentration of the anabolics in the final extract was determined by TLC scanning in the fluorescence mode using the antidiagonal technique of *Beljaars et al.* [9]. In **Table 5** the recoveries of the unlabeled steroids are shown.

Table 5

Overall recovery of hormones added to urine.

Substance	Recovery (%) (mean ± *SD*)	Concentration range* added (µg/l)
Estrogens		
Benzestrol (5)	70 ± 5.6	5 –10
Dienestrol (6)	71 ± 7.5	1 – 3
Diethylstilboestrol (6)	75 ± 2.3	0.5– 3
17β-Estradiol (6)	88 ± 2.1	2 –12
Estriol	< 10	5 –10
Estrone (3)	31 ± 6.9	8 –10
Ethynylestradiol (6)	76 ± 6.5	2 – 4
Equilinin (3)	80 ± 7.3	4 – 8
Hexestrol (3)	78 ± 5.1	10 –12
Zeralanone (4)	61 ± 4.5	2 – 6
Zeralenone (6)	55 ± 9.7	4 – 8
Zeranol (4)	55 ± 5.5	2 – 6
Talarenol (6)	61 ± 3.8	4 –12
Androgens + Gestagens		
1-Dehydrotestosterone (3)	82 ± 4.6	4 – 8
5-Dihydrotestosterone (3)	77 ± 3.8	4 – 8
Epitestosterone (4)	74 ± 12.0	2 – 4
Ethisterone (6)	76 ± 6.2	4 – 8
Medroxyprogesterone (4)	86 ± 7.9	4 – 8
Medroxyprogesterone acetate (5)	75 ± 7.1	2 – 4
Melengestrol acetate (4)	72 ± 7.7	4 –10
17-Methyltestosterone (12)	80 ± 6.5	0.5– 3
4,9,11-Methyltestosterone (6)	70 ± 8.2	2 – 4
19-Nortestosterone (6)	87 ± 6.0	2 – 4
Progesterone (4)	87 ± 5.0	4 –10
Testosterone (6)	87 ± 4.5	2 – 4
Trenbolone (7)	75 ± 4.5	1 – 4
Trenbolone acetate (6)	69 ± 16.5	1 – 4
Vinyltestosterone (6)	6 ± 6.0	4 – 8

n = Number of individual experiments. Concentration range added and determined by TLC scanning.

The recoveries are high (70–90%) for all the androgens and gestagens and for most estrogens. For estriol and estrone considerable lower extraction yields were found. These lower yields are probably due to partial adsorption of these products on the Polyclar column. The recovery for zeranol and analogues is moderate but still acceptable.

4 Conclusions

The described method permits the routine detection of various anabolic residues in bovine urine at levels of 0.5–10 ppb. The extraction procedure is as reproducible and reliable as the method described before [4, 15]. The results obtained with this method on various samples of urine were compared with those obtained by the method of *Verbeke* [4], which has been accepted by the EEC [15]. In all cases the same positive

and negative results with equal sensitivity were obtained. However, the present method is considerably faster: one analyst is able to clean up at least 30 urines in one week (6 in a working day).

By combination of the faster clean-up with the faster "4 × 4" HPTLC developing mode [11, 12] the delay between sampling and obtaining results is considerably shortened. This will help to reduce the hold-up of carcasses at the slaughterhouse.

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