Afdeling Additieven 1982-05-15

VERSLAG 82.48 Pr.nr. 505.0620

Onderwerp: The determination of DES and other anabolics in urine and faeces by LC-EC and by HPTLC

Verzendlijst: direkteur, sektorhoofd (3x), direktie VKA, afd. Additieven (25x), afd. Normalisatie (Humme), Projektbeheer, Projektleider (De Ruig).

8248

Afdeling Additieven 1982-05-15

VERSLAG 82.48 Pr.nr. 505.0620

Projekt: Ontwikkeling methoden voor het aantonen en bepalen van hormonen

Onderwerp: The determination of DES and other anabolics in urine and faeces by LC-EC and by HPTLC.

Poster presented at Analytica 82, Munich 1982-04-27/30.

Summary

Methods have been developed for the determination of DES and other anabolics. The sample pretreatment before detection is of great importance. The following methods are described.

- DES in a small amount of urine (up to about 5 ml), detection by LC-EC: scheme 1.
- DES in a small amount of faeces, detection by LC-EC: scheme 2.
- DES in a large amount of urine (25-250 ml), using a specially shaped HPLC pre-column, detection by LC-EC: scheme 3.
- Hexestrol, DES and dienestrol in urine, column pretreatment, detection by either HPLTC or LC-EC scheme 4, outline of method on page 14, Results:

Conclusion

HPTLC and LC-EC methods can act as reliable methods of analyses of DES and related compounds at 1 ppb level, in between RIA and GC-MS techniques.

Verantwoordelijk: dr W.G. de Ruig

Medewerkers: T.D.B. van der Struijs, H. Hooijerink, J.M. Weseman, G.M. Binnendijk, A.G. van Leeuwen and D.J. Vonk

Projektleider: dr W.G. de Ruig

The determination of DES and other anabolics in urine and faeces by LC-EC and by HPTLC.

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Poster presented at Analytica 82, Munich 1982-04-27/30.

Naturel and synthetic hormones are used worldwide to improve meat production. The benefit for the producer is obvious: application of these anabolic agents may save 5-28% of the feeding stuffs.
There are, however, possible hazards for the consumer.
Therefore in most European countries the use of hormonal anabolics for fattening purposes is restricted or even prohibited by law. Moreover consumer organizations call for import boycott of meat from countries with insufficient inspection.
To control legal and illegal use extensive enforcement and surveillance programmes are being set up. For economic reasons analytical results should be available shortly after sampling.
Among the banned synthetic anabolics diethylstilbestrol (DES) takes the most important place at the moment.

Analytical control is carried out in various ways. Histologic control is an easy and rapid method, but can be carried out in the slaughterhouse only, and is not decisive for adult animals. Bioassays are time-consuming (10 days).
Radioimmunoassay (RIA) is most attractive and widely used now for screening purposes of DES.
Physicochemical methods include GLC, HPLC, TLC, GC-MS. GC-MS [1] is the most conclusive for confirmation, but expensive and laborious.
Although anabolic agents are electroactive and can be detected qualitatively and quantitatively by electrochemistry, until now little attention has been payed to this technique.
Frischkorn and Smyth [2,3] have described an HPLC method with electrochemical detection for determination of growth promoting hormones in chicken meat; Kenyhercz and Kissinger [4] for DES in liver and kidney.

Urine and faeces are suitable matrices to control (mis)use on the farms, and can also be used as indicator for the absence of anabolics in the meat in the slaughterhouse. (The concentrations of anabolics in urine and faeces are higher than in meat).

Experimental

We have developed the method based on HPLC separation followed by electrochemical detection (LC-EC) for the determination of DES in urine and faeces, and optimized it with respect to sensitivity, convenience and rapidity.

Flow schemes of the method for small amounts of urine or faeces (up to about 5 ml) are given in scheme 1 and 2.

For large amounts of sample (25-250 ml) a modified treatment is applied, using a specially shaped HPLC pre-column having a large capacity [5], see scheme 3.

Results

In fig. 1 a chromatovoltammogram of 25 pg DES is given. Fig. 2 and 3 give the results of 5 and 0,1 µg/kg DES in urine after treatment of 50 ml urine with the specially shaped HPLC column (scheme 3). Fig. 4 is the result of 5 g faeces with 5 µg/kg DES treated according to scheme 2.

Sensitivity

Using the guideline that a detectable signal has to be at least 3x the noise, the limit of detection for DES as such is 5 pg. For DES in a sample the noise level will be higher depending on the matrix, the prepurification and the quantity of treated sample. Starting with 5 g of sample 1 µg/kg can be detected. Using larger amounts this concentration can be lowered.

Convenience

Sample preparation is most simple, only a few actions are required, and the use of chemicals is minimized.

Rapidity

The time required for one complete analysis for small volumes is about 3 hours, including hydrolysis, for large amounts of sample about 4 hours. By comparison: a radioimmunoassay requires about 28 hours; a GC-MS analysis 48 hours.

Other anabolic agents

By the method as described, other anabolics can be detected qualitatively and quantitatively as well. Besides differences in HPLC retention time, they differ in electrochemical behaviour. Combination of both phenomena offers an additional possibility for distinguishing the various anabolics.

Other methods

We also have optimized the two dimensional HPTLC [6] with respect to prepurification and developing solvents [7]. It offers an alternative convenient method for detection of various anabolics. After pretreatment hexestrol, DES and dienestrol are separated on a basic celite column (scheme 4). The three separated fractions are analysed by LC-EC (figure 6) and/or by HPTLC (figure 7). The HPTLC spots can be measured by densitometry (figure 7).

Acknowledgement

The authors thank A.G. van Leeuwen and D.J. Vonk for their valuable assistance.

Literature

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2. C.G.B. Frischkorn, M.R. Smyth, H.E. Frischkorn and J. Golimowski - Fresenius Z. anal. Chem. 300 (1980) 407-412.

3. M.R. Smyth and C.G.B. Frischkorn - Fresenius Z. anal. Chem. 301 (1980) 220-223.

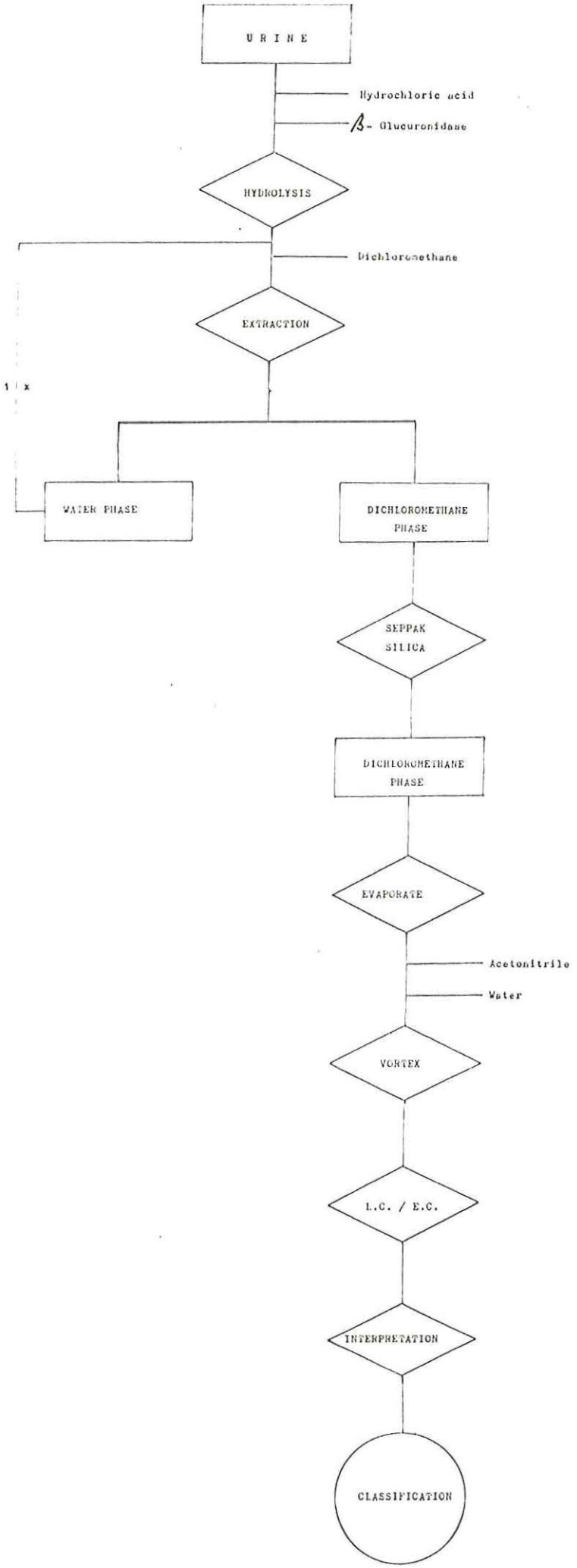
4. T.M. Kenyhercz and P.T. Kissinger - J. anal. toxicol. 2 (1978) 1-2.

5. H.M. Ruijten, P.H. van Amsterdam and H. de Bree - Joint NL-UK symposium on quantitative organic analysis - Noordwijkerhout (NL), 1981.

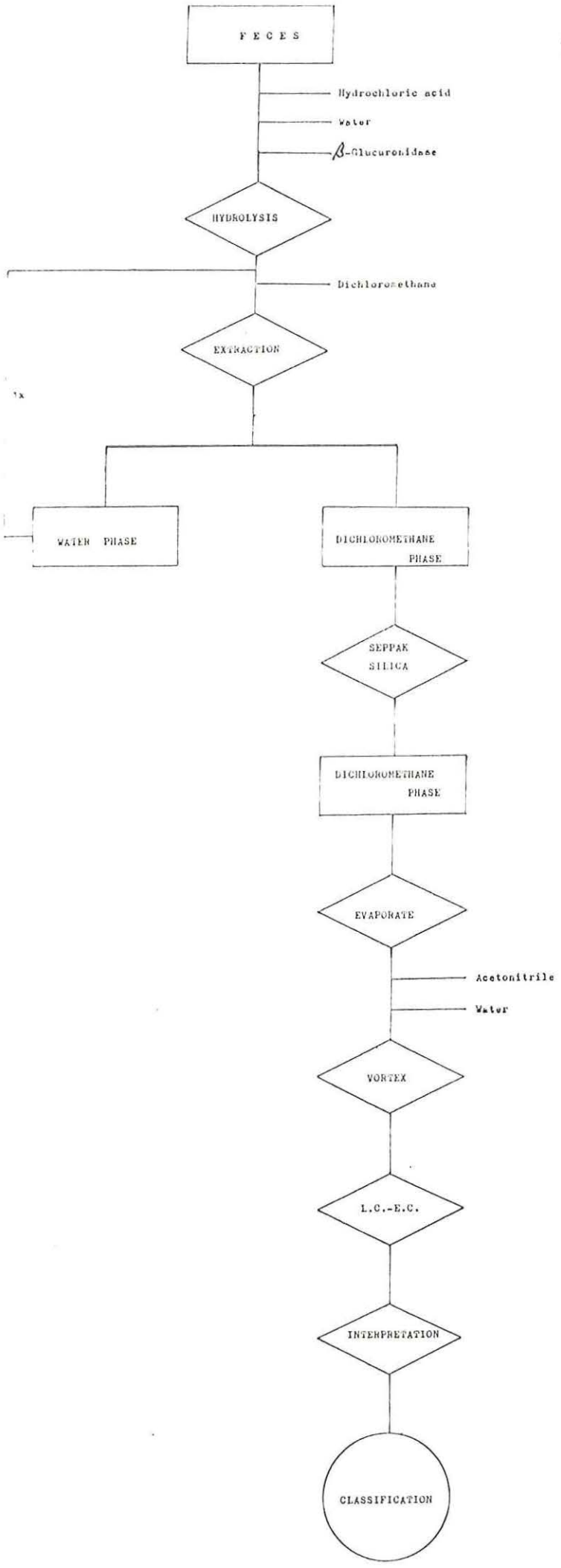
6. P.L. Schuller - J. Chromatogr. 31 (1967) 237-240.

7. W.G. de Ruig and J. Weseman - in press.

Scheme 1


URINE
Hydrochloric acid
β- Glucuronidase
HYDROLYSIS
Dichloromethane
EXTRACTION
1 x
WATER PHASE
DICHLOROMETHANE PHASE
SEPPAK SILICA
DICHLOROMETHANE PHASE
EVAPORATE
Acetonitrile
Water
VORTEX
L.C. / E.C.
INTERPRETATION
CLASSIFICATION

Scheme 2


FECES
Hydrochloric acid
Water
β-Glucuronidase
HYDROLYSIS
Dichloromethane
EXTRACTION
WATER PHASE
DICHLOROMETHANE PHASE
SEPPAK SILICA
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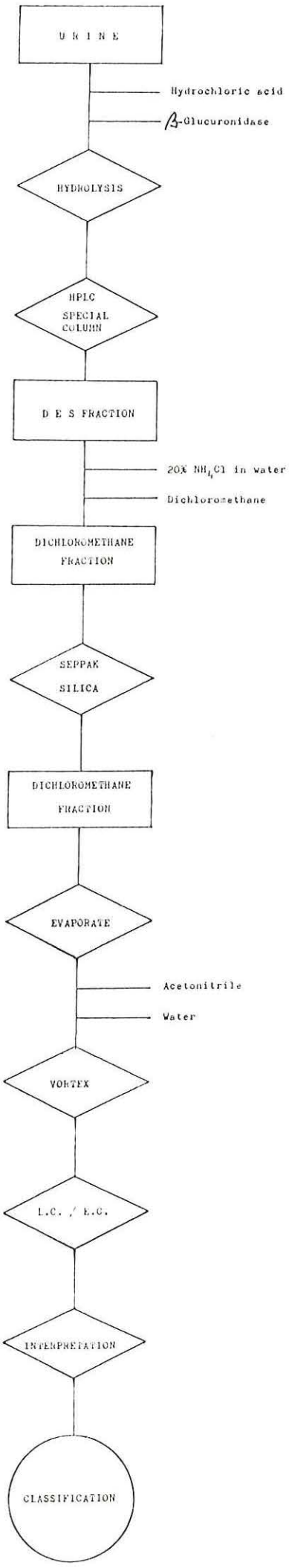
EVAPORATE
Acetonitrile
Water
VORTEX
L.C.-E.C.
INTERPRETATION
CLASSIFICATION

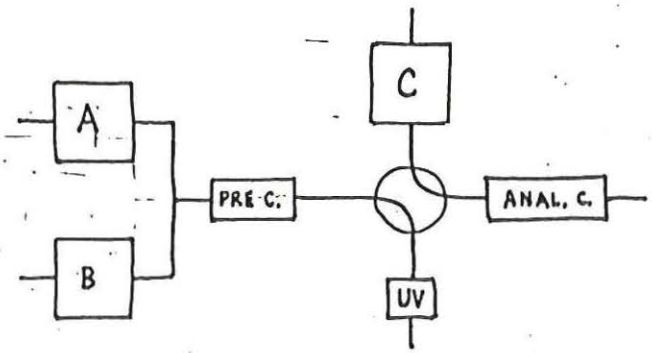
Scheme 3


URINE
Hydrochloric acid
β-Glucuronidase
HYDROLYSIS
HPLC
SPECIAL
COLUMN
D E S FRACTION
20% NH_4Cl in water
Dichloromethane
DICHLOROMETHANE
FRACTION
SEPPAK
SILICA
DICHLOROMETHANE
FRACTION
EVAPORATE
Acetonitrile
Water
VORTEX
L.C. / E.C.
INTERPRETATION
CLASSIFICATION

DES in urine and faeces by LC-EC Flow schemes 1, 2, 3

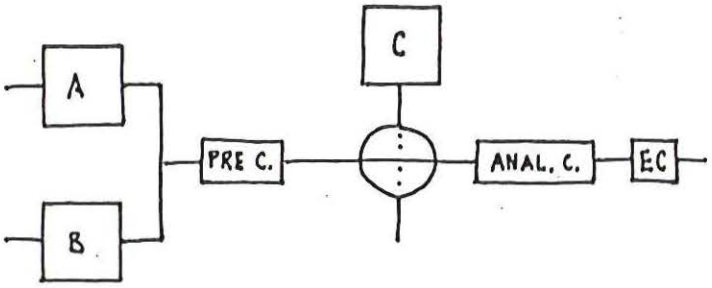
I.

Preconcentration on pre-column



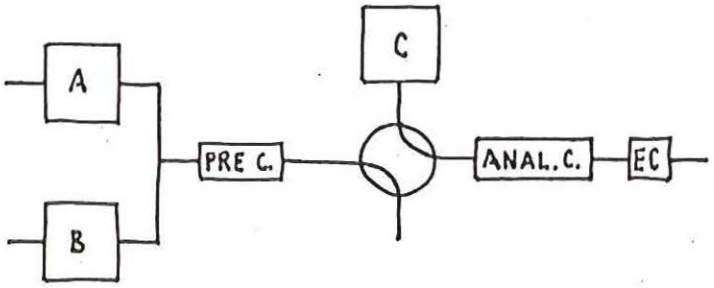
II.

DES eluted from pre-column



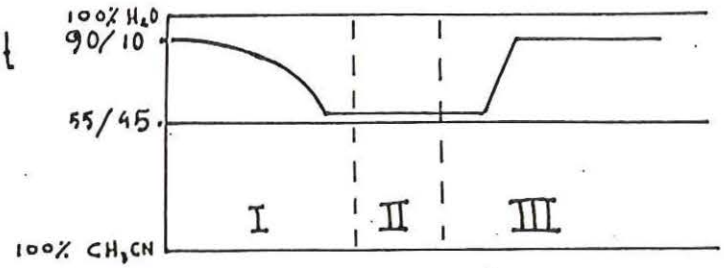
III.

DES eluted from analytical column and electrochem. detected



A, B } gradient

C isocratic



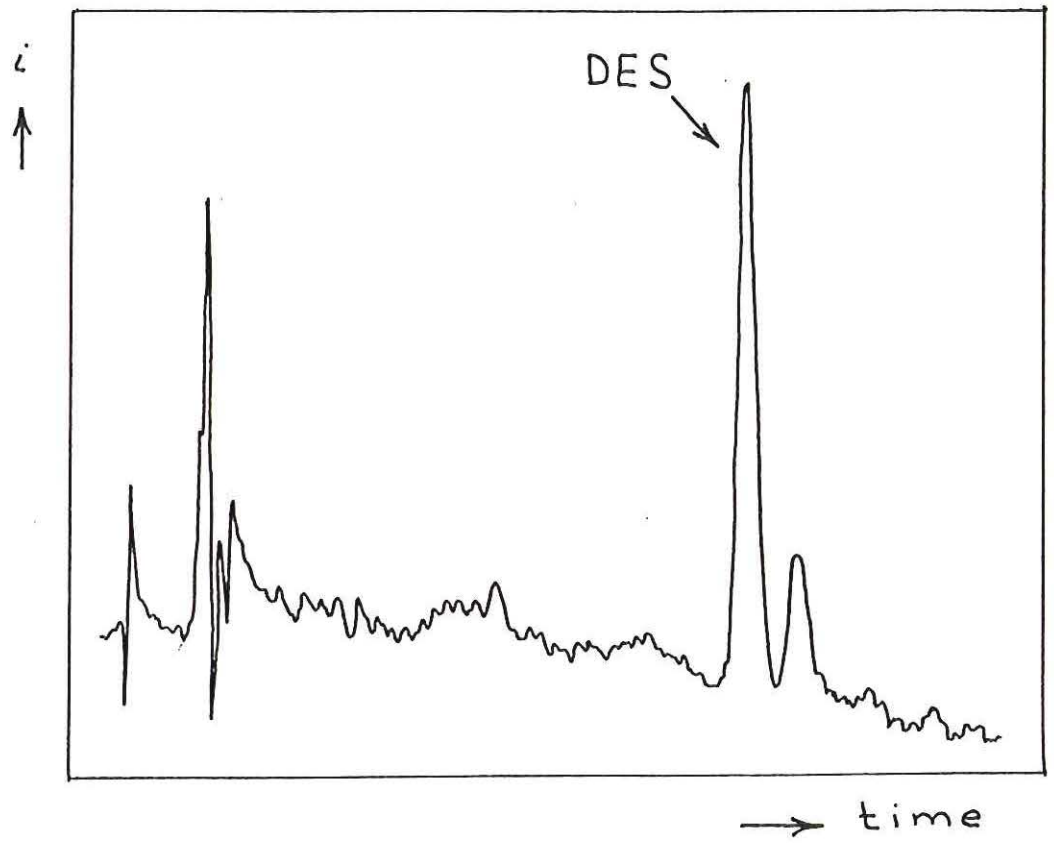


Fig. 1 Chromatovoltammogram of 25 pg of DES.

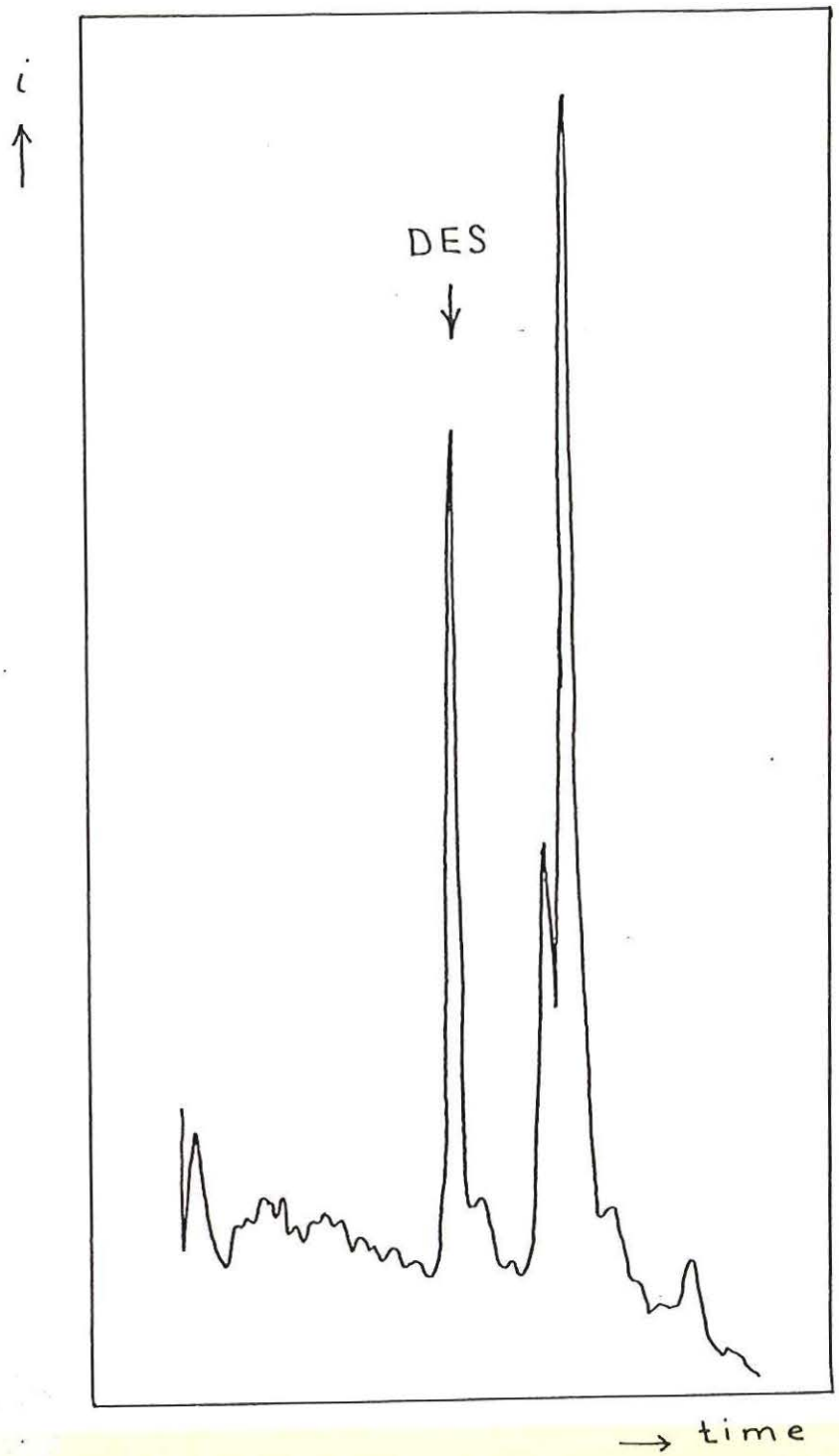


Fig. 2 Chromatovoltammogram of 5 µg/kg DES in cow's urine, starting with 50 ml of sample for pre-treatment and using 1/10 th for determination, according to scheme 3.

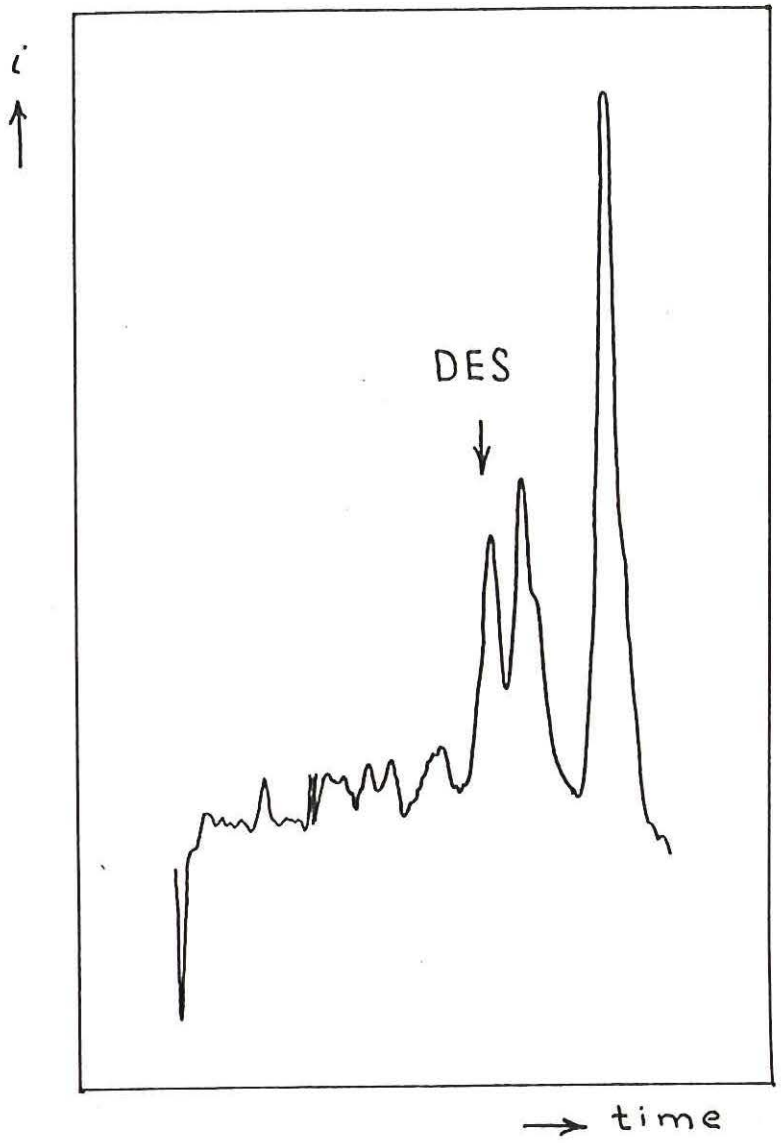


Fig. 3 Chromatovoltammogram of 0,1 µg/kg DES in cow's urine, starting with 50 ml of sample, according to scheme 3.

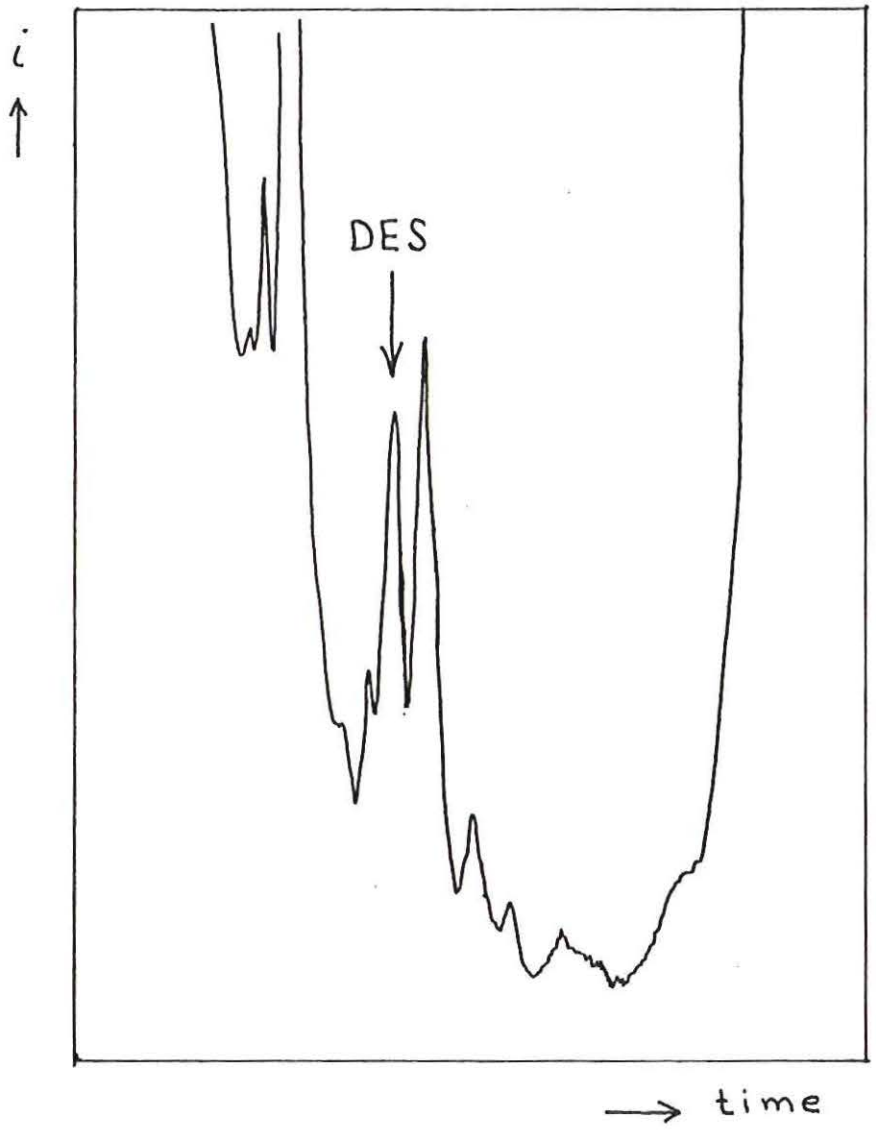


Fig. 4 Chromatovoltammogram of 3,5 µg/kg DES in cow's faeces, starting with 1 g of sample.

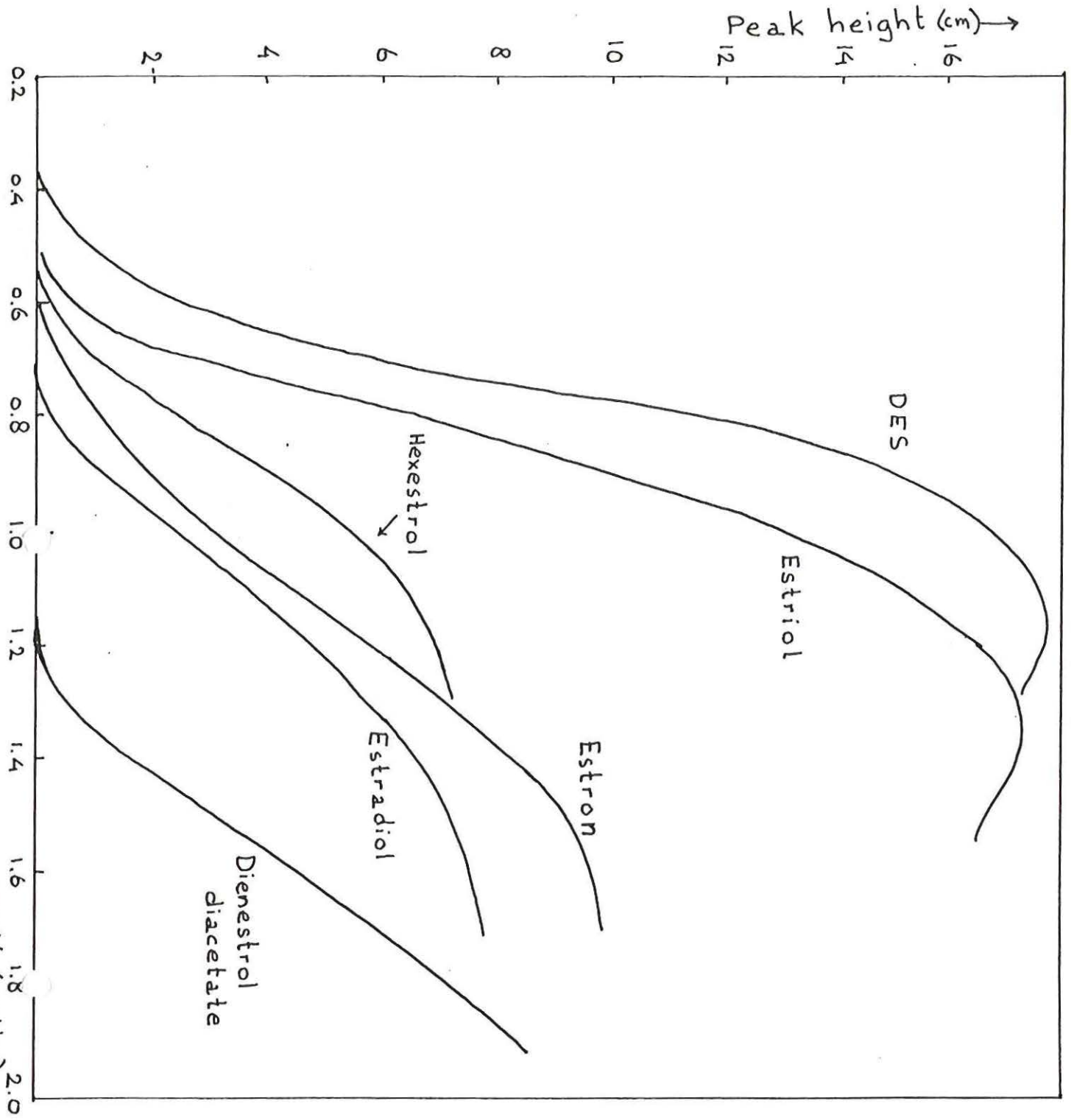
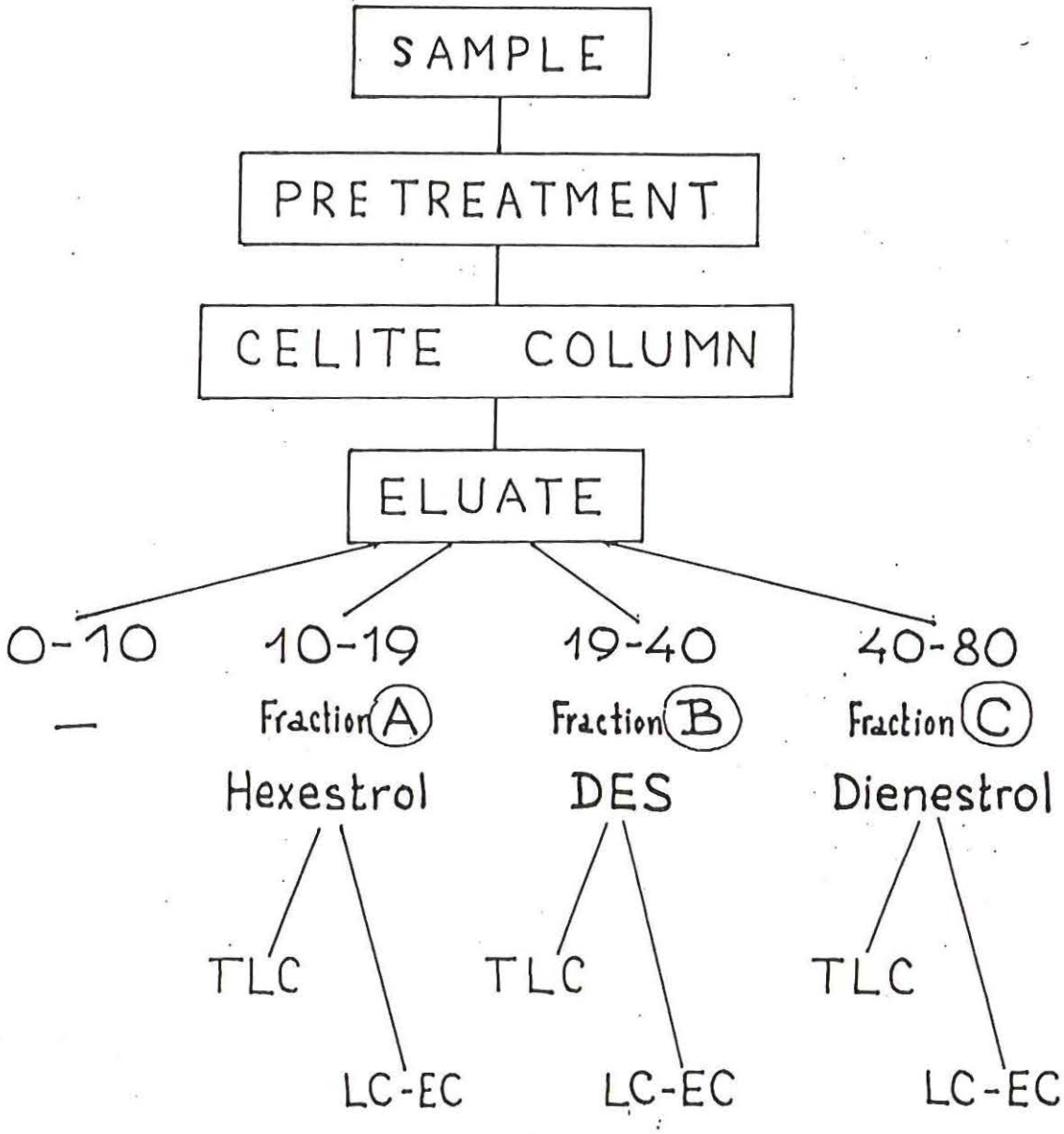
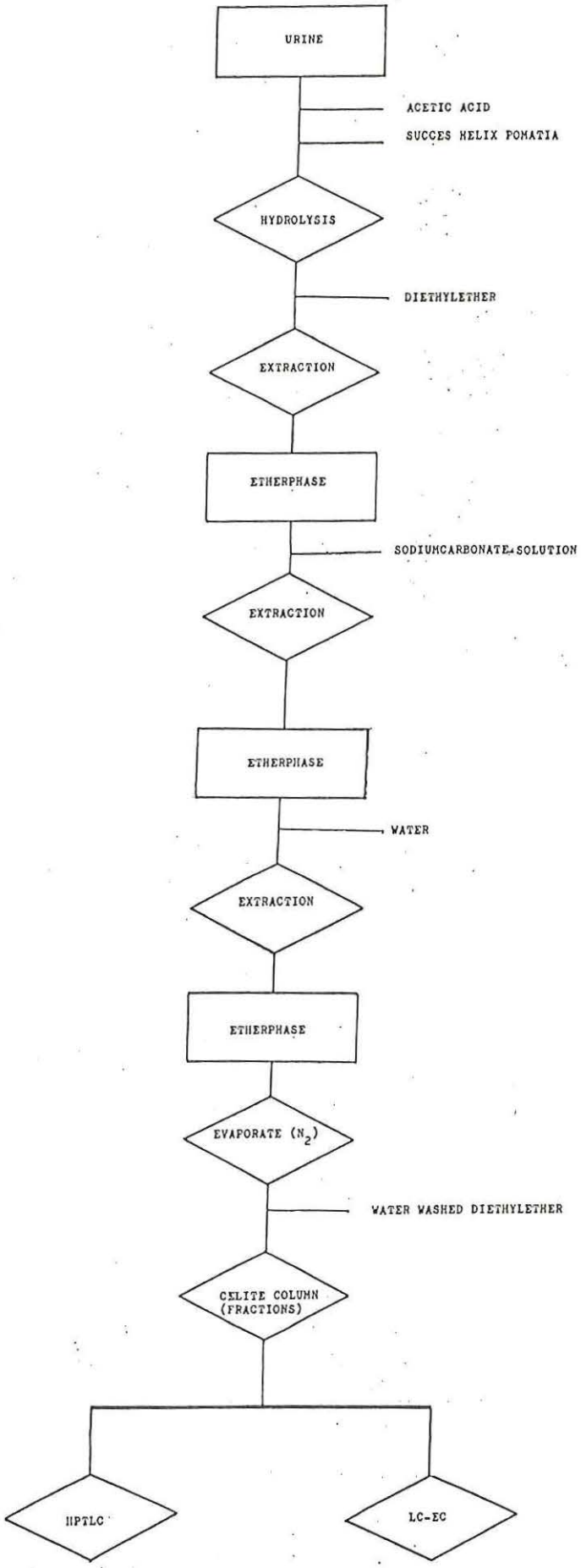


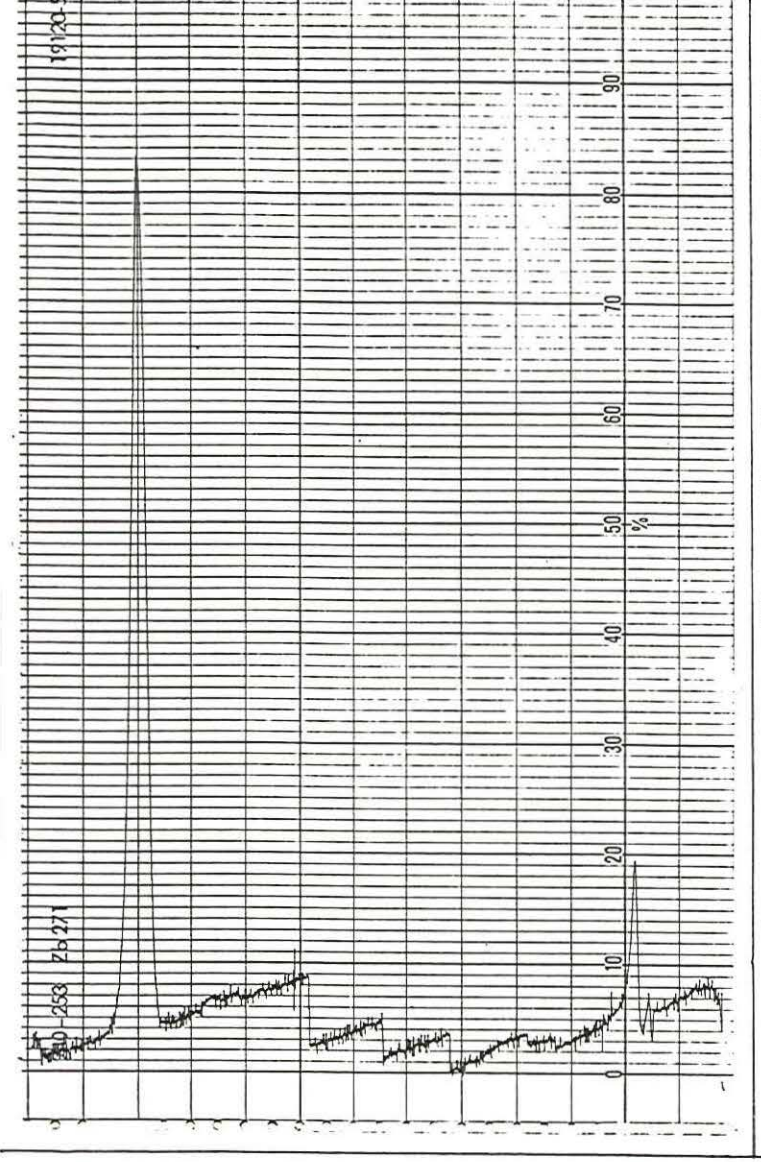
Fig. 5 Voltammetric behaviour of some anabolic agents. Peakhight as a function of the applied voltage vs. Ag/AgCl in 3M LiCl methanol.

Hexestrol
DES
Dienestrol
} in urine by { HPTLC
LC-EC

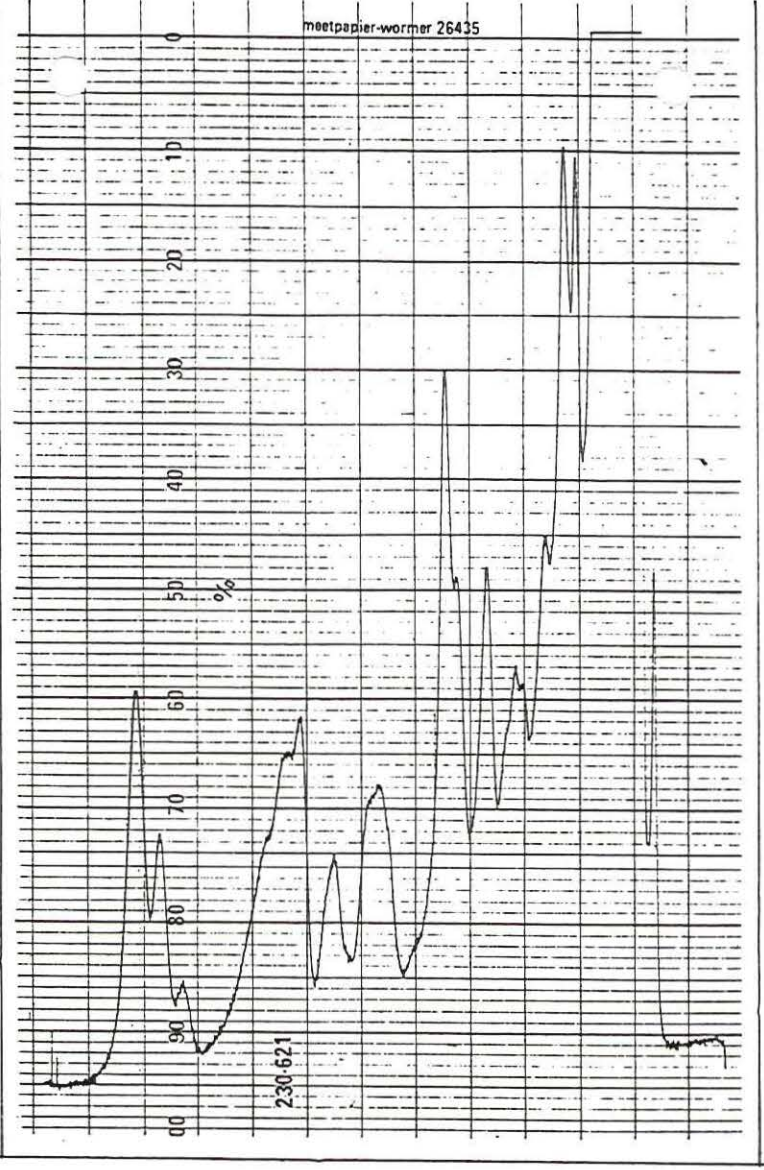


Scheme 4

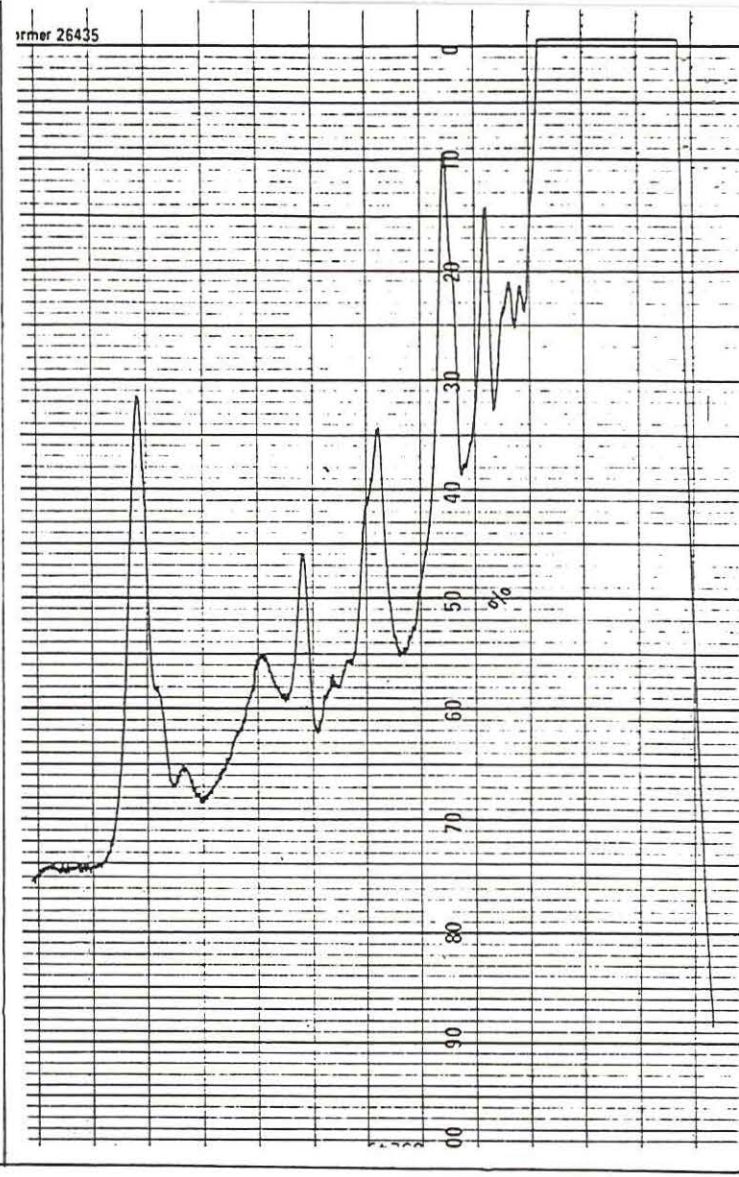

URINE
ACETIC ACID
SUCCES HELIX POMATIA
HYDROLYSIS
DIETHYLETHER
EXTRACTION
ETHERPHASE
SODIUMCARBONATE SOLUTION
EXTRACTION
ETHERPHASE
WATER
EXTRACTION
ETHERPHASE
EVAPORATE (N_2)
WATER WASHED DIETHYLETHER
CELITE COLUMN (FRACTIONS)
HPTLC
LC-EC



hexestrol
standard 25 ng



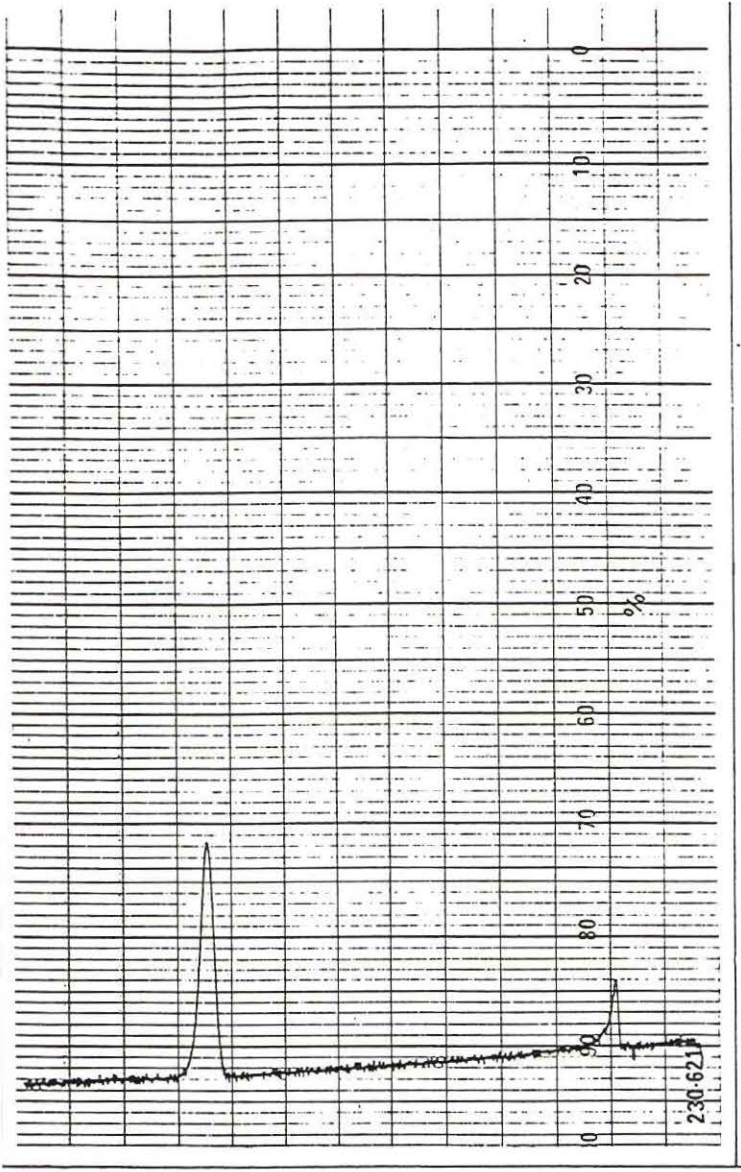
hexestrol
sample urine 1 µg/kg



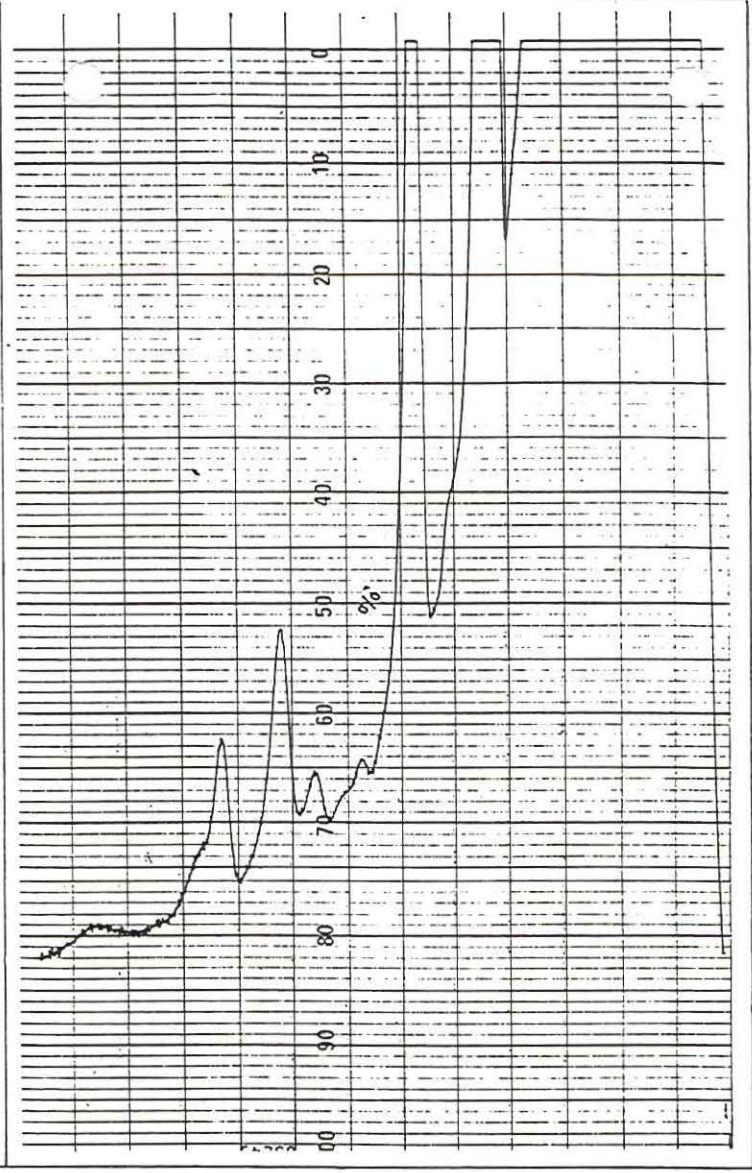
hexestrol
sample urine 4 µg/kg

FRACTION A

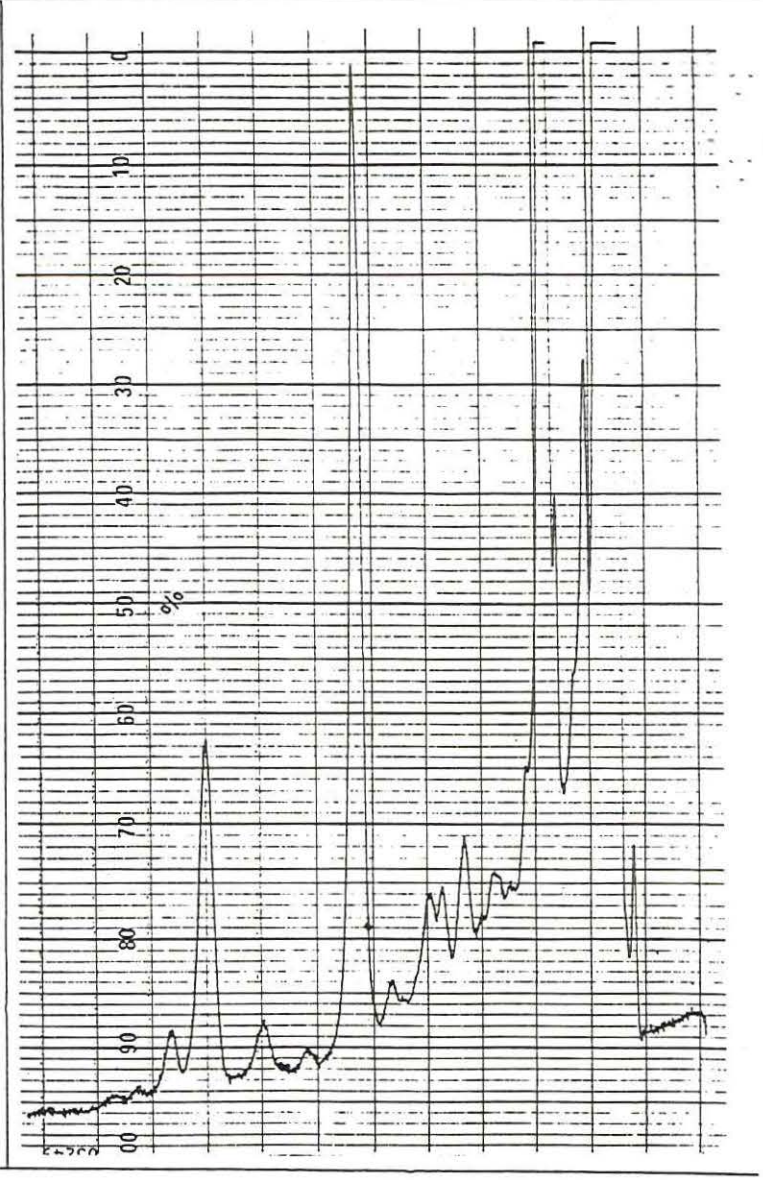
LC-EC



diethylstilbestrol
standard 25 ng



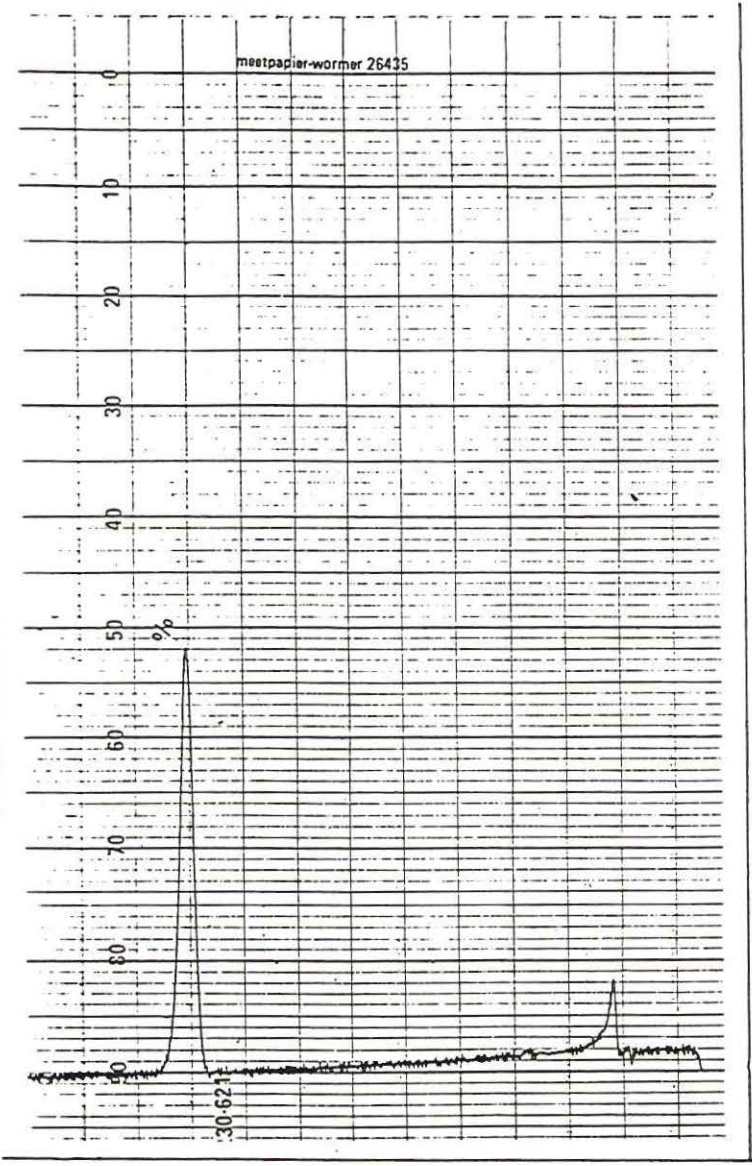
diethylstilbestrol
sample urine 1 µg/kg



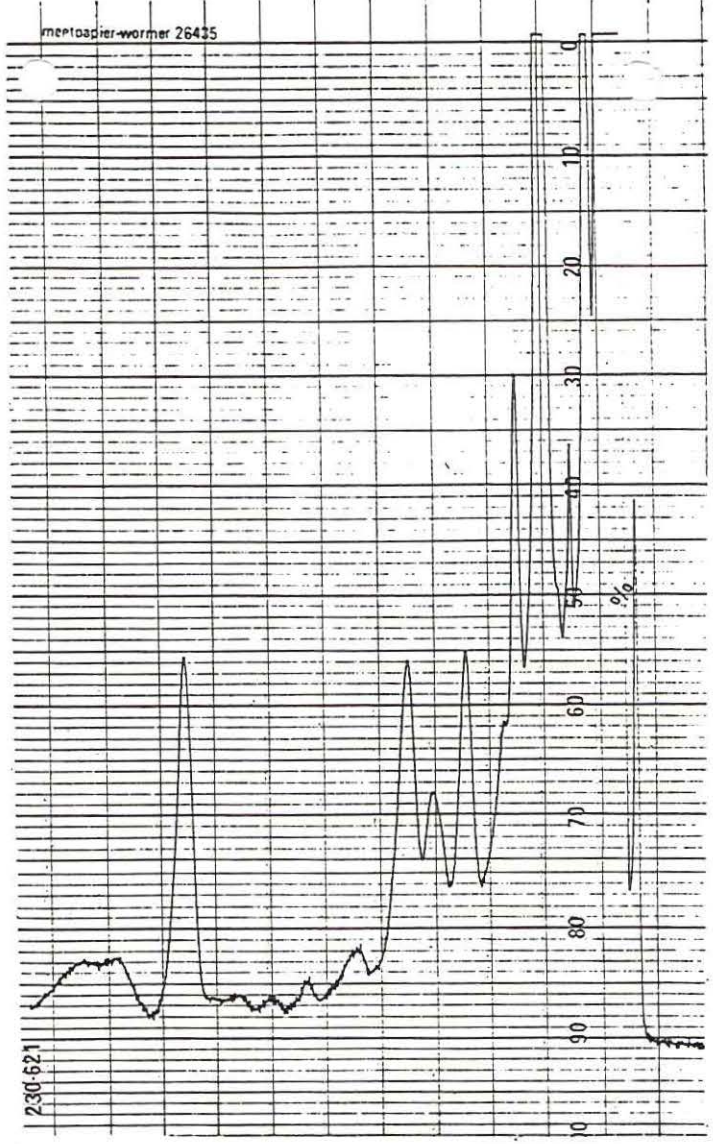
diethylstilbestrol
sample urine 4 µg/kg

FRACTION B

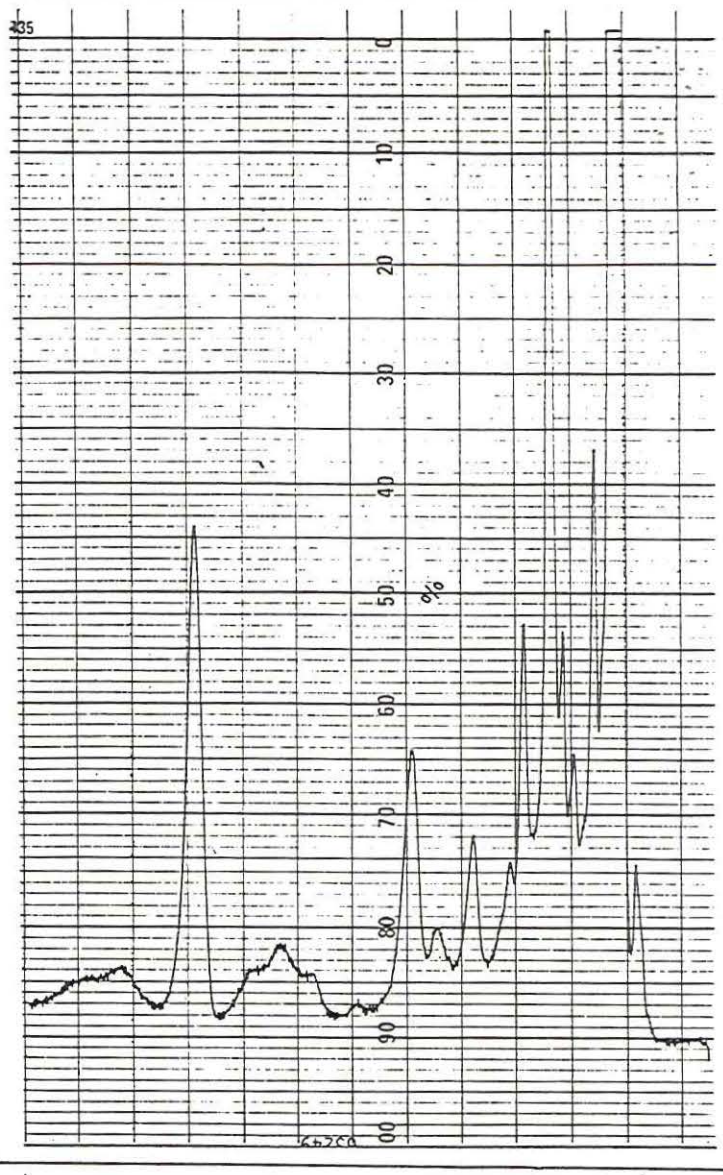
LC-EC



dienestrol
standard 25 ng



dienestrol
sample urine 1 µg/kg



dienestrol
sample urine 4 µg/kg

FRACTION C

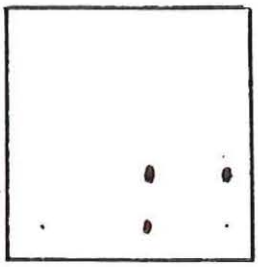
LC-EC

HPTLC results

Fraction (A)

Hexestrol

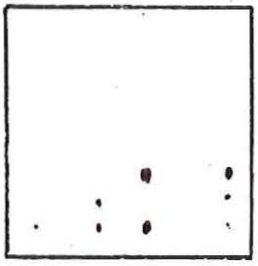
1 µg/l



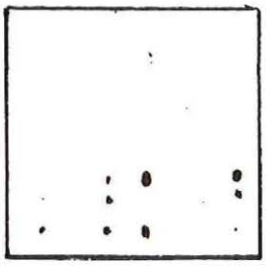
4 µg/l urine

Fraction (B)

DES



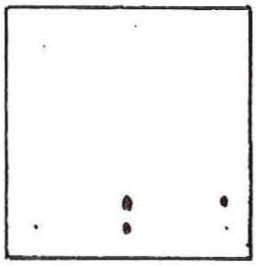
1 µg/l



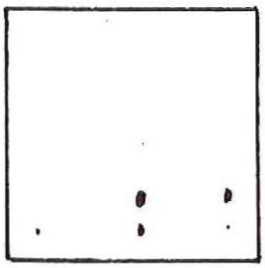
4 µg/l

Fraction (C)

Dienestrol



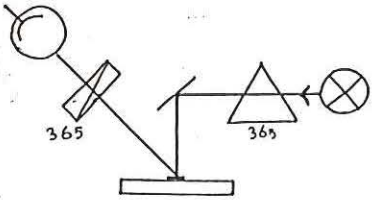
1 µg/l



4 µg/l

DENSITOMETRY OF HPTLC PLATES

ZEISS CHROMATOGRAM SPECTRAL PHOTOMETER KM 3



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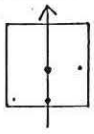
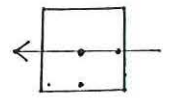
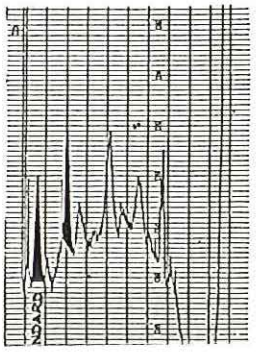
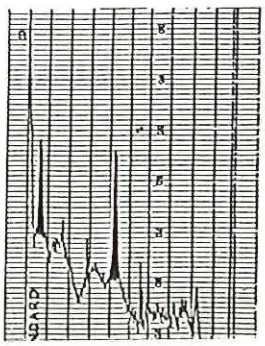




Fraction (A)

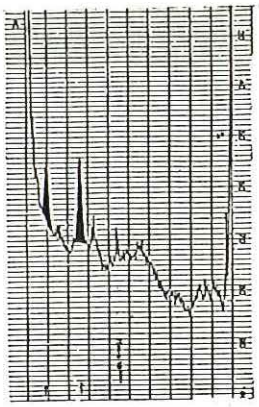
4 µg/l hexestrol

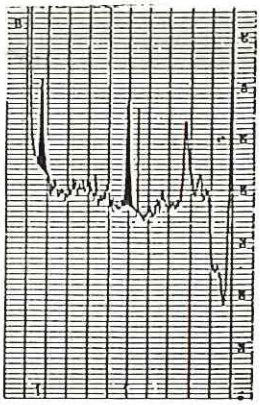




Fraction (B)

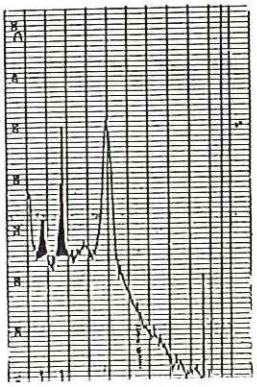
4 µg/l DES

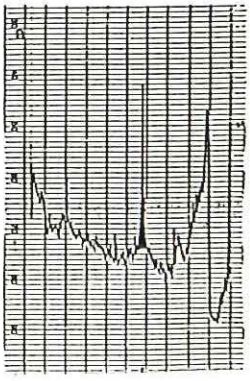




Fraction (C)

4 µg/l dienestrol





ANABOLICS GROWTH PROMOTORS

Benefit for the producer

Anabolics may save 5 - 28 % feeding stuffs

Production (carcass weigt)	Cattle	Swine	Poultry	$\times 10^6$ tons
EEC	7	10	4	
USA Canada Australia N.Zealand	13	8	7	
Total :	~ 50, 000, 000 tons			

Production value : 180 Giga DM 70 Giga US $ 1.3 Tera A Shilling
In USA poultry : 1950 - 1967 : $ 230 000 000 saved

Possible hazards for consumer

direct toxic effect

allergenetic reaction to the drug

effects from tumorigenic compounds

alteration of drug sensitization to micro organisms

Legal restrictions in most countries

"anti - oestrogen - laws"

EEC : all synthetic hormones forbidden, except zeranol and trenbolone

Consumer organisations

Call for import boycott of meat from countries with insufficient control

Illegal treatment

↓

ANALYTICAL CONTROL

METHODS OF ANALYSIS

Histological control

In slaughterhouse only

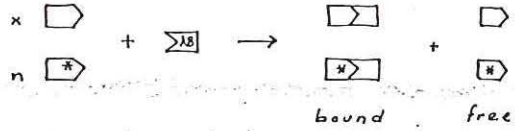
Bio assay

Mouse uterus test : sample is incorporated into the diet of immature female mice, and the weight change of the uterus noted, with respect to control group

Detection limit ~2 µg/kg
Lack of specificity
10 days to perform

Radio immuno assay (RIA)

Competative binding of anabolic compound and the same radiolabelled compound with antibody



Bound fraction is radiocounted

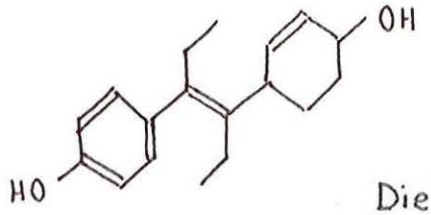
Sensitive
False positive in case of cross reaction

Recently found in the Netherlands :
Straightforward RIA → too much false positive results
RIA no longer accepted by the court

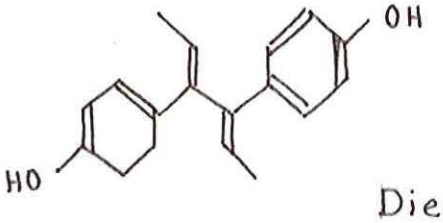
} Need of chromatographic prepurification
Confirmation by other method

Physico-chemical methods

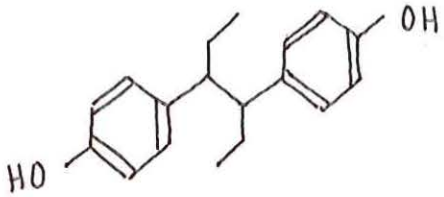
	Sensitivity	Screening	Confirmation	Multimethod	Output	Cost
GLC	–					
HPLC (UV det.)	–					
LC-EC	+	~	+	+	~/+	~
HPTLC	+	~/+	+	+	~/+	~
GC-MS	+	–	+ +	–	–	– – –
(RIA)	+ +	+ +	–	–	+ +	+ +



Diethylstilbestrol



Dienestrol



Hexestrol