HPTLC of Anabolic Compounds in Injection Sites

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Summary

A rapid method, comprising methanolic extraction with a Stomacher extractor followed by HPTLC analysis, is described for the qualitative determination of anabolic compounds in injection sites. The aim is to separate and identify a large number of hormones and their esters under standard conditions and, if necessary, different concentration steps and thin layer plates coated with different adsorbents may be used to achieve this.

After fluorescence induction with sulfuric acid the spots are identified by comparing their R_f values and colors with those of standard compounds. R_f values of 46 anabolic compounds on three types of plates and with seven different solvent systems are presented, together with the colors of the spots in UV and visible light.

1 Introduction

Notwithstanding the total prohibition of the use of hormones as growth promoters in the EEC, these products are still used illegally because of the considerable economic profit.

The so-called hormone cocktails, mixtures of different exogenic and endogenic anabolic compounds (mostly esterified), may even be injected intramuscularly and, as the residue concentration in the injection site may be very high, the risk to the health of the consumer can be considerable. It is, therefore, necessary to locate suspect zones from the carcasses and analyze them for illicit anabolic compounds.

Immunoassay detection methods exist for the most commonly used anabolic compounds but not for the esterified forms usually present in the illegal cocktails [1 – 3]. The high concentrations of the analytes also makes possible the use of HPLC with on-line recording of UV spectra by diode-array detection [4, 5]. GC-MS is often used to confirm preliminary results [3, 6].

This paper describes the detection of a large number of anabolic compounds in injection sites by means of an HPTLC method in the 4 x 4 mode [7]. By comparing the R_f values of unknowns and standards on different layers the method can be considered as a multi-residue procedure.

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In practical application on injection sites anabolic compounds can be detected at the ppm level. Below this level complex purification and concentration steps are necessary.

The method described here has been used in Belgium for three years for the official analysis of samples taken from slaughterhouses.

2 Experimental

2.1 Standards and Reagents

Most of the reference compounds were obtained from Steraloids (Wilton, NY, USA) or Sigma (St. Louis, MO, USA)

All solvents used were analytical grade or Resi-analyzed from Baker or Pro-analysis grade from Merck.

Stock solutions were prepared in ethanol at a concentration of 1 mg/ml. For routine control purposes working solutions were prepared by dilution of stock solutions to 50 ng/μl; the same concentration was used for the following solutions of mixtures of the most frequently used anabolic compounds:

mixture A estradiol benzoate
trenbolone acetate
chlormadinone acetate
ethinylestradiol
diethylstilbestrol

mixture B nortestosterone decanoate
testosterone propionate
methyltestosterone
medroxyprogesterone acetate
zeranol

mixture C stanozolol
boldenone
methylboldenone
chlortestosterone acetate

2.2 Chromatography

HPTLC was performed on silica gel Si 60 octadecyl- and cyanosilica; all were obtained from Merck (Darmstadt, FRG).

Plates were developed with the following solvent systems:
1 *n*-hexane – diethyl ether – dichloromethane (25 + 45 + 30, *v/v*)

2 chloroform – acetone (90 + 10, *v/v*)
3 cyclohexane – ethyl acetate – ethanol (77.5 + 20 + 2.5, *v/v*)
4 *n*-hexane – dichloromethane – acetonitrile (80 + 10 + 5, *v/v*)
5 methanol – toluene (95 + 5, *v/v*)
6 petroleum ether (30 – 60 °C) – acetone (80 + 20, *v/v*)
7 acetone – water (60 + 40, *v/v*)

2.3 Procedure

2.3.1 Sampling

Tissue samples were submitted to a radical visual examination to find suspicious zones: occasionally it was necessary to cut the tissue into slices to locate the injected liquid. Such zones were isolated by dissection, cut into small pieces, and 5 g weighed into a disposable Stomacher bag (Stomacher-80, Seward Laboratories). If the total weight was less than 5 g the whole sample was taken for analysis.

2.3.2 Extraction

25 ml methanol (or a volume 5 times the weight of the sample in grams) was added to the sample as obtained in Section 2.3.1. The plastic bag was then placed in the extraction compartment of the Stomacher and mechanical extraction performed for one minute. Bag and contents were left at ambient temperature for at least 24 h to complete the extraction and obtain maximum recovery; the supernatant was then decanted into a 25 ml disposable flask or vial (*e.g.* scintillation vial) and stored at 4 °C.

2.3.3 HPTLC

Chromatographic analysis can be performed in four stages.

Stage 1: Screening Purposes (Monodimensional Elution)

This stage of the analysis was performed on silica gel or RP-18 plates using the eluents listed. 1 µl of the extract from Section 2.3.2 was applied with a disposable micropipet (graduated capillary) to a 10 x 10 cm plate, 0.5 cm from the edge. If the distance between spots is 0.5 cm, up to fifteen samples can be analyzed (positions 1, 7, 13, and 19 being reserved for standards) and as an elution distance of 4 cm is sufficient for the separation both halves of the plate can be used for the analysis.

After elution the plates were dipped into a 5 % ethanolic solution of sulfuric acid for 30 s [8]; induction of fluorescence was accelerated by heating the plates in an oven at 95 °C for 10 min and the spots were identified by viewing by transillumination under UV (λ = 366 nm) and visible light.

A positive result from stage 1 was indicative of the presence of the steroid in the sample at a concentration greater than 25 ppm (average sensitivity of the different compounds). Following a negative result the second stage of the analysis was performed.

Stage 2: Concentration

5 ml of the extract obtained from Section 2.3.2 was transferred through a 0.5 µm filter (*e.g.* Millex, Millipore) into a 10 ml disposable glass tube. The solvent was evaporated using either a stream of nitrogen or a vacuum concentrator (*e.g.* Speed-vac) and the residue re-dissolved in 50 µl methanol. 1 µl was chromatographed as described for stage 1; a positive result was indicative of the presence of the compound at a level higher than 250 ppb.

This stage may also be used to confirm the results obtained in stage 1. If so, the use of two-dimensional cochromatography on silica or RP-18, or a combination of both, should be regarded as mandatory.

Stage 3: Re-examination

2 – 10 µl of the concentrated extract (stage 2) was chromatographed, preferably in two dimensions, using solvent

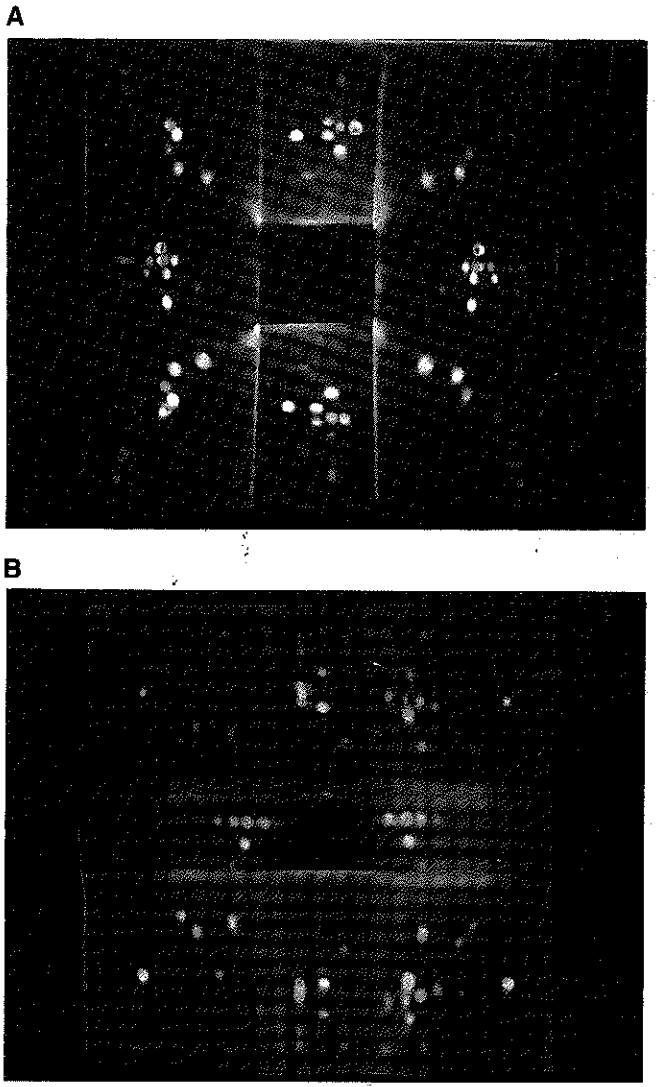

Figure 1

(A) "4 x 4" HPTLC chromatogram of a real sample on silica gel; elution solvents 1 and 2; (B) "4 x 4" HPTLC chromatogram of the same sample as in figure a. but on RP-18; elution solvents 4 and 5. Cochromatography with the reference mixtures A, B, and C on the right-hand-side of the plate and reference mixtures A, B, and C in the middle lanes. Compounds identified: stanozolol, testosterone, estradiol, chlortestosterone acetate, estradiol benzoate, testosterone decanoate, testosterone cypionate.

systems 3 and 4 on RP-18 plates (owing to the better separation of the different anabolic esters on this type of plate).

Stage 4: Confirmation of Re-examination

If (exceptionally) the result from re-examination contradicts that obtained from the initial analysis, further concentration with additional purification is obligatory. Solid phase extraction (SPE), a simple and rapid means of achieving this, is performed as follows:

The concentrated extract (from stage 2) is dissolved in methanol – water (40 + 60, 2 ml) by first adding the methanol (to ensure complete solution of the residue) and then the water. The solution is then passed through a C 18 octadecyl SPE column (by means, *e.g.*, of a ten-place vacuum manifold, *e.g.* Vac-Elut) which has previously been conditioned by washing with two 2 ml portions of methanol and similarly with water.

After washing with methanol-water (40 + 60, 4 ml), the column is eluted with methanol – water (70 + 30, *v/v*), to isolate the free anabolic compounds, followed by methanol, which elutes the esters.

Both fractions are concentrated, and chromatographed by HPTLC as described above.

2.4 Detectable Compounds

The method has been tested with several reference compounds and found to be suitable for the analysis of the following:

estrogens: stilbenes and their esters, estradiol and its esters (benzoate, cypionate, a.o.), ethinylestradiol, and mestranol;

androgens: methyltestosterone, testosterone and its esters (propionate, cypionate, a.o.), nortestosterone and its esters (decanoate, a.o.), trenbolone and trenbolone acetate, stanozolol, boldenone, methylboldenone, and chlortestosterone acetate; and

gestagens: progesterone, medroxyprogesterone acetate, chlormadinone acetate, melengestrol acetate, and megestrol acetate.

A more detailed list is given in the tables.

3 Results and Discussion

3.1 Sampling

The use of mechanical homogenization for sampling injection sites can cause problems: owing to differences in analyte concentration over the small dimensions of such sites it is almost impossible to prepare two identical portions of the same sample, one for primary analysis and one for re-analysis. This can cause problems when two laboratories are working with the same sample but analyzing different portions.

The problem is best overcome by chemical homogenization, carried out either by the veterinary taking the sample, or by the laboratory designated to perform the primary analysis. The extract may then be divided into as many portions as necessary, each with similar homogeneity.

The stability of anabolic compounds in such extracts has been studied [9]: identical results were obtained after 3 to 6 months.

3.2 Extraction

Extraction using the Stomacher is very simple. We use disposable plastic bags to avoid cross-contamination which might arise from the use of re-usable glassware, *etc.*; for similar reasons we do not recommend the use of ultrasonic extraction, which we have, in addition, found to be more time-consuming and less efficient than use of the Stomacher.

Methanol was selected as a result of the good solubility of most of the compounds studied, its low volatility, and the stability of the analytes in this solvent.

3.3 HPTLC

The main advantage of monodimensional TLC is its applicability to the fast screening of large numbers of samples. Unknown spots may be identified by comparison of their R_f values and appearance (*e.g.* fluorescence color) with those of reference compounds.

Some of the compounds studied, especially different esters of the same compound, could not be separated by one-dimensional elution and so for unequivocal identification two-dimensional separation is essential. This may usually be achieved by cochromatography on silica gel; remaining doubt about the identity of any spot may be resolved by use of a modified silica (*e.g.* RP-18 or CN, see **Figure 1**).

The R_f values measured (in duplicate, and calculated relative to that of nortestosterone decanoate) for the compounds studied are listed in **Tables 1, 2** and **3** for silica gel, RP-18, and CN plates, respectively; a two-dimensional separation on silica gel is illustrated in **Figure 2.** The average sensitivity was, with the exceptions listed, 10 – 50 ng on the plate.

4 Conclusion

The aim of this paper has been to describe a "standard" method for use in all Belgian laboratories involved in regulatory control. The procedure is simple, relatively quick, and, in order to reduce the possibility of cross-contamination, includes as few steps as possible. It may, therefore, be applied for routine analysis.

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Table 1

R_f values, relative to nortestosterone decanoate*, of the most important anabolic compounds on silica gel with different solvent systems.

Substance	Solvent system 1	2	3	Color[a] in UV light (λ = 366 nm)	Color in visible light
Reference mixture A					
1 Diethylstilbestrol, *cis*-	0.67	0.36	0.41	RD	RD
2 Diethylstilbestrol, *trans*-	0.93	0.68	0.60	RD	RD
3 Ethinylestradiol	0.85	0.55	0.56	RD	RD
4 Estradiol benzoate	0.74	0.78	0.68	YW	OE
5 Chlormadinone acetate	0.71	0.87	0.35	RD	BE
6 Trenbolone acetate	0.80	0.88	0.69	GN-BE	YW
Reference mixture B					
7 Zeranol (100 ng)	0.35	0.34	0.19	GN	YW-BN
8 Nortestosterone decanoate	1.00	1.00	1.00	GY-YW	GY-GN
9 Testosterone propionate	0.91	0.97	0.81	YW	GY-BE
10 Methyltestosterone	0.47	0.58	0.38	YW-GN	YW
11 Medroxyprogesterone acetate	0.76	0.88	0.43	YW	GN
Reference mixture C					
12 Stanozolol	0.13	0.17	0.13	BE	OE-BN
13 Boldenone (100 ng)	0.27	0.42	0.20	OE	OE
14 Methylboldenone	0.34	0.47	0.20	OE-BN	OE
15 Chlortestosterone acetate	1.03	1.09	1.01	YW	GY-GN
Other anabolic compounds					
16 Trenbolone	0.37	0.51	0.27	BE-GN	
17 Estradiol	0.63	0.46	0.42	YW	OE
18 – 17β-cypionate	1.16	0.91	1.09	YW	OE
19 – diacetate	1.20	1.07	1.39	YW	OE
20 – dipropionate	1.23	1.10	1.55	YW	OE
21 – valerate	1.14	0.90	1.12	YW	OE
22 Mestranol	1.08	0.91	1.05	RD	RD
23 Testosterone	0.43	0.60	0.28	YW	GY-BE
24 – acetate	0.85	0.94	0.68	YW	GY-BE
25 – benzoate	0.93	0.99	0.81	YW	GY-BE
26 – enanthate	1.00	1.01	0.98	YW	GY-BE
27 – tosylate	0.84	0.96	0.48	YW	GY-GN
28 – cypionate	0.98	1.01	0.98	YW	GY-BE
29 – cyclohexylpropionate	0.97	0.99	0.98	YW	GY-BE
30 – isobutyrate	0.95	0.97	0.89	YW	GY-BE
31 – valerate	0.96	0.96	0.95	YW	GY-BE
32 – undecylenate	1.02	0.99	1.07	YW	GY-BE
33 – decanoate	1.04	1.04	1.06	YW	GY-BE
34 – φ-propionate	0.96	1.01	0.87	YW	GY-BE
35 Ethinyltestosterone	0.64	0.71	0.47	RD	GY-BN
36 Mibolerone	0.57	0.54	0.29	BE	YW-BN
37 Vinyltestosterone	0.60	0.68	0.50	YW	YW-BN
38 19-Nortestosterone	0.39	0.57	0.23	YW	BN
39 – laurate	1.02	1.01	1.03	GY-YW	GY-GN
40 – φ-propionate	0.85	0.98	0.80	GY-YW	GY-GN
41 – benzoate	0.90	0.95	0.74	GY-YW	GY-GN
42 – hexyloxy-φ-propionate	0.99	1.03	0.91	GY-YW	GY-GN
43 Norethisterone	0.64	0.64	0.39	YW	YW-BN
44 Progesterone (200 ng)	0.80	0.88	0.55	GY-BE	
45 Megestrol acetate	0.70	0.82	0.43	YW	GN
46 Melengestrol acetate	0.71	0.85	0.43	BN	BN
* R_f of Nortestosterone decanoate	0.83	0.90	0.50		

[a] YW, yellow; GN, green; BE, blue; OE, orange; RD, red; BN, brown; GY, grey

Table 2

R_f values, relative to nortestosterone decanoate*, of the most important anabolic compounds on RP-18 plates with different solvent systems.

Substance	Solvent system 4	5	Color[a] in UV light (λ = 366 nm)	Color in visible light
Reference mixture A				
1 Diethylstilbestrol, *cis*-	0.43	2.07	RD	RD
2 Diethylstilbestrol, *trans*-	0.65	2.27	RD	RD
3 Ethinylestradiol	0.40	2.32	OE	OE
4 Estradiol benzoate	0.66	1.58	OE	OE
5 Chlormadinone acetate	0.65	2.10	OE	YW-GN
6 Trenbolone acetate	0.55	1.83	BE-GN	YW-BN
Reference mixture B				
7 Zeranol (100 ng)	0.22	2.28	GN	YW-BN
8 Nortestosterone decanoate	1.00	1.00	GY	GY-BN
9 Testosterone propionate	0.76	1.62	YW	OE
10 Methyltestosterone	0.37	1.97	YW-GN	BN
11 Medroxyprogesterone acetate	0.67	2.12	OE	GY-GN
Reference mixture C				
12 Stanozolol	0.02	1.79	BE	OE-BN
13 Boldenone (100 ng)	0.19	1.98	RD-OE	RD
14 Methylboldenone	0.25	1.91	RD-BN	RD-BN
15 Chlortestosterone acetate	0.99	1.72	YW-OE	BN
Other anabolic compounds				
16 Trenbolone	0.14	2.06	BE-GN	YW-BN
17 Estradiol	0.37	2.16	OE	OE
18 – 17β-cypionate	0.92	1.38	OE	OE
19 – diacetate	1.40	1.49	OE	OE
20 – dipropionate	1.64	1.42	OE	OE
21 – valerate	0.90	1.65	OE	OE
22 Mestranol	1.06	1.84	RD	OE
23 Testosterone	0.32	1.93	YW	BN
24 – acetate	0.70	1.70	YW	OE
25 – benzoate	0.80	1.40	YW	OE

Substance	Solvent system 4	Solvent system 5	Color[a] in UV light (λ = 366 nm)	Color in visible light
26 – enanthate	0.93	1.20	YW	OE
27 – tosylate	0.67	1.87	YW	OE
28 – cypionate	0.91	1.13	YW	OE
29 – cyclohexylpropionate	0.96	1.09	YW	OE
30 – isobutyrate	0.83	1.53	YW	OE
31 – valerate	0.89	1.46	YW	OE
32 – undecylenate	1.02	1.02	YW	OE
33 – decanoate	1.04	0.91	YW	OE
34 – φ-propionate	0.83	1.45	YW	OE
35 Ethinyltestosterone	0.48	2.17	YW-OE	BN
36 Mibolerone	0.32	2.02	BE	YW-BN
37 Vinyltestosterone	0.47	1.96	YW	OE
38 19-Nortestosterone	0.18	2.02	YW-GY	BN
39 – laurate	1.01	0.89	GY	GY-BN
40 – φ-propionate	0.87	1.15	GY	GY-BN
41 – benzoate	0.79	1.54	GY	GY-BN
42 – hexyloxy-φ-propionate	0.87	1.15	GY	GY-BN
43 Norethisterone	0.36	2.23	OE	BN
44 Progesterone (200 ng)	0.61	1.76	GY-BE	YW
45 Megestrol acetate	0.61	2.19	YW	YW-GN
46 Melengestrol acetate	0.64	1.95	OE-BN	OE-BN
* R_f of Nortestosterone decanoate	0.55	0.38		

[a] YW, yellow; GN, green; BE, blue; OE, orange; RD, red; BN, brown; GY, grey

Table 3

R_f values, relative to nortestosterone decanoate*, of the most important anabolic compounds on CN plates with different solvent systems.

Substance	Solvent system 6	Solvent system 7	Color[a] in UV light (λ = 366 nm)	Color in visible light
Reference mixture A				
1 Diethylstilbestrol, *cis*-				
2 Diethylstilbestrol, *trans*-	0.68	3.16	RD	RD
3 Ethinylestradiol	0.50	3.81	OE	RD
4 Estradiol benzoate	0.79	2.00	YW	OE
5 Chlormadinone acetate	0.58	2.61	OE	RD
6 Trenbolone acetate	0.75	3.23	BE-GN	YW
Reference mixture B				
7 Zeranol (100 ng)	0.30	4.21	GY-GN	GY-BN
8 Nortestosterone decanoate	1.00	1.00	GY-YW	
9 Testosterone propionate	0.95	2.59	GY-YW	
10 Methyltestosterone	0.66	4.25	YW-GN	YW
11 Medroxyprogesterone acetate	0.62	3.16	OE	YW-BN
Reference mixture C				
12 Stanozolol	0.32	4.13	YW	
13 Boldenone (100 ng)	0.52	4.45	RD	RD
14 Methylboldenone	0.59	4.85	GY	OE
15 Chlortestosterone acetate	0.79	2.28	OE	
Other anabolic compounds				
16 Trenbolone	0.48	4.40	BE-GN	YW
17 Estradiol	0.50	3.81	YW	OE
18 – 17β-cypionate	0.71	1.61	YW	OE
19 – diacetate	1.09	1.90	YW	OE
20 – dipropionate	1.16	1.61	YW	OE
21 – valerate	0.70	2.01	YW	OE
22 Mestranol	0.72	2.92		
23 Testosterone	0.59	4.17	YW-OE	
24 – acetate	0.76	3.22	GY-YW	
25 – benzoate	0.75	2.11	GY-YW	
26 – enanthate	0.95	1.71	GY-YW	
27 – tosylate	0.55	2.33	GY-YW	
28 – cypionate	0.84	1.56	GY-YW	
29 – cyclohexylpropionate	0.98	1.19	GY-YW	
30 – isobutyrate	0.92	2.01	GY-YW	
31 – valerate	0.97	2.00	GY-YW	
32 – undecylenate	1.06	1.00	GY-YW	
33 – decanoate	0.98	0.86	GY-YW	
34 – φ-propionate				
35 Ethinyltestosterone	0.59	3.91	YW	OE-BN
36 Mibolerone	0.67	3.90	GY-GN	BN
37 Vinyltestosterone	0.68	3.92	YW	YW
38 19-Nortestosterone	0.54	4.21	GY-YW	
39 – laurate	1.01	0.89	GY-YW	
40 – φ-propionate	0.77		GY-YW	
41 – benzoate	0.83	2.08	GY-YW	
42 – hexyloxy-φ-propionate	0.85	0.98	GY-YW	
43 Norethisterone	0.54	3.81	OE	BN
44 Progesterone (200 ng)	0.78	2.90	GY-GN	
45 Megestrol acetate	0.60	3.29	OE	OE
46 Melengestrol acetate	0.64	3.24	OE	BN
* R_f of Nortestosterone decanoate	0.89	0.13		

[a] YW, yellow; GN, green; BE, blue; OE, orange; RD, red; BN, brown; GY, grey

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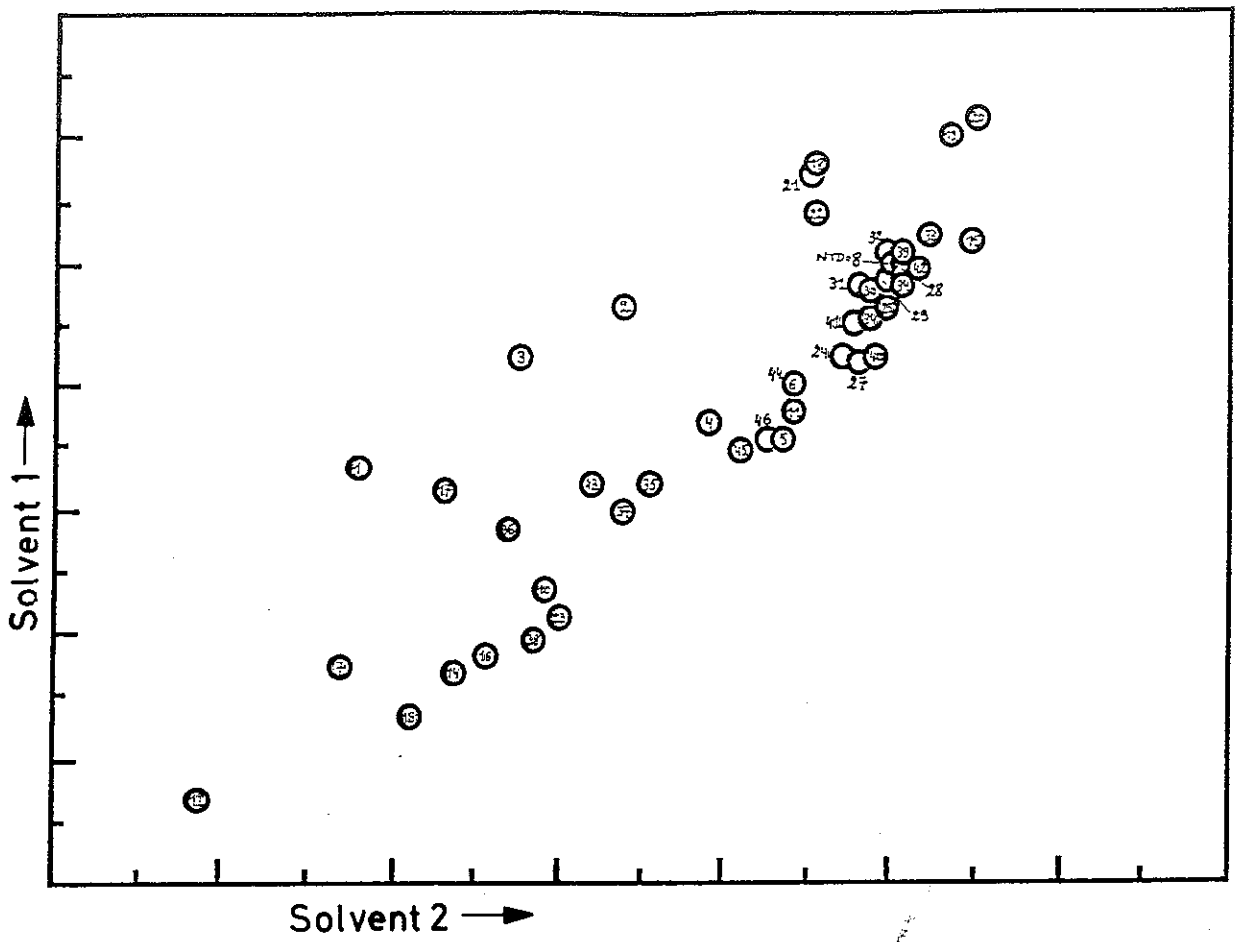

Figure 2

Schematic TLC chromatogram, on silica gel, of the anabolic compounds listed in Table 1.

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