QUALITATIVE, QUANTITATIVE AND PREPARATIVE CHROMATOGRAPHY OF STEROIDS ON FULLY ACETYLATED PAPER*

I. QUALITATIVE CHROMATOGRAPHY OF Δ^4- AND $\Delta^{1,4}$-3-KETOSTEROIDS

F. J. RITTER AND J. HARTEL
Central Laboratory T.N.O., Delft (The Netherlands)

INTRODUCTION

During the last decade many methods have been developed for the paper-chromatographic separation of steroids[1]. In most of them use is made of a relatively polar, non-volatile stationary phase, such as propyleneglycol[2], phenylcellosolve[3] or formamide[2] and a nonpolar mobile phase, such as toluene, benzene or heptane. Some reversed phase techniques have also been applied, in which the paper is rendered hydrophobic with, *e.g.*, stearato-chromylchloride (Quilon)[4], paraffin oil[5,6], kerosene[7] or silicone[8] and the mobile phase is a relatively polar liquid, such as glacial acetic acid, an alcohol, or an alcohol–water mixture.

In all these cases the paper has to be impregnated with the stationary phase. This has the following disadvantages:

1. Humidity[3] and temperature[7b] of the air, time and temperature of application of the substances[9], the way of blotting or drying the papers after the impregnation and other factors affect the amount and condition of the impregnating substance on the paper.

Moreover, the spots of the substances to be chromatographed must be applied after the impregnation and local displacement of the stationary phase by the solvent used for these substances may result[7b].

As a consequence of all these factors the reproducibility of the results in general and the R_F values in particular, is usually rather bad.

2. A non-volatile impregnating substance may interfere in quantitative determinations.

3. In reversed phase chromatography the influence of the polar OH groups cannot be completely eliminated by impregnation with a hydrophobic solvent. These groups may cause undesirable adsorption effects, for example incomplete elution in quantitative determinations.

* Some preliminary data were presented to the International Congress on Clinical Chemistry at Stockholm, August 1957, in a paper by Dr. F. J. Ritter.

A short communication on the same subject has appeared as a "Letter to the Editor" in *Nature*, 181 (1958) 765.

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Reversed phase chromatography has also been applied on acetylated paper, in which case impregnation with a nonpolar liquid is not necessary as the acetylation renders the paper hydrophobic. The separation of various lipophilic substances on *partially* acetylated papers has been described by several authors[10,11,12]. It is, however, difficult to obtain a homogeneous partially acetylated paper with a constant degree of esterification. A commercially available partially acetylated paper (*e.g.*, Schleicher and Schüll 2043b "acetyliert") has been found to be very heterogeneous. The saponification number varied not only for different sheets, but also within one paper as much as from 200 to 300 mg of KOH per gramme[13] and, consequently, the R_F values obtained with it are not reproducible.

For investigations carried out in this laboratory, in which large numbers of chromatograms had to be compared as a part of a screening programme, well-reproducible R_F values were desired. We therefore developed methods for the separation of steroids on fully acetylated paper, prepared by the method of ZIJP[14]. This paper is very homogeneous and has a saponification number of at least 570 (calculated for cellulose triacetate: 583). ZIJP used this paper for the identification and quantitative determination of antioxidants in rubber[15].

For the separation of steroids special phase systems and solvent mixtures had to be worked out. Three different types of chromatography were developed, *viz.* reversed phase "adsorption" chromatography with a one-phase mixture, reversed phase partition chromatography with a two-phase mixture and "conventional" partition chromatography with a two-phase mixture of benzene, methanol and water. Good separations of various groups of steroids have been obtained by these methods.

This paper describes the qualitative chromatography of Δ^4- and $\Delta^{1,4}$-3-keto-steroids on fully acetylated paper. The chromatography of other steroids, the quantitative and the preparative chromatography of steroids on fully acetylated paper will be described in subsequent papers.

PHASE SYSTEMS AND TYPES OF CHROMATOGRAPHY

Method A. Reversed phase "adsorption" chromatography with a one-phase mixture of benzene, methanol and water

In the first experiments a one-phase mixture of benzene, methanol and water, 1:10:3, was used as the mobile phase. No accommodation was applied, as equilibration with the mobile phase yielded less regular spots. The method should perhaps be regarded as adsorption chromatography rather than as partition chromatography. Well-reproducible R_F values are obtained with it, but some tailing is usually observed (Fig. 1). The chromatograms show a reversed phase pattern, *i.e.* the more polar steroids have higher R_F values than the less polar ones, as is usual for chromatography on acetylated paper.

Method B. Reversed phase partition chromatography with a two-phase mixture of benzene, methanol and water

To prevent the tailing effects, efforts were made to find a method by which accommo-

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dation of the paper could be performed, without losing the separating capacities of the system.

This proved to be possible when the ratio of benzene, methanol and water is chosen in such a manner that two phases are obtained. One phase is then used for the equilibration, by hanging the paper in its vapours, and the other is used as the mobile phase.

As the solvent moves very fast over the paper, especially during the first few

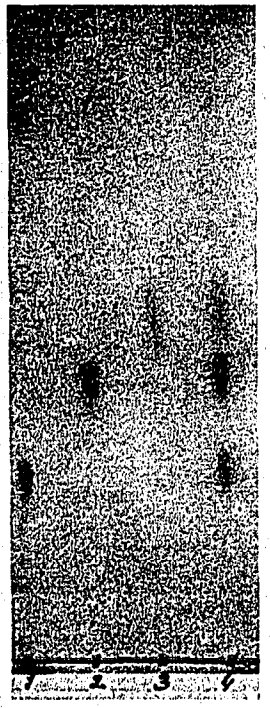

Fig. 1

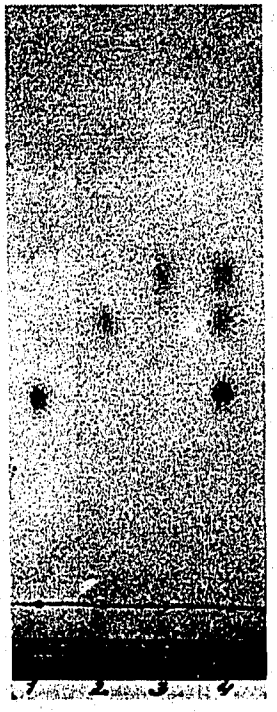

Fig. 2

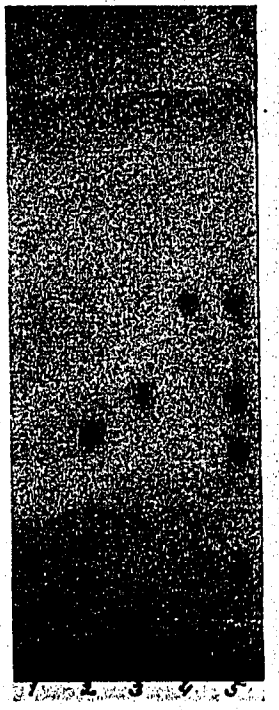

Fig. 3a

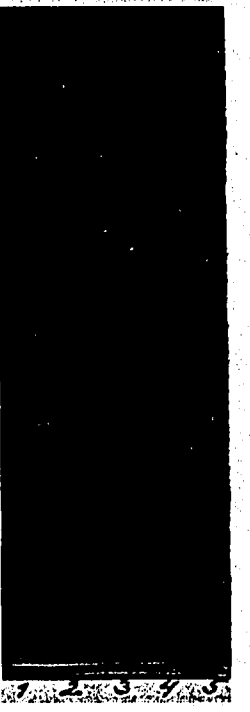

Fig 3b

Fig. 1. Phase system A. Detection with DNPH reagent. 1 = cholestenone; 2 = progesterone; 3 = cortisone; 4 = mixture. 40 γ spots.

Fig. 2. Phase system B. Detection with DNPH reagent. Separation of some corticosteroid (C_{21}) acetates. 1 = 11-deoxycorticosterone acetate; 2 = cortisone acetate; 3 = hydrocortisone acetate; 4 = mixture. 60 γ spots.

Fig. 3. Phase system B. a. Detection with DNPH reagent. b. Detection: fluorescence reaction of BUSH. Separation of C_{19} steroids (acetate) and cholestenone (C_{27}). 1 = cholestenone; 2 = testosterone acetate; 3 = Δ^4-androstene-3,17-dione; 4 = testosterone; 5 = mixture. 40 γ spots.

minutes, it may be convenient to sew a "retardation strip" of unacetylated paper along the edge of the acetylated paper. This is most clearly demonstrated in Figs. 2 and 3a*. In this way more compact and regular spots are obtained.

Successful separations have been obtained with the two-phase mixture benzene–methanol–water 4:4:1. When the lower layer (mainly benzene) is used for the accommodation and the upper layer (mainly methanol) as the mobile phase, reversed phase chromatograms are obtained. Just as in method A, the more polar steroids have higher R_F values than the less polar ones (Figs. 2, 3a, 3b and 4a).

* In Figs. 3b, 4a and 4b retardation strips are also present, but they are less clearly visible on the photograph.

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Method C. "Conventional" partition chromatography with a two-phase mixture of benzene, methanol and water

It is also possible to use the upper (more polar) layer of the mixture benzene–methanol–water 4:4:1 for accommodation and the lower layer as the mobile phase. In that case chromatograms are obtained, in which the weakly polar steroids have higher R_F

Fig. 4a

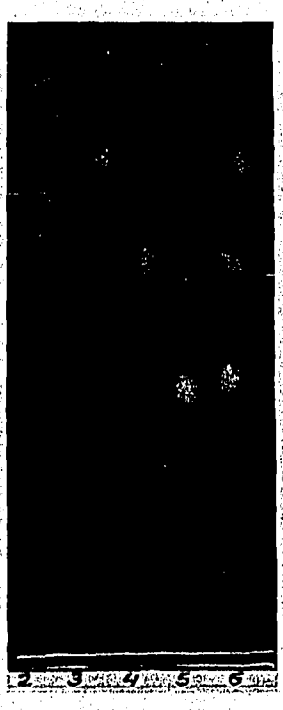

Fig. 4b

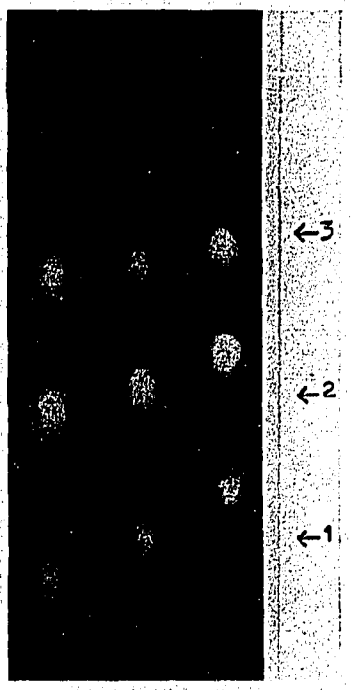

Fig. 5

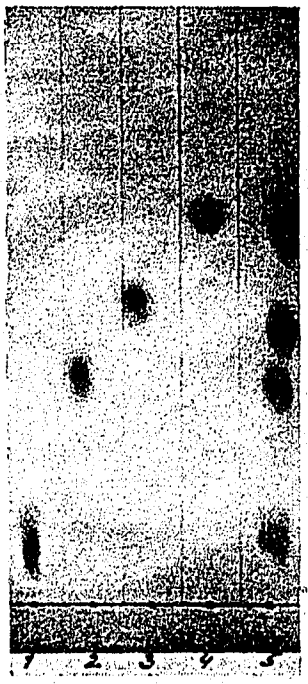

Fig. 6

Fig. 4. a. Phase system B. b. Phase system C. Detection: fluorescence reaction of BUSH. Separation of some C_{21} steroids and cholestenone. 1 = cholestenone; 2 = progesterone; 3 = 17α-hydroxyprogesterone (progesterone + 1 OH); 4 = Reichstein's Substance S (progesterone + 2 OH); 5 = hydrocortisone (progesterone + 3 OH); 6 = mixture. 40 γ spots.

Fig. 5. Phase system B, without retardation strip. Influence of distance from edge to starting-points, on size and form of spots. Detection: fluorescence reaction of BUSH. Mixtures of cholestenone (1), progesterone (2) and testosterone (3). 40 γ spots.

Fig. 6. Phase system B, without retardation strip. Detection with DNPH reagent. 1 = cholestenone; 2 = testosterone acetate; 3 = Δ^4-androstene-3,17-dione; 4 = testosterone; 5 = mixture. 80 γ spots.

values than the more strongly polar ones. As this type of chromatogram was the normal one in the early days of chromatography, it is often referred to as being "conventional". It should, however, be noted that in the case of chromatography on acetylated paper it is in fact rather unusual (Fig. 4b, *cf.* Fig. 4a).

Whereas in method A the chromatography takes only 5 hours, methods B and C require 16 hours for accommodation, followed by 8 hours for chromatography, if in all cases the ascending technique is used. In methods B and C, however, tailing of the spots is largely prevented, the separations are better and the spots more compact. In methods B and C a prolongation of the time of equilibration has no harmful effects and these methods may, in contrast to method A, be regarded as partition chromatography rather than as adsorption chromatography.

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A wide range of steroids, from weakly polar (Δ^4-cholesten-3-one) to strongly polar (hydrocortisone), may be chromatographed by methods A and B. With method C strongly polar steroids are better separated, but steroids less polar than progesterone move with the front.

A similar improvement to that obtained by using a retardation strip can be achieved by choosing the starting-line at a greater distance from the lower edge of the acetylated paper. If a distance of 9 cm is chosen, rather compact spots may also be obtained. The R_F values in this case are not very different from those which are obtained if a retardation strip of unacetylated paper is used (Fig. 5).

However, without any retardation, fairly good chromatograms are sometimes obtained with the phase system of method B (Fig. 6).

In all the methods mentioned so far, the ascending technique was applied. Descending chromatography may also be carried out, if the differences in R_F values between the components of a mixture are not sufficient. The mobile phase may then be allowed to drip off the paper or, more conveniently, be absorbed in a roll of paper, attached to the lower edge of the acetylated paper. The resolution may often be enhanced in this way, but no R_F values can be determined and the reproducibility is inferior to that obtained with the ascending technique.

RESULTS

A great variety of steroids has been chromatographed by the methods mentioned. The phase systems have been summarized in Table I and the R_F values of a number of Δ^4- and $\Delta^{1,4}$-3-ketosteroids are summarized in Table II.

From Table II and the figures it will be seen that satisfactory separations of various groups of steroids have been obtained.

The methods may be used for the separation of sex hormones, adrenal cortex hormones and their acetates, and also for weakly polar steroids such as testosterone acetate and cholestenone.

By similar methods other steroids, such as cholesterol and its oxidation products, bile acids, androsterone, etc., may be separated, as will be described in a later paper.

As the figures show, very regular and compact spots are obtained, especially

TABLE I

PHASE SYSTEMS AND TYPES OF CHROMATOGRAPHY

Ratio C_6H_6:CH_3OH:H_2O	*Stationary phase*	*Mobile phase*	*Type*
A. 1:10:3 (one phase)	acetylated paper	total solvent mixture	adsorption (?), reversed phase
B. 4:4:1 (two phases)	acetylated paper, accommodated with lower layer	upper (most polar) layer	partition, reversed phase
C. 4:4:1 (two phases)	acetylated paper, accommodated with upper layer	lower layer	partition, conventional

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TABLE II

MEAN R_F VALUES OF Δ^4- AND $\Delta^{1,4}$-3-KETOSTEROIDS AFTER CHROMATOGRAPHY ON FULLY ACETYLATED PAPER

Steroid	*Methods used Phase system (see Table I)*		
	A	*B*	*C*
C_{27} steroid			
Δ^4-Cholesten-3-one	0.28	0.09	—
C_{21} steroids			
Progesterone	0.48	0.35	0.88
11-Deoxycorticosterone	0.54	0.45	0.77
17α-Hydroxyprogesterone	0.54	0.47	0.75
Reichstein's Substance S	—	0.57	0.57
Cortisone	0.61	0.60	0.43
Hydrocortisone	0.68	0.68	0.36
Prednisone	—	0.65	—
Prednisolone	—	0.73	—
17α-Ethynyltestosterone	—	0 45	—
C_{21} steroid acetates			
11-Deoxycorticosterone acetate	—	0.30	—
Cortisone acetate	—	0.46	—
Hydrocortisone acetate	—	0.56	—
C_{20} steroid			
17-Methyltestosterone	—	0.51	—
C_{19} steroids			
Δ^4-Androstene-3,17-dione	0.52	0.41	—
$\Delta^{1,4}$-Androstadiene-3,17-dione	—	0.47	—
Δ^4-Androsten-17α-ol-3-one	—	0.52	—
Testosterone	0.60	0.57	—
C_{19} steroid acetate			
Testosterone acetate	0.27	0.32	—
C_{18} steroid			
19-Nortestosterone	—	0.55	—

with methods B and C. A fine example of the influence of the introduction of 1, 2 and 3 OH groups into the progesterone molecule is found in Figs. 4a and 4b. In method B, the R_F value is approximately proportional to the number of OH groups. As the table shows, the increase in the R_F value on the introduction of each OH group is 0.10–0.12. In method C the corresponding decrease is larger (0.11–0.21), but not constant. In that case the separations are the better, the more polar the steroids.

EXPERIMENTAL

Apparatus

The chromatography was usually carried out in cylinders, which had the following dimensions: height 50 cm, internal diameter 19 cm*. For the ascending technique, which was usually applied, two troughs with a capacity of 50 ml each, were placed

* If other cylinders or cabinets are used, the amount of solvents should be changed in proportion to the internal volume of the vessel.

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at the bottom. The cover of the cylinder had one centric and one excentric hole, which could be closed with corks. The cork in the centric hole carried an all-glass paper-hanger provided with hooks, which could carry two parallel sheets of paper. The cylinders were placed in a conditioned room at 25°.

Two ultra-violet light sources were used for the detection of the spots, namely a Hanovia Chromatolite* and a Chromatolux**.

Acetylated papers

Generally Whatman paper No. 1 was used and acetylated by the method of ZIJP[14], with only minor modifications:

16 sheets of paper of 46.7 × 14 cm each (the longer side perpendicular to the machine direction of the paper), in total about 90 g, are loosely rolled together and placed in a cylindrical vessel of about 2 l. The papers are covered with glacial acetic acid and after 16 hours the acid is decanted. An acetylating mixture containing 440 ml acetic anhydride, 545 ml glacial acetic acid, 1250 ml toluene and 1.25 ml 60% perchloric acid, and cooled to about 10°, is poured into the vessel until the papers are entirely covered by the mixture. The vessel is cooled in an ice-bath and the contents are gently agitated by occasional stirring. The temperature should not rise above 20°. The next day the mixture is poured off and the vessel is put upside down on a Büchner funnel to drain the papers. One by one the papers are then removed cautiously and blotted between ordinary filter paper. Thereupon they are washed 3–4 h in running water and subsequently dried, first at room temperature and then in a drying oven during 10 min at 100°.

After the acetylation the papers are about 44 × 13 cm and have become about three times as thick.

For methods B and C a retardation strip of Whatman paper No. 4 of 3 × 13 cm (shorter side perpendicular to the machine direction of the paper) is sewn to the end of the acetylated paper by means of a sewing machine, with an overlap of 1 cm.

Some experiments have also been made with Schleicher and Schüll paper 2043a "vollacetyliert", which has recently become commercially available. The results obtained were slightly different from those described in this paper. For qualitative experiments we prefer the papers prepared in this laboratory, with which somewhat more compact spots were obtained. For preparative purposes, especially in the "chromatopile", the S & S paper has some definite advantages, as will be described in a subsequent paper.

Partially acetylated S & S paper has also been tested. Compact spots were obtained, but apart from the inferior homogeneity, it turned out to have the disadvantage of giving less satisfactory separations and, moreover, the detection method of BUSH was less sensitive on this paper, possibly as a result of the stronger fluorescence of the background.

* Hanovia Ltd., Slough, England.
** Gerard Pleuger N.V., Rotterdam, The Netherlands.

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Reagents

a. Distilled benzene.
b. Distilled methanol.
c. Distilled water.
d. Alcoholic DNPH reagent: 0.15 g 2,4-dinitrophenylhydrazine (DNPH) is dissolved in 25 ml concentrated hydrochloric acid and subsequently adjusted to 100 ml with distilled water. Before use the solution is mixed with an equal volume of 96% ethanol.
e. Reagent of BUSH: 10 g of NaOH is dissolved in 100 ml 60% aqueous methanol.

Chromatographic procedures

Usually two sheets of fully acetylated paper of about 44 × 13 cm are run together in one cylinder. The spots of the steroids or mixtures are preferably applied in a 1% methanol or ether solution. It should be noted that the paper is soluble in acetone and chloroform. The spots may contain up to 150 μg of each of the individual steroids. In methods B and C larger quantities may be applied but then some "tailing" will result. The papers are attached to the hanger in such a way, that they dip about 3 mm into the solvent. After chromatography the papers are dried in a drying oven for 15 min at 60°, before the detection is carried out.

Method A. The spots are applied at 3 cm from the lower edge of the paper. The two troughs are filled with 35 ml each, of a mixture of benzene, methanol and water, 1:10:3. The papers are attached and the solvent is allowed to ascend for about 5 hours. The front is then about 36 cm from the starting line.

Method B. A mixture of benzene, methanol and water, 4:4:1 is shaken in a separating funnel. On standing two phases separate. The sheets of acetylated paper (44 × 13 cm) are provided with a retardation strip, as described before, and the spots are applied at 3 cm from the lower edge of the acetylated paper, *i.e.* 5 cm from the lower edge of the retardation strip. The papers are attached to the hanger and 120 ml of the lower layer of the solvent mixture is poured on the bottom of the cylinder. The papers are accommodated for 16 hours and subsequently 35 ml of the upper layer is poured into each of the two troughs, through the excentric hole in the cover, by means of a long pipette. The mobile phase is allowed to ascend for 8 hours. The front is then about 32 cm from the starting line.

Method C. This method is carried out in the same way as method B, but now the upper layer of the solvent mixture is used for accommodation and the lower layer as the mobile phase.

Detection

Method 1. The detection of Δ^4-3-ketosteroids on the chromatogram is most easily performed by examination of the dry chromatogram in ultra-violet light of a sufficiently low wave length, such as that obtained with the Hanovia Chromatolite (253.7 mμ). The steroids then appear as dark spots on a purple background. In contrast to the

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method mentioned hereafter, $\Delta^{1,4}$-3-ketosteroids and other substances with conjugated systems are also detected by this method.

Method 2. Far more sensitive and more specific than method 1 is the fluorescence method of BUSH[16]. It seems to be quite specific for Δ^4-3-ketosteroids, of which 0.25–2 μg may be detected after development of the chromatogram*. The papers are sprayed with the reagent of BUSH and are subsequently dried for about 10 min at 80° until the paper turns slightly yellow. The chromatograms are then observed in the ultra-violet light of the Chromatolux (maximum at 360 mμ). The Δ^4-3-ketosteroids appear as yellow to orange spots on a purple background. No reaction is obtained with $\Delta^{1,4}$-3-ketosteroids.

Method 3. The detection of Δ^4-3-ketosteroids may also be carried out with 2,4-dinitrophenylhydrazine (DNPH). This reagent should be applied in alcoholic solution, as an aqueous solution does not wet the hydrophobic acetylated paper. Unconjugated ketosteroids, such as androsterone, are not detected by this method, probably because the paper is also coloured by the reagent. The Δ^4-3-ketosteroids, however, appear as orange spots on a yellow background.

$\Delta^{1,4}$-3-Ketosteroids also give orange spots, but they react more slowly with DNPH and the spots are sometimes rather faint.

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SUMMARY

A wide range of Δ^4- and $\Delta^{1,4}$-3-ketosteroids, from weakly polar to strongly polar, may be chromatographed and detected on cellulose triacetate paper, with benzene–methanol–water mixtures as developing solvents. Three systems have been worked out. In the first a one-phase mixture of benzene, methanol and water is used as the mobile phase and the paper is not accommodated. A "reversed phase" type of chromatogram is obtained, *i.e.* the more polar compounds have higher R_F values than the less polar ones, as is usual for chromatography on acetylated paper. If, however, two-phase mixtures of benzene, methanol and water are used, as was the case in the other two methods, "reversed phase" as well as "conventional" type chromatograms may be obtained. This depends upon whether the lower or the upper layer is used as the mobile phase, the other layer being used for accommodation of the paper. Perhaps the first-mentioned method should, in contrast to the other two, be regarded as adsorption chromatography, rather than as partition chromatography.

Well-reproducible R_F values are obtained and, especially with the two-phase mixtures, compact spots are found. Detection methods and R_F data are given for various Δ^4- and $\Delta^{1,4}$-3-ketosteroids, ranging from C_{18} to C_{27} compounds and including sex hormones and adrenocortical hormones.

* The sensitivity may be further enhanced by cooling. In a recent article[17], attention was drawn to the fact that many substances which show no fluorescence at room temperature, do so on cooling. We found that the sensitivity of the method of BUSH is improved by dipping the chromatogram in liquid nitrogen. The spots then become brighter and show an afterglow. The colour shifts in the direction from orange → yellow → green.

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