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REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY FOR THE SEPARATION AND ANALYSIS OF ECDYSTEROIDS

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SUMMARY

Reversed-phase thin-layer chromatography plates of different types and different manufacture are all suitable for the chromatography of ecdysteroids (insect moulting hormones). A number of solvent systems have been examined, and elution with methanol–water mixtures from C_{18}-coated plates provides a suitable general system. The detection limit of ecdysteroids on reversed-phase plates is 10^{-7} g, using fluorescence quenching or vanillin–sulphuric acid spray. The recovery of ecdysteroids, which is inefficient from normal silica plates, is much more efficient from reversed-phase plates, especially when residual silanol groups are "capped" with organosilane reagents.

INTRODUCTION

A variety of chromatographic techniques have been used in the isolation, identification and analysis of ecdysteroids, the moulting hormones of insects and crustaceans. The existence of 17 zooecdysteroids and over 50 phytoecdysteroids, all of closely related structure[1], makes heavy demands upon the separation powers available to an investigator. Adsorption columns, thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC) have all been used. TLC has been a popular method with ecdysteroids because it is simple to use, inexpensive, gives good resolution and R_F properties can be varied widely by exploiting the choice of solvent systems available[2]. TLC in this area suffers from two disadvantages, first, relatively poor sensitivity of detection compared with GC and HPLC, and secondly, losses of compounds due to irreversible adsorption on silica may be high when small quantities of compound are available[3]. Now that reversed-phase (RP) TLC plates are available from a number of manufacturers and can be made easily in the laboratory, we decided to explore their usefulness for ecdysteroids. RP-TLC is highly suitable for such polar compounds, and problems of irreversible adsorption should be reduced or eliminated. It provides another system with different

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separation properties for the qualitative analysis and identification of ecdysteroids in biological systems.

We have here examined the separation of several ecdysteroids on commercially available RP-TLC plates and the recovery of ecysteroids from them, and conducted some preliminary experiments with "homemade" RP-TLC plates.

EXPERIMENTAL

TLC plates

Absorption TLC plates were prepared by coating 5 × 20 cm glass plates with a 0.3-mm layer of silica gel G (E. Merck, Darmstadt, G.F.R.). The plates were allowed to dry in air and stored over $CaCl_2$.

RP-TLC plates were obtained from three commercial sources and were made in the laboratory. Precoated plates (10 × 10 cm) containing short, medium and long carbon chains bonded to the silica surface (RP-2, RP-8 and RP-18 respectively) were obtained from Merck. One type of plate (20 × 5 cm), KC_{18}, precoated with C_{18} carbon chains was obtained from Whatman (Springfield Mill, Great Britain). Three types of Macherey, Nagel & Co. (Düren, G.F.R.) plates were used, which have different degrees of silanization with octadecylsilane. These plates (10 × 10 cm) are 50, 75 or 100% silanized.

RP-TLC plates were prepared in the laboratory by immersing precoated silica TLC plates (10 × 10 cm, Whatman high-performance silica, Type HP-K) in a solution of 2% trichlorooctadecylsilane in toluene (dried, molecular sieves). The plates were sonicated three times for 30-sec intervals in the first 15 min of immersion and left in the bath overnight, then washed for 10 min in each of toluene, acetone and methanol. Plates were dried at 110°C for 1 h before use. Capping was carried out in the same way using trimethylchlorosilane or dimethyldichlorosilane.

All plates had an acid-stable fluorescent indicator incorporated.

Use of the plates

Ecdysone (95% pure by HPLC with UV detection) and 20-hydroxyecdysone were obtained from Simes (Milan, Italy). Solvents were of analytical grade.

Amounts of ecdysteroid from 10^{-6} to 10^{-7} g were spotted on the plates using a 10-μl SGE syringe. The reversed-phase plates were conditioned for 30 min in the atmosphere of the tank before use. They were then developed in 95% ethanol–water (1:1) for quantitative studies or the appropriate solvent for R_F studies. The adsorption plates were developed in chloroform–methanol (8:2).

After development, the plates were visualized under a UV lamp at 254 nm. Fluorescence was induced in ecdysteroids by heating for 1 h at 110°C in the presence of solid ammonium carbonate, and viewing under a UV lamp at 366 nm. Vanillin spray reagent was prepared by dissolving vanillin in a mixture of concentrated sulphuric acid and 95% ethanol (5:70:25, w/v/v). After spraying with the reagent, plates were heated to 100–120°C for 10 min and observed in normal light.

Quantification studies

Where recovery from the plates was measured, after visualization in UV light, the appropriate R_F bands were scraped off. The silica was transferred to a test-tube

and extracted with methanol (1 ml). The silica was separated by centrifugation and the clear supernatant liquid decanted into a Reacti-vial (Pierce & Warrner, Chester, Great Britain), where it was evaporated to dryness with a stream of warm nitrogen. The residue was re-dissolved in a known volume of methanol for quantification by HPLC, using a Pye LC3 XP pump, a Pye gradient elution system and Chromatronix 220 fixed-wavelength (254 nm) absorption detector. The analysis was carried out on a Shandon Southern 100 × 5 mm column packed with 5-μm particles of Hypersil ODS under isocratic conditions (methanol–water, 45:55) at a flow-rate of 1.0 ml min^{-1}. Samples of 10 μl were injected onto the column. The quantity of compound present was calculated from a standard curve obtained by plotting peak area against quantity of ecdysone injected. The least detectable amount was 10 ng.

RESULTS AND DISCUSSION

At present, a range of RP-TLC plates are available commercially. Merck produce plates with ethyl (C_2), *n*-octyl (C_8) or *n*-octadecyl (C_{18}) groups chemically bonded to the silica surface, gibing a range of partitioning properties. Macherey, Nagel & Co. produce a similar range of properties by controlling the degree of silanizing to produce plates which are 50, 75 and 100% covered with C_{18} groups. Whatman also manufacture a C_{18}-coated plate.

We have determined the R_F values of ecdysone and 20-hydroxyecdysone, the commonest ecdysteroids encountered, on each of the Merck plates, using a range of organic solvents (methanol, ethanol, 2-propanol, acetone and acetonitrile) together with water. The variation of R_F value with solvent composition on the three types of plate are shown in Table I. Only the upper and lower limits of R_F values measured are given.

Good resolution of ecdysone and 20-hydroxyecdysone was possible using all the solvent systems tested. The eluotropic series formed by this group of organic

TABLE I

VARIATION OF R_F VALUES OF ECDYSONE (α) AND 20-HYDROXYECDYSONE (β) ON MERCK RP-TLC PLATES, IN DIFFERENT SOLVENT SYSTEMS

Solvent	*Proportions*	C_2		C_8		C_{18}	
		α	β	α	β	α	β
Methanol–water*	50:50	0.38	0.53	0.28	0.44	0	0
	80:20	0.88	0.88	0.75	0.82	0.76	0.82
Ethanol–water	40:60	0.38	0.53	0.34	0.52	0.41	0.57
	70:30	0.89	0.89	0.88	0.88	0.88	0.88
2-Propanol–water	20:80	0.18	0.46	0.19	0.46	0.24	0.46
	60:40	0.87	0.87	0.83	0.83	0.81	0.81
Acetonitrile–water	30:70	0.44	0.60	0.33	0.53	0.35	0.50
	60:40	0.86	0.86	0.90	0.90	0.90	0.90
Acetone–water	30:70	0.35	0.58	0.16	0.53	0.26	0.45
	70:30	0.79	0.87	0.86	0.86	0.84	0.91

* With methanol–water (60:40) on C_{18} plates, $\alpha = 0.39$ and $\beta = 0.54$. With methanol–water (90:10), R_F values were 0.9 for both compounds on all three types of plate.

solvents is as follows: isopropanol > acetonitrile > acetone > ethanol > methanol. An R_F of approximately 0.5 (on all three types of plate) was obtained with the following solvent compositions: isopropanol–water (30:60); acetonitrile–water (35:65); acetone–water (40:60); ethanol–water (45:55) and methanol–water (65:35). The largest separations between ecdysone and 20-hydroxyecdysone were achieved with the minimum possible organic modifier which still allowed solvent migration. The best separation, of 0.29 R_F units was obtained on a C_2 plate using isopropanol–water (20:80).

The effect of alcohol chain length on the quantity of organic modifier required to achieve the same separations is illustrated in Fig. 1. Differences resulting from the length of the carbon chain bonded to the silica were most noticeable at low R_F, where less organic modifier was present. With larger proportions of organic modifier in the solvent R_F values on all three types of plate were similar. There was no trend with chain length, the R_F values on the C_8 plates sometimes being lower than on the C_2 or C_{18} types. Bearing in mind that the greater the proportion of the organic component, the less viscous the mixture, and the shorter the elution time, none of the systems showed any distinct advantage over methanol–water mixtures, and for further work, attention was confined to this solvent system.

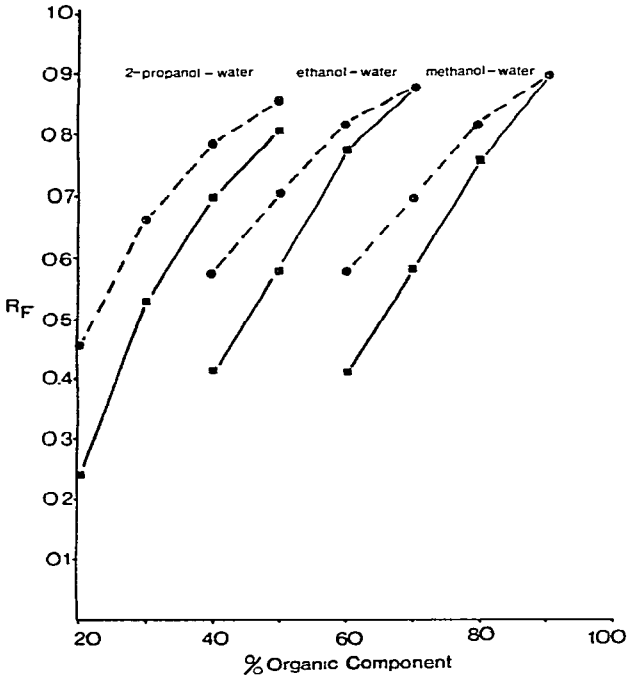

Fig. 1. The variation of R_F values for ecdysone (solid line) and 20-hydroxyecdysone (dashed line) on Merck C_{18} RP-TLC plates in alcohol–water solvent systems.

Examination of the Macherey, Nagel & Co. plates, coated with 50, 75 and 100% of C_{18} hydrocarbon chains, showed that their R_F properties were very similar to those of the Merck plates (Table II). There was greater separation between ecdysone and 20-hydroxyecdysone at low proportions of methanol (methanol–water, 50:50), and

TABLE II

VARIATION OF R_F VALUES OF ECDYSONE (α) AND 20-HYDROXYECDYSONE (β) ON MACHEREY, NAGEL & CO. AND WHATMAN RP-TLC PLATES

Proportions of methanol–water	*Extent of coating, Macherey, Nagel & Co., C_{18}*						*Whatman C_{18}*	
	50%		*75%*		*100%*			
	α	β	α	β	α	β	α	β
50:50	0.25	0.38	0.20	0.34	0.13	0.19	*	*
90:10	0.84	0.84	0.89	0.89	0.86	0.86	0.78	0.78

* Not wetted by solvent; in methanol–water (60:40), R_F = 0.38 (α) and 0.5 (β).

R_F values of approximately 0.5 were achieved with solvent proportions 55:45 on the 50% coated plates and 65:35 on the 75% and 100% coated plates. A trend towards lower R_F values is seen, the greater the extent of coverage by the organic phase. The Whatman C_{18} plates had similar R_F values (Table II), but like the Merck C_{18} plates, were not wetted by methanol–water (50:50).

One of the factors which limits the use of RP-TLC plates is their hydrophobic nature, which limits the choice of solvent in which the sample can be applied, as well as limiting the choice of mobile phase. RP-TLC plates with a non-absorbent application zone, of the type already available for silica plates would be helpful where it is desired to apply aqueous solutions (*e.g.*, insect haemolymph or extracts containing a high proportion of water).

The quantity of water which can be accommodated in the mobile phase depends not only on the organic phase but also on the process of manufacture, Macherey, Nagel & Co. C_{18} 100% plates can be used with methanol–water (50:50) whereas there is no solvent migration with this mixture on Merck plates, and it causes adsorbant to come away from the glass backing of the Whatman plates.

The high cost of the commercial RP-TLC plates encouraged us to explore the preparation of our own plates in the laboratory. Normal high-performance silica plates were silanized in a bath of toluene and trichlorooctadecylsilane overnight[4]. The plates prepared in this way compared favourably with commercial plates, however, the fluorescent marker in the silica is lost during silanizing. The plates were also "capped", by causing remaining surface silanol groups to react with either dimethyldichlorosilane or trimethylchlorosilane. Some R_F values are given in Table III. It is noteworthy that the plates treated with trimethylchlorosilane gave the highest R_F values, and also had the most non-polar surface (see *Recovery of ecdysteroids from reversed-phase plates*).

Separation of a large number of ecdysteroids

The separations achieved on Merck C_2, C_8 and C_{18} plates for a range of twelve ecdysteroids, in three solvent systems are given in Table IV. It is interesting that significant changes in selectivity are apparent between alcohol–water and acetonitrile–water based solvent systems. In particular poststerone and cyasterone appear in alcohol–water to be more polar than ecdysone, whilst in acetonitrile–water they are

TABLE III

R_F VALUES OF ECDYSONE (α) AND 20-HYDROXYECDYSONE (β) ON LABORATORY-MADE REVERSED-PHASE PLATES

Solvent system: ethanol–water (60:40). DMCS = Dimethyldichlorosilane; TMCS = trimethylchlorosilane.

	Uncapped	*DMCS treated*	*TMCS treated*
α	0.75	0.81	0.84
β	0.78	0.85	0.89

less polar. It is perhaps noteworthy that these two compounds differ from all the other ecdysteroids examined in that they posess two, rather than one keto group. Muristerone and ajugasterone C (which are unusual in possessing a hydroxyl at C-11), also exhibit some differences in behaviour, relative to ecdysone, in different solvent systems. It should therefore be possible to manipulate both the type of plate used, and the solvent system employed to obtain a wide range of separations amongst the ecdysteroids, as both polarity and structural details seem to be important in their RP-TLC.

Detection of ecdysteroids on reversed-phase plates

The 7-en-6-one group common to the ecdysteroids strongly absorbs UV light at 240 nm. Consequently, on TLC plates incorporating a fluorescent marker, the ecdysteroids are readily visible as dark spots against the fluorescent background. By this method, the smallest amount of ecdysteroid we could detect was $1 \cdot 10^{-7}$ g on RP-TLC plates, and about the same quantity on normal silica TLC plates (*cf.*, ref. 2).

On silica the ecdysteroids have customarily been identified with the aid of a number of visualizing reagents. The one most widely used has been vanillin–sulphuric acid spray. The colour produced (blue to green to violet) can vary with the ecdysteroid structure. We have examined the usefulness of this reagent on RP-TLC plates. After spraying and heating, the typical colours were produced with ecdysone and hydroxyecdysone, discolouration of the plates was minimal and the detection limit was again about 10^{-7} g.

The formation of fluorescent derivatives can be used to lower detection limits in chromatography. Ecdysteroids form fluorescent derivatives in ethanol–sulphuric acid[5–7] but we have found the fluorescence intensity is variable with conditions, and therefore not reliable for quantitation. An alternative is to heat the plate in an atmosphere of ammonium carbonate at 110°C. When viewed under UV light of 366 nm, the ecdysteroids show a blue-white fluorescence, detectable to $5 \cdot 10^{-8}$ g, moreover the fluorescence appears to be stable for several days, but the fluorescence is rather non-specific[8], so ecdysteroids could not be detected in only partially purified biological samples.

Recovery of ecdysteroids from reversed-phase plates

It has been demonstrated in a number of cases that polar organic compounds are not recovered efficiently from silica or alumina chromatographic materials because of irreversible adsorption[3]. This loss through adsorption is negligible in most

TABLE IV

R_F VALUES OF VARIOUS ECDYSTEROIDS CHROMATOGRAPHED ON THREE TYPES OF MERCK RP-TLC PLATES, USING THREE SOLVENT SYSTEMS

Solvent systems: PrOH = 2-propanol; MeOH = methanol; ACN = acetonitrile; in each case the other component is water.

*Compound**	C_2			C_8			C_{18}		
	30% PrOH	*60% MeOH*	*40% ACN*	*30% PrOH*	*60% MeOH*	*40% ACN*	*30% PrOH*	*60% MeOH*	*40% ACN*
Ecdysone	0.38	0.52	0.66	0.39	0.38	0.61	0.43	0.31	0.64
20-Hydroxyecdysone	0.53	0.65	0.75	0.58	0.53	0.72	0.59	0.47	0.75
2-Deoxy-20-hydroxyecdysone	0.36	0.53	0.62	0.42	0.32	0.6	0.39	0.33	0.57
Inokosterone	0.49	0.67	0.71	0.53	0.53	0.72	0.55	0.50	0.73
Muristerone A	0.27	0.57	0.56	0.28	0.38	0.57	0.29	0.30	0.55
Makisterone A	0.45	0.63	0.67	0.5	0.47	0.72	0.51	0.39	0.71
Cyasterone	0.45	0.66	0.60	0.53	0.53	0.65	0.57	0.47	0.64
Polypodine B	0.36	0.63	0.57	0.47	0.47	0.61	0.49	0.40	0.59
Ponasterone A	0.14	0.44	0.41	0.13	0.21	0.41	0.14	0.16	0.39
Polypodine B	0.52	0.71	0.70	0.63	0.55	0.78	0.61	0.48	0.77
Ajugasterone C	0.2	0.60	0.60	0.26	0.36	0.57	0.26	0.31	0.59
Carpesterol	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

* For structures, see ref. 1.

TABLE V

RECOVERY (%) OF ECDYSONE FROM RP-TLC PLATES COMPARED WITH NORMAL-PHASE SILICA ADSORPTION TLC PLATES, IN QUANTITIES NEAR THE LIMIT OF DETECTION

Results were single determinations for laboratory-made plates, others are the average of two determinations.

Type of TLC plates	*Amount applied (g)*		
	$1 \cdot 10^{-6}$	$5 \cdot 10^{-7}$	$1 \cdot 10^{-7}$
Adsorption TLC	56	56	51
Merck C_2	84	84	79
Merck C_8	82	79	80
Merck C_{18}	87	84	82
Macherey, Nagel & Co. 100% C_{18}	88	83	75
Whatman C_{18}	76	76	71
Laboratory-made, not capped	82	69	*
capped DMCS	87	83	*
capped TMCS	91	80	*

* Not tested.

cases, but working in the μg and ng range, this loss can become significant to serious. With a non-polar surface bonded to silica this irreversible adsorption loss should be reduced, and RP-TLC plates should be more suitable for the quantitative separation and recovery of ecdysteroids.

We have therefore compared the recovery of small samples of ecdysone in the range 10^{-6}–10^{-7} g (near the limit of detection) from normal-phase silica TLC plates with that from various kinds of RP-TLC plates. The results are given in Table V. While recoveries varied from 50 to 60% on normal phase, all the RP-TLC plates showed much higher recovery, up to 90%. Recoveries are slightly better in all cases for the larger amounts. Doubtless larger errors would be found for still smaller quantities. The longer the carbon chain (see the Merck plates) the better the recovery, but the difference is small. The recovery from plates coated with C_{18} groups in the laboratory was improved by "capping" the unreacted surface silanol groups with dimethyldichlorosilane or trimethylchlorosilane. Both Merck and Macherey, Nagel & Co. plates are available with or without a fluorescent indicator added, and we have examined the effect of the indicator on recovery. No difference was found in recovery of ecdysone for the same manufacturer's plates with or without fluorescer, but the indicator plates contain elutable components absorbing UV light, which give a number of peaks when the sample is analysed later by HPLC.

We draw attention again to the poor recovery of such compounds as the ecdysteroids from an activated silica surface, whether column, thin layer or HPLC column is used. Clearly, RP-TLC is preferable for these compounds when all other considerations are equal. The best material for this chromatography is non-fluorescent plates, made by coating silica plates in the laboratory and then "capping" them with trimethylchlorosilane.

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