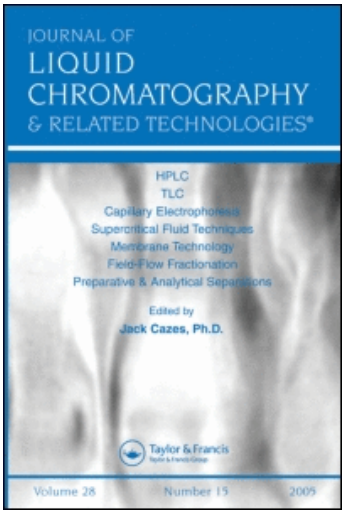

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Thin-Layer Chromatography of Phytoecdysteroids

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ABSTRACT

Planar chromatography of ecdysteroids is reviewed. Separation of various ecdysteroids is detailed using both straight-phase and reversed-phase thin-layer chromatography (RP-TLC). The generally used special techniques, such as two-dimensional TLC (2D-TLC), forced-flow TLC (FF-TLC), displacement mode of development, etc., are also specified. The particular behavior of certain ecdysteroids is discussed.

Key Words: Phytoecdysteroids; Thin-layer chromatography; Two-dimensional TLC; Forced-flow TLC; Hyphenated techniques.

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INTRODUCTION

The history of thin-layer chromatography (TLC) started with the analysis of biologically active ingredients of some tinctures and has continued with constant focus on biologically active substances. Thin-layer chromatography has several advantages in comparison to the other chromatographic procedures. Its advantages can be fully utilized when active components of a plant extract or that of a phytochemical medication is investigated, such as in the analytical and preparative separation of ecdysteroids.

An essential feature of TLC is the wide availability of references. Analytical Chemistry regularly contains critical review of the publications dealing with TLC.[1] The CAMAG Bibliography Service (CBS) publishes semi-annually a special section on ecdysteroids.[2] Several excellent books[3–5] summarize the essence of the progress of TLC, and also a special journal[6] is publishing details on the newest results. Naturally, literature searches through the electronic networks of the natural sciences also provide information based on key words. There is an excellent on-line source describing the continuously updated knowledge about ecdysteroids accessible through the Internet.[7]

Ecdysteroids are insect molting hormones. They were discovered by Butenandt and Karlson,[8] who isolated ecdysone from *Silk worm* pupae. The chemical structure of ecdysteroids is characterized by a cyclopentano-perhydrophenantrene skeleton with an alkyl side chain at C17, and containing a 7-en-6-one chromophore and several (minimum 2, maximum 8) hydroxyls. Certain other steroids are closely related to ecdysteroids, such as brassinolids and sterols. The chemical structures of ecdysteroids dealt with in this paper are given in Fig. 1.

Ecdysteroids show an intensive absorbance in the ultraviolet (UV) region at 240–260 nm. Therefore, a general detection method is given by viewing the dark spots under 254 nm light on TLC containing a fluorescent indicator. Furthermore, the majority of ecdysteroids show certain color reactions, which are more or less specific. Therefore, even a virtual separation can be generated.

The number of known ecdysteroids is over 330. Plant ecdysteroids number about 240. Phytoecdysteroids are important not only due to their diversity, but also because their level in the plant is higher than the level of ecdysteroids in insects.

The large number and variety of ecdysteroids is mainly due to the variation of the number, position, and orientation of the hydroxyl groups. Certain anomalies of lipophilicity of ecdysteroids can be sometimes misleading from the chromatographic point of view. The number of hydroxyl substitutions does not unconditionally decrease lipophilicity, but sometimes increases it. Polypodine B is characterized by seven hydroxyls, and 20-hydroxyecdysone contains one less (six). However, the extra hydroxyl of polypodine B is located at C5. Therefore, an intra-molecular hydrogen bond

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is formed with the hydrogen of C5 hydroxyl and the oxygen of the oxo group (on C6), and this five-member ring seems to be quite stable and to increase the lipophilicity of polypodine B. This is the reason that polypodine B, with seven hydroxyl groups, has a chromatographic behavior similar to 2-deoxyintegristerone A, which contains only six hydroxyls (Fig. 1, Table 1).

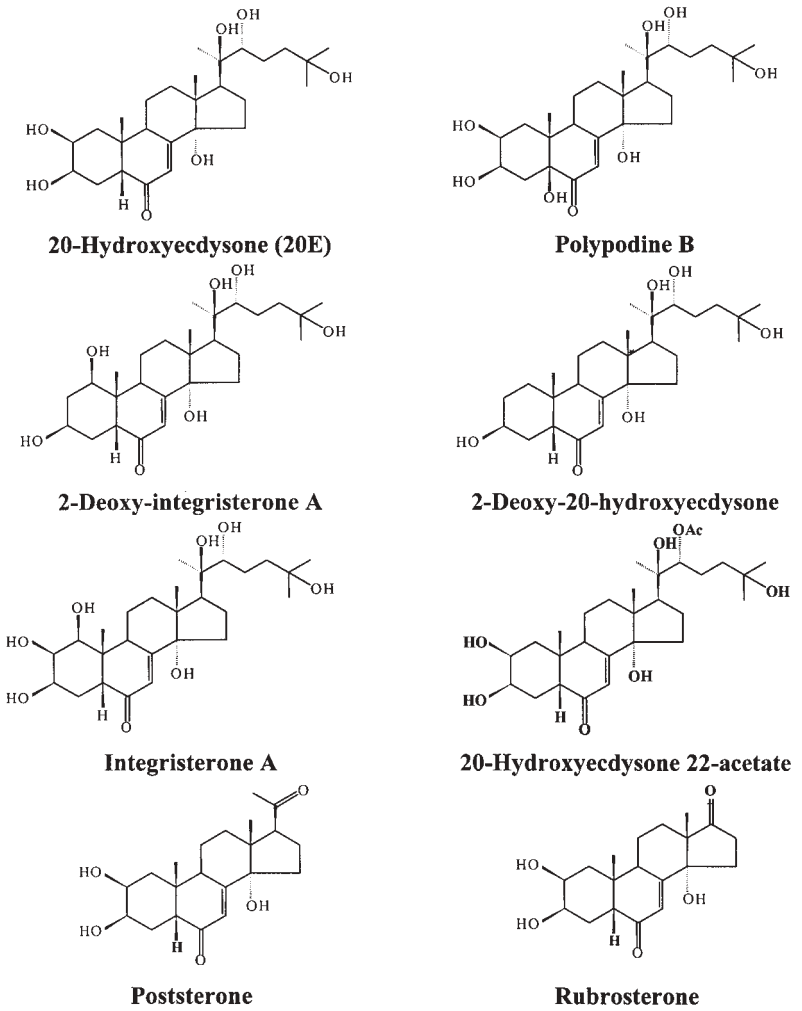

Figure 1. Chemical structure of some plant ecdysteroids.

(*continued*)

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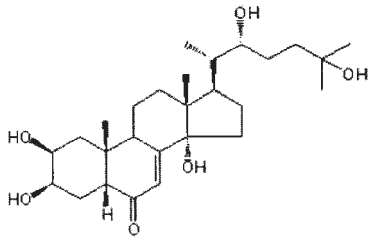
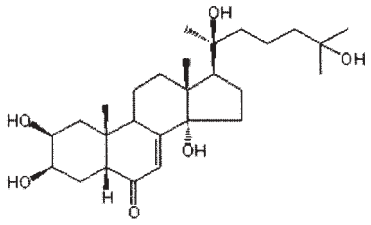
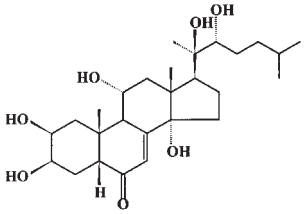


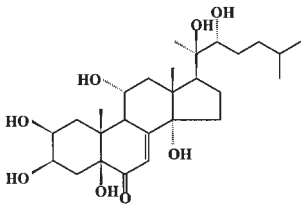
22-Deoxy-20-hydroxyecdysone



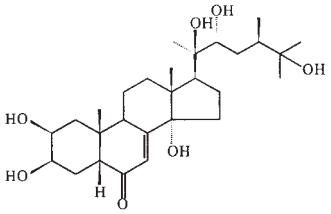
Ecdysone



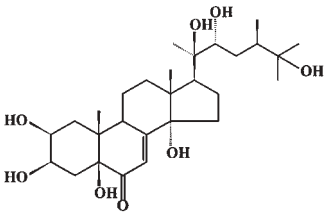
Ajugasterone C



Muristerone A



Makisterone A



Dacrysterone

Figure 1. Continued.

Also, the diversity of ecdysteroids is given by the potential substituents on the hydroxyls, which can be a glycosyl forming sugar (such as glucose, galactose, or xylose), or an ester forming organic acid (such as benzoate, acetate, cinnamate, coumarate, or tiglynat), or an ester forming inorganic acid (such as sulfate or phosphate), and acetonide.

20-Hydroxeecdysone is the major phytoecdysteroid. It can be present in an overwhelming quantity in all known ecdysteroid-containing plants of the continents. Side ecdysteroids occur in one magnitude less concentration than the major ecdysteroids, such as 2-deoxy-20-hydroxyecdysone, integristerone A, and 20-hydroxyecdysone-22-acetate in the case of *Silene otites* (L.) Wib. Minor ecdysteroids occur at a level two to several magnitudes less than the major ecdysteroids (e.g., less than 0.001% of the dry weight of plant).

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Table 1. $R_F \times 100$ values of ecdysteroids using silica gel as stationary phase.

	$R_F \times 100$ using silica gel stationary phase and mobile phases as given				
Name of the ecdysteroids	A	B	C	D	E
Viticosterone E	71	50	nda	52	55
2-Deoxy-E 22-acetate	65	58	59	58	68
2-Deoxy-20E 22-acetate	59	52	56	55	62
2-Deoxy-E	54	49	55	51	58
Rubrosterone	46	27	37	48	55
Poststerone	46	27	37	49	56
2-Deoxy-20E	43	42	49	46	53
Muristerone	38	24	28	39	57
20E 22-acetate	34	24	32	40	44
Ecdysone	33	27	35	40	41
Polypodine B	32	20	25	35	38
20E 22-benzoate	32	37	27	39	42
2-Deoxy-integristerone A	32	21	36	33	39
22-Deoxy-20E	26	23	30	34	42
20-Hydroxyecdysone	24	21	27	31	37
22-Deoxy-integristerone A	22	16	18	25	36
Integristerone A	18	15	16	24	29
5α-2-Deoxy-integristerone A	16	24	30	21	32
26-Hydroxy-polypodine B	12	10	11	15	16
2-Deoxy-20E 22-glucoside	10	7	8	14	17
2-Deoxy-polypodine B	nda	nda	46	49	60
Makisterone C	nda	nda	41	40	57
24(28)-Dehydromakisterone A	nda	nda	32	36	48
9α,20-Dihydroxyecdysone	nda	nda	35	38	47
Makisterone A	nda	nda	31	38	40
5α-20E	nda	nda	32	38	38

Note: E, Ecdysone; 20E, 20-Hydroxyecdysone; nda, no data available.

The first crystalline structure of ecdysone was determined by x-ray diffraction by Huber and Hope.[9] Subsequently, Báthori et al.[10] described that 20-hydroxyecdysone has a pseudo-polymorph crystalline structure, giving various crystalline forms. One of them consists of one methanol and one water molecule per each 20-hydroxyecdysone molecule, and it stabilizes the 20-hydroxyecdysone molecule. Another crystalline form does not have any additional molecule, and the loose space filling permits rotation (vibration) of the side chain of 20-hydroxyecdysone. In this way, two distinct positions of the side chain are observable in the x-ray picture.[10]

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Thin-layer chromotography of ecdysteroids is preferred to other methods in several cases, such as:

Screening and identification of ecdysteroids in plant extracts.

Monitoring the ecdysteroid level in vegetation.

Monitoring the purification of ecdysteroids and removal of impurities.

Quality control of pure ecdysteroid preparations.

Scouting the optimum stationary phase–mobile phase combination.

Analytical TLC requires a certain amount of ecdysteroids, called the minimum detectable amount. Using UV densitometry, no ecdysteroid spot under 0.01 μg can be detected, and 0.1 μg is generally required for reliable detection. Linearity of calibration curves starts between 0.1 and 1 μg of the ecdysteroid spot. As the usual volume of load is 10 μL, the concentration limit for ecdysteroid detection is between 1 and 10 $\mu g\,mL^{-1}$. Any other sample requires pre-concentration by the use of either a clean-up, or the use of essential enrichment.

MATERIALS AND METHODS FREQUENTLY USED FOR ECDYSTEROID SEPARATION

Set-up for Thin-Layer Chromatography of Ecdysteroids

All-glass chambers or glass chambers with stainless steel lids are used for the development of plates, however, both automated multiple development, and overpressured layer chromatography can be used for development.

Stationary Phases

Thin-layer chromatography plates (silica, RP18, alumina, cyanopropyl, F_{254}, and without fluorescent indicator) can be purchased from BDH (Poole, Dorset, UK), Macherey-Nagel (Düren, Germany), Merck (Darmstadt, Germany), or Whatman (Clifton, NJ) under various trade names.

Paraffin impregnation of TLC silica can be carried out by a 20 h continuous development of silica plates with 10% paraffin in hexane. The samples can be applied immediately after drying the plates.

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Mobile Phases

Several solvent systems have been used in our laboratory for ecdysteroid separation by normal-phase TLC:[11–13]

(A) Dichloromethane–ethanol (96%) (8 : 2).
(B) Ethyl acetate–methanol–ammonia (25%) (85 : 10 : 5).
(C) Toluene–acetone–ethanol (96%)–ammonia (25%) (100 : 140 : 32 : 9).
(D) Chloroform–methanol–benzene (25 : 5 : 3).
(E) Ethyl acetate–ethanol (96%)–water (16 : 2 : 1).
(F) Ethyl acetate–formic acid–water (85 : 10 : 5).

Two-dimensional (2D) TLC is possible using any two of the above list, but the combinations (C) and (F), and (C) and (D) are preferred in the first and second dimensions compared to the others. Mobile phase (D) was water-free, and (C) contained about 3% water. This water may remain on the stationary phase if (C) is used for the first dimensional development.

Reversed-phase TLC (RP-TLC) systems are:

(G) Methanol–water (6 : 4).
(H) Acetonitrile–water (35 : 65).
(I) Acetonitrile–water–trifluoroacetic acid (35 : 65 : 0.1).
(J) Tetrahydrofuran–water (45 : 55).

Displacement TLC can be carried out using silica gel stationary phase with dichloromethane-isopropanol carrier and either dimethylamino-propylamine or triethanolamine as the displacer. The mobile phase contains the mixture of both the carrier and the displacer, as given here:

(L) Dichloromethane–isopropanol–dimethylaminopropylamine (110 : 30 : 5).
(M) Dichloromethane–isopropanol–dimethylaminopropylamine (100 : 30 : 5).
(N) Dichloromethane–isopropanol–dimethylaminopropylamine (95 : 30 : 5).
(O) Dichloromethane–isopropanol–triethanolamine (70 : 20 : 2).
(P) Dichloromethane–isopropanol–triethanolamine (50 : 20 : 2).
(R) Dichloromethane–isopropanol–triethanolamine (40 : 20 : 2).

In the case of displacement chromatography, there are two fronts, successively running through the stationary phase. The faster moving is the

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carrier front, while the slower moving (i.e., the secondary front) is the displacer front.

Detection Methods

After development, the plates are dried and observed visually at 254 nm. Then the plates are sprayed with vanillin–sulfuric acid (1 : 100, w/v), and observed in daylight and at 354 nm. The spots are colored in daylight from turquoise green through yellow, orange, and brown to violet, and violet to orange under 354 nm light. The compositions of a number of other color reagents useful for ecdysteroid detection are described by Stahl[14] and Lafont et al.[15]

SCREENING AND IDENTIFICATION OF PLANT ECDYSTEROIDS

The ecdysteroids in the plant extract can have little differences in their pK_a values, therefore, a selective system is required for their separation. The separation power of chromatographic systems can be further improved by the use of triple detection, which is specific for ecdysteroids. This triple detection consists of: (1) observation under UV light to find the spots absorbing at 254 nm; (2) spraying with vanillin–sulfuric acid and observing the vividly colored spots in daylight; and (3) viewing induced fluorescence (after spraying with vanillin–sulfuric acid) under 354 nm light. Fluorescence of the ecdysteroid spots gives specificity, and also increases the sensitivity of the detection.

Successful separation of ecdysteroids from each other and, also, from the contaminating compounds highly improves the reliability of their identification. Lafont et al.[15] presented an overview of the chromatographic analysis of ecdysteroids, using both paper- and TLC. The mobile phases were composed of either binary or ternary mixtures. Either a chlorinated hydrocarbon (chloroform or dichloromethane) or ethyl acetate was the organic modifier. One component was generally an alcohol (methanol, ethanol, or propanol), and sometimes water was also added to the mixture. In special cases, boron-complexing TLC[16] and RP-TLC[17] have also been used.

Some of the TLC systems contain either benzene, toluene, or acetone. These solvents have strong UV absorbance at 240–260 nm, the favorite monitoring region of high-performance liquid chromatography (HPLC) separations. However, these solvents are totally removed between TLC development and detection steps, therefore, their interference with detection is completely eliminated. Moreover, the majority of the mobile phases

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mentioned here contain water up to several percent. The water content definitely improves selectivity for certain compounds. Both the water and the ammonia (25%) can reverse the order of elution (Table 1).

ANALYSIS OF 20-HYDROXYECDYSONE THROUGH THE VEGETATION OF PLANTS

The 20-hydroxyecdysone content of plants can reach 3% of their dry weight, and can be as low as 0.004%.[18] This drastic variation is the major reason to monitor 20-hydroxyecdysone production of the plant through the vegetation period, such as at the rosette stage, stem formation, shoot formation, blossoming, and fruit ripening. To reach a proper selectivity, several mobile phases of Table 1 were checked. The (A) and (B) chromatograms did not show proper separation. System (E) retained the spots stronger than system (C), therefore, the scanning time became longer. The R_S (selectivity) and $T_{0.05}$ (tailing) values are given in Table 2 for mobile phases (C) and (D). The selectivity of (C) was lower than that of (D), but (C) gave better symmetry (Fig. 2). The final selection was based on the peak homogeneity established by in situ comparison of the UV spectra, and it resulted in the use of mobile phase (D) for routine work (Fig. 3). It is necessary to emphasize that both the preceding and the following peaks were definitely smaller than the peak of 20-hydroxyecdysone. Therefore, their interference in the quantitative evaluation was almost negligible assuming their adequate separation. Furthermore,

Table 2. Comparison of selectivity (R_S) and tailing factor ($T_{0.05}$) given by two TLC mobile phases.

	Mobile phase				
	R_S (C)		R_S (D)		$T_{0.05}$ (C)
	R_{SI}	R_{SII}	R_{SI}	R_{SII}	T_{20E}
Month and day					
May 31	1.55	0.90	1.06	1.2	1.1
June 27	0.88	1.20	1.10	1.2	1.1
July 26	0.80	1.07	1.00	1.6	1.1
August 12	2.13	1.00	1.00	1.6	1.1
September 26	0.90	1.20	1.00	1.2	1.1

Note: R_{SI} and R_{SII} are resolutions calculated from the proceeding and following components. Is the tailing factor of 20-hydroxyecdysone at 5% of its peak.

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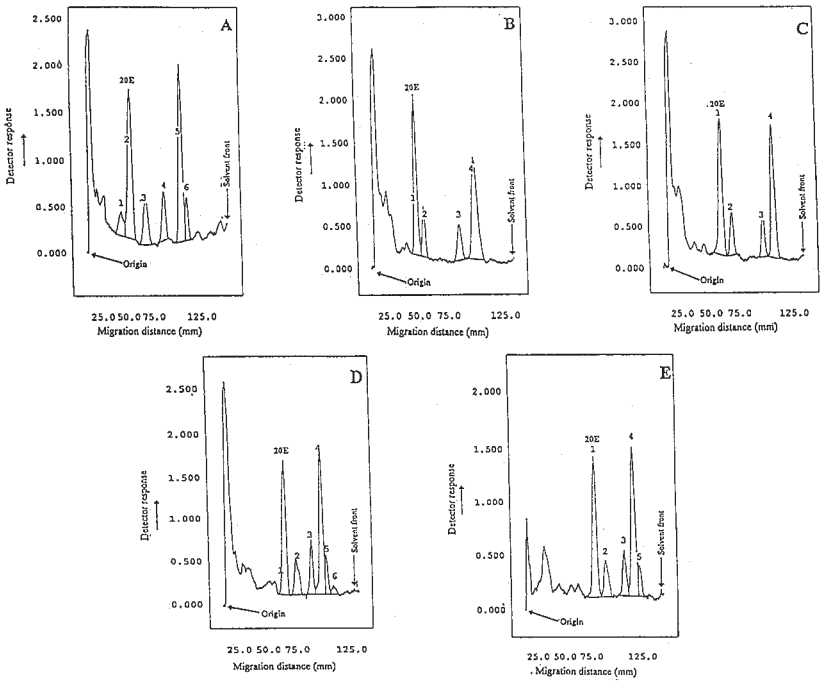

Figure 2. Densitograms of ecdysteroid separation using A, B, C, D, and E mobile phases (see text for composition). The sample was the extract of *S. otites* (L.) Wib., and the major goal was quantitative determination of 20-hydroxyecdysone (20E).

the spot of 20-hydroxyecdysone was scraped from the plate and extracted from the silica, and the purity of the dissolved 20-hydroxyecdysone was evaluated by the use of HPLC. The HPLC analysis showed the purity of 20-hydroxyecdysone to be over 93%. Thin-layer chromatography/densitometry has been used to investigate the seasonal dependence of 20-hydroxyecdysone production. According to our experience, TLC was the proper method for the determination of several hundred samples through a working day.

LIPOPHILICITY OF ECDYSTEROIDS

Lipophilicity of ecdysteroids has been investigated on paraffin-impregnated TLC silica plates.[19] The decreasing order of lipophilicity for 12 ecdysteroids was as follow: cyasterone > 22-deoxy-20-hydroxyecdysone > 2-deoxyecdysone > vitikosterone E > makisterone

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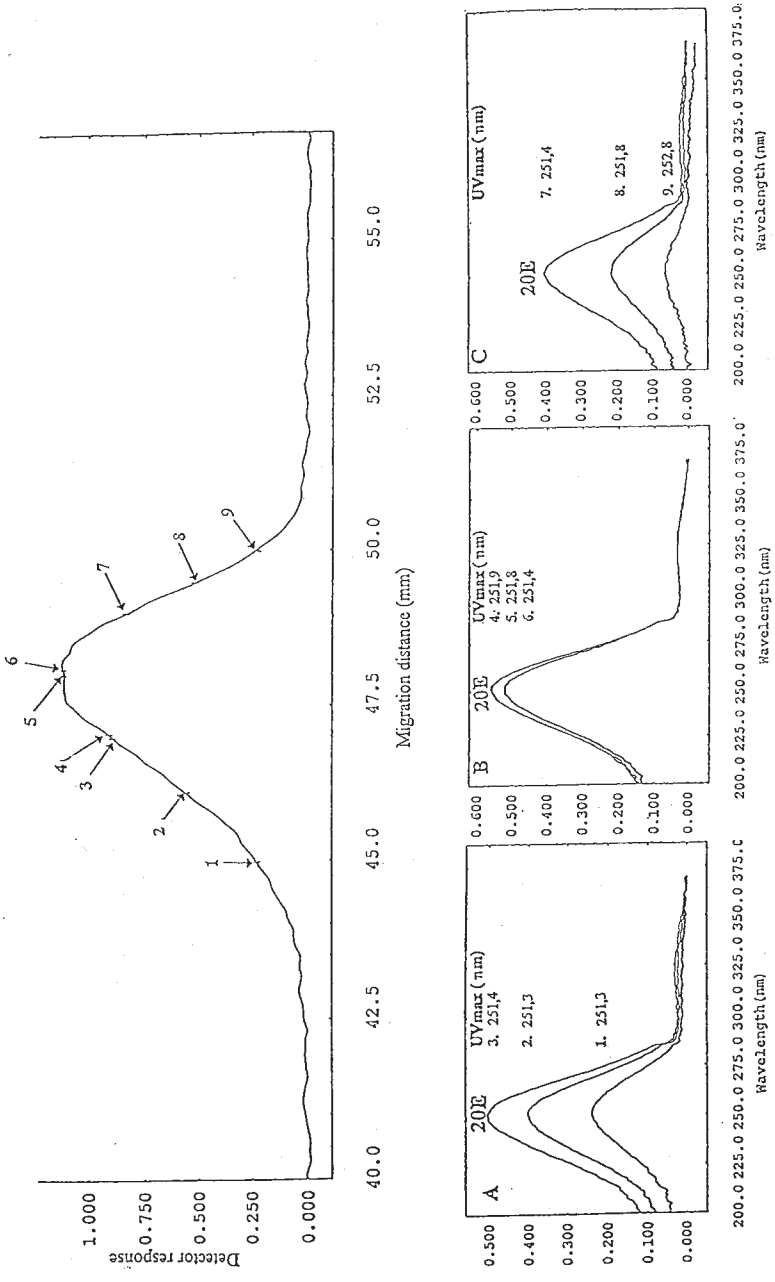

Figure 3. In situ UV absorption curves for homogeneity of the spots on TLC plates.

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A > 2-doxy 20-hydroxyecdysone > 20-hydroxyecdysone-22 acetate > rubrosterone > polypodine B > 20-hydroxyecdysone > integristerone A.

ANALYSIS OF ECDYSTEROIDS ON REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY PLATES

Reversed-phase TLC is used to complement the separation by normal-phase TLC (NP-TLC). There are several ecdysteroid pairs with no or only limited separation using NP-TLC, such as the poststerone from rubrosterone and 20-hydroxyecdysone from 22-deoxy-20-hydroxyecdysone. These pairs are adequately separated by RP-TLC, as shown in Table 3. The mobile phase used for RP-TLC gave good results. These mobile phases have also been used for the HPLC of ecdysteroids. Changing the organic modifier, the separation can be optimized (Table 3). The order of retention has remained, but the selectivity became better when tetrahydrofuran was used as the organic modifier.

Reversed-phase materials such as C18, C12, C8, C2, aminopropyl, and cyano-propyl bonded phases, as well as paraffin-impregnated TLC-silica plates, are used. One shortcoming of RP-TLC is the poor resolving power between 5β-OH and 5β-H analogs, such as when polypodine B had to be separated from 20-hyroxyecdysone.

Thin-layer chromatography is generally carried out on silica plates; therefore, RP-HPLC is followed by TLC on silica. However, RP-TLC is also a usual way to scout for the optimum of mobile phase composition for HPLC experiments. Wilson[17] examined the effect of the organic modifier (methanol) on different TLC plates. The dependence of R_F on the methanol content remained parallel for ecdysone and 20-hydroxyecdysone, even when using a wide variety of plates (ready made plates with C18 bonded stationary phase from Macherey-Nagel and Whatman; cyano-propyl and diphenyl bonded stationary phases from Merck). This means that resolution will not be improved by changing the methanol content. However, the use of RP-TLC has two major advantages: lipophilicity can be determined in an RP-TLC system in a simple and practical way, and also RP-TLC may indicate whether the effect of hydroxylation of ecdysteroids alters their physicochemical properties. The addition of a hydroxyl to ecdysone at C20 position produces 20-hydroxyecdysone, a much more hydrophilic compound than ecdysone. However, an additional hydroxyl at the 5β-position (which produces polypodine B from 20-hydroxyecdysone) results in minor or no changes in the RP-chromatographic behavior. Both the biological effects and the crystalline structure[16,20] of 20-hydroxyecdysone and polypodine B show similarities. The methanol solvate hydrate crystals of 20-hydroxyecdysone and the crystals

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Table 3. $R_F \times 100$ values of ecdysteroids using RP-18 silica gel as stationary phase.

	$R_F \times 100$ using silica gel stationary phase and mobile phases as given			
Name of the ecdysteroids	G	H	I	J
2-Deoxy-E 22-acetate	07	06	06	18
2-Deoxy-20E 22-acetate	nda	23	18	nda
2-Deoxy-E	18	19	21	47
Rubrosterone	59	52	47	66
Poststerone	44	40	35	56
2-Deoxy-20E	29	33	31	52
Muristerone	36	36	32	42
20E 22-acetate	39	38	34	56
Ecdysone	33	38	35	61
Polypodine B	48	55	49	66
20E 22-benzoate	30	nda	nda	nda
2-Deoxy-integristerone A	44	52	47	58
22-Deoxy-20E	30	34	32	58
20-Hydroxyecdysone	47	56	47	66
22-Deoxy-integristerone A	39	42	39	61
Integristerone A	55	63	56	70
5α-2-Deoxy-integristerone A	58	61	53	70
26-Hydroxy-polypodine B	59	69	61	78
2-Deoxy-20E 22-glucoside	46	53	51	69
2-Deoxy-polypodine B	24	32	30	46
Makisterone C	26	33	30	45
24(28)-Dehydromakisterone A	30	36	31	52
9α,20-Dihydroxyecdysone	38	53	46	62
Makisterone A	57	49	41	nda
5α-20E	nda	54	50	65

Note: E, Ecdysone; 20E, 20-Hydroxyecdysone; nda, no data available.

of polypodine B hydrate (with three water) comprise iso-structural pairs.[10,20] The specific side-chain rotation of the water-free crystals of 20-hydroxyecdysone has not been found to exist in the case of polypodine B.

It is almost a general rule that 5β-hydroxylation has a negligible effect on the chromatographic behavior. Ajugasterone C and its 5β-hydroxy analog, muristerone A, as well as makisterone A and its 5β-hydroxy analog, dacrysterone, show similar R_F values in any RP-TLC system. Fortunately, these 5β-hydroxylated compounds can be easily separated from the 5β-H ecdyster-

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oids when silica stationary phase is used. In certain other cases, the R_F difference depends on the plate. Using a Whatman C18 plate, integristerone A had a much higher R_F value than 20-hydroxyecdysone (integristerone A corresponds to 20-hyroxyecdysone hydroxylated on C1), while integristerone A shows a lower R_F value on the other octadecyl and cyano phases.[17]

FORCED-FLOW THIN-LAYER CHROMATOGRAPHY OF ECDYSTEROIDS

Ecdysteroids were also separated by the use of forced-flow TLC (FF-TLC), including forced-flow elution chromatography[21] and forced-flow displacement (FF-DTLC).[22] In FF-DTLC, the separation is carried out in a closed system, therefore, the effect of the vapor phase is eliminated and the development can be completed in several minutes. If the supply rate of the mobile phase was between 1.2 and 0.3 mL min^{-1}, the chromatogram was developed in 2 and 8 min, respectively. An interesting phenomenon of FF-DTLC was the generation of a double displacement front when the mobile phase supply went down.[22]

AUTOMATED MULTIPLE DEVELOPMENT THIN-LAYER CHROMATOGRAPHY OF ECDYSTEROIDS

Separation of ecdysteroids by automated multiple development (AMD) was introduced by Wilson and Lewis.[23] The method results in good separation, but is a time-consuming procedure.

TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY OF ECDYSTEROIDS

One directional separation can be carried out using either two or more developments. The basic condition is that the spot capacity has to be high enough for the separation of the spots. Wilson et al.[23,24] published two variations of the one-directional multiple development.

Double-development method in the same direction served to differentiate the radiolabeled metabolites of 20-hydroxyecdysone. Wilson and Lafont[24] looked for the insect metabolism of [^{3}H]-labeled-20-hydroxyecdysone. As biotransformation of insects can yield both hydroxylated and conjugated (phosphate and acetate conjugated) metabolites, a wide spectrum of the

polar and apolar ecdysteroids has to be separated. The first development was carried out in ethanol–ethyl acetate–water (80 : 20 : 5), while the second development (in the same direction) was carried out in chloroform–ethanol (4 : 1). The first dimensional run results in separation of polar ecdysteroids, and also impregnates the TLC plate with water. Our retrospective explanation is that the silica gel retains this water even during drying before the second run. In the second development, the ecdysteroids are subjected to partition chromatography between the water-impregnated silica and the chloroform–ethanol.

Figure 4 shows a 2D-RP-TLC ecdysteroid separation from *S. otites* (L.) Wib. extract using RP-TLC stationary phase from Merck and the mobile phases methanol–water (6 : 4) and tetrahydrofuran–water (45 : 55) in the first and second dimensional runs, respectively. The spread of the spots was good, as their location was far from the diagonal.

Two-dimensional TLC serves for the differentiation of numerous spots at the same time. The second dimensional development can multiply the spot capacity of the first dimensional run. Either the identification or screening of one substance (or one substance group) is possible, or numerous components

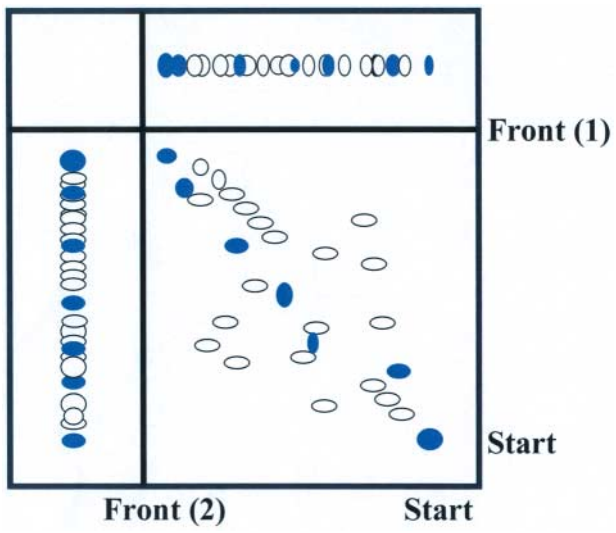

Figure 4. The employment of 2D-TLC for ecdysteroid separation. The sample was the extract of *S. otites* (L.) Wib. RP-TLC silica stationary phase of Merck was used and the mobile phases were methanol–water (6 : 4) and tetrahydrofuran–water (45 : 55) in the first and second dimensional runs, respectively.

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can be separated on a TLC plate. There are four different reasons for the use of 2D-TLC:

Identification of the major component.

Separation of ecdysteroids from the abundant amount of flavonoids and also giving an idea of the identity of the flavonoids (Fig. 5).

Detecting one component during the essential enrichment by displacement chromatography.

Finding all ecdysteroids in a plant extract or after metabolism of a radiolabelled ecdysteroid.

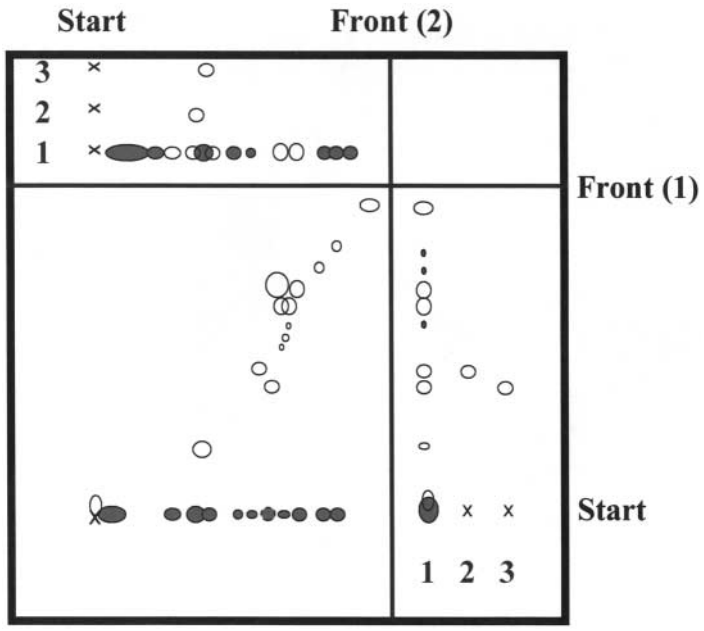

Figure 5. Sesqui-dimensional separation of components of the extract of *Serratula tinctoria* (L.). Samples were the plant extract (1), 20-hydroxyecdysone (2), and polypodine B (3). The ecdysteroids are separated, using two-dimensional TLC, from each other and also from the abundant amount of flavonoids. However, the system separates flavonoids only in the second dimensional run. Silica gel was the stationary phase, and © [toluene–acetone–ethanol (96%)–ammonia (25%) (100 : 140 : 32 : 9)] and (F) [ethyl acetat–formic acid–water (85 : 10 : 5)] mobile phases were used in the first and second dimensional developments, respectively. Open spots represent ecdysteroids, the dark spots indicate flavonoids.

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DISPLACEMENT THIN-LAYER CHROMATOGRAPHY OF ECDYSTEROIDS

Figure 6 shows displacement TLC of 20-hydroxyecdysone. Three zones are easily recognizable. Displacement TLC is taking place in the center zone (Dis). It is located in the front of the displacer front marked with a dashed line. As the displacer train is fully developed in a short distance, the displaced spot represents a well-concentrated band, free of any impurity. By changing the carrier, different compounds can be part of the displacement train. Here, 20-hydroxyecdysone was subjected to displacement chromatography in the second dimension. The other two zones contain the eluted components. Some components can be eluted with the carrier itself (El2), and their migration is faster than that of the displacer front, while other components show very limited migration. These components are neither eluted with the carrier nor displaced by the displacer front, but they remain behind the displacer front, either at the start or eluted with the displacer in the carrier (they are behind the

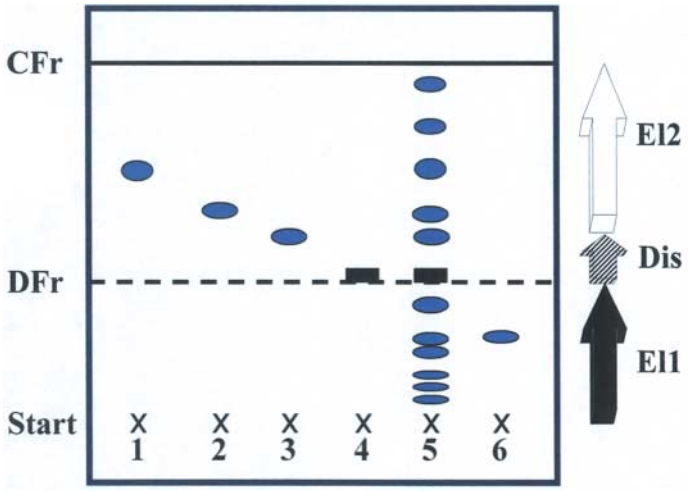

Figure 6. Displacement TLC using silica gel stationary phase and dichloromethane–*n*-propanol–dimethylaminopropylamine (80 : 30 : 5) mobile phase. The spots were 2-deoxyecdysone (1), 2-deoxy-20-hydroxyecdysone (2), 20-hydroxyecdysone 22-acetate (3), 20-hydroxyecdysone (4), extract of *S. otites* (L.) Wib. (5), and integristerone A (6). CFr and DFr indicate the front of carrier and displacer, respectively. Three distinct zones characterize displacement chromatography, such as the center zone (Dis) where the displacement is taking place; components eluted with the carrier itself (El2); components eluted with the displacer in the carrier (El1).

displacer zone, El1). Changing either the carrier or the displacer concentration (not shown here), the majority of the substances can be placed in the displacement train.

HYPHENATED TECHNIQUES

The most usual combination is TLC separation with UV absorbance detection. Certain sophisticated devices are able to produce the UV spectrum of the spots.[25] The recorded spectrum serves an important function, it confirms the suitability of the TLC system. Therefore, the complete UV spectra of both the sample and the standard are recorded directly on the TLC plate. Using silica gel stationary phase and chloroform–methanol–benzene (25 : 5 : 3) as the mobile phase, 20-hydroxyecdysone was separated from the contaminating flavonoids, and the TLC system was suitable for the direct determination of 20-hydroxyecdysone in the extract of *S. otites* (L.) Wib.

The off-line method[24] was based on removing a small area from the TLC stationary phase, mulling with the co-solvents glycerol and dimethyl sulfoxide, and applying to the surface of a FAB (fast atom bombardment) target. TLC separated various peaks from the *S. otites* extract. Glass-backed HPTLC plates developed with chloroform–ethanol (4 : 1) were used. The peaks were subjected to FAB-MS (fast atom bombardment mass spectrometry), and the chemical structures were elucidated. Another example presented was the TLC separation of *S. otites* extract and use of negative FAB-MS. Although they used the most sophisticated methods (Wilson's laboratory remains the sole place to use FAB-MS for ecdysteroid research), location of ecdysteroid spots was definitely supported by their R_F values on the TLC silica plate.

Wilson et al. used two on-line methods combined with TLC. Both directly coupled mass spectrometry[26,27] and on-line NMR spectroscopy[28] were successfully used for either identification or tracing the minor ecdysteroids behind the major ecdysteroid components separated on the TLC plates. In the on-line TLC-MS,[27] a VG Analytic 70SEQ tandem mass spectrometer (Manchester, UK) was used. The separated ecdysteroids were located by their UV absorbance on TLC plates with fluorescent indicator (aluminum-backed HPTLC silica gel was used, and the mobile phase was chloroform–ethanol, 4 : 1).

CONCLUSIONS

Thin-layer chromatography is an essential tool for the qualitative and quantitative analysis of ecdysteroids. The method is easy, flexible, and reliable.

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Special features of TLC include that specific detection permits unequivocal location of ecdysteroid spots, cleanup before TLC can be partially or totally neglected, and several spectroscopic methods can be combined off- or on-line with TLC.

Special modes of development can also be carried out, including pretreatment of the stationary phase, the use of FF-TLC with overpressured layer chromatography (OPLC) set-up, and also displacement type of developments.

Thin-layer chromatography has kept its role as an essential method to identify ecdysteroids. The spots can be located on the TLC plate by the use of a specific procedure, which differentiates the ecdysteroids from any other compounds.

The chemical structure of the ecdysteroids identified by TLC can be confirmed or rejected by the use of other methods (mainly HPLC, but sometimes capillary electrophoresis), and also by the employment of various spectroscopic methods, such as NMR, MS, x-ray, etc. However, both the HPLC and the spectroscopic methods are rather preferred to TLC when minor ecdysteroids are to be looked for and isolated. These ecdysteroids are present in trace amount (e.g., under 0.001%), and, therefore, the sample requirement for TLC could take the majority of their gross amount.

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