Journal of Chromatography A, 658 (1994) 31–53
Elsevier Science B.V., Amsterdam

CHROMSYMP. 2905

Review

Chromatographic procedures for phytoecdysteroids

R. Lafont

Departement de Biologie, Laboratoire de Biochimie, CNRS URA 686, École Normale Supérieure, 46 Rue d'Ulm, 75230 Paris Cedex 05 (France)

E.D. Morgan* and I.D. Wilson

Department of Chemistry, Keele University, Keele, Staffordshire, ST5 5BG (UK)

ABSTRACT

The complexity of the mixtures of ecdysteroids in plants and the close similarities in their chemical structures have challenged chemists to find suitable ways to separate and identify them. Great ingenuity has been applied to these problems and consequently a wide range of separation and methods are available today. These methods have been reviewed with assessment of their strengths and limitations, with the intention to guide investigators towards the methods most useful to their purpose.

CONTENTS

* Corresponding author.

0021-9673/94/$26.00
SSDI 0021-9673(93)E0838-L

1. INTRODUCTION

The short period between 1965 and 1967 was one of remarkable advances in the study of the insect moulting hormones or ecdysteroids. In 1965, eleven years after their initial isolation of the compound, Karlson *et al.* [1], using an improved isolation procedure, obtained sufficient crystalline ecdysone from silkworm pupae for Huber and Hoppe to determine that it was a polyhydroxysterol [2]. The next year Hampshire and Horn [3] showed their crustecdysone, isolated from a crayfish, *Jasus lalandii* was 20-hydroxyecdysone and Hocks and Wiechert [4] isolated 20-hydroxyecdysone from the silkworm *Bombyx mori*, but more important for our present subject, the data becoming available made others realize that compounds they were examining from plants had the same or similar structures. Very quickly Nakanishi *et al.* [5] reported ponasterone A from the leaves of the coniferous tree *Podocarpus nakai*, and Galbraith and Horn [6] isolated 20-hydroxyecdysone from the Australian pine *Podocarpus elatus*. In the following year Takemoto *et al.* [7] isolated 20-hydroxyecdysone and inokosterone from the common Japanese weed *Achyranthes fauriei* and Jizba *et al.* [8] reported the isolation of 20-hydroxyecdysone from the roots of the fern *Polypodium vulgare*.

Thus began the remarkable series of investigations of plants for ecdysteroids, which still continues today. Galbraith and Horn [6] suggested in their paper that the ecdysteroids in plants, or *phytoecdysteroids* may protect plants from insect attack. Thus, too began the controversy, still unsettled, over the function, if any, of ecdysteroids in plants.

The isolations referred to above were all carried out using solvent extractions, partitions and low-pressure column chromatography of various kinds, with only a little help from thin layer chromatography. The best of these methods are listed here. The most efficient methods of separation and the most sensitive methods of detection were developed subsequently, largely stimulated by the search through insect and plant materials for ecdysteroids.

2. SAMPLE PREPARATION

2.1. General considerations

The ecdysteroids form a group of rather polar compounds and as a consequence the initial extraction step is best performed using a polar solvent such as methanol (MeOH). Alternative solvents include ethanol (EtOH), acetone, acetonitrile and methanol–water mixtures. Supercritical fluid extraction would also be adequate, but it was not used up to now in this case. Having obtained and concentrated an extract, the next step usually involves one or more solvent partitions with the aim of removing the bulk of polar and non-polar contaminants prior to chromatography (Fig. 1).

2.2. Partition techniques

2.2.1. Solvent partitioning

In the early studies [9], the initial partition was made between an aqueous concentrate and *n*-butanol (BuOH). The BuOH residue, into which the ecdysteroids were extracted, was then partitioned between aqueous MeOH and hexane to remove non-polar material such as lipids. However, reversing this order of operation was found to be beneficial and led to reduced emulsion formation during the partition step and fewer problems with frothing during evaporation.

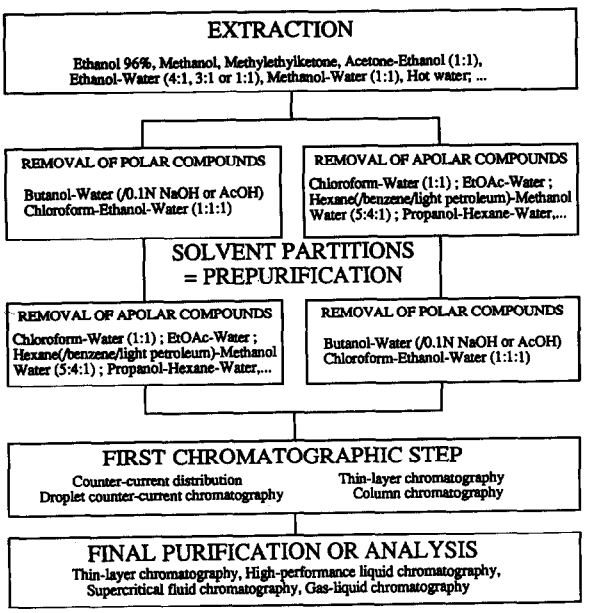

Fig. 1. General extraction and purification chart for ecdysteroids.

Other suitable partitioning systems for removing lipids include hexane–aqueous methanol (7:3, v/v) or light petroleum (b.p. 40–60°C)–aqueous methanol. Mixtures of water–propanol (PrOH) (3:1, v/v) and hexane can also be used to remove non-polar contaminants, the ecdysteroids remaining in the aqueous phase. The addition of $(NH_4)_2SO_4$ to promote the formation of the two phases may be required with the PrOH mixture. The separation of polar impurities from the ecdysteroids can be achieved by partition between water and BuOH (ecdysteroids partition into the organic phase) and water and ethyl acetate (EtOAc) (ecdysteroids remain in the aqueous phase).

The major factors governing the choice of solvent partition system are the type of contaminants to be removed (*i.e.* mainly lipids or mainly polar, etc.) and the nature of the ecdysteroids to be isolated. Thus the addition or removal of one OH group, or conjugation to polar (*e.g.* sulphate) or non-polar (*e.g.* acetate or fatty acyl) groups can significantly affect partition ratios. Partition coefficients of representative ecdysteroids and precursors in a number of systems are given in Table 1.

TABLE 1

PARTITION COEFFICIENTS OF ECDYSTEROIDS AND VARIOUS PRECURSORS

K = Concentration in the non-polar phase/concentration in the polar phase.

Ecdysteroid	K	Ref.
Cyclohexane–n-butanol–water (5:5:10)		
Ecdysone	3.54	71
Makisterone A	1.27	72
20-Hydroxyecdysone	0.52	71
3-Epi-20-hydroxyecdysone	0.52	72
26-Hydroxyecdysone	0.39	73
20,26-Dihydroxyecdysone	0.06	72
n-Butanol–water (1:1)		
Ecdysone	*ca.* 10	74
20-Hydroxyecdysone	5.3	9
Ethyl acetate–water (1:1)		
2,22-Dideoxyecdysone	20	116
2-Deoxyecdysone	4	
Ecdysone	0.4	
20-Hydroxyecdysone	0.1	
Chloroform–methanol–water (2:1:1)		
2,22-Dideoxyecdysone	13	116
2-Deoxyecdysone	2.7	
Ecdysone	0.4	
20-Hydroxyecdysone	0.1	
Chloroform–ethanol–water (1:1:1)		
Ecdysone	4.6	116
20-Hydroxyecdysone	1.5	
Chloroform–water (1:1)		
2,22,25-Trideoxyecdysone (Ketodiol)	90	116
2,22-Dideoxyecdysone	20	
2-Deoxyecdysone	2.9	
Ecdysone	0.06	
20-Hydroxyecdysone	0.015	
Hexane–acetonitrile (1:1)		
Cholesterol	1.5	116
2,22,25-Trideoxyecdysone	0.06	
2,22-Dideoxyecdysone	<0.01	
2-Deoxyecdysone	<0.01	
Ecdysone	<0.01	

2.2.2. Liquid–liquid partition techniques

2.2.2.1. Counter-current distribution (CCD). Counter-current distribution between BuOH and water is effective for removing polar contami-

nants (the addition of a small amount of salt reduces emulsion formation) and $CHCl_3$–MeOH–water or hexane–PrOH–water.

This technique, as is the case for all other liquid–liquid partitions systems, has the advantage of a 100% sample recovery. The technique is not of wide use at the present time. It has been advantageously replaced by the more recent DCCC and RLCC techniques (see below).

2.2.2.2. Droplet counter-current chromatography (DCCC). This technique provides an efficient means for purifying samples up to the gram range. It belongs to the family of liquid–liquid partition chromatographic methods.

DCCC equipment is compact, it can be used with rather crude samples, and in favourable cases it can allow the preparation of "pure" compounds [10–13]. In our hands, it seemed however to require a subsequent HPLC step in order to get fully pure ecdysteroids. Several systems in the ascending or descending mode have been described (Table 2).

Rotation locular counter-current chromatography (RLCC) was designed as an alternative to DCCC [14]. It was applied to the purification of *Vitex strickeri* methanolic extracts [15], in combination with recycling HPLC.

Although efficient, DCCC and RLCCC are rather time-consuming, and processing a single sample usually requires 1–5 days [14]. This is because of the need to allow good exchange to proceed between the mobile droplets and the stationary phase. To overcome this drawback, HSCCC (high-speed counter-current chromatography) was proposed [14]. In that case, the column is a multi-layer coil and efficient mixing is achieved through planetary rotation. Centrifugal force replaces normal gravity and allows separation to be achieved in hours rather than days.

2.3. Low-pressure column chromatography

2.3.1. Analytical scale: disposable cartridges for sample clean-up

2.3.1.1. Normal-phase cartridges. The use of low pressure chromatography on a small column has been used very early in phytoecdysteroid research for the fractionation of crude extracts. It was at that time either silica or alumina which was used in such columns (normal phase systems), generally eluted with binary mixtures —a step-gradient of alcohol in chloroform or benzene. Use of these disposable cartridges is now generally called solid-phase extraction (SPE).

2.3.1.2. Reversed-phase cartridges. The availability of hydrophobic phases (resins like Amberlite or hydrocarbon-bonded silica) had led to a complete renewal of the procedures [16]. Moreover, the design of small cartridges or syringes containing 0.2–1 g of HPLC phase has led to an ideally suited material for a rapid clean-up of small samples.

Among them, Sep-Pak cartridges from Waters are the most widely used (Fig. 2 [17–21]. In fact their use has been extended not only for biological extracts but also for desalting purposes, *e.g.* direct adsorption of ecdysteroids from cul-

TABLE 2

SOLVENT SYSTEMS FOR DROPLET COUNTER-CURRENT CHROMATOGRAPHY

A = Ascending mode; D = descending mode.

Solvent system	Mode	Ref.
$CHCl_3$–MeOH–water (13:7:4)	A	10, 11, 13, 50, 75
$CHCl_3$–C_6H_6–EtOAc–MeOH–water (45:2:3:60:40)	D	23
C_6H_6–$CHCl_3$–MeOH–water (5:5:7:2)	D	24
$CHCl_3$–MeOH–water (65:20:20)	D	24, 50

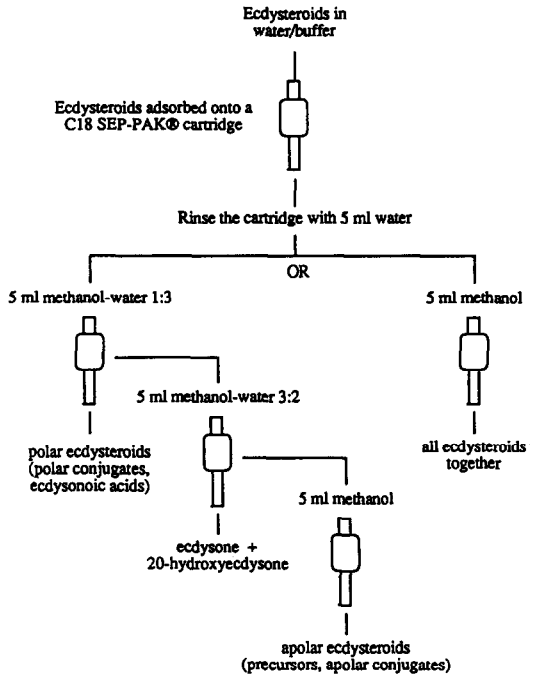

Fig. 2. Utilization of reversed-phase cartridges for ecdysteroid purification.

ture media or from reverse phase HPLC fractions containing an involatile buffer.

The use of C_{18} cartridges may be extended to more refined separations, provided that rigorous protocols are used regarding solvent composition and volume. By that way, it appears possible to separate ecdysteroids that differ by the presence or absence of a single –OH group (provided that its position on the molecule evokes a significant polarity change).

2.3.1.3. Immobilized phenylboronic acid (PBA) cartridges. Recently, cartridges filled with immobilized phenylboronic acid on silica, agarose or cellulose have become available. Such systems are able to retain selectively some compounds bearing vicinal diols. Ecdysteroids may contain such diol functions, *i.e.* at C2–C3, C20–C22 and/or C25–C26. Up to now, experiments have been made with compounds bearing no 26-OH, and therefore were limited to the two first cases [22].

It appears that the 2,3-diol does not readily form cyclic boronates (at least with a 2-OH_{ax} and a 3-OH_{eq}), and the adsorption process is essentially determined by the presence or absence of the 20,22-diol, *e.g.* it is particularly efficient to distinguish between ecdysone and 20-hydroxyecdysone series. It is thus possible to adsorb ecdysteroids and to elute them sequentially, first ecdysone-type and then 20-hydroxyecdysone-type compounds (Fig. 3).

This procedure can be combined with other solid phase systems to separate various classes of ecdysteroids [22] and it could have several specific developments.

2.4. Preparative scale

Low-pressure column chromatography uses either normal phases or (less often) reversed phases (Table 3). The size of the column has to be related to the size of samples (at least ten times the dry weight of the sample), and elution is usually performed with a step-gradient. Most often, silica or alumina are used with a step-gradient of methanol or ethanol in a chlorinated solvent (chloroform or methylene chloride). In the reversed-phase mode, Sephadex LH-20 may be used to remove non-polar contaminants, and polyamide is used specifically to remove yellow pigments.

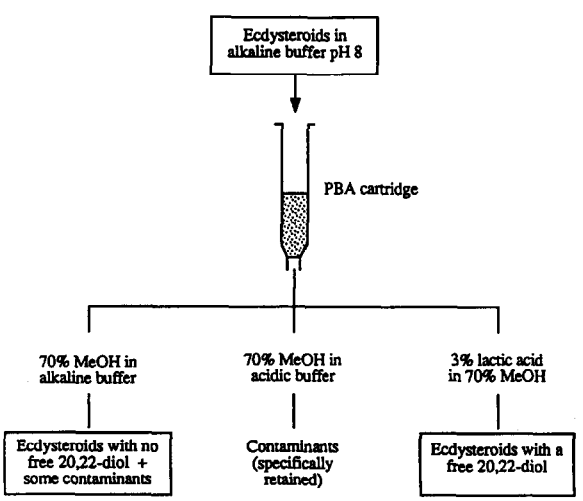

Fig. 3. Extraction procedure for ecdysteroids using immobilized phenylboronic acid cartridges.

TABLE 3

USE OF LOW-PRESSURE CHROMATOGRAPHY FOR MEDIUM- OR LARGE-SCALE PURIFICATION

Type of stationary phase	Solvent system	Ref.
Normal phases		
Silica (silica gel, silicic acid or celite)	$CHCl_3$–MeOH (step gradient)	76
	$CHCl_3$–MeOH (step gradient)	77
	$CHCl_3$–MeOH (step gradient)	78
	$CHCl_3$–MeOH (step gradient) (+1% CH_3COOH)	79
	$CHCl_3$–MeOH (8:2)	80
	$CHCl_3$–MeOH (95:5)	81
	$CHCl_3$–MeOH (100:3)	82
	$CHCl_3$–EtOH (19:1)	83
	Me_2CO–CH_2Cl_2–water (2:8:1), then EtOH	84
	$CHCl_3$–MeOH–water (8:2:1 or 7.5:2:1, lower phase)	85
	CH_2Cl_2–EtOH (step gradient)	49
	EtOAc	86
	EtOAc–MeOH (step gradient)	87
Alumina (containing usually 10% water)	$CHCl_3$–MeOH (step gradient)	77
	$CHCl_3$–MeOH (step gradient)	78
	$CHCl_3$–MeOH (step gradient)	88
	$CHCl_3$–MeOH (step gradient)	89
	$CHCl_3$–MeOH (2:1)	90
	$CHCl_3$–EtOH (step gradient)	91
	CH_2Cl_2–EtOH (step gradient)	92, 93
	EtOAc–EtOH (step gradient)	94
	Me_2CO–CH_2Cl_2–water (62.5:15:10)	84
	CH_2Cl_2–EtOH (9:1)	95
	EtOAc–MeOH (1:1)	76
	EtOAc–EtOH (1:1)	83
	EtOAc–EtOH (2:1)	96
Sephadex LH-20	CH_2Cl_2–Me_2CO	83, 97
	CH_2Cl_2–MeOH (step gradient)	83, 97
	$CHCl_3$–EtOH (88:12)	96
Reversed-phases[a]		
Amberlite XAD-2 (medium-pressure LC)	Water–MeOH (step gradient)	98, 99
Sephadex LH-20	EtOH–water (7:3)	81
	MeOH	85, 92, 93
Celite impregnated with BuOH + cyclohexane	Water (saturated with BuOH + cyclohexane)	76
Polyamide (for removal of flavonoids)	Water	80, 93, 100

[a] RP is used in a broad sense, to include less conventional systems.

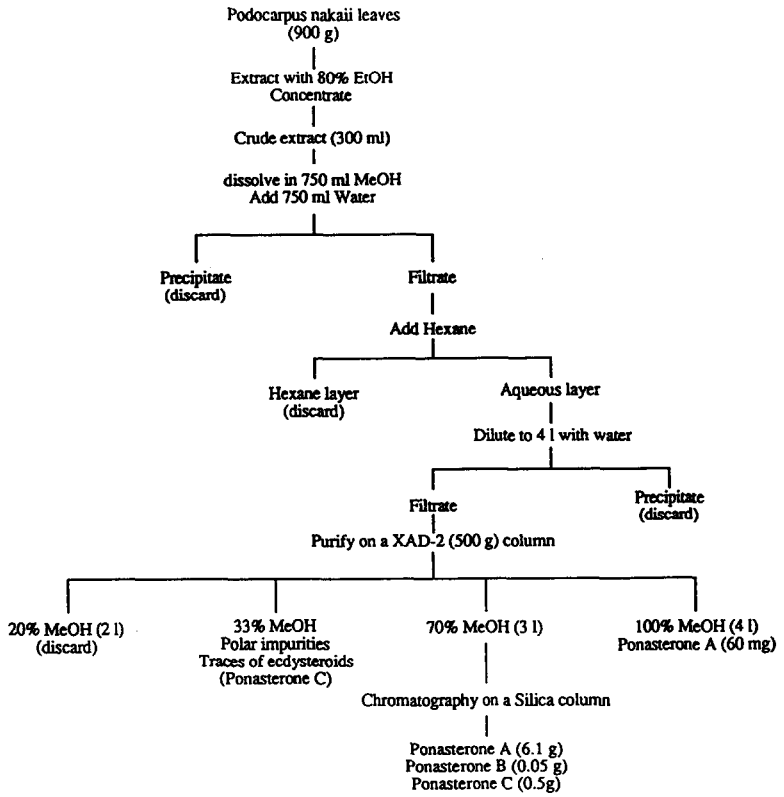

Fig. 4. Isolation of ecdysteroids from *Podocarpus nakaii* leaves [16].

2.5. Selected examples of protocols

Some representative protocols are given in Figs. 4–7, that each use a part of the above techniques. Other complex processing schemes can be found elsewhere [23–25].

3. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC is the most popular technique for ecdysteroid separations, both for analytical and preparative purposes. It offers wide choice of techniques, that are adapted for polar or non-polar metabolites. The identification of any ecdysteroid by co-migration with a reference compound must rely on the simultaneous use of several (at least two) different HPLC systems, generally one normal-phase and one reversed-phase system. Given the very large ecdysteroid family, there is no guarantee that these criteria provide unambiguous evidence for the identity of a given compound.

When facing a separation problem it is possible to make a logical choice, according to the results of a preliminary TLC step. Current HPLC techniques have been designed about ten years ago and the HPLC of ecdysteroids may now be considered to be a mature technique (for reviews see refs. 26–31).

3.1. Correspondence between TLC and HPLC

It is known that relationships between TLC and HPLC may help to design adequate HPLC solvent systems. The migration of a given compound by TLC is given by its R_F (=retardation factor). Another parameter also used for TLC is the R_M, defined as

$$R_M = \log(1/R_F - 1)$$

Any change on a molecule evokes a change of

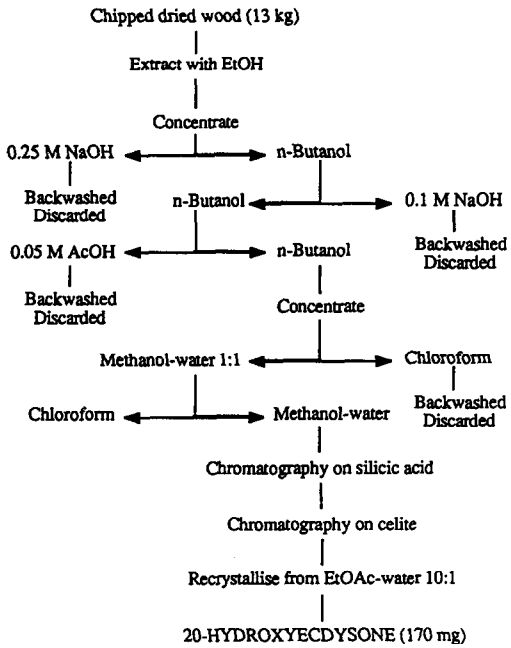

Fig. 5. Isolation of 20-hydroxyecdysone from *Podocarpus* bark [115].

the R_F or R_M and the same change on two molecules of the same family may evoke the same ΔR_M. Accordingly, the ΔR_M values are cumulative, and two independent changes 1 and 2 evoke a final $\Delta R_M = \Delta R_{M1} + \Delta R_{M2}$.

When using HPLC, the behaviour of a given compound is expressed by its retention time (or volume) t_R or better by its capacity factor k', defined as $k' = (t_R - t_0/t_0)$, where t_0 is the retention time of a compound eluted in the void volume. The correspondence with TLC is easy to make, as

$t_R = t_0/R_F$, $k' = (1/R_F - 1)$ and thus $R_M = \log k'$

Therefore, provided that the chromatographic conditions and the phases are the same, it is easy to deduce the HPLC behaviour from the TLC data, and the relationship between R_F and k' is exemplified in Fig. 8. It is clearly apparent from it that HPLC solvents might be chosen from among those giving R_F between 0.1 and 0.3, in order to avoid a too rapid elution or extended analysis times.

Moreover, the selectivity factor α for a pair of compounds 1 and 2 analyzed by HPLC can be defined as $\alpha = k'_2/k'_1$, and from this

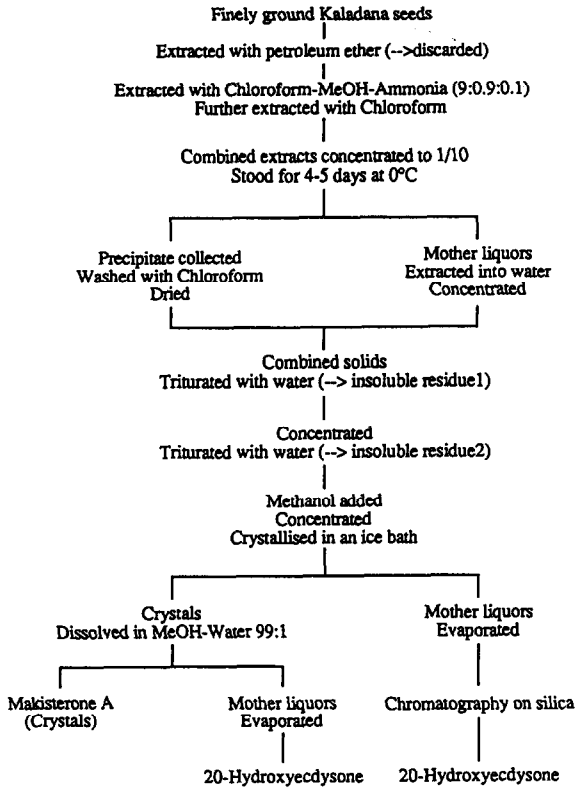

Fig. 6. Isolation of ecdysteroids from *Kaladana* seeds [108].

$\Delta R_M = \log k'_2 - \log k'_1 = \log(k'_2/k'_1)$, or

$\Delta R_M = \log \alpha$

The consequence of this is that a given modification (*e.g.* presence or absence of a given –OH group) evokes a similar effect on various compound pairs; for instance the α value for the ecdysone:20-hydroxyecdysone pair is the same as for the 2-deoxyecdysone:2-deoxy-20-hydroxyecdysone one, provided of course that *isocratic* conditions are used. Moreover, it seems possible to determine the expected retention time of a compound where no reference is available [32]. Similar calculations apply to acetates, conjugates, etc. and may prove useful in many cases.

3.2. Chromatographic procedures (Table 4)

3.2.1. Normal-phase systems

Normal-phase (NP) systems generally use silica columns (sometimes aminopropyl- or diol-bonded columns) and a standard system of

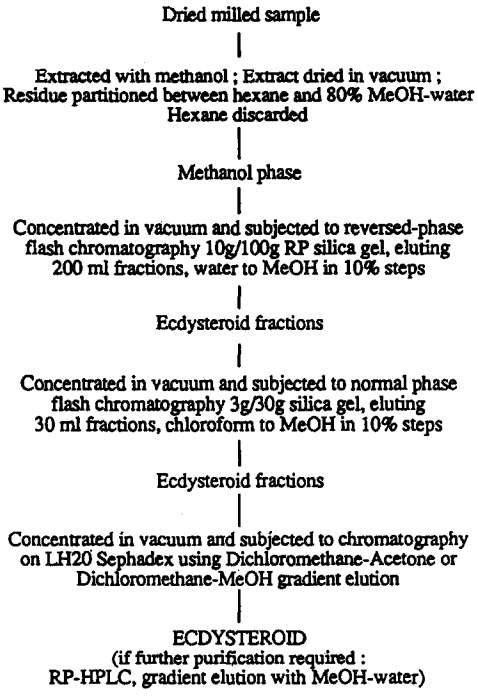

Fig. 7. Isolation of ecdysteroids from plants (after Russel and Greenwood [97]).

dichloromethane–isopropanol–water mixtures as initially designed by Lafont *et al.* [33]. The respective proportions of the three components can vary, according to sample polarity. Thus, specific ternary mixtures can be prepared for non-polar compounds, *e.g.* acetates or ecdysone precursors (*e.g.* 125:15:1, v/v/v) for medium-polarity compounds (125:25:2 or 125:30:2 for ecdysone and 20-hydroxyecdysone) or for more polar ecdysteroids (125:40:3 for 26-hydroxy-ecdysteroids; 100:40:3 for glucosides).

With normal-phase systems, k' is particularly

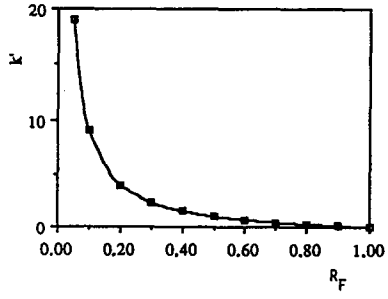

Fig. 8. Inverse relationship between k' (HPLC) and R_F (TLC).

TABLE 4

CHROMATOGRAPHIC SYSTEMS COMMONLY USED FOR THE HPLC ANALYSIS OF ECDYSTEROIDS

See also ref. 26 for a more extensive review of data before 1980.

Mode of chromatography	Ref.
Normal-phase chromatography (silica or diol-, aminopropyl or TMS-bonded silica)	
Chloroform–95% aqueous ethanol	101
Chloroform–ethanol (gradient)	96
Chloroform–isopropanol (gradient)	81
Chloroform–methanol	79
Dichloromethane–tetrahydrofuran–methanol	42
Dichloromethane–methanol	42
Dichloromethane–ethanol–water	33
Dichloromethane–isopropanol–water	33
Dichloromethane–methanol–water–acetic acid	103
Dichloroethane–methanol–isopropanol	
Hexane–ethanol–methanol–acetonitrile	104
Isooctane–isopropanol–water	105
Isooctane–isopropanol–water (with grad. methanol)	38
Cyclohexane–isopropanol–water	32
Reversed-phase chromatography [Amberlite XAD-2 or (mainly) C_{18} bonded silica]	
Ethanol–water	98
Methanol–water	16
Methanol–water	106
Methanol–water	39
Methanol–water	85
Acetonitrile–water	107
Acetonitrile–water	81
Acetonitrile–water	39
Acetonitrile–methanol–water	75
Acetonitrile–0.1% TFA	75
Dioxan–water	39
Tetrahydrofuran–water	39
Methanol–triethylammonium phosphate + 1-butanesulfonic acid	95
Isopropanol–water, 50°C	108
Recycling HPLC (Asahipack GS-320)	
Methanol	15, 40

sensitive to temperature. Low temperatures may result in greatly *decreased* retention times, possibly because they reduce ecdysteroid solubility in the stationary water phase adsorbed onto the silica particles. This effect is particularly striking between 10°C and 20°C. As a consequence, it is highly recommended to place the HPLC system in a room where temperature is controlled.

Polar-bonded columns (diol or aminopropyl

silica) can also be used instead of silica ones [27,34]. Diol-bonded columns have been used with *Chenopodium album* and *Kochia scoparia* (Chenopodiaceae) extracts [35,36] whereas aminopropyl ones proved particularly efficient for the separation of mixtures of 3α-OH, 3β-OH and 3-oxoecdysteroids [37] —indeed these separations were better than those achieved on silica columns [3]. In addition, bonded phases allow the use of gradients without the problems linked with long re-equilibration times encountered with silica columns.

Non-polar bonded-phase columns can also be used with the above solvents, and they provide somewhat efficient separations with reduced retention times [38]. In this respect, trimethylsilane (TMS) bonded phases seem particularly interesting: they give very symmetrical peaks and a selectivity that differs from silica columns. The retention of ecdysteroids on non-polar bonded columns results from the presence of remaining free silanol groups on bonded phases (this becomes especially evident when columns have previously been used over a long period) which could in theory permit both NP- and RP-HPLC with a single column! There exist some HPLC columns (cyanopropyl or nitrile-bonded) that have been designed both for NP and RP purposes and these might probably be used for normal-phase chromatography of polar (but of course non-ionic) ecdysteroids.

Dichloromethane-based solvents, although very efficient for chromatographic separations, suffer from a high UV-cutoff and quenching properties of this compound, which preclude the use of diode-array detectors or in-line radioactivity monitors. This problem may be overcome with isooctane-based mixtures [29] which in counterpart suffer from a lower efficiency (plate number) and poor ecdysteroid solubility, which may become inconvenient for (1) polar ecdysteroids and (2) preparative purposes. The selectivity of such solvent systems is also very different from that of dichloromethane-based ones. More recently, [32] cyclohexane-based mixtures were tested: they provide much better solubilities and allow the use of diode-array detectors. On the other hand, these mixtures display a significantly higher viscosity and hence working pressure, a problem that can be overcome by increasing solvent temperature.

3.2.2. Reversed-phase systems

Reversed-phase HPLC with C_{18}-bonded columns is the most widely used system, and it provides efficient separations. Methanol-water mixtures are usual, and they represent the easiest way to obtain satisfactory separations, even if they are not the most efficient ones. Other water-miscible organic solvents may equally be used, and acetonitrile provides the advantage of forming mixtures with water that have a much lower viscosity. On the other hand, 50% methanol is a much better solvent than 20–25% acetonitrile for preparative purposes.

Selectivity linked with the nature of the organic phase is an important parameter for determining the most adequate solvent mixture for a given separation: depending upon the specific problem, better results may be obtained either with methanol, acetonitrile or THF [32,39].

Recycling HPLC using methanol as eluent may provide an interesting method for the resolution of closely migrating compounds and it was successfully used for the separation of ecdysteroids from the bark of *Vitex strickeri* [15]. This method appears to have several advantages, regarding its ability to separate closely related compounds at the preparative scale and its low solvent consumption [40].

4. PLANAR CHROMATOGRAPHY

Planar chromatography, particularly thin-layer chromatography (TLC) has been used extensively for the qualitative analysis and isolation of phytoecdysteroids. Paper chromatography, whilst now generally considered to be obsolete, has been used for the separation of ecdysteroids [41] and for completeness these results are presented in Table 5.

NP-TLC systems using silica as stationary phase have most frequently been used to separate ecdysteroids. A widely used general solvent system has been chloroform–ethanol (4:1, v/v) with a single ascending development, however, many normal phase solvent systems have been described for these and details of a number of

TABLE 5

R_F OF ECDYSTEROIDS ON PAPER CHROMATOGRAPHY [41]

Solvent systems are prepared by saturating the non-polar component (A) with the mixture (B + C) of polar solvents.

Compound	Solvent system			Ratio B:C	R_F
	A	B	C		
Ecdysone	Benzene	propan-2-ol	water	55:45	0.69
	Toluene	propan-2-ol	water	50:50	0.34
	Toluene	butan-2-ol	water	50:50	0.79
	Water	butan-1-ol		Saturated[a]	0.68
20-Hydroxyecdysone	Benzene	propan-2-ol	water	55:45	0.34
	Benzene	butan-2-ol	water	55:45	0.29
	Toluene	butan-2-ol	water	50:50	0.45
	Toluene	butan-2-ol	water	60:40	0.29
	Toluene	propan-2-ol	water	50:50	0.12
	Water	butan-1-ol		Saturated[a]	0.80
	Butan-1-ol	water		Saturated[a]	0.86
20-Hydroxyecdysone 2-acetate	Toluene	propan-2-ol	water	50:50	0.46
Inokosterone	Benzene	propan-2-ol	water	55:45	0.45
	Benzene	butan-2-ol	water	55:45	0.38
	Toluene	butan-2-ol	water	50:50	0.53
	Toluene	butan-2-ol	water	60:40	0.37
	Toluene	propan-2-ol	water	50:50	0.15
	Water	butan-1-ol		Saturated[a]	0.74
	Butan-1-ol	water		Saturated[a]	0.88
Makisterone A	Benzene	propan-2-ol	water	55:45	0.52
	Toluene	propan-2-ol	water	50:50	0.22
	Toluene	butan-2-ol	water	50:50	0.64
Ponasterone A	Benzene	propan-2-ol	water	55:45	0.93
	Toluene	propan-2-ol	water	50:50	0.74
	Toluene	butan-2-ol	water	50:50	0.92

[a] First liquid saturated with the second.

solvent systems used to separate the ecdysteroids are given in Table 6 [42]. The R_F values obtained for some 20 compounds in the chloroform-ethanol (4:1) solvent system are provided in Table 7 [43]. Following separation by NPTLC the detection of ecdysteroids on TLC plates can be accomplished in a number of ways. Where the plates incorporate a suitable fluorescence indicator that can provide a general, if rather non-specific method of detection. Spray reagents have been used to provide a higher degree of specificity, and the vanillin-sulphuric acid reagent has been particularly widely employed. This reagent gives a range of colour with different ecdysteroids as indicated in Table 8. The colours produced range from pink (*e.g.* cyasterone) and red (*e.g.* 20-hydroxyecdysone) to dark green (*e.g.* ecdysone), but like many such colour reactions are subject to quite wide inter-laboratory variations. Table 8 also lists a number of additional chromogenic reactions which have been utilised for these compounds. Scanning densitometry now provides a convenient method for obtaining *in situ* UV spectra, and this can provide increased confidence in the identification of sample components as ecdysteroids (*e.g.* see

TABLE 6

SOLVENT SYSTEMS FOR TLC OF ECDYSTEROIDS ON SILICA GEL

Data from ref. 42.

Solvent system	Composition	R_F	
		Ecdysone	20-Hydroxyecdysone
$CHCl_3$–95% aqueous EtOH	7:3	0.39	0.34
$CHCl_3$–95% aqueous EtOH	13:7	–	0.31
$CHCl_3$–EtOH	3:2	–	0.5
$CHCl_3$–MeOH	9:1	0.10	0.07
$CHCl_3$–MeOH	5:1	–	0.23
$CHCl_3$–MeOH	3:2	–	0.40
$CHCl_3$–MeOH	1:1	–	0.55
$CHCl_3$–MeOH–25% aqueous NH_3–water	12:7:1:1	–	–
$CHCl_3$–MeOH–AcOH	4:1:0.05	–	0.36
$CHCl_3$–propan-1-ol	9:5	0.21	0.12
$CHCl_3$–MeOH–Me_2CO	6:2:1	–	0.48
$CHCl_3$–MeOH–Me_2CO	6:2:1	–	0.33
CH_2Cl_2–Me_2CO–MeOH	2:1:1	0.69	0.62
CH_2Cl_2–Me_2CO–EtOH	16:4:5	0.32	0.10
CH_2Cl_2–MeOH–C_6H_6	25:5:3	–	0.19
CH_2Cl_2–Me_2CO–water	15:62.5:10	0.65	–
CH_2Cl_2–MeOH–water	7.9:15.1	0.32	0.19
CH_2Cl_2–MeOH–25% aqueous NH_3–water	77:20:2:1	0.47	0.40
CH_2Cl_2–MeOH	7:3	–	–
C_6H_6–MeOH	7:3	0.40	–
EtOAc–EtOH	4:1	–	0.60
EtOAc–EtOH	4:1	0.49	0.46
EtOAc–EtOH	4:1	–	0.32
EtOAc–EtOH–water	2:8:1	–	–
EtOAc–MeOH–NH_3–water	10:2:1:1	–	–

Fig. 9). As discussed in more detail later, *in situ* mass spectrometry has also been used to good effect for the identification of phytoecdysteroids following NP-TLC.

RP-TLC chromatography on bonded phases (C_2, C_8, C_{12}, C_{18}, aminopropyl and cyanopropyl) as well as on paraffin-impregnated silica gel (*e.g.* refs. 39, 44 and 45) has also been employed for ecdysteroids and typical results, obtained using various C_{18} bonded phases, for a range of structures are provided in Table 9 [45]. In general, methanol–water (1:1, v/v) solvent systems provide good separations but other organic modifiers can be used to achieve different selectivities [44]. Reversed-phase systems, like their HPLC equivalents are poor at separating 5β-OH compounds from their 5β-H analogues (*e.g.* polypodine B from 20-hydroxyecdysone). The effects of various substitution patterns on the RP-TLC of ecdysteroids are discussed in detail elsewhere [45]. Detection methods following RP-TLC are essentially the same as those employed for NP-TLC separations, although RP-TLC–MS has not proved to be so readily achieved as NP-TLC–MS.

Separations can also be modified, in both NP- and RP-TLC systems by esterifying 20,22-diol-containing ecdysteroids with boronic acids (*e.g.* phenylboronic acid) [46,47]. This has the effect of reducing the polarity of such esters with a consequent effect on the chromatographic separation of an ecdysteroid mixture. This can be

TABLE 7

R_F AND $R_{ecdysone}$ VALUES OBTAINED FOR ECDYSTEROIDS BY NP-TLC ON SILICA GEL TLC PLATES USING CHLOROFORM–ETHANOL (4:1)

Data from ref. 43.

Compound	R_F	$R_{ecdysone}$	Compound	R_F	$R_{ecdysone}$
Poststerone	0.32	152	22-Isoecdysone	0.13	62
2-Deoxyecdysone	0.38	180	Calonysterone	0.42	200
Ecdysone	0.21	100	Pterosterone	0.32	152
20-Hydroxyecdysone	0.15	71	Kaladasterone	0.49	233
Muristerone A	0.27	129	Pinnasterol	0.56	267
Dacrysterone	0.27	129	20-Hydroxyecdysone 2-cinnamate	0.53	252
2-Deoxy-20-hydroxyecdysone	0.31	141	Polypodine B 2-cinnamate	0.56	267
Makisterone A	0.20	95	Acetylpinnasterol	0.68	324
Polypodine B	0.22	104	Ponasterone C	0.38	181
Inokosterone	0.17	77	Ponasterone C 2-cinnamate	0.65	310
2-Deoxy-3-epiecdysone	0.44	209	Ponasterone A	0.42	200
Ajugasterone C	0.22	104	Carpesterol	0.86	410
Cyasterone	0.33	157			

used to good effect to resolve ponasterone A and 2-deoxyecdysone, which are otherwise difficult to separate [4,6].

Of the more recently developed techniques for planar chromatography, automated multiple development (AMD) and over-pressure layer chromatography (OPLC) have been applied to the resolution of ecdysteroids [48,49]. AMD provided good separations but was somewhat time-consuming [48]. The use of OPLC for the separation of phytoecdysteroids of *Silene nutans* [49] enabled very rapid analysis to be performed, and it could easily be used for preparative work. However, the time required for plate preparation, etc., means that this is unlikely to supplant conventional separation methods.

The current practice of TLC for the analysis of phytoecdysteroids is to use it for qualitative applications such as monitoring extractions and purification (see refs. 50 and 51 for some recent examples). In these applications the ease of TLC and the generally rugged nature of the separation system are well suited to a rapid and high throughput of often difficult samples. However, there is no practical reason why scanning densitometry could not be used for quantitative analysis of plant extracts expecially with the high concentrations of material frequently encountered in such samples. It therefore seems likely that, with the wider availability of scanning densitometers that the use of TLC and HPTLC for quantitative analysis of these compounds will increase.

5. GAS CHROMATOGRAPHY

Ecdysteroids are too polar and have too little thermal stability to be suitable subjects for gas chromatography (GC) but by protecting some or all of the hydroxyl groups as silyl ethers they can be thermally stabilized and their polarity reduced so that they can be chromatographed in the gaseous phase. The gas chromatography of ecdysone as a trimethylsilyl ether derivative was first described by Katz and Lensky [52], although the exact nature of the product was not stated. Morgan and Woodbridge [53,54] developed conditions for the preparation of trimethylstyl ether O-methyloximes for the quantitative study of ecdysteroids, but later it was found that protection of the ketone group as an O-methyloxime was not necessary. Poole *et al.* [55] and Borst

TABLE 8

REACTIONS OF ECDYSTEROIDS AND THEIR DERIVATIVES WITH SPRAY REAGENTS

Compound	Reagent	Colour	Ref.
Cyasterone	Vanillin–sulphuric acid	Pink	9
Dacrysterone	Vanillin–sulphuric acid	Mauve-brown	109
2-Deoxy-20-hydroxyecdysone	Vanillin–sulphuric acid	Olive green then yellow	9
Ecdysone	Vanillin–sulphuric acid	Blue then red brown	9
	Vanillin–sulphuric acid	Turquoise	9
	Vanillin–sulphuric acid	Red-brown	110
	2,4-Dinitropheny hydrazine, then $K_3Fe(CN)_6$	Slightly yellow	110
	Phosphotungstic acid	Blue	110
20,26-Dihydroxyecdysone	Vanillin–sulphuric acid	Turquoise	111
20-Hydroxyecdysone	Vanillin–sulphuric acid	Olive green then brown	9
	Vanillin–sulphuric acid	Yellow-green	9
	Vanillin–sulphuric acid	Turquoise-grey blue	110
	2,4-Dinitrophenyl hydrazine, then $K_3Fe(CN)_6$	Slightly yellow	
	Phosphotungstic acid	Blue	
20-Hydroxyecdysone 2-cinnamate	Vanillin–sulphuric acid	Olive green	112
Inokosterone	Vanillin–sulphuric acid	Orange	9
Makisterone A	Vanillin–sulphuric acid	Mauve-brown	9
	Vanillin–sulphuric acid	Mauve-brown	109
Makisterone C	Vanillin–sulphuric acid	Dark green	9
	Vanillin–sulphuric acid	Green	109
Ponasterone A	Vanillin–sulphuric acid	Mauve then grey-green	9
Ponasterone B	Vanillin–sulphuric acid	Blue-mauve then brown	9
Ponasterone C	Vanillin–sulphuric acid	Green then brown	9
	Vanillin–sulphuric acid	Dark green	112
Ponasterone C 2-cinnamate	Vanillin–sulphuric acid	Dark green	112
Polypodine B 2-cinnamate	Vanillin–sulphuric acid	Olive green	112
Pterosterone	Vanillin–sulphuric acid	Dark green	112
		Green	9
Rubrosterone	Vanillin–sulphuric acid	Brown	9
20-Hydroxyecdysone 2-acetate	Vanillin–sulphuric acid	Olive green then yellow	113
20-Hydroxyecdysone 3-acetate	Vanillin–sulphuric acid	Olive green then yellow	113
20-Hydroxyecdysone 22-acetate	Vanillin–sulphuric acid	Dark green	113
20-Hydroxyecdysone 2,3-diacetate	Vanillin–sulphuric acid	Olive green then yellow	113
20-Hydroxyecdysone 2,22-diacetate	Vanillin–sulphuric acid	Dark green	113

TABLE 8 (*continued*)

Compound	Reagent	Colour	Ref.
20-Hydroxyecdysone 3,22-diacetate	Vanillin–sulphuric acid	Dark green	113
20-Hydroxyecdysone 2,3,22-triacetate	Vanillin–sulphuric acid	Dark green	113
20-Hydroxyecdysone 2,3,22,25-tetracetate	Vanillin–sulphuric acid	Dark green	113
20-Hydroxyecdysone 20,22-acetonide	Vanillin–sulphuric acid	Olive green then brown	113
20-Hydroxyecdysone 2,3,20,22-diacetonide	Vanillin–sulphuric acid	Olive green then brown	113

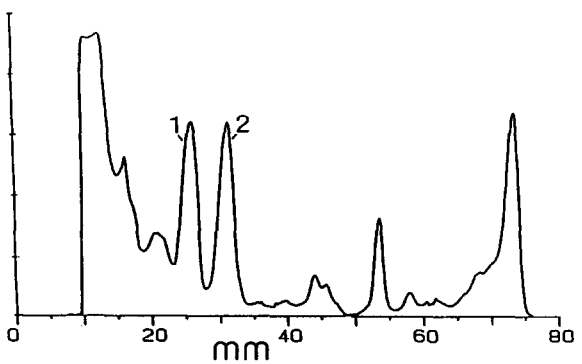

Fig. 9. HP-TLC of an extract from the plant *Silene schafta*, silica gel, chloroform–ethanol (4:1, v/v), 254 nm. Peaks: 1 = 20-hydroxyecdysone; 2 = polypodine B.

and O'Connor [56] almost simultaneously discovered that ecydsone trimethylsilyl ethers were suitable subjects for the electron capture detector which made the sensitivity and selectivity of detection of ecdysteroids much greater. This work was done with packed columns, and was reviewed in 1976 [42], but the difficulties of preparing trimethylsilyl ethers in a reproducible and quantitative manner [57] have discouraged the use of gas chromatography in ecdysteroid work.

The introduction of fused-silica capillary columns increased the resolution of GC enormously so that the resolving power of GC and the sensitivity of electron-capture detection have yet to be bettered by any physical methods for ecdysteroids. Because other chromatographic methods described here which do not require derivative formation have replaced GC we are not aware of the use of GC in any phytoecdysteroid work in the past decade.

An example of capillary column operating conditions for trimethylsilylated ecdysteroids is given by Evershed and co-workers [58,59]. They used a 25 m × 0.22 mm fused-silica capillary column, coated with 0.1 μm film of BP-1 (a non-polar dimethylsiloxane polymer) with oven temperature 50°C at injection, then raised immediately to 200°C and programmed at 8°C min^{-1} to 320°C and held at that temperature. Using helium as the moving phase at a linear velocity of 100 cm s^{-1}, they found ecdysone trimethylsilyl ether had a retention time of 15.2 min, 20-hydroxyecdysone 16.0 min, makisterone A 17.2 min and 20,26-dihydroxyecdysone 18.2 min [58].

6. SUPERCRITICAL FLUID CHROMATOGRAPHY

Separation of complex mixtures can be achieved by chromatography using a moving liquid or gaseous phase. It was pointed out by Lovelock in 1958 (see ref. 60) that a supercritical fluid should also act as a mobile phase for chromatography. The supercritical fluid used in most cases is carbon dioxide or carbon dioxide "modified by" (*i.e.* mixed with) methanol, or some other small-molecule polar substance to increase the polarity of the supercritical fluid.

The instrumentation is either an adaptation of GC, using fused-silica capillary columns and a flame ionization detector or an adaptation of HPLC, using packed columns and a UV detec-

TABLE 9

RETENTION OF PHYTOECDYSTEROIDS ON RP-TLC PLATES OF DIFFERENT ORIGINS

Solvent system: methanol–water (50:50, v/v). Data from ref. 45.

Compound	Merck C_{18} bonded TLC plates		Whatman C_{18} bonded TLC plates		Macherey–Nagel C_{18} bonded TLC plates	
	hR_F	$\%R_F$ ecdysone	hR_F	$\%R_F$ ecdysone	hR_F	$\%R_F$ ecdysone
Ecdysone	29	100.0	28	100.0	32	100.0
20-Hydroxyecdysone 2-cinnamate	4	13.8	3	10.7	5	15.6
Inokosterone	44	151.8	37	132.0	45	140.6
20-Hydroxyecdysone	44	151.8	38	135.7	49	153.1
Muristerone A	32	110.3	31	110.7	45	140.6
Carpesterol	0	0.0	0	0.0	0	0.0
2-Deoxy-20-hydroxyecdysone	21	72.4	29	103.6	29	90.6
Ponasterone C 2-cinnamate	0	0.0	0	0.0	0	0.0
Pterosterone	29	100.0	38	135.7	37	115.6
Abutasterone	42	144.8	56	200.0	52	162.5
Integristerone A	45	155.2	63	225.0	52	162.5
Calyonysterone	20	68.9	37	132.1	24	75.0
Kaladasterone	17	58.6	30	107.1	30	93.8
Ponasterone A	16	55.2	18	64.3	24	75.0
Makisterone A	31	106.9	40	142.9	40	125.0
Dacrysterone	31	106.9	37	132.1	40	125.0
Polypodine B	42	144.8	44	157.1	49	153.1
Cyasterone	40	137.9	51	182.1	49	153.1
Acetylpinnasterol	0	0.0	0	0.0	0	0.0
2-Deoxyecdysone	15	51.7	17	60.7	17	53.1
Ajugasterone C	38	131.0	38	135.7	44	137.5
Ponasterone C	29	100.0	37	132.1	40	125.0
Poststerone	37	127.6	38	135.7	46	143.8

tor. The flame detector precludes the use of organic modifiers but has great sensitivity and resolution. Raynor *et al.* [61] have used capillary supercritical fluid chromatography (SFC) to chromatograph some relatively non-polar ecdysteroid precursors but the retention times of ecdysone and hydroxyecdysone were too great for practical purposes. Perhaps these experiments would be worth repeating using higher pressures and possibly slightly higher temperature. Now that the electron capture detector is becoming adaptable to SFC conditions, it becomes more important to attempt capillary SFC of ecdysteroids. SFC with electron-capture detection could become a superior method for ecdysteroids in terms of resolution, sensitivity and selectivity. Morgan *et al.* [62] have shown that packed-column SFC using carbon dioxide–methanol mixtures is a practical procedure, both for pure ecdysteroids and for plant extracts (see Fig. 10) [63]. Very short columns can be used with advantages of short retention times and high resolution. The sensitivity of detection is greater than with HPLC probably as a result of the sharper peaks and greater UV transparency of the fluid [64]. Packed-column SFC has the retention characteristics of a NP-HPLC system. A variety of columns from silica to ODS can be used. In practice, aminopropyl silica and cyanopropyl silica seem best for ecdysteroids. Some conditions are given in Table 10. A further advantage of SFC over HPLC is the avoidance of costly high purity solvents and the problems of their recovery and disposal. Since many ecdysteroids contain vicinal diols, particularly 2,3- and 20,22-diols, reagents specific for vicinal diols

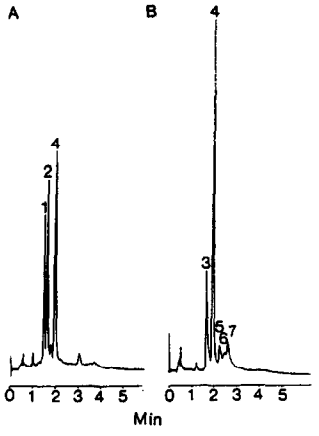

Fig. 10. Supercritical fluid chromatograms of plant extracts. (A) *Silene otites* and (B) *Silene nutans* [63]. Peaks: 1 = 2-deoxyecdysone; 2 = 2-deoxy-20-hydroxyecdysone; 3 = polypodine B; 4 = 20-hydroxyecdysone; 5 = 26-hydroxypolypodine B; 6 = integristerone A; 7 = 20,26-dihydroxyecdysone. Conditions: column 5 μm cyanopropyl silica (20 × 4.6 mm) CO_2–MeOH (9:1) at 3 ml min^{-1}, 60°C and 290 bar, detection at 235 nm.

can be useful in selecting out such compounds from mixtures. We have described the use of solid-phase extraction with immobilized boronic acids for the selective retention of ecdysteroids containing 20,22-diols [22]. We have recently extended this to the preparation and separation by SFC of methyl, butyl and phenylboronic esters of ecdysteroids with 20,22-diol groups [65].

7. CAPILLARY ELECTROPHORESIS

Capillary zone electrophoresis (CZE) has been shown to provide very efficient separations for a wide range of substances. As the bulk of the ecdysteroids are uncharged, simple CE, which relies on the presence of an ionised group to attain separations, cannot be used. However, micellar capillary electrophoresis (MCE), whereby a molecule such as sodium dodecyl sulphate is added to the buffer at a concentration above its critical micelle concentration, has been shown to be suitable for such compounds. In a series of preliminary studies we have evaluated MCE [66,67] for a range of sample types, including plant extracts of various degrees of purity. These studies clearly demonstrated that MCE could be used for the separation of phytoecysteroids. MCE was performed using fused-silica polyimide-coated capillaries of 50 or 75 μm I.D. and 72 or 50 cm in length, respectively. The run buffer employed was 40 m*M* sodium dihydrogen phosphate–20 m*M* disodium borate–20 m*M* sodium dodecyl sulphate–methanol (5%, v/v) at pH 9.4. Depending upon the system, separations were performed at 20 or 8.5 kV and a run temperature of 50°C. Increasing either the applied voltage or temperature reduced migration times. The organic modifier content of the mobile phase also affected the migration of the ecdysteroids with elution time increasing with organic modifier content.

The migration times of a number of common phytoecdysteroids, in the two CZE systems examined, with the run buffer described above are given in Table 11. Using a 50 μm I.D. capillary, migration times ranged from 7.2 min for cyasterone up to 25.9 min for 2-deoxyecdysone. The observed elution order is similar to that seen in RP-HPLC. This is not surprising as in MCE the separation mechanism for ecdysteroids is based on hydrophobic partitioning. It is interesting to note that there is an excellent separation between ponasterone A (16.5 min) and 2-deoxyecdysone (25.9 min) which have been difficult to separate by RP-HPLC, TLC, etc. The results obtained with a 75 μm capillary were similar. MCE gave a linear calibration curve over the range 0 to 560 μg/ml, corresponding to 0 to 2.8 ng injected on-column. The sensitivity of the instrument was such that the detection of *ca.* 175 pg (35 μg/ml) on-column of a sample of pure ecdysone was possible without difficulty. Ecdysteroid-rich extracts from both plant and insect sources were subjected to analysis by MCE. Samples with a range of purities were examined with different degrees of success. Attempted MCE of simple methanol extracts of *Silene nutans* were unsuccessful because large and distorted peaks were obtained, apparently due to column overloading and the presence of interfering compounds. The analysis of plant extracts after more extensive purification gave good results as illustrated in Fig. 11 for an extract of *Silene otites*. This shows the sample to contain 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdy-

TABLE 10

EXAMPLES OF CONDITIONS USED FOR SUPERCRITICAL FLUID CHROMATOGRAPHY OF ECDYSTEROIDS

1 p.s.i. = 6894.76 Pa. For many other conditions, see ref. 114.

Column type	Column dimensions	Mobile phase	Flow-rate	Pressure or density	Temperature (°C)	Compound	t_R (min)	Ref.
Capillary								
Cyanopropyl–dimethylsiloxane (1:1)	10 m × 5 μm	CO_2	–	0.4–0.71 g cm^{-3}	120	14,22,25-Trideoxyecdysone	20	61
						2-Deoxyecdysone	31	61
Packed								
Cyanopropyl Spherisorb 5 μm	250 × 4.6 mm	CO_2–MeOH (90:10)	3.25 l min^{-1} gas	300 bar	50	14,22,25-Trideoxyecdysone	1	61
						2-Deoxyecdysone	2.3	61
						Ponasterone A	2.5	61
						Ecdysone	3.2	61
						Polypodine B	3.2	61
						Makisterone A	3.2	61
						20-Hydroxyecdysone	4.0	61
						Cyasterone	7.0	61
Hypersil 5 μm	100 × 4.6 mm	CO_2–MeOH (80:20)	4 ml min^{-1}	300 bar	80	Ecdysone	1.68	62
						20-Hydroxyecdysone	1.88	62
						Inokosterone	2.10	62
						Cyasterone	1.71	62
Cyanopropyl Spherisorb	250 × 46 mm	CO_2–MeOH (90:10)	3 ml min^{-1}	290 bar	60	2-Deoxyecdysone	1.5	63
						2-Deoxy-20-hydroxy-ecdysone	1.6	63
						Polypodine B	1.8	63
						20-Hydroxyecdysone	2.0	63
						26-Hydroxypolypodine B	2.2	63
						Integristerone A	2.4	63
						20,26-Dihydroxyecdysone	2.6	63
Silica 5 μm	250 × 4.6	CO_2–MeOH (75:25)	3.0	2000 p.s.i.	60	Ecdysone	3.0	64
						20-Hydroxyecdysone	3.2	64
		(80:20)	2.0	2000 p.s.i.	60	Ecdysone	7.1	64
						20-Hydroxyecdysone	8.1	64
ODS 5 μm	250 × 4.6	CO_2–MeOH (75:25)	3.0	2000 p.s.i.	60	Ecdysone	2.3	64
						20-Hydroxyecdysone	2.3	64
		(86:14)	3.0	2000 p.s.i.	60	Ecdysone	4.3	64
						20-Hydroxyecdysone	4.7	64
Aminopropyl	250 × 4.6	CO_2–MeOH (80:20)	2.0	2000 p.s.i.	60	Ecdysone	4.5	64
						20-Hydroxyecdysone	4.8	64
Cyanopropyl	250 × 4.6	CO_2–MeOH (80:20)	2.0	2000 p.s.i.	80	Ecdysone	5.2	64
						20-Hydroxyecdysone	6.0	64
Aminopropyl	250 × 4.6	CO_2–MeOH (80:20)	2.0	2000 p.s.i.	40	Ecdysone	11.0	64
						20-Hydroxyecdysone	13.9	64

sone and 2-deoxyecdysone as major components. The electropherogram obtained for the analysis of an extract of the roots of *Silene nutans* is shown in Fig. 12. This extract contained 20-hydroxyecdysone, polypodine B and 2-deoxy-20-hydroxyecdysone. Under these conditions 20-hydroxyecdysone and polypodine B co-migrate (Fig. 12a). This lack of separation between

TABLE 11

RETENTION TIME DATA FOR ECDYSTEROIDS FOR MICELLAR CAPILLARY ELECTROPHORESIS

MCE conditions as in text, data from ref. 67. A = ABI 270A CZE system; B = Beckman P/ACE 2000 CZE system.

Compound	Retention time (min)	
	A	B
Calonysterone	14.43	–
Cyasterone	7.17	–
2-Deoxyecdysone	25.91	21.74
2-Deoxy-20-hydroxyecdysone	12.84	11.90
Ecdysone	11.30	10.65
20-Hydroxyecdysone	7.35	7.34
20-Hydroxyecdysone 2-cinnamate	13.57	–
Kaladasterone	13.19	–
Polypodine B	–	7.34
Ponasterone A	16.45	16.02
Pterosterone	10.07	–
Makisterone A	8.89	9.00
Muristerone	8.37	–

polypodine B and 20-hydroxyecdysone is similar to that found in RP-HPLC. However, the resolution of these compounds is possible by MCE providing that run buffers containing over 50% (v/v) of methanol are used, albeit with long analysis times (over 30 min). Increasing the applied voltage did enable analysis times to be reduced without loss of resolution as shown in Fig. 12b.

MCE therefore is capable of providing efficient separations of phytoecdysteroids in plant extracts. On-column sensitivity is excellent, and clearly adequate for these concentrated samples. MCE can provide an alternative to HPLC or TLC in this type of application and the small on-column sample requirements may also prove useful. However, relatively pure extracts seem to be required for good peak shape. Sensitivity is limited by the amount of material (a few nanolitres) that can be introduced into the capillary. The relatively low concentration, as opposed to on-column sensitivity of MCE, may therefore be a source of difficulty for some

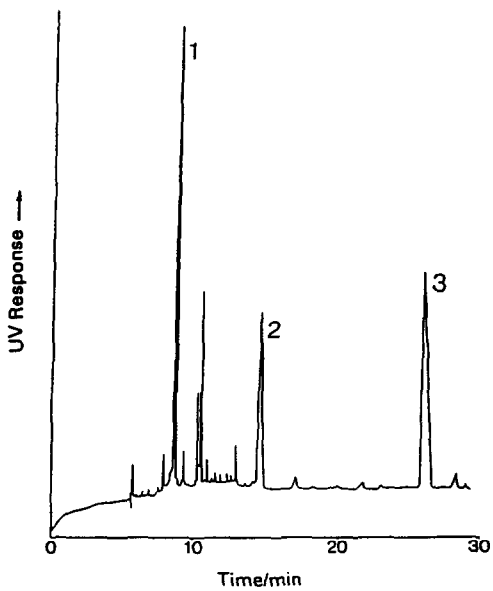

Fig. 11. MCE of an ecdysteroid-rich extract of the plant *Silene otites* using the ABI 270A. The extract contained, in order of elution, (1) 20-hydroxyecdysone, (2) 2-deoxy-20-hydroxyecdysone and (3) 2-deoxyecdysone. Conditions: UV absorption at 240 nm, mobile phase 5% methanol and 95% buffer containing 40 m*M* NaH_2PO_4, 20 m*M* Na_2HBO_3 and 20 m*M* sodium dodecyl sulphate, pH 9.4, 20 kV.

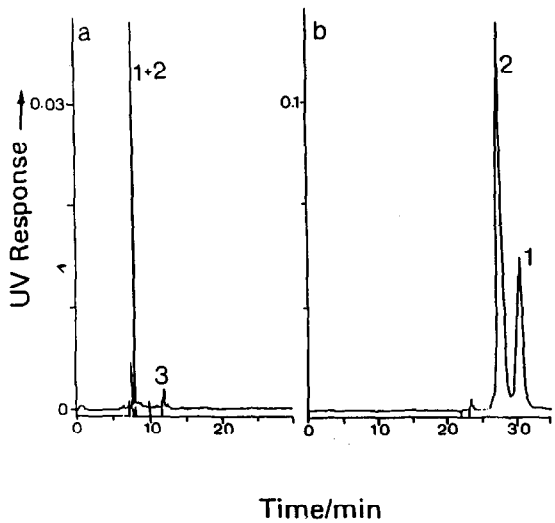

Fig. 12. MCE, using the Beckman P/ACE, of an ecdysteroid-rich extract of the plant *Silene nutans* using (a) standard conditions in which polypodine B (1) and 20-hydroxyecdysone (2) co-migrate, but are resolved from 2-deoxy-20-hydroxyecdysone (3); and (b) 52.5% of methanol in the run buffer when 20-hydroxyecdysone elutes before polypodine B. Conditions as in Fig. 11, but varying the mobile phase in (b) and using 8.5 kV throughout.

sample types. Whilst these initial studies are promising it is too early to say whether or not MCE will be generally useful for the analysis of phytoecdysteroids. Further work is required to enable a rigorous comparison with other analytical techniques to be made.

8. HYPHENATED TECHNIQUES OF CHROMATOGRAPHY AND MASS SPECTROMETRY

The large number of phytoecdysteroids that have been encountered in plant extracts clearly poses problems in their identification based merely on chromatographic properties alone. Whilst selectivity can be attained by the use of a number of different chromatographic systems (see also Lafont *et al.* [32]), linked chromatography–mass spectroscopy provides an alternative system for unequivocally establishing identity. The use of GC–MS for ecdysteroids is well established for insect and other arthropod samples but has not been widely applied to plant samples. The reason for this is that in the case of arthropod samples it is the sensitivity as well of the specificity of the mass spectrometer as a detector which is the driving force for its use. In the case of plant samples the quantities of material are usually such that it is considered to be easier to isolate material for mass spectrometry rather than go through the process of derivatisation which is necessary before GC can be used. The introduction of SFC has also enabled the ecdysteroids to be introduced into the mass spectrometer without the need for derivatisation, and SFC–MS has been used for the identification of a number of ecdysteroids in extracts of *Silene nutans* and *Silene otites* [63]. The results obtained were either chemical ionization- or electron-impact-like, depending upon the temperature of the jet block, with higher temperatures associated with greater fragmentation. Conditions could thus be varied to give molecular mass or diagnostic fragmentation data.

In the case of HPLC–MS for phytoecdysteroids progress has been limited by the available interfaces. In Fig. 13 the chromatograms obtained for HPLC–MS of an extract of *Silene otites* with UV and MS data, monitoring the ions

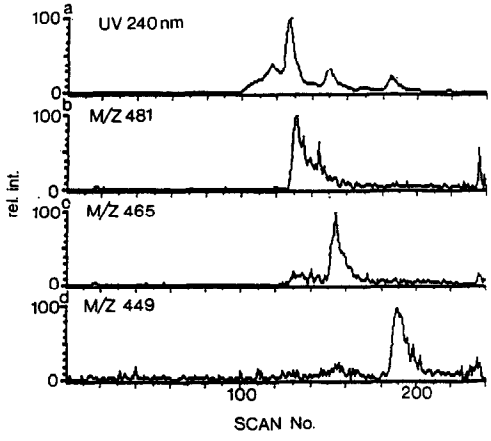

Fig. 13. HPLC–MS of ecdysteroids present in an extract of *Silene otites* separated by RP-HPLC on a C_{18} bonded column (ODS Spherisorb 20 × 0.46 cm) using acetonitrile–0.1 *M* ammonium acetate (40:60) at 1 ml min^{-1} with a thermospray interface (VG Quatro, capillary temperature 270°C). (a) UV trace at 240 nm; (b) ion current for *m/z* 481 for 20-hydroxyecdysone; (c) ion current *m/z* 465 for 2-deoxy-2-hydroxyecdysone and (d) ion current *m/z* 449 for 2-deoxyecdysone.

at *m/z* 481, 465 and 449 are shown using the thermospray interface. The resulting spectrum for 20-hydroxyecdysone (*m/z* 481) is given in Fig. 14A. It is typical of the results obtained with this interface following reversed-phase HPLC (methanol–ammonium acetate buffer, see caption for details). A number of ions are associated with the 20-hydroxyecdysone peak corresponding to the protonated molecular (MH) ion (481), MH − 18 (463) and MH − 2 × 18 (445) (loss of one or two molecules of water) and an ion corresponding to $M + MeCN + NH_4^+$ (539), highlighting the difficulties of interpretation encountered with this mode of ionisation. In order to characterise more fully such compounds with this interface we have recently employed MS–MS techniques. An example of the results obtained is shown in Fig. 14B where the daughter ion spectrum obtained for the ion at *m/z* 481 is shown. The weak ion at *m/z* 463 (not marked) and ions at *m/z* 445 and 427 correspond to loss of 1, 2 and $3H_2O$ respectively, but no simple explanation can be given for the other ions. Similar spectra were obtained for 2-deoxy-20-hydroxyecdysone and 2-deoxyecdysone.

In addition to the hyphenation of SFC and

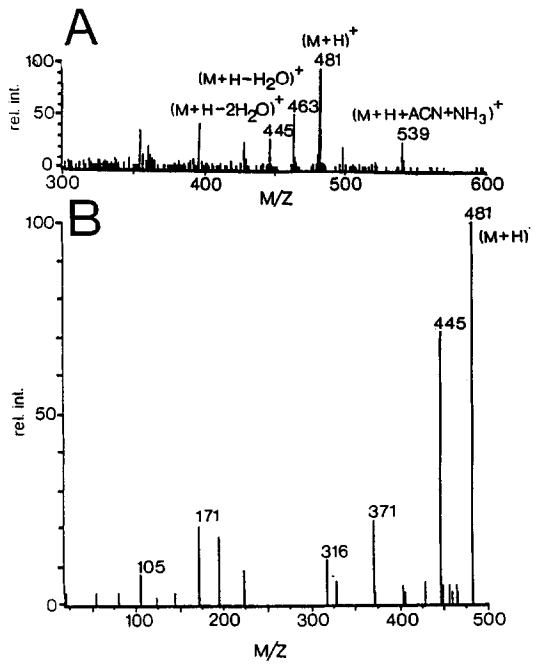

Fig. 14. (A) The mass spectrum obtained for 20-hydroxyecdysone using the conditions outlined in Fig. 13. (B) The daughter ion spectrum obtained for the M + 1 ion (*m/z* 481) for 20-hydroxyecdysone.

HPLC with MS and MS–MS techniques we have also experienced considerable success in combining TLC separations with fast atom bombardment MS [68] and MS–MS [69,70], using both "in-line" and "off-line" techniques. The inherent simplicity of this approach (especially the off-line technique) makes it an attractive and readily implemented alternative to the method of hyphenation outlined above. Furthermore, as the TLC plate effectively stores the separation, confirmation of identity by MS need not be performed at the time of separation (or indeed at the same site).

9. FUTURE PROSPECTS

It would appear that all the stratagems and inventions of the chromatographer have been applied to the separation of phytoecdysteroids from their matrix of plant substances. Techniques are still developing quickly and we can expect to see these advances being applied to phytoecdysteroids too. The prospects of supercritical fluid extraction combined with supercritical fluid chromatography and electron capture detection is a particularly attractive long term goal. In the shorter term we should see advances in LC–MS to make it a more sensitive and diagnostically more useful system and we should also see greater use of TLC with UV scanning and mass spectral analysis. A further system, not yet discussed in detail will be the combination of HPLC and NMR spectroscopy. We can begin to foresee the day when ecdysteroids will be separated, quantified and structurally identified all in one operation without ever having been isolated as pure substances.

REFERENCES

1 P. Karlson, H. Hoffmeister, H. Hummel, P. Hocks and G. Spiteller, *Chem. Ber.*, 98 (1965) 2394.
2 R. Huber and W. Hoppe, *Chem. Ber.*, 98 (1965) 2403.
3 F. Hampshire and D.H.S. Horn, *Chem. Commun.*, (1966) 37.
4 P. Hocks and R. Wiechert, *Tetrahedron Lett.*, 26 (1966) 2989.
5 K. Nakanishi, M. Koreeda, S. Sasaki, M.L. Chang and H.Y. Hsu, *Chem. Commun.*, (1966) 915.
6 M.N. Galbraith and D.H.S. Horn, *Chem. Commun.*, (1966) 905.
7 T. Takemoto, S. Ogawa and N. Nishimoto, *Yakagaku Zasshi*, 87 (1967) 325.
8 J. Jizba, V. Herout and F. Sorm, *Tetrahedron Lett.*, 18 (1967) 1689.
9 D.H.S. Horn, in M. Jacobson and D.G. Crosby (Editors), *Naturally Occurring Insecticides*, Marcel Dekker, New York, 1971, p. 333.
10 I. Kubo, J.A. Klocke, I. Ganjian, N. Ishikawa and T. Matsumoto, *J. Chromatogr.*, 257 (1983) 157.
11 I. Kubo, A. Matsumoto and S. Asano, *Insect Biochem.*, 15 (1984) 45.
12 I. Kubo, A. Matsumoto and J.J. Ayafor, *Agric. Biol. Chem.*, 48 (1984) 1683.
13 I. Kubo, A. Matsumoto, F.J. Hanke and J.J. Ayafor, *J. Chromatogr.*, 321 (1985) 246.
14 Y. Ito, *Trends Anal. Chem.*, 5 (1986) 142.
15 M. Zhang, M.J. Stout and I. Kubo, *Phytochemistry*, 31 (1992) 247.
16 D.A. Schooley, G. Weiss and K. Nakanishi, *Steroids*, 19 (1972) 377.
17 I.D. Wilson, C.R. Bielby, E.D. Morgan and A.E.M. McLean, *J. Chromatogr.*, 194 (1980) 343.

18 R. Lafont, J.-L. Pennetier, M. Andrianjafintrimo, J. Claret, J.-F. Modde and C. Blais, *J. Chromatogr.*, 236 (1982) 137.
19 R.D. Watson and E. Spaziani, *J. Liq. Chromatogr.*, 5 (1982) 525.
20 G.D. Primprikar, M.J. Coign, H. Sakurai and J.R. Heitz, *J. Chromatogr.*, 317 (1984) 413.
21 H.H. Rees and R.E. Issac, *Methods Enzymol.*, 111 (1985) 377.
22 S.J. Murphy, E.D. Morgan and I.D. Wilson, in A.R. McCaffery and I.D. Wilson (Editors), *Chromatography and Isolation of Insect Hormones and Pheromones*, Plenum Press, London, 1990, p. 131.
23 J.-P. Girault, M. Bathori, E. Varga, K. Szendrei and R. Lafont, *J. Nat. Products*, 53 (1990) 279.
24 D. Rudel, M. Bathori, J. Gharbi, J.-P. Girault, I. Racz, K. Melis, K. Szendrei and R. Lafont, *Planta Med.*, 58 (1992) 358.
25 M. Wessner, B. Champion, J.-P. Girault, N. Kaouadji, B. Saidi and R. Lafont, *Phytochemistry*, 31 (1992) 3785.
26 R. Lafont, G. Sommé-Martin, B. Mauchamp, B.F. Maume and J.-P. Delbecque, in J.A. Hoffmann (Editor), *Progress in Ecdysone Research*, Elsevier/North-Holland, Amsterdam, 1980, p. 45.
27 R. Lafont, P. Beydon, B. Mauchamp, G. Sommé-Martin, M. Andrianjafintrimo and P. Krien, in F. Sehnal, A. Zabaz, J.J. Menn and B. Cymborowski (Editors), *Regulation of Insect Development and Behaviour*, Wroclaw Technical University Press, Wroclaw, 1981, p. 125.
28 J.C. Touchstone, in G. Zweig and J. Sherma (Editors), *CRC Handbook of Chromatography, Steroids*, CRC Press, Boca Raton, FL, 1986, p. 119.
29 R. Lafont, in H. Kalasz and S. Ettre (Editors), *Chromatography '87*, Akademia Kiado, Budapest, 1988, p. 1.
30 M.J. Thompson, J.A. Svoboda and M.F. Feldlaufer, in W.D. Nes and E.J. Parish (Editors), *Analysis of Sterols and Other Biologically Significant Steroids*, Academic Press, San Diego, CA, 1989, p. 81.
31 R. Lafont and I.D. Wilson, in A.R. McCaffery and I.D. Wilson (Editors), *Chromatography and Isolation of Insect Hormones and Pheromones*, Plenum Press, London, New York, 1990, p. 79.
32 R. Lafont, N. Kaouadji, E.D. Morgan and I.D. Wilson, *J. Chromatogr. A*, 658 (1994) 55.
33 R. Lafont, G. Sommé-Martin and J.-C. Chambet, *J. Chromatogr.*, 170 (1979) 185.
34 L.N. Dinan, P.L. Donnahey, H.H. Rees and T.W. Goodwin, *J. Chromatogr.*, 205 (1981) 139.
35 L.N. Dinan, *Phytochem. J.*, 3 (1992) 132.
36 L.N. Dinan, *J. Chromatogr. A*, 658 (1994) 69.
37 C. Blais and R. Lafont, *Hoppe-Seylers' Z. Physiol. Chem.*, 365 (1984) 809.
38 N. Kaouadji and R. Lafont, *J. Chromatogr.*, 505 (1990) 408–412.
39 I.D. Wilson, C.R. Bielby and E.D. Morgan, *J. Chromatogr.*, 238 (1982) 97.
40 I. Kubo and T. Nakatsu, *LC·GC*, 8 (1992) 933.
41 M.W. Gilgan and T.E. Farquharson, *Steroids*, 22 (1973) 366.
42 E.D. Morgan and C.F. Poole, *Adv. Insect Physiol.*, 12 (1976) 17; and references cited therein.
43 I.D. Wilson, *J. Chromatogr.*, 318 (1985) 373.
44 I.D. Wilson, S. Scalia and E.D. Morgan, *J. Chromatogr.*, 212 (1981) 211.
45 I.D. Wilson, *J. Planar Chromatogr.*, 1 (1988) 116.
46 J. Pis and J. Harmatha, *J. Chromatogr.*, 596 (1992) 271.
47 I.D. Wilson, *J. Planar Chromatogr.*, 5 (1992) 316.
48 I.D. Wilson and S. Lewis, *J. Chromatogr.*, 408 (1987) 445.
49 H. Read, I.D. Wilson and R. Lafont, in A.R. McCaffery and I.D. Wilson (Editors), *Chromatography and Isolation of Insect Hormones and Pheromones*, Plenum Press, London, New York, 1990, p. 129.
50 M. Bathori, K. Szendrei, H. Kalasz, R. Lafont and J.-P. Girault, *Chromatographia*, 25 (1988) 627.
51 H. Kalasz, M. Bathori, L. Kerecsen and L. Toth, *J. Planar Chromatogr.*, 6 (1993) 38.
52 M. Katz and Y. Lensky, *Experientia*, 26 (1970) 1043.
53 E.D. Morgan and A.P. Woodbridge, *Chem. Commun.*, (1971) 475.
54 E.D. Morgan and A.P. Woodbridge, *Org. Mass Spectrom.*, 9 (1974) 102.
55 C.F. Poole, E.D. Morgan and P.M. Bebbington, *J. Chromatogr.*, 104 (1975) 172.
56 D.W. Borst and J.D. O'Connor, *Steroids*, 24 (1974) 637.
57 C.R. Bielby, A. Gande, E.D. Morgan and I.D. Wilson, *J. Chromatogr.*, 194 (1980) 43.
58 R.P. Evershed, J.G. Mercer and H.H. Rees, *J. Chromatogr.*, 390 (1987) 357.
59 R.P. Evershed, M. Kabbouh, M.C. Prescott, J.L. Maggs and H.H. Rees, in A.R. McCaffery and I.D. Wilson (Editors), *Chromatography and Isolation of Insect Hormones and Pheromones*, Plenum Press, London, New York, 1990, p. 103.
60 J.E. Lovelock, in C.M. White (Editor), *Modern Supercritical Fluid Chromatography*, VCH, Weinheim, 1988.
61 M.W. Raynor, J.P. Kithinji, I.K. Barker, K.D. Bartle and I.D. Wilson, *J. Chromatogr.*, 436 (1988) 497.
62 E.D. Morgan, S.J. Murphy, D.E. Games and I.C. Mylchreest, *J. Chromatogr.*, 441 (1988) 165.
63 M.W. Raynor, J.P. Kithinji, K.D. Bartle, D.E. Games, I.C. Mylchreest, E.D. Morgan, R. Lafont and I.D. Wilson, *J. Chromatogr.*, 467 (1989) 292.
64 E.D. Morgan, H.-P. Huang and I.D. Wilson, in A.R. McCaffery and I.D. Wilson (Editors), *Chromatography and Isolation of Insect Hormones and Pheromones*, Plenum, London, New York, 1990, p. 95.
65 J.-H. Shim, E.D. Morgan and I.D. Wilson, *J. Chromatogr.*, 639 (1993) 281.
66 T. Large, R. Lafont, E.D. Morgan and I.D. Wilson, *Anal. Proc.*, 29 (1992) 386.
67 P. Davis, R. Lafont, T. Large, E.D. Morgan and I.D. Wilson, *Chromatographia*, 37 (1993) 37.
68 I.D. Wilson, R. Lafont and P. Wall, *J. Planar Chromatogr.*, 1 (1988) 357.
69 I.D. Wilson, R. Lafont, R.G. Kingston and C.J. Porter, *J. Planar Chromatogr.*, 3 (1990) 359.

70 R. Lafont, C.J. Porter, E. Williams, H. Read, E.D. Morgan and I.D. Wilson, *J. Planar Chromatogr.*, in press.
71 J.N. Kaplanis, M.J. Thompson, R.T. Yamamoto, W.E. Robbins and S.J. Louloudes, *Steroids*, 8 (1966) 605.
72 J.N. Kaplanis, S.R. Dutky, W.E. Robbins, M.J. Thompson, E.L. Lindquist, D.H.S. Horn and M.N. Galbraith, *Science*, 190 (1975) 681.
73 J.N. Kaplanis, W.E. Robbins, M.J. Thompson and S.R. Dutky, *Science*, 180 (1973) 307.
74 P. Karlson, *Angew. Chem., Int. Ed. Engl.*, 2 (1963) 175.
75 I. Kubo and F.J. Hanke, in J.R. Miller and T.A. Miller (Editors), *Insect-Plant Interactions (Springer Series in Experimental Entomology)*, Springer, New York, 1986, p. 225.
76 M.N. Galbraith and D.H.S. Horn, *Aust. J. Chem.*, 22 (1969) 1045.
77 S. Imai, M. Hori, S. Fujioka, E. Murata, M. Goto and K. Nakanishi, *Tetrahedron Lett.*, 36 (1968) 3883.
78 A. Banerji, G.J. Chintalwar, N.K. Joshi and M.S. Chadha, *Phytochemistry*, 10 (1971) 2225.
79 M.L. Colombo, F. Tome, O. Servettaz and C. Bugatti, *Int. J. Crude Drug Res.*, 28 (1990) 219.
80 J. Jizba, L. Dolejs and V. Herout, *Phytochemistry*, 13 (1974) 1915.
81 T. Yokota, M. Arima and N. Takahashi, *Tetrahedron Lett.*, 23 (1982) 1275.
82 A. Fukuzawa, M. Miyamoto, Y. Kumagai and T. Masamune, *Phytochemistry*, 25 (1986) 1305.
83 G.B. Russel, P.G. Fenemore, D.H.S. Horn and E.J. Middleton, *Aust. J. Chem.*, 25 (1972) 1935.
84 G. Heinrich and H. Hoffmeister, *Experientia*, 23 (1967) 995.
85 N. Nishimoto, Y. Shiobara, M. Kujino, S.S. Inoue, T. Takemoto, F. De Oliveira, G. Akisue, M.K. Akisue, G. Hashimoto, O. Tanaka, R. Kasai and H. Matsuura, *Phytochemistry*, 26 (1987) 2505.
86 A.C. Santos, M.T. Chua, N. Eufemio and C. Abela, *Experientia*, 26 (1970) 1053.
87 H. Hikino, K. Mohri, T. Okuyama and K.-Y. Yen, *Steroids*, 28 (1976) 649.
88 F. Camps, J. Coll and O. Dargallo, *Ann. Quim.*, 81C (1975) 74.
89 R. Hardman and S.M.A. Mahar, *Planta Med.*, 33 (1978) 278.
90 A.U. Mamatkhanov, M.I. Shamsutdinov and T.T. Shakirov, *Khim. Prir. Soedin.*, 5 (1967) 667.
91 Z. Saatov, M.B. Gorovits, N.D. Abdullaev, B.Z. Usmanov and N.K. Abubakirov, *Khim. Prir. Soedin.*, 5 (1985) 611.
92 I. Tóth, M. Báthory, K. Szendrei, E. Minker and G. Blazsó, *Fitoterapia*, 52 (1981) 77.
93 M. Bathori, I. Toth, K. Szendrei and J. Reisch, *Phytochemistry*, 10 (1982) 2225.
94 T. Takemoto, S. Ogawa, N. Nishimoto, K.-Y. Yen, K. Abe, T. Sato, K. Osawa and M. Takahashi, *Yakugaku Zasshi*, 87 (1967) 1521.
95 R. Hardman and T.V. Benjamin, *J. Chromatogr.*, 131 (1977) 468.
96 A. Suksamrarn, J.S. Wilkie and D.H.S. Horn, *Phytochemistry*, 25 (1986) 1301.
97 G.B. Russel and D.R. Greenwood, in J. Koolman (Editor), *Ecdysone, From Chemistry to Mode of Action*, Georg Thieme Verlag, Stuttgart, 1989, p. 97.
98 M. Hori, *Seroids*, 14 (1969) 33.
99 D.A. Schooley, G. Weiss and K. Nakanishi, *Steroids*, 19 (1973) 377.
100 R. Hardman and T.V. Benjamin, *Planta Med.*, 39 (1980) 148.
101 H.N. Nigg, M.J. Thompson, J.N. Kaplanis, J.A. Svoboda and W.E. Robbins, *Steroids*, 23 (1974) 507.
102 E.D. Morgan and C.F. Poole, *J. Chromatogr.*, 116 (1976) 333.
103 B. Stay, L.S. Ostedgaard, S.S. Tobe, A. Strambi and E. Spaziani, *J. Insect Physiol.*, 30 (1984) 643.
104 A. Moribayashi, H. Kurahashi and T. Ohtaki, *Arch. Insect Biochem. Physiol.*, 2 (1985) 237.
105 P. Beydon and R. Lafont, in A.P. Gupta (Editor), *Recent Advances in Comparative Arthropod Morphology, Physiology and Development*, Vol. I, Rutgers University Press, New Brunswick, NJ, 1989, p. 455.
106 P.V. Ripa, E.A. Martin, S.M. Cocciolone and J.H. Adler, *Phytochemistry*, 29 (1990) 425.
107 G.M. Holman and R.W. Meola, *Insect Biochem.*, 8 (1978) 275.
108 L. Canonica, B. Danieli, G. Ferrari, J. Krepinsky and I. Weisz-Venze, *Phytochemistry*, 14 (1975) 525–527.
109 G.B. Russel and J.G. Fraser, *Aust. J. Chem.*, 1805, (1973).
110 J. Koolman and K.-D. Spindler, *Hoppe-Seyler's Z. Physiol. Chem.*, 358 (1977) 1339.
111 M.J. Thompson, J.N. Kaplanis, W.E. Robbins and R.T. Yamamato, *Chem. Commun.*, (1967) 650.
112 W.-S. Chou and M.-S. Lu, in J.A. Hoffmann (Editor), *Progress in Ecdysone Research*, Elsevier, Amsterdam, 1980, p. 281.
113 A. Faux, M.N. Galbraith, D.H.S. Horn, E.J. Middleton and J.A. Thomson, *Chem. Commun.*, (1970) 243.
114 H.P. Huang, *Ph.D. Thesis*, Keele University, Keele, 1992.
115 G.B. Russell, D.G. Fenemore, D.H.S. Horn and E.J. Middleton, *Chem. Commun.*, (1968) 971.
116 R. Lafont, unpublished results.