Selectivity in the high-performance liquid chromatography of ecdysteroids

R. Lafont* and N. Kaouadji

Département 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, University of Keele, Keele, Staffordshire ST5 5BG (UK)

ABSTRACT

Phytoecdysteroids are a large family of plant compounds structurally related to ecdysone, and the complete HPLC separation of all these compounds requires the development of various complementary chromatographic systems. This paper describes a set of reversed-phase (RP) and normal-phase (NP) HPLC systems that can be used to separate ecdysteroids efficiently. A suitable combination of these NP- and RP-HPLC systems may allow the complete resolution of complex mixtures, but clearly the use of only two systems is not sufficient. Several original solvent systems are described and some examples are given to illustrate their selectivity towards the most common modifications of the ecdysteroid molecule.

INTRODUCTION

HPLC is by far the most widely used separation technique for many natural compounds, including phytoecdysteroids (*e.g.*, [1–6]). Many HPLC systems have been applied to the separation of ecdysteroids [7–11]. Both reversed-phase (RP) and normal-phase (NP) systems are used, and it is generally considered that a combination of one NP and one RP system will allow the complete resolution of individual components of complex ecdysteroid mixtures, as is most often the case with plant extracts. Usually, plants contain one or a few major components (mainly 20-hydroxyecdysone and, *e.g.*, polypodine B) and, in addition, a wide range of minor components, which seem to result from a random combination of a limited number of individual modifications [12,13]. Each structural modifica-

tion may result in a significant change in the chromatographic behaviour, which may be more or less marked when using RP or NP systems. As a consequence, when individual peaks collected during RP analysis are run on an NP system, previously co-eluting compounds are usually resolved [14]. For this reason, it is generally accepted that co-migration of a given ecdysteroid with the same reference compound in two solvent systems (generally one RP and one NP) can be considered as sufficient evidence for establishing their identity.

However, the size of the ecdysteroid family has increased continuously in recent years, and over 150 different compounds have now been described [12,13]. As a consequence, it has become necessary to develop several RP and NP systems to ensure all the required separations, because in a given HPLC system many ecdysteroids may elute very close together. Of course, very efficient chromatographic systems are required, but efficiency alone is not sufficient

---

* Corresponding author.

56

R. Lafont et al. / J. Chromatogr. A 658 (1994) 55–67

to ensure good resolution, and selectivity is a major parameter to be considered. The selectivity of a given chromatographic system results from both the stationary phase (column type) and the mobile phase. Changing one or both parameters may result in dramatic changes in separations (*i.e.*, the elution order of a given set of ecdysteroids). This important topic has already been addressed [11]. Published data concern mainly RP systems, where columns from various suppliers and mobile phases that contained either methanol, acetonitrile, tetrahydrofuran or dioxane were compared [15]. This problem has nevertheless not led to systematic developments that would provide general recipes to be used when faced with a given problem of separation. Moreover, this approach has not concerned NP systems. This paper reviews published data and includes original data, paying special attention to NP systems.

EXPERIMENTAL

Ecdysteroids

Ecdysone, 20-hydroxyecdysone (Fig. 1) and makisterone A were obtained from Simes (Milan, Italy). 2-Deoxyecdysone, 2-deoxy-20-hydroxyecdysone and ponasterone A were generous gifts from Dr. D. Horn (Acheron, Australia). Abutasterone was a gift from Dr. M. Pinheiro (Manaus, Brazil). Turkesterone was a gift from Dr. A. Suksamrarn (Bangkok, Thailand). 11α-Hydroxyecdysone (and its dehydra-
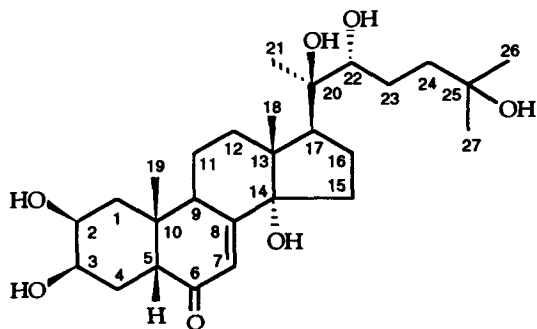
tion by-product) was a gift from Professor I. Kubo (Berkeley, CA, USA). Makisterone C, rubrosterone, poststerone, polypodine B, taxisterone, 20,26-dihydroxyecdysone, 22-oxo-20-hydroxyecdysone, 20-hydroxyecdysone 25-acetate, dacryhainansterone and 24,28-dehydromakisterone A were isolated from various plant sources [16–19]. 24-Epimakisterone was prepared by reduction of 24,28-dehydromakisterone A [20]. 3-Dehydro-20-hydroxyecdysone was prepared from 20-hydroxyecdysone by chemical oxidation [21]. 22-Epi-20-hydroxyecdysone and 3-epi-20-hydroxyecdysone were prepared from the 22-oxo and 3-oxo compounds, respectively, by chemical reduction [22]. Monoacetates of 20-hydroxyecdysone were prepared according to Horn [23]. 5α-Isomers were obtained from the corresponding 5β-compounds on equilibration under alkaline conditions [23]. 25-Deoxyecdysone and various dehydration products of ecdysone and 20-hydroxyecdysone were prepared according to Heinrich [24–26].

HPLC systems

HPLC equipment from Waters or DuPont was used. Isocratic conditions were always used, so that they can more readily be reproduced in other laboratories. Analytical columns were either (1) a Spherisorb 5 ODS-2 (Biochrom), 25 cm × 4.6 mm I.D., eluted with water–0.1% (final concentration) trifluoroacetic acid and either 23% acetonitrile (solvent system 1), 50% methanol (solvent system 2), 30% ethanol (solvent system 3), 18% 2-propanol (solvent system 4), 23% acetonitrile–2-propanol (5:2) (solvent system 5), 30% acetonitrile–methanol (1:1) (solvent system 6), or (2) a Zorbax-Sil column (DuPont), 25 cm × 4.6 mm I.D., eluted with either dichloromethane–2-propanol–water (125:30:2) (solvent system 7) or (125:40:3) (solvent system 8), cyclohexane–2-propanol–water (100:40:3) (solvent system 9) or (80:40:3) (solvent system 10), or isooctane–2-propanol–water (100:40:3) (solvent system 11) or (100:30:2) (solvent system 12). The flow-rate was 1 ml min^{-1} for all systems. Solvents (HPLC grade) were obtained from Carlo Erba (2-propanol, methanol, trifluoroacetic acid), Scharlau (iso-

Fig. 1. Structure and carbon numbering of 20-hydroxyecdysone.

octane, acetonitrile), Janssen (cyclohexane) and Prolabo (dichloromethane). Ultrapure water was obtained from a standard Millipore Milli-RO/ Milli-Q system.

RESULTS AND DISCUSSION

Fundamental structural changes

Usually, 20-hydroxyecdysone (Fig. 1) represents the major phytoecdysteroid found in most ecdysteroid-containing plants. It is therefore logical to consider how variations in the basic structure of 20-hydroxyecdysone result in changed chromatographic properties. Any combination of several of these individual changes (Table I) might be expected to be found in plants, even if not yet described. Mainly phytoecdysteroids will be considered here (for a more extensive list of known ecdysteroids, see ref. 27).

Towards general rules for the HPLC behaviour of ecdysteroids

Both isocratic RP and NP systems can separate complex ecdysteroid mixtures, and the use of solvent gradients would increase the possibilities of these systems. In order to evaluate the effects of single modifications on the HPLC behaviour of ecdysteroids, we selected a set of available reference ecdysteroids and used four different HPLC systems, i.e., two RP and two NP. The results of this study are given in Table II.

Effect of changing the number of OH groups. We examined most of the already described possibilities, i.e., structural variations at positions 1, 2, 5, 11, 20, 22, 24, 25 and 26, in NP- and RP-HPLC, and several conclusions can be drawn. First, it is clear that the addition of one OH group generally results in increased polarity, which is not surprising. 5β-OH is an exception to this rule, and indeed polypodine B shows unusu-

TABLE I

FUNDAMENTAL CHANGES TO THE 20-HYDROXYECDYSONE MOLECULE

Type	Positions on the molecule
Hydroxyl groups:	
Additional	1, 5, 11, 19, 23, 24, 26
Less	2, 20, 22, 25
Oxidation $\left(\diagdown\!\!CHOH \rightarrow \diagdown\!\!C = O \right)$	3, 22
Epimerization	$3\alpha/\beta, 5\alpha/\beta$
Alkyl substitution	24 (methyl, ethyl, methylene,...)
Esterification:	
Acetates	2, 3, 22, 25
Benzoates	20, 22, 25
Cinnamates	2
Coumarates	3
Sulphates	22
Etherification:	
Intramolecular	Between C-22 and C-25
Methoxy ether	25
Galactosides	3, 22
Glucosides	3, 25
Acetonides	2-3, 20-22
Dehydration	$\Delta^{9(11)}$, $\Delta^{14(15)}$, $\Delta^{24(25)}$, $\Delta^{25(26)}$
Side-chain cleavage	C-20/C-22, C-17/C-20
Presence of a lactone ring	Concerns essentially C_{28} or C_{29} ecdysteroids

TABLE II

INFLUENCE OF SOME REPRESENTATIVE ELEMENTARY STRUCTURAL CHANGES ON THE HPLC BEHAVIOUR OF VARIOUS ECDYSTEROIDS USING TWO RP AND TWO NP SYSTEMS

Relative retention times (20-hydroxyecdysone = 100). Retention times of 20-hydroxyecdysone = 5.15 min (system 1), 5.40 min (system 2), 28.9 min (system 7) and 21.3 min (system 9). For solvent systems, see Experimental.

Ecdysteroid	System 1	System 2	System 7	System 9
20-hydroxyecdysone	100	100	100	100
Hydroxylation/dehydroxylation at various positions				
Integristerone A (+1β-OH)	81	81	134	135
2-Deoxy-20-hydroxyecdysone (−2β-OH)	243	219	48	55
Polypodine B (+5β-OH)	105	100	65	101
Turkesterone (+11α-OH)	86	68	311	171
Ecdysone (−20-OH)	188	155	65	71
Taxisterone (−22-OH)	262	217	75	72
Abutasterone (+24-OH)	86	89	123	127
Ponasterone A (−25-OH)	636	324	23	37
20,26-Dihydroxyecdysone (+26-OH)	72	75	311	221
Oxidation/isomerization at C-3				
3-Epi-20-hydroxyecdysone	108	100	89	92
3-Oxo-20-hydroxyecdysone	127	107	32	76
Oxidation/isomerization at C-22				
22-Epi-20-hydroxyecdysone	90	87	173	121
22-Oxo-20-hydroxyecdysone	241	181	58	71
Isomerization of A/B ring junction				
5α,20-Hydroxyecdysone	95	89	86	113
Alkyl substitutions at C-24				
Makisterone C (+24α-C$_2$H$_5$)	326	238	39	53
Makisterone A (+24α-CH$_3$)	136	128	71	83
24-Epimakisterone A (+24β-CH$_3$)	152	127	58	73
$\Delta^{24(28)}$-Makisterone A (+24=CH$_2$)	155	138	47	64
Side-chain cleavage				
Rubrosterone	109	142	29	24
Poststerone	181	112	29	23
Acetylation				
20-Hydroxyecdysone 2-acetate	283	210	37	65
20-Hydroxyecdysone 3-acetate	202	143	39	70
20-Hydroxyecdysone 22-acetate	233	141	60	81
20-Hydroxyecdysone 25-acetate	349	175	30	54

al behaviour. In the RP mode, it may elute slightly before or after 20-hydroxyecdysone, whereas in the NP mode it may migrate close to either 20-hydroxyecdysone or ecdysone. In the latter instance (solvent system 7, Table II), this means that adding one OH group may result in a decrease in polarity, possibly owing to hydrogen bonding with the 6-ketone.

The changes in polarity induced by additional hydroxyl groups are strongly dependent on their position in the molecule (Fig. 2). Clearly posi-

tions 11, 25 and 26 have the greatest effects, owing to their location in hydrophobic parts of the molecule, whereas other positions, e.g., 1 or 24, which are in close vicinity to α-diols at C-2/C-3 or C-20/C-22, have more limited effects. The combined effects of two elementary changes depend whether they are located in adjacent or remote areas, so that they might be considered as linked or independent. In the latter instance the effect (in isocratic systems) can be approximated by a calculation that takes into account
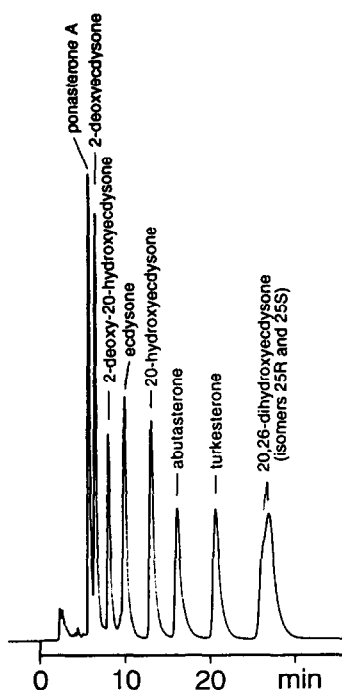
Fig. 2. Separation of a mixture of ecdysteroids (differing in the number of hydroxyl groups) by NP-HPLC (solvent system 10).

the equivalent of the property of additivity of elementary ΔR_m values used in TLC [28,29]. Some examples are given in Table III.

Effect of changing functions at C-3 (3β-OH, 3α-OH and 3-oxo). Oxidation of the secondary hydroxyl group to a ketone results in a significant decrease in polarity when studied by NP-HPLC, especially with solvent system 7 (Fig. 3). Using RP-HPLC, the decrease in polarity is less pronounced, and indeed when using methanol–water mixtures some workers were unable to obtain any separation of 3-oxo from 3β-OH compounds [30]. More generally, oxidation or epimerization at C-3 has only a limited effect on RP-HPLC when using methanol in the mobile phase. On the other hand, 3β-OH, 3α-OH and 3-oxo compounds are baseline resolved using acetonitrile in the mobile phase [21], with elution in that order. On NP columns, the separation is usually more efficient: 3-oxo compounds show a much decreased polarity, and the elution order is 3-oxo, 3α-OH, 3β-OH using silica [21].

Effect of changing functions at C-22 (22R-OH,

22S-OH and 22-oxo). Changes in stereochemistry at C-22 (to 22-isoecdysteroids) have so far not been described for naturally occurring ecdysteroids, although their occurrence cannot be excluded, as 22-oxoecdysteroids have recently been discovered [18,19]. Clearly changes at C-22 result in significant changes in polarity when studied using NP-HPLC, and the 22-epimer (22-iso-20-hydroxyecdysone) elutes much later than the parent compound.

Effect of changing the stereochemistry of the A/B ring junction (5β = cis; 5α = trans). With 20-hydroxyecdysone, this change results in limited effects on polarity, which vary according to the solvent system (Table II). In fact, the magnitude of these effects is more or less pronounced, depending on the number and stereochemistry of OH groups present on ring A at C-2 (or C-1; data not shown), and solvent selectivity is also important (see below).

Effect of alkyl substituents at C-24. The presence of substituents at C-24 results in significant decreases in polarity with both NP and RP systems (effect of ethyl > effect of methyl or methylene), as expected from the introduction of an additional hydrophobic group (Fig. 4).

Effect of side-chain cleavage (between C-20/C-22 or C-17/C-20). Side-chain cleavage at C-17/C-20 has little effect on the RP-HPLC behaviour (Table II). This can be interpreted as some compensation between the effects of the simultaneous removal of both the polar hydroxyls and the hydrophobic groups present on the side-chain. On the other hand, it results in a large decrease of polarity when studied by NP-HPLC, which is of the same order as that produced by the absence of a 25-OH group when the side-chain is present.

Effect of esterification: example of acetates. Acetylation results in a significant decrease in polarity, which varies according to the position involved (Fig. 5) On NP-HPLC, esterification, absence of the corresponding OH group or its conversion into a ketone seem to have similar effects (Table II). Acetates at C-2 and C-3 are poorly separated by NP-HPLC, whereas this separation is easily achieved by RP-HPLC (Table II). The effect of esterifying a given OH group may depend on the presence or absence of

TABLE III

EFFECT OF CHANGING THE NUMBER OF –OH GROUPS ON k' AND α (RELATIVE TO 20-HYDROXYECDYSONE) OF ECDYSTEROIDS ON NP-HPLC (SOLVENT SYSTEM 8)

k' = Capacity factor $[=(t_r - t_0)/t_0]$; α = selectivity factor $(=k'_2/k'_1)$. Ln α is equivalent to ΔR_m used in TLC [26]. According to the same additivity rules as those used in the case of TLC with the elementary ΔR_m, we can calculate the expected ln α in HPLC when double changes are made to the 20-hydroxyecdysone molecule.

Position/compound	k'	Ln α	
20-Hydroxyecdysone	5.00		
		observed	
Single changes			
–C-2 (2-deoxy-20-hydroxyecdysone)	2.35	–0.752	
–C-20 (ecdysone)	3.29	–0.420	
–C-22 (taxisterone)	3.78	–0.278	
–C-25 (ponasterone A)	0.88	–1.740	
+C-1β (integristerone A)	7.53	+0.410	
+C-5 (polypodine B)	3.29	–0.420	
+C-11α (turkesterone)	14.7	+1.077	
+C-24 (abutasterone)	6.14	+0.205	
+C-26 (20,26-dihydroxyecdysone)	14.7	+0.077	
		Observed	Calculated
Double changes: independent?			
–C-20 – C-2 (2-deoxyecdysone)	1.57	–1.157	–1.172
–C-20 – C-25 (25-deoxyecdysone)	0.63	–2.071	–2.160
+C-26 + C-5 (26-hydroxy-polypodine B)	9.9	+0.682	+0.657
+C-26 – C-20 (26-hydroxyecdysone)	7.8	+0.445	+0.657
+C-26 – C-22 (22-deoxy-26-hydroxyecdysone)	10.6	+0.750	+0.799
+C-11α – C-20 (11α-hydroxyecdysone)	7.53	+0.410	+0.657
+C-11α – C-25 (ajugasterone C)	2.27	–0.789	–0.663
Double changes: linked?			
+C-1 – C-2 (2-deoxy-integristerone A)	3.82	–0.269	–0.342
–C-20 – C-22 (22-deoxyecdysone)	1.43	–1.25	–0.698
–C-25 + C-24 (pterosterone)	2.62	–0.644	–1.535
–C25 + C-26 (inokosterone)	4.48	–0.110	–0.663

another OH group in its close vicinity. For instance, when studied by RP-HPLC (solvent system 1 in Table II), 20E22Ac elutes *between* 20E3Ac and 20E2Ac, whereas in the ecdysone series (data not shown) E22Ac elutes well *after* E3Ac and E2Ac. Methanol and acetonitrile result in differences in the order of elution of acetates, and in the former system 20E3Ac and 20E22Ac are not resolved.

Effect of an additional double bond. The presence of an additional double bond either on the nucleus [$\Delta^{9(11)}$] or on the side-chain [$\Delta^{24(25)}$ and $\Delta^{25(26)}$] results in limited changes in the retention times, with the exception of solvent system 2

(Table IV). The presence of a $\Delta^{9(11)}$ double bond results in a slightly increased polarity in all the tested systems, whereas double bonds on the side-chain have more pronounced effects. Clearly, NP-HPLC solvents based on dichloromethane are very poor in that case. Surprisingly, the 24-methylene group appears less polar than the methyl group in NP-HPLC [compare makisterone A and its $\Delta^{24(28)}$ derivative].

Selectivity in NP-HPLC

Selectivity due to the column. Usual NP-HPLC columns are packed with either silica (*e.g.*, Partisil, Zorbax-Sil) or polar-bonded silica (*e.g.*,
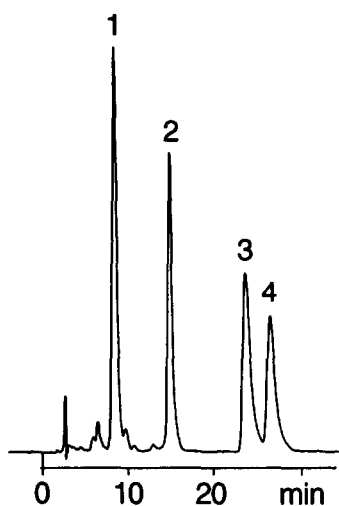
Fig. 3. Separation of a mixture of ecdysteroids modified at position 3 or 22 by NP-HPLC on a Zorbax-Sil column (solvent system 7). Peaks: 1 = 3-dehydro-20-hydroxy-ecdysone; 2 = 22-oxo-20-hydroxyecdysone; 3 = 3-epi-20-hydroxyecdysone; 4 = 20-hydroxyecdysone.

diol, Polyol, NH_2). Using diol instead of silica columns does not seem to introduce any large changes; the retention times may vary, but the elution order usually remains the same; diol
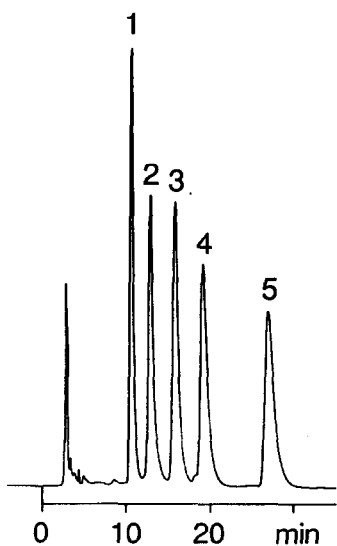

Fig. 4. Separation of a mixture of ecdysteroids bearing substitutions at C-24 by NP-HPLC on a Zorbax-Sil column (solvent system 7). Peaks: 1 = makisterone C; 2 = 24(28)-dehydromakisterone A; 3 = 24-epi-makisterone A; 4 = makisterone A; 5 = 20-hydroxyecdysone.
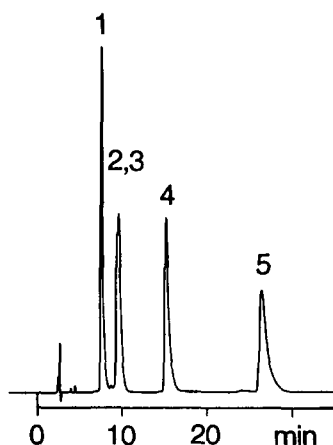

Fig. 5. Separation of a mixture of 20-hydroxyecdysone monoacetates by NP-HPLC on a Zorbax-Sil column (solvent system 7). Peaks: 1 = 20-hydroxyecdysone 25-acetate; 2 = 20-hydroxyecdysone 2-acetate; 3 = 20-hydroxyecdysone 3-acetate; 4 = 20-hydroxyecdysone 22-acetate; 5 = 20-hydroxyecdysone.

columns provide the advantage of allowing gradients to be used. On the other hand, NH_2 (or APS) columns may interact in a different way with some ecdysteroids and therefore introduce a different selectivity [30]. We have also used TMS-bonded phases [31]; they behave very like silica columns but with less peak tailing. However, these data were obtained with an "old" column and they could not be reproduced with a new column. Clearly, the properties of such columns depend strongly on the percentage of free silanol groups, and a lower percentage is connected with shorter retention times. Nevertheless, changing the usual solvents to less polar types still allows the efficient use of "new" TMS columns.

The retention times obtained with silica columns are not completely stable. They may decrease on prolonged use, especially with water-containing solvents which will slowly deactivate the column. In this event, reactivation with anhydrous solvents allows the complete recovery of previous retention times. Among the columns tested, it seems that Zorbax-Sil is the least affected by the prolonged use of water-containing solvents.

Other types of NP stationary phases are also available (alumina, graphitic carbon), but there

TABLE IV

SEPARATION OF COMPOUNDS DIFFERING BY THE PRESENCE/ABSENCE OF DOUBLE BONDS

Retention times in minutes. For solvent systems, see Experimental.

Compound	System 1	System 2	System 7	System 9	System 12
Ponasterone A (25d20E)	6.45[a]	17.6	6.85	7.4	15.1
Dacryhainansterone [$\Delta^{9(11)}$]	5.6[a]	14.3	7.3	7.7	15.9
Stachysterone C [$\Delta^{24(25)}$]	5.25[a]	13.4	7.3	8.1	16.8
"Iso"-stachysterone C [$\Delta^{25(26)}$]	5.25[a]	12.2	7.3	8.2	16.8
25-Deoxyecdysone (25dE)	12.2[a]	32.8	6.1	6.4	11.6
$\Delta^{24(25)}$-25dE	9.5[a]	24.5	6.2	6.8	12.9
$\Delta^{25(26)}$-25dE	8.8[a]	21.8	6.3	6.9	13.5
Ecdysone (E)	9.45	8.4	18.8	15.1	–
$\Delta^{9(11)}$-E	8.1	7.2	20.9	16.9	–
Makisterone A	7.7	7.0	20.5	17.7	–
$\Delta^{24(28)}$-makisterone A	6.9	7.5	13.6	13.6	–

[a] 35% instead of 23% acetonitrile.

are no descriptions of their use with ecdysteroids.

Selectivity due to solvents. Many solvent systems have already been described for the NP-HPLC of ecdysteroids [7–11,29]. They are less numerous than those described for TLC, however, simply because UV detection (242 or 254 nm) precludes the use of solvents with a high UV cut-off (*e.g.*, ethyl acetate, benzene or acetone). Therefore, the usual basis for the solvent is a chlorinated hydrocarbon (chloroform, methylene chloride, ethylene dichloride [29]), and the modifier is an alcohol (methanol, ethanol, propanol or 2-propanol). Adding water just below saturation is useful, because it results in reduced peak tailing [32]. We proposed some years ago [32] the use of such tertiary mixtures based on dichloromethane, 2-propanol and water (*e.g.*, 125:25:2, v/v/v) to separate complex ecdysteroid mixtures. Later, we proposed the replacement of dichloromethane with isooctane, as the latter allowed the in-line use of diode-array detectors or radioactivity monitors [10]. However, isooctane is a poor solvent for ecdysteroids, and we encountered recovery problems, which were overcome by using cyclohexane.

Cyclohexane-based ternary mixtures have a significantly different selectivity, and their combination with dichloromethane-based solvents allows many separations to be achieved (Table II). They are highly viscous, and working pressures are above 100 bar with analytical columns at a flow-rate of 1 ml min^{-1}. Raising the temperature to 50°C can overcome this problem, however, as it results in a *ca.* 40% decrease in working pressure without affecting the separation.

Some examples. Clearly dichloromethane- or cyclohexane-based solvents (Table II) provide very different selectivities, as exemplified by the separation of ecdysone, 20-hydroxyecdysone and polypodine B, or by turkesterone and 20,26-dihydroxyecdysone. When considering the separation of 5α–5β pairs (Table V), the differences are striking.

The separation of 3-oxo, 3β-OH and 3α-OH compound mixtures is achieved on silica columns, but with aminopropyl-bonded phases 3α-OH compounds elute *after* 3β-OH compounds [30] and the overall separation is more efficient (Table VI).

The separation of compounds with or without double bonds has clearly shown (see above) that NP-HPLC is inefficient in this respect, whereas RP-HPLC allows their easy resolution.

Another way of modifying chromatographic

TABLE V

CHROMATOGRAPHIC DATA FOR 5α–5β PAIRS OF ECDYSTEROIDS

Relative retention times (20-hydroxyecdysone = 100). Retention times of 20-hydroxyecdysone: 5.15 min (system 1), 5.40 min (system 2), 5.80 min (system 3), 6.0 min (system 4), 7.5 min (system 6), 28.9 min (system 7), 21.3 min (system 9) and 27.8 min (system 11). For solvent systems, see Experimental. 2dE = 2-deoxyecdysone; 2d20E = 2-deoxy-20-hydroxyecdysone; E = ecdysone; 20E = 20-hydroxyecdysone.

Solvent	2dE		2d20E		E		20E	
	5α	5β	5α	5β	5α	5β	5α	5β
RP-HPLC								
System 1	586	620	225	237	181	189	95	100
System 2	401	419	198	219	135	155	89	100
System 3	650	721	240	260	167	207	84	100
System 4	639	735	216	248	165	215	84	100
System 6	869	901	311	320	195	232	89	100
NP-HPLC								
System 7	32	33	47	48	52	65	86	100
System 9	47	42	64	55	76	71	113	100
System 11	47	40	62	53	77	74	110	100

mobility in NP-HPLC, specific for 20,22- but not 2,3-diols, is the formation of cyclic boronates as described by Pis and Harmatha [33].

Selectivity in RP-HPLC

Selectivity due to the column. The user is faced with a profusion of available columns that differ according to the bond chain length or type (C_6, C_8, C_{18}, C_{22}, phenyl, CN, . . .), the extent of bonding (percentage of carbon load, usually 5–15%) which may be indicated by the commercial names (*e.g.*, ODS-1, ODS-2, ODS-3) or the porosity of silica used. All these parameters may change the selectivity of the column, and for that reason it is not always possible to reproduce data from the literature except by using exactly the same conditions, including the column type. Comparison of columns from various suppliers has been addressed previously [15] and slightly

TABLE VI

SELECTIVITY CHANGES OF APS COLUMNS WITH VARIOUS TERNARY SOLVENT MIXTURES

After ref. 30. Column, APS-Hypersil (5 μm), 25 cm × 4.6 mm I.D., eluted at 1 ml min^{-1} with various dichloromethane–methanol–2-propanol mixtures. Retention times relative to 20-hydroxyecdysone = 100.

Mobile phase composition	2dE		E			20E		
	3α	3β	3α	3β	3-Oxo	3α	3β	3-Oxo
95:1:4	28	21	110	63	19	190	100	28
95:2:3			105	68	18	169	100	
95:3:2				67			100	
95:4:1				67		124	100	

64

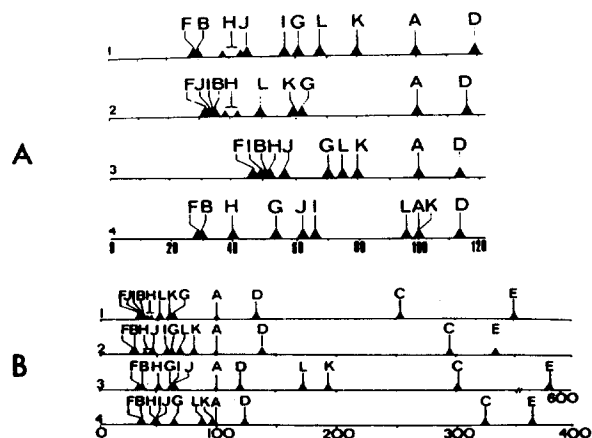R. Lafont et al. / J. Chromatogr. A 658 (1994) 55–67

Fig. 6. Selectivity of RP-HPLC is linked to both the columns and the solvent systems (from ref. 15). (A) Selectivity differences between Spherisorb ODS eluted with (1) acetonitrile–water (15:85) or (2) methanol–water (35:65) and Nucleosil-ODS eluted with (3) methanol–water (50:50) or (4) acetonitrile–water (20:80). (B) Comparison of different solvents with a Spherisorb ODS column: (1) methanol–water (35:65); (2) acetonitrile–water (15:85); (3) tetrahydrofuran–water (10:90); (4) dioxane–water (20:80). Retention is given relative to ecdysone (A) which is given a value of 100. A = ecdysone; B = 20-hydroxyecdysone; C = 2-deoxyecdysone; D = 2-deoxy-20-hydroxyecdysone; E = ponasterone A; F = polypodine B; G = makisterone A; H = inokosterone; I = cyasterone; J = poststerone; K = ajugasterone C; L = muristerone A.

different separations were obtained with standard ecdysteroid mixtures (Fig. 6A).

The most usual columns for the separation of ecdysteroids are the C_{18} (or ODS) bonded type,

but C_8 columns are also used. Recently, the use of β-cyclodextrin-bonded silica was proposed [34]. Such stationary phases are expected to interact in a specific way with some ecdysteroids and thus display new kinds of selectivities, but so far they have been used with only a few ecdysteroids and this clearly requires further investigations.

Selectivity due to the solvents. RP-HPLC solvents consist of water and a water-miscible organic modifier (*e.g.*, acetonitrile, methanol, ethanol, 2-propanol, tetrahydrofuran, dioxane). When tested with ecdysteroid mixtures, these solvent systems gave significantly different results (Fig. 6B).

Methanol and acetonitrile are the most widely used organic modifiers. Acetonitrile–water mixtures may give excessive peak tailing, which can be suppressed by replacing water with a buffer or simply by adding trifluoroacetic acid (0.1%, v/v). Recently, very interesting separations with 2-propanol have been reported [11,35] and, when applied to 20-hydroxyecdysone–polypodine B mixtures, they clearly resulted in very efficient separations (Table VII).

Similarly, we tested several RP solvent systems on compounds differing by one OH group, and the results are given in Table VIII. Clearly, the retention is greatly affected by changing the organic modifier in the mobile phase. 2-Propanol–water provides the best separation for 5α–5β pairs (Table V). Methanol is particularly

TABLE VII

EFFECT OF ORGANIC MODIFIERS ON THE SEPARATION OF 20-HYDROXYECDYSONE AND POLYPODINE B USING RP-HPLC

After ref. 11. Column, Spherisorb 10 ODS-2, 25 cm × 4.6 mm I.D.; mobile phase flow-rate 1 ml min^{-1}. k' = Capacity factor; α = selectivity factor (k' ratio); N = efficiency (theoretical plates per metre column length); R_s = resolution $\left(= \frac{1}{4} \sqrt{N} \cdot \frac{k'}{1+k'} \cdot \frac{\alpha-1}{\alpha} \right)$.

Organic modifier	k'		α	N	R_s
	PolB	20E			
20% acetonitrile	7.73	7.73	1	13 751	0
45% methanol	2.21	2.21	1	1860	0
35% methanol	7.95	8.45	1.06	5261	0.60
15% 2-propanol	2.22	2.63	1.18	16 920	2.85
11% 2-propanol	5.42	6.90	1.27	5009	2.93

TABLE VIII

RP-HPLC OF ECDYSTEROIDS USING FIVE DIFFERENT RP SYSTEMS

Relative retention times (20-hydroxyecdysone = 100). Retention times of 20-hydroxyecdysone: 5.15 min (system 1), 5.8 min (system 3), 6.0 min (system 4), 5.95 min (system 5) and 7.5 min (system 6). For solvent systems, see Experimental.

Ecdysteroid	System 1	System 3	System 4	System 5	System 6
20-Hydroxyecdysone	100	100	100	100	100
Integristerone A (+1β-OH)	81	69	84	77	68
2-Deoxy-20-hydroxyecdysone (−2β-OH)	243	260	247	250	320
Polypodine B (+5β-OH)	105	97	92	97	100
Turkesterone (+11α-OH)	86	59	62	63	53
Ecdysone (−20-OH)	188	207	216	201	232
Taxisterone (−22-OH)	262	324	308	297	373
Abutasterone (+24-OH)	86	83	84	85	81
Ponasterone A (−25-OH)	636	679	814	678	687
20,26-Dihydroxyecdysone (+26-OH)	72	69	72	69	61

efficient towards extra double bonds (Table IV), as it may baseline separate $\Delta^{24(25)}$ and $\Delta^{25(26)}$ pairs, but on the other hand it cannot separate 3α–3β isomers, which acetonitrile can do. Extra OH groups generally increase the polarity (but see polypodine B) and their effect depends on both their position *and* the solvent system used (Table VIII). Positions 11α and 26 are located in hydrophobic regions of the molecule, and introducing an extra OH group in these positions results in the most conspicuous effects.

Among the solvents tested, we used acetonitrile–methanol–water and acetonitrile–2-propanol–water mixtures. As can be seen from Table VIII, these mixtures do not exactly possess intermediate properties between those which these organic solvents display when used alone,

but they have their own selectivity towards some ecdysteroids.

Effects of temperature. A temperature increase results in increased efficiency, decreased pressure and a decrease in the capacity factor, k'. This has been particularly investigated using 2-propanol–water mixtures [11,35] and it allowed the calculation of the temperature dependence of k' for a set of reference ecdysteroids (Table IX). There were only small differences in the exponents for the various ecdysteroids, which means that selectivity is not much affected.

CONCLUSIONS

It is necessary to be very cautious regarding the conclusions that can be drawn from the co-

TABLE IX

VARIATIONS IN THE CAPACITY FACTOR (k') WITH TEMPERATURE (T) (ADJUSTED CURVES)

From ref. 35. Column, Spherisorb 5 ODS-2, 10 cm × 4 mm I.D.; mobile phase, 2-propanol–water (7:93); flow-rate, 1 ml min^{-1}.

Ecdysteroid	Equation of exponential decay	$T_{1/2}$	r
29-Norsengosterone	$k' = 21.6\,e^{-0.045T} + 3.0$	15.20	0.999
29-Norcyasterone	$k' = 32.0\,e^{-0.049T} + 3.3$	14.07	0.999
Polypodine B	$k' = 19.3\,e^{-0.38T} + 3.7$	17.87	0.998
20-Hydroxyecdysone	$k' = 29.1\,e^{-0.042T} + 4.1$	16.20	0.999
Cyasterone	$k' = 39.0\,e^{-0.046T} + 4.8$	15.06	0.999
Makisterone A	$k' = 75.7\,e^{-0.044T} + 7.1$	15.60	1
Ajugalactone	$k' = 76.8\,e^{-0.044T} + 3.0$	15.61	1

66

R. Lafont et al. / J. Chromatogr. A 658 (1994) 55–67

migration of ecdysteroids in only one or even two solvent systems. As shown here (Fig. 7), this may result in erroneous identifications (or provide too optimistic conclusions regarding the purity of a compound). Clearly, using one NP and one RP system gives better selectivity than using two NP or two RP systems.

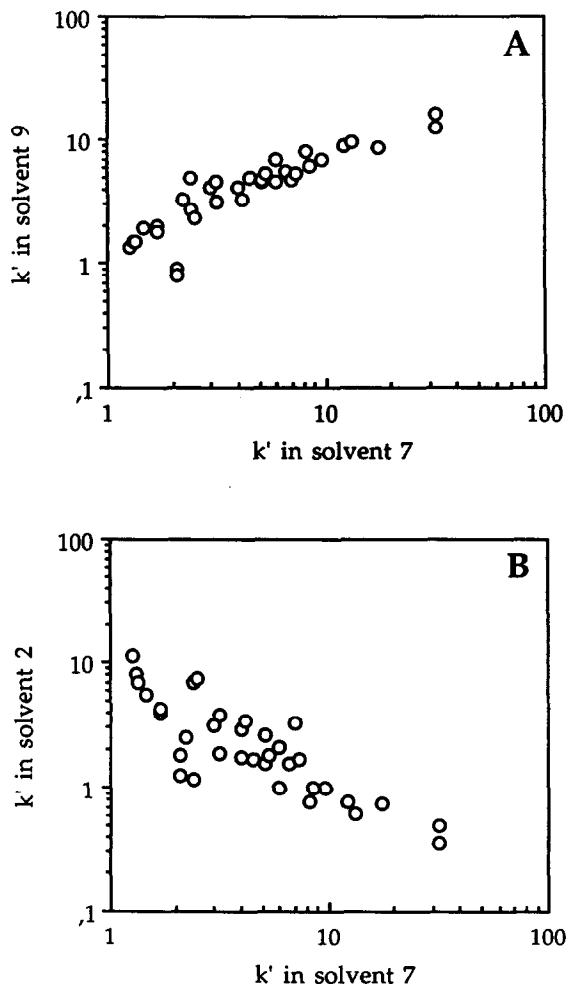In order to overcome the problem connected with the existence of a very large family of such

closely related compounds, it seems important to recommend the use of more sophisticated criteria. We can see here (Table II) that when using (1) two solvent systems for NP (dichloromethane- and cyclohexane-based) and (2) two solvent systems for RP (acetonitrile- and methanol- or 2-propanol-based), the probability of the identification being incorrect becomes very low.

In fact, what we describe here is only a transposition of what has been used for a long time in the TLC analysis of steroid molecules, for which many solvent systems have been proposed to achieve the resolution of specific pairs of compounds. In HPLC, the use of in-line UV detection precludes the use of many of the usual TLC solvents (e.g., ethyl acetate, acetone, pyridine, toluene), but this is counter-balanced by a greater efficiency. In spite of this, a single system is clearly unable to resolve all compounds, and it remains of interest to take advantage of selective effects of the mobile phases to improve the efficiency of HPLC separations.

The approach may provide a basis from which to develop a computer program that would predict the behaviour of any new ecdysteroid in various solvent systems and therefore help to identify ecdysteroids when no reference compounds are available, and when amounts are inadequate for MS or NMR analyses.

Fig. 7. The use of two different solvent systems increases the possibility of resolving complex ecdysteroid mixtures, but it does not allow a complete resolution of the 35 ecdysteroids tested in this study. (A) Use of two solvents systems of the same type (here two NP systems): some selective effects are apparent, although restricted to a few ecdysteroids, and overlapping of many compounds is observed. (B) Use of one RP and one NP system increases the efficiency of separations, but still a few overlapping compounds remain.

REFERENCES

1 I. Kubo, J.A. Klocke, I. Ganjian, N. Ichikawa and T. Matsumoto, J. Chromatogr., 257 (1983) 157.
2 M. Bathori, K. Szendrei and I. Herke, Chromatographia, 21 (1986) 234.
3 I. Kubo and F.J. Hanke, in J.R. Miller and T.A. Miller (Editors), Insect–Plant Interactions (Springer Series in Experimental Entomology), Springer, Berlin, 1986, pp. 225–249.

4 P.V. Ripa, E.A. Martin, S.M. Cocciolone and J.D. Adler, *Phytochemistry*, 29 (1990) 425.

5 M.L. Colombo, F. Tome, O. Servettaz and C. Bugatti, *Int. J. Crude Drug Res.*, 28 (1990) 219.

6 L. Dinan, *J. Chromatogr. A*, 658 (1994) 69.

7 R. Lafont, P. Beydon, B. Mauchamp, G. Sommé-Martin, M. Andrianjafintrimo and P. Krien, in F. Sehnal, A. Zabza, J.J. Menn and B. Cymborowski (Editors), *Regulation of Insect Development and Behaviour*, Wroclaw Technical University Press, Wroclaw, 1981, pp. 125–144.

8 J.C. Touchstone, in G. Zweig and J. Sherma (Editors), *CRC Handbook of Chromatography. Steroids*, CRC Press, Boca Raton, FL, 1986, pp. 119–135.

9 R. Lafont, in H. Kalász and S. Ettre (Editors), *Chromatography'87*, Akadémiai Kiadó, Budapest, 1988, pp. 1–15.

10 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, 1990, pp. 79–94.

11 E.D. Morgan and M.P. Marco, *Invert. Reprod. Dev.*, 18 (1990) 55.

12 R. Lafont and D.H.S. Horn, in J. Koolman (Editor), *Ecdysone, from Chemistry to Mode of Action*, Georg Thieme, Stuttgart, 1989, pp. 39–64.

13 F. Camps, in J.B. Harborne and F.A. Thomas-Barberan (Editors), *Ecological Chemistry and Biochemistry of Plant Terpenoids*, Clarendon Press, Oxford, 1991, pp. 331–376.

14 M.W. Gilgan, *J. Chromatogr.*, 129 (1976) 447.

15 I.D. Wilson, C.R. Bielby and E.D. Morgan, *J. Chromatogr.*, 238 (1982) 97.

16 J.-P. Girault, R. Lafont, E. Varga, Zs. Hajdu, I. Herke and K. Szendrei, *Phytochemistry*, 27 (1988) 737.

17 J.-P. Girault, M. Bathori, E. Varga, K. Szendrei and R. Lafont, *J. Nat. Prod.*, 53 (1990) 279.

18 D. Rudel, M. Bathori, J. Gharbi, J.-P. Girault, I. Racz, K. Melis, K. Szendrei and R. Lafont, *Planta Med.*, 58 (1992) 358.

19 M. Wessner, B. Champion, J.-P. Girault, N. Kaouadji, B. Saidi and R. Lafont, *Phytochemistry*, 31 (1992) 3785.

20 P. Maurer, J.-P. Girault, M. Larchevêque and R. Lafont, *Arch. Insect Biochem. Physiol.*, 23 (1993) 29.

21 J.-P. Girault, C. Blais, P. Beydon, C. Rolando and R. Lafont, *Arch. Insect. Biochem. Physiol.*, 10 (1989) 199.

22 L.N. Dinan and H.H. Rees, *Steroids*, 32 (1979) 629.

23 D.H.S. Horn, in M. Jacobson and D.G. Crosby (Editors), *Naturally Occurring Insecticides*, Marcel Dekker, New York, 1971, pp. 333–459.

24 G. Heinrich, *Ph.D. Dissertation*, Math. Nat. Fakultät, Universität Hamburg, Hamburg, 1970.

25 L. Dinan, *Arch. Insect Biochem. Physiol.* 2 (1985) 295.

26 R. Lafont, J. Pis, M. Larchevêque and J.-P. Girault, in preparation.

27 R. Lafont and I.D. Wilson, *The Ecdysone Handbook*, Chromatographic Society Press, Nottingham, 1992.

28 J. Koolman, L. Reum and P. Karlson, *Hoppe-Seyler's Z. Physiol. Chem.*, 360 (1979) 1351.

29 R. Lafont, E.D. Morgan and I.D. Wilson, *J. Chromatogr. A*, 658 (1994) 31.

30 L.N. Dinan, P.L. Donnahey, H.H. Rees and T.W. Goodwin, *J. Chromatogr.*, 205 (1981) 139.

31 N. Kaouadji and R. Lafont, *J. Chromatogr.*, 505 (1990) 408.

32 R. Lafont, G. Sommé-Martin and J.-C. Chambet, *J. Chromatogr.*, 170 (1979) 185.

33 J. Pis and J. Harmatha, *J. Chromatogr.*, 596 (1992) 271.

34 T. Vaisar and T. Vanek, *J. Chromatogr.*, 547 (1991) 443.

35 F. Camps, J. Coll, M.P. Marco and J. Tomás, *J. Chromatogr.*, 514 (1991) 199.