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Article *in* Journal of Chromatography A · December 2001

DOI: 10.1016/S0021-9673(01)00992-X · Source: PubMed

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Review

Chromatographic procedures for the isolation of plant steroids

Laurence Dinan[a,*], Juraj Harmatha[b], René Lafont[c]

[a]*Department of Biological Sciences, University of Exeter, Hatherly Laboratories, Prince of Wales Road, Exeter, Devon EX4 4PS, UK*
[b]*Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Flemingovo nám. 2, 166 10 Prague, Czech Republic*
[c]*Laboratoire d'Endocrinologie Moléculaire et Evolution, Université Pierre et Marie Curie, 7 Quai St. Bernard, 75252 Paris 05, France*

Abstract

In this review, we consider the general principles and specific methods for the purification of different classes of phytosteroids which have been isolated from plant sources: brassinosteroids, bufadienolides, cardenolides, cucurbitacins, ecdysteroids, steroidal saponins, steroidal alkaloids, vertebrate-type steroids and withanolides. For each class we give a brief summary of the characteristic structural features, their distribution in the plant world and their biological effects and applications. Most classes are associated with one or a few plant families, e.g., the withanolides with the Solanaceae, but others, e.g., the saponins, are very widespread. Where a compound class has been extensively studied, a large number of analogues are present across a range of species. We discuss the general principles for the isolation of plant steroids. The predominant methods for isolation are solvent extraction/partition followed by column chromatography and thin-layer chromatography/HPLC.

Keywords: Reviews; Steroids; Alkaloids; Brassinosteroids; Bufadienolides; Cardenolides; Cucurbitacins; Ecdysteroids; Saponins; Withanolides; Sapogenins; Glycoalkaloids

Contents

*Corresponding author. Tel.: +44-1392-264-605; fax: +44-1392-263-700.
E-mail address: l.n.dinan@exeter.ac.uk (L. Dinan).

0021-9673/01/$ – see front matter
PII: S0021-9673(01)00992-X

1. Introduction

Plants produce a wide array of steroid molecules (Fig. 1) which can be divided into three groups, based on their biological relevance:

(1) Substances which have physiological roles in the plant itself, as hormones or pheromones. Thus, brassinosteroids (**1**) are growth-promoting phytohormones, whereas antheridiol (**2**) and oogoniol (**3**) are pheromones in an aquatic filamentous fungus.

(2) Allelochemical substances related to animal hormones: ecdysteroids (**4**) are analogues of insect moulting hormones, whereas androgens, oestrogens, progestagens (**5**), corticosteroids and cholecalciferols (**6**) are related to vertebrate hormones.

(3) Plant-specific allelochemical substances, which often display protective (repellent, antifeedant, toxic) actions towards phytophagous animals or parasitic fungi: these are, e.g., cucurbitacins (**7**), cardenolides (**8**), bufadienolides (**9**), sapogenins (**10**), withanolides (**11**) and steroidal alkaloids (**12**).

The first point to be considered is the **scale** of the experiment: isolating 1 mg or several grams requires appropriate methods. It should be noted that thanks to the progress of spectroscopic methods [both mass spectrometry (MS) and nuclear magnetic resonance (NMR)], 1 mg of any pure steroid is usually sufficient to establish its structure, whereas the required amounts only 10 years ago were at least one or two orders of magnitude higher. The purity to be reached is also of importance: obtaining a substance pure at 90%, 95% or >99% is not the same task at all, and this has important consequences for the protocols to be used.

A second point to be considered is the **concentration** of the compound within the plant. While hormones and pheromones are present only at very low concentrations (pg/g to ng/g), allelochemical substances (secondary metabolites) may reach much higher concentrations (i.e., μg/g to mg/g) and sometimes even represent up to 1–5% of the plant's dry mass. In the case of substances present in minute

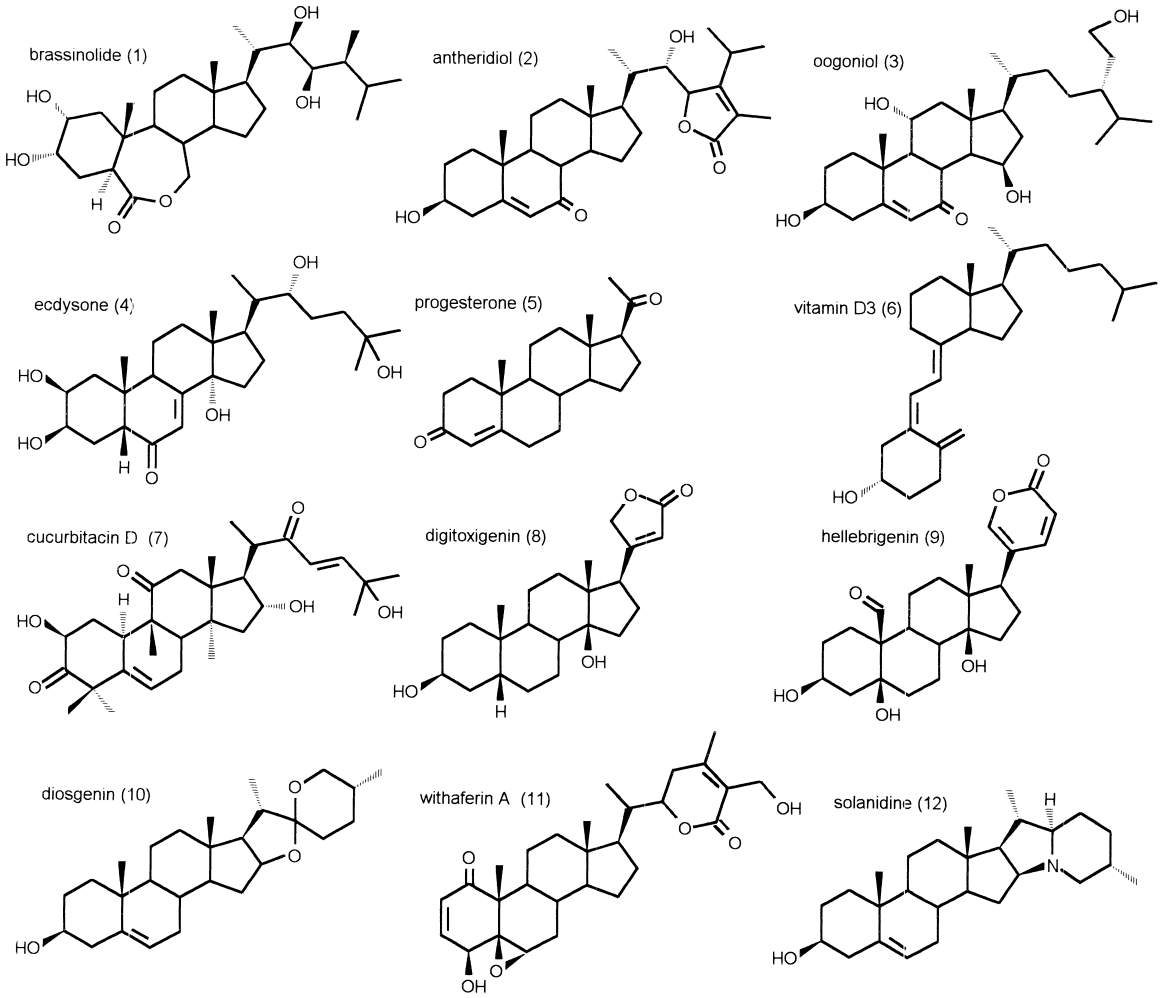

Fig. 1. Some representative structures of plant steroids. Only aglycones are shown. The configurations are 8β-H, 9α-H and 14α-H, unless otherwise depicted.

amounts, large samples (i.e., kg, or even more) have to be processed, whereas for major components a few grams are enough. Consequently, the scale and complexity of the protocols to be used are totally different in such cases.

Finally, the third point to be considered is related to the **polarity** of the compound(s) of interest: among plant steroids, we can find apolar, polar or even very polar (water-soluble) molecules (Table 1), and of course purification methods must be adapted for each specific case.

2. General purification strategy

From plant sample to pure compounds, the purification strategy always comprises a multi-step procedure including extraction, prepurification and then one or several chromatographic steps. Thus, the fresh or dry sample will be cut up or ground to a powder before (i) extraction with organic solvents (perhaps in a sequence of increasing polarity), water or supercritical CO_2, (ii) solvent partitions to remove less polar and/or more polar compounds, (iii) initial

Table 1
Different classes of polarity of plant steroids

Property	Examples
Non-polar	BS (fatty acyl esters), BU, CA (genins), VS
Moderately polar	BS, CU, ES, SA, WI
Polar to very polar	BS (glucosides), CA (glycosides), ES (glucosides/sulphates), SS

Abbreviations for the various classes of steroids: BS: brassinosteroids; BU: bufadienolides; CA: cardenolides; CU: cucurbitacins; ES: ecdysteroids; SA: steroidal alkaloids; SS: steroidal saponins; VS: vertebrate-type steroids; WI: withanolides.

chromatographic steps (flash chromatography, counter-current chromatography or low-pressure column chromatography on silica or alumina) and (iv) final purification by thin-layer chromatography (TLC) and/or high-performance liquid chromatography (HPLC). This general strategy is similar to that used for other classes of plant molecules, such as polypeptides [1]. Extraction (of milled, dry material) can be performed using a large range of solvents (with ca. 10% vol/w), among which alcohols are the most widely used. For polar steroids (Table 1), it is possible to first extract with apolar solvents (e.g., a hydrocarbon) which will extract non-polar compounds, and then with a more polar one (alcohol). This procedure has some connection with the partition step as described thereafter.

After concentration, a second step usually involves a partition between two non-miscible solvents. The purpose of such a step is that it can be used whatever the sample size, and it can be very efficient in removing both more polar and less polar contaminants, if two complementary partition steps are being used (Table 2). Moreover, the recovery of compounds of interest in nearly quantitative. For example, ecdysteroids can be purified using isobutyl acetate–water (they remain in the water phase), then *n*-butanol–water (they go into the butanol phase) [2–4]. The choice of partition system relies on the polarity of the compounds of interest, which can roughly be estimated from the number of –OH groups (Table 3), although it also depends of course on their position on the molecule. In Table 3, the

Table 2
Some solvent partition systems

Solvent system[a]	Organic phase	Water or aq. MeOH phase
n-Hexane–80% MeOH	Non-polar lipids, sterols	BS, ES, WI
Diethyl ether–aq. MeOH	WI	
Chloroform–water	BS, VS, CU, WI	ES
Isobutyl acetate–water	VS	ES
Ethyl acetate–water	(ES), VS, CA	ES
Chloroform–MeOH–water	ES	
n-BuOH–water	ES, SS, SA	

[a] These systems are listed according to an increasing polarity of the organic phase. Abbreviations for steroid classes as in Table 1.

Table 3
Partition coefficients of ecdysteroids in three different systems [95]

Number of –OH	$CHCl_3$–water (1:1)	EtOAc–water (1:1)	*n*-BuOH–water (1:1)
2	90	n.d.	n.d.
3	20	20	n.d.
4	2.9	4	n.d.
5	0.06	0.4	10
6	0.015	0.1	5.3

n.d.: Not determined.

Table 4
Some counter-current distribution systems

Example	Type[a]	Compound	Ref.
$CHCl_3$–MeOH–water (13:4:4)	D	ES	[79]
$CHCl_3$–MeOH–water (13:7:2)	A	SS	[169]
$CHCl_3$–MeOH–water (13:7:4)	A	ES	[88]
$CHCl_3$–MeOH–water (4:4:4)	D	BU	[34]
$CHCl_3$–MeOH–water (7:13:8)	D	SS	[170]
$CHCl_3$–MeOH–water (5:10:6)	A	BU	[24]
$CHCl_3$–MeOH–PrOH–water–NH_4OH (35:65:40:5:1)	A	SA	[171]
$CHCl_3$–C_6H_6–EtOAc–MeOH–water (45:2:3:60:40)	D	ES	[172]
C_6H_6–$CHCl_3$–MeOH–water (5:5:7:2)	D	ES	[79]

[a] A: Ascending; D: descending.

approximate proportions of extracted compounds (with reference to ecdysteroids) in the organic layer are shown for three partition systems.

At the third step, chromatographic procedures start to be used. Small-scale experiments can use preparative TLC, but larger samples are usually purified by low-pressure column chromatography (CC) on alumina or silica. Alternatively, several counter-current chromatographic (CCC) techniques can be used, which are based on the distribution of compounds between organic and water phases (Table 4): e.g., droplet counter-current chromatography (DCCC) and rotation locular counter-current chromatography (RLCC). Examples can be found for ecdysteroids [2,3,5,6] and cardenolides [7]. These techniques require the availability of specific equipment.

Low-pressure column chromatography generally uses normal-phase systems, i.e., a polar stationary phase (alumina, silica) eluted with organic solvent mixtures of increasing eluting power (e.g., a step-gradient of MeOH in $CHCl_3$). Alternatively, reversed-phase systems are sometimes used with a non-polar stationary phase (C_{18}-bonded silica, Sephadex LH-20 or a resin such as Amberlite) eluted with a step-gradient of methanol in water. In fact, normal-phase systems are more generally used, but possibly on historical grounds rather than because of better performance. After one or possibly two such steps, major compounds can usually be considered as satisfactorily pure, especially if they are crystallised from the corresponding fractions. However, in the case of complex mixtures of structurally-related compounds, these methods are not efficient enough, and further chromatographic steps are needed.

TLC can then be used both to check compound purity and also sometimes as an additional purification step (for examples, see Table 5); several visualisation procedures can be used to observe the migra-

Table 5
Some major thin-layer chromatography solvents

TLC type	Examples of solvents	Compounds[a]
Silica gel	Hexane–Et_2O	VS
	Toluene–EtOAc	CA, CU
	$CHCl_3$–MeOH	CA,WI, ES, SA, SS
	CH_2Cl_2–MeOH (97:3)	VS
	$CHCl_3$–MeOH–formamide (93:6:1)	CA
	EtOAc–MeOH–water (81:11:8)	BU
	EtOAc–MeOH (97:3)	CA
	$CHCl_3$–MeOH–water (14:6:1)	SS, SA
Non-polar bonded silica	MeOH–water (7:3)	CU, ES, WI

[a] The abbreviations for the steroid classes are as in Table 1.

Table 6
Various methods for steroid visualisation after TLC

Method	Operating mode	Compounds[a]	Ref.
Fluorescence quenching	ZnS-containing silica	ES, CA, WI, VS	[94,173]
Non-specific colour reactions	Anisaldehyde	ES, SS	[118,174]
	H_2SO_4	ES, SA, SS	[116,175,176]
	$SbCl_3$	BU, WI, SS, SA WI CA, ES, SS, VS	[36,115,122,165]
	Vanillin–95% EtOH–H_2SO_4	SA, VS	[44,117]
	Chloramine T–H_2SO_4	VS	[177]
	Carbazole–H_2SO_4		[178]
''Specific'' reactions	3,5-Dinitrobenzoic acid in alcohol	CA	[179]
	$CeSO_4$ in H_2SO_4	CA	[180]
	2,4,2′,4′-Tetradinitrophenyl in toluene	CA	[51]
	Various methods	CU	[71]
	$(NH_4)_2CO_3$ (fluorescence induction)	ES	[181]
	Folin–Ciocalteu⇒blue (for 3-keto groups)	ES	[182]
	2,4-Dinitrophenylhydrazine⇒yellow (for keto groups)	ES	[182]
	Triphenyltetrazolium chloride⇒red (for keto groups)	ES	[182]
	Dragendorff's reagent	WI, SA	[123,149]
	4-(4-Nitrobenzyl)-pyridine (for epoxides)	WI	[149]

[a] The abbreviations for the steroid classes are as in Table 1.

tion of compounds during analytical experiments (Table 6). HPLC is more widely used, as it represents the most powerful technique and allows the recovery of pure compounds in the 1–100 mg range. Again mixtures of related compounds will possibly require several HPLC steps using systems of different selectivities (for a discussion see Ref. [99]).

2.1. Brassinosteroids

Since the isolation of brassinolide from rape pollen (4 mg from 40 kg pollen) [8], more than 40 different compounds of the brassinosteroid family have been isolated [9,10]. All are derivatives of 5α-cholestane with two vicinal diols (2α,3α and 22*R*,23*R*), a 6-keto or a 6-oxalactone group on ring-B, and various substituents in position 24 (24*S*-methyl, 24*R*-methyl, 24-methylene, etc.). Their occurrence seems general in the plant kingdom, as besides spermaphytes they have also been isolated from pteridophytes and chlorophytes [10]. Brassinosteroids represent a unique class of plant growth regulators belonging to the steroid family. Their biosynthetic pathway has been almost fully elucidated by a combination of metabolic studies with suitable radiolabelled precursors and the use of various *Arabidopsis* dwarf mutants [11–13]. Brassinosteroids share some chemical resemblance to ecdysteroids, and they may bind to insect ecdysteroid receptors, acting either as weak agonists or antagonists (e.g., Refs. [14,15]).

2.1.1. Extraction

With the exception of rape pollen, brassinosteroid concentrations are usually very low (ng to μg/kg – e.g., 360 ng/kg of castasterone in *Arabidopsis* seeds [16]), so their purification requires a multi-step procedure. For example [17], the plant material is dried and milled, before extraction with methanol. The methanolic extract is dried in vacuo and the residue is partitioned between water and chloroform. The chloroform phase is dried and the residue partitioned between hexane and 80% aq. MeOH. The residue from the aq. MeOH phase is subjected sequentially to (i) silica gel chromatography with a step-gradient from $CHCl_3$ to MeOH, (ii) Sepahadex LH-20 chromatography, (iii) DEAE ion-exchange chromatography and (iv) preparative HPLC. The

brassinosteroids are finally analysed by gas chromatography–mass spectrometry (GC–MS) after derivatisation. This procedure shares some resemblance with that used for ecdysteroids, although the latter are more polar and do not partition in the same phase when using chloroform–water (see later). Their isolation is based on bioassay-directed procedures [9], and their analysis from plant extracts uses mainly GC–MS [10,17,18].

2.1.2. HPLC

HPLC has been used for metabolic studies performed with explants or cell suspension cultures. Those have shown both hydroxylations (at C-20, C-25 or C-26), reduction of the 6-oxo into 6β-OH, conjugation with either fatty acids (at C-3) or sugars (at C-2, C-3 and C-25) and side-chain cleavage between C-20 and C-22 [19]. This wide array of metabolites can be resolved by classical reversed-phase (RP) HPLC procedures.

As brassinosteroids lack any chromophore or fluorophore, a major problem for their HPLC analysis concerns their detection. In such cases, pre-column derivatization can be used to produce detectable derivatives. The reactions used take advantage of the presence of two vicinal *cis*-diols at positions 2α,3α and 22*R*,23*R* which can readily react with boronic acid derivatives (bisnaphthaleneboronates, bisphenanthreneboronates or bisferroceneboronates) which can be detected by UV, fluorescence or electrochemical detectors, and are efficiently separated by RP-HPLC with acetonitrile–water mixtures [20,21].

In the case of the preparative isolation of brassinosteroids from plant samples, fractions are collected and analyzed by a suitable bioassay [9,17].

2.1.3. GC–MS

Analytical studies of small biological samples require very sensitive techniques, and GC–MS in the selected ion monitoring (SIM) mode has proved particularly efficient in this respect [17]. Volatile derivatives are bismethaneboronates when molecules have two *cis*-diols; where an odd hydroxyl group is present, it has to be converted into a trimethylsilyl ether. Subsequent analysis uses capillary GC coupled with a mass spectrometric detection of specific fragments. The technique has proved invaluable for studies of the distribution of brassinosteroids in the plant kingdom.

2.2. Bufadienolides

Bufadienolides are typically polyhydroxy C_{24} steroids and their glycosides. They are characterised by the presence of a six-membered lactone (α-pyrone) ring located at C-17β. On account of this chromophoric ring, they possess a characteristic UV absorption [λ_{max} at 296–299 in methanol (ϵ=5000 l mol^{-1} cm^{-1}) and a second absorption at 200–220 nm]. Many possess a 5β-hydroxyl (A/B-*cis* ring junction), a *trans*-B/C ring junction, a 14β-hydroxyl (C/D-*cis* ring junction) and an aldehydic group at C-19 (e.g., hellebrigenin, **9**: Fig. 1). Bufadienolides have been isolated from both plant and animal sources [22,23]. Over 250 bufadienolides have been identified, of which ca. 160 have been isolated from plants [23]. They have been detected in representatives of six plant families (*Cotyledon* and *Kalanchoe* in the Crassulaceae, *Urginea*/*Scilla* and *Bowiea* in the Hyacinthaceae, *Holmeria* and *Moraea* in the Iridaceae, *Bersama* and *Melianthus* in the Melianthaceae, *Helleborus* in the Ranunculaceae, *Thesium* in the Santalaceae). Concentrations of total bufadienolides may be over 1% of the dry mass [24]. Most bufadienolides from plant sources are glycosides with one to three sugars in a chain linked to the 3-hydroxyl of the genin, although branched chain glycosides and sugar attachment to the 5β-OH are also found [23]. Recently, 19-nor bufadienolides have been isolated from *Helleborus torquatus* [25]. They are important for their activity as cardiac glycosides, where they increase the contractile force of the heart by inhibiting Na+/K+-ATPase [26]. Helleborin, isolated from *Helleborus* spp. was previously used medicinally in the treatment of heart rhythm irregularities [27]. It has even been suggested that endogenous cardiotonic steroids (cardenolides and bufadienolides) are produced by the adrenal cortex in mammals and that these may be a new class of steroid hormone involved in the regulation of hypertension [28]. Certain bufadienolides possess antitumour activity [29]. They also show toxic activity towards livestock [30]. They possess insecticidal properties [31] and potent insect antifeedant

properties [32]. They also possess antimicrobial activity [33].

2.2.1. Extraction and prepurification

Generally, bufadienolides (free and glycosides) are extracted from plant sources with methanol [32,34]. Purification may be achieved by chromatography on silica gel eluted with $CHCl_3$–MeOH mixtures (for aglycones) or EtOAc–MeOH mixtures for glycosides. DCCC (with $CHCl_3$–MeOH–water, 4:4:4, descending procedure) has also been used for purification of *Helleborus* bufadienolides [34], and with $CHCl_3$–MeOH–water (5:10:6 ascending) for the purification of bufadienolide glycosides from *Urginea* spp. [24]. Final purification may be by TLC or by HPLC.

2.2.2. TLC

TLC has been used for the qualitative analysis of bufadienolides and this approach is finding application in the consideration of the chemotaxonomic significance of bufadienolides in plants; silica gel 60 F_{254}, developed with EtOAc–MeOH–water (81:11:8) [35] and detected by spraying with $SbCl_3$ reagent and heating [36]. RP-high-performance (HP) TLC has been applied to the separation of toad bufadienolides [37].

2.2.3. HPLC

RP-HPLC appears more appropriate for separation of bufadienolides, since strong peak trailing is observed on silica columns [38]. These authors investigated several RP (C_{18}) systems for the analysis of a complex bufadienolide mixture from *Urginea maritima*. Tittel and Wagner [39] developed reversed-phase methods suitable for the separation of bufadienolides from pharmaceutical preparations of *Scilla* spp. They used C_{18} columns eluted with a gradient from 10 to 60% MeOH in water and monitored the separations at 280 or 300 nm. The elution sequence of the various bufadienolides was determined by the number and type of functional groups on the aglycone, rather than the number of sugar units present. For quantitative analyses, isocratic elution with 25% acetonitrile (ACN) in water was found to be most applicable. Tittel [40] developed a method for the sophisticated analysis of cardiac glycosides (bufadienolides and cardenolides) in two pharmaceutical preparations based on plant material. A linear gradient from 10 to 50% ACN in water was used to elute a C_{18} column and the effluent was monitored by photodiode-array detector. The absorbance at 220 nm was used to detect the cardenolides present, that at 300 nm was used to detect bufadienolides and the absorbance at 340 nm indicated if flavonoids were present which would interfere in the detection and quantification of the bufadienolides. The retention times of a large number of plant bufadienolides on a C_{18} column eluted with an ACN–water gradient have been determined [41].

2.3. Cardenolides

Cardenolides are structurally closely related to bufadienolides, but possess a five-membered lactone (butenolide) ring located at C-17β. This imparts a characteristic UV absorption at ca. 220 nm in methanol. The A/B and C/D ring junctions are *cis*-fused, with a 14β-hydroxyl and a 5β-H (e.g., digitoxigenin, **8**: Fig. 1). Cardenolides are fairly widely distributed in the plant world, but are particularly associated with the Asclepiadaceae (*Periploca*, *Calotropis*), Apocynaceae (*Nerium*, *Strophanthus*), Scrophulariaceae (*Digitalis*), Ranunculaceae (*Adonis*) and Convallariaceae (*Convallaria*, *Speirantha*) [42]. In plants, cardenolide 3-*O*-glycosides predominate, but 2,3-doubly-linked glycosides also occur (e.g., Ref. [43]) and 3-*O*-sulfate esters have recently been identified [44]. The most important application is the therapeutic use of digoxin and its derivatives (acetyl- and methyldigoxin) as inhibitors of Na^+/K^+-ATPase to regulate heart contractions. Owing to their medical significance, these compounds have received a large amount of research attention with the characterisation of the mode of action and extensive structure–activity studies using natural and synthetic cardenolide analogues [42]. Cardenolides are also the major active ingredients in the poisons applied to arrow/dart tips by indigenous peoples in Africa, South America and Southeast Asia [45]. The most well-known cardenolide is ouabain, and, as mentioned above in the section on bufadienolides, endogenous hormone-like cardiac glycosides have been identified from human plasma, including an ouabain-like compound [46]. Cardenolides also possess anti-

bacterial activity [47,48] and can act as insect deterrents [49].

2.3.1. Extraction

Generally, cardenolides are extracted from plant material with EtOH, MeOH or 70% aq. MeOH. This is usually followed by partition between EtOAc and water, with the cardenolides partitioning into the EtOAc phase.

2.3.2. TLC

TLC on both silica gel (with $CHCl_3$–MeOH, 19:1, or toluene–EtOAc, 1:1 [44]) and RP-8 [50] plates have been applied to cardenolides. For detection methods, see Table 6. TLC has been used to compare the cardenolide content of two morphologically similar species of *Asclepias* {silica gel G with multiple development with (i) $CHCl_3$–MeOH–formamide (93:6:1) or (ii) EtOAc–MeOH (97:3), followed by spraying with 0.4% 2,4,2′,4′-tetradinitrodiphenyl in toluene and 10% KOH in 50% MeOH [51]}.

2.3.3. HPLC

HPLC methods have been developed for the analysis of cardenolides present in *Convallaria majalis* using a C_{18} column with ACN–water gradient elution and detection at 220 nm [52,53]. For the analysis of cardenolides in *Digitalis obscura*, a C_{18} column with a non-linear gradient from 30 to 60% ACN in water with detection at 230 nm was found to be appropriate [54]. The complex mixture of cardenolides present in *Asclepias fruticosa* have been separated by an ACN–water gradient on a C_{18} column with fixed wavelength (220 nm) and photodiode array monitoring [55]. Solid-phase extraction (SPE) has been used as a prepurification step prior to HPLC for cardenolides from *Digitalis lanata* [56].

2.4. Cucurbitacins

The cucurbitacins are a group of highly oxygenated C_{30} triterpenoids possessing a 19(10→9β)-abeo-10α-lanost-5-ene skeleton (including a *gem*-dimethyl group at C-4 and further methyls at C-9 and C-14). All cucurbitacins also possess a 5(6)-double bond (e.g., cucurbitacin D, **7**: Fig. 1). Strictly, they are not steroidal, because a methyl group is located at C-9 rather than at C-10. The cucurbitacins are most commonly associated with species in the Cucurbitaceae, but they have also been detected in members of the Begoniaceae, Cruciferae, Datiscaceae, Desfontainaceae, Elaeocarpaceae, Polemoniaceae, Primulaceae, Rosaceae, Rubiaceae, Scrophulariaceae and Sterculiaceae. The structural diversity amongst cucurbitacins has been reviewed previously [57–59]. Approximately 50 analogues have been identified, but cucurbitacins B and D are most commonly found in nature. In growing plants cucurbitacins are generally present as glycosides (often as 2β-*O*-glucosides), but these are frequently hydrolysed on extraction to give the genin, if elaterase activity is present in the plant. Free cucurbitacins occur naturally in seeds. The cucurbitacins have many medicinal and therapeutic effects. They are extremely bitter tasting and have been used for centuries for their purgative properties and cucurbitacin-containing *Momordica* spp. are still used for this purpose today. Cucurbitacins have strong cytotoxic activity [60] and antitumour [61] action. Cucurbitacins also possess antimicrobial [62], antihepatoxic [63], antiinflammatory [64], antigiberellin [65] and antihelminthic activities. Many insect species are also deterred or killed by the presence of cucurbitacins in the diet, although diabroticite beetles (which specialise on cucurbits) are attracted by low to moderate concentrations [66,67]. Recently, cucurbitacins have been shown to act as ecdysteroid receptor antagonists [68]. Cucurbitacins also have chemotaxonomic value in differentiating between plant species in cucurbitacin-containing genera [69].

2.4.1. Extraction

Cucurbitacins are generally extracted from plant material with methanol or ethanol. The genins have a low solubility in water, but significant solubility in $CHCl_3$. Partition between these two solvents is frequently used to partially purify cucurbitacins from alcoholic plant extracts. Subsequently, cucurbitacins have generally been purified by open-column chromatography on silica gel, alumina or florisil, or by TLC with the same media.

2.4.2. TLC

The frequent occurrence of α,β-unsaturated ketones in the A-ring and/or the side-chain impart a

significant UV-absorbance with a λ_{max} at ca. 230 nm, but many cucurbitacin analogues exist which differ from this or do not have a maximum above 210 nm [57,70]. The relative mobilities of a number of frequently encountered cucurbitacins (B, D, E, I, J, K, L and tetrahydro-I) in various solvent systems have been compared [71]. This paper also summarises various detection methods, suitable for cucurbitacins on TLC plates. Reversed-phase (C_{18} F_{254}) HPTLC (developed with MeOH–water, 7:3) and normal-phase (silica F_{254}) HPTLC (developed with toluene–EtOAc, 25:75) systems have been applied to the analysis of a number of frequently encountered cucurbitacins [72].

2.4.3. HPLC

Cucurbitacins are polar to medium-polar compounds, so reversed-phase systems are most appropriate. Owing to the significant variations in UV spectra amongst the cucurbitacins, absorption maxima vary considerably, but monitoring separations at 230 nm appears to be a good compromise, since almost all cucurbitacins possess some absorption at this wavelength. HPLC methods for cucurbitacins have been described: C_{18} column, eluted with a linear gradient from 20 to 50% [70] or 20% to 45% ACN in water [72], which are useful for the analysis of several frequently encountered cucurbitacins (free and glycosides). However, prior sample purification (solvent partitions or SPE) of plant extracts is required to remove interfering substances. Recently, a method has been developed for the analysis of cucurbitacins by HPLC–MS, using a C_{18} column with a gradient of ACN in 0.01% trifluoroacetic acid (TFA) [73]. The greater selectivity of this method will reduce complications arising from interfering substances.

2.4.4. Bioassays

The antigiberellin activity of cucurbitacins has been used as the basis of a bioassay for the compounds by co-application of giberellin and test extracts to rice seedlings or cucumber hypocotyls [58]. The B_{II} bioassay based on an ecdysteroid-responsive permanent cell line of *Drosophila melanogster* [74] can be used for the qualitative and quantitative assessment of cucurbitacins via their antiecdysteroid activity. This microplate-based bioassay is convenient for monitoring chromatographic fractions [75].

2.5. Ecdysteroids

Ecdysteroids are present both in animals (mainly Arthropods) and plants and this family comprises more than 300 different molecules related to ecdysone (**4**; Fig. 1) [76,77]. Their common characteristics are: (1) a 7-en-6-one chromophore (with a maximum absorbance at 242–243 nm in MeOH), (2) a 5β-hydrogen (*cis* A/B-ring junction), (3) often, but not always, the retention of the entire skeleton of cholesterol (C_{27} ecdysteroids) or of homologues bearing a methyl (or ethyl) group at the C-24 position and (4) the presence of several hydroxyl groups which render them rather water-soluble. They are present in significant amounts in 5–6% of plant species investigated so far [78], their concentration ranging from 50 ng/g to as much as 30 mg/g dry mass (i.e., 3%).

Plants usually contain one or a few major ecdysteroids together with a wide array of closely related molecules present in lower amounts [79,80] and these molecules are thought to protect plants against non-adapted phytophagous insects [78,81–83].

The isolation of phytoecdysteroids is performed according with the general strategy described above. Methods for the isolation and analysis of ecdysteroids are well documented and the reader can find many reviews of the field (e.g., Refs. [84–87]).

2.5.1. Extraction

Dried milled samples are usually extracted with MeOH or EtOH. The crude extracts are defatted by a partition between hexane (or light petroleum) and 80% aqueous MeOH [85]. Then extracts are partitioned between 1-BuOH and water, and ecdysteroid (ES) partition into the 1-BuOH phase. Further purification is achieved by column chromatography (silica or alumina), eluted with a step-gradient of MeOH (or 96% EtOH) in chloroform [86] or reversed-phase flash chromatography with a step-gradient of water in MeOH [85], but various countercurrent distribution procedures have been used [88–91] or chromatography on Sephadex LH-20 [85]. Affinity chromatography on immobilized phenylbor-

onic acid may provide a means to retain selectively ESs bearing a 20,22-diol [92].

2.5.2. TLC

TLC on silica plates is an efficient way to separate ESs [93]. Many different solvent systems have been described [86,87,94,95]. The most usual ones are $CHCl_3$–MeOH or $CHCl_3$–96% EtOH mixtures. Several efficient RP-TLC systems are also available, that use paraffin-coated or ODS-bonded silica developed with various MeOH–water mixtures [96]. The most usual detection procedures are fluorescence quenching and the vanillin–sulphuric acid spray reagent (Table 6). Overpressure thin-layer chromatography (OPTLC) has also been used; this method shortens the time for TLC development [97]. HPTLC is also an interesting technique for analytical purposes [98].

2.5.3. HPLC

The strongly UV-absorbing chromophore (λ_{max} ca. 242 nm, ϵ=ca. 12 000 l mol^{-1} cm^{-1}) allows sensitive detection of ESs. Normal-phase HPLC on silica columns provides very efficient separations, using mobile phases such as CH_2Cl_2–2-PrOH–water (125:25:2 or 125:40:3) or cyclohexane–2-PrOH–water (e.g., 100:40:3) mixtures [86,87,95]. Increasing the 2-PrOH–water proportion or using a trimethylsilyl (TMS)-bonded silica allows a convenient analysis of polar ESs (glucosides) [99].

RP-HPLC is widely used, and many different solvent systems have been described [99,87]. The most common ones are MeOH–water mixtures (isocratic or gradient mode), but MeOH can be replaced by MeCN or 2-PrOH which display different selectivities and allow some specific separations to be achieved.

Other separation methods, e.g., supercritical fluid chromatography (SFC) [95,100,101] and capillary zone electrophoresis (CZE) [95] are very efficient and fast separation methods which work only on an analytical scale and, consequently, will not be discussed here.

2.6. Steroid saponins/sapogenins

Steroid saponins are glycosides consisting of one or more oligosaccharide chains, linked to a steroid aglycone with specific skeletal features and typically localised substituents. The sugar chain is usually attached to C-3 and contains predominantly 2–5 linear or branched monosaccharide units. The classical definition of saponins is based on their surfactant or detergent properties, reflected in their stable foam formation in water solutions. This arises because they contain both water-soluble and fat-soluble molecular moieties.

Occurrence and distribution of saponins in plant kingdom is extremely frequent and high, both in a qualitative and quantitative sense. It is known that over 90 plant families contain saponins [102] and there are still many new occurrences being reported. Saponin content depends on many factors, e.g., the cultivar, the age, or the geographic locality of the plant. Considerable variation (mainly quantitative) can be observed in organs; high contents were found mostly in reproductive organs (flowers, seeds) or in lateral roots (root hairs), medium levels in stems and leaves and low levels in roots or bulbs. Steroidal saponins are not so widely distributed in nature than are the more frequent triterpenoid type. The main sources of steroid saponins are species in the Liliaceae, Dioscoreaceae and Agavaceae, mainly the genera *Allium*, *Asparagus*, *Lilium*, *Agave*, *Yucca* and *Dioscorea*.

Steroid saponins can be divided into two main groups. The largest group are spirostanol glycosides, comprising aglycones of the spirostane type (e.g., diosgenin, **10**: Fig. 1) with a sugar chain generally situated in position C-3. For spirostanols, it is typical that the spiroketal arrangement is linked at C-22. Structural variations of spirostanols depend mainly on the stereochemistry at position C-5 with a 5α-H or 5β-H configuration (A/B ring *trans* or *cis* annelation, respectively) and at position C-25 with the methyl group in *R* or *S* configuration. The presence of a double bond in position 5(6) or 25(27) increases the variability, which can be multiplied by the presence of one to four hydroxyls at almost any position, mostly at 1, 2, 3, 5, 6, 11, 12 and 15 (and again in α- or β-configuration). Over 45 spirostanol-type aglycones have been structurally identified [102,103] and when taking into consideration also the oxidised forms (usually carbonyls at positions 6 and 12, or a lactone forming carbonyl at 26), then the types can amount to over 100 [104]. The number

of structural types rises even more with the variable sugar chains linked to the hydroxyl at position C-3 [105].

The second typical group comprises furostanol glycosides with aglycones of spirostanol-like skeleton, but with an open side-chain (transformed ring F by hydrolysis of C-22 acetal to 22,26-diol). Fewer furostanol aglycone structural forms have been identified than spirostanols, but more glycoside conjugates (saponins), as the sugar chains can be linked not only to position C-3, but often also to C-26. Furostanols can be transformed during enzymatic hydrolysis to spirostanols and under certain conditions also spirostanols can be transformed by acid hydrolysis to furostanols. Moreover, eterification at position C-22 can also occur (i.e., formation of methyl ethers during methanol extractions), or dehydration of hydroxyl at position C-6 can form 6(7)-ene derivatives. Such side reactions can form many artefacts, which have to be taken into consideration when selecting methods for saponin analysis or separation.

An unusual and small group of steroid saponins are osladin and polypodosaponin types [106,107] with open ring E and preserved six-membered hemiacetal ring F and with sugar chains linked to both C-3 and C-26 hydroxyls. These saponins are responsible for the very sweet taste of *Polypodium vulgare* (sweet flag) and *P. glycyrhiza* (licorice fern).

Saponins often occur in plants which are used in human and animal nutrition [108], in traditional or modern medicine [109], or as herbal remedies in food, pharmacy, cosmetics, etc. [110], or other commercially important preparations and products [102]. For such purposes they are produced on a large scale using general or specially modified separation processes (e.g., the formation water insoluble complexes with cholesterol). To investigate a wide variety of biological and pharmacological activities [111], chemoecological activities in plant–insect interactions [112] or other antimicrobial, molluscicidal, antifertility activities often require several specially adapted analytic and separation processes.

2.6.1. Extraction and separation

Steroid saponins are usually extracted from dry or fresh plant material with aqueous methanol. After evaporating a major part of methanol, the water portion is partitioned against ethyl acetate (to remove non-polar constituents) and then against *n*-butanol to extract saponins (together with other polar constituents), and remove sugars, salts and other highly water-soluble components. After removal of the solvent, the saponins can be separated by open-column chromatography on silica by gradient solvent system $CHCl_3$–MeOH–water (87:12:1–14:6:1), or by HPLC.

2.6.2. TLC

TLC on silica is suitable for monitoring saponins during fractionation or for final small-scale purification. $CHCl_3$–MeOH–water (14:6:1) or $CHCl_3$–MeOH (4:1) are frequently used solvent systems for saponins [113,114] and $CHCl_3$–EtOH (20:1), $CHCl_3$–Me_2CO (9:1), *n*-hexane–EtOAc (1:1) and several others for aglycones [115]. They may be detected after chromatography by various visualisation reagents as colour spots: (i) spraying with sulphuric acid [116], (ii) with vanillin–sulphuric acid [117], (iii) with anisaldehyde–acetic acid–sulphuric acid [118], (iv) with $SbCl_3$ in chloroform and heating gives characteristic coloured spots [115], (iv) spraying with Ehrlich reagent (*p*-dimethylaminobenzaldehyde–conc. HCl–EtOH) and heating (red colours for furostanol derivatives).

2.6.3. HPLC

The highly polar nature and high molecular mass of saponins, as well as their close structural similarities (isomers or epimers of the aglycone or sugar parts) can cause difficulties in TLC or CC, but the greater resolution of HPLC makes this the method of choice. The single difficulty is the lack of a suitable chromophore for UV detection. Therefore derivatisation, refractive detection or MS detection [119] are applied to HPLC separations. A large number of mobile phases is reviewed in the monograph by Hostetmann and Marston [102].

2.7. Steroid alkaloids/glycoalkaloids

Steroid alkaloids represent a large group characterised by the presence of an intact or modified steroid skeleton with nitrogen integrated either into a ring, or attached as a side-chain or substituent. Depending on the location of the nitrogen and the skeletal

arrangement, several subgroups exist (e.g., Ref. [120]). Nitrogen can be attached as a primary NH_2 group in position 3 or 20 (free or methylated) forming simple steroidal bases, can be ring-closed to skeletal or side-chain carbon (as a secondary NH) or annelated in two rings as a tertiary N (e.g., solanidine, **12**: Fig. 1). This often influences the chemical character of the compound.

Plants contain alkaloids often in glycosidic form as glycoalkaloids. Structural variation in the family of plant steroidal glycoalkaloids is limited to two main groups, based on the skeletal type of the aglycone. One is the spirosolan type, similar to spirostan (as diosgenin, **10**: Fig. 1), but with nitrogen in place of oxygen in ring F (forming tetrahydrofuran and piperidine spiro-linked bicyclic system). Second is the solanidane type, where N connects spirostan rings E and F in the place of both oxygens forming C–N annelated E and F rings (see solanidine, **12**: Fig. 1). Moreover, all types can contain double bonds and hydroxyls in various positions and also sugar chains, as it is in the case of steroid saponins. However, the number and variety of naturally occurring glycoalkaloids is much lower than is the number of steroid glycosides. Their distribution is limited to the Solanaceae family, which includes many important agricultural crop plants, such as potato, tomato, eggplant and capsicum. The best known, solasodine, has been found in about 200 *Solanum* species [121]. Glycoalkaloids are usually found in all plant organs, but with the highest concentrations in flowers, unripe berries, young leaves or shoots (metabolic active parts). They are generally toxic, but in fruits they gradually decompose to nitrogen-free non-toxic constituents during ripening.

2.7.1. Extraction and separation

For isolation of single glycoalkaloids or their aglycones, the same extraction and separation procedures are used as for steroid saponins or sapogenins. However, there is one advantage that can be used in certain cases effectively; alkaloids can form water-soluble salts with acids. Crude alkaloids can be then obtained from the weakly acidic water portions of extracts by neutralisation with ammonia. When applying this procedure, it is important to maintain mild conditions and consider the possible formation of artefacts.

2.7.2. TLC

For TLC monitoring of steroid glycoalkaloids the same methods and solvent systems can be used as for steroid saponins. For detection and colour visualisation, sulfuric acid [116] or $SbCl_3$–$CHCl_3$ [122] have been used. Alkaloid-specific spraying reagents can be also applied as, e.g., Dragendorff's reagent (e.g., Ref. [123]).

2.7.3. HPLC

For HPLC analyses and preparations, the well-tried steroid saponin systems can be applied with modifications necessitated by the presence of alkaloid nitrogen. In some approaches NH_2 or carbohydrate columns were tested [124], or C_{18} columns with addition of ethanolamine to the mobile phase [125]. Some authors used NH_2 columns with a CH_3CN–water–KH_2PO_3 solvent system [126] or C_{18} column with MeOH–water–H_3PO_3 [127]. For quantitative HPLC analysis of solanidine glycoalkaloids, a generally applicable method for working with small sample amounts was developed [128].

2.8. Vertebrate-type steroids

The presence of vertebrate-type steroids in plants is not so well documented, although there seems little doubt about their occurrence as natural components in certain plant species [129,130]. In many instances, their occurrence has been established on the basis of colour reactions and low-efficiency chromatographic methods, which cannot be regarded as fully conclusive. Concentrations in the low ppb range (μg/kg) have been reported, and in a few species only. The evidence for their biosynthesis in some plant species has also been documented through labelling experiments with cholesterol or mevalonate (e.g., Refs. [131–133]).

Two points should be noted here: (1) some of these steroids can be regarded as intermediates in the biosynthesis of other plant steroids (thus progesterone is an intermediate in the biosynthesis of cardenolides) and (2) substances with a biological activity comparable with oestrogens (thus termed ''phytoestrogens'') are found in plants, which how-

ever do not belong to steroids (e.g., flavones, isoflavones).

Progesterone can be reduced in position C-20 and conjugated into steroidal glucosides (e.g., Ref. [134]). More generally, steroidal glucosides can either still bear the original 3-one-4-ene moiety, or they can be fully reduced (as 3α/3β-OH and 5α/5β-H) into pregnanes and the C-3 position can then be linked to various sugars [135–137].

Progesterone [138] and oestrogens [132] have been isolated from apple seeds, whereas testosterone was isolated from scotch pine pollen [139], but in fact such examples are scarce. Secosteroids related to vitamin D_3 (**6**: Fig. 1) have been isolated in several instances [140–143]. All these compounds are present in trace amounts, and their isolation relied upon immunoassay/radioreceptor assay-directed detection. On the other hand, steroidal glycosides are more abundant metabolites, and they can be purified on the basis of their UV absorbance [134–137,144].

2.8.1. Extraction

In the case of vitamin D_3 metabolites from *Nicotiana glauca*, extraction was performed with a $CHCl_3$–MeOH (1:2) mixture. Adding $CHCl_3$ and water allows two phases to separate, and the steroids partition in the organic phase [143]. Further purification was performed on a Sephadex LH-20 column (eluted with hexane–$CHCl_3$–MeOH, 9:1:1). For a more polar steroidal glucoside from *Lepisorus ussuriensis*, the MeOH extract was evaporated, the residue partitioned between water and successively hexane, $CHCl_3$ and *n*-BuOH. The *n*-BuOH extract was then purified by column chromatography on silicagel eluted by a gradient of $CHCl_3$ in MeOH [145].

2.8.2. TLC

This technique has been extensively used for the separation of vertebrate hormones, especially for metabolic studies which used radioactive molecules, and many visualization procedures have been designed (see Table 6). Similar TLC techniques have also been used for metabolic studies in plants, e.g., by Bennett and Heftmann [146], using CH_2Cl_2–MeOH (97:3) or hexane–Et_2O (3:7) as mobile phases.

2.8.3. HPLC

For seco-steroids (vitamin D), Aburjai et al. [142] used a Nova-Pak C_{18} column eluted with a gradient of MeOH in water, with UV monitoring at 264 nm. Various MeOH–water mixtures (75:25 to 90:10) were used to isolate Vitamin D_3 metabolites from *Nicotiana glauca* [143]. For the isolation of a pregn-4-en-3-one monoglucoside from *Centaurea moschata,* a combination of RP-HPLC on a C_{18} column (solvent MeOH–water, 1:1) and then either RP-HPLC on a C_6 column (solvent MeOH–water, 40:60) or NP-HPLC on a diol-bonded silica (solvent $CHCl_3$–MeOH, 96:4) was used [134]. For other steroidal glycosides, the systems used were either C_{18} RP columns eluted with gradients of MeCN or MeOH in water [147] or isocratic MeOH–water (1:1) [137].

2.9. Withanolides (withasteroids)

Withanolides are typically C_{28} ergostane-type steroids possessing a 22,26-δ-lactone. The side-chain may be linked to the steroid nucleus 17α or 17β. These molecules are also characterised by the presence of a large number of oxygen-containing functional groups (hydroxyls, ketones, epoxides, cyclic ethers). The withanolides are also often subdivided on the basis of whether they possess a hydroxyl group at C-20. 90% of all known withanolides possess a 1-oxo-group (e.g., withaferin A, **11**: Fig. 1). Over 200 structural analogues have been isolated. Withanolides occur predominantly as aglycones, although glycosides are known from some sources. They occur mainly in the leaves of withanolide-containing species. Their structural diversity, biosynthesis and biological activities have been reviewed [148–152]. Withanolides are predominantly associated with members of the Solanaceae (16 out of 96 genera), but they have recently also been detected in *Tacca plantaginea* (Taccaceae [153]), *Cassia siamae* (Leguminosae [154]) and *Ajuga parviflora* (Labiatae [155–157]). Several withanolide-containing species are medicinally or therapeutically important: *Datura* spp., *Withania* spp. and *Physalis* spp. Withanolides possess biological activity as anti-tumour, immunosuppressive and hepatoprotective agents, antibacterial properties [151], induction of cell differentiation [158], insect feeding

deterrents [159–161] and as ecdysteroid receptor antagonists [162].

2.9.1. Extraction

Withanolides are generally extracted from plant material with methanol or ethanol, which is then mixed with water before being partitioned against hexane to remove plant pigments. The aqueous methanol phase is then partitioned against $CHCl_3$, CH_2Cl_2 or diethyl ether to extract out the withanolides. After removal of the solvent, the withanolide mixture can be separated by open column/flash chromatography on silica or aluminium oxide, or by HPLC.

2.9.2. TLC

TLC on silica is convenient and suitable for the analysis of withanolides. It is often used to monitor fractionations or for final purification of withanolides. $CHCl_3$–MeOH (95:5) is a frequently used solvent system for aglycones (e.g., Ref. [163]) and $CHCl_3$–MeOH (90:10) for glycosides (e.g., Ref. [164]). They may be detected after chromatograpy by: (i) UV quenching, (ii) spraying with Dragendorff's reagent (even though they are not N-containing), (iii) an epoxide reagent (4-[4-nitrobenzyl]-pyridine) or (iv) spraying with a saturated $CHCl_3$ solution of $SbCl_3$ (+heating) [165]. For the withanolides from *Iochroma gesnerioides*, TLC analysis on SiO_2 F_{254} plates (with isobutyl methyl ketone–hexanol–hexane–acetic acid, 30:30:40:1, saturated with water) and RP_{18} F_{254} plate (with MeOH–water, 7:3) has been carried out [166].

2.9.3. HPLC

The UV absorption spectrum varies considerably as a number of permutations of chromophoric groups may occur in withanolides [151]. Withanolides with α,β-unsaturated ketones in the side-chain and in ring-A possess UV maxima near 220 nm (ϵ=ca. 18 000 l mol^{-1} cm^{-1}). HPLC appears to have been infrequently used in the purification of withanolides. This is probably a consequence of the low UV maxima associated with most withanolides which makes monitoring of HPLC separations more difficult. Also, the concentrations of withanolides present in withanolide-positive species is normally high enough that the lower sensitivity of TLC is not a problem. However, RP (C_{18})-HPLC has been applied to the separation of withanolides [158,160,166,167] and *Physalis* withanolides have been separated by normal-phase (NP) HPLC on a diol column [168].

3. Conclusions

(1) Plants elaborate a very wide array of steroidal compounds, partly as endogenous hormones (in low amounts) or as allelopathic defence compounds (in much higher concentrations).

(2) The distributions of the various classes of defence steroids varies between plant families, between species within the family, within the species (ecotypes) and within the organs of the plants which contain them.

(3) The availability of efficient, modern procedures for purification and analysis of natural products permits the identification of an ever-growing number of phytosteroids in each class with relative ease.

(4) In spite of (3), systematic and extensive studies on the chromatographic behaviour of phytosteroids (especially on HPLC) have only been performed for phytoecdysteroids. There is considerable scope for investigations in this area for the other classes of phytosteroids.

(5) The huge diversity of analogues found among phytosteroids reflects the ''combinatorial biochemistry'' at work within plants. The similarities between the structural modifications observed in the various classes of steroids indicate the same (or very similar) enzymes may be involved in the later stages of the various biosynthetic pathways, with the major difference being the identity of the early precursor which is fed into the pathway. This would provide a biosynthetic system for steroidal secondary products in plants which is both efficient and flexible, minimising redundancy at both the genetic and protein levels.

(6) It is clear that phytosteroids possess many interesting medicinal, pharmaceutical and agrochemical activities. For this reason alone, further studies are warranted on these compounds to identify lead compounds. Further, plants are a good source of large numbers of varied analogues suitable for structure–activity studies. Modification of the levels or profiles of phytosteroids by genetically modified

(GM) or non-GM means in crop species may result in improved growth and yields (brassinosteroids) or better allelopathic effects (deterrence of vertebrate or invertebrate predators, greater resistance to microbial attack, etc.: bufadienolides, cardenolides, cucurbitacins, ecdysteroids, steroidal alkaloids, sapogenins, vertebrate-type steroids and withanolides).

Acknowledgements

The research group in Exeter was supported by BBSRC, EU-INTAS and Rohm & Haas Co. and the group in Prague by GA CR, grant No. 203/98/0451.

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